939328

# THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

*July 21, 2018*

**THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.**

**APPLICATION NUMBER:** ***62/532,297***
**FILING DATE:** ***July 13, 2017***
**RELATED PCT APPLICATION NUMBER:** ***PCT/US18/41579***

**THE COUNTRY CODE AND NUMBER OF YOUR PRIORITY APPLICATION, TO BE USED FOR FILING ABROAD UNDER THE PARIS CONVENTION, IS** ***US62/532,297***

Certified by

Michelle K. Lee

Under Secretary of Commerce
for Intellectual Property
and Director of the United States
Patent and Trademark Office

PTO/AIA/14 (11-15)
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| Application Data Sheet 37 CFR 1.76 | Attorney Docket Number | VRD-3002-PV |
|---|---|---|
| | Application Number | |
| Title of Invention | BIOLOGICAL METHODS FOR PREPARING TERPENES | |

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

## Secrecy Order 37 CFR 5.2:

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

## Inventor Information:

**Inventor 1** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | Kimberly | Ann | Aeling | |

**Residence Information (Select One)** ◉ US Residency ○ Non US Residency ○ Active US Military Service

| City | San Clemente | State/Province | CA | Country of Residence | US |
|---|---|---|---|---|---|

**Mailing Address of Inventor:**

| Address 1 | 324 Plaza Estival | | |
|---|---|---|---|
| Address 2 | | | |
| City | San Clemente | State/Province | CA |
| Postal Code | 92672 | Country | US |

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

## Correspondence Information:

**Enter either Customer Number or complete the Correspondence Information section below.**
**For further information see 37 CFR 1.33(a).**

☐ **An Address is being provided for the correspondence Information of this application.**

| | | | |
|---|---|---|---|
| Customer Number | 69403 | | |
| Email Address | docketing@cpaglobal.com | Add Email | Remove Email |
| Email Address | bgrant@grantip.com | Add Email | Remove Email |
| Email Address | kdickinson@grantip.com | | Remove Email |

## Application Information:

| Title of the Invention | BIOLOGICAL METHODS FOR PREPARING TERPENES | | |
|---|---|---|---|
| Attorney Docket Number | VRD-3002-PV | Small Entity Status Claimed | ☒ |
| Application Type | Provisional | | |
| Subject Matter | Utility | | |
| Total Number of Drawing Sheets (if any) | 59 | Suggested Figure for Publication (if any) | |

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| | Application Number | |
| Title of Invention | BIOLOGICAL METHODS FOR PREPARING TERPENES | |

## Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

| Application number of the previously filed application | Filing date (YYYY-MM-DD) | Intellectual Property Authority or Country |
|---|---|---|
| | | |

## Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

## Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

| Please Select One: | ◉ Customer Number | ○ US Patent Practitioner | ○ Limited Recognition (37 CFR 11.9) |
|---|---|---|---|
| Customer Number | 69403 | | |

## Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the "Application Number" field blank.

| Prior Application Status | | | Remove |
|---|---|---|---|
| Application Number | Continuity Type | Prior Application Number | Filing or 371(c) Date (YYYY-MM-DD) |
| | | | |

Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button.

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|---|---|---|
| | Application Number | |
| Title of Invention | BIOLOGICAL METHODS FOR PREPARING TERPENES | |

## Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)[i] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

Remove

| Application Number | Country[i] | Filing Date (YYYY-MM-DD) | Access Code[i] (if applicable) |
|---|---|---|---|
| | | | |

Additional Foreign Priority Data may be generated within this form by selecting the **Add** button.

## Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

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| Application Data Sheet 37 CFR 1.76 | Attorney Docket Number | VRD-3002-PV |
|---|---|---|
| | Application Number | |
| Title of Invention | BIOLOGICAL METHODS FOR PREPARING TERPENES | |

## Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

**NOTE**: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

**1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)**

**A. Priority Document Exchange (PDX)** - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

**B. Search Results from U.S. Application to EPO** - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

**2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)**

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

**NOTE:** Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

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| Application Data Sheet 37 CFR 1.76 | Attorney Docket Number | VRD-3002-PV |
|---|---|---|
| | Application Number | |
| Title of Invention | BIOLOGICAL METHODS FOR PREPARING TERPENES | |

## Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

**Applicant 1**

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

| | | |
|---|---|---|
| ◉ Assignee | ○ Legal Representative under 35 U.S.C. 117 | ○ Joint Inventor |
| ○ Person to whom the inventor is obligated to assign. | ○ Person who shows sufficient proprietary interest | |

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

| Organization Name | VERDEZYNE, INC. | | |
|---|---|---|---|
| **Mailing Address Information For Applicant:** | | | |
| **Address 1** | 2715 Loker Avenue West | | |
| Address 2 | | | |
| **City** | Carlsbad | **State/Province** | CA |
| **Country** | US | Postal Code | 92010 |
| Phone Number | 760-707-5200 | Fax Number | 760-707-5100 |
| Email Address | info@verdezyne.com | | |

Additional Applicant Data may be generated within this form by selecting the Add button.

## Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

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|---|---|---|
| | Application Number | |
| Title of Invention | BIOLOGICAL METHODS FOR PREPARING TERPENES | |

**Assignee 1**

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

If the Assignee or Non-Applicant Assignee is an Organization check here. ☐

| Prefix | **Given Name** | Middle Name | **Family Name** | Suffix |
|---|---|---|---|---|
| | | | | |

**Mailing Address Information For Assignee including Non-Applicant Assignee:**

| | | | |
|---|---|---|---|
| **Address 1** | | | |
| Address 2 | | | |
| **City** | | **State/Province** | |
| **Country** i | | Postal Code | |
| Phone Number | | Fax Number | |
| Email Address | | | |

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button.

## Signature:

NOTE: This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

| **Signature** | /Kari A. Dickinson/ | | | Date (YYYY-MM-DD) | 2017-07-13 |
|---|---|---|---|---|---|
| First Name | Kari A. | Last Name | Dickinson | Registration Number | 60632 |

Additional Signature may be generated within this form by selecting the Add button.

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| Application Data Sheet 37 CFR 1.76 | Attorney Docket Number | VRD-3002-PV |
|---|---|---|
| | Application Number | |
| Title of Invention | BIOLOGICAL METHODS FOR PREPARING TERPENES | |

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

# Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent CooperationTreaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

PATENT
VRD-3002-PV

# BIOLOGICAL METHODS FOR PREPARING TERPENES

## Related Patent Applications

This patent application is related to a U.S. provisional patent application, filed simultaneously herewith, entitled BIOLOGICAL METHODS FOR MODIFYING CELLULAR CARBON FLUX, naming Tom Beardslee as inventor, and designated by Attorney Docket No. VRD-3001-PV. This patent application is also related to U.S. provisional patent application no. 61/222,902 filed on July 2, 2009, entitled BIOLOGICAL METHODS FOR PREPARING ADIPIC ACID, naming Stephen Picataggio as inventor, and designated by Attorney Docket No. VRD-1001-PV. This patent application also is related to International patent application no. PCT/US2010/040837 filed on July 1, 2010, entitled BIOLOGICAL METHODS FOR PREPARING ADIPIC ACID, naming Stephen Picataggio and Tom Beardslee as inventors, and designated by Attorney Docket No. VRD-1001-PC. This patent application is related to U.S. provisional patent application no. 61/430,097 filed on January 5, 2011, entitled BIOLOGICAL METHODS FOR PREPARING ADIPIC ACID, naming Stephen Picataggio and Tom Beardslee as inventors, and designated by Attorney Docket No. VRD-1001-PV2. This patent application also is related to U.S. provisional patent application no. 61/482,160 filed on May 3, 2011, entitled BIOLOGICAL METHODS FOR PREPARING ADIPIC ACID, naming Stephen Picataggio and Tom Beardslee as inventors, and designated by Attorney Docket No. VRD-1001-PV3. This patent application also is related to U.S. patent application no. 13/245,777 filed on September 26, 2011, entitled BIOLOGICAL METHODS FOR PREPARING ADIPIC ACID, naming Stephen Picataggio and Tom Beardslee as inventors, and designated by Attorney Docket No. VRD-1001-CT. This patent application also is related to U.S. patent application no. 13/245,780 filed on September 26, 2011, entitled BIOLOGICAL METHODS FOR PREPARING ADIPIC ACID, naming Stephen Picataggio and Tom Beardslee as inventors, and designated by Attorney Docket No. VRD-1001-UT. This patent application also is related to U.S. patent application no. 13/245,782 filed on September 26, 2011, entitled BIOLOGICAL METHODS FOR PREPARING ADIPIC ACID, naming Stephen Picataggio and Tom Beardslee as inventors, and designated by Attorney Docket No. VRD-1001-UT2. This patent application also is related to International patent application no. PCT/US2012/020230 filed on January 4, 2012, entitled BIOLOGICAL METHODS FOR PREPARING ADIPIC ACID, naming Stephen Picataggio and Tom Beardslee as inventors, and designated by Attorney Docket No. VRD-1001-PC2. This patent application also is related to International patent application no. PCT/US2012/056562 filed on September



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21, 2012, entitled BIOLOGICAL METHODS FOR PREPARING ADIPIC ACID, naming Stephen Picataggio and Tom Beardslee as inventors, and designated by Attorney Docket No. VRD-1001-PC3. This patent application also is related to U.S. provisional patent application no. 62/136,350 filed on March 20, 2015, entitled BIOLOGICAL METHODS FOR PREPARING 3-HYDROXYPROPIONIC ACID, naming Eric Michael Knight as inventor, and designated by Attorney Docket No. VRD-2001-PV. This patent application also is related to International patent application no. PCT/US2016/023243 filed on March 18, 2016, entitled BIOLOGICAL METHODS FOR PREPARING 3-HYDROXYPROPIONIC ACID, naming Eric Michael Knight as inventor. This patent application also is related to U.S. provisional patent application no. 61/505,092 filed on July 6, 2011, entitled BIOLOGICAL METHODS FOR PREPARING SEBACIC ACID naming Stephen Picataggio and Tom Beardslee as inventors, and designated by Attorney Docket No. VRD-1005-PV. This patent application also is related to U.S. provisional patent application no. 61/523,216 filed August 12, 2011, entitled BIOLOGICAL METHODS FOR PREPARING DODECANEDIOIC ACID naming Stephen Picataggio and Tom Beardslee as inventors, and designated by Attorney Docket No. VRD-1006-PV. This patent application also is related to International patent application no. PCT/US2012/045615 filed on July 5, 2012, entitled BIOLOGICAL METHODS FOR PREPARING A FATTY DICARBOXYLIC ACID, naming Tom Beardslee, Stephen Picataggio, L. Dudley Eirich and Jose Miguel Laplaza as inventors, and designated by Attorney Docket No. VRD-1005-PC. This patent application also is related to International patent application no. PCT/US2012/045622 filed on July 5, 2012, entitled BIOLOGICAL METHODS FOR PREPARING A FATTY DICARBOXYLIC ACID, naming Tom Beardslee, Stephen Picataggio, Alex Hutagalung and Tom Fahland as inventors, and designated by Attorney Docket No. VRD-1006-PC. This patent application also is related to U.S. patent application no. 14/131,170 filed on April 14, 2014 entitled BIOLOGICAL METHODS FOR PREPARING A FATTY DICARBOXYLIC ACID, naming Tom Beardslee, Stephen Picataggio, L. Dudley Eirich and Jose Miguel Laplaza as inventors. This patent application also is related to U.S. patent application no. 14/131,174 filed on April 28, 2014, entitled BIOLOGICAL METHODS FOR PREPARING A FATTY DICARBOXYLIC ACID, naming Tom Beardslee, Stephen Picataggio, Alex Hutagalung and Tom Fahland as inventors. This patent application also is related to U.S. provisional patent application no. 61/739,656 filed December 19, 2012, entitled BIOLOGICAL METHODS FOR PREPARING A FATTY DICARBOXYLIC ACID naming Jose Laplaza, Tom Beardslee, Dudley Eirich and Stephen Picataggio as inventors, and designated by Attorney Docket No. VRD-1007-PV. This patent application also is related to U.S. provisional patent application no. 61/739,661 filed December 19, 2012, entitled

BIOLOGICAL METHODS FOR PREPARING A FATTY DICARBOXYLIC ACID naming Tom Beardslee, Alex Hutagalung and Stephen Picataggio as inventors, and designated by Attorney Docket No. VRD-1008-PV. This patent application also is related to International patent application no. PCT/US2013/076664 filed on December 19, 2013, entitled BIOLOGICAL METHODS FOR PREPARING A FATTY DICARBOXYLIC ACID, naming Jose Laplaza, Tom Beardslee, Dudley Eirich and Stephen Picataggio as inventors. This patent application also is related to International patent application no. PCT/US2013/076739 filed on December 19, 2013, entitled BIOLOGICAL METHODS FOR PREPARING A FATTY DICARBOXYLIC ACID, naming Tom Beardslee, Alex Hutagalung and Stephen Picataggio as inventors. This patent application also is related to U.S. patent application no. 14/654,442 filed on June 19, 2015 entitled BIOLOGICAL METHODS FOR PREPARING A FATTY DICARBOXYLIC ACID, naming Jose Laplaza, Tom Beardslee, Dudley Eirich and Stephen Picataggio as inventors. This patent application also is related to U.S. patent application no. 14/654,458 filed on June 19, 2015, entitled BIOLOGICAL METHODS FOR PREPARING A FATTY DICARBOXYLIC ACID, naming Tom Beardslee, Alex Hutagalung and Stephen Picataggio as inventors. This patent application is also related to U.S. provisional patent application no. 62/011,500 filed on June 12, 2014 entitled PURIFICATION OF POLYCARBOXYLIC ACIDS, naming Jose Laplaza as inventor. This patent application is also related to U.S. patent application no. 14/738,600 filed on June 12, 2015 entitled PURIFICATION OF POLYCARBOXYLIC ACIDS, naming Jose Laplaza, William Andrew Evanko and Jason H. Radany as inventors. This patent application also is related to International patent application no. PCT/US2015/035634 filed June 12, 2015 entitled PURIFICATION OF POLYCARBOXYLIC ACIDS, naming Jose Laplaza as inventor.
The entire content of each of the foregoing patent applications is incorporated herein by reference, including, without limitation, all text, tables and drawings.

## Field

The technology relates in part to biological methods for producing terpenes and to engineered cells and microorganisms capable of such production.

## Background

Cells and microorganisms employ various enzyme-driven biological pathways to support metabolism and growth. A cell synthesizes native proteins, including enzymes, in vivo based on



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the sequence of deoxyribonucleic acid (DNA) encoding the protein. DNA first is transcribed into a complementary ribonucleic acid (RNA) that contains a ribonucleotide sequence encoding the protein. RNA then directs translation of the encoded protein by interaction with various cellular components, such as ribosomes. When the resulting protein is an enzyme, it can participate as a biological catalyst in biochemical pathways involved in producing a variety of organic molecules by the cell or organism.

These pathways can be exploited for the harvesting of naturally produced organic molecules. The pathways also can be altered to increase production or to produce specific molecules that may be commercially valuable. Advances in recombinant molecular biology methodology allow researchers to isolate DNA from one cell or organism and insert it into another cell or organism, thus altering the cellular synthesis of enzymes or other proteins. Advances in recombinant molecular biology methodology also allow endogenous genes, carried in the genomic DNA of a cell or microorganism, to be increased in copy number, thus altering the cellular synthesis of enzymes or other proteins. Such genetic engineering can change the biological pathways within the host cell or organism, causing it to produce a desired product. Microorganic industrial production can minimize the use of caustic chemicals and the production of toxic byproducts, thus providing a "clean" source for certain compounds. The use of appropriate plant-derived feedstocks allows production of "green" compounds while further minimizing the need for and use of petroleum-derived compounds.

Summary

Provided herein in certain aspects are genetically modified microorganisms comprising one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides, where expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide. Also provided herein in certain aspects are genetically modified microorganisms comprising one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides, and a genetic modification that alters the expression of a polypeptide providing for transport of acetyl-carnitine in the microorganisms. Also provided herein in certain aspects are methods for producing a terpene comprising contacting a genetically modified microorganism provided herein with a feedstock comprising a carbon source, and culturing the microorganism under conditions in which the terpenes are produced from the feedstock.



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Also provided herein in certain aspects is a genetically modified *Candida viswanathii* yeast, comprising one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides. Also provided herein in certain aspects are methods for producing a terpene comprising contacting a genetically modified *Candida viswanathii* yeast provided herein with a feedstock comprising a carbon source, and culturing the microorganism under conditions in which the terpenes are produced from the feedstock.

Also provided in certain aspects is a genetically modified yeast comprising one or more heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase. Also provided in certain aspects is a genetically modified yeast comprising one or more heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase. Also provided in certain aspects is a genetically modified yeast comprising one or more heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, and cytochrome p450 reductase.

Certain embodiments are described further in the following description, examples, claims and drawings.

Incorporation by Reference

All publications, patents and patent applications, GENBANK sequences (e.g., available at the World Wide Web Uniform Resource Locator (URL) ncbi.nlm.nih.gov of the National Center for Biotechnology Information (NCBI), sequences available through other databases, and websites and other published materials referred to throughout the entire disclosure herein, unless noted otherwise, are incorporated by reference in their entirety. Citation of any publications, patents and patent applications, GENBANK (and other database) sequences, websites and other published materials herein is not an admission that any of the foregoing is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents.

Brief Description of the Drawings

The drawings illustrate certain embodiments of the technology and are not limiting. For clarity and ease of illustration, the drawings are not made to scale and, in some instances, various aspects may be shown exaggerated or enlarged to facilitate an understanding of particular embodiments.

Fig. 1 is a diagrammatic representation of a cassette for the addition of a gene of interest (GOI) into a host non-functional ura3 locus using the single crossover integration method. The core of the cassette contains the GOI gene with a promoter ($P_{XXX}$) and terminator ($T_{XXX}$) for controlling transcription of the GOI gene. The *URA3* gene selectable marker is split with a 3' portion of the gene at one end of the cassette and a 5' portion positioned at the other end of the cassette. The segment of the expression cassette containing the gene of interest (GOI) is positioned between the *URA3* promoter ($P_{URA3}$) and terminator ($T_{URA3}$). Parts (A) and (B) of Fig. 1 show results of integration of one copy (A) and two copies (B) of the cassette. Integration of one cassette generates an added, functional GOI expression unit and may or may not provide for expression of a functional Ura3p, depending on the nature of the ura3 locus and the location of the split in the *URA3* selectable marker. Integration of two copies of the cassette generates a complete, functional *URA3* sequence by combining the 5' end of *URA3* from one copy of the cassette and the 3' end of *URA3* from the second copy of the cassette. Additional copies may also be integrated. Transformants are selected for by growth on uracil-free media. This integration method thus favors selection of transformants containing multiple copies of the GOI.

Fig. 2 is a diagrammatic illustration of an exemplary gene cassette for use in a double crossover homologous recombination integration referred to as "knock out" mutagenesis. Two slightly different cassettes are depicted for use in separately disrupting each of the two *FAT1* alleles in a diploid yeast. The two cassette-containing nucleic acid segments are referred to as "Deletion 1" and "Deletion 2," respectively. Each cassette contains a *URA3* gene including a URA3 promoter ($P_{URA3}$) and terminator ($T_{URA3}$). Additionally, each cassette contains a repeat of the $P_{URA3}$ sequence immediately downstream of the terminator sequence. The two separate deletion cassette-containing fragments differ in the sequences of the target gene that they contain on each side of the *URA3* cassette.



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Fig. 3A, Fig. 3B, and Fig. 3C show diagrammatic illustrations of a "knock in" gene disruption method which disrupts one target gene ("GOI1") and also adds a desired gene of interest ("GOI2") at the disrupted locus. The basic URA3 disruption cassette is the same as that described in Fig. 2, except for an additional expression cassette immediately downstream of the second $P_{URA3}$ repeat sequence. This expression cassette contains the gene of interest, GOI2, for adding to the endogenous GOI1 locus and includes a promoter ($P_{XXX}$) and terminator ($T_{XXX}$) for controlling transcription of GOI2. Immediately upstream of the first $P_{URA3}$ sequence is a sequence of nucleotides of the GOI1 gene, and immediately downstream of the terminator ($T_{XXX}$) for GOI2 is another sequence of the GOI1 gene. These sequences are for use in integration of the cassettes into the GOI1 locus. Fig. 3B shows the locus after the integration of the knock-in cassette which incorporates the functional URA3 selection gene. To remove the *URA3* gene, transformants are grown in the presence of 5-FOA to facilitate a "loop-out" event that is driven by the direct repeat sequences on either side of the *URA3* gene (in this case $P_{URA3}$). The result of that event is shown in Fig. 3C which depicts the $P_{URA3}$ sequence that remains followed by the functional GOI2 cassette.

Fig. 4 is a schematic illustration of the general, unmodified flow of carbon from a fatty acid carbon source in a wild-type eukaryotic cell, such as, for example, a yeast cell. "FA": fatty acid; "Co-A": coenzyme A; "PL": phospholipid; "TAG": triacylglyceride; FA-CoA": fatty acyl-CoA "*FAA1*" and "*FAT1*": acyl-CoA synthetase genes; "*PEX11*": peroxisomal biogenesis factor gene; "*PXA1*": peroxisomal transport protein gene; "β-Ox": β-oxidation; "Ac-CoA": acetyl-CoA; "*CAT2*": carnitine acetyltransferase gene; "Carn": carnitine; "Ac-Carn": acetyl-carnitine; "*CRC1*": mitochondrial acetyl-carnitine transport protein; "Cit": citrate; "TCA": tricarboxylic acid cycle; "Iso-Cit": isocitrate; "*ICL1*": isocitrate lyase gene; "Succ": succinate; "*MLS1*": malate synthase gene; "Glx": glyoxylate; "Mal": malate.

Fig. 5 is a schematic illustration of an engineered carbon flux pathway of a modified cell for use in producing a target molecule. The figure depicts cellular modifications in some embodiments of a eukaryotic (i.e., yeast in this example) platform system for developing particular target molecule production systems. The platform system contains an acetyl group carbon recycle loop that diverts acetyl moieties generated in the breakdown of fatty acids in peroxisomal β-oxidation("β-ox") into cytosolic fatty acid synthesis to regenerate a fatty acid that can be subjected to another cycle of peroxisomal β-oxidation. The recycle loop is depicted by the dark, solid reaction arrows beginning with extracellular fatty acid ("FA") internalization in the upper left

corner of the figure. Free fatty acids that have entered the cell can undergo oxidation to dicarboxylic acids (DCA) through ω-oxidation ("ω-ox"). Multiple modifications introduced via genetic manipulation, as well as unmodified activities of the cell, are indicated as follows: acyl-CoA synthetase gene deletions shown as "faa1Δ" and "fat1Δ" and resulting disruption of cytosolic activation of fatty acids (indicated as a lightly shaded dotted line reaction arrow below the gene deletion symbols and extending from "FA" to "FA-CoA") and diminished entry of FA-CoA into lipid (triacylglycerides ("TAG") and phospholipids ("PL")) biosynthesis; endogenous, unmodified peroxisomal enzymes acyl-CoA synthetase ("FAA2") and thioesterase ("TES"); unmodified glyoxylate cycle ("GlyOx") showing endogenous isocitrate lyase enzyme ("ICL1"); unmodified endogenous peroxisomal carnitine acetyltransferase ("CAT2") for conversion of acetyl-CoA ("Ac-CoA") to acetyl-carnitine ("AC-Carn"); modified (indicated by diagonal hatch lines) cytosolic carnitine acetyltransferase ("CAT2$^{cyt}$") and acetyl-CoA carboxylase ("ACC1") enzymes; unmodified endogenous fatty acid synthase enzyme complex ("FAS"); modified and added cytosolic thioesterase enzyme ("TES$^{cyt}$") showing added activity as solid, dark reaction arrow extending from FA-CoA to FA (which represents the final segment of the recycle loop); modified (gene deletion) peroxisomal transport protein ("pxa1Δ") showing disrupted (lightly shaded dotted line reaction arrow) acyl-CoA ("FA-CoA") import activity; modified peroxisomal biogenesis factor ("PEX11") activity; modified (promoter replacement) mitochondrial acetyl-carnitine transport protein ("CRC1") showing diminished (lightly shaded dotted line reaction arrow) acetyl-carnitine import activity; modified mitochondrial carnitine acetyltransferase ("CAT2") activity showing decreased conversion (lightly shaded dotted line reaction arrow) of AC-Carn to AC-CoA; unmodified mitochondrial tricarboxylic acid cycle ("TCA"); lightly shaded dashed lines reflect unmodified cellular activities that are not part of the carbon recycle loop shown in dark, solid lines. The details of the modifications in this exemplary engineered platform system are provided in the Detailed Description that follows.

Fig. 6 is a schematic illustration of an engineered carbon flux pathway of a modified cell for use in producing a target molecule. The figure depicts cellular modifications in some embodiments of a eukaryotic (i.e., yeast in this example) platform system for developing particular target molecule production systems. The platform system is similar to that shown in Fig. 5 except for the following: modified (gene deletion) peroxisomal carnitine acetyltransferase ("cat2Δ") showing disrupted (lightly shaded dotted line reaction arrow) generation of peroxisomal acetyl-carnitine (AC-Carn shown with an "X" over it); modified and added peroxisomal acetyl-CoA hydrolase for converting acetyl-CoA (AC-CoA) to acetate ("Ac"); modified (promoter



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replacement) cytosolic acetyl-CoA synthetase ("ACS") activity. The details of the modifications in this exemplary engineered platform system are provided in the Detailed Description that follows.

Fig. 7 is a schematic illustration of an engineered carbon flux pathway of a modified cell for use in producing a target molecule. The figure depicts cellular modifications in some embodiments of a eukaryotic (i.e., yeast in this example) platform system for developing particular target molecule production systems. The carbon recycle loop in this platform system, depicted by the dark, solid reaction arrows, extends through mitochondrial metabolism and differs from that shown in Figs. 5 and 6. The mitochondrial acetyl-carnitine transporter ("CRC1") and carnitine acetytransferase ("CAT2") are unmodified in this exemplary platform system. A cytosolic ATP citrate lyase ("ACL1/2") activity is added to the system. The details of the modifications in this exemplary engineered platform system are provided in the Detailed Description that follows.

Fig. 8 is a schematic illustration of an engineered carbon flux pathway of a modified cell for use in producing a target molecule. The figure depicts cellular modifications in some embodiments of a eukaryotic (i.e., yeast in this example) platform system for for the enhanced production of malonyl-CoA and various target molecules that can be synthesized using malonyl-CoA as a precursor. The details of the modifications in this exemplary engineered platform system are provided in the Detailed Description that follows.

Fig. 9 is a schematic illustration of an example of an engineered production pathway for cell- or microbial-based synthesis of 3-hydroxypropionic acid ("3HP"). Added cytosolic malonyl-CoA reductase ("MCR") activity and modified 3-hydroxy-propionate-dehydrogenase ("HPD1") activities for 3HP synthesis are shown as well as modified (gene deleted) endogenous semialdehyde dehydrogenase ("ald6Δ") activity. The details of the modifications in this exemplary engineered platform system are provided in the Detailed Description that follows.

Fig. 10 is a schematic illustration of an example of an engineered production pathway for cell- or microbial-based synthesis of triacetic acid lactone ("TAL"). Added 2-pyrone synthase ("2PS") activity for TAL synthesis is shown. The details of the modifications in this exemplary engineered platform system are provided in the Detailed Description that follows.

Fig. 11 is a schematic illustration depicting cellular modifications in some embodiments of a eukaryotic (i.e., yeast in this example) platform system for the enhanced generation of acetyl-CoA and the production of a diverse array of target molecules (e.g., terpenes). In one aspect, Fig. 11 differs from Fig. 8 in that it shows an embodiment of the platform system in which target molecule production pathways extend from acetyl-CoA, instead of malonyl-CoA , as a precursor molecule. ("Mev" refers to the mevalonate pathway; "IPP" refers to isopentenyl diphosphate; "DMAPP" refers to dimethylallyl diphosphate.) The details of the modifications in this exemplary engineered platform system are provided in the Detailed Description that follows.

Fig. 12 is a restriction endonuclease site map of plasmid pAA061 showing the relative placement of the following nucleic acid sequences: *Candida* strain ATCC 20336 orotidine-5'-phosphate decarboxylase (*URA3*) gene promoter (Prom), open-reading frame and terminator (Term); β-lactamase (ampicillin-resistance) gene promoter (P(BLA)) and ORF ($AP^r$); and the *Escherichia coli* origin of replication (ORI). Also shown are the *Candida* strain ATCC 20336 phosphoglycerate kinase (*PGK*) gene promoter and terminator that were added to pAA061 to form pAA105.

Fig. 13 is a restriction endonuclease site map of plasmid pAA105 which was constructed by ligating the *Candida* strain ATCC 20336 phosphoglycerate kinase (*PGK*) gene promoter and terminator with the PstI/NdeI fragment of pAA061 (Fig. 12).

Fig. 14 is a restriction endonuclease site map of plasmid pAA219 which was constructed by inserting the *Candida* strain ATCC 20336 cytochrome P450 reductase (*CPRB*) ORF between the *PGK* gene promoter and terminator in pAA105 (Fig. 13).

Fig. 15 is a restriction endonuclease site map of a PstI/NdeI fragment of plasmid pAA073 which contains the *Candida* strain ATCC 20336 acyl-CoA oxidase (*POX4*) gene promoter and terminator with restriction sites between them for incorporating ORFs to be controlled by the inducible *POX4* promoter.

Fig. 16 is a restriction endonuclease site map of a fragment of plasmid pAA073. Plasmid pAA073 was constructed by ligating the PstI/NdeI fragment shown in Fig. 15 with the PstI/NdeI fragment of pAA061 (Fig. 12).



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Fig. 17 is a restriction endonuclease site map of plasmid pAA153 which was constructed by inserting the *Candida* strain ATCC 20336 cytochrome P450 monooxygenase (*CYP52A14*) ORF between the *POX4* gene promoter and terminator in pAA073.

Fig. 18 is a diagrammatic representation of pAA153 (Fig. 17) linearized by endonuclease cutting of the plasmid at the *Cla*I site to yield a cassette for use in the addition of a *Candida* strain ATCC 20336 *CYP52A14* gene into a host non-functional ura3 locus using the single crossover integration method. The core of the cassette contains the *CYP52A14* gene with a *POX4* promoter and terminator for controlling transcription of the gene. Cutting of the plasmid at the *Cla*I site splits the *URA3* selectable marker and yields a linear DNA fragment with the *CYP52A14* gene expression cassette positioned between the *URA3* promoter (URA3 Prom) and terminator (URA3 Term).

Fig. 19 is a diagrammatic representation of plasmid pAA367 generated by (1) PCR amplification of two separate fragments of pAA153 (Fig. 17), one fragment containing a 3' URA3 sequence and the URA3 terminator and another fragment containing a *CYP52A14* gene expression cassette with a *POX4* promoter and terminator followed by the URA3 promoter and a 5' URA3 sequence, (2) joining of the two amplicons by overlap extension PCR to generate a single amplified fragment and (3) cloning of the single fragment into pCR-BluntII-TOPO.

Fig. 20 is a diagrammatic representation of a linear DNA expression cassette obtained by amplification from pAA367 (Fig. 19) that does not contain nucleic acid encoding an antibiotic selection marker (i.e., antibiotic-free).

Fig. 21A, Fig. 21B, and Fig. 21C show diagrammatic illustrations of a "knock out" gene disruption method which disrupts a target gene ("GOI"). Fig. 21A shows a double-crossover gene knock-out cassette for knocking out the function of a GOI containing a *URA3* selectable marker gene (including the gene promoter ($P_{URA3}$) and terminator ($T_{URA3}$)) between 5' and 3' homologous sequences for the GOI. The *URA3* selectable marker also has DNA sequence direct repeats ($P_{URA3}$) at the beginning and at the end of the gene sequence. After transformation of the double-crossover gene knock-out cassette into a $Ura^-$ mutant, the *URA3* marker allows selection on SC-URA plates for colonies that have integrated the construct (Fig. 21B) disrupting the GOI and generating a $Ura^+$ phenotype. Subsequent growth of $Ura^+$ transformants on 5-fluoroorotic acid (5-FOA) yields $Ura^-$ cells resulting from removal of the



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*URA3* selectable marker from the genome by a second crossover homologous recombination between the DNA sequence direct repeats ($P_{URA3}$) (Fig. 21C). A DNA sequence direct repeat remains in the genome as a "scar" left behind at the gene knock out site. The *URA3* selection marker may now be used again for further genetic modifications.

Fig. 22 is a diagrammatic depiction of how the knock out gene disruption method illustrated in Figs. 21A-21C, which regenerates an auxotrophic ($Ura^-$) cell after the second homologous recombination event, enables the same *URA3*-based selection method to be used repeatedly on the same cell, for example, such as in the disruption of the second allele ("Deletion 2") of a gene of interest (GOI) following the disruption of the first allele.

Fig. 23 is a restriction endonuclease site map of plasmid pAA298 containing a double-crossover gene knock-out cassette for knocking out the function of a *FAT1* gene. As shown in the figure, the double-crossover gene knock-out cassette includes a *URA3* selectable marker gene (including the gene promoter and terminator) between 5' and 3' homologous sequences ("N-Fat1" and "C-Fat1," respectively) for the *FAT1* gene. The plasmid also contains elements from pCR-BluntII-TOPO.

Fig. 24 shows restriction endonuclease site maps of plasmids pAA1519 and pAA1520 each containing a double-crossover gene knock-out cassette for knocking out the function of a *CAT2* gene. Each *CAT2* gene deletion cassette includes a 5' *Candida viswanathii* strain ATCC 20336 *CAT2* DNA fragment ("Cv CAT2 5' homology"), a 3' *Candida viswanathii* strain ATCC 20336 *CAT2* DNA fragment ("Cv CAT2 3' homology") and a *Candida viswanathii* strain ATCC 20336 *URA3* gene fragment containing a *URA3* ORF ("Cv URA3"), *URA3* promoter and a *URA3* terminator followed by a repeat of the promoter. The gene knock-out cassettes are contained within pCR-BluntII-TOPO.

Fig. 25A and Fig. 25B show a comparison of the N-terminal (Fig. 25A) and C-terminal (Fig. 25B) amino acid sequences of an unmodified *Candida* strain ATCC 20336 carnitine acetyltransferase ("Cv-CAT2 from pAA426") protein and of modified carnitine acetyltransferase proteins lacking one or both of the N-terminal mitochondrial targeting sequence (mts) and the C-terminal peroxisomal targeting sequence (pts). ("Cv-CAT2(-mts)" refers to the protein lacking only an N-terminal mitochondrial targeting sequence; "Cv-CAT2(-pts)" refers to the protein lacking only a C-terminal peroxisomal targeting sequence; "Cv-CAT2(-mts-pts)" refers to the protein lacking

the N-terminal mitochondrial targeting sequence and the C-terminal peroxisomal targeting sequence.)

Fig. 26 is a map of plasmid pAA1164 containing all the elements of the pCR-BluntII-TOPO vector, two separate portions of a URA3 gene selectable marker originally cloned from *Candida* strain ATCC 20336, nucleic acid encoding a modified *Candida* strain ATCC 20336 Pox5p (i.e., Pox5(F98G)) and the *HDE* gene promoter and *POX4* gene terminator, both from *Candida* strain ATCC 20336. Also shown are the locations of sequences corresponding to oligonucleotides oAA4722 and oAA4723 which were used as primers in PCR amplification of the larger fragment sequence between these two sites. The amplified DNA fragment was used in the construction of pAA1610 (see details provided in the Examples herein). The "CvURA3" segment positioned following the 3' *URA3* fragment ("CvURA3 F23 to Stop") corresponds to the URA3 terminator, whereas the "CvURA3" segment positioned in front of the 5' *URA3* fragment ("CvURA3 M1 to L22") corresponds to the URA3 promoter.

Fig. 27 shows a comparison of the N-terminal amino acid sequences of an unmodified *Candida* strain ATCC 20336 cytosolic carnitine acetyltransferase ("Cv-Yat1p") protein and of modified Yat1p carnitine acetyltransferase proteins containing an added N-terminal mitochondrial targeting sequence (mts) in place of the initiating methionine of the unmodified Yat1p. ("Cv-Yat1p+CAT2mts" refers to the protein with an added N-terminal mitochondrial targeting sequence from the *Candida* strain ATCC 20336 mitochondrial carnitine acetyltransferase; "Cv-Yat1p+CIT1mts" refers to the protein with an added N-terminal mitochondrial targeting sequence from the *Candida* strain ATCC 20336 citrate synthase; "Cv-Yat1p+COX4mts" refers to the protein with an added N-terminal mitochondrial targeting sequence from the *Candida* strain ATCC 20336 cytochrome c oxidase.)

Fig. 28 is a restriction endonuclease site map of plasmid pAA245 which contains all the elements of the pCR-BluntII-TOPO vector, DNA encoding a *Candida* strain ATCC 20336 acetyl-CoA carboxylase ("CvACC1") enzyme and a 5' partial intron ("CvACC1 5' Intron partial") at the 5' end of the ACC1 ORF. Also shown are the locations of sequences corresponding to oligonucleotides oAA0784 and oAA0785 which can be used as primers in PCR amplification of the Acc1-encoding DNA fragment from genomic DNA.



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Fig. 29 is a restriction endonuclease site map of plasmid pAA326 which was generated by cutting pAA245 (Fig. 28) with BspQI and ligating the resulting *ACC1* gene fragment including 5' partial intron into BspQI-cut plasmid pAA105 (Fig.13) to put the gene under the control of the *PGK* promoter and terminator from *Candida* strain ATCC 20336.

Fig. 30 is a restriction endonuclease site map of plasmid pAA1634 generated by ligating a SpeI/XbaI fragment of pAA326 (Fig. 29) containing DNA encoding amino acids R643 to the STOP codon of Candida strain ATCC 20336 *ACC1* with SpeI/XbaI-digested pAA601 (Fig. 12). A series of site-directed mutagenesis reactions was performed on pAA1634 to introduce mutations into the truncated *ACC1* coding sequence as described in the Examples herein. Also shown in the figure is an illustration of two DNA fragments, one containing an *HDE* gene promoter and one containing DNA encoding amino acids M1 – S642 of the Acc1p (both from *Candida* strain ATCC 20336) that were subsequently ligated with pAA1634 to generate plasmids containing DNA encoding full-length mutant Acc1p.

Fig. 31 is a map of plasmid pAA2247 which was generated by ligating a SbfI/MluI fragment of plasmid pAA1908 containing DNA encoding a *Candida* strain ATCC 20336 Acc1p mutant (S1158A) surrounded by the *HDE* gene promoter ("Prom") and *PGK* gene terminator ("Term") from *Candida* strain ATCC 20336 with SbfI/MluI-digested plasmid pAA2153. Plasmid pAA2153 contains DNA encoding a *Candida* strain ATCC 20336 *URA3* selectable marker with a direct repeat of the $T_{URA3}$ sequence located just upstream of the *URA3* gene promoter sequence ($P_{URA3}$) to yield $T_{URA3}$-$P_{URA3}$-*URA3*-$T_{URA3}$. The *URA3* selectable marker in pAA2153 is placed between genomic DNA sequence elements ("IGR5 5' homology" and "IGR5 3' homology") from *Candida* strain ATCC 20336 which are named IGR5. The IGR5 homology regions target integration of the intervening DNA into genomic DNA by homologous recombination. Also shown are the locations of DNA corresponding to primers oAA7259 and oAA7260 which can be used to amplify pAA2247 to generate a linear DNA for transformation of host cells.

Fig. 32 shows maps of plasmids pAA1613 and pAA1701 which contain double-crossover *Candida* strain ATCC 20336 *CRC1* gene knock-out cassettes that can be used to disrupt *Candida CRC1* genes. The cassette in each plasmid contains a 5' *Candida* strain ATCC 20336 *CRC1* DNA fragment ("CRC1 5' homology"), a 3' *Candida* strain ATCC 20336 *CRC1* DNA fragment ("CRC1 3' homology") and a *Candida* strain ATCC 20336 *URA3* gene fragment containing a *URA3* promoter, *URA3* ORF, and a *URA3* terminator followed by a repeat of the



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*URA3* promoter. The cassettes in the two plasmids differ in the sizes of the *CRC1* gene homology regions and the orientation of the *URA3* selectable marker between the homology regions. The gene knock-out cassettes are contained within pCR-BluntII-TOPO. Also shown are the locations of DNA corresponding to primers oAA5511 and oAA5512 which can be used to PCR amplify linear cassettes from each plasmid to generate a linear DNA for transformation of host cells.

Fig. 33 is a map of plasmid pAA2214 which was generated by ligating a plasmid backbone containing the IGR5 homology regions and *URA3* selectable marker (with $T_{URA3}$ repeat) amplified from plasmid pAA2247 (Fig. 31) with a 1,816-bp DNA fragment containing a *Candida* strain ATCC 20336 modified *CRC1* gene expression cassette. The expression cassette contains the glucose-6-phosphate isomerase (*G6PI*) promoter and *POX4* gene terminator from ATCC 20336 for controlling expression of the Crc1p. Also shown are the locations of DNA corresponding to primers oAA7259 and oAA7260 which can be used to PCR amplify a linear cassette from pAA2214 to generate a linear DNA for transformation of host cells.

Fig. 34 is a map of plasmid pAA2311 which contains a single-crossover cassette with nucleic acid encoding a *Candida* strain ATCC 20336 *CRC1* gene protein linked to a *Candida* strain ATCC 20336 *G6PI* low-expression promoter. The *CRC1* expression elements, $P_{G6PI}$-*CRC1*-$T_{POX4}$, were obtained as a DNA fragment amplified from plasmid pAA2214 (Fig. 33). This fragment was ligated with a fragment amplified from pAA1164 (Fig. 26) containing all the elements of the pCR-BluntII-TOPO vector, two separate portions of a URA3 selectable marker and a *POX4* gene terminator to yield pAA2311. Also shown are the locations of sequences corresponding to oligonucleotides oAA2206 and oAA2209 which can be used as primers in PCR amplification of a 3,307-bp linear, antibiotic-free DNA fragment for use in transforming host cells for expression of the Crc1p.

Fig. 35 is a map of plasmid pAA879 which contains a double-crossover knock-in cassette with nucleic acid encoding a *Candida* strain ATCC 20336 cytochrome P450 reductase (*CPRB*) gene protein. The plasmid contains all the elements of the pCR-BluntII-TOPO vector, two separate portions ("FAA1 5' homology" and "CvFAA1 3' homology") of a *Candida* strain ATCC 20336 *FAA1* gene, elements for the expression of CprB protein ("POX4 Promoter," "CvCPRB" and "POX4 term") and a *URA3* selectable marker with $P_{URA3}$ repeat (URA3 Prom-URA3-URA3 Term-URA3 Prom). Also shown are the locations of sequences corresponding to oligonucleotides



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oAA3557 and oAA3564 which can be used as primers in PCR amplification of a linear DNA fragment for use in transforming host cells for disruption of the *FAA1* gene and expression of CPRB protein.

Fig. 36 is a map of plasmid pAA208 which includes two separate portions ("POX4 5' homology" and "POX4 3' homology") of a *Candida* strain ATCC 20336 *POX4* gene and a *Candida* strain ATCC 20336 *URA3* gene selectable marker with $P_{URA3}$ repeat (URA3 Prom-URA3-URA3 Term-URA3 Prom).

Fig. 37 is a map of plasmid pAA850 which contains a double-crossover knock-in cassette with nucleic acid encoding a *Candida* strain ATCC 20336 *PEX11* gene protein. The plasmid contains all the elements of the pCR-BluntII-TOPO vector, two separate portions ("POX4 5' homology" and "POX4 3' homology") of a *Candida* strain ATCC 20336 *POX4* gene, elements for the expression of Pex11 protein ("POX4 Prom," "PEX11" and "POX4 term") and a *URA3* selectable marker with $P_{URA3}$ repeat (URA3 Prom-URA3-URA3 Term-URA3 Prom). Also shown are the locations of sequences corresponding to oligonucleotides oAA3355 and oAA3357 which can be used as primers in PCR amplification of a linear DNA fragment for use in transforming host cells for disruption of the *POX4* gene and expression of Pex11 protein.

Fig. 38 shows a partial amino acid sequence of *Candida* strain ATCC 20336 Pox5p acyl-CoA oxidase and the results of analysis of the amino acid sequence using HotSpot Wizard (a software program tool for identifying sites for engineering of substrate specificity and/or activity of enzymes using a combination of structural, functional and sequence analysis). HotSpot Wizard identified several amino acid positions, or "hotspots," of Pox5p to mutate, with each position given a score from 1 (cold) to 9 (hot). The different HotSpot residues identified are highlighted in the figure and shaded according to the score assigned to the residue.

Fig. 39 is an illustration of an overlap extension PCR-based method for generating nucleic acids encoding mutants (F98G and W429F) of a *Candida* strain ATCC 20336 Pox5 acyl-CoA oxidase. The oligonucleotides ("Oligos") used in the PCR amplifications are listed in the table shown in the figure. Oligos B and C contain the desired point mutations. Sequences for each of the oligonucleotides are provided in the Examples herein.



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Fig. 40 shows maps of plasmids pAA1117 and pAA1155 which contain *Candida* strain ATCC 20336 *PXA1* gene knock-out cassettes that can be used to disrupt alleles of *PXA1* genes. Both plasmids contain all the elements of the pCR-BluntII-TOPO vector, two separate portions ("PXA1 5' homology" and "PXA1 3' homology") of a *Candida* strain ATCC 20336 *PXA1* gene, and a *URA3* selectable marker with $P_{URA3}$ repeat (URA3 Prom-URA3-URA3 Term-URA3 Prom). Also shown are the locations of sequences corresponding to oligonucleotides oAA2679/oAA2684 and oAA2914/oAA2919 which can be used as primers in PCR amplification of pAA1117 and pAA1155, respectively, to obtain a linear DNA fragment for use in transforming host cells for disruption of the *PXA1* gene. Plasmids pAA1117 and pAA1155 differ in the sizes of *PXA1* gene 5' and 3' homology sequences and the orientation of the *URA3* selectable marker nucleic acid sequence contained in the plasmids.

Fig. 41 is a flow diagram showing the parent-daughter relationship for exemplary engineered yeast strains that can be used in generating cells and organisms for use in target molecule platform and production systems described herein. Strains in bold type are $Crc^-$ strains.

Fig. 42 shows a restriction endonuclease site map of plasmid pAA276 which contains a *Candida* strain ATCC 20336 *FAA1* gene knock-out cassette that can be used to disrupt alleles of *FAA1* genes. The plasmid contains all the elements of the pCR-BluntII-TOPO vector, two separate portions ("FAA1 N terminal" and "FAA1 C terminal") of a *Candida* strain ATCC 20336 *FAA1* gene, and a *URA3* selectable marker with URA3 Promoter repeat (Promoter URA3 -URA3 - Terminator URA3 – Promoter URA3).

Fig. 43 shows a restriction endonuclease site map of plasmid pAA918 which contains a *Candida* strain ATCC 20336 *POX5* gene knock-out cassette that can be used to disrupt alleles of *POX5* genes. The plasmid contains all the elements of the pCR-BluntII-TOPO vector, two separate portions ("5' FR POX5" and "3' FR POX5") of a *Candida* strain ATCC 20336 *POX5* gene, and a *URA3* selectable marker with URA3 terminator repeat (Tura3 – Pura3 - URA3 -Tura3).

Fig. 44 shows photographs of the agar plates obtained from spot growth assays of wild-type *Candida* strain ATCC 20336 ("CAT2/CAT2") and mutant strains as follows: *cat2-Δ1::*$P_{URA3}$*/cat2-Δ2::URA3* (sAA4594) and a $Cat2^-$ *Candida* strain (*cat2-Δ1::*$P_{URA3}$*/cat2-Δ2::*$P_{URA3}$) that had been transformed with either pAA1610 or pAA1876. The upper and lower photographs on the left side of the figure are control agar plates containing synthetic complete media with dextrose

minus uracil ("SCD-URA"), and the upper and lower photographs on the right side of the figure are plates containing yeast nitrogen base without amino acids, plus phosphate and 2% oleic acid ("YNBP + 2% oleic acid"). Each row of "spots" corresponds to serial dilutions of cells of the strain designated to the right of each row (increasing dilutions from right-to-left for the control agar plates and from left-to-right for the plates containing YNBP + 2% oleic acid).

Fig. 45 shows photographs of the agar plates obtained from spot growth assays of wild-type *Candida* strain ATCC 20336 ("CAT2/CAT2") and mutant strains as follows: *cat2-Δ1::*$P_{URA3}$*/cat2-Δ2::URA3* (sAA4594) and a Cat2$^-$ *Candida* strain (*cat2-Δ1::*$P_{URA3}$*/cat2-Δ2::*$P_{URA3}$) that had been transformed with one or more of plasmids pAA1610, pAA1967, pAA1968 and pAA1969. The photograph on the left side of the figure is of control agar plates containing synthetic complete media with dextrose minus uracil ("SCD-URA"), and the photograph on the right side of the figure is of plates containing yeast nitrogen base without amino acids, plus phosphate and 2% oleic acid ("YNBP + 2% oleic acid"). Each row of "spots" corresponds to serial dilutions of cells of the strain designated to the right of each row (increasing dilutions from right-to-left for the control agar plates and from left-to-right for the plates containing YNBP + 2% oleic acid).

Fig. 46 shows photographs of the agar plates obtained from spot growth assays of wild-type *Candida* strain ATCC 20336 ("CAT2/CAT2") and mutant strains as follows: *cat2-Δ1::*$P_{URA3}$*/cat2-Δ2::URA3* (sAA4594) and a Cat2$^-$ *Candida* strain (*cat2-Δ1::*$P_{URA3}$*/cat2-Δ2::*$P_{URA3}$) that had been transformed with pAA1846, pAA1875 and one or more of plasmids pAA1967, pAA1968, pAA1969 and pAA1847. The photograph on the left side of the figure is of control agar plates containing synthetic complete media with dextrose minus uracil ("SCD-URA"), and the photograph on the right side of the figure is of plates containing yeast nitrogen base without amino acids, plus phosphate and 2% oleic acid ("YNBP + 2% oleic acid"). Each row of "spots" corresponds to serial dilutions of cells of the strain designated to the right of each row (increasing dilutions from right-to-left for the control agar plates and from left-to-right for the plates containing YNBP + 2% oleic acid).

Fig. 47 shows photographs of the agar plates obtained from spot growth assays of wild-type *Candida* strain ATCC 20336 and mutant strains as follows: *ura3/ura3 pox4a::ura3/pox4b::ura3 faa1::* $P_{URA3}$*/faa1::*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::URA3* (sAA875), *ura3/ura3 pox4a::ura3/pox4b::ura3 faa1::*$P_{URA3}$*/faa1::*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::*$P_{URA3}$ *crc1-Δ1::URA3/CRC1* (sAA4057), *ura3/ura3 pox4a::ura3/pox4b::ura3 faa1::*$P_{URA3}$*/faa1::*$P_{URA3}$ *fat1-*

*Δ1::P$_{URA3}$/fat1-Δ2::P$_{URA3}$ crc1-Δ1::P$_{URA3}$/crc1-Δ2::URA3* (sAA4281), *ura3/ura3 crc1-Δ1::URA3/CRC1* (sAA4368), and *ura3/ura3 crc1-Δ1::P$_{URA3}$/crc1-Δ2::URA3* (sAA9398). Also shown are the agar plates obtained from spot growth assays of strains sAA5916, sAA5917 and sAA5918 generated by transforming strain sAA4377 (*ura3/ura3 pox4a::ura3/pox4b::ura3 faa1::P$_{URA3}$/faa1::P$_{URA3}$ fat1-Δ1::P$_{URA3}$/fat1-Δ2::P$_{URA3}$ crc1-Δ1::P$_{URA3}$/crc1-Δ2::P$_{URA3}$*) with a double-crossover integration cassette containing DNA encoding a *Candida* strain ATCC 20336 Crc1p linked to the *G6PI*, *PIC2* or *SUL2* promoter, respectively. The photographs on the left side of the figure are of control agar plates containing synthetic complete media with dextrose minus uracil ("SCD-URA"), and the photographs on the right side of the figure are of plates containing yeast nitrogen base without amino acids, plus phosphate and 2% oleic acid ("YNBP + 2% oleic acid"). Each row of "spots" corresponds to serial dilutions of cells of the strain designated to the right of each row (increasing dilutions from right-to-left for the control agar plates and from left-to-right for the plates containing YNBP + 2% oleic acid).

Fig. 48A shows GC-MS of valencene production from sAA7449 with its library match shown in Fig. 48B.

Fig. 49A shows GC-MS of valencene production from sAA7453 with its library match shown in Fig. 49B.

Fig. 50 shows the yeast upper mevalonate pathway. The enzymes acetyl-CoA C-acetyltransferase (*ERG10*), HMG-CoA synthase (*ERG13*), and HMG-CoA reductase (*HMG1*), are native to yeast described herein. The bracketed number under each enzyme refers to the IUBMB enzyme nomenclature for the enzyme.

Fig. 51 shows the yeast lower mevalonate pathway. The enzymes Mevalonate kinase (*ERG12*), Phosphomevalonate kinase (*ERG8*), Diphosphomevalonate decarboxylase (*MVD1*; *ERG19*), Isopentenyl diphosphate delta isomerase (*IDI1*), are native to yeast described herein. The bracketed number under each enzyme refers to the IUBMB enzyme nomenclature for the enzyme.

Fig. 52 shows a biosynthetic pathway to ergosterol or geranylgeranyl diphosphate. *ERG20*, *ERG9*, and *BTS1* are native to yeast described herein.



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Fig. 53 shows carotenoid production from farnesyl pyrophosphate. *CrtE*, *CrtB*, *CrtI, CrtY, CrtZ, and CrtW* are heterologous genes.

Fig. 54 shows an alternative route to lutein, canthaxanthin, and astaxanthin from β-carotene by the cytochrome P450 hydroxylase *CrtS*. *CrtS* is a heterologous enzyme which requires the activity of *CrtR*, a heterologous cytochrome p450 reductase.

Fig. 55 shows a route from farnesyl pyrophosphate to valencene by TPS1, a heterologous valencene synthase.

Fig. 56 show an absorption spectra of extracted carotenoids from sAA001, sAA7443, sAA7444, and sAA7445. Strains sAA7443, aAA7444 and sAA7445 are generated from sAA001 plus CsCrtE, CsCrtB and CsCrtI each under a glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter. Lycopene absorption maxima are expected at 443, 472, and 502 nm.

Fig. 57 shows an HPLC chromatogram of lycopene production in sAA001 and sAA8503.

Detailed Description

There are multiple cellular metabolic pathways that utilize carbon-containing molecules for varying purposes, such as, for example, biomass production, energy generation and growth. Different metabolic pathways can occur in distinct areas of the cell. For example, in eukaryotic microorganisms metabolic processes such as glycolysis, the pentose phosphate pathway and gluconeogenesis occur in the cytoplasm, whereas β-oxidation, the tricarboxylic acid (TCA) cycle and glyoxylate cycle are carried out, in whole or in part, in cellular organelles. The different metabolic pathways can be differentially utilized to maintain the basic functions required for survival of a microorganism under a variety of conditions (e.g., varying carbon source, nutrient, and oxygen availabilities). These features contribute to the ability of microorganisms to readily adapt to a variety of environmental conditions. The adaptability of microorganisms facilitates manipulation of microbial metabolic processes for the production of commercially useful materials (e.g., proteins, lipids, and organic acids).

There are a number of molecules that are valuable as final products, and/or as raw materials in generating a desired product, that incorporate carbon atoms resulting from catabolic and

anabolic carbon-metabolism pathways in microorganisms. A desired molecule that can be produced in cells and microorganisms is referred to herein as a "target" molecule or product. Some of these molecules are generated in wild-type microorganisms, whereas others that are not produced in a native microorganism can be generated through modification of a wild-type organism. In either case, the goal in the development of cellular and microbial production systems is to maximize yield and efficiency and minimize loss for process economy. Biological cell- or organism-based systems for production of carbon-containing molecules, such as, for example, polymers of substituted or unsubstituted hydrocarbons, may not be optimally efficient due to loss of carbon atoms that are transferred into other, non-target-producing, metabolic processes in the cell. In order for a bioproduction system to be cost-efficient and economically viable, it generally should meet certain metrics of titer, rate and yield with minimal by-product formation.

In order to minimize carbon loss and increase process efficiency of biological cell-based production systems, provided herein are modified cells and organisms (e.g., microorganisms) in which the flux of cellular carbon has been altered relative to an unmodified cell or organism. In some aspects, the cells or organisms are modified to redirect carbon from entering one or more growth and/or energy production metabolic pathways so that it is available for use in other inherent and/or engineered production processes. Alteration of carbon flux facilitates engineering of the cells or organisms for enhanced production of desired target molecules, including, for example, organic acids, terpenes and precursor molecules that can be used in the production of industrial chemicals. As such, modified cells and microorganisms provided herein are useful as platform systems that can be used for enhanced production of many different desired target molecules (e.g., terpenes) either singly or multiply in co-production microbial systems. Also provided herein are methods of modifying cellular carbon flux and methods of generating cells or microorganisms in which carbon flux has been optimized for production of target molecules, e.g., carbon-containing compounds, terpenes. Further provided herein are methods of producing target molecules (e.g., terpenes) using cell-based or microbial biosynthesis systems, including, for example, modified cells or microorganisms in which the flux of cellular carbon has been altered relative to an unmodified microorganism. Also provided are compositions, including, but not limited to, nucleic acids and chemical media and combinations, that can be used in the methods provided herein.



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*Terpenes*

Provided herein are methods for producing terpenes and engineered cells and microorganisms capable of producing terpenes. Terpenes are compounds generally made from isoprene units $(C_5H_8)_n$. Isoprene, also known as 2-methyl-1,3-butadiene or 2-Methylbuta-1,3-diene, is an organic compound having the formula $CH_2=C(CH_3)-CH=CH_2$. Isoprene units may be assembled in various combinations to produce thousands of possible terpene compounds. Terpenes may include hemiterpenes (one isoprene unit), monoterpenes (two isoprene units), sesquiterpenes (three isoprene units), diterpenes (four isoprene units), sesterterpenes (five isoprene units), triterpenes (six isoprene units), sesquarterpenes (seven isoprene units), tetraterpenes (eight isoprene units), and polyterpenes (many isoprene units; more than eight isoprene units). Hemiterpenes may include, for example, isoprene, prenol, isovaleric acid, benzoate, and prenyl acetate. Monoterpenes may include, for example, α-ocimene, β-ocimene, α-myrcene, β-myrcene, geraniol, citronellal, citronellol, linalool, citral A, halomon, S-limonene, R-limonene, phellandrene, α-terpinene, menthol, S-carvone, R-carvone, safranal, terpineol, thymol, carvacrol, umbellulone, piperitone, pulegone, rose oxide, lactisole, sabinene, camphene, eucalyptol, thujene, thujone, pinene, nepetalactone, ascaridole, borneol, verbenone, camphor, ethyl fenchol, and cyclosantene. Sesquiterpenes may include, for example, farnesene, farnesol, nerolidol, zingiberene, humulene, bisabolane, bisabolene, elemol, caryophyllene, guaiol, vetivazulene, cadinene, caryophyllene, cuparane, laurene, laurane, opposítane, mutisianthol, thapsane, lepidozanes, chiloscyphane, pinguisanes, herbertanes, botrydial, ngaione, longifolene, copaene, patchoulol, norpatchoulenol, santalol, cortisol, progesterone, oestrogen, testosterone, and 11-ketotestosterone. Diterpenes may include, for example, phytol, cembrene A, retinol, retinal, labdane, abietic acid, ferruginol, tetrahydrocannabinol, forskolin, cafestol, and gibberellins. Sesterterpenes may include, for example, geranylfarnesol and amyrin. Triterpenes may include, for example, squalene, sapelenins, tangshenoside I, cholecalciferol, dihydrotachysterol, lanosterol, cholesterol, cycloartenol, lovastatin, cucurbitacin, 20-hydroxyecdysone, campesterol, stigmasterol, ergosterol, bile acids, betulinic acid, withaferin A, ginsenosides, eleutherosides, astragalosides, bacoside A, araloside A, ziziphin, and gymnemic acids. Tetraterpenes may include, for example, phytoene, phytofluene, lycopene, torulene, γ-carotene, δ-carotene, citranaxanthin, rubixanthin, α-carotene, β-carotene, astaxanthin, cryptoxanthins, canthaxanthin, violaxanthin, antheraxanthin, zeaxanthin, lutein, diadinoxanthin, diatoxanthin, neoxanthin, fucoxanthins and flavoxanthins. Polyterpenes may include, for example, polypropylene, rubber and latex.



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Terpenes may include molecules containing only carbon (C) and hydrogen (H) atoms and also may include molecules containing carbon (C) and hydrogen (H) atoms in addition to other atoms (e.g. oxygen (O)). Terpene molecules containing atoms in addition to carbon (C) and hydrogen (H) may be referred to as terpenoids. In certain instances, compounds referred to as terpenoids contain only carbon (C) and hydrogen (H). Use of the term terpene herein includes terpenoids. Terpene structures may be acyclic (no carbon rings), monocyclic (one carbon ring), bicyclic (two carbon rings), tricyclic (three carbon rings), tetracyclic (four carbon rings), pentacyclic (five carbon rings), hexacyclic (six carbon rings), heptacyclic (seven carbon rings), or octacyclic (eight carbon rings). Terpene structures may contain more than eight carbon rings.

Terpenes may include carotenoids. Carotenoids (also referred to as tetraterpenoids) generally are pigments that provide red, orange, and yellow pigmentation to certain organisms, and include certain 40-carbon molecules. Over 700 carotenoids are known, which include, for example, carotenes (e.g., γ-carotene, β-carotene, α-carotene, ε-carotene, δ-carotene), xanthophylls (e.g., lutein, zeaxanthin, neoxanthin, violaxanthin, flavoxanthin, and α- and β-cryptoxanthin), torulene, lycopene, lutein epoxide, hydroxyechinenone, phoenicoxanthin, astaxanthin, fucoxanthin, 19′-butanoyloxyfucoxanthin, 19'-hexanoyloxyfucoxanthin, diatoxanthin, diadinoxanthin 19-hexanoyloxyparacentrone 3-acetate, gyroxanthin, canthaxanthin, citranaxanthin, and apo-caroten-ester. About 40 to 50 carotenoids can be found in food, and about 6 carotenoids can be found in human serum. Carotenoids may be synthesized de novo by organisms including, for example, archae, bacteria, fungi, algae, plants, aphids & spider mites. Carotenoids have various uses including, for example, antioxidants, color attractants, protection of plants from sun damage, precursors to vitamins, inflammatory disease protection, eye health, bone growth, and immune function.

Provided herein are microorganisms genetically modified to produce terpenes. For example, a genetically modified microorganism may comprise one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides. Terpene biosynthesis polypeptides may include any polypeptide capable of producing a terpene molecule or a terpene precursor. Terpene biosynthesis polypeptides may include one or more enzymes. For example, terpene biosynthesis polypeptides may include one or more of terpene synthase, phytoene synthase, geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin

synthase, zeaxanthin glucosyltransferase, valencene synthase, and cytochrome p450 reductase. In some embodiments, a genetically modified microorganism comprises heterologous nucleic acids encoding one or more of phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase. In some embodiments, a genetically modified microorganism comprises heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase. In some embodiments, a genetically modified microorganism comprises heterologous nucleic acids encoding one or more of geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase. In some embodiments, a genetically modified microorganism comprises heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase. In some embodiments, a genetically modified microorganism comprises heterologous nucleic acids encoding one or more of geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, and phytoene synthase. In some embodiments, a genetically modified microorganism comprises heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, and phytoene synthase. In some embodiments, a genetically modified microorganism comprises a heterologous nucleic acid encoding valencene synthase.

In some embodiments, a genetically modified microorganism comprises heterologous nucleic acids encoding one or more of geranylgeranyl diphosphate synthase, phytoene desaturase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, and cytochrome p450 reductase. In some embodiments, a genetically modified microorganism comprises heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, and cytochrome p450 reductase. In some embodiments, a genetically modified microorganism comprises heterologous nucleic acids encoding one or more of geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, and cytochrome p450 reductase. In some embodiments, a genetically modified microorganism comprises heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, and cytochrome p450 reductase.



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Terpene biosynthesis polypeptides may include any polypeptide (e.g., enzyme) capable of producing a terpene precursor. In certain embodiments, terpene biosynthesis polypeptides include one or more polypeptides (e.g., enzymes) in a mevalonate pathway (e.g., upper mevalonate pathway, lower mevalonate pathway). Typically, a mevalonate pathway begins with cytoplasmic acetyl-CoA, and the end product of a mevalonate pathway is isopentenyl diphosphate (IPP), which can serve as a monomer unit for terpene production. Polypeptides (e.g., enzymes) in a mevalonate pathway may include, for example, one or more of acetyl-CoA C-acetyltransferase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, isopentyl diphosphate delta isomerase, dimethylallyltranstransferase, and farnesyl diphosphate synthetase. In some embodiments, amounts and/or activities of one or more proteins chosen from acetyl-CoA C-acetyltransferase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, isopentyl diphosphate delta isomerase, dimethylallyltranstransferase, and farnesyl diphosphate synthetase are increased in a genetically modified microorganism provided herein. In some embodiments, a genetically modified microorganism comprises heterologous nucleic acids encoding one or more of acetyl-CoA C-acetyltransferase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, isopentyl diphosphate delta isomerase, dimethylallyltranstransferase, and farnesyl diphosphate synthetase.

In some embodiments, a heterologous nucleic acid encoding a terpene biosynthesis polypeptide is endogenously expressed in a microorganism that is different from the microorganism genetically modified to produce terpenes. In some embodiments, a heterologous nucleic acid encoding a terpene biosynthesis polypeptide is endogenously expressed in a microorganism that is of a different species, genus, family, order, class, phylum or kingdom than the microorganism genetically modified to produce terpenes. For example, a heterologous nucleic acid encoding a terpene biosynthesis polypeptide may be endogenously expressed in a microorganism chosen from *Cronobacter* spp, *Callitropsis* spp, *Xanthophyllomyces* spp, *Agrobacterium* spp, and *Pantoea* spp.

A heterologous nucleic acid encoding a terpene biosynthesis polypeptide may include an endogenous nucleic acid linked to a promoter or other regulatory element that is not normally functionally linked to the endogenous nucleic acid sequence. Promoters may include, for example, a mutated form of an endogenous promoter, an endogenous promoter replaced with a

modified version of the endogenous promoter, and an endogenous promoter replaced with a promoter located elsewhere in the same genome or from a different genome. A modification may be performed by directed mutagenesis, and the mutated promoter may be referred to as "heterologous" because it is no longer native. In certain instances, a modification may be performed by replacing an endogenous promoter with a modified promoter, the latter of which would be considered heterologous because the modified promoter is not normally functionally linked to an endogenous nucleic acid.

In some embodiments, a heterologous nucleic acid encoding a terpene biosynthesis polypeptide is regulated according to an inducible system. For example, a heterologous nucleic acid encoding a terpene biosynthesis polypeptide may be regulated by a nucleic acid that provides for fatty acid induction of expression of the terpene biosynthesis polypeptide. The fatty acid that can induce expression of a terpene biosynthesis polypeptide may be a saturated fatty acid or an unsaturated fatty acid. For example, a fatty acid may be chosen from one or more of oleic acid, palmitoleic acid, erucic acid, linoleic acid, palmitic acid, caproic acid, enanthic acid, caprylic acid pelargonic acid, capric acid, undecylic acid, lauric acid, myristic acid, pentadecanoic acid, margaric acid, stearic acid arachidic acid, behenic acid, tridecylic acid, and linolenic acid.

In some embodiments, a nucleic acid that provides for fatty acid induction of expression of a terpene biosynthesis polypeptide is a promoter or a portion of a promoter. In some embodiments, a nucleic acid that provides for fatty acid induction of expression of a terpene biosynthesis polypeptide comprises a fatty acid response element. In some embodiments, a nucleic acid that provides for fatty acid induction of expression of a terpene biosynthesis polypeptide is a promoter, or a portion of a promoter, comprising a fatty acid response element. In some embodiments, a fatty acid response element comprises an oleic acid response element. In some embodiments, a nucleic acid that provides for fatty acid induction of expression of a terpene biosynthesis polypeptide comprises a promoter region of a heterologous gene. In some embodiments, a nucleic acid that provides for fatty acid induction of expression of a terpene biosynthesis polypeptide comprises a promoter region of an endogenous gene. For example, a promoter region may be a promoter region of a gene encoding hydratase-dehydrogenase-epimerase (HDE), acyl co-A oxidase (POX; e.g., POX4), acyl co-A thiolase (POT), peroxin (PEX), peroxisomal protein POX18, or peroxisomal adenine nucleotide transporter protein (ANT1). In some embodiments, a nucleic acid that provides for



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fatty acid induction of expression of a terpene biosynthesis polypeptide comprises a promoter region of a gene encoding hydratase-dehydrogenase-epimerase (HDE).

In some embodiments, a nucleic acid that provides for fatty acid induction of expression of a terpene biosynthesis polypeptide comprises a promoter region of a gene from *Candida*. For example, a promoter region may be a promoter region of a gene encoding *Candida* hydratase-dehydrogenase-epimerase (HDE), *Candida* acyl co-A oxidase (POX; e.g., POX4), *Candida* acyl co-A thiolase (POT), *Candida* peroxin (PEX), peroxisomal protein POX18, or *Candida* peroxisomal adenine nucleotide transporter protein (ANT1). In some embodiments, a nucleic acid that provides for fatty acid induction of expression of a terpene biosynthesis polypeptide comprises a promoter region of a gene encoding *Candida* hydratase-dehydrogenase-epimerase (HDE).

In some embodiments, a heterologous nucleic acid encoding a terpene biosynthesis polypeptide is regulated by a nucleic acid that provides for alkane induction of expression of the terpene biosynthesis polypeptide. An alkane may be chosen from one or more of hexane, heptane, nonane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, hexadecane, heptadecane, and octadecane, for example. In some embodiments, a nucleic acid that provides for alkane induction of expression of a terpene biosynthesis polypeptide is a promoter or a portion of a promoter. In some embodiments, a nucleic acid that provides for alkane induction of expression of a terpene biosynthesis polypeptide comprises an alkane response element. In some embodiments, a nucleic acid that provides for alkane induction of expression of a terpene biosynthesis polypeptide is a promoter, or a portion of a promoter, comprising an alkane response element. In some embodiments, an alkane response element comprises an alkane response element 1 (ARE1) sequence or an ARE1-like sequence as described herein. In some embodiments, a nucleic acid that provides for alkane induction of expression of a terpene biosynthesis polypeptide comprises a promoter region, or a portion of a promoter region, of a heterologous gene.

In some embodiments, a heterologous nucleic acid encoding a terpene biosynthesis polypeptide is regulated by a nucleic acid that provides for glucose induction of expression of the terpene biosynthesis polypeptide. In some embodiments, a nucleic acid that provides for glucose induction of expression of a terpene biosynthesis polypeptide comprises a promoter region of a gene encoding glyceraldehyde-3-phosphate dehydrogenase (GPD). In some embodiments, a



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nucleic acid that provides for glucose induction of expression of a terpene biosynthesis polypeptide comprises a promoter region of a gene from *Candida*. In some embodiments, a nucleic acid that provides for glucose induction of expression of a terpene biosynthesis polypeptide comprises a promoter region of a gene encoding *Candida* glyceraldehyde-3-phosphate dehydrogenase (GPD).

*Modification of an acetyl-CoA C-acetyltransferase activity*

An initial step in the upper mevalonate pathway is the conversion of 2 acetyl-CoA molecules to acetoacetyl-CoA and CoA. An acetyl-CoA C-acetyltransferase enzyme (also referred to as ERG10, acetoacetyl-CoA thiolase, acetyl-CoA acetyltransferase, ergosterol biosynthesis protein 10, beta-acetoacetyl coenzyme A thiolase, 2-methylacetoacetyl-CoA thiolase, 3-oxothiolase, acetyl coenzyme A thiolase, acetyl-CoA:N-acetyltransferase, thiolase II, LPB3, TSM0115; EC 2.3.1.9) is a cytosolic and/or peroxisomal enzyme that can transfer an acetyl group from one acetyl-CoA molecule to another, forming acetoacetyl-CoA. In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of an acetyl-CoA C-acetyltransferase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of an acetyl-CoA C-acetyltransferase, may be modified to decrease the amount and/or activity of an acetyl-CoA C-acetyltransferase, or may be modified to alternately increase and decrease the amount and/or activity of an acetyl-CoA C-acetyltransferase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of an acetyl-CoA C-acetyltransferase in a cell is increased. Increasing the amount and/or activity of an acetyl-CoA C-acetyltransferase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway (e.g., mevalonate production, isopentenyl diphosphate production, geranyl diphosphate production, farnesyl diphosphate production, geranylgeranyl diphosphate production, terpene production, carotenoid production, beta carotene production, lycopene production, astaxanthin production) and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, acetyl-CoA C-acetyltransferase activity is unchanged in a host or engineered cell or organism. In some embodiments, the amount and/or activity of a host acetyl-



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CoA C-acetyltransferase can be increased by increasing the number of copies of a nucleic acid encoding an acetyl-CoA C-acetyltransferase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid); by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding an acetyl-CoA C-acetyltransferase; or by increasing the number of copies of a nucleic acid encoding an acetyl-CoA C-acetyltransferase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding an acetyl-CoA C-acetyltransferase. In some embodiments, an acetyl-CoA C-acetyltransferase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of acetyl-CoA C-acetyltransferase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding an acetyl-CoA C-acetyltransferase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding an acetyl-CoA C-acetyltransferase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed.

In some embodiments, an acetyl-CoA C-acetyltransferase enzyme can be a fungal or bacterial protein. In a particular embodiment, the acetyl-CoA C-acetyltransferase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*), *Fusarium* (e.g., *F. oxysporum*), *Bacillus* (e.g., *B. megaterium*, *B. subtilis*), *Chronobacter* (e.g., *C. sakazakii*), *Xanthophyllomyces* (e.g., *X. dendrorhous*), or *Pantoea* (e.g., *P. ananatis*) protein. An example of a *Candida viswanathii* nucleotide sequence (SEQ ID NO: 371) encoding a polypeptide (*ERG10*; SEQ ID NO: 337) having an acetyl-CoA C-acetyltransferase activity is provided herein. Additional nonlimiting examples of nucleotide sequences encoding polypeptides having acetyl-CoA C-acetyltransferase activity include: *Candida tropicalis CTPACTB* gene for acetoacetyl-CoA thiolase A (Genbank accession no. D13471.1), *Candida tropicalis CTPACTA* gene for acetoacetyl-CoA thiolase A (Genbank accession no. D13470.1), and *Candida tropicalis MYA-3404* acetyl-CoA acetyltransferase IB (NCBI Reference Sequence: XM_002547232.1).

Presence, absence or amount of acetyl-CoA C-acetyltransferase activity can be detected by any suitable method known in the art. For example, detection can be performed by using an acetyltransferase activity kit (e.g., ENZO, ADI-907-026). Nucleic acid sequences encoding native and/or modified acetyl-CoA C-acetyltransferase sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like

and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a nucleic acid (e.g., a heterologous nucleic acid) encoding an acetyl-CoA C-acetyltransferase can be modified. For example, the amount of an acetyl-CoA C-acetyltransferase protein expressed in a particular cellular location may be increased by including in the nucleic acid (e.g., heterologous nucleic acid) a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* oleate-induced peroxisomal protein (*POX18*) gene promoter. Another example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequences of a *Candida viswanathii* HDE gene promoter and a *Candida* POX18 gene promoter are provided herein as are examples of additional fatty acid-inducible promoters. In some embodiments, the promoter is modified by replacing the endogenous promoter with a promoter comprising an alkane response element (ARE1). Non-limiting examples of assays suitable for assessing induction of acetyl-CoA C-acetyltransferase expression by a carbon source or feedstock include RT-PCR or qRT-PCR after the host cell or microorganism has been exposed to the chosen carbon source or feedstock for varying amounts of time.

Decreasing acetyl-CoA C-acetyltransferase activity in a cell can be accomplished by modifying the amount of acetyl-CoA C-acetyltransferase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous acetyl-CoA C-acetyltransferase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type acetyl-CoA C-acetyltransferase such that the encoded modified or substituted acetyl-CoA C-acetyltransferase protein has a reduced enzyme activity.

*Modification of an HMG-CoA synthase activity*

A further step in the upper mevalonate pathway is the reaction in which acetyl-CoA condenses with acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). An HMG-CoA synthase enzyme (also referred to as ERG13, hydroxymethylglutaryl-CoA synthase, (S)-3-



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hydroxy-3-methylglutaryl-CoA acetoacetyl-CoA-lyase, (CoA-acetylating), 3-hydroxy-3-methylglutaryl CoA synthetase, 3-hydroxy-3-methylglutaryl coenzyme A synthase, 3-hydroxy-3-methylglutaryl coenzyme A synthetase, 3-hydroxy-3-methylglutaryl-CoA synthase, 3-hydroxy-3-methylglutaryl-coenzyme A synthase, beta-hydroxy-beta-methylglutaryl-CoA synthase, acetoacetyl coenzyme A transacetase, hydroxymethylglutaryl coenzyme A synthase, and hydroxymethylglutaryl coenzyme A-condensing enzyme; EC 2.3.3.10) contains a catalytic cysteine residue that acts as a nucleophile in the acetylation of the enzyme by acetyl-CoA (first substrate) to produce an acetyl-enzyme thioester, releasing the reduced coenzyme A. A subsequent nucleophilic attack on acetoacetyl-CoA (second substrate) forms HMG-CoA. In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of an HMG-CoA synthase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of an HMG-CoA synthase, may be modified to decrease the amount and/or activity of an HMG-CoA synthase, or may be modified to alternately increase and decrease the amount and/or activity of an HMG-CoA synthase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of an HMG-CoA synthase in a cell is increased. Increasing the amount and/or activity of an HMG-CoA synthase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway (e.g., mevalonate production, isopentenyl diphosphate production, geranyl diphosphate production, farnesyl diphosphate production, geranylgeranyl diphosphate production, terpene production, carotenoid production, beta carotene production, lycopene production, astaxanthin production) and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, HMG-CoA synthase activity is unchanged in a host or engineered cell or organism. In some embodiments, the amount and/or activity of a host HMG-CoA synthase can be increased by increasing the number of copies of a nucleic acid encoding an HMG-CoA synthase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid); by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding an HMG-CoA synthase; or by increasing the number of copies of a nucleic acid encoding an HMG-CoA synthase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding an HMG-CoA synthase. In some embodiments, an HMG-CoA synthase is



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endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of HMG-CoA synthase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding an HMG-CoA synthase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding an HMG-CoA synthase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed.

In some embodiments, an HMG-CoA synthase enzyme can be a fungal or bacterial protein. In a particular embodiment, the HMG-CoA synthase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*), *Fusarium* (e.g., *F. oxysporum*), *Bacillus* (e.g., *B. megaterium*, *B. subtilis*), *Chronobacter* (e.g., *C. sakazakii*), *Xanthophyllomyces* (e.g., *X. dendrorhous*), or *Pantoea* (e.g., *P. ananatis*) protein. An example of a *Candida viswanathii* nucleotide sequence (SEQ ID NO: 372) encoding a polypeptide (*ERG13*; SEQ ID NO: 338) having an HMG-CoA synthase is provided herein. Additional nonlimiting examples of nucleotide sequences encoding polypeptides having HMG-CoA synthase activity include: *Candida tropicalis MYA-3404* hydroxymethylglutaryl-CoA synthase (NCBI Reference Sequence: XM_002546412.1), and *Candida tanzawaensis NRRL Y-17324* hydroxymethylglutaryl-CoA synthase (NCBI Reference Sequence: XM_020207398.1).

Presence, absence or amount of HMG-CoA synthase activity can be detected by any suitable method known in the art. For example, detection can be performed by using a visible wavelength spectrophotometric assay for HMG-CoA synthase (see e.g., Skaff et al. (2010) Anal. Biochem. 396(1):96-102). Nucleic acid sequences encoding native and/or modified HMG-CoA synthase sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a nucleic acid (e.g., a heterologous nucleic acid) encoding an HMG-CoA synthase can be modified. For example, the amount of an HMG-CoA synthase protein expressed in a particular cellular location may be increased by including in the nucleic acid (e.g., heterologous nucleic acid) a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An

example of one such heterologous promoter is a *Candida* oleate-induced peroxisomal protein (*POX18*) gene promoter. Another example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequences of a *Candida viswanathii* HDE gene promoter and a *Candida* POX18 gene promoter are provided herein as are examples of additional fatty acid-inducible promoters. In some embodiments, the promoter is modified by replacing the endogenous promoter with a promoter comprising an alkane response element (ARE1). Non-limiting examples of assays suitable for assessing induction of HMG-CoA synthase expression by a carbon source or feedstock include RT-PCR or qRT-PCR after the host cell or microorganism has been exposed to the chosen carbon source or feedstock for varying amounts of time.

Decreasing HMG-CoA synthase activity in a cell can be accomplished by modifying the amount of HMG-CoA synthase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous HMG-CoA synthase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type HMG-CoA synthase such that the encoded modified or substituted HMG-CoA synthase protein has a reduced enzyme activity.

*Modification of an HMG-CoA reductase activity*

A further (rate controlling) step in the upper mevalonate pathway is the conversion of HMG-CoA to mevalonate by HMG-CoA reductase. HMG-CoA reductase (EC 1.1.1.34) may also be referred to as HMG1, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, HMGCR, LDLCQ3, 3-hydroxy-3-methylglutaryl-CoA reductase, and hydroxymethylglutaryl-CoA reductase. In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of an HMG-CoA reductase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of an HMG-CoA reductase, may be modified to decrease the amount and/or activity of an HMG-CoA reductase, or may be modified to alternately increase and decrease the amount and/or activity of an HMG-CoA reductase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of an HMG-CoA reductase in a cell is increased. Increasing the amount and/or activity of an HMG-CoA reductase may be particularly beneficial

in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway (e.g., mevalonate production, isopentenyl diphosphate production, geranyl diphosphate production, farnesyl diphosphate production, geranylgeranyl diphosphate production, terpene production, carotenoid production, beta carotene production, lycopene production, astaxanthin production) and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, HMG-CoA reductase activity is unchanged in a host or engineered cell or organism. In some embodiments, the amount and/or activity of a host HMG-CoA reductase can be increased by increasing the number of copies of a nucleic acid encoding an HMG-CoA reductase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid); by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding an HMG-CoA reductase; or by increasing the number of copies of a nucleic acid encoding an HMG-CoA reductase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding an HMG-CoA reductase. In some embodiments, an HMG-CoA reductase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of HMG-CoA reductase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding an HMG-CoA reductase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding an HMG-CoA reductase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed.

In some embodiments, an HMG-CoA reductase enzyme can be a fungal or bacterial protein. In a particular embodiment, the HMG-CoA reductase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*), *Fusarium* (e.g., *F. oxysporum*), *Bacillus* (e.g., *B. megaterium*, *B. subtilis*), *Chronobacter* (e.g., *C. sakazakii*), *Xanthophyllomyces* (e.g., *X. dendrorhous*), or *Pantoea* (e.g., *P. ananatis*) protein. An example of a *Candida viswanathii* nucleotide sequence (SEQ ID NO: 373) encoding a polypeptide (*HMG1*; SEQ ID NO: 339) having an HMG-CoA reductase activity is provided herein. An additional nonlimiting example of a nucleotide sequence encoding a polypeptide having HMG-CoA reductase activity includes: *Candida tropicalis MYA-3404* 3-hydroxy-3-methylglutaryl-coenzyme A reductase (NCBI Reference Sequence: XM_002550004.1).

Presence, absence or amount of HMG-CoA reductase activity can be detected by any suitable method known in the art. For example, detection can be performed by using an HMG-CoA Reductase Assay Kit (e.g., SIGMA-ALDRICH, CS1090 Sigma); and/or a Colorimetric HMG-CoA Reductase Activity Assay Kit (ABCAM, ab204701). Nucleic acid sequences encoding native and/or modified HMG-CoA reductase sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a nucleic acid (e.g., a heterologous nucleic acid) encoding an HMG-CoA reductase can be modified. For example, the amount of an HMG-CoA reductase protein expressed in a particular cellular location may be increased by including in the nucleic acid (e.g., heterologous nucleic acid) a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* oleate-induced peroxisomal protein (*POX18*) gene promoter. Another example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequences of a *Candida viswanathii* HDE gene promoter and a *Candida* POX18 gene promoter are provided herein as are examples of additional fatty acid-inducible promoters. In some embodiments, the promoter is modified by replacing the endogenous promoter with a promoter comprising an alkane response element (ARE1). Non-limiting examples of assays suitable for assessing induction of HMG-CoA reductase expression by a carbon source or feedstock include RT-PCR or qRT-PCR after the host cell or microorganism has been exposed to the chosen carbon source or feedstock for varying amounts of time.

Decreasing HMG-CoA reductase activity in a cell can be accomplished by modifying the amount of HMG-CoA reductase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous HMG-CoA reductase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type HMG-CoA reductase such that the encoded modified or substituted HMG-CoA reductase protein has a reduced enzyme activity.

*Modification of a mevalonate kinase activity*

The initial step in the lower mevalonate pathway is the conversion of mevalonate to phosphomevalonate by mevalonate kinase. Mevalonate kinase (EC 2.7.1.36) may also be referred to as ERG12, MVK, LRBP, MK, MVLK, and POROK3. In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of a mevalonate kinase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of a mevalonate kinase, may be modified to decrease the amount and/or activity of a mevalonate kinase, or may be modified to alternately increase and decrease the amount and/or activity of a mevalonate kinase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of a mevalonate kinase in a cell is increased. Increasing the amount and/or activity of a mevalonate kinase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway (e.g., isopentenyl diphosphate production, geranyl diphosphate production, farnesyl diphosphate production, geranylgeranyl diphosphate production, terpene production, carotenoid production, beta carotene production, lycopene production, astaxanthin production) and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, mevalonate kinase activity is unchanged in a host or engineered cell or organism. In some embodiments, the amount and/or activity of a host mevalonate kinase can be increased by increasing the number of copies of a nucleic acid encoding a mevalonate kinase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid); by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a mevalonate kinase; or by increasing the number of copies of a nucleic acid encoding a mevalonate kinase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a mevalonate kinase. In some embodiments, a mevalonate kinase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of mevalonate kinase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding a mevalonate kinase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding a



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mevalonate kinase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed.

In some embodiments, a mevalonate kinase enzyme can be a fungal or bacterial protein. In a particular embodiment, the mevalonate kinase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*), *Fusarium* (e.g., *F. oxysporum*), *Bacillus* (e.g., *B. megaterium*, *B. subtilis*), *Chronobacter* (e.g., *C. sakazakii*), *Xanthophyllomyces* (e.g., *X. dendrorhous*), or *Pantoea* (e.g., *P. ananatis*) protein. An example of a *Candida viswanathii* nucleotide sequence (SEQ ID NO: 374) encoding a polypeptide (*ERG12*; SEQ ID NO: 340) having a mevalonate kinase activity is provided herein.

Presence, absence or amount of mevalonate kinase activity can be detected by any suitable method known in the art. For example, detection can be performed by using a mevalonate kinase assay (see e.g., Green et al. (1970) Anal. Biochem. 38(1):130-138; Gibson et al. (1989) Enzyme 41(1):47-55). Nucleic acid sequences encoding native and/or modified mevalonate kinase sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a nucleic acid (e.g., a heterologous nucleic acid) encoding a mevalonate kinase can be modified. For example, the amount of a mevalonate kinase protein expressed in a particular cellular location may be increased by including in the nucleic acid (e.g., heterologous nucleic acid) a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* oleate-induced peroxisomal protein (*POX18*) gene promoter. Another example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequences of a *Candida viswanathii* HDE gene promoter and a *Candida* POX18 gene promoter are provided herein as are examples of additional fatty acid-inducible promoters. In some embodiments, the promoter is modified by replacing the endogenous promoter with a promoter comprising an alkane response element (ARE1). Non-limiting examples of assays suitable for assessing induction of mevalonate kinase expression by a carbon source or feedstock include

RT-PCR or qRT-PCR after the host cell or microorganism has been exposed to the chosen carbon source or feedstock for varying amounts of time.

Decreasing mevalonate kinase activity in a cell can be accomplished by modifying the amount of mevalonate kinase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous mevalonate kinase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type mevalonate kinase such that the encoded modified or substituted mevalonate kinase protein has a reduced enzyme activity.

*Modification of a phosphomevalonate kinase activity*

A further step in the lower mevalonate pathway is the conversion of phosphomevalonate to diphosphomevalonate (mevalonate-5-phosphate to mevalonate-5-pyrophosphate; 5-phosphomevalonate to 5-diphosphomevalonate) by phosphomevalonate kinase. Phosphomevalonate kinase (ERG8; EC 2.7.4.2) may also be referred to as 5-phosphomevalonate phosphotransferase and PMVK. In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of a phosphomevalonate kinase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of a phosphomevalonate kinase, may be modified to decrease the amount and/or activity of a phosphomevalonate kinase, or may be modified to alternately increase and decrease the amount and/or activity of a phosphomevalonate kinase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of a phosphomevalonate kinase in a cell is increased. Increasing the amount and/or activity of a phosphomevalonate kinase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway (e.g., isopentenyl diphosphate production, geranyl diphosphate production, farnesyl diphosphate production, geranylgeranyl diphosphate production, terpene production, carotenoid production, beta carotene production, lycopene production, astaxanthin production) and away from other cellular metabolic pathways not involved in target molecule production.



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In certain embodiments, phosphomevalonate kinase activity is unchanged in a host or engineered cell or organism. In some embodiments, the amount and/or activity of a host phosphomevalonate kinase can be increased by increasing the number of copies of a nucleic acid encoding a phosphomevalonate kinase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid); by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a phosphomevalonate kinase; or by increasing the number of copies of a nucleic acid encoding a phosphomevalonate kinase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a phosphomevalonate kinase. In some embodiments, a phosphomevalonate kinase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of phosphomevalonate kinase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding a phosphomevalonate kinase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding an phosphomevalonate kinase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed.

In some embodiments, a phosphomevalonate kinase enzyme can be a fungal or bacterial protein. In a particular embodiment, the phosphomevalonate kinase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*), *Fusarium* (e.g., *F. oxysporum*), *Bacillus* (e.g., *B. megaterium*, *B. subtilis*), *Chronobacter* (e.g., *C. sakazakii*), *Xanthophyllomyces* (e.g., *X. dendrorhous*), or *Pantoea* (e.g., *P. ananatis*) protein. An example of a *Candida viswanathii* nucleotide sequence (SEQ ID NO: 375) encoding a polypeptide (*ERG8*; SEQ ID NO: 341) having a phosphomevalonate kinase activity is provided herein.

Presence, absence or amount of phosphomevalonate kinase activity can be detected by any suitable method known in the art. For example, detection can be performed by using a microplate assay for phosphomevalonate kinase activity (see e.g., Schulte et al. (1999) Anal. Biochem. 269(2):245-54). Nucleic acid sequences encoding native and/or modified phosphomevalonate kinase sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or



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organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a nucleic acid (e.g., a heterologous nucleic acid) encoding a phosphomevalonate kinase can be modified. For example, the amount of a phosphomevalonate kinase protein expressed in a particular cellular location may be increased by including in the nucleic acid (e.g., heterologous nucleic acid) a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* oleate-induced peroxisomal protein (*POX18*) gene promoter. Another example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequences of a *Candida viswanathii* HDE gene promoter and a *Candida* POX18 gene promoter are provided herein as are examples of additional fatty acid-inducible promoters. In some embodiments, the promoter is modified by replacing the endogenous promoter with a promoter comprising an alkane response element (ARE1). Non-limiting examples of assays suitable for assessing induction of phosphomevalonate kinase expression by a carbon source or feedstock include RT-PCR or qRT-PCR after the host cell or microorganism has been exposed to the chosen carbon source or feedstock for varying amounts of time.

Decreasing phosphomevalonate kinase activity in a cell can be accomplished by modifying the amount of phosphomevalonate kinase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous phosphomevalonate kinase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type phosphomevalonate kinase such that the encoded modified or substituted phosphomevalonate kinase protein has a reduced enzyme activity.

*Modification of a diphosphomevalonate decarboxylase activity*

A further step in the lower mevalonate pathway is the conversion of mevalonate 5-diphosphate (diphosphomevalonate; mevalonate-5-pyrophosphate; 5-diphosphomevalonate) to isopentenyl diphosphate. A diphosphomevalonate decarboxylase enzyme (also referred to as MVD1, ERG19, pyrophosphomevalonate decarboxylase, mevalonate-5-pyrophosphate decarboxylase, pyrophosphomevalonic acid decarboxylase, 5-pyrophosphomevalonate decarboxylase,



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mevalonate 5-diphosphate decarboxylase, and ATP:(R)-5-diphosphomevalonate carboxy-lyase (dehydrating); EC 4.1.1.33 ) can convert mevalonate 5-diphosphate to isopentenyl diphosphate through ATP dependent decarboxylation. In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of a diphosphomevalonate decarboxylase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of a diphosphomevalonate decarboxylase, may be modified to decrease the amount and/or activity of a diphosphomevalonate decarboxylase, or may be modified to alternately increase and decrease the amount and/or activity of a diphosphomevalonate decarboxylase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of a diphosphomevalonate decarboxylase in a cell is increased. Increasing the amount and/or activity of a diphosphomevalonate decarboxylase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway (e.g., isopentenyl diphosphate production, geranyl diphosphate production, farnesyl diphosphate production, geranylgeranyl diphosphate production, terpene production, carotenoid production, beta carotene production, lycopene production, astaxanthin production) and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, diphosphomevalonate decarboxylase activity is unchanged in a host or engineered cell or organism. In some embodiments, the amount and/or activity of a host diphosphomevalonate decarboxylase can be increased by increasing the number of copies of a nucleic acid encoding a diphosphomevalonate decarboxylase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid); by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a diphosphomevalonate decarboxylase; or by increasing the number of copies of a nucleic acid encoding a diphosphomevalonate decarboxylase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a diphosphomevalonate decarboxylase. In some embodiments, a diphosphomevalonate decarboxylase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of diphosphomevalonate decarboxylase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding a diphosphomevalonate decarboxylase into a

cell or microorganism. For example, introduction of heterologous nucleic acid encoding a diphosphomevalonate decarboxylase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed.

In some embodiments, a diphosphomevalonate decarboxylase enzyme can be a fungal or bacterial protein. In a particular embodiment, the diphosphomevalonate decarboxylase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*), *Fusarium* (e.g., *F. oxysporum*), *Bacillus* (e.g., *B. megaterium*, *B. subtilis*), *Chronobacter* (e.g., *C. sakazakii*), *Xanthophyllomyces* (e.g., *X. dendrorhous*), or *Pantoea* (e.g., *P. ananatis*) protein. An example of a *Candida viswanathii* nucleotide sequence (SEQ ID NO: 376) encoding a polypeptide (*MVD1* (*ERG19)*; SEQ ID NO: 342) having a diphosphomevalonate decarboxylase activity is provided herein. Additional nonlimiting examples of nucleotide sequences encoding polypeptides having diphosphomevalonate decarboxylase activity include: *Candida tropicalis MYA-3404* diphosphomevalonate decarboxylase (NCBI Reference Sequence: XM_002549937.1) and *Candida tanzawaensis NRRL Y-17324* Diphosphomevalonate decarboxylase (NCBI Reference Sequence: XM_020210701.1).

Presence, absence or amount of diphosphomevalonate decarboxylase activity can be detected by any suitable method known in the art. Nucleic acid sequences encoding native and/or modified diphosphomevalonate decarboxylase sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a nucleic acid (e.g., a heterologous nucleic acid) encoding a diphosphomevalonate decarboxylase can be modified. For example, the amount of a diphosphomevalonate decarboxylase protein expressed in a particular cellular location may be increased by including in the nucleic acid (e.g., heterologous nucleic acid) a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* oleate-induced peroxisomal protein (*POX18*) gene promoter. Another example of one

such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequences of a *Candida viswanathii* HDE gene promoter and a *Candida* POX18 gene promoter are provided herein as are examples of additional fatty acid-inducible promoters. In some embodiments, the promoter is modified by replacing the endogenous promoter with a promoter comprising an alkane response element (ARE1). Non-limiting examples of assays suitable for assessing induction of diphosphomevalonate decarboxylase expression by a carbon source or feedstock include RT-PCR or qRT-PCR after the host cell or microorganism has been exposed to the chosen carbon source or feedstock for varying amounts of time.

Decreasing diphosphomevalonate decarboxylase activity in a cell can be accomplished by modifying the amount of diphosphomevalonate decarboxylase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous diphosphomevalonate decarboxylase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type diphosphomevalonate decarboxylase such that the encoded modified or substituted diphosphomevalonate decarboxylase protein has a reduced enzyme activity.

*Modification of an isopentenyl diphosphate delta isomerase activity*

A further step in the lower mevalonate pathway is the conversion of isopentenyl diphosphate (isopentenyl pyrophosphate, IPP) to dimethylallyl diphosphate (dimethylallyl pyrophosphate, DMAPP). An isopentenyl diphosphate delta isomerase enzyme (also referred to as IDI1, isopentenyl pyrophosphate isomerase, IPP isomerase; EC 5.3.3.2) can catalyze the isomerization of isopentenyl diphosphate to dimethylallyl diphosphate by an antarafacial transposition of hydrogen. In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of an isopentenyl diphosphate delta isomerase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of an isopentenyl diphosphate delta isomerase, may be modified to decrease the amount and/or activity of an isopentenyl diphosphate delta isomerase, or may be modified to alternately increase and decrease the amount and/or activity of an isopentenyl diphosphate delta isomerase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.



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In certain aspects, the amount and/or activity of an isopentenyl diphosphate delta isomerase in a cell is increased. Increasing the amount and/or activity of an isopentenyl diphosphate delta isomerase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway (e.g., geranyl diphosphate production, farnesyl diphosphate production, geranylgeranyl diphosphate production, terpene production, carotenoid production, beta carotene production, lycopene production, astaxanthin production) and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, isopentenyl diphosphate delta isomerase activity is unchanged in a host or engineered cell or organism. In some embodiments, the amount and/or activity of a host isopentenyl diphosphate delta isomerase can be increased by increasing the number of copies of a nucleic acid encoding an isopentenyl diphosphate delta isomerase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid); by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding an isopentenyl diphosphate delta isomerase; or by increasing the number of copies of a nucleic acid encoding an isopentenyl diphosphate delta isomerase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding an isopentenyl diphosphate delta isomerase. In some embodiments, an isopentenyl diphosphate delta isomerase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of isopentenyl diphosphate delta isomerase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding an isopentenyl diphosphate delta isomerase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding an isopentenyl diphosphate delta isomerase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed.

In some embodiments, an isopentenyl diphosphate delta isomerase enzyme can be a fungal or bacterial protein. In a particular embodiment, the isopentenyl diphosphate delta isomerase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*), *Fusarium* (e.g., *F. oxysporum*), *Bacillus* (e.g., *B. megaterium*, *B. subtilis*), *Chronobacter* (e.g., *C. sakazakii*), *Xanthophyllomyces* (e.g., *X. dendrorhous*), or *Pantoea* (e.g., *P. ananatis*) protein. An example of a *Candida viswanathii* nucleotide sequence (SEQ ID NO:

377) encoding a polypeptide (*IDI1*; SEQ ID NO: 343) having an isopentenyl diphosphate delta isomerase activity is provided herein. An additional nonlimiting example of a nucleotide sequence encoding a polypeptide having isopentenyl diphosphate delta isomerase activity is a *Candida tropicalis MYA-3404* isopentenyl-diphosphate delta-isomerase (NCBI Reference Sequence: XM_002545339.1).

Presence, absence or amount of isopentenyl diphosphate delta isomerase activity can be detected by any suitable method known in the art. For example, detection can be performed by using an isopentenyl diphosphate delta isomerase activity assay (see e.g., Diaz et al. (2012) Insect Biochem. Mol. Bio. 42(10):751-757). Nucleic acid sequences encoding native and/or modified isopentenyl diphosphate delta isomerase sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a nucleic acid (e.g., a heterologous nucleic acid) encoding an isopentenyl diphosphate delta isomerase can be modified. For example, the amount of an isopentenyl diphosphate delta isomerase protein expressed in a particular cellular location may be increased by including in the nucleic acid (e.g., heterologous nucleic acid) a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* oleate-induced peroxisomal protein (*POX18*) gene promoter. Another example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequences of a *Candida viswanathii* HDE gene promoter and a *Candida* POX18 gene promoter are provided herein as are examples of additional fatty acid-inducible promoters. In some embodiments, the promoter is modified by replacing the endogenous promoter with a promoter comprising an alkane response element (ARE1). Non-limiting examples of assays suitable for assessing induction of isopentenyl diphosphate delta isomerase expression by a carbon source or feedstock include RT-PCR or qRT-PCR after the host cell or microorganism has been exposed to the chosen carbon source or feedstock for varying amounts of time.

Decreasing isopentenyl diphosphate delta isomerase activity in a cell can be accomplished by modifying the amount of isopentenyl diphosphate delta isomerase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous isopentenyl diphosphate delta isomerase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type isopentenyl diphosphate delta isomerase such that the encoded modified or substituted isopentenyl diphosphate delta isomerase protein has a reduced enzyme activity.

*Modification of a terpene synthase activity*

Provided herein are one or more modifications to one or more terpene synthase activities. Terpene synthases may include one or more enzymes in a family of enzymes involved in terpene biosynthesis, such as, for example, terpene synthases known in the art and described in Yamada et al. (2015) PNAS 112(3):857-862. In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of a terpene synthase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of a terpene synthase, may be modified to decrease the amount and/or activity of a terpene synthase, or may be modified to alternately increase and decrease the amount and/or activity of a terpene synthase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of a terpene synthase in a cell is increased. Increasing the amount and/or activity of a terpene synthase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway (e.g., mevalonate production, isopentenyl diphosphate production, geranyl diphosphate production, farnesyl diphosphate production, geranylgeranyl diphosphate production, terpene production, carotenoid production, beta carotene production, lycopene production, astaxanthin production) and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, terpene synthase activity is unchanged in a host or engineered cell or organism. In some embodiments, the amount and/or activity of a host terpene synthase can be increased by increasing the number of copies of a nucleic acid encoding a terpene synthase

(e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid); by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a terpene synthase; or by increasing the number of copies of a nucleic acid a terpene synthase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a terpene synthase. In some embodiments, a terpene synthase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of terpene synthase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding a terpene synthase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding a terpene synthase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed. In some embodiments, a terpene synthase is not endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, a terpene synthase protein can be expressed in a cell by introducing heterologous nucleic acid encoding a terpene synthase into a cell or microorganism.

In some embodiments, a terpene synthase enzyme can be a fungal or bacterial protein. In a particular embodiment, the terpene synthase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*), *Fusarium* (e.g., *F. oxysporum*), *Bacillus* (e.g., *B. megaterium*, *B. subtilis*), *Chronobacter* (e.g., *C. sakazakii*), *Xanthophyllomyces* (e.g., *X. dendrorhous*), or *Pantoea* (e.g., *P. ananatis*) protein.

Presence, absence or amount of terpene synthase activity can be detected by any suitable method known in the art. For example, detection can be performed by using an appropriate terpene synthase activity assay. Nucleic acid sequences encoding native and/or modified terpene synthase sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a nucleic acid (e.g., a heterologous nucleic acid) encoding a terpene synthase can be modified. For example, the amount of a terpene synthase protein expressed in a particular cellular location may be increased by including in the



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nucleic acid (e.g., heterologous nucleic acid) a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* oleate-induced peroxisomal protein (*POX18*) gene promoter. Another example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequences of a *Candida viswanathii* HDE gene promoter and a *Candida* POX18 gene promoter are provided herein as are examples of additional fatty acid-inducible promoters. In some embodiments, the promoter is modified by replacing the endogenous promoter with a promoter comprising an alkane response element (ARE1). Non-limiting examples of assays suitable for assessing induction of terpene synthase expression by a carbon source or feedstock include RT-PCR or qRT-PCR after the host cell or microorganism has been exposed to the chosen carbon source or feedstock for varying amounts of time.

Decreasing terpene synthase activity in a cell can be accomplished by modifying the amount of terpene synthase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous terpene synthase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type terpene synthase such that the encoded modified or substituted terpene synthase protein has a reduced enzyme activity.

*Modification of a dimethylallyltranstransferase activity*

One component of terpene biosynthesis is the conversion of dimethylallyl diphosphate (dimethylallyl pyrophosphate) and isopentenyl diphosphate (isopentenyl pyrophosphate) into farnesyl diphosphate (farnesyl pyrophosphate). A dimethylallyltranstransferase enzyme (EC 2.5.1.1) can convert dimethylallyl diphosphate and isopentenyl diphosphate into farnesyl diphosphate. Dimethylallyltranstransferase may also be referred to as ERG20, farnesyl pyrophosphate synthase, farnesyl diphosphate synthase, or farnesyl diphosphate synthetase (EC 2.5.1.10). Geranyl diphosphate (geranyl pyrophosphate) often is created in an intermediate step. In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of a dimethylallyltranstransferase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of a dimethylallyltranstransferase, may be modified to decrease the amount and/or activity of a dimethylallyltranstransferase, or may be modified to alternately increase and

decrease the amount and/or activity of a dimethylallyltranstransferase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of a dimethylallyltranstransferase in a cell is increased. Increasing the amount and/or activity of a dimethylallyltranstransferase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway (e.g., geranyl diphosphate production, farnesyl diphosphate production, geranylgeranyl diphosphate production, terpene production, carotenoid production, beta carotene production, lycopene production, astaxanthin production) and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, dimethylallyltranstransferase activity is unchanged in a host or engineered cell or organism. In some embodiments, the amount and/or activity of a host dimethylallyltranstransferase can be increased by increasing the number of copies of a nucleic acid encoding a dimethylallyltranstransferase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid); by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a dimethylallyltranstransferase; or by increasing the number of copies of a nucleic acid encoding a dimethylallyltranstransferase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a dimethylallyltranstransferase. In some embodiments, a dimethylallyltranstransferase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of dimethylallyltranstransferase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding a dimethylallyltranstransferase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding a dimethylallyltranstransferase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed. In some embodiments, a dimethylallyltranstransferase is not endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, a dimethylallyltranstransferase protein can be expressed in a cell by introducing heterologous nucleic acid encoding a dimethylallyltranstransferase into a cell or microorganism.



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In some embodiments, a dimethylallyltranstransferase enzyme can be a fungal or bacterial protein. In a particular embodiment, the dimethylallyltranstransferase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*), *Fusarium* (e.g., *F. oxysporum*), *Bacillus* (e.g., *B. megaterium*, *B. subtilis*), *Chronobacter* (e.g., *C. sakazakii*), *Xanthophyllomyces* (e.g., *X. dendrorhous*), or *Pantoea* (e.g., *P. ananatis*) protein. An example of a *Candida viswanathii* nucleotide sequence (SEQ ID NO: 378) encoding a polypeptide (*ERG20*; SEQ ID NO: 344) having a dimethylallyltranstransferase activity is provided herein. An additional nonlimiting example of a nucleotide sequence encoding a polypeptide having dimethylallyltranstransferase activity is *Candida tropicalis MYA-3404* farnesyl pyrophosphate synthetase (NCBI Reference Sequence: XM_002547254.1).

Presence, absence or amount of dimethylallyltranstransferase activity can be detected by any suitable method known in the art. For example, detection can be performed by using a dimethylallyltranstransferase ELISA kit (e.g., MYBIOSOURCE, MBS943684). Nucleic acid sequences encoding native and/or modified dimethylallyltranstransferase sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a nucleic acid (e.g., a heterologous nucleic acid) encoding a dimethylallyltranstransferase can be modified. For example, the amount of a dimethylallyltranstransferase protein expressed in a particular cellular location may be increased by including in the nucleic acid (e.g., heterologous nucleic acid) a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* oleate-induced peroxisomal protein (*POX18*) gene promoter. Another example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequences of a *Candida viswanathii* HDE gene promoter and a *Candida* POX18 gene promoter are provided herein as are examples of additional fatty acid-inducible promoters. In some embodiments, the promoter is modified by replacing the endogenous promoter with a promoter comprising an alkane response element (ARE1). Non-limiting examples of assays suitable for assessing induction of dimethylallyltranstransferase expression by a carbon source or feedstock

include RT-PCR or qRT-PCR after the host cell or microorganism has been exposed to the chosen carbon source or feedstock for varying amounts of time.

Decreasing dimethylallyltranstransferase activity in a cell can be accomplished by modifying the amount of dimethylallyltranstransferase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous dimethylallyltranstransferase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type dimethylallyltranstransferase such that the encoded modified or substituted dimethylallyltranstransferase protein has a reduced enzyme activity.

*Modification of a geranylgeranyl diphosphate synthase activity*

Another component of terpene biosynthesis is the synthesis of geranylgeranyl diphosphate from farnesyl diphosphate and isopentenyl diphosphate. A geranylgeranyl diphosphate synthase enzyme (also referred to as BTS1, CrtE, GGPS1, GGPPS, GGPPS1, geranylgeranyl diphosphate synthase 1; EC 2.5.1.29) can catalyze the synthesis of geranylgeranyl diphosphate from farnesyl diphosphate and isopentenyl diphosphate. In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of a geranylgeranyl diphosphate synthase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of a geranylgeranyl diphosphate synthase, may be modified to decrease the amount and/or activity of geranylgeranyl diphosphate synthase, or may be modified to alternately increase and decrease the amount and/or activity of an geranylgeranyl diphosphate synthase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of a geranylgeranyl diphosphate synthase in a cell is increased. Increasing the amount and/or activity of a geranylgeranyl diphosphate synthase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway (e.g., mevalonate production, isopentenyl diphosphate production, geranyl diphosphate production, farnesyl diphosphate production, geranylgeranyl diphosphate production, terpene production, carotenoid production, beta



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carotene production, lycopene production, astaxanthin production) and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, geranylgeranyl diphosphate synthase activity is unchanged in a host or engineered cell or organism. In some embodiments, the amount and/or activity of a host geranylgeranyl diphosphate synthase can be increased by increasing the number of copies of a nucleic acid encoding a geranylgeranyl diphosphate synthase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid); by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a geranylgeranyl diphosphate synthase; or by increasing the number of copies of a nucleic acid encoding a geranylgeranyl diphosphate synthase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a geranylgeranyl diphosphate synthase. In some embodiments, a geranylgeranyl diphosphate synthase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of geranylgeranyl diphosphate synthase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding a geranylgeranyl diphosphate synthase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding a geranylgeranyl diphosphate synthase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed. In some embodiments, a geranylgeranyl diphosphate synthase is not endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, a geranylgeranyl diphosphate synthase protein can be expressed in a cell by introducing heterologous nucleic acid encoding a geranylgeranyl diphosphate synthase into a cell or microorganism.

In some embodiments, a geranylgeranyl diphosphate synthase enzyme can be a fungal or bacterial protein. In a particular embodiment, the geranylgeranyl diphosphate synthase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*), *Fusarium* (e.g., *F. oxysporum*), *Bacillus* (e.g., *B. megaterium*, *B. subtilis*), *Chronobacter* (e.g., *C. sakazakii*), *Xanthophyllomyces* (e.g., *X. dendrorhous*), or *Pantoea* (e.g., *P. ananatis*) protein. An example of a *Candida viswanathii* nucleotide sequence (SEQ ID NO: 379) encoding a polypeptide (*BTS1*; SEQ ID NO: 345) having a geranylgeranyl diphosphate synthase activity is provided herein. Additional nonlimiting examples of nucleotide sequences encoding polypeptides having geranylgeranyl diphosphate synthase activity are provided herein which include: *Chronobacter sakazakii CrtE* gene (SEQ ID NO:357), which encodes *CsCrtE*

(SEQ ID NO:326); *Xanthophyllomyces dendrorhous CrtE* gene (SEQ ID NO: 360, which encodes *XdCrtE* (SEQ ID NO:329); and *Pantoea ananatis CrtE* gene (SEQ ID NO: 363), which encodes *PaCrtE* (SEQ ID NO:332).

Presence, absence or amount of geranylgeranyl diphosphate synthase activity can be detected by any suitable method known in the art. For example, detection can be performed by using a geranylgeranyl diphosphate synthase ELISA kit (e.g., MYBIOSOURCE, MBS929545). Nucleic acid sequences encoding native and/or modified geranylgeranyl diphosphate synthase sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a nucleic acid (e.g., a heterologous nucleic acid) encoding a geranylgeranyl diphosphate synthase can be modified. For example, the amount of a geranylgeranyl diphosphate synthase protein expressed in a particular cellular location may be increased by including in the nucleic acid (e.g., heterologous nucleic acid) a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* oleate-induced peroxisomal protein (*POX18*) gene promoter. Another example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequences of a *Candida viswanathii* HDE gene promoter and a *Candida* POX18 gene promoter are provided herein as are examples of additional fatty acid-inducible promoters. In some embodiments, the promoter is modified by replacing the endogenous promoter with a promoter comprising an alkane response element (ARE1). Non-limiting examples of assays suitable for assessing induction of geranylgeranyl diphosphate synthase expression by a carbon source or feedstock include RT-PCR or qRT-PCR after the host cell or microorganism has been exposed to the chosen carbon source or feedstock for varying amounts of time.

Decreasing geranylgeranyl diphosphate synthase activity in a cell can be accomplished by modifying the amount of geranylgeranyl diphosphate synthase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous geranylgeranyl diphosphate



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synthase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type geranylgeranyl diphosphate synthase such that the encoded modified or substituted geranylgeranyl diphosphate synthase protein has a reduced enzyme activity.

*Modification of a phytoene synthase activity*

Another component of terpene biosynthesis is the conversion of geranylgeranyl diphosphate (geranylgeranyl pyrophosphate) to phytoene. A phytoene synthase enzyme (also referred to as CrtB, prephytoene-diphosphate synthase, 15-cis-phytoene synthase, PSase, geranylgeranyl-diphosphate geranylgeranyltransferase; EC 2.5.1.32) is a transferase enzyme that can catalyze the conversion of geranylgeranyl diphosphate to phytoene. In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of a phytoene synthase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of a phytoene synthase, may be modified to decrease the amount and/or activity of a phytoene synthase, or may be modified to alternately increase and decrease the amount and/or activity of a phytoene synthase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of a phytoene synthase in a cell is increased. Increasing the amount and/or activity of a phytoene synthase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway (e.g., terpene production, carotenoid production, beta carotene production, lycopene production, astaxanthin production) and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, phytoene synthase activity is unchanged in a host or engineered cell or organism. In some embodiments, the amount and/or activity of a host phytoene synthase can be increased by increasing the number of copies of a nucleic acid encoding a phytoene synthase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid); by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a phytoene synthase; or by increasing the number of copies of a nucleic acid encoding a phytoene synthase and increasing the activity of a promoter that regulates transcription of a



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nucleic acid encoding a phytoene synthase. In some embodiments, a phytoene synthase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of phytoene synthase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding a phytoene synthase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding a phytoene synthase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed. In some embodiments, a phytoene synthase is not endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, a phytoene synthase protein can be expressed in a cell by introducing heterologous nucleic acid encoding a phytoene synthase into a cell or microorganism.

In some embodiments, a phytoene synthase enzyme can be a fungal or bacterial protein. In a particular embodiment, the phytoene synthase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*), *Fusarium* (e.g., *F. oxysporum*), *Bacillus* (e.g., *B. megaterium*, *B. subtilis*), *Chronobacter* (e.g., *C. sakazakii*), *Xanthophyllomyces* (e.g., *X. dendrorhous*), or *Pantoea* (e.g., *P. ananatis*) protein. An example of a *Chronobacter sakazakii* nucleotide sequence (SEQ ID NO: 359) encoding a polypeptide (*CsCrtB*; SEQ ID NO: 328) having a phytoene synthase activity is provided herein. An example of a *Pantoea ananatis* nucleotide sequence (SEQ ID NO: 365) encoding a polypeptide (*PaCrtB*; SEQ ID NO: 334) having a phytoene synthase activity is provided herein.

Presence, absence or amount of phytoene synthase activity can be detected by any suitable method known in the art. For example, detection can be performed by using phytoene synthase activity assay (see e.g., Lopez-Emparan et al. (2014) PLoS One 9(12):e114878; Schledz et al. (1996) The Plant Journal 10(5):781-792). Nucleic acid sequences encoding native and/or modified phytoene synthase sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.



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The promoter used for regulating transcription of a nucleic acid (e.g., a heterologous nucleic acid) encoding a phytoene synthase can be modified. For example, the amount of a phytoene synthase protein expressed in a particular cellular location may be increased by including in the nucleic acid (e.g., heterologous nucleic acid) a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* oleate-induced peroxisomal protein (*POX18*) gene promoter. Another example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequences of a *Candida viswanathii* HDE gene promoter and a *Candida* POX18 gene promoter are provided herein as are examples of additional fatty acid-inducible promoters. In some embodiments, the promoter is modified by replacing the endogenous promoter with a promoter comprising an alkane response element (ARE1). Non-limiting examples of assays suitable for assessing induction of phytoene synthase expression by a carbon source or feedstock include RT-PCR or qRT-PCR after the host cell or microorganism has been exposed to the chosen carbon source or feedstock for varying amounts of time.

Decreasing phytoene synthase activity in a cell can be accomplished by modifying the amount of phytoene synthase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous phytoene synthase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type phytoene synthase such that the encoded modified or substituted phytoene synthase protein has a reduced enzyme activity.

*Modification of a phytoene desaturase activity*

Another component of terpene biosynthesis is the conversion of phytoene to lycopene. A phytoene desaturase enzyme can catalyze the conversion of phytoene to lycopene. In some embodiments, a phytoene desaturase is a 3,4-didehydrolycopene-forming phytoene desaturase (also referred to as 5-step phytoene desaturase, five-step phytoene desaturase, Al-1, 15-cis-phytoene:acceptor oxidoreductase (3,4-didehydrolycopene-forming); EC 1.3.99.30). In some embodiments, a phytoene desaturase is a neurosporene-forming phytoene desaturase (also referred to as CrtI, 3-step phytoene desaturase, three-step phytoene desaturase, 15-cis-phytoene:acceptor oxidoreductase (neurosporene-forming); EC 1.3.99.28). In some embodiments of the cells, organisms, compositions and methods provided herein, the amount



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and/or activity of a phytoene desaturase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of a phytoene desaturase, may be modified to decrease the amount and/or activity of a phytoene desaturase, or may be modified to alternately increase and decrease the amount and/or activity of a phytoene desaturase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of a phytoene desaturase in a cell is increased. Increasing the amount and/or activity of a phytoene desaturase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway (e.g., terpene production, carotenoid production, beta carotene production, lycopene production, astaxanthin production) and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, phytoene desaturase activity is unchanged in a host or engineered cell or organism. In some embodiments, the amount and/or activity of a host phytoene desaturase can be increased by increasing the number of copies of a nucleic acid encoding a phytoene desaturase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid); by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a phytoene desaturase; or by increasing the number of copies of a nucleic acid encoding a phytoene desaturase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a phytoene desaturase. In some embodiments, a phytoene desaturase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of phytoene desaturase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding a phytoene desaturase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding a phytoene desaturase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed. In some embodiments, a phytoene desaturase is not endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, a phytoene desaturase protein can be expressed in a cell by introducing heterologous nucleic acid encoding a phytoene desaturase into a cell or microorganism.



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In some embodiments, a phytoene desaturase enzyme can be a fungal or bacterial protein. In a particular embodiment, the phytoene desaturase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*), *Fusarium* (e.g., *F. oxysporum*), *Bacillus* (e.g., *B. megaterium*, *B. subtilis*), *Chronobacter* (e.g., *C. sakazakii*), *Xanthophyllomyces* (e.g., *X. dendrorhous*), or *Pantoea* (e.g., *P. ananatis*) protein. An example of a *Chronobacter sakazakii* nucleotide sequence (SEQ ID NO: 358) encoding a polypeptide (*CsCrtI*; SEQ ID NO: 327) having a phytoene desaturase activity is provided herein. An example of a *Xanthophyllomyces dendrorhous* nucleotide sequence (SEQ ID NO: 361) encoding a polypeptide (*XdCrtI*; SEQ ID NO: 330) having a phytoene desaturase activity is provided herein. An example of a *Pantoea ananatis* nucleotide sequence (SEQ ID NO: 364) encoding a polypeptide (*PaCrtI*; SEQ ID NO: 333) having a phytoene desaturase activity is provided herein.

Presence, absence or amount of phytoene desaturase activity can be detected by any suitable method known in the art. For example, detection can be performed by using a phytoene desaturase activity assay (see e.g., Xu et al. (2007) Microbiology 153:1642-52). Nucleic acid sequences encoding native and/or modified phytoene desaturase sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a nucleic acid (e.g., a heterologous nucleic acid) encoding a phytoene desaturase can be modified. For example, the amount of a phytoene desaturase protein expressed in a particular cellular location may be increased by including in the nucleic acid (e.g., heterologous nucleic acid) a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* oleate-induced peroxisomal protein (*POX18*) gene promoter. Another example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequences of a *Candida viswanathii* HDE gene promoter and a *Candida* POX18 gene promoter are provided herein as are examples of additional fatty acid-inducible promoters. In some embodiments, the promoter is modified by replacing the endogenous promoter with a promoter

comprising an alkane response element (ARE1). Non-limiting examples of assays suitable for assessing induction of phytoene desaturase expression by a carbon source or feedstock include RT-PCR or qRT-PCR after the host cell or microorganism has been exposed to the chosen carbon source or feedstock for varying amounts of time.

Decreasing phytoene desaturase activity in a cell can be accomplished by modifying the amount of phytoene desaturase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous phytoene desaturase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type phytoene desaturase such that the encoded modified or substituted phytoene desaturase protein has a reduced enzyme activity.

*Modification of a lycopene cyclase activity and/or modification of a bifunctional lycopene cyclase/phytoene synthase activity*

Another component of terpene biosynthesis is the conversion of lycopene to beta carotene. A lycopene cyclase enzyme (also referred to as lycopene beta-cyclase, CrtY, CrtL (beta-ionone end group producing), CrtL (eta-ionone end group producing) and CrtL (capsanthin/capsorubin synthase); EC 5.5.1.19) can catalyze the conversion of lycopene to beta carotene. The cyclization of lycopene is typically the final step in carotenoid biosynthesis and may proceed via one of two pathways: the formation of a beta ring by beta-cyclase, or an epsilon ring by epsilon-cyclase. Epsilon-cyclase adds one ring, forming a monocyclic delta-carotene, and beta-cyclase introduces a ring at both ends of lycopene to form a bicyclic beta-carotene. In some embodiments, a lycopene cyclase is a bifunctional lycopene cyclase/phytoene synthase (also referred to as CrtYB), which includes a lycopene beta-cyclase domain (EC 5.5.1.19) and a phytoene synthase domain (EC 2.5.1.32). A bifunctional lycopene cyclase can catalyze the reaction from geranylgeranyl diphosphate to phytoene (phytoene synthase) and the reaction from lycopene to beta-carotene via an intermediate gamma-carotene (lycopene cyclase). A bifunctional enzyme containing lycopene cyclase activity and phytoene synthase activity may be referred to herein as a bifunctional lycopene cyclase/phytoene synthase or a bifunctional lycopene cyclase.

In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of a lycopene cyclase in a cell is modified. For example, in some



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aspects, a cell or microorganism may be modified to increase the amount and/or activity of a lycopene cyclase, may be modified to decrease the amount and/or activity of a lycopene cyclase, or may be modified to alternately increase and decrease the amount and/or activity of a lycopene cyclase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of a bifunctional lycopene cyclase/phytoene synthase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of a bifunctional lycopene cyclase/phytoene synthase, may be modified to decrease the amount and/or activity of a bifunctional lycopene cyclase/phytoene synthase, or may be modified to alternately increase and decrease the amount and/or activity of a bifunctional lycopene cyclase/phytoene synthase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of a lycopene cyclase in a cell is increased. Increasing the amount and/or activity of a lycopene cyclase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway (e.g., terpene production, carotenoid production, beta carotene production, astaxanthin production) and away from other cellular metabolic pathways not involved in target molecule production.

In certain aspects, the amount and/or activity of a bifunctional lycopene cyclase/phytoene synthase in a cell is increased. Increasing the amount and/or activity of a bifunctional lycopene cyclase/phytoene synthase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway (e.g., terpene production, carotenoid production, beta carotene production, astaxanthin production) and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, lycopene cyclase activity is unchanged in a host or engineered cell or organism. In some embodiments, the amount and/or activity of a host lycopene cyclase can be increased by increasing the number of copies of a nucleic acid encoding a lycopene cyclase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid); by increasing the

activity of a promoter that regulates transcription of a nucleic acid encoding a lycopene cyclase; or by increasing the number of copies of a nucleic acid encoding a lycopene cyclase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a lycopene cyclase. In some embodiments, a lycopene cyclase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of lycopene cyclase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding a lycopene cyclase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding a lycopene cyclase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed. In some embodiments, a lycopene cyclase is not endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, a lycopene cyclase protein can be expressed in a cell by introducing heterologous nucleic acid encoding a lycopene cyclase into a cell or microorganism.

In certain embodiments, bifunctional lycopene cyclase/phytoene synthase activity is unchanged in a host or engineered cell or organism. In some embodiments, the amount and/or activity of a host bifunctional lycopene cyclase/phytoene synthase can be increased by increasing the number of copies of a nucleic acid encoding a bifunctional lycopene cyclase/phytoene synthase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid); by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a bifunctional lycopene cyclase/phytoene synthase; or by increasing the number of copies of a nucleic acid encoding a bifunctional lycopene cyclase/phytoene synthase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a bifunctional lycopene cyclase/phytoene synthase. In some embodiments, a bifunctional lycopene cyclase/phytoene synthase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of bifunctional lycopene cyclase/phytoene synthase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding a bifunctional lycopene cyclase/phytoene synthase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding a bifunctional lycopene cyclase/phytoene synthase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed. In some embodiments, a bifunctional lycopene cyclase/phytoene synthase is not endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, a bifunctional lycopene cyclase/phytoene synthase protein can be expressed in a cell by



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introducing heterologous nucleic acid encoding a bifunctional lycopene cyclase/phytoene synthase into a cell or microorganism.

In some embodiments, a lycopene cyclase enzyme and/or a bifunctional lycopene cyclase/phytoene synthase enzyme can be a fungal or bacterial protein. In a particular embodiment, the lycopene cyclase enzyme and/or the bifunctional lycopene cyclase/phytoene synthase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*), *Fusarium* (e.g., *F. oxysporum*), *Bacillus* (e.g., *B. megaterium*, *B. subtilis*), *Chronobacter* (e.g., *C. sakazakii*), *Xanthophyllomyces* (e.g., *X. dendrorhous*), or *Pantoea* (e.g., *P. ananatis*) protein. An example of a *Xanthophyllomyces dendrorhous* nucleotide sequence (SEQ ID NO: 362) encoding a polypeptide (*XdCrtYB*; SEQ ID NO: 331) having a bifunctional lycopene cyclase/phytoene synthase activity is provided herein.

Presence, absence or amount of lycopene cyclase activity and/or bifunctional lycopene cyclase/phytoene synthase activity can be detected by any suitable method known in the art. For example, detection can be performed by using a lycopene cyclase activity assay (see e.g., Yu et al. (2010) J. Biol. Chem. 285(16):12109-12120). Nucleic acid sequences encoding native and/or modified lycopene cyclase sequences and/or bifunctional lycopene cyclase/phytoene synthase sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a nucleic acid (e.g., a heterologous nucleic acid) encoding a lycopene cyclase and/or a bifunctional lycopene cyclase/phytoene synthase can be modified. For example, the amount of a lycopene cyclase protein and/or a bifunctional lycopene cyclase/phytoene synthase protein expressed in a particular cellular location may be increased by including in the nucleic acid (e.g., heterologous nucleic acid) a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* oleate-induced peroxisomal protein (*POX18*) gene promoter. Another example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequences of a *Candida viswanathii* HDE gene promoter and a *Candida* POX18

gene promoter are provided herein as are examples of additional fatty acid-inducible promoters. In some embodiments, the promoter is modified by replacing the endogenous promoter with a promoter comprising an alkane response element (ARE1). Non-limiting examples of assays suitable for assessing induction of lycopene cyclase expression and/or a bifunctional lycopene cyclase/phytoene synthase expression by a carbon source or feedstock include RT-PCR or qRT-PCR after the host cell or microorganism has been exposed to the chosen carbon source or feedstock for varying amounts of time.

Decreasing lycopene cyclase activity in a cell can be accomplished by modifying the amount of lycopene cyclase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous lycopene cyclase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type lycopene cyclase such that the encoded modified or substituted lycopene cyclase protein has a reduced enzyme activity.

Decreasing bifunctional lycopene cyclase/phytoene synthase activity in a cell can be accomplished by modifying the amount of bifunctional lycopene cyclase/phytoene synthase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous bifunctional lycopene cyclase/phytoene synthase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type bifunctional lycopene cyclase/phytoene synthase such that the encoded modified or substituted bifunctional lycopene cyclase/phytoene synthase protein has a reduced enzyme activity.

*Modification of a beta carotene ketolase activity*

Additional components of terpene biosynthesis include, for example, astaxanthin biosysnthesis (e.g., the conversion of beta carotene to astaxanthin), canthaxanthin biosynthesis, and zeaxanthin biosynthesis. Canthaxanthin, for example, may be produced using a beta carotene ketolase enzyme (also referred to as CrtW, beta carotene monoketolase, beta-carotene oxygenase; EC 1.3.5.B4). Astaxanthin, for example, may be produced using a beta carotene ketolase enzyme in conjunction with a beta carotene hydroxylase (discussed below). In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of a beta carotene ketolase in a cell is modified. For example, in some aspects, a

cell or microorganism may be modified to increase the amount and/or activity of a beta carotene ketolase, may be modified to decrease the amount and/or activity of a beta carotene ketolase, or may be modified to alternately increase and decrease the amount and/or activity of a beta carotene ketolase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of a beta carotene ketolase in a cell is increased. Increasing the amount and/or activity of a beta carotene ketolase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway (e.g., terpene production, carotenoid production, astaxanthin production) and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments beta carotene ketolase activity is unchanged in a host or engineered cell or organism. In some embodiments, the amount and/or activity of a host beta carotene ketolase can be increased by increasing the number of copies of a nucleic acid encoding an beta carotene ketolase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid); by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a beta carotene ketolase; or by increasing the number of copies of a nucleic acid encoding a beta carotene ketolase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a beta carotene ketolase. In some embodiments, a beta carotene ketolase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of beta carotene ketolase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding a beta carotene ketolase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding a beta carotene ketolase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed. In some embodiments, a beta carotene ketolase is not endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, a beta carotene ketolase protein can be expressed in a cell by introducing heterologous nucleic acid encoding a beta carotene ketolase into a cell or microorganism.

In some embodiments, a beta carotene ketolase enzyme can be a fungal or bacterial protein. In a particular embodiment, the beta carotene ketolase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*), *Fusarium* (e.g.,

*F. oxysporum*), *Bacillus* (e.g., *B. megaterium*, *B. subtilis*), *Chronobacter* (e.g., *C. sakazakii*), *Xanthophyllomyces* (e.g., *X. dendrorhous*), *Pantoea* (e.g., *P. ananatis*), or *Agrobacterium* (e.g., *A. aurantiacum*) protein. An example of an *Agrobacterium aurantiacum* nucleotide sequence (SEQ ID NO: 369) encoding a polypeptide (*AaCrtW*; SEQ ID NO: 351) having a beta carotene ketolase activity is provided herein.

Presence, absence or amount of beta carotene ketolase activity can be detected by any suitable method known in the art. For example, detection can be performed by using an appropriate beta carotene ketolase activity assay. Nucleic acid sequences encoding native and/or modified beta carotene ketolase sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a nucleic acid (e.g., a heterologous nucleic acid) encoding a beta carotene ketolase can be modified. For example, the amount of a beta carotene ketolase protein expressed in a particular cellular location may be increased by including in the nucleic acid (e.g., heterologous nucleic acid) a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* oleate-induced peroxisomal protein (*POX18*) gene promoter. Another example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequences of a *Candida viswanathii* HDE gene promoter and a *Candida* POX18 gene promoter are provided herein as are examples of additional fatty acid-inducible promoters. In some embodiments, the promoter is modified by replacing the endogenous promoter with a promoter comprising an alkane response element (ARE1). Non-limiting examples of assays suitable for assessing induction of beta carotene ketolase expression by a carbon source or feedstock include RT-PCR or qRT-PCR after the host cell or microorganism has been exposed to the chosen carbon source or feedstock for varying amounts of time.

Decreasing beta carotene ketolase activity in a cell can be accomplished by modifying the amount of beta carotene ketolase protein expression in the cell, for example, by replacing the

wild-type promoter of an endogenous beta carotene ketolase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type beta carotene ketolase such that the encoded modified or substituted beta carotene ketolase protein has a reduced enzyme activity.

*Modification of a beta carotene hydroxylase activity*

In certain instances, astaxanthin and/or zeaxanthin may be produced using a beta carotene hydroxylase enzyme (also referred to as CrtZ, beta-carotene 3-hydroxylase, beta-carotene 3,3'-monooxygenase, beta-carotene, NADH:oxygen 3-oxidoreductase; EC 1.14.13.129). In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of a beta carotene hydroxylase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of a beta carotene hydroxylase, may be modified to decrease the amount and/or activity of a beta carotene hydroxylase, or may be modified to alternately increase and decrease the amount and/or activity of a beta carotene hydroxylase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of a beta carotene hydroxylase in a cell is increased. Increasing the amount and/or activity of a beta carotene hydroxylase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway (e.g., terpene production, carotenoid production, astaxanthin production) and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, beta carotene hydroxylase activity is unchanged in a host or engineered cell or organism. In some embodiments, the amount and/or activity of a host beta carotene hydroxylase can be increased by increasing the number of copies of a nucleic acid encoding a beta carotene hydroxylase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid); by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a beta carotene hydroxylase; or by increasing the number of copies of a nucleic acid encoding a beta carotene hydroxylase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a beta carotene hydroxylase. In some embodiments, a beta carotene hydroxylase is endogenous to the host cell or microorganism. In

one aspect of the cell or microbial systems and methods provided herein, the amount of beta carotene hydroxylase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding a beta carotene hydroxylase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding a beta carotene hydroxylase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed. In some embodiments, a beta carotene hydroxylase is not endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, a beta carotene hydroxylase protein can be expressed in a cell by introducing heterologous nucleic acid encoding a beta carotene hydroxylase into a cell or microorganism.

In some embodiments, a beta carotene hydroxylase enzyme can be a fungal or bacterial protein. In a particular embodiment, the beta carotene hydroxylase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*), *Fusarium* (e.g., *F. oxysporum*), *Bacillus* (e.g., *B. megaterium*, *B. subtilis*), *Chronobacter* (e.g., *C. sakazakii*), *Xanthophyllomyces* (e.g., *X. dendrorhous*), *Pantoea* (e.g., *P. ananatis*) or *Agrobacterium* (e.g., *A. aurantiacum*) protein. An example of an *Agrobacterium aurantiacum* nucleotide sequence (SEQ ID NO: 368) encoding a polypeptide (*AaCrtZ*; SEQ ID NO: 350) having a beta carotene hydroxylase activity is provided herein.

Presence, absence or amount of beta carotene hydroxylase activity can be detected by any suitable method known in the art. For example, detection can be performed by using an appropriate beta carotene hydroxylase activity assay. Nucleic acid sequences encoding native and/or modified beta carotene hydroxylase sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a nucleic acid (e.g., a heterologous nucleic acid) encoding a beta carotene hydroxylase can be modified. For example, the amount of a beta carotene hydroxylase protein expressed in a particular cellular location may be increased by including in the nucleic acid (e.g., heterologous nucleic acid) a strong heterologous promoter

and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* oleate-induced peroxisomal protein (*POX18*) gene promoter. Another example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequences of a *Candida viswanathii* HDE gene promoter and a *Candida* POX18 gene promoter are provided herein as are examples of additional fatty acid-inducible promoters. In some embodiments, the promoter is modified by replacing the endogenous promoter with a promoter comprising an alkane response element (ARE1). Non-limiting examples of assays suitable for assessing induction of beta carotene hydroxylase expression by a carbon source or feedstock include RT-PCR or qRT-PCR after the host cell or microorganism has been exposed to the chosen carbon source or feedstock for varying amounts of time.

Decreasing beta carotene hydroxylase activity in a cell can be accomplished by modifying the amount of beta carotene hydroxylase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous beta carotene hydroxylase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type beta carotene hydroxylase such that the encoded modified or substituted beta carotene hydroxylase protein has a reduced enzyme activity.

*Modification of an astaxanthin synthase activity*

In certain instances, astaxanthin may be produced using an astaxanthin synthase enzyme (also referred to as CrtS, astaxanthin synthetase, cytochrome-450 hydroxylase, cytochrome-P450 hydroxylase/astaxanthin synthase). In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of an astaxanthin synthase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of an astaxanthin synthase, may be modified to decrease the amount and/or activity of an astaxanthin synthase, or may be modified to alternately increase and decrease the amount and/or activity of an astaxanthin synthase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.



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In certain aspects, the amount and/or activity of an astaxanthin synthase in a cell is increased. Increasing the amount and/or activity of an astaxanthin synthase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway (e.g., terpene production, carotenoid production, astaxanthin production) and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, astaxanthin synthase activity is unchanged in a host or engineered cell or organism. In some embodiments, the amount and/or activity of a host astaxanthin synthase can be increased by increasing the number of copies of a nucleic acid encoding an astaxanthin synthase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid); by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding an astaxanthin synthase; or by increasing the number of copies of a nucleic acid encoding an astaxanthin synthase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding an astaxanthin synthase. In some embodiments, an astaxanthin synthase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of astaxanthin synthase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding an astaxanthin synthase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding an astaxanthin synthase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed. In some embodiments, a astaxanthin synthase is not endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, a astaxanthin synthase protein can be expressed in a cell by introducing heterologous nucleic acid encoding a astaxanthin synthase into a cell or microorganism.

In some embodiments, an astaxanthin synthase enzyme can be a fungal or bacterial protein. In a particular embodiment, the astaxanthin synthase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*), *Fusarium* (e.g., *F. oxysporum*), *Bacillus* (e.g., *B. megaterium*, *B. subtilis*), *Chronobacter* (e.g., *C. sakazakii*), *Xanthophyllomyces* (e.g., *X. dendrorhous*), or *Pantoea* (e.g., *P. ananatis*) protein. An example of a *Xanthophyllomyces dendrorhous* nucleotide sequence (SEQ ID NO: 367) encoding a polypeptide (*XdCrtS*; SEQ ID NO: 336) having an astaxanthin synthase activity is provided herein.

Presence, absence or amount astaxanthin synthase activity can be detected by any suitable method known in the art. For example, detection can be performed by using an appropriate astaxanthin synthase activity assay. Nucleic acid sequences encoding native and/or modified astaxanthin synthase sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a nucleic acid (e.g., a heterologous nucleic acid) encoding an astaxanthin synthase can be modified. For example, the amount of an astaxanthin synthase protein expressed in a particular cellular location may be increased by including in the nucleic acid (e.g., heterologous nucleic acid) a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* oleate-induced peroxisomal protein (*POX18*) gene promoter. Another example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequences of a *Candida viswanathii* HDE gene promoter and a *Candida* POX18 gene promoter are provided herein as are examples of additional fatty acid-inducible promoters. In some embodiments, the promoter is modified by replacing the endogenous promoter with a promoter comprising an alkane response element (ARE1). Non-limiting examples of assays suitable for assessing induction of astaxanthin synthase expression by a carbon source or feedstock include RT-PCR or qRT-PCR after the host cell or microorganism has been exposed to the chosen carbon source or feedstock for varying amounts of time.

Decreasing astaxanthin synthase activity in a cell can be accomplished by modifying the amount of astaxanthin synthase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous astaxanthin synthase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type astaxanthin synthase such that the encoded modified or substituted astaxanthin synthase protein has a reduced enzyme activity.



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*Modification of a zeaxanthin glucosyltransferase activity*

Another component of terpene biosynthesis is the conversion of zeaxanthin to zeaxanthin diglucoside. A zeaxanthin glucosyltransferase enzyme (also referred to as CrtX, UDP-glucose:zeaxanthin beta-D-glucosyltransferase; EC 2.4.1.276) can catalyze the conversion of zeaxanthin to zeaxanthin diglucoside. In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of a zeaxanthin glucosyltransferase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of a zeaxanthin glucosyltransferase, may be modified to decrease the amount and/or activity of a zeaxanthin glucosyltransferase, or may be modified to alternately increase and decrease the amount and/or activity of a zeaxanthin glucosyltransferase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of a zeaxanthin glucosyltransferase in a cell is increased. Increasing the amount and/or activity of a zeaxanthin glucosyltransferase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway (e.g., terpene production, carotenoid production, xanthophyll production, zeaxanthin diglucoside production), and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, zeaxanthin glucosyltransferase activity is unchanged in a host or engineered cell or organism. In some embodiments, the amount and/or activity of a host zeaxanthin glucosyltransferase can be increased by increasing the number of copies of a nucleic acid encoding a zeaxanthin glucosyltransferase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid); by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a zeaxanthin glucosyltransferase; or by increasing the number of copies of a nucleic acid encoding a zeaxanthin glucosyltransferase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a zeaxanthin glucosyltransferase. In some embodiments, a zeaxanthin glucosyltransferase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of zeaxanthin glucosyltransferase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding a zeaxanthin glucosyltransferase into a cell or microorganism. For example, introduction of heterologous



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nucleic acid encoding a zeaxanthin glucosyltransferase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed. In some embodiments, a zeaxanthin glucosyltransferase is not endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, a zeaxanthin glucosyltransferase protein can be expressed in a cell by introducing heterologous nucleic acid encoding a zeaxanthin glucosyltransferase into a cell or microorganism.

In some embodiments, a zeaxanthin glucosyltransferase enzyme can be a fungal or bacterial protein. In a particular embodiment, the zeaxanthin glucosyltransferase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*), *Fusarium* (e.g., *F. oxysporum*), *Bacillus* (e.g., *B. megaterium*, *B. subtilis*), *Chronobacter* (e.g., *C. sakazakii*), *Xanthophyllomyces* (e.g., *X. dendrorhous*), or *Pantoea* (e.g., *P. ananatis*) protein.

Presence, absence or amount of zeaxanthin glucosyltransferase activity can be detected by any suitable method known in the art. For example, detection can be performed by using an appropriate zeaxanthin glucosyltransferase activity assay. Nucleic acid sequences encoding native and/or modified zeaxanthin glucosyltransferase sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a nucleic acid (e.g., a heterologous nucleic acid) encoding a zeaxanthin glucosyltransferase can be modified. For example, the amount of a zeaxanthin glucosyltransferase protein expressed in a particular cellular location may be increased by including in the nucleic acid (e.g., heterologous nucleic acid) a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* oleate-induced peroxisomal protein (*POX18*) gene promoter. Another example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequences of a *Candida viswanathii* HDE gene promoter and a *Candida* POX18 gene promoter are provided herein as are examples of additional fatty acid-inducible promoters.

In some embodiments, the promoter is modified by replacing the endogenous promoter with a promoter comprising an alkane response element (ARE1). Non-limiting examples of assays suitable for assessing induction of zeaxanthin glucosyltransferase expression by a carbon source or feedstock include RT-PCR or qRT-PCR after the host cell or microorganism has been exposed to the chosen carbon source or feedstock for varying amounts of time.

Decreasing zeaxanthin glucosyltransferase activity in a cell can be accomplished by modifying the amount of zeaxanthin glucosyltransferase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous zeaxanthin glucosyltransferase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type zeaxanthin glucosyltransferase such that the encoded modified or substituted zeaxanthin glucosyltransferase protein has a reduced enzyme activity.

*Modification of a valencene synthase activity*

Another component of terpene biosynthesis is the synthesis of valencene. A valencene synthase enzyme (also referred to as TPS1, (2E,6E)-farnesyl-diphosphate diphosphate-lyase (valencene-forming); EC 4.2.3.73) can catalyze the conversion of farnesyl diphosphate (farnesyl pyrophosphate) to valencene. In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of a valencene synthase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of a valencene synthase, may be modified to decrease the amount and/or activity of a valencene synthase, or may be modified to alternately increase and decrease the amount and/or activity of a valencene synthase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of a valencene synthase in a cell is increased. Increasing the amount and/or activity of a valencene synthase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway (e.g., terpene production, valencene production, nootkatone production) and away from other cellular metabolic pathways not involved in target molecule production.



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In certain embodiments, valencene synthase activity is unchanged in a host or engineered cell or organism. In some embodiments, the amount and/or activity of a host valencene synthase can be increased by increasing the number of copies of a nucleic acid encoding a valencene synthase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid); by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a valencene synthase; or by increasing the number of copies of a nucleic acid encoding a valencene synthase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a valencene synthase. In some embodiments, a valencene synthase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount valencene synthase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding a valencene synthase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding a valencene synthase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed. In some embodiments, a valencene synthase is not endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, a valencene synthase protein can be expressed in a cell by introducing heterologous nucleic acid encoding a valencene synthase into a cell or microorganism.

In some embodiments, a valencene synthase enzyme can be a fungal or bacterial protein. In a particular embodiment, the valencene synthase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*), *Fusarium* (e.g., *F. oxysporum*), *Bacillus* (e.g., *B. megaterium*, *B. subtilis*), *Chronobacter* (e.g., *C. sakazakii*), *Xanthophyllomyces* (e.g., *X. dendrorhous*), *Pantoea* (e.g., *P. ananatis*), or *Callitropsis* (e.g., *C. nootkatensis*) protein. An example of a *Callitropsis nootkatensis* nucleotide sequence (SEQ ID NO: 370) encoding a polypeptide (*TPS1*; SEQ ID NO: 352) having a valencene synthase activity is provided herein.

Presence, absence or amount of valencene synthase activity can be detected by any suitable method known in the art. For example, detection can be performed by using an appropriate valencene synthase activity assay. Nucleic acid sequences encoding native and/or modified valencene synthase sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot



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analysis, the like and combinations thereof), where the engineered cell or organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a nucleic acid (e.g., a heterologous nucleic acid) encoding a valencene synthase can be modified. For example, the amount of a valencene synthase protein expressed in a particular cellular location may be increased by including in the nucleic acid (e.g., heterologous nucleic acid) a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* oleate-induced peroxisomal protein (*POX18*) gene promoter. Another example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequences of a *Candida viswanathii* HDE gene promoter and a *Candida* POX18 gene promoter are provided herein as are examples of additional fatty acid-inducible promoters. In some embodiments, the promoter is modified by replacing the endogenous promoter with a promoter comprising an alkane response element (ARE1). Non-limiting examples of assays suitable for assessing induction of valencene synthase expression by a carbon source or feedstock include RT-PCR or qRT-PCR after the host cell or microorganism has been exposed to the chosen carbon source or feedstock for varying amounts of time.

Decreasing valencene synthase activity in a cell can be accomplished by modifying the amount of valencene synthase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous valencene synthase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type valencene synthase such that the encoded modified or substituted valencene synthase protein has a reduced enzyme activity.

*Modification of RAS2 activity*

RAS2 is a guanine nucleotide-binding protein that can be activated by binding GTP (e.g., in the presence of glucose). In some embodiments of the microorganisms, compositions and methods provided herein, the amount and/or activity of RAS2 in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase RAS2 and/or RAS2 activity, may be modified to decrease RAS2 and/or RAS2 activity, or may be modified to alternately increase



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and decrease RAS2 and/or RAS2 activity depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of a RAS2 in a cell is decreased. Reducing or eliminating the amount and/or activity of a RAS2 may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway and away from other cellular metabolic pathways involving activated fatty acids. In certain embodiments, an endogenous microbial gene encoding RAS2 (e.g., yeast *RAS2* gene) can be disrupted or deleted in a host microorganism to reduce or eliminate RAS2 activity in the host relative to a microorganism in which the gene has not been modified. Methods for decreasing the amount and/or activity of RAS2 in a cell include, but are not limited to, modifying the amount of RAS2 protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous RAS2 gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting one or both copies of an endogenous gene, and/or replacing or modifying a gene encoding a wild-type RAS2 such that the encoded modified or substituted RAS2 protein has a reduced enzyme activity. For example, expression of a host RAS2 activity can be decreased or eliminated by disruption (e.g., knockout, insertion mutagenesis, the like and combinations thereof) of a host gene encoding the protein, or by decreasing the activity of the promoter (e.g., through addition of repressor sequences to the promoter or 5'UTR or replacing the promoter) that controls transcription of a RAS2 gene using recombinant molecular biology techniques known in the art and/or described herein. In one embodiment, a diploid yeast, such as, for example, a *Candida* yeast, when used as a host microorganism can be subjected to genetic modification in which one of the two alleles of a RAS2 gene is disrupted or deleted. In so doing, a single allele of the gene remains for a reduced amount of RAS2 expression in the microorganism and a reduced amount of the protein in the cell.

One method for disrupting an endogenous RAS2 gene is by recombinantly inserting a heterologous nucleic acid (e.g., a nucleotide sequence encoding a selectable marker such as an enzyme that restores an auxotrophic host organism to prototrophy) into the endogenous gene, thereby generating an engineered organism deficient in RAS2 activity. This can be done, for example, through homologous recombination in which a heterologous nucleic acid containing sequences of an endogenous RAS2 gene and a disrupting sequence (e.g., a knock-out gene cassette such as described herein) is introduced into a host cell or microorganism. Nucleic acids encoding a RAS2 can be obtained from a number of sources, including, for

example, yeast cells. Genomic DNA from cell sources can be amplified using oligonucleotide primers based on the nucleotide sequence of a RAS2 encoding gene, including examples provided herein.

Presence, absence or amount of RAS2 activity can be detected by any suitable method known in the art. For example, detection can be performed by using an appropriate RAS2 activity assay (e.g., enzymatic assays, PCR based assays (e.g., qPCR, RT-PCR), immunological detection methods (e.g., antibodies specific for RAS2), the like and combinations thereof). Nucleic acid sequences encoding native and/or modified RAS2 sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

*Carbon-containing products*

Certain organic acids and polyketides are chemical intermediates in manufacturing processes used to make polyamides, polyurethanes and plasticizers, all of which have wide applications in producing items such as antiseptics, carpets, elastomers, food packaging, lubricants, top-grade coatings, hot-melt coating and adhesives, painting materials, corrosion inhibitor, surfactant, engineering plastics and can also be used as a starting material in the manufacture of fragrances. Some large-scale synthetic processes for making organic acids and polyketides include the use of noxious chemicals and/or solvents, some require high temperatures, and all require significant energy input. In addition, some of the processes emit toxic byproducts (such as nitrous oxide) and give rise to environmental concerns. Furthermore, chemical synthesis and extraction of desirable chemical compounds, such as terpenes, for example, from natural sources yields low product levels and is often not economically feasible. Provided herein are methods for producing organic acid and other organic chemical intermediate target molecules using biological systems provided herein. Such production systems may have significantly less environmental impact and could be economically competitive with synthetic manufacturing systems.



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*Organic Acids*

Examples of organic acid target molecules that can be produced using compositions and methods provided herein include, but are not limited to, fatty acids, diacids and β-hydroxy acids (e.g., hydroxyalkanoate monomers) and salts and esters thereof. Fatty acids generally tend to be aliphatic acids of varying carbon chain lengths. Naturally occurring fatty acids in biological systems generally contain an even number of carbon atoms, typically between about 12 to about 24, or about 14 to about 24, and most commonly, 16 or 18 carbon atoms. Based on the number of carbons in a fatty acid carbon chain, it can be categorized as a short-, medium- or long-chain fatty acid. Generally, short-chain fatty acids have a chain length of about 2 to about 6 carbon atoms, medium-chain fatty acids have a chain length of about 8 to about 10 carbon atoms, long-chain fatty acids have a chain length of about 12 to about 20 carbon atoms and very long-chain length fatty acids have a chain length of about 22 or about 24 or more carbon atoms. The carbon atom bonds in the alkyl chain may all be single bonds (i.e., a saturated fatty acid) or may contain one or more double bonds (i.e., an unsaturated fatty acid). Unsaturated fatty acids having one double bond are also referred to as monoenoic; unsaturated fatty acids having two or more double bonds in the carbon chain are also referred to as polyenoic and polyunsaturated (PUFA). The carbon chain in a fatty acid may also be substituted with hydroxyl, methyl, or other groups in place of a hydrogen. Carboxylic acids, such as fatty acids, can partially dissociate in aqueous media and exist as undissociated, uncharged molecules and as a dissociated, anionic form.

Fatty acids containing one carboxyl group can also be referred to as monocarboxylic fatty acids. A fatty acid containing two carboxyl groups (e.g., α,ω-dicarboxylic acids) is a fatty dicarboxylic acid, also referred to herein as a diacid. An example of a diacid is adipic acid (hexanedioic acid) which contains six carbon atoms. A diacid sometimes is a C4 to a C24 diacid (i.e., a diacid containing 4 carbons to 24 carbons) and sometimes is a C8, C10, C12, C14, C16, C18, or C20 diacid. Diacids can contain an even as well as an odd number (e.g., C5, C7, C9, C11, C13, C15, C17, C19, C21 or C23) of carbons. A hydrocarbon portion of a diacid sometimes is fully saturated and sometimes a diacid includes one or more unsaturations (e.g., double bonds).

Non-limiting examples of diacids include octadecanedioic acid, decanedioic acid, dodecanedioic acid, tetradecanedioic acid, hexadecanedioic acid, octadecanedioic acid, eicosanedioic acid and other organic intermediates using biological systems. Non-limiting examples of fatty

dicarboxylic acids include adipic acid (hexanedioic acid, 1,4-butanedicarboxylic acid), suberic acid (i.e., octanedioic acid, 1,8-octanedioic acid, octanedioic acid, octane-1,8-dioic acid, 1,6-hexanedicarboxylic acid, capryllic diacids), azelaic acid, sebacic acid (i.e., 1,10-decanedioic acid, decanedioic acid, decane-1,10-dioic acid, 1,8-octanedicarboxylic acid, capric diacid), undecanedioc acid, dodecanedioic acid (i.e., DDDA, 1,12-dodecanedioic acid, dodecanedioic acid, dodecane-1,12-dioic acid, 1,10-decanedicarboxylic acid, decamethylenedicaboxylic acid, 1,10-dicarboxydecane, lauric diacid), tetradecanedioic acid (i.e., TDDA, 1,14-tetradecanedioic acid, tetradecanedioic acid, tetradecane-1,14-dioic acid, 1,12-dodecanedicarboxylic acid, myristic diacid), thapsic acid (i.e., hexadecanedioic acid, 1,16-hexadecanedioic acid, hexadecanedioic acid, hexadecane-1,16-dioic acid, 1,14-tetradecanedicarboxylic acid, palmitic diacid), cis-9-hexadecenedioic acid (i.e., palmitoleic diacids), octanedioic acid (i.e., 1,18-octadecanedioic acid, octadecanedioic acid, octadecane-1,18-dioic acid, 1,16-hexadecanedicarboxylic acid, stearic diacid), cis-9-octadecenedioic acid (i.e., oleic diacids), cis-9,12-octadecenedioic acid (i.e., linoleic diacids), cis-9,12,15-octadecenedioic acid (i.e., linolenic diacids), arachidic diacid (i.e., eicosanoic diacid, icosanoic diacid), 11-eicosenoic diacid (i.e., cis-11-eicosenedioic acid), 13-eicosenoic diacids (i.e., cis-13-eicosenedioic acid), arachidonic diacid (i.e., cis-5,8,11,14-eicosatetraenedioic acid) and salts and esters of fatty acids, including, for example, any of the foregoing diacids.

Adipic acid and suberic acid are 6- and 8-carbon dicarboxylic acids, respectively, that are chemical intermediates in manufacturing processes used to make certain polyamides, polyurethanes and plasticizers. Azelaic acid, a 9-carbon dicarboxylic acid, is also used in therapeutic compositions due to its antibacterial and keratolytic activities. Sebacic acid, a 10-carbon dicarboxylic acid, is also used in cosmetics and candles and as an intermediate in producing aromatics and antiseptics. Dodecandioic acid (DDDA), a 12-carbon dicarboxylic acid, is widely used in forming polyamides, such as nylon. Some large-scale industrial processes for making adipic acid include (i) liquid phase oxidation of ketone alcohol oil (KA oil); (ii) air oxidation/hydration of cyclohexane with boric acid to make cyclohexanol, followed by oxidation with nitric acid; and (iii) hydrocyanation of butadiene to a pentenenitrile mixture, followed by hydroisomerization of adiponitrile, followed by hydrogenation. Suberic acid can be synthetically manufactured by oxidation of cyclooctene with ozone oxygen or ozone $H_2O_2$. Methods of chemical synthesis of sebacic acid include alkaline cleavage of ricinoleic acid and electrolytic dimerization of monomethyl adipate. DDDA is synthetically produced from butadiene in a

multistep chemical process. These energy-requiring processes involve the use and/or production of toxic chemicals and/or solvents.

3-hydroxypropionic acid (3-HP or 3HP, used interchangeably herein, which collectively refers to 3-hydroxypropionic acid, a 3-hydroxypropionate salt or ester thereof, or mixtures thereof in any proportion) is a platform chemical that can be converted into a variety of valuable products such as poly(hydroxypropionate), 1,3-propanediol, ethyl 3-ethoxypropionate (EEP), malonic acid and acrylic acid. For example, 3-HP can be dehydrated to produce acrylic acid, which in turn can be esterified to produce methyl acrylate or aminated to produce acrylamide. Acrylamide can further be converted by dehydration to acrylonitrile, acrylonitrile can be condensed to produce adiponitrile and adiponitrile can be hydrolysed to produce hexamethylenediamine (HMDA). In addition, polymerized acrylic acid (with itself or with other monomers such as acrylamide, acrylonitrile, vinyl, styrene, or butadiene) can produce a variety of homopolymers and copolymers that are used in the manufacture of various plastics, coatings, adhesives, elastomers, latex applications, emulsions, leather finishings, and paper coating, as well as floor polishes and paints. Acrylic acid also can be used as a chemical intermediate for the production of acrylic esters such as ethyl acrylate, butyl acrylate, methyl acrylate, and 2-ethyl hexyl acrylate and superabsorbent polymers (glacial acrylic acid).

*Polyketides*

Polyketides are secondary metabolites polymerized from short-chain carboxylic acid units (e.g., acetate, proprionate, malonate and butyrate). Many polyketide-derived molecules are valuable pharmaceuticals such as antibiotics, antitumor agents and cholesterol-lowering drugs. Non-limiting examples of polyketides include triacetic acid lactone (TAL or 4-hydroxy-6-methyl-2-pyrone, used interchangeably herein) and 6-methylsalicylic acid (6-MSA or 2-hydroxy-6-methylbenzoic acid). TAL can be converted into end products such as sorbic acid and 1,3-pentadiene, and can serve as a precursor in the synthesis of compounds (e.g, phloroglucinol and resorcinol) used in production of resin and adhesive formulations.

*Terpenes*

Terpenes are made up of units of isoprene ($C_5H_8$) (isoprene, methylbuta-1,3-diene, hemiterpene are used interchangeably herein) that can be joined together in a variety of different

combinations to generate thousands of terpene compounds. Terpenes can be categorized according to the number of isoprene units contained in the molecule: monoterpenes (2 isoprene units), sesquiterpenes (3 isoprene units), diterpenes (4 isoprene units), sesterterpenes (5 isoprene units), triterpenes (6 isoprene units), sesquaterpenes (7 isoprene units), tetraterpenes (8 isoprene units), polyterpenes (many isoprene units). The isoprene units can be joined "head-to-tail" in a linear chain or arranged in rings. Terpenes can be hydrocarbons or can contain other atoms, such as oxygen (e.g., alcohols, aldehydes and ketones) which are typically referred to as terpenoids. Terpenes are commercially valuable compounds with a variety of uses in the healthcare, food, cosmetics and chemical industries, including, but not limited to, uses as pharmaceuticals (e.g., anticancer and antimalarial drugs), nutraceuticals, supplements, antioxidants, fragrances, flavoring agents, food colorants and agricultural pest control agents. Included in the tetraterpenes are organic pigments (e.g., β-carotene and astaxanthin) referred to as carotenoids. These terpenes have many uses such as, for example, additives in food and feed stocks, precursors to vitamin A, antioxidants and supplements (e.g., lutein and lycopene).

*Cellular carbon flux*

Cells can obtain carbon atoms from external carbon sources such as, for example, carbohydrates, hydrocarbons, acids and alcohols. Upon entering the cell, the source molecule is metabolized through various chemical reactions depending on the carbon source, as well as other factors (e.g., oxygen and nutrient availability). Carbon atoms flow or flux through these metabolic reactions and are utilized in generating energy and in the production of cellular materials. The multiple series of coordinated reactions involved in metabolizing different carbon sources for different purposes are referred to as metabolic pathways and can be catabolic or anabolic. In catabolic pathways, the carbon source is broken down through oxidative reactions in which electrons are removed from substrates or intermediates, and, in the process, energy is generated and stored as adenosine triphosphate (ATP). Glycolysis is an example of a catabolic pathway in which a carbohydrate carbon source (e.g., glucose) is converted to pyruvate which is oxidatively decarboxylated to acetyl-CoA by the pyruvate dehydrogenase multi-enzyme complex in the mitochondria of eukaryotic cells and in the cytosol of prokaryotic cells. In this series of reactions, a multi-carbon source molecule is degraded into a 2-carbon acetyl group and carbon dioxide. The 2 carbons of the acetyl group are then incorporated into a citrate molecule in the tricarboxylic acid cycle (also referred to as the TCA, citric acid and Krebs cycle) in which additional ATP molecules are generated. Intermediates in the TCA cycle (e.g., citrate, α-

ketoglutarate, succinyl-CoA and oxaloacetate) provide precursors in the synthesis of essential cellular components such as amino acids, fatty acids, nucleotides and porphyrins. The TCA cycle is considered an amphibolic pathway which combines both catabolic and anabolic functions. In another catabolic pathway, β-oxidation, a fatty acid carbon source is broken down into acetyl-CoA and chain-shortened acyl-CoA which in turn can enter another cycle of β-oxidation for further degradation. The acetyl-CoA molecules generated in β-oxidation, which occurs in peroxisomes in eukaryotic microorganisms and the cytosol of prokaryotic cells, are then utilized in ATP generation in the TCA cycle.

Acetyl-CoA generated through β-oxidation in microorganisms (e.g., yeast and bacteria) can also be used in the glyoxylate cycle, which is an anabolic pathway wherein 2-carbon acetyl units are converted to 4-carbon molecules that can be used for the biosynthesis of macromolecules. The glyoxylate cycle thus allows these microorganisms to utilize non-fermentable carbon sources, such as fatty acids, acetate and ethanol, as a sole carbon source. In the glyoxylate cycle, which is similar to the TCA cycle, isocitrate is cleaved directly into the 4-carbon succinate molecule, and the 2-carbon glyoxylate molecule, through the enzyme isocitrate lyase without the two decarboxylation steps that occur in the same conversion in the TCA cycle. Glyoxylate then condenses with acetyl-CoA generated through β-oxidation to produce malate which in turn is converted to oxaloacetate and then isocitrate. Succinate generated in the glyoxylate cycle can also reenter the TCA cycle to produce oxaloacetate. Malate and oxaloacetate produced in the glyoxylate cycle can be converted into phosphoenolpyruvate, which is the product of the first enzyme-catalyzed reaction in gluconeogenesis. Gluconeogenesis is another anabolic pathway and provides for synthesis of carbohydrates when non-carbohydrate carbon sources are available to cells. Microorganisms growing on non-fermentable carbon sources utilize gluconeogenesis to synthesize glucose-6-phosphate which is used in the synthesis of ribonucleotides and deoxyribonucleotides. The carbon skeletons for generation of glucose-6-phosphate are contained within oxaloacetate from the glyoxylate and TCA cycles. In gluconeogenesis, oxaloacetate is converted into pyruvate through phosphoenolpyruvate carboxykinase, followed by several reactions that ultimately yield glucose-6-phosphate.

Additional anabolic pathways in cells include reactions in the synthesis of lipids, including, for example, triacylglycerols (referred to interchangeably as triglycerides and TAG) and phospholipids. Lipids are a diverse group of compounds that are soluble in non-polar organic solvents but not in water. Fatty acids serve as building blocks in the synthesis of storage lipids

(e.g., triacylglycerols and steryl esters) and membrane lipids (e.g., phospholipids and sphingolipids). For example, triacylglycerol is an ester of glycerol and three fatty acids. In the synthesis of triacylglycerols from free fatty acids internalized into microbial cells from the environment, the fatty acids are first activated with coenzyme A to form an acyl-CoA. The acyl-CoA is involved in two pathways of triacylglycerol synthesis: the glycerol-3-phosphate (G3P) pathway and the dihydroxyacetone phosphate (DHAP) pathway. Both pathways proceed through formation of phosphatidic acid and subsequently diacylglycerol which is then acetylated to form triacylglycerol. Phosphatidic acid can also be converted to cytidine diphosphate-diacylglycerol which is the precursor of all major phospholipids in cells.

*Modification of Cellular Carbon Flux*

The multiple routes of carbon metabolism in cells provide opportunities for loss of carbon from a production pathway for desired organic molecules in a cell. Such losses can result in decreased product yields, increased production times and costs, and overall decreased production process efficiency and economy. Cells, organisms and microorganisms and methods described herein provide systems for enhanced production of target molecules. In one aspect, production is enhanced through alteration of carbon flux in cell-based and microbial production systems. Through alteration of cellular carbon flux, carbon atoms that may have flowed or been transported into other metabolic processes (e.g., energy and/or cellular composition generation) in a cell are redirected and/or recycled and made available for use in organic target molecule production processes. In so doing, starting material loss is reduced and carbon sources are utilized to a fuller extent in the production of the desired molecules.

*Acetyl coenzyme A*

Acetyl coenzyme A (acetyl-CoA; used interchangeably herein) is a major precursor in cell-based or microbial production of many industrially important chemicals. The fatty acid biosynthesis pathway begins with the conversion of acetyl-CoA to malonyl-CoA. Similarly, organic acids, such as, for example 3-hydroxypropionic acid, and polyketides, such as triacetic acid lactone, can be synthesized using acetyl-CoA as a starting material. Additional high-value products that can be synthesized in reactions beginning with acetyl-CoA include terpenes, which can be generated from isopentenyl diphosphate produced through microbial mevalonate pathways in cells. Acetyl-CoA is formed from an acetyl group and coenzyme A (a derivative of pantothenate



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and cysteine) which are linked through a thioester bond. Acetyl-CoA is a central metabolite in carbon metabolism. It is the final carbon form resulting from the catabolism of external carbon sources and is the initial precursor carbon form in many of the cellular anabolic pathways and energy generation processes. Acetyl-CoA is formed in multiple locations of a eukaryotic cell depending on the metabolic pathway and/or carbon source through which it is generated. For example, acetyl-CoA generated through glycolysis is localized in the mitochondria, whereas acetyl-CoA generated through peroxisomal β-oxidation is localized to peroxisomes. Acetyl-CoA generated through metabolism of acetate or ethanol is localized to the cytoplasm. Typically, acetyl-CoA formed in any of these cellular locations is transferred to the mitochondrial matrix for use in the TCA cycle for the generation of energy and precursors of cellular constituents, although some acetyl-CoA localized to the cytoplasm can be used in the synthesis of oxaloacetate via initial conversion to malate.

Acetyl-CoA, due to its chemical nature, cannot freely cross biological membranes. Therefore, acetyl-CoA formed in peroxisomes and the cytoplasm is modified for transport to the mitochondria. The primary mechanism for transfer of acetyl-CoA into the mitochondria in eukaryotic cells is the carnitine shuttle in which the acetyl group of acetyl-CoA is reversibly linked to a carrier molecule, carnitine, which is able to traverse biological membranes. Acetyl-carnitine is generated and degraded by the action of carnitine acetyltransferases (e.g., EC 2.3.1.7). Peroxisomal acetyl-CoA not destined for the glyoxylate cycle is converted to acetyl-carnitine by carnitine O-acetyltransferase. Due to its smaller size compared to acetyl-CoA, acetyl-carnitine is able to diffuse through pores in the peroxisomal membrane, across the cytoplasm to mitochondria where it is converted back to acetyl-CoA by mitochondrial carnitine O-acetyltransferase. Yeast also have carnitine acetyltransferases that localize to the cytosol and/or to the outer mitochondrial membrane which, in some species, are encoded by *YAT1* genes. The enzymes encoded by these genes may convert cytosolic acetyl-carnitine to acetyl-CoA and carnitine.

Acetyl-carnitine uptake into mitochondria involves an acetyl-carnitine translocase which, in some yeast species, is encoded by a *CRC1* gene. Mitochondria possess two membranes with the outer membrane allowing free diffusion of metabolites and the inner membrane controlling metabolite transport with multiple membrane transport proteins. A mitochondrial inner-membrane transport protein (e.g., Crc1p) may function as an acetyl-carnitine transporter providing for transport of acetyl-carnitine into the mitochondrial matrix.



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Certain aspects of the cells, microorganisms, compositions and methods provided herein involve cellular carbon flux modifications to capture the carbon atoms in the acetyl group of acetyl-CoA formed in cellular metabolism. In some embodiments, carbon flux is modified to capture acetyl group carbon atoms generated in organelles (e.g., peroxisomes) or membranes as they are transported through the cytosol in the form of acetyl-carnitine. In other embodiments, acetyl group carbons of organelle-generated acetyl-CoA are re-directed from the carnitine-assisted transport system and toward conversion to acetate. Acetate, unlike acetyl-CoA, is able to traverse membranes and enter the cytosol from organelles. In further embodiments, carbon atoms of acetyl groups in mitochondrial acetyl-CoA can also be captured from intermediates of the TCA cycle that move into the cytosol.

*Acetyl-carnitine capture/conversion*

Included in embodiments of the cells, microorganisms, compositions and methods provided herein are cell-based or microbial production platform systems and components thereof in which the amount of (a) acetyl-carnitine in the cell cytosol is modified and/or (b) carnitine acetyltransferase and/or carnitine acetyltransferase activity in the cell cytosol is/are modified. In some instances, the amount of (a) acetyl-carnitine in the cell cytosol is increased and/or decreased and/or (b) carnitine acetyltransferase and/or carnitine acetyltransferase activity in the cell cytosol is/are increased and/or decreased. For example, in some aspects, a cell or microorganism may be modified to increase cytosolic acetyl-carnitine, may be modified to decrease cytosolic acetyl-carnitine or may be modified to alternately increase and decrease cytosolic acetyl-carnitine depending on the conditions in which the modified cell or microorganism is cultured.

In one embodiment, the amount of acetyl group carbons in the cytosol in the form of acetyl-carnitine in transit from the peroxisome and other areas to the mitochondria is increased in a cell or microorganism through a reduction in, and/or slowing of, the entry of acetyl-carnitine into mitochondria from the cytosol. This provides an increased availability of substrate for cytosolic carnitine acetyltransferase to convert to acetyl-CoA, and effectively results in an increase in the generation of cytosolic acetyl-CoA. Some of the acetyl group carbons are thereby diverted from the mitochondria, and from utilization in metabolic processes therein, and are instead retained in the cytosol. In some embodiments, the amount and/or activity of carnitine acetyltransferase in



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the cytosol of a cell or microorganism is/are increased. This provides an increased conversion of acetyl-carnitine, such as that which is in transit from the peroxisome to the mitochondria, into acetyl-CoA in the cytosol. In some embodiments, the entry of acetyl-carnitine into mitochondria from the cytosol is reduced in a cell or microorganism, and the amount and/or activity of carnitine acetyltransferase in the cytosol of the cell or microorganism is/are increased.

*Acetate capture/conversion*

Included in embodiments of the microorganisms, compositions and methods provided herein are microbial production platform systems and components thereof in which acetyl group carbons of organelle-generated acetyl-CoA are directed toward conversion to acetate. Modification of carbon flux in this manner provides for a tight and precise control of the movement of the acetyl carbons because acetate may pass through some membranes, e.g., peroxisomal membranes, more readily than other membranes, e.g., mitochondrial inner membranes. Therefore, carbon atoms captured in the form of cytosolic acetate will be less readily transported into mitochondria for further metabolism, unlike carbon atoms captured in the form of cytosolic acetyl-carnitine. Provided herein are cells, microorganisms, compositions and methods in which cellular carbon flux has been modified through the altered de novo generation of cellular acetate. In particular embodiments, cellular carbon flux has been modified to increase the production of acetate in a cell and/or a particular cellular location. For example, in certain aspects, cells are modified to increase the production of acetate in peroxisomes. In some embodiments, acetyl group carbons are directed toward conversion to acetate and away from the carnitine-carrier transport system.

In embodiments in which modification of cellular acetate generation yields increased amounts of cytosolic acetate, the amount and/or activity of cytosolic acetyl-CoA synthetase (also referred to as ACS or acetate-CoA ligase and used interchangeably herein) can also be increased to provide for increased conversion of acetate to acetyl-CoA. For example, the genomic copy number of nucleic acids encoding acetyl-CoA synthetase can be increased and/or the promoter for the acetyl-CoA synthetase-encoding nucleic acid can be replaced with a stronger promoter or one that provides for a different pattern of expression in the cell or microorganism.



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*Citrate capture/conversion*

Carbon atoms of acetyl groups in mitochondrial acetyl-CoA can also be captured from intermediates of the TCA cycle such as, for example, citrate molecules generated in the first step of the cycle through the citrate synthase-catalyzed condensation of acetyl-CoA and oxaloacetate. In another embodiment of the cell and microbial production systems and methods provided herein, carbon atoms incorporated into citrate that has been transferred to the cytosol are captured through the cleavage of citrate to oxaloacetate and acetyl-CoA by the enzyme ATP citrate lyase (i.e., ACL, used interchangeably herein). The capture of metabolite carbon in this manner diverts it from use in other metabolic processes and also results in an increase in the level cytoplasmic acetyl-CoA.

*Acyl coenzyme A*

Acyl-CoA synthetases (e.g., EC 6.2.1.3) are enzymes that catalyze the activation of free fatty acids in the cytoplasm into CoA esters (fatty acyl-CoA) which are involved in several metabolic pathways. For example, free fatty acids internalized into cells that become activated with coenzyme A to form an acyl-CoA are used in the synthesis of triacylglycerols via two pathways: the glycerol-3-phosphate (G3P) pathway and the dihydroxyacetone phosphate (DHAP) pathway. When free fatty acids are activated and used in cellular processes, such as lipid biosynthesis, the carbon atoms in the free fatty acids are not available for use in cell or microbial production of commercially important chemicals. Certain aspects of the cells, microorganisms, compositions and methods provided herein include one or more modifications to reduce or eliminate cytosolic activation of free fatty acids into acyl-CoA. An example of a modification to reduce or eliminate activation of cytosolic free fatty acids is reduction or elimination of the amount and/or activity of acyl-CoA synthetase in the cytoplasm.

*Malonyl-CoA*

Malonyl-CoA is a coenzyme A derivative of the dicarboxylic acid malonic acid that can serve as a precursor in the synthesis of numerous valuable organic molecules, including fatty acids and polyketides. Cytoplasmic acetyl-CoA can be converted to malonyl-CoA by the enzyme acetyl-CoA carboxylase (e.g., EC 6.4.1.2). A modification of cellular carbon flux that increases cytosolic acetyl-CoA alone may not be optimal for enhancing fatty acid or other organic acid



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production in an engineered cell or microbial system if there is not a concurrent increase in conversion of acetyl-CoA to malonyl-CoA. To maximize production efficiencies, included in the cells, microorganisms, compositions and methods provided herein are cellular carbon flux modifications that increase the amount of cytosolic malonyl-CoA.

*Cells and organisms*

Provided herein are modified cells and organisms. In particular embodiments, the modified cells and organisms have been manipulated in ways designed to alter the cellular flux of carbon to direct carbon atoms toward one or more biochemical events or cellular locations and/or away from other metabolic pathways or locations. Also provided herein are methods of producing modified cells and organisms.

*Host cells and organisms*

Modified cells and organisms provided herein can be generated by manipulation of an existing cell or organism. The terms "host," "starting" or "parental" as used herein in reference to a cell or organism refers to such an existing cell or organism. Host cells and organisms include, for example, wild-type or native cells or organisms as they occur in nature in their genetically unmodified, predominant form, and mutant cells or organisms that have one or more genetic differences compared to a wild-type cell or organism. A host cell or organism can also be a cell or organism that has been genetically modified. A host cell or organism thus serves as a reference cell or organism with respect to a modified or engineered cell or organism obtained by manipulation of a host. Organisms or cells that can be used as host organisms or cells, or as a source for a nucleic acid, are publicly available, from, for example, American Type Culture Collection (Manassas, Virginia), Thermo Fisher Scientific (Waltham, MA) and Agricultural Research Culture Collection (NRRL; Peoria, Illinois).

Host or modified organisms include multicellular and single cell, or unicellular, organisms. Microscopic organisms, referred to interchangeably herein as a "microorganism," "microbial cell" or "microbe," are an example of a host or modified organism and are included in the term "organism." Many microorganisms are unicellular and often are capable of dividing and proliferating. Cells from non-microbial organisms can also be utilized as a host or modified organism or source for a heterologous polynucleotide.

Organisms can be prokaryotic (e.g., bacteria) and non-prokaryotic (e.g., eukaryotic). Examples of eukaryotic organisms include yeast, filamentous fungi, protists, plants, algae and amoeba. An organism or microorganism can include one or more of the following features: aerobe, anaerobe, filamentous, non-filamentous, monoploid, haploid, diploid, oleaginous, non-oleaginous, auxotrophic and/or non-auxotrophic.

Host cells or organisms or modified cells or organisms can be selected based on a variety of criteria depending, for example, on the methods of generating modified cells or organisms therefrom and the uses of the modified cells or organisms from which they are derived. Selection criteria can include inherent metabolic mechanisms, suitability for genetic manipulation, adaptability to a variety of or particular growth or culture conditions, and ease of large-scale maintenance for use in industrial production processes. For example, microorganisms often can be cultured at cell densities useful for industrial production of a target product, including in a fermentation device. Included among microorganisms that may be selected as a host or modified organism or source for a heterologous polynucleotide are fungi. Examples of fungi include, but are not limited to, yeast, *Aspergillus* fungi (e.g., *A. parasiticus*, *A. nidulans*), *Thraustochytrium* fungi, *Schizochytrium* fungi and *Rhizopus* fungi (e.g., *R. arrhizus*, *R. oryzae*, *R. nigricans*). In some embodiments, a host organism can be a fungus such as a yeast strain, an *A. parasiticus* strain that includes, but is not limited to, strain ATCC 24690, and in certain embodiments, a fungus is an *A. nidulans* strain that includes, but is not limited to, strain ATCC 38163.

In some embodiments, a modified cell or microorganism provided herein can be derived from any one of the following cell lines: ATCC 20362, ATCC 8862, ATCC 18944, ATCC 20228, ATCC 76982, LGAM S(7)1, ATCC 20336, ATCC 20913, SU-2 (ura3-/ura3-), ATCC 20962, ATCC 24690, ATCC 38164, ATCC 38163, H5343, ATCC 8661, ATCC 8662, ATCC 9773, ATCC 15586, ATCC 16617, ATCC 16618, ATCC 18942, ATCC 18943, ATCC 18944, ATCC 18945, ATCC 20114, ATCC 20177, ATCC 20182, ATCC 20225, ATCC 20226, ATCC 20228, ATCC 20237, ATCC 20255, ATCC 20287, ATCC 20297, ATCC 20306, ATCC 20315, ATCC 20320, ATCC 20324, ATCC 20341, ATCC 20346, ATCC 20348, ATCC 20362, ATCC 20363, ATCC 20364, ATCC 20372, ATCC 20373, ATCC 20383, ATCC 20390, ATCC 20400, ATCC 20460, ATCC 20461, ATCC 20462, ATCC 20496, ATCC 20510, ATCC 20628, ATCC 20688, ATCC 20774, ATCC 20775, ATCC 20776, ATCC 20777, ATCC 20778, ATCC 20779, ATCC

20780, ATCC 20781, ATCC 20794, ATCC 20795, ATCC 20875, ATCC 22421, ATCC 22422, ATCC 22423, ATCC 22969, ATCC 32338, ATCC 32339, ATCC 32340, ATCC 32341, ATCC 32342, ATCC 32343, ATCC 32935, ATCC 34017, ATCC 34018, ATCC 34088, ATCC 34922, ATCC 38295, ATCC 42281, ATCC 44601, ATCC 46025, ATCC 46026, ATCC 46027, ATCC 46028, ATCC 46067, ATCC 46068, ATCC 46069, ATCC 46070, ATCC 46330, ATCC 46482, ATCC 46483, ATCC 46484, ATCC 48436, ATCC 60594, ATCC 62385, ATCC 64042, ATCC 74234, ATCC 76598, ATCC 76861, ATCC 76862, ATCC 90716, ATCC 90806, ATCC 90811, ATCC 90812, ATCC 90813, ATCC 90814, ATCC 90903, ATCC 90904, ATCC 90905, ATCC 96028, ATCC 201089, ATCC 201241, ATCC 201242, ATCC 201243, ATCC 201244, ATCC 201245, ATCC 201246, ATCC 201247, ATCC 201248, ATCC 201249, ATCC 201847, ATCC MYA-165, ATCC MYA-166, ATCC MYA-2613, and ATCC MYA-4467. That is, in certain embodiments, an engineered cell or microorganism described herein can be generated from one or more of the aforementioned ancestral cell lines.

Yeast that can serve as a host organism, and that can be modified organisms, include, but are not limited to, ascomycetes, non-Saccharomyces ascomycetes, and basidiomycetes. Non-limiting examples of yeast include *Yarrowia* yeast (e.g., *Y. lipolytica* (formerly classified as *Candida lipolytica*)), *Candida* yeast (e.g., *C. revkaufi*, *C. viswanathii*, *C. pulcherrima*, *C. tropicalis*, *C. utilis*), *Blastobotrys* (formerly classified as *Arxula*) (e.g., *Blastobotrys adeninivorans* (formerly classified as *Arxula adeninivorans*), *Blastobotrys mokoenaii*), *Rhodotorula* yeast (e.g., *R. glutinus*, *R. graminis*), *Rhodosporidium* yeast (e.g., *R. toruloides*), *Saccharomyces* yeast (e.g., *S. cerevisiae*, *S. bayanus*, *S. pastorianus*, *S. carlsbergensis*), *Cryptococcus* yeast, *Trichosporon* yeast (e.g., *T. pullans*, *T. cutaneum*), *Pichia* yeast (e.g., *P. pastoris*) and *Lipomyces* yeast (e.g., *L. starkeyii*, *L. lipoferus*). In some embodiments, a suitable yeast is of the genus *Arachniotus*, *Aspergillus*, *Aureobasidium*, *Auxarthron*, *Blastobotrys*, *Blastomyces*, *Candida*, *Chrysosporuim*, *Debaryomyces*, *Coccidiodes*, *Cryptococcus*, *Gymnoascus*, *Hansenula*, *Histoplasma*, *Issatchenkia, Kluyveromyces*, *Lipomyces*, *Lssatchenkia*, *Microsporum*, *Myxotrichum*, *Myxozyma*, *Oidiodendron*, *Pachysolen*, *Penicillium*, *Pichia*, *Rhodosporidium*, *Rhodotorula*, *Saccharomyces*, *Schizosaccharomyces*, *Scopulariopsis*, *Sepedonium*, *Trichosporon*, or *Yarrowia*. In some embodiments, a suitable yeast is of the species *Arachniotus flavoluteus*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aureobasidium pullulans*, *Auxarthron thaxteri*, *Blastobotrys adeninivorans*, *Blastomyces dermatitidis*, *Candida albicans*, *Candida dubliniensis*, *Candida famata*, *Candida glabrata*, *Candida guilliermondii*, *Candida kefyr*, *Candida krusei*, *Candida lambica*, *Candida lipolytica*,

*Candida lustitaniae*, *Candida parapsilosis*, *Candida pulcherrima*, *Candida revkaufi*, *Candida rugosa*, *Candida tropicalis*, *Candida utilis*, *Candida viswanathii*, *Candida xestobii*, *Chrysosporuim keratinophilum*, *Coccidiodes immitis*, *Cryptococcus albidus* var. *diffluens*, *Cryptococcus laurentii*, *Cryptococcus neofomans*, *Debaryomyces hansenii*, *Gymnoascus dugwayensis*, *Hansenula anomala*, *Histoplasma capsulatum*, *Issatchenkia occidentalis*, *Isstachenkia orientalis*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Kluyveromyces thermotolerans*, *Kluyveromyces waltii*, *Lipomyces lipoferus*, *Lipomyces starkeyii*, *Microsporum gypseum*, *Myxotrichum deflexum*, *Oidiodendron echinulatum*, *Pachysolen tannophilis*, *Penicillium notatum*, *Pichia anomala*, *Pichia pastoris*, *Pichia stipitis*, *Rhodosporidium toruloides*, *Rhodotorula glutinus*, *Rhodotorula graminis*, *Saccharomyces cerevisiae*, *Saccharomyces kluyveri*, *Schizosaccharomyces pombe*, *Scopulariopsis acremonium*, *Sepedonium chrysospermum*, *Trichosporon cutaneum*, *Trichosporon pullans*, *Yarrowia lipolytica*, or *Yarrowia lipolytica* (formerly classified as *Candida lipolytica*). In some embodiments, a yeast is a *Y. lipolytica* strain that includes, but is not limited to, ATCC 20362, ATCC 8862, ATCC 18944, ATCC 20228, ATCC 76982 and LGAM S(7)1 strains (Papanikolaou S., and Aggelis G., *Bioresour. Technol*. 82(1):43-9 (2002)).

In certain embodiments, a yeast is a *C. tropicalis* strain, a *C. viswanathii* strain, a *Y. lipolytica* strain or a yeast strain that includes, but is not limited to, ATCC 20336, ATCC 20913, SU-2 (ura3-/ura3-), ATCC 20962, H5343 (beta oxidation blocked; US Patent No. 5648247) ATCC 20362, ATCC 8862, ATCC 18944, ATCC 20228, ATCC 76982, LGAM S(7)1, ATCC 8661, ATCC 8662, ATCC 9773, ATCC 15586, ATCC 16617, ATCC 16618, ATCC 18942, ATCC 18943, ATCC 18944, ATCC 18945, ATCC 20114, ATCC 20177, ATCC 20182, ATCC 20225, ATCC 20226, ATCC 20228, ATCC 20237, ATCC 20255, ATCC 20287, ATCC 20297, ATCC 20306, ATCC 20315, ATCC 20320, ATCC 20324, ATCC 20341, ATCC 20346, ATCC 20348, ATCC 20362, ATCC 20363, ATCC 20364, ATCC 20372, ATCC 20373, ATCC 20383, ATCC 20390, ATCC 20400, ATCC 20460, ATCC 20461, ATCC 20462, ATCC 20496, ATCC 20510, ATCC 20628, ATCC 20688, ATCC 20774, ATCC 20775, ATCC 20776, ATCC 20777, ATCC 20778, ATCC 20779, ATCC 20780, ATCC 20781, ATCC 20794, ATCC 20795, ATCC 20875, ATCC 22421, ATCC 22422, ATCC 22423, ATCC 22969, ATCC 32338, ATCC 32339, ATCC 32340, ATCC 32341, ATCC 32342, ATCC 32343, ATCC 32935, ATCC 34017, ATCC 34018, ATCC 34088, ATCC 34922, ATCC 38295, ATCC 42281, ATCC 44601, ATCC 46025, ATCC 46026, ATCC 46027, ATCC 46028, ATCC 46067, ATCC 46068, ATCC 46069, ATCC 46070, ATCC 46330, ATCC 46482, ATCC 46483, ATCC 46484, ATCC 48436, ATCC 60594, ATCC

62385, ATCC 64042, ATCC 74234, ATCC 76598, ATCC 76861, ATCC 76862, ATCC 90716, ATCC 90806, ATCC 90811, ATCC 90812, ATCC 90813, ATCC 90814, ATCC 90903, ATCC 90904, ATCC 90905, ATCC 96028, ATCC 201089, ATCC 201241, ATCC 201242, ATCC 201243, ATCC 201244, ATCC 201245, ATCC 201246, ATCC 201247, ATCC 201248, ATCC 201249, ATCC 201847, ATCC MYA-165, ATCC MYA-166, ATCC MYA-2613, and ATCC MYA-4467.

In certain embodiments, a yeast is a *Candida* species (i.e., *Candida* spp.) yeast. In some embodiments, suitable *Candida* species include, but are not limited to *Candida albicans*, *Candida dubliniensis*, *Candida famata*, *Candida glabrata*, *Candida guilliermondii*, *Candida kefyr*, *Candida krusei*, *Candida lambica*, *Candida lipolytica*, *Candida lustitaniae*, *Candida parapsilosis*, *Candida pulcherrima*, *Candida revkaufi*, *Candida rugosa*, *Candida tropicalis*, *Candida utilis*, *Candida viswanathii*, *Candida xestobii* and any other *Candida* spp. yeast described herein. Non-limiting examples of *Candida* spp. strains include, but are not limited to, sAA001 (ATCC 20336), sAA002 (ATCC 20913), sAA003 (ATCC 20962), sAA496 (US2012/0077252), sAA106 (US2012/0077252), SU-2 (ura3-/ura3-), H5343 (beta oxidation blocked; US Patent No. 5648247) strains. Any suitable strains from *Candida* spp. yeast may be utilized as parental strains for modification.

Examples of ascomycetes fungi include, but are not limited to, *Candida* spp., *Yarrowia* spp., *Blastobotrys* spp., *Aspergillus* spp., *Penicillium* spp., *Saccharomyces* spp., *Debaryomyces* spp., *Lipomyces* spp., *Fusarium* spp., *Paecilomyces* spp., *Trichoderma* spp., *Cladosporium* spp., *Pichia* spp., and *Neurospora* spp. Examples of basidiomycetes fungi include, but are not limited to, *Trichosporon* spp., *Rhodotorula* spp., *Rhodosporidium* spp., *Cryptococcus* spp., *Phaffia* spp., and *Xanthophyllomyces* spp.

Prokaryote organisms that can serve as host organisms, and that can be modified organisms, include, for example, Gram negative or Gram positive bacteria. Examples of bacteria include, but are not limited to, *Bacillus* (e.g., *B. subtilis*, *B. megaterium*), *Acinetobacter*, *Norcardia*, *Xanthobacter*, *Escherichia* (e.g., *E. coli* (e.g., strains DH10B, Stbl2, DH5-alpha, DB3, DB3.1), DB4, DB5, JDP682 and ccdA-over (e.g., U.S. Application No. 09/518,188))), *Streptomyces*, *Erwinia*, *Klebsiella*, *Serratia* (e.g., *S. marcessans*), *Pseudomonas* (e.g., P. aeruginosa), *Salmonella* (e.g., *S. typhimurium*, *S. typhi*), *Megasphaera* (e.g., *Megasphaera elsdenii*). Bacteria also include, but are not limited to, photosynthetic bacteria (e.g., green non-sulfur

bacteria (e.g., *Choroflexus* (e.g., *C. aurantiacus*), *Chloronema* (e.g., *C. gigateum*)), green sulfur bacteria (e.g., *Chlorobium* bacteria (e.g., *C. limicola*), *Pelodictyon* (e.g., *P. luteolum*), purple sulfur bacteria (e.g., *Chromatium* (e.g., *C. okenii*)), and purple non-sulfur bacteria (e.g., *Rhodospirillum* (e.g., *R. rubrum*), *Rhodobacter* (e.g., *R. sphaeroides*, *R. capsulatus*), and *Rhodomicrobium* (e.g., *R. vanellii*)).

Examples of cells from non-microbial organisms that can be utilized as a host cell or organism, engineered cell or organism or source for a heterologous polynucleotide include, but are not limited to, insect cells (e.g., *Drosophila* (e.g., *D. melanogaster*), *Spodoptera* (e.g., *S. frugiperda* Sf9 or Sf21 cells) and *Trichoplusa* (e.g., High-Five cells); nematode cells (e.g., *C. elegans* cells); avian cells; amphibian cells (e.g., *Xenopus laevis* cells); reptilian cells; mammalian cells (e.g., NIH3T3, 293, CHO, COS, VERO, C127, BHK, Per-C6, Bowes melanoma and HeLa cells); and plant cells (e.g., *Arabidopsis thaliana*, *Nicotania tabacum*, *Cuphea acinifolia*, *Cuphea aequipetala*, *Cuphea angustifolia*, *Cuphea appendiculata*, *Cuphea avigera*, *Cuphea avigera* var. pulcherrima, *Cuphea axilliflora*, *Cuphea bahiensis*, *Cuphea baillonis*, *Cuphea brachypoda*, *Cuphea bustamanta*, *Cuphea calcarata*, *Cuphea calophylla*, *Cuphea calophylla* subsp. *mesostemon*, *Cuphea carthagenensis*, *Cuphea circaeoides*, *Cuphea confertiflora*, *Cuphea cordata*, *Cuphea crassiflora*, *Cuphea cyanea*, *Cuphea decandra*, *Cuphea denticulata*, *Cuphea disperma*, *Cuphea epilobiifolia*, *Cuphea ericoides*, *Cuphea flava*, *Cuphea flavisetula*, *Cuphea fuchsiifolia*, *Cuphea gaumeri*, *Cuphea glutinosa*, *Cuphea heterophylla*, *Cuphea hookeriana*, *Cuphea hyssopifolia* (Mexican-heather), *Cuphea hyssopoides*, *Cuphea ignea*, *Cuphea ingrata*, *Cuphea jorullensis*, *Cuphea lanceolata*, *Cuphea linarioides*, *Cuphea llavea*, *Cuphea lophostoma*, *Cuphea lutea*, *Cuphea lutescens*, *Cuphea melanium*, *Cuphea melvilla*, *Cuphea micrantha*, *Cuphea micropetala*, *Cuphea mimuloides*, *Cuphea nitidula*, *Cuphea palustris*, *Cuphea parsonsia*, *Cuphea pascuorum*, *Cuphea paucipetala*, *Cuphea procumbens*, *Cuphea pseudosilene*, *Cuphea pseudovaccinium*, *Cuphea pulchra*, *Cuphea racemosa*, *Cuphea repens*, *Cuphea salicifolia*, *Cuphea salvadorensis*, *Cuphea schumannii*, *Cuphea sessiliflora*, *Cuphea sessilifolia*, *Cuphea setosa*, *Cuphea spectabilis*, *Cuphea spermacoce*, *Cuphea splendida*, *Cuphea splendida* var. viridiflava, *Cuphea strigulosa*, *Cuphea subuligera*, *Cuphea teleandra*, *Cuphea thymoides*, *Cuphea tolucana*, *Cuphea urens*, *Cuphea utriculosa*, *Cuphea viscosissima*, *Cuphea watsoniana*, *Cuphea wrightii*, *Cuphea lanceolata*).

In some embodiments, host organisms, or modified organisms, can be hydrocarbon-utilizing (e.g. alkane-utilizing), fatty acid-utilizing and/or fatty alcohol-utilizing microorganisms. These

organisms are able to assimilate hydrocarbons, fatty acids and/or fatty alcohols for energy and biomass generation. Many of these organisms are able to utilize hydrocarbons, fatty acids and/or fatty alcohols as a sole carbon source. Some examples of hydrocarbon-, fatty acid- and/or fatty alcohol-utilizing microorganisms include some species of fungi (including, e.g., yeast), bacteria and algae. Non-limiting examples of such organisms include *Yarrowia* (e.g., *Y. lipolytica* (formerly classified as *Candida lipolytica*)), *Candida* (e.g., *C. apicola*, *C. maltosa*, *C. tropicalis*, *C. utilis*, *C. viswanathii*, *C. catenulate*, *C. rugose*, *C. vini*, *C. entamophila*, *C. intermedia*), *Aspergillus* (e.g., *A. niger*, *A. versicolor*, *A. ustus*, *A. fumigatus*, *A. oryzae*, *A. flavus*, *A. ficuum*, *A. terricola*, *A. japonicas*, *A. wentii*, *A. clavatus*, *A. terreus*), *Penicillium* (*P. cyclopium*, *P. chrysogenum*, P. *italicum*), *Fusarium* (e.g., *F. oxysporum*, *F. moniliforme*, *F. solani*), *Paecilomyces* (e.g., *Paec. lilacinus*), *Trichoderma* (e.g., *T. koningii*, *T. viride*, *T. virens*), *Cladosporium* (e.g., *C. herbarum*), *Stachybotrys*, *Trichosporon* (e.g., *T. veenhuisii*, *T. asahii*, *T. jirovecii*, *T. monteviblankiideense*), *Rhodotorula* (e.g., *R. glutinous*, *R. mucilaginosa*), *Rhodosporidium* (e.g., *R. toruloides*), *Cryptococcus* (e.g., *C. neoformans*, *C. albidus*), *Pichia* (e.g., *P. farinosa*, *P. stipitis*), *Debaryomyces* (e.g., *D. hansenii*), *Blastobotrys* (e.g., *Blastobotrys adeninivorans*), *Saccharomyces* (e.g., *S. cerevisiae*, *S. bayanus*, *S. pastorianus*, *S. carlsbergensis*), *Lipomyces* (e.g., *L. starkeyii*, *L. lipoferus*) and *Chlorella* algae (e.g., *Chlorella protothecoides*).

In some embodiments, a host organism or modified organism can be an oleaginous organism (e.g., an oleaginous microorganism). As used herein, an "oleaginous" organism is an organism capable of accumulating at least about 20% or more of its cell mass (by dry weight) as intracellular lipids (e.g., oil). In oleaginous organisms, a significant carbon flux towards lipid synthesis occurs and is enhanced under certain conditions (e.g., limited supply of nitrogen). These lipid-accumulating organisms can be characterized by the endogenous expression of cytosolic ATP citrate lyase, which catalyzes the degradation of citrate generated in the TCA cycle into acetyl-CoA and oxaloacetate, and/or a dependence on AMP concentration for the activity of isocitrate dehydrogenase in the TCA cycle. Generally, under certain conditions (e.g., limited nitrogen), AMP deaminase is activated in oleaginous yeast which can lead to a decrease in mitochondrial AMP concentration and isocitrate dehydrogenase activity. This, in turn, can cause an accumulation of mitochondrial citrate from the TCA cycle which is then exported to the cytosol and can serve as substrate for ATP citrate lyase. The acetyl-CoA that may be generated through the action of ATP citrate lyase can be used in synthesizing fatty acyl-CoA that can be converted into lipids which may be stored in lipid bodies in the cells. A "non-

oleaginous" organism, as used herein, is an organism that is not capable of accumulating at least about 20% or more of its cell mass (by dry weight) as intracellular lipids. In some embodiments, a host organism or modified organism can be a non-oleaginous organism.

Oleaginous microorganisms include species of fungi, bacteria and algae. Examples of oleaginous fungi include, but are not limited to, *Blastobotrys* (e.g., *Blastobotrys adeninivorans*), *Yarrowia* (e.g., *Y. lipolytica*), *Trichosporon* (e.g., *T. fermentans*, *T. porosum*, *T. pullulan*), *Rhodotorula* (e.g., *R. graminis*, *R. glutinous*, *R. araucariae*, *R. minuta*, *R. bogoriensis*, *R. mucilaginosa*, *R. colostri*), *Rhodosporidium* (e.g., *R. toruloides*, *R. kractochvilovae*, *R. paludigenum*, *R. fluviale*, R. *babjevae*), *Lipomyces* (e.g., *L. starkeyii*, *L. lipofer*), *Debaryomyces* (e.g., *D. hansenii*), *Cryptococcus* (e.g., *C. podzolicus*, *C. phenolicus*, *C. curvatus*), *Pichia* (e.g., *P. segobiensis*), *Cystofilobasidium* (e.g., *C. informiminiatum*), *Leucosporidium* (e.g., *L. scottii*), *Sporobolomyces* (e.g., *S. singularis*, *S. poonsookiae*, *S. odoratus*, *S. metaroseus*, *S. bannaenis*), *Sporidiobolus* (e.g., *S. ruineniae*, *S. carnicolor*, S. pararoseus, *S. johnsonii*), *Schwanniomyces* (e.g., *S. occidentalis*), *Occultifur* (e.g., *O. externus*), *Blakeslea*, *Cunninghamella*, *Mortirella*, *Mucor*, *Phycomyces* and *Pythium*. Nonlimiting examples of oleaginous bacteria include *Morrococcus*, *Bacillus subtilis* and *Rhodococcus opacus*. Examples of oleaginous algae include, but are not limited to *Nannochloropsis* (e.g., *N. oceania*), *Chlorella* (e.g., *C. vulgaris*), *Thraustochyrtium* and *Schizochytrium*.

In particular embodiments, a host organism or modified organism can be a non-oleaginous yeast. In some embodiments, a host organism or modified organism can be a non-oleaginous, non-*Saccharomyces* yeast. Included among such yeast are non-oleaginous, non-*Saccharomyces* ascomycetes yeast as well as non-oleaginous, basidiomycetes yeast and non-oleaginous, ascomycetes yeast. In certain aspects, a host organism or modified organism can be an oleaginous, non-*Yarrowia* yeast, a non-*Yarrowia* ascomycetes yeast, or a non-*Yarrowia*, non-*Saccharomyces*, ascomycetes yeast. In another aspect, a host organism or modified organism can be an oleaginous yeast that accumulates 20% to 65% or 20% to 60% or 20% to 58%, or 20% to 55%, or 20% to 50% or 20% to 45%, or 20% to 40% or 20% to 35%, or 20% to 30% or 20% to 25% of its cell mass (by dry weight) as intracellular lipids (e.g., oil). In another embodiment, a host organism or modified organism can be an oleaginous yeast that accumulates at least 20% or at least about 25% of its cell mass (by dry weight) as intracellular lipids (e.g., oil). In a further embodiment, a host organism or modified organism can be an oleaginous yeast that accumulates at least 20% but less than 70%, or at least 20% but less than

60%, or at least 20% but less than 50%, or at least 20% but less than 40%, or at least 20% but less than 30%, of its cell mass (by dry weight) as intracellular lipids (e.g., oil). In another embodiment, a host organism or modified organism can be an oleaginous yeast in which linoleic acid is less than 50% or less than 45% or less than 40% or less than 35% or less than 30% or less than 25% or less than 20% of the intracellular accumulated lipid composition.

In some embodiments, a host cell or organism or modified cell or organism is one that is capable of ω-oxidation of alkanes and/or fatty acids. Such cells or organisms can endogenously produce enzymes of the ω-oxidation pathway. This pathway includes steps of ω-hydroxylation, oxidation and dehydrogenation of ω-carbon. The ω-hydroxylation step can be catalyzed by a hydroxylase complex including a cytochrome P450 monooxygenase (such as, for example, an alkane-inducible cytochrome P450, e.g., CYP52) and a cytochrome P450:NADPH oxidoreductase which yields an alcohol. In a subsequent oxidation step, the alcohol is further oxidized to an aldehyde in a reaction catalyzed by a fatty alcohol oxidase. A dicarboxylic acid is generated through dehydrogenation of the aldehyde by a fatty aldehyde dehydrogenase. In some aspects, a host cell or organism endogenously expresses proteins having cytochrome P450 monooxygenase and cytochrome P450:NADPH oxidoreductase activity. In some embodiments, a host cell or organism or modified cell or organism is one that is capable of synthesizing dicarboxylic acids, such as, for example, α,ω-dicarboxylic acids.

In some embodiments, the host cell or organism or modified cell or organism is a diploid cell or organism. In certain aspects, the host cell or organism or modified cell or organism is an anamorphic ascomycetes yeast.

In some embodiments, a host organism or modified organism can be a thermotolerant and/or osmotolerant organism. As used herein, "thermotolerant," in reference to an organism, e.g., a microorganism, refers to the ability of the organism to survive at elevated temperatures. For example, a thermotolerant organism, e.g., a microorganism, such as yeast, is one that is able to survive and/or grow and/or assimilate fatty acids and/or aliphatic carbon sources at temperatures greater than 30°C, greater than 31°C, greater than 32°C, greater than 33°C, greater than 34°C, greater than 35°C, greater than 36°C, greater than 37°C, greater than 38°C, greater than 39°C, greater than 40°C, greater than 41°C, greater than 42°C, greater than 43°C, greater than 44°C, greater than 45°C, greater than 46°C, greater than 47°C, or greater than 48°C. A thermotolerant organism can be one that is able to survive and/or grow and/or



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assimilate fatty acids and/or aliphatic carbon sources at temperatures up to about 30°C, 32°C, 34°C, 35°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C or 50°C or more. As used herein, "osmotolerant," in reference to an organism, e.g., a microorganism, refers to the ability of the organism to survive in elevated external osmotic pressure environments, e.g., high solute (such as salt or sugar) concentrations). For example, an osmotolerant organism, e.g., a microorganism, such as yeast, is one that is able to survive and/or grow and/or assimilate fatty acids and/or aliphatic carbon sources in media containing up to about 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25% or greater NaCl. Thermotolerant and/or osmotolerant microorganisms include, for example, species of *Blastobotrys* yeast (e.g., *Blastobotrys adeninivorans*), *Candida* yeast (e.g., *C. Mexicana, C. glycerinogenes*, *C. zemplinina*), *Pichia* yeast (e.g., *P. mississippiensis*, *P. mexicana*, *P. farinosa*, *P. sorbitophila*), *Clavispora* yeast (e.g., *C. opuntiae*, *C. lusitaniae*), *Kluyveromyces* yeast (e.g., *K. thermotolerans*), *Debaryomyces* (e.g., *D. hansenii*), *Rhodotorula* (e.g., *R. mucilaginosa*), *Zygosaccharomyces* (e.g., *Z. rouxii*) and *Issatchenkia* (e.g., *I. orientalis*). Thermotolerant and/or osmotolerant organisms can be well suited for use in industrial production systems operating at elevated temperatures and/or osmotic pressures that would impair growth and/or metabolism and/or completely inactivate organisms that are not thermotolerant and/or osmotolerant. Furthermore, in many instances, production efficiency can be improved and production costs reduced in using such organisms due to decreases in losses and avoidance of implementation of cooling processes.

Host cells and microorganisms and engineered cells and microorganisms may be provided in any suitable form. For example, such cells and microorganisms may be provided in liquid culture or solid culture (e.g., agar-based medium), which may be a primary culture or may have been passaged (e.g., diluted and cultured) one or more times. Microorganisms and cells also may be provided in frozen form or dry form (e.g., lyophilized). Microorganisms and cells may be provided at any suitable concentration.

*Modified cells and organisms*

Provided herein are cells and organisms (including microorgansims) that have been modified in one or more aspects relative to the unmodified cell or organism (i.e., the cell or organism prior to the modification). For example, a cell or organism can be modified by altering one or more cellular activities and/or the sum total of a cell's or organism's activities. Thus, in this example, modifications can include alteration of cellular activities, addition of cellular activities and/or

elimination of cellular activities. A "cellular activity," as used herein, refers to any process, functioning, or operation that can occur in a cell. In particular embodiments provided herein, a cell or organism has been modified to alter cellular carbon flux. Such modified cells and organisms have been manipulated in ways designed to direct carbon atoms toward one or more biochemical events, cellular activities or cellular locations and/or away from other metabolic pathways, cellular activities or locations. The alteration(s) can involve a single modification or multiple modifications of the original, or host, cell or organism in which carbon flux is altered. Also provided herein are methods of producing such modified cells and organisms. As described herein, there are multiple methods of altering cellular carbon flux by modifying one or more aspects of carbon processing in cells. Aspects of cellular carbon processing include, for example, but are not limited to, fatty acid metabolism, including fatty acid catabolism and synthesis, ω-oxidation, β-oxidation, fatty acid transport, acetyl group transfer/transport and processing, the TCA cycle, metabolite processing and triacylglyceride and lipid biosynthesis. For example, in altering carbon flux, certain cellular activities may be reduced, slowed or eliminated and/or other activities may be increased, accelerated, added or relocated. In particular embodiments, the amount and/or activity of one or more enzymes and/or transport proteins is/are modified in cells or microorganisms.

As such, the cells and organisms provided herein are "modified" or "engineered." The terms "engineered" or "modified," as used interchangeably herein, in reference to a cell, organism or microorganism refer to a cell or organism (including a microorganism) that has been manipulated or altered such that it is distinct (e.g., detectably changed or physically different) from a naturally occurring cell or organism. For example, the sum total of the cellular activities of a modified or engineered cell or microorganism can be distinct from those of a naturally occurring cell or microorganism, e.g., a modified cell or microorganism may include or lack one or more activities relative to the activities present in an unmodified cell or microorganism utilized as a starting point (e.g., host cell, host organism or host microorganism) for modification. In another example, one or more cellular activities of a modified or engineered cell or microorganism may be altered relative to the cellular activity or activities of the host cell or microorganism. A modified or engineered cell or organism can be genetically modified through any alteration in its genetic composition. For example, a genetically modified cell or organism can include one or more heterologous polynucleotides, can have one or more endogenous nucleic acid deletions and/or can have one or more genetic mutations. Mutations include point mutations, insertions and deletions of a single or multiple residues in a nucleic acid. In some



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embodiments, an engineered cell, organism or microorganism includes a heterologous polynucleotide, and in certain embodiments, an engineered cell, organism or microorganism has been subjected to selective conditions that alter an activity, or introduce an activity, relative to the host cell or microorganism. Thus, a modified or engineered cell, organism or microorganism has been altered directly or indirectly by a human being. It is understood that the terms "modified cell," "modified organism," "modified microorganism," "engineered cell," " engineered organism," " engineered microorganism," refer not only to the particular cell or organism but to the progeny or potential progeny of such a cell or organism. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

For example, a "genetically modified" or "genetically engineered" cell, organism or microorganism is one in which the genetic make-up of the cell, organism or microorganism has been modified. Genetic modification encompasses a variety of alterations and can be accomplished in numerous ways. A genetic modification includes, but is not limited to, any of the following alterations: modification of the expression of an endogenous gene (e.g., the amount, pattern, timing and/or regulation (e.g., inducibility) of expression of a gene), disruption or deletion of an endogenous gene, increasing the copy number of an endogenous gene, mutation of an endogenous gene (including the regulatory components, exons, introns and/or peptide- or protein-encoding portions of a gene), and introduction of heterologous nucleic acid in to a cell or cells. These genetic modifications, and others, are described herein.

A genetic modification of a cell or organism can be one that modifies the expression of one or more nucleic acids or polypeptides in the cell or organism. A genetic modification of a cell or organism can be one that modifies the amount and/or activity of a polypeptide in the cell or organism. For example, modified expression of a nucleic acid or protein (e.g., modified rate, amount and/or level of expression) or modified amount or activity of a polypeptide may be a reduction, slowing, decrease or elimination, or increase, acceleration, addition or elevation in expression of a nucleic acid or protein or in the amount and/or activity of a polypeptide. Modified expression of a nucleic acid or modification of the amount and/or activity of a polypeptide may be a relocation of expression or activity within a cell.



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In one example, a genetic modification of a cell or organism can be one that alters the expression of, or the amount and/or activity of, a polypeptide involved in a reaction that generates a product (e.g., cytosolic acetyl-CoA, cytosolic malonyl-CoA, peroxisomal acetate) in a cell or organism. A "polypeptide involved in a reaction that generates," as used herein with respect to generation of a product, refers to a polypeptide that participates in the direct generation of the product from reactants. A reaction that directly generates a particular product can be a single-step reaction or a multi-step reaction involving transient reaction intermediates. For example, a polypeptide involved in a reaction that generates cytosolic acetyl-CoA is one that participates in a reaction that directly yields acetyl-CoA in the cytosol. Exemplary polypeptides (e.g., enzymes) involved in a reaction that generates cytosolic acetyl-CoA include, but are not limited to, cytosolic carnitine acetyltransferase, cytosolic acetyl-CoA synthetase and cytosolic ATP citrate lyase. A polypeptide involved in a reaction that generates cytosolic malonyl-CoA is one that participates in a reaction that directly yields malonyl-CoA in the cytosol. A non-limiting example of a polypeptide involved in a reaction that generates cytosolic malonyl-CoA is acetyl-CoA carboxylase. A polypeptide involved in a reaction that generates peroxisomal acetate is one that participates in a reaction that directly yields acetate in the peroxisome. A non-limiting example of a polypeptide involved in a reaction that generates peroxisomal acetate is acetyl-CoA hydrolase.

The term "endogenous," as used herein in reference to an aspect (e.g., a gene, nucleic acid, peptide, polypeptide, activity, genetic composition, gene expression, and the like) of a cell or organism or microorganism refers to the inherent aspect, or condition thereof, in the cell, organism or microorganism that has not been modified or engineered (i.e., the reference cell, organism or microorganism). The term "heterologous," "exogenous" or "foreign" as used herein with respect to a composition or quality (e.g., a gene, nucleic acid, peptide, polypeptide, cellular activity, genetic composition, gene expression, and the like) refers to the composition or quality not being a physically existing part or attribute of a reference cell, organism or microorganism. For example, a heterologous, exogenous or foreign nucleic acid can be any nucleic acid that is introduced into a cell or microorganism as part of a genetic modification of the cell or microorganism. A heterologous, exogenous or foreign composition (such as, for example, a nucleic acid) includes compositions that may be identical to an endogenous composition (e.g., a nucleic acid gene sequence that is introduced into a cell or microorganism to increase the copy number and/or alter the positioning or expression of the same nucleic acid sequence existing therein) or may be different from an endogenous composition.



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*Coordination of carbon source, host organism and regulatory mechanisms to optimize carbon flux modification*

In developing cell- and organism-based systems for enhanced production of target molecules, there are multiple factors, in addition to the design of cellular modifications for altering carbon flux, that can affect the overall efficiency and economics of the production process. These additional considerations include the sources of carbon available to the microorganism, the organism's ability to utilize various forms of carbon in the sources and the cellular regulatory systems that can be used in controlling carbon flux. Coordination of these factors can play a significant role in optimization of carbon flux alteration and, in turn, the efficiency of target molecule production.

Carbon sources used for culturing cells and microorganisms and/or fermentation processes sometimes are referred to as feedstocks. The term "feedstock" as used herein refers to a composition containing a carbon source that is provided to a cell or organism, which is used by the cell or organism to produce energy and metabolic products useful for growth. In order for cells and organisms to utilize carbon in vital processes, the carbon source is processed intracellularly in catabolic pathways to a form(s) that can be accommodated by energy generation and biosynthetic pathways. For example, glucose is processed in glycolytic pathways in cells whereas fatty acids are processed through β-oxidation. Thus, the carbon source used in microbial-based methods of target molecule production can influence which metabolic pathways will be involved in assimilating the carbon. A target molecule production system that incorporates elements of endogenous cellular metabolic pathways may not perform optimally if those pathways are not utilized in processing the carbon source.

Some cells and microorganisms are able to utilize a variety of carbon sources. However, many cells and microorganisms preferentially utilize particular carbon sources over others, and some cells and microorganisms are unable to utilize certain carbon sources. For example, *Saccharomyces cerevisiae* can utilize xylulose but not xylose. *Blastobotrys adeninivorans* and *Arxula terestre* are able to utilize carbon- and nitrogen-containing compounds, e.g., adenine, uric acid, butylamine and pentylamine, as a sole source of carbon and nitrogen.



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One consideration in the design of an economically feasible cell-based system for the production of target molecules is production-associated costs. The carbon source used in cultivating cells and organisms can be a significant factor contributing to production costs. Many microorganisms, including yeast, preferentially use glucose over other carbon sources. However, glucose is a relatively high-cost carbon source. Therefore, from an economic perspective, it can be beneficial to utilize lower-cost sources of carbon in bioproduction systems. Non-fermentable carbon sources, including, for example, glycerol and fatty acids, may be lower-cost alternatives to glucose and other carbohydrates in feedstocks. For example, waste materials, such as waste cooking oil, can be used as feedstocks containing non-fermentable carbon sources.

Therefore, in developing a cost-effective, efficient cell- or microbial-based target molecule production system, the modifications made to an organism to alter carbon flux should be coordinated and compatible with, and complementary to, the carbon source and cell or organism that will be employed in the production methods. Additionally, for optimal target molecule production, the regulatory mechanisms that are used in the cell or organism for controlling the individual elements (e.g., enzyme expression) being modified should provide for timing and extent of each element's activity that correlates with the desired carbon flux alterations at each stage of the production process.

For example, some embodiments of the cell- and microbial-based methods for producing target molecules provided herein include cells or organisms in which carbon processing activities have been engineered to enhance carbon flow through cellular oxidative metabolism pathways, e.g, ω-oxidation and/or β-oxidation. One advantage of such modified bioproduction systems is that they are well suited for use with lower cost, alternative carbon sources, including, for example, non-carbohydrate and non-fermentable carbon sources such as aliphatic compounds and hydrocarbons (e.g, alkanes, fatty acids and fatty alcohols). Use of such carbon sources is not only more cost-effective but can also have the added advantage of reducing the environmental impact of harmful wastes (e.g., agro-industrial by-products, waste cooking oil and waste motor oil) that can be used as feedstocks in target molecule production instead of being discarded. Cells or organisms particularly compatible with such methods are those that are able to utilize non-fermentable, as well as fermentable, carbon sources. Generally, such cells and organisms contain endogenous metabolic pathways that form part of the basis for the desired carbon flux modifications. As also described herein, embodiments of the cell- and microbial-based systems



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in which carbon processing activities have been engineered to direct carbon flow through oxidative metabolism and away from mitochondrial metabolism can be controlled to provide for maximal, coordinated and highly efficient target molecule production based on, for example, use of carbon source-dependent transcription regulation of modified activities in the cells.

Transcription regulatory elements, including promoters, for some genes are responsive to the carbon source available to the cells. For example, transcription of some genes is subject to glucose repression in which the gene may not be expressed, or is less expressed, in the presence of glucose. Thus, in contrast to unregulated constitutive promoters, transcription regulatory elements for genes such as these are repressed, derepressible and/or inducible by varying carbon sources. When glucose is depleted, genes that were subject to glucose repression are then transcribed in a process referred to as glucose derepression. For some of these genes, this increase in transcription due to derepression represents the extent to which the genes will be expressed because they are not subject to induction and further increased transcription. For others of these genes, transcription may be increased several-fold over the derepressed level upon induction by, for example, certain carbon sources. Examples of such carbon sources include, but are not limited to, vegetable oils, triglycerides, fatty acids, e.g, oleic acid, esters of fatty acids and n-alkanes. Some genes encoding peroxisomal proteins (including enzymes involved in fatty acid catabolism) are subject to glucose repression/derepression. As described herein, the transcription regulatory elements for genes subject to glucose repression can advantageously be used in cell- and microbial-based methods for target molecule production involving alternative carbon sources.

Different carbon sources or feedstocks may be used in culturing cells or microorganisms at different phases of a target molecule production process. For example, one carbon source, e.g., glucose, may be used in preparing an initial starter culture of modified cells or microorganisms to establish a foundation of growing cells and a different carbon source (e.g., a lower-cost alternative such as fatty acids) may be used in a target molecule production phase subsequent to establishment of the starter culture. Accordingly, carbon source utilization can vary depending on the goal of a particular time or phase of a culture process.

In some embodiments of cell- and microbial-based target molecule production systems provided herein, modifications made to the cells or microorganisms include use of carbon source-dependent regulatory elements in altering carbon flux to enhance production efficiency. As



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described herein, in some embodiments, cytosolic activities for generating acetyl-CoA and/or malonyl-CoA (e.g., carnitine acetyltransferase, acetyl-CoA carboxylase, acetyl-CoA synthetase and/or ATP citrate lyase) can be increased for target molecule production during fatty acid or alkane assimilation, while mitochondrial and/or cytosolic activities for uptake and utilization of acetyl group carbons (e.g., cytosolic acyl-CoA synthetase, mitochondrial acetyl-carnitine transporters and/or mitochondrial carnitine acetyltransferase) are decreased. For optimal coordination of these activities with the overall production process, in some embodiments, the expression of proteins involved in some of the target molecule production activities can be engineered to be controlled by particular glucose-repressible and/or fatty acid-inducible transcription regulatory elements. For example, during initial cell-growth stages of a production method, a preferred carbon source may be, for example, glucose. Activities participating in target molecule production can be suppressed during this stage by using glucose-repressible elements, e.g., promoters, to regulate transcription of nucleic acids encoding proteins involved in those activities. At the same time, activities (e.g., mitochondrial metabolism) involved in cellular energy generation for growth can be permitted to function and/or elevated by using constitutive or glucose-inducible elements, e.g., promoters, to regulate transcription of nucleic acids encoding proteins involved in those activities. On the other hand, activities participating in target molecule production will be permited and/or increased following growth stages and during target molecule production stages when glucose is depleted and fatty acid carbon sources are provided by using glucose-repressible and/or fatty acid-inducible elements, e.g., promoters, to regulate transcription of nucleic acids encoding proteins involved in those activities. Also during those stages, the activities (e.g., mitochondrial metabolism) involved in cellular energy generation and growth can be unchanged or reduced or minimized by using weak, constitutive and/or glucose-inducible/fatty acid-inhibited elements, e.g., promoters, to regulate transcription of nucleic acids encoding proteins involved in those activities. This type of coordination of gene expression regulation with cellular modifications to alter carbon flux and use of alternative carbon sources can greatly enhance target molecule production efficiency and economy.

In some embodiments of cell- and microbial-based target molecule production systems provided herein, such as those in which target molecule production involves cellular oxidative metabolism pathways, e.g, ω-oxidation and/or β-oxidation, for processing of fatty acids and/or alkanes, modifications made to the cells or microorganisms may include optimization of carbon source-dependent regulatory elements within the pathways. For example, although expression of unmodified genes encoding some of the polypeptides (e.g., enzymes) in these pathways is



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regulated by glucose-repressible and/or fatty acid (and/or alkane)-inducible transcriptional control elements, it may be beneficial to utilize heterologous stronger, more active, fatty acid (and/or alkane)-inducible transcriptional control elements to increase expression and/or activity of pathway polypeptides (e.g., enzymes) in modified cells and organisms. Thus, for example, the promoter of an endogenous glucose-repressible and/or fatty acid-inducible gene (e.g., an acyl-CoA oxidase (such as Pox5p), a peroxisomal protein (such as Pex11p), a cytochrome P450 monooxygenase or reductase (such as CYP52A17 or CPRB proteins)) can be replaced with a glucose-repressible and/or fatty acid-inducible promoter from another gene (e.g., an *HDE* gene) to enhance carbon processing through oxidative metabolism in modified cells or organisms.

In some embodiments of cell- and microbial-based target molecule production systems provided herein, carbon flux can be altered to reduce acetyl carbon processing in mitochondria during target molecule production occurring with fatty acid or alkane assimilation. In these embodiments, transcriptional control elements of some endogenous glucose-repressible and/or fatty acid-inducible genes encoding mitochondria-assoicated polypepties (e.g., mitochondrial transporter proteins (such as Crc1p) and carnitine acetyltransferases (such as Cat2p)) can be replaced with a promoter that is not fatty acid-inducible (e.g., a weaker and/or constitutive promoter) from another gene (e.g., glucose-6-phosphate isomerase gene) to reduce acetyl carbon uptake by and/or metabolism in mitochondria in modified cells or organisms. As also described herein, acetyl carbon uptake by and/or metabolism in mitochondria can be reduced in modified cells and organisms by replacing genes encoding one or more endogenous mitochondria-associated polypeptides (e.g., enzymes, such as carnitine acetyltransferase) with nucleic acid encoding a less active polypeptide. The nucleic acid encoding a less active polypeptide may also be linked to a transcriptional control element that provides for weak and/or not inducible expression of the polypeptide. If, however, the activity of the less-active polypeptide is insufficient for optimal cell functioning, a more active, inducible (e.g., fatty acid-inducible) promoter can be used to regulate expression of the less-active polypeptide.

*Aliphatic and hydrophobic carbon sources*

The ability of cells and microorganisms to utilize alternative carbon sources for energy generation and growth is based in the multiple enzyme-mediated metabolic pathways and gene regulation systems in microbial cells. In general, glucose often is a preferred carbon and energy



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source for many cells and microorganisms, e.g., yeast. A number of genes encoding products, such as certain enzymes, involved in cellular pathways and processes that are not used in carbohydrate metabolism may be repressed when glucose is present in culture media. If glucose is depleted in the media, some of these genes may then be derepressed. If alternative carbon sources, e.g., non-fermentable carbon sources, are available, some of the genes may be induced, and may be induced by several-fold. For example, when aliphatic or hydrophobic carbon sources, (e.g., alkanes, alkenes, fatty acids) are the external carbon source, the expression of genes encoding enzymes involved in β-oxidation and proteins involved in peroxisome proliferation can be induced. One example of a gene that is repressed in glucose media, derepressed in non-fermentable carbon source-containing media and induced in fatty acid-containing media is the gene encoding the peroxisomal trifunctional enzyme hydratase-dehydogenase-epimerase (HDE) in yeast, such as, for example, *Candida* (see, e.g., Sloots et al. (1991) *Gene* 105:129-134). The upstream regulatory regions of the HDE gene include a glucose-responsive region controlling glucose repression, a non-fermentable carbon-responsive region controlling derepression and an oleic acid-responsive region controlling fatty acid induction of transcription of the gene.

In some embodiments of the methods for producing a target molecule provided herein, a carbon source used during the production phase of a culturing step in the method includes an aliphatic or hydrophobic carbon source. In particular embodiments, an aliphatic or hydrophobic carbon source is the primary carbon source or may be the sole, or only, carbon source used during the production phase of a culturing step in the method. In some embodiments, the carbon source is a fatty acid and/or alkane. In certain aspects, the carbon source is a fatty acid. In some embodiments, the carbon source is an 18-carbon fatty acid such as, for example, oleic acid (C18:1), linoleic acid (C18:2) or linolenic acid (C18:3). Embodiments of the methods in which an aliphatic or hydrophobic carbon source is used are particularly advantageous when target molecule production involves oxidation pathways such as ω-oxidation and/or β-oxidation pathways and/or involves peroxisomes.

Additional advantages of using aliphatic or hydrophobic carbon sources in some embodiments of the methods of producing target molecules as provided herein are reduced costs and positive environmental effects realized through their use.



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*Methods of modifying cellular carbon flux*

Included in the cells, organisms and microorganisms and methods described herein are those that provide for enhanced production of desired target molecules. In one aspect, production is enhanced through modification of carbon flux in cell or microbial production systems. Through modification of cellular carbon flux, carbon atoms that may have flowed or been transported into other metabolic processes (e.g., energy and/or cellular composition generation) in the cell are redirected for use in a target molecule production process. Described herein are multiple cellular modifications that can be employed to beneficially alter carbon flux. A modification can be used alone or in combination with one or more other modifications depending on the target molecule produced and the carbon flux alteration that is best suited to maximize its production.

*Modification of acetyl-carnitine entry into mitochondria*

Included in embodiments of the cells, microorganisms, compositions and methods provided herein are cell and microbial production platform systems and components thereof in which the amount of (a) acetyl-carnitine in the cell cytosol and/or (b) carnitine acetyltransferase and/or carnitine acetyltransferase activity in the cell cytosol is/are modified. Carnitine and carnitine acetyltransferase are the primary elements of the carnitine shuttle system in which acetyl carbons from acetyl-CoA are transferred across intracellular membranes and transported throughout eukaryotic cells. For example, the carnitine shuttle is a mechanism through which acetyl carbons from acetyl-CoA generated in peroxisomes flow to mitochondria. Acetyl groups from peroxisomal acetyl-CoA can be transferred to carnitine in a reaction catalyzed by carnitine acetyltransferase and then move across the peroxisomal membrane and into the cytosol in the form of acetyl-carnitine. Cytosolic acetyl-carnitine can then be transported into mitochondria where mitochondrial carnitine acetyltransferase catalyzes the reverse reaction to transfer the acetyl moiety from carnitine to free coenzyme A to generate acetyl-CoA. The carnitine shuttle provides a main "highway" for the flow of carbon atoms into mitochondria, particularly in the assimilation of fatty acids and other non-carbohydrate and non-fermentable carbon source molecules by cells. It is thus one target for modification of carbon flux in cells and microorganisms provided herein.

In some embodiments of the cells, microorganisms, compositions and methods provided herein, the amount of (a) acetyl-carnitine in the cell cytosol is increased and/or decreased, and/or (b)



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carnitine acetyltransferase and/or carnitine acetyltransferase activity in the cell cytosol is/are increased and/or decreased. For example, in some aspects, a cell or microorganism may be modified to increase cytosolic acetyl-carnitine, may be modified to decrease cytosolic acetyl-carnitine or may be modified to alternately increase and decrease cytosolic acetyl-carnitine depending on the conditions in which the modified cell or microorganism is cultured.

In certain embodiments, a host cell or microorganism is modified to increase the amount of (a) acetyl-carnitine in the cell cytosol and/or (b) carnitine acetyltransferase and/or carnitine acetyltransferase activity in the cell cytosol. Increasing the amount and/or activity of cytosolic carnitine acetyltransferase provides for an increased conversion of acetyl-carnitine into acetyl-CoA in the cytosol. Increasing the amount of acetyl-carnitine in the cytosol provides an increased availability of substrate for cytosolic carnitine acetyltransferase to convert to acetyl-CoA. These modifications effectively result in an increase in the generation and amount of cytosolic acetyl-CoA which can then be used in the production of desired carbon-containing molecules.

*Modification of a mitochondrial acetyl-carnitine transporter*

In one embodiment, cells or organisms provided herein are modified to increase and/or decrease the amount of acetyl group carbons in the cytosol in the form of acetyl-carnitine in transit from the peroxisome and other areas to the mitochondria by increasing or reducing the entry of acetyl-carnitine into mitochondria from the cytosol. In one aspect, the amount and/or rate of acetyl-carnitine transfer into the mitochondria in a cell can be increased or reduced by increasing or decreasing the expression of an acetyl-carnitine translocase protein localized in mitochondrial inner membranes. This protein carrier transports acetyl-carnitine across the mitochondrial inner membrane and into the mitochondrial matrix in exchange for carnitine. For example, a mitochondrial carnitine translocase is encoded by a *CRC1* gene in *Saccharomyces* (see, e.g, Palmieri *et al.* (1999) *FEBS Lett* 462:472-276), which contains an oleate-responsive element in the promoter region, and by an *AcuH* gene in *Asperillgus* (see, e.g., De Lucas *et al.* (2001) *FEMS Microbiol Lett* 201:193-198). Carnitine carrier proteins belong to a family of mitochondrial carrier proteins which generally contain three tandemly repeated ~100-amino acid domains. Each of the three domains typically contains two hydrophobic regions, spanning the membrane as α-helices, linked by a hydrophobic loop that extends into the mitochondrial matrix. Each domain also typically contains a version of a motif (PX[DE]XX[RK]XRK) involved in



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forming a salt bridge that closes off the matrix side of a channel generated by the α-helices (see, e.g., Indiveri et al. (2011) *Molecular Aspects of Medicine* 32:223-233).

There are a number of ways to increase or reduce expression of an acetyl-carnitine translocase in a cell. For example, a host acetyl-carnitine translocase activity can be decreased by disruption (e.g., knockout, insertion mutagenesis, the like and combinations thereof) of a host gene encoding the protein, or by decreasing the activity of the promoter (e.g., through addition of repressor sequences to the promoter or 5'UTR or replacing the promoter) that controls transcription of an acetyl-carnitine translocase gene using recombinant molecular biology techniques known in the art and/or described herein. One method for disrupting an endogenous acetyl-carnitine translocase gene is by recombinantly inserting a heterologous nucleic acid (e.g., a nucleotide sequence encoding a selectable marker such as an enzyme that restores an auxotrophic host cell or organism to prototrophy) into the endogenous gene, thereby generating an engineered cell or organism deficient in acetyl-carnitine translocase activity. This can be done, for example, through homologous recombination in which a heterologous nucleic acid containing sequences of the endogenous acetyl-carnitine translocase gene and a disrupting sequence (e.g., a knock-out gene cassette such as described herein) is introduced into a host cell or microorganism. Nucleic acids encoding an acetyl-carnitine translocase can be obtained from a number of sources, including, for example, yeast cells. For example, genomic DNA from cell sources can be amplified using oligonucleotide primers based on the nucleotide sequence of an acetyl-carnitine translocase-encoding gene. Provided herein, for example, are a nucleotide sequence (SEQ ID NO: 71) that encodes a *Candida viswanathii* acetyl-carnitine translocase and the corresponding amino acid sequence (SEQ ID NO: 14). Nucleotide sequences encoding additional acetyl-carnitine translocase proteins include, but are not limited to: *Saccharomyces cerevisiae CRC1* (Genbank accession number AJ250124) and *Aspergillus nidulans AcuH* (Genbank accession number AJ011563).

Presence, absence or amount of an acetyl-carnitine translocase activity can be detected by any suitable method known in the art and/or described herein. For example, detection can be performed using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cells or organisms exhibit increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism. Methods of evaluating the activity

of an acetyl-carnitine translocase include, for example, measuring carnitine uptake into and/or efflux from liposomes reconstituted with acetyl-carnitine translocase protein purified from microbial cells expressing the protein (see, e.g, , Palmieri *et al*. (1999) *FEBS Lett* 462:472-276).

In another example, a host acetyl-carnitine translocase activity can be increased, for example, by increasing the number of copies of an acetyl-carnitine translocase gene (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the gene), by increasing the activity of a promoter that regulates transcription of an acetyl-carnitine translocase gene, or by increasing the number of copies of an acetyl-carnitine translocase gene and increasing the activity of a promoter that regulates transcription of an acetyl-carnitine translocase gene. In some embodiments, an acetyl-carnitine translocase is endogenous to the host microorganism. Acetyl-carnitine translocase activities can also be increased, for example, by using an inducible promoter, e.g, a glucose- or fatty acid-inducible promoter for regulating transcription of an acetyl-carnitine translocase-encoding nucleic acid, and culturing the recombinant cell or microorganism in media containing a transcription-inducing carbon source.

Thus, in another example of modifying the expression of an acetyl-carnitine translocase in a cell, the promoter used for expression of nucleic acid encoding a mitochondrial acetyl-carnitine transport protein can be modified relative to an endogenous promoter encoding a transport protein. A promoter that is weaker, stronger and/or differently regulated than any endogenous mitochondrial acetyl-carnitine translocase gene promoter will provide for modified expression levels of the translocase protein. To achieve such modified expression, an endogenous promoter of a gene encoding a mitochondrial inner membrane acetyl-carnitine translocase can, in effect, be replaced with another promoter. This can be accomplished, for example, by introducing into a cell or microorganism a heterologous nucleic acid construct that includes a translocase-encoding sequence of nucleotides operably linked to a promoter that provides modified transcription or expression in the cell or microorganism relative to the endogenous promoter. The cell or microorganism can be one in which the endogenous gene(s) encoding a mitochondrial acetyl-carnitine translocase has been disrupted or deleted. For example, a host organism could be a yeast, e.g., a *Candida* yeast, in which the endogenous promoter of the mitochondrial acetyl-carnitine translocase includes an oleate-responsive element allowing for fatty acid induction. An example of a weaker promoter that would not be fatty acid inducible and provide for decreased acetyl-carnitine translocase expression, particularly when exposed to fatty acids as a carbon source, could be a yeast glucose-6-phosphate isomerase gene



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promoter. Modifying a promoter in this way provides another method for decreasing the amount and/or activity of acetyl-carnitine translocase protein in a host. This method is particularly advantageous when decreasing or eliminating acetyl-carnitine translocase activity through gene disruption is detrimental to cell growth and/or viability.

*Modification of mitochondrial carnitine acetyltransferase activity*

Acetyl-carnitine can be generated and degraded by the action of carnitine acetyltransferases (e.g., EC 2.3.1.7). In another embodiment provided herein, the amount of acetyl group carbons in the cytosol in the form of acetyl-carnitine in transit from the peroxisome to the mitochondria can be modified through altering the amount and/or activity of mitochondrial carnitine acetyltransferase. For example, by decreasing the activity level of mitochondrial carnitine acetyltransferase, there can be a corresponding decrease in conversion of acetyl-carnitine to acetyl-CoA in the mitochondria. This can introduce a bottleneck in acetyl-carnitine processing in the mitochondria which can have the effect of diverting acetyl-carnitine from entering the mitochondria from the cytoplasm. Alternatively, by increasing the activity level of mitochondrial carnitine acetyltransferase, there can be a corresponding increase in conversion of acetyl-carnitine to acetyl-CoA in the mitochondria which can augment acetyl-carnitine processing and avoid slowing of mitochondrial entry of acetyl-carnitine due to bottlenecks that might occur in the presence of increased amounts of acetyl-carnitine in the cytoplasm.

In some organisms, such as, for example, certain yeast species, carnitine acetyltransferase is dually targeted to mitochondria and peroxisomes by N-terminal and C-terminal targeting signals, respectively (see, e.g., Elgersma *et al.* (1995) *EMBO J.* 14: 3472-3479 and Kawachi *et al.* (1996) *Eur. J. Biochem.* 238: 845-852). An N-terminal sequence is referred to as the mitochondrial targeting signal (mts) and a C-terminal sequence is referred to as the peroxisomal targeting sequence (pts). An example of such an enzyme is the carnitine O-acetyltransferase enzyme encoded by some yeast *CAT2* genes.

Modifying carnitine acetyltransferase activity in mitochondria can be accomplished by modifying the amount of mitochondrial carnitine acetyltransferase protein expression in a cell, for example, by replacing the wild-type promoter of an endogenous gene in a cell or organism with a weaker or stronger heterologous promoter, and/or replacing or modifying a gene encoding a wild-type carnitine acetyltransferase such that the encoded modified or substituted carnitine



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acetyltransferase protein has a reduced or increased enzyme activity. For example, a host carnitine acetyltransferase activity can be decreased by disruption (e.g., knockout, insertion mutagenesis, the like and combinations thereof) of a host gene encoding the protein, or by decreasing the activity of the promoter (e.g., through addition of repressor sequences to the promoter or 5'UTR or replacing the promoter) that controls transcription of a carnitine acetyltransferase gene using recombinant molecular biology techniques known in the art and/or described herein. In one embodiment, a diploid yeast, such as, for example, a *Candida* yeast, when used as a host microorganism can be subjected to genetic modification in which one of the two alleles of a mitochondrial carnitine acetyltransferase gene is disrupted or deleted. In so doing, a single allele of the gene remains for a reduced amount of carnitine acetyltransferase expression in the microorganism and a reduced amount of the protein in the mitochondria. This can effectively reduce and/or slow the amount of acetyl-carnitine that is processed into acetyl-CoA in the mitochondria without completely eliminating a minimal supply of acetyl carbons that may be required for cellular respiration that occurs in the mitochondria, yet provides for increased retention of acetyl-carnitine in the cytosol. In some instances, the amount of carnitine acetyltransferase activity remaining after disruption of a single allele of a mitochondrial carnitine acetyltransferase gene of a diploid cell or organism may be at a higher level than desired. In such cases, both alleles may be disrupted. A heterologous nucleic acid encoding a carnitine acetyltransferase that is less active than an endogenous carnitine acetyltransferase (or nucleic acid encoding a carnitine acetyltransferase that is linked to a weak promoter) can be introduced into host cells or organisms in which all alleles of the endogenous gene have been disrupted.

One method for disrupting an endogenous carnitine acetyltransferase gene is by recombinantly inserting a heterologous nucleic acid (e.g., a nucleotide sequence encoding a selectable marker such as an enzyme that restores an auxotrophic host cell or organism to prototrophy) into the endogenous gene, thereby generating an engineered cell or organism deficient in carnitine acetyltransferase activity. This can be done, for example, through homologous recombination in which a heterologous nucleic acid containing sequences of the endogenous carnitine acetyltransferase gene and a disrupting sequence (e.g., a knock-out gene cassette such as described herein) is introduced into a host cell or microorganism. Nucleic acids encoding a carnitine acetyltransferase can be obtained from a number of sources, including, for example, yeast cells. For example, genomic DNA from cell sources can be amplified using oligonucleotide primers based on the nucleotide sequence of a carnitine acetyltransferase-encoding gene. Provided herein, for example, are a nucleotide sequence (SEQ ID NO: 59) that



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encodes a *Candida viswanathii* carnitine acetyltransferase (*CAT2* gene) and the corresponding amino acid sequence (SEQ ID NO: 2). Nucleotide sequences encoding additional carnitine acetyltransferase proteins include, but are not limited to: *Saccharomyces cerevisiae CAT2* (Genbank accession numbers Z14021, NM_001182400), *Candida tropicalis CAT2* (Genbank accession number D84549), *Candida glabrata CAT2* (Genbank accession number AF2811), *Candida albicans CAT2* (Genbank accession numbers AF525684), *Aspergillus nidulans AcuJ* (Genbank accession number XM_658791) and *Cyberlindnera jadinii* (Genbank accession number AB641826).

In cells or organisms in which a mitochondrial carnitine acetyltransferase is encoded by a gene that generates a protein containing mitochondrial and peroxisomal targeting sequences, it may be desired to modify only the mitochondrial enzyme, and continue expression of the peroxisomal enzyme. In this instance, an endogenous mitochondrial/peroxisomal carnitine acetyltransferase gene can be disrupted or deleted and heterologous nucleic acids separately encoding a mitochondrial-targeted enzyme and a peroxisomal-targeted enzyme can be introduced into the cell or microorganism. For example, a peroxisomal-targeted enzyme that would not be expressed in the mitochondria can be produced in a cell or microorganism by introducing a heterologous nucleic acid that encodes a carnitine acetyltransferase that includes a peroxisomal targeting sequence of amino acids but lacks a mitochondrial targeting sequence of amino acids. An example of such a modified *Candida viswanathii* nucleic acid sequence (*CAT2*$^{\Delta mts}$; SEQ ID NO: 60), and the amino acid sequence encoded thereby (Cat2p$^{\Delta mts}$; SEQ ID NO: 3), are provided herein. A mitochondrial-targeted carnitine acetyltransferase that would not be expressed in peroxisomes can be produced in a cell or microorganism by introducing a heterologous nucleic acid that encodes a carnitine acetyltransferase that includes a mitochondrial targeting sequence of amino acids but lacks a peroxisomal targeting sequence of amino acids. An example of such a modified *Candida viswanathii* nucleic acid sequence (*CAT2*$^{\Delta pts}$; SEQ ID NO: 62), and the amino acid sequence encoded thereby (Cat2p$^{\Delta pts}$; SEQ ID NO: 5), are provided herein. A heterologous nucleic acid encoding a mitochondrial-targeted carnitine acetyltransferase that would not be expressed in peroxisomes can also include modifications that alter its expression and or activity in the mitochondria as described herein. For example, regulatory sequences of nucleic acids (e.g, promoter sequences, repressor sequences) can be included that provide for decreased or increased expression of the enzyme and/or an altered pattern of expression of the enzyme. A heterologous nucleic acid encoding a mitochondrial-targeted carnitine acetyltransferase can include modifications that alter its activity,



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e.g., providing for more active or less active enzymatic activity relative to an endogenous mitochondrial carnitine acetyltransferase. The carnitine acetyltransferase activities of host and modified cells and microorganisms can be evaluated and monitored using methods known in the art. For example, methods of isolating peroxisomal and mitochondrial components of yeast cells and of extracting carnitine acetyltransferase from subcellular fractions have been described by Ueda et al. [(1982) *Eur. J. Biochem.*124:205-210] and Kozulic et al. [(1987) *Eur. J. Biochem.*168:245-250]. Methods of measuring the enzymatic activity of carnitine acetyltransferase are also known in the art, see, e.g., Fritz and Schultz (1965) *J. Biol. Chem.* 240:2188-2192; Chase (1969) *Meth. Enzymol.*13:387-393.

In one embodiment provided herein, a heterologous nucleic acid encoding a yeast cytoplasmic carnitine acetyltransferase that has a reduced carnitine acetyltransferase activity relative to the activity of the enzyme encoded by a host microorganism's endogenous mitochondrial carnitine acetyltransferase gene can be introduced into a microbial host in which the endogenous mitochondrial carnitine acetyltransferase gene(s) has been disrupted or deleted. The heterologous nucleic acid encoding the less active carnitine acetyltransferase can be modified to include nucleotides encoding a mitochondrial targeting sequence for expression of the enzyme in the mitochondria. For example, in one aspect, a heterologous nucleic acid encoding a *Candida viswanathii* cytosolic carnitine acetyltransferase (*YAT1*) with added nucleotides encoding a mitochondrial targeting sequence (*YAT1*$^{+mts}$) can be introduced into a host cell or microorganism (e.g., a *Candida viswanathii* cell). Any sequence encoding a mitochondrial targeting from a protein that is localized to mitochondria can be used in generating the heterologous nucleic acid. Examples include, but are not limited to, nucleotides encoding mitochondrial targeting sequences from mitochondrial cytochrome oxidase subunit IV (Cox4p), mitochondrial citrate synthase (Cit1p) and mitochondrial carnitine acetyltransferase (Cat2p) proteins. Nucleotide sequences encoding (and the amino acid sequences of) *Candida viswanathii* Yat1p (amino acid SEQ ID NO: 6 and nucleotide SEQ ID NO: 63), YAT1$^{+mts}$p (amino acid SEQ ID NOS: 10, 11 and 12 and nucleotide SEQ ID NOS: 67, 68 and 69), and the mitochondrial targeting sequences of Cox4p (amino acid SEQ ID NO: 7 and nucleotide SEQ ID NO: 64), Cit1p (amino acid SEQ ID NO: 8 and nucleotide SEQ ID NO: 65) and Cat2p (amino acid SEQ ID NO: 9 and nucleotide SEQ ID NO: 66) are provided herein. Additional non-limiting examples of nucleic acids encoding cytoplasmic carnitine acetyltransferase include: *Saccharomyces cerevisiae YAT1* (Genbank accession number X74553), *Aspergillus nidulans FacC* (Genbank accession number AF023156), *Cyberlindnera jadinii YAT1* (Genbank accession



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number AB641829), *Candida dubliniensis YAT1* (Genbank accession number XM_002416790) and *Candida albicans* (Genbank accession number AF525683). Additional non-limiting examples of nucleic acids encoding mitochondrial targeting sequences include *Saccharomyces cerevisiae Cit1* (nucleotides in Genbank accession number NM_001183178 encoding N-terminal 37 amino acids) and *Saccharomyces cerevisiae Cox4* (nucleotides in Genbank accession number NM_001181052 encoding N-terminal 25 amino acids).

In another example, the amount and/or activity of carnitine acetyltransferase in a cell or microorganism can be increased, for example, by increasing the number of copies of a carnitine acetyltransferase gene (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the gene), by increasing the activity of a promoter that regulates transcription of a carnitine acetyltransferase gene, or by increasing the number of copies of a carnitine acetyltransferase gene and increasing the activity of a promoter that regulates transcription of a carnitine acetyltransferase gene. In some embodiments, a carnitine acetyltransferase is endogenous to the host cell or microorganism. Carnitine acetyltransferase activities can also be increased, for example, by using an inducible promoter, e.g, a glucose- or fatty acid-inducible promoter for regulating transcription of a carnitine acetyltransferase-encoding nucleic acid, and culturing the recombinant cell or microorganism in media containing a transcription-inducing carbon source.

Thus, in another example of modifying the expression of a carnitine acetyltransferase in a cell, the promoter used for expression of nucleic acid encoding a carnitine acetyltransferase protein can be modified relative to an endogenous promoter encoding a carnitine acetyltransferase protein. A promoter that is weaker, stronger and/or differently regulated than any endogenous carnitine acetyltransferase gene promoter will provide for modified expression levels of the protein. To achieve such modified expression, an endogenous promoter of a gene encoding a carnitine acetyltransferase can, in effect, be replaced with another promoter. This can be accomplished, for example, by introducing into a cell or microorganism a heterologous nucleic acid construct that includes a carnitine acetyltransferase-encoding sequence of nucleotides operably linked to a promoter that provides modified expression in the cell or microorganism relative to the endogenous promoter. The cell or microorganism can be one in which the endogenous gene(s) encoding a carnitine acetyltransferase has been disrupted or deleted. For example, a host organism could be a yeast, e.g., a *Candida* yeast, in which the endogenous promoter includes an oleic acid-responsive element allowing for fatty acid induction. An example of a weaker promoter that would not be fatty acid inducible and provide for decreased



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carnitine acetyltransferase expression, particularly when exposed to fatty acids as a carbon source, could be a yeast glucose-6-phosphate isomerase gene promoter.

Different combinations of transcription regulatory elements (e.g., promoters) and enzymes can be utilized to achieve an optimal level of activity of carnitine acetyltransferase (or other enzyme being modified) in a cell or microorganism modified to alter carbon flux therein. For example, in embodiments in which a decreased level, but not an elimination, of an activity, such as mitochondrial carnitine acetyltransferase, in a cell or organism is desired, an optimal activity level may be achieved by using a strong and/or inducible promoter to express nucleic acid encoding a protein having a decreased activity. In one embodiment described herein, the mitochondrial carnitine acetyltransferase activity of a host organism (e.g., *Candida* yeast) is decreased by disrupting both alleles of the endogenous gene encoding mitochondrial carnitine acetyltransferase and introducing heterologous nucleic acid encoding a mitochondrial-targeted carnitine acetyltransferase having a lower enzyme activity than the endogenous mitochondrial carnitine acetyltransferase. To ensure that the level of enzyme activity provided by the less active mitochondrial-targeted carnitine acetyltransferase is sufficient and optimal in the modified cell, a strong, fatty acid-inducible promoter (e.g., an *HDE* gene promoter) can be linked to the nucleic acid encoding the less active enzyme to regulate transcription and production of a desired amount of the enzyme.

*Modification of cytosolic carnitine acetyltransferase activity*

Included in embodiments of the cells, microorganisms, compositions and methods provided herein are microbial production platform systems and components thereof in which the amount of carnitine acetyltransferase in the cell cytosol and/or carnitine acetyltransferase activity in the cell cytosol is modified. In some instances, the amount of carnitine acetyltransferase in the cell cytosol is increased and/or decreased, and/or carnitine acetyltransferase activity in the cell cytosol is increased and/or decreased. For example, in some aspects, a cell or microorganism may be modified to increase cytosolic carnitine acetyltransferase and/or carnitine acetyltransferase activity, may be modified to decrease cytosolic carnitine acetyltransferase and/or carnitine acetyltransferase activity, or may be modified to alternately increase and decrease cytosolic carnitine acetyltransferase and/or carnitine acetyltransferase activity depending on the conditions in which the modified cell or microorganism is cultured.



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In some embodiments, the capture of carbon atoms in the acetyl group of acetyl-CoA generated from metabolic processes such as peroxisomal β-oxidation can be accomplished by increasing the amount of carnitine acetyltransferase protein and/or activity in the cell cytosol of a cell or microorganism. In so doing, there is an increased conversion of acetyl-carnitine, such as that which is in transit from the peroxisome to the mitochondria, into acetyl-CoA in the cytoplasm. In one aspect, the amount and/or activity of a host cytosolic carnitine acetyltransferase can be increased, for example, by increasing the number of copies of a gene encoding a cytoplasmic carnitine acetyltransferase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the gene), by increasing the activity of a promoter that regulates transcription of a gene encoding a cytoplasmic carnitine acetyltransferase, or by increasing the number of copies of a gene encoding a cytoplasmic carnitine acetyltransferase and increasing the activity of a promoter that regulates transcription of a gene encoding a cytoplasmic carnitine acetyltransferase. In some embodiments, a cytoplasmic carnitine acetyltransferase is endogenous to the host cell or microorganism.

In one embodiment of the cell and microbial systems and methods provided herein, the amount of carnitine acetyltransferase protein expressed in the cytosol can be increased by introducing heterologous nucleic acid encoding a cytoplasmic carnitine acetyltransferase into a cell or microorganism. In some cells and microorganisms, e.g., some yeast strains, cytoplasmic carnitine acetyltransferase is encoded by a gene that is distinct from the gene(s) encoding mitochondrial and/or peroxisomal carnitine acetyltransferase. For example, in some yeast strains, a cytoplasmic carnitine acetyltransferase is encoded by a *YAT* gene, whereas a mitochondrial and/or peroxisomal carnitine acetyltransferase is encoded by a *CAT* gene. Nucleotide sequences encoding (and the amino acid sequences of) *Candida viswanathii* Yat1p (amino acid SEQ ID NO: 6 and nucleotide SEQ ID NO: 63) are provided herein. Additional non-limiting examples of nucleic acids encoding cytoplasmic carnitine acetyltransferase include *Saccharomyces cerevisiae YAT1* (Genbank accession number X74553), *Aspergillus nidulans FacC* (Genbank accession number AF023156), *Cyberlindnera jadinii YAT1* (Genbank accession number AB641829), *Candida dubliniensis YAT1* (Genbank accession number XM_002416790) and *Candida albicans* (Genbank accession number AF525683).

In another embodiment, the amount of cytoplasmic carnitine acetyltransferase can be modified by modifying the activity of a promoter that regulates transcription of a gene encoding a cytoplasmic carnitine acetyltransferase. Thus, in another example of modifying the expression

of a cytosolic carnitine acetyltransferase in a cell, the promoter used for expression of nucleic acid encoding a cytosolic carnitine acetyltransferase protein can be modified relative to an endogenous promoter encoding a cytosolic carnitine acetyltransferase protein. A promoter that is weaker, stronger and/or differently regulated than any endogenous cytosolic carnitine acetyltransferase gene promoter will provide for modified expression levels of the protein. To achieve such modified expression, an endogenous promoter of a gene encoding a cytosolic carnitine acetyltransferase can, in effect, be replaced with another promoter. This can be accomplished, for example, by introducing into a cell or microorganism a heterologous nucleic acid construct that includes a cytosolic carnitine acetyltransferase-encoding sequence of nucleotides operably linked to a promoter that provides modified expression in the cell or microorganism relative to the endogenous promoter. The cell or microorganism can be one in which the endogenous gene(s) encoding a cytosolic carnitine acetyltransferase has been disrupted or deleted. For example, a host organism could be a yeast, e.g., a *Candida* yeast, in which the endogenous promoter does not include an oleic acid-responsive element allowing for fatty acid induction. An example of a stronger promoter that would be fatty acid inducible and provide for increased cytosolic carnitine acetyltransferase expression, particularly when exposed to fatty acids as a carbon source, is a peroxisomal protein gene and/or β-oxidation enzyme gene promoter, e.g., a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequence of a *Candida viswanathii* HDE gene promoter (SEQ ID NO: 113) is provided herein as are examples of additional fatty acid-inducible promoters.

In a further embodiment, cytosolic carnitine acetyltransferase activity can be modified by introducing into a cell or microorganism a heterologous nucleic acid encoding a carnitine acetyltransferase that is more active or less active than an endogenous cytosolic carnitine acetyltransferase. For example, a heterologous nucleic acid encoding an enzyme that has an increased carnitine acetyltransferase activity relative to the activity of a cytosolic carnitine acetyltransferase expressed in the host cell or microorganism can be introduced into a host to provide for increased generation of cytosolic acetyl-CoA from acetyl-carnitine. The host can be one in which the endogenous cytosolic carnitine acetyltransferase gene(s) has been disrupted or deleted. The heterologous nucleic acid encoding the more active carnitine acetyltransferase can, if required, be altered to exclude any nucleotides encoding a cell localization (e.g., mitochondria, peroxisomes) targeting sequence in order to provide for expression of the enzyme in the cytosol.



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Thus, for example, engineered carnitine O-acetyltransferase proteins lacking amino acid sequence targeting signals that direct the enzyme to one or more cellular locations other than the cytoplasm can be expressed in host cells thereby increasing the amount of carnitine O-acetyltransferase in the cytoplasm. Such engineered proteins will remain in the cytoplasm after being produced by the cell or organism. One such modified carnitine O-acetyltransferase protein is a yeast Cat2p$^{\Delta mts\Delta pts}$ lacking a mitochondrial targeting signal (mts) and a perioxisomal targeting signal (pts). In some instances, a mitochondrial and/or peroxisomal carnitine acetyltransferase (e.g., a yeast Cat2p) may be more active than an endogenous cytosolic carnitine acetyltransferase (e.g., a yeast Yat1p). A more active Cat2p enzyme can be expressed in the cytosol of a host upon introduction of heterologous nucleic acid (e.g., *CAT2*$^{\Delta mts\Delta pts}$) encoding the more active enzyme lacking mitochondrial- and peroxisomal-targeting sequences. In a particular embodiment, the Cat2p enzyme can be a *Candida* yeast protein. An example of a *Candida viswanathii* nucleotide sequence (*CAT2*$^{\Delta mts\Delta pts}$; SEQ ID NO: 61) encoding a carnitine acetyltransferase lacking mitochondrial- and peroxisomal-targeting sequences (Cat2p$^{\Delta mts\Delta pts}$; SEQ ID NO: 4) is provided herein.

Additional examples of nucleotide sequences encoding carnitine acetyltransferase proteins include: *Saccharomyces cerevisiae CAT2* (Genbank accession numbers Z14021, NM_001182400), *Candida tropicalis CAT2* (Genbank accession number D84549), *Candida glabrata CAT2* (Genbank accession number AF2811), *Candida albicans CAT2* (Genbank accession numbers AF525684), *Aspergillus nidulans AcuJ* (Genbank accession number XM_658791), *Neurospora crassa* (Genbank accession number XM_957579) and *Cyberlindnera jadinii CAT2* (Genbank accession number AB641826). Any of these, and other such carnitine acetyltransferase-encoding nucleic acids, can be analyzed for the presence of 5' and 3' ORF nucleotides encoding possible mitochondrial- or peroxisomal-targeting sequences of amino acids and modified to eliminate such sequences. For example, the initial approximately 66 base pairs of the *Saccharomyces cerevisiae CAT2* or the *Candida tropicalis CAT2* coding sequence can be excluded to eliminate mitochondrial targeting of the enzyme, while deletion of the terminal 9 base pairs of the coding sequence that encode a 3-amino acid persoxisomal targeting sequence, i.e., PTS1, (AKL or PKL motif) eliminates peroxisomal targeting of the enzyme (see, e.g., Elgersma *et al.* (1995) *EMBO J.* 14: 3472-3479 and Kawachi et al. (1996) *J. Biochem.* 120:731-735). Similarly, the initial approximately 120 base pairs of the *Aspergillus nidulans AcuJ* coding sequence can be excluded to possibly eliminate mitochondrial targeting of the enzyme, while deletion of the terminal 9 base pairs of the coding sequence that encode a 3-



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amino acid PTS1 (AKL motif) may eliminate peroxisomal targeting of the enzyme (see, e.g., Hynes et al. (2011) *Eukarot. Cell* 10:547-555). In general, yeast mitochondrial targeting sequences occur within the initial 10-90 N-terminal amino acid residues of a mitochondrial protein, have a significant arginine composition and very few to no negatively charged residues. Prediction tools, e.g., MitoProt, TargetP, Predotar and TPpred2, can be used in evaluating an amino acid sequence for identification of possible mitochondrial targeting sequences (see, e.g., Claros (1995) *Comput. Appl. Sci.* 11:441-447; Emanuelsson et al. (2000) *J. Mol. Biol*. 300:1005-1016; Small et al. (2004) *Proteomics* 4:1581-1590; Savojardo et al. (2014) *Bioinformatics* 30:2973-2974). Yeast peroxisomal targeting sequences generally occur at the C-terminus of a peroxisomal protein. Generally, the 3-amino acid consensus sequence of a yeast PTS1 has an initial amino acid containing a small, uncharged side chain (e.g., serine, alanine and cysteine), followed by a positively charged residue (e.g., lysine, arginine and histidine) and ending with a leucine residue; however, variants (e.g., PKL and others) of the consensus sequence do occur. Another example of a peroxisomal targeting signal sequence is the tripeptide AKI of the *Candida tropicalis* trifunctional enzyme hydratase-dehydrogenase-epimerase (HDE) (see, e.g., Aitchison et al. (1991) *J. Biol. Chem*. 266(34):23197-23203. Prediction tools, e.g., PTSI Predictor (mendel.imp.ac.at/mendeljsp/sat/pts1/PTS1predictor.jsp), can be used in evaluating an amino acid sequence for identification of possible peroxisomal targeting signal sequences (see, e.g., Brocard and Hartig (2006) *Biochim. Biophys. ACTA* 1763:1565-1573).

The promoter used for regulating transcription of a heterologous nucleic acid encoding a carnitine acetyltransferase that is more active or less active than an endogenous cytosolic carnitine acetyltransferase can also be modified. For example, the amount of a more active carnitine acetyltransferase protein expressed in the cytosol may be increased by including in the heterologous nucleic acid a stronger heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism.

Alternatively, decreasing carnitine acetyltransferase activity in the cytosol can be accomplished by modifying the amount of cytosolic carnitine acetyltransferase protein expression in a cell, for example, by replacing the wild-type promoter of an endogenous cytosolic carnitine acetyltransferase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type cytosolic carnitine acetyltransferase such that the encoded modified or substituted carnitine acetyltransferase protein has a reduced enzyme activity. For example, expression of a host



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cytosolic carnitine acetyltransferase activity can be decreased by disruption (e.g., knockout, insertion mutagenesis, the like and combinations thereof) of a host gene encoding the protein, or by decreasing the activity of the promoter (e.g., through addition of repressor sequences to the promoter or 5'UTR or replacing the promoter) that controls transcription of a cytosolic carnitine acetyltransferase gene using recombinant molecular biology techniques known in the art and/or described herein. In one embodiment, a diploid yeast, such as, for example, a *Candida* yeast, when used as a host microorganism can be subjected to genetic modification in which one of the two alleles of a cytosolic carnitine acetyltransferase gene is disrupted or deleted. In so doing, a single allele of the gene remains for a reduced amount of carnitine acetyltransferase expression in the microorganism and a reduced amount of the protein in the cytosol. This effectively reduces and/or slows the amount of acetyl-carnitine that is processed into acetyl-CoA in the cytosol.

One method for disrupting an endogenous carnitine acetyltransferase gene is by recombinantly inserting a heterologous nucleic acid (e.g., a nucleotide sequence encoding a selectable marker such as an enzyme that restores an auxotrophic host organism to prototrophy) into the endogenous gene, thereby generating an engineered organism deficient in cytosolic carnitine acetyltransferase activity. This can be done, for example, through homologous recombination in which a heterologous nucleic acid containing sequences of the endogenous cytosolic carnitine acetyltransferase gene and a disrupting sequence (e.g., a knock out gene cassette as described herein) is introduced into a host cell or microorganism. Nucleic acids encoding a cytosolic carnitine acetyltransferase can be obtained from a number of sources, including, for example, yeast cells. Genomic DNA from cell sources can be amplified using oligonucleotide primers based on the nucleotide sequence of a cytosolic carnitine acetyltransferase-encoding gene. For example, in some yeast strains, a cytosolic carnitine acetyltransferase is encoded by a *YAT* gene. Nucleotide sequences encoding (and the amino acid sequences of) *Candida viswanathii* Yat1p (amino acid SEQ ID NO: 6 and nucleotide SEQ ID NO: 63) are provided herein. Additional non-limiting examples of nucleic acids encoding cytoplasmic carnitine acetyltransferase include *Saccharomyces cerevisiae YAT1* (Genbank accession number X74553), *Aspergillus nidulans FacC* (Genbank accession number AF023156), *Cyberlindnera jadinii YAT1* (Genbank accession number AB641829), *Candida dubliniensis YAT1* (Genbank accession number XM_002416790) and *Candida albicans* (Genbank accession number AF525683).



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*Modification of acetyl-CoA generation through oxidative metabolism*

Included in the biological production platform systems and components thereof provided herein are embodiments in which the generation of acetyl-CoA in a cell or organism is modified. In some embodiments, the cellular processing of fatty acids, such as those obtained from external carbon sources (e.g., non-fermentable carbon sources) and internal, cell-generated sources (including, for example, but not limited to, fatty acids generated by catabolism of alkanes, fatty alcohols and fatty aldehydes), can be directed toward acetyl-CoA-generating oxidative metabolism pathways in cells. In some embodiments, the processing of fatty acids can be directed toward oxidative metabolism (e.g., ω- and/or β-oxidation) and away from cellular pathways, such as lipid synthesis pathways, that may not be involved in target molecule production. Accordingly, provided herein are cells, organisms, compositions and methods in which cellular carbon flux has been modified through alterations in cellular oxidative metabolism and/or fatty acid activation. In particular embodiments, cellular carbon flux has been modified to increase the production of acetyl-CoA in a cell through altering oxidative metabolism and/or fatty acid activation. Carbon flux modifications involving oxidative metabolism are particularly useful in embodiments in which alternative, non-carbohydrate carbon sources (e.g., some non-fermentable carbon sources) are used as a feedstock for modified cells and organisms in target molecule production.

For example, some organisms (e.g., some species of *Candida*, *Yarrowia*, *Pichia*, *Debaryomyces*, *Acinetobacter, Bacillus, Mycobaterium, Pseudomonas, Sphingomonas, Alcanivorax and Rhodococcus*) are able to endogenously assimilate alkanes as a carbon source. A primary pathway for alkane assimilation (also referred to as monoterminal alkane oxidation), which occurs in association with the endoplasmic reticulum and peroxisomes in eukaryotes, is through the initial conversion of alkanes to fatty acids which can then be metabolized in the cells. The conversion can occur through a three-step process as follows: (1) terminal hydroxylation of alkane by a cytochrome P450-dependent monooxygenase system (e.g, *ALK* gene products of the CYP52 family as a terminal oxidase and an NADPH-dependent cytochrome P450 reductase (e.g., *CPR1*-encoded) for electron transfer) which yields a fatty alcohol; (2) conversion of the terminal hydroxy group of the alcohol to a fatty aldehyde in reactions involving fatty alcohol dehydrogenase (e.g., *ADH*) or fatty alcohol oxidase (e.g., *FAO*); and (3) conversion of the fatty aldehyde to a fatty acid by a fatty aldehyde dehydrogenase. The



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resulting fatty acid can then be subject to the same metabolic processing as is a fatty acid taken up directly by the organism.

Fatty acids can be metabolized in several ways depending on the type of cell or organism. Many fatty acid metabolic pathways, including β-oxidation, lipid biosynthesis, and protein acylation, require that a fatty acid be activated by thioesterification to coenzyme A (i.e., acyl-CoA), or to an acyl carrier protein (i.e., acyl-ACP), prior to being metabolized. As used herein, "activation" with reference to fatty acids refers to the thioesterification of a fatty acid with a carrier molecule such as coenzyme A (Co-A) or acyl carrier protein (ACP). A fatty acid that has undergone activation into an acyl-CoA or fatty acid-ACP molecule is referred to as an activated fatty acid. The thioesterification reaction can be catalyzed by acyl-CoA synthetase enzymes. There are multiple enzymes having acyl-CoA synthetase activity in cells which differ based on cellular localization (e.g., plasma membrane, cytosol, endoplasmic reticulum membrane, peroxisomes) and substrate (e.g., fatty acid carbon chain length) specificity. In general, plasma membrane-associated acyl-CoA synthetases often are more specific for very long chain fatty acids and are involved in transport of these hydrophobic molecules across the membrane concurrent with activation of the fatty acids to acyl-CoA. Once activated, the acyl-CoA can then be used in a number of metabolic pathways, only one of which is β-oxidation. Thus, fatty acids activated at the plasma membrane and/or in the cytosol can represent possible "losses" of carbon atoms to cellular synthesis pathways (e.g., lipid synthesis) at the expense of other target molecule production pathways (e.g., oxidative metabolism, malonyl-CoA production and organic acid, polyketide and/or terpene synthesis). Therefore, in some embodiments of cells and organisms provided herein for use in target molecule production systems, it can be beneficial to capture the carbon atoms of free fatty acids for use in target molecule production and decrease activated fatty acid (e.g., acyl-CoA) flow into cellular pathways not associated with target molecule production. As described herein, methods of enhancing fatty acid carbon flow through oxidative metabolism include, but are not limited to, modification of activities of cellular ω- and/or β-oxidation systems and acyl-CoA synthetase activities.

*Modification of ω-oxidation*

In the oxidative metabolism pathway referred to as ω-oxidation (or diterminal oxidation), fatty acids can be converted to dicarboxylic acids (diacids). Several enzyme activities (e.g., cytochrome P450 hydroxylase complex, fatty alcohol oxidase and fatty aldehyde



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dehydrogenase) can be involved in the process of ω-oxidation. The term "ω-oxidation pathway" as used herein, refers to a cellular metabolic pathway constituted by a series of enzymatic activities through which fatty acids and alkanes are converted to dicarboxylic acids. Some cells and microorganisms (e.g., species of yeast and bacteria) endogenously express the enzyme activities, and those that do not can be genetically modified to provide a heterologous ω-oxidation pathway by introducing nucleic acids encoding the required enzymes into cells and expressing the proteins therein. Free fatty acids internalized into cells, or generated within cells (e.g., by oxidation of internalized alkanes), can directly enter into and be processed in the ω-oxidation pathway without prior activation to acyl-CoA. In some embodiments of the cell-based production systems and methods provided herein, dicarboxylic acids can be a target molecule. In some embodiments, e.g., in which some shorter-chain dicarboxylic acids are a target molecule, or dicarboxylic acids are not a target molecule (or a co-target molecule along with one or more other desired products), dicarboxylic acids generated by ω-oxidation can be further oxidized through β-oxidation. Dicarboxylic acids can traverse peroxisomal membranes in eukaryotic cells and be metabolized to yield acetyl-CoA that can be used in target molecule generation. Because free fatty acids can be processed through ω-oxidation without being activated by thioesterification with Co-A, and diacids resulting from ω-oxidation of fatty acids readily move into peroxisomes, the ω-oxidation pathway can serve as a cellular gateway for funneling internalized fatty acids into oxidative metabolism and away from cytosolic activation that is required for use of fatty acids in other cellular pathways that may not be involved in target molecule production.

The term "ω-oxidation activity" refers to any of the activities in the ω-oxidation pathway utilized to metabolize alkanes and fatty acids. The activities that may be utilized in this metabolic pathway include, but are not limited to, monooxygenase activity (e.g., cytochrome P450 activity), monooxygenase reductase activity (e.g., cytochrome P450 reductase activity), alcohol dehydrogenase activity (e.g., fatty alcohol dehydrogenase activity or long-chain alcohol dehydrogenase activity), fatty alcohol oxidase activity and fatty aldehyde dehydrogenase activity. In some embodiments of the cells, organisms, compositions and methods provided herein, the ω-oxidation activity of a cell or organism is modified. In one embodiment, one or more of the activities in the ω-oxidation pathway can be modified. In particular embodiments, one or more of the activities in the ω-oxidation pathway can be increased.



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*Modification of a monooxygenase activity*

The initial step in the ω-oxidation pathway is the conversion of a fatty acid to a corresponding fatty alcohol and involves NADPH and molecular oxygen. A cytochrome P450 enzyme (e.g., monooxygenase activity, EC 1.14.14.1) often catalyzes the insertion of one atom of oxygen bound to the heme group in cytochrome P450 into an organic substrate (RH) while the other oxygen atom is reduced to water. A cytochrome P450 reductase catalyzes the reductive splitting of the oxygen and transfer of electrons from NADPH to cytochrome P450. Insertion of the oxygen atom near the omega carbon of a substrate yields an alcohol derivative of the original starting substrate (e.g., yields a fatty alcohol). In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of a monooxygenase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of a monooxygenase, may be modified to decrease the amount and/or activity of a monooxygenase, or may be modified to alternately increase and decrease the amount and/or activity of a monooxygenase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of a monooxygenase in a cell is increased. Increasing the amount and/or activity of a monooxygenase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway involving oxidative metabolism and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, the monooxygenase activity is unchanged in a host or engineered cell or organism. In one embodiment, the amount and/or activity of a host monooxygenase can be increased, for example, by increasing the number of copies of a nucleic acid encoding a monooxygenase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid), by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a monooxygenase, or by increasing the number of copies of a nucleic acid encoding a monooxygenase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a monooxygenase. In some embodiments, a monooxygenase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of monooxygenase protein expressed in a cell can be



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increased by introducing heterologous nucleic acid encoding a monooxygenase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding a monooxygenase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed.

In some embodiments, a cytochrome P450 monooxygenase enzyme can be a fungal or bacterial protein. In a particular embodiment, the monooxygenase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*), *Fusarium* (e.g., *F. oxysporum*), *Bacillus* (e.g., *B. megaterium*, *B. subtilis*) protein. *Candida tropicalis* contains a family of cytochrome P450 genes referred to as *CYP* genes. Examples of *Candida viswanathii* nucleotide sequences encoding polypeptides having monooxygenase activities are provided herein (nucleotide SEQ ID NO: 99 and amino acid SEQ ID NO: 45) and in International patent application no. PCT/US2012/045615 (publication no. WO 2013/106730). Additional nonlimiting examples of nucleotide sequences encoding polypeptides having monooxygenase activity include: *Candida tropicalis CYP52A12* (Genbank accession no. AY230498), *Candida tropicalis CYP52A13* (Genbank accession no. AY230499), *Candida tropicalis CYP52A14* (Genbank accession no. AY230500), *Candida tropicalis CYP52A15* (Genbank accession no. AY230501), *Candida tropicalis CYP52A16* (Genbank accession no. AY230502), *Candida tropicalis CYP52A17* (Genbank accession no. AY230504), *Candida tropicalis CYP52A18* (Genbank accession no. AY230505), *Candida tropicalis CYP52A19* (Genbank accession no. AY230506), *Candida tropicalis CYP52A20* (Genbank accession no. AY230507), *Candida tropicalis CYP52D2* (Genbank accession no. AY230503), *Bacillus megaterium CYPBM3* (Genbank accession no. KC839476) and *Fusarium oxysporum CYP505* (Genbank accession no. AB030037).

Monooxygenase activity can be provided by any suitable polypeptide, such as a cytochrome P450 polypeptide (CYP450) in certain embodiments. Examples of a polypeptide having CYP450 activity include CYP52A12, a CYP52A13, a CYP52A14, a CYP52A15, a CYP52A16, a CYP52A17, a CYP52A18, a CYP52A19, a CYP52A20, a CYP52D2, and/or a BM3. In some embodiments, the activity can be a single polypeptide with both monooxygenase and monooxygenase reductase activities (e.g., *B. megaterium* cytochrome P450:NADPH P450 reductase, *Fusarium oxysporum* CYP505). Presence, absence or amount of cytochrome P450 activity can be detected by any suitable method known in the art. For example, detection can be performed by assaying a reaction containing cytochrome P450 (CYP52A family) and NADPH

- cytochrome P450 reductase (see, e.g., Craft et al. (2003) *Appl. Environ. Microbiol.* 69: 5983 and 5992). Nucleic acid sequences encoding native and/or modified CYP450 sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a heterologous nucleic acid encoding a monooxygenase can also be modified. For example, the amount of a monooxygenase protein expressed in a particular cellular location may be increased by including in the heterologous nucleic acid a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequence of a *Candida viswanathii* HDE gene promoter is provided herein as are examples of additional fatty acid-inducible promoters. Promoter elements from different monooxygenase-encoding genes can have differing responsiveness to induction by various carbon sources. Thus, the amount of a monooxygenase protein expressed in a cell or organism can be modified by using heterologous promoters from different cytochrome P450 monoxygenase genes to regulate transcription of a monoxgenase-encoding nucleic acid that is introduced into a host cell and by the carbon source provided to the modified cell or organism. Non-limiting examples of assays suitable for assessing induction of cytochrome P450 (or other protein) expression by a carbon source or feedstock include RT-PCR or qRT-PCR after the host cell or microorganism has been exposed to the chosen carbon source or feedstock for varying amounts of time.

Decreasing monooxygenase activity in a cell can be accomplished by modifying the amount of monooxygenase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous monooxygenase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type monooxygenase such that the encoded modified or substituted monooxygenase protein has a reduced enzyme activity.



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*Modification of a cytochrome P450 reductase activity*

A cytochrome P450 reductase (e.g., monooxygenase reductase activity or NADPH:cytochrome oxidoreductase (NCP); EC 1.6.2.4) can catalyze the reduction of the heme-thiolate moiety in cytochrome P450 by transferring electrons to the cytochrome P450. This activity recycles cytochrome P450 and makes it available for further use in catalyzing reactions that occur in ω-oxidation of fatty acids. In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of a cytochrome P450 reductase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of a cytochrome P450 reductase, may be modified to decrease the amount and/or activity of a cytochrome P450 reductase, or may be modified to alternately increase and decrease the amount and/or activity of a cytochrome P450 reductase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of a cytochrome P450 reductase in a cell is increased. Increasing the amount and/or activity of a cytochrome P450 reductase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway involving oxidative metabolism and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, the cytochrome P450 reductase activity is unchanged in a host or engineered cell or organism. In one embodiment, the amount and/or activity of a host cytochrome P450 reductase can be increased, for example, by increasing the number of copies of a nucleic acid encoding a cytochrome P450 reductase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid), by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a cytochrome P450 reductase, or by increasing the number of copies of a nucleic acid encoding a cytochrome P450 reductase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a cytochrome P450 reductase. In some embodiments, a cytochrome P450 reductase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of cytochrome P450 reductase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding a cytochrome P450 reductase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding a cytochrome

P450 reductase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed.

In some embodiments, a cytochrome P450 reductase enzyme can be a yeast or bacterial protein. In a particular embodiment, the reductase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*) or *Bacillus* (e.g., *B. megaterium*) protein. In a particular embodiment, the cytochrome P450 reductase enzyme can be a *Candida* yeast protein. *Candida tropicalis* contains two alleles of a cytochrome P450 reductase gene referred to as *CPRa* and *CPRb*. Examples of *Candida viswanathii* nucleotide sequences encoding cytochrome P450 reductase activities are provided herein (nucleotide SEQ ID NO: 90 and amino acid SEQ ID NO: 45) and in International patent application no. PCT/US2012/045615 (publication no. WO 2013/106730). Additional non-limiting examples of nucleotide sequences encoding polypeptides having cytochrome P450 reductase activity include: *Candida tropicalis* (Genbank accession nos. AY705446, AY823228), *Candida bombicola* (Genbank accession no. EF050789), *Bacillus megaterium CYPBM3* (Genbank accession no. KC839476), *Bacillus megaterium* (Genbank accession no. FJ859036).

Presence, absence or amount of cytochrome P450 reductase activity can be detected by any suitable method known in the art. For example, detection can be performed by assaying a reaction containing cytochrome c and NADPH and measuring the rate of cytochrome reduction by monitoring absorbance (see, e.g., He and Chen (2005) *Yeast* 22:481-491; Van Bogaert et al. (2007) *Yeast* 7:922-928). Nucleic acid sequences encoding native and/or modified cytochrome P450 reductase also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a heterologous nucleic acid encoding a cytochrome P450 reductase can also be modified. For example, the amount of a cytochrome P450 reductase protein expressed in a particular cellular location may be increased by including in the heterologous nucleic acid a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene

promoter. The nucleotide sequence of a *Candida viswanathii* HDE gene promoter is provided herein as are examples of additional fatty acid-inducible promoters.

Alternatively, decreasing cytochrome P450 reductase activity in a cell can be accomplished by modifying the amount of cytochrome P450 reductase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous cytochrome P450 reductase gene in an organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type cytochrome P450 reductase such that the encoded modified or substituted cytochrome P450 reductase protein has a reduced enzyme activity.

*Modification of an alcohol dehydrogenase activity*

A second step in the ω-oxidation pathway generally is the conversion of a fatty alcohol to a corresponding fatty aldehyde and involves $NAD^+$- or $NADP^+$-dependent fatty alcohol dehydrogenases and/or hydrogen peroxide-producing fatty alcohol oxidases. Oxidation of the alcohol to an aldehyde may be performed by an enzyme in the fatty alcohol oxidase family (e.g., long-chain fatty alcohol oxidase EC 1.1.3.20), or an enzyme in the alcohol dehydrogenase family (e.g., fatty alcohol dehydrogenase; EC 1.1.1.1). An alcohol dehydrogenase (e.g., fatty alcohol dehydrogenase, long-chain alcohol dehydrogenase) can catalyze the removal of a hydrogen from an alcohol to yield an aldehyde or ketone and a hydrogen atom and NADH.

In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of an alcohol dehydrogenase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of an alcohol dehydrogenase, may be modified to decrease the amount and/or activity of an alcohol dehydrogenase, or may be modified to alternately increase and decrease the amount and/or activity of an alcohol dehydrogenase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of an alcohol dehydrogenase in a cell is increased. Increasing the amount and/or activity of an alcohol dehydrogenase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a



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particular target product pathway involving oxidative metabolism and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, the alcohol dehydrogenase activity is unchanged in a host or engineered cell or organism. In one embodiment, the amount and/or activity of a host alcohol dehydrogenase can be increased, for example, by increasing the number of copies of a nucleic acid encoding an alcohol dehydrogenase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid), by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding an alcohol dehydrogenase, or by increasing the number of copies of a nucleic acid encoding an alcohol dehydrogenase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding an alcohol dehydrogenase. In some embodiments, an alcohol dehydrogenase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of alcohol dehydrogenase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding an alcohol dehydrogenase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding an alcohol dehydrogenase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed.

In some embodiments, an alcohol dehydrogenase enzyme can be a yeast or bacterial protein. In a particular embodiment, the alcohol dehydrogenase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*) or *Bacillus* (*B. stearothermophilus*) protein. In a particular embodiment, the alcohol dehydrogenase enzyme can be a *Candida* yeast protein. *Candida tropicalis* contains at least 6 genes encoding alcohol dehydrogenases. Examples of *Candida viswanathii* nucleotide sequences encoding polypeptides having alcohol dehydrogenase activities are provided herein (nucleotide SEQ ID NO: 100 and amino acid SEQ ID NO: 46) and in International patent application no. PCT/US2012/045615 (publication no. WO 2013/106730). Additional examples of nucleotide sequences encoding polypeptides having alcohol dehydrogenase activity include, but are not limited to: *Candida tropicalis ADH1* (Genbank accession no. XM_002546589), *Candida utilis ADH1* (Genbank accession no. DQ397054), *Candida albicans ADH1* (Genbank accession no. X81694), *Aspergillus flavus ADH1* (Genbank accession no. L27434), *Yarrowia lipolytica ADH1* (Genbank accession no. AF175271), *Yarrowia lipolytica ADH2* (Genbank accession no. AF175272), *Yarrowia lipolytica ADH3* (Genbank accession no. AF175273), *Bacillus*



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*stearothermophilus ADH-HT* (Genbank accession no. Z27089), *Pseudomonas putida ADHA* (Genbank accession no. AF052750).

Presence, absence or amount of alcohol dehydrogenase activity can be detected by any suitable method known in the art. For example, detection can be performed using spectrophotometric assays (see, e.g., Gatter et al. (2014) *FEMS Yeast Res.* 14:858-872). Nucleic acid sequences encoding native and/or modified alcohol dehydrogenase also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a heterologous nucleic acid encoding an alcohol dehydrogenase can also be modified. For example, the amount of an alcohol dehydrogenase protein expressed in a particular cellular location may be increased by including in the heterologous nucleic acid a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequence of a *Candida viswanathii* HDE gene promoter is provided herein as are examples of additional fatty acid-inducible promoters.

Alternatively, decreasing alcohol dehydrogenase activity in a cell can be accomplished by modifying the amount of alcohol dehydrogenase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous alcohol dehydrogenase gene in an organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type alcohol dehydrogenase such that the encoded modified or substituted alcohol dehydrogenase protein has a reduced enzyme activity.

*Modification of fatty alcohol oxidase activity*

A fatty alcohol oxidase (e.g., long-chain alcohol oxidase, EC 1.1.3.20) enzyme can catalyze the oxidation of a fatty alcohol to yield a fatty aldehyde in the peroxisome of a cell. In some embodiments of the cells, organisms, compositions and methods provided herein, the amount



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and/or activity of a fatty alcohol oxidase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of a fatty alcohol oxidase, may be modified to decrease the amount and/or activity of a fatty alcohol oxidase, or may be modified to alternately increase and decrease the amount and/or activity of a fatty alcohol oxidase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of a fatty alcohol oxidase in a cell is increased. Increasing the amount and/or activity of a fatty alcohol oxidase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway involving oxidative metabolism and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, the fatty alcohol oxidase activity is unchanged in a host or engineered cell or organism. In one embodiment, the amount and/or activity of a host fatty alcohol oxidase can be increased, for example, by increasing the number of copies of a nucleic acid encoding a fatty alcohol oxidase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid), by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a fatty alcohol oxidase, or by increasing the number of copies of a nucleic acid encoding a fatty alcohol oxidase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a fatty alcohol oxidase. In some embodiments, a fatty alcohol oxidase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of fatty alcohol oxidase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding a fatty alcohol oxidase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding a fatty alcohol oxidase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed.

In some embodiments, a fatty alcohol oxidase enzyme can be a yeast or bacterial protein. In a particular embodiment, the fatty alcohol oxidase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*), *Yarrowia* (e.g., *Y. lipolytica*) or *Bacillus* (e.g, *B. stearothermophilus*) protein. In a particular embodiment, the fatty alcohol oxidase enzyme can be a *Candida* yeast protein. *Candida tropicalis* contains two genes encoding fatty alcohol oxidase. Examples of *Candida viswanathii* nucleotide sequences encoding polypeptides having



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fatty alcohol oxidase activities are provided herein (nucleotide SEQ ID NO: 101 and amino acid SEQ ID NO: 47) and in International patent application no. PCT/US2012/045615 (publication no. WO 2013/106730). Additional examples of nucleotide sequences encoding polypeptides having fatty alcohol oxidase activity include, but are not limited to: *Candida tropicalis FAO1* (Genbank accession no. AY538780), *Candida tropicalis FAO2a* (Genbank accession no. AY538781), *Candida tropicalis FAO2b* (Genbank accession no. AY538782).

Presence, absence or amount of fatty alcohol oxidase activity can be detected by any suitable method known in the art. For example, detection can be performed using a two-enzyme coupled reaction assay (see, e.g., Eirich et al. (2004) *Appl. Environ. Microbiol.* 70(8):4872-4879). Nucleic acid sequences encoding native and/or modified fatty alcohol oxidase also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a heterologous nucleic acid encoding a fatty alcohol oxidase protein expressed in a particular cellular location may be increased by including in the heterologous nucleic acid a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequence of a *Candida viswanathii* HDE gene promoter is provided herein as are examples of additional fatty acid-inducible promoters.

Alternatively, decreasing fatty alcohol oxidase activity in a cell can be accomplished by modifying the amount of fatty alcohol oxidase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous fatty alcohol oxidase gene in an organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type fatty alcohol oxidase such that the encoded modified or substituted fatty alcohol oxidase protein has a reduced enzyme activity.



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*Modification of aldehyde dehydrogenase activity*

A third step in the ω-oxidation pathway is generally the conversion of a fatty aldehyde to a corresponding fatty acid and involves $NAD^+$- or $NADP^+$-dependent fatty aldehyde dehydrogenases (e.g., long-chain-aldehyde dehydrogenase or fatty aldehyde dehydrogenase; EC 1.2.1.48). In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of a fatty aldehyde dehydrogenase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of a fatty aldehyde dehydrogenase, may be modified to decrease the amount and/or activity of a fatty aldehyde dehydrogenase, or may be modified to alternately increase and decrease the amount and/or activity of a fatty aldehyde dehydrogenase depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of a fatty aldehyde dehydrogenase in a cell is increased. Increasing the amount and/or activity of a fatty aldehyde dehydrogenase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway involving oxidative metabolism and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, the fatty aldehyde dehydrogenase activity is unchanged in a host or engineered cell or organism. In one embodiment, the amount and/or activity of a host fatty aldehyde dehydrogenase can be increased, for example, by increasing the number of copies of a nucleic acid encoding a fatty aldehyde dehydrogenase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid), by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a fatty aldehyde dehydrogenase, or by increasing the number of copies of a nucleic acid encoding a fatty aldehyde dehydrogenase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a fatty aldehyde dehydrogenase. In some embodiments, a fatty aldehyde dehydrogenase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of fatty aldehyde dehydrogenase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding a fatty aldehyde dehydrogenase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding a



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fatty aldehyde dehydrogenase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed.

In some embodiments, a fatty aldehyde dehydrogenase enzyme can be a yeast protein. In a particular embodiment, the fatty aldehyde dehydrogenase enzyme can be a *Candida* (e.g., *C. tropicalis*, *C. viswanathii*, *C. maltosa*, *C. cloacae*) or a *Yarrowia* (e.g., *Y. lipolytica*) yeast protein. In a particular embodiment, the fatty aldehyde dehydrogenase enzyme can be a *Candida* yeast protein. Examples of *Candida viswanathii* nucleotide sequences encoding polypeptides having fatty aldehyde dehydrogenase activities are provided, for example, in International patent application no. PCT/US2012/045615 (publication no. WO 2013/106730). Additional examples of nucleotide sequences encoding polypeptides having fatty aldehyde dehydrogenase activity include, but are not limited to: *Yarrowia lipolytica HFD1* (Genbank accession no. AB935099), *Yarrowia lipolytica HFD2A* (Genbank accession no. AB935101), *Yarrowia lipolytica HFD2B* (Genbank accession no. AB935103), *Yarrowia lipolytica HFD3* (Genbank accession no. AB935104), *Yarrowia lipolytica HFD4* (Genbank accession no. AB935106).

Presence, absence or amount of fatty aldehyde dehydrogenase activity can be detected by any suitable method known in the art. For example, detection can be performed using enzyme activity assays (see, e.g., Iwama et al. (2014) *J. Biol. Chem.* 289(48):33275-33286). Nucleic acid sequences encoding native and/or modified fatty aldehyde dehydrogenase also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cell or organism exhibits decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a heterologous nucleic acid encoding an fatty aldehyde dehydrogenase can also be modified. For example, the amount of a fatty aldehyde dehydrogenase protein expressed in a particular cellular location may be increased by including in the heterologous nucleic acid a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequence of a *Candida viswanathii* HDE gene promoter is provided herein as are examples of additional fatty acid-inducible promoters.

Alternatively, decreasing fatty aldehyde dehydrogenase activity in a cell can be accomplished by modifying the amount of fatty aldehyde dehydrogenase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous fatty aldehyde dehydrogenase gene in an organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type fatty aldehyde dehydrogenase such that the encoded modified or substituted fatty aldehyde dehydrogenase protein has a reduced enzyme activity.

*Modification of β-oxidation*

Another oxidative metabolism pathway, referred to as β-oxidation, is generally a degradative pathway through which fatty acids, typically in the form of fatty acid-CoA esters, can be broken down to shorter chain acyl-CoA and acetyl-CoA. In fungi and plant cells, β-oxidation can occur in peroxisomes, whereas in animal cells it additionally can take place in mitochondria. The β-oxidation pathway generally includes four main reaction steps resulting in an acyl-CoA that is shortened by two carbon atoms which are released as acetyl-CoA. The shortened acyl-CoA molecule can re-enter the pathway after each cycle and be subjected to another removal of two carbons from the acyl carbon chain. As such, the β-oxidation pathway can generate significant amounts of acetyl-CoA and is a major source of acetyl-CoA in cells. Alteration of enzyme activities in the the β-oxidation pathway can also provide for the generation of fatty acid or diacid target molecules including, but not limited to, adipic acid, suberic acid, sebacic acid and dodecanedioic acid (DDDA). Provided herein are cells, organisms, compositions and methods in which cellular carbon flux has been modified through one or more alterations in the β-oxidation pathway. In some embodiments, the β-oxidation pathway is modified by modifying one or more activities in the pathway. In particular embodiments, the β-oxidation pathway is modified to increase the generation of acetyl-CoA in a cell for use in target molecule production. For example, the β-oxidation pathway can be modified to increase one or more activities in the pathway. In some embodiments, β-oxidation can be manipulated (e.g., decreasing one or more pathway activities and/or altering the specificity of one or more activities) to be used as a pathway for production of target fatty acids and diacids (e.g., adipic acid) of a particular carbon chain length.



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The term "β-oxidation pathway" as used herein, refers to a series of cellular enzymatic activities utilized to metabolize fatty alcohols, fatty acids, or dicarboxylic acids. The activities utilized to metabolize fatty alcohols, fatty acids, or dicarboxylic acids can include, but are not limited to, acyl-CoA oxidase activity, acyl-CoA hydrolase activity, enoyl-CoA hydratase activity, 3-hydroxyacyl-CoA dehydrogenase activity, and acetyl-CoA C-acyltransferase activity. The term "β-oxidation activity" refers to any and/or all of the activities in the β-oxidation pathway utilized to metabolize fatty alcohols, fatty acids or dicarboxylic acids. Additional activities, referred to as β-oxidation peripheral or auxillary activities, can be involved in degradation of unsaturated fatty acids (i.e., fatty acid chains containing double bonds) and fatty acids containing modifications (e.g., hydroxyl, methyl, phenoxy groups) including, but not limited to enoyl CoA isomerase ((ECI) or enoyl-CoA delta isomerase 1, dodecenoyl-CoA isomerase, 3,2 trans-enoyl-CoA isomerase, acetylene-allene isomerase, delta3, delta2-enoyl-CoA isomerase, dodecenoyl-CoA delta isomerase, and EC 5.3.3.8), dienoyl CoA Isomerase (DCI, e.g., EC 5.3.3, Δ3,5,Δ2,4-dienoyl-CoA isomerase, Δ3,5,Δ2,4-dienoyl-coenzyme A isomerase) and 2,4-dienoyl-CoA reductase (DCR, e.g., EC 1.3.1.34).

There are also cellular compositions and activities that can be closely associated with β-oxidation. These include peroxisomal- and mitochondrial-related compositions and activities. For example, as described herein, such compositions and activities include, but are not limited to, acyl-CoA synthetases, thioesterases, peroxisomal transport proteins and peroxisomal biogenesis factors. Included in the cells, organisms, systems and methods provided herein are embodiments in which one or more of these β-oxidation-associated compositions and/or activities are modified. In some embodiments, a β-oxidation-associated composition or activity is modified to enhance β-oxidation activity.

*Modification of acyl-CoA oxidase activity*

Typically, the first step in the β-oxidation pathway is oxidation of acyl-CoA, which is carried out by the enzyme acyl-CoA oxidase (e.g., EC 1.3.3.6). This step can be a rate-limiting step in β-oxidation. The term "acyl-CoA oxidase activity" as used herein refers to the enzymatic activity (e.g., catalytic activity) of an acyl-CoA oxidase. An acyl-CoA oxidase can catalyze the following chemical reaction:

$$\text{acyl-CoA} + O_2 \leftrightarrow \text{trans-2-enoyl-CoA} + H_2O_2$$



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Acyl-CoA oxidase enzymes generally contain FAD from which two electrons are transferred to oxygen to generate $H_2O_2$.

Different cells contain different types, and numbers of types, of acyl-CoA oxidase activities. For example, *Saccharomyces cerevisiae* expresses only one acyl-CoA oxidase, Pox1p/Fox1p, which has activity on acyl-CoA substrates with a broad range of carbon chain lengths. In contrast, other organisms, e.g., species of *Candida*, *Yarrowia*, *Arabidopsis*, can have multiple genes encoding different proteins having acyl-CoA oxidase activities with varying substrate specificities. In some embodiments, acyl-CoA oxidase activity refers to its enzyme activity (or lack thereof) on a selective set of substrates. The activity of an acyl-CoA oxidase can be affected by its ability to bind a substrate, oxidize a substrate and/or release a product. In some embodiments, an acyl-CoA oxidase is active in one compartment of a cell and not in another compartment of the cell. In some embodiments, an acyl-CoA oxidase activity is from a peroxisome.

Different Acyl-CoA oxidases can display different carbon chain-length substrate specificities. Some acyl-CoA oxidases display broad chain-length specificity and can accept any fatty acyl-CoA (or diacyl-CoA) as a substrate. However, some acyl-CoA oxidases can display narrow chain-length specificity. For example, the acyl-CoA oxidase activity encoded by the *POX4* gene of *Candida* strain ATCC 20336 has a relatively broad carbon chain-length specificity and exhibits a higher specific activity for acyl-CoA molecules with shorter carbon chain lengths (e.g., less than 10 carbons). The Pox5 enzyme from *Candida* strain ATCC 20336 displays optimal activity on fatty acid substrates having 12 to 18 carbons (C12 - C18) in the carbon chain, a decreased activity on substrates having less than 10 carbons (C10) in the carbon chain and has low activity on C6 and C8 substrates. In a cell with such a *Candida* Pox5 as the only functional acyl-CoA oxidase, long chain fatty acyl-CoA or diacyl-CoA substrates can be shortened to about 8 carbons and do not typically enter another cycle of β-oxidation. The shorter substrates (e.g., a C8 fatty acid or dicarboxylic acid) are not typically recognized as a substrate by Pox5. In this instance, an acyl-CoA would not be completely broken down to acetyl-CoA units. Instead, the chain-length substrate specificity of the acyl-CoA oxidase in such a cell (which would limit further degradation of an acyl-CoA once it has been broken down into about an 8-carbon chain) effectively controls the chain length of an acid or diacid produced through break down of fatty acids through β-oxidation. The shorter substrates (e.g., a C8 acyl-CoA) would remain intact, the



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CoA would be removed by peroxisomal thioesterases and the fatty acid or dicarboxylic acid (e.g., an α,ω - dicarboxylic acid) product is secreted from the cell. In this way, β-oxidation can be manipulated to be used as a pathway for production of target fatty acids and diacids (e.g., adipic acid) of a particular carbon chain length.

In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of one or more acyl-CoA oxidases in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of an acyl-CoA oxidase, may be modified to decrease the amount and/or activity of an acyl-CoA oxidase, or may be modified to alternately increase and decrease the amount and/or activity of one or more acyl-CoA oxidases depending, for example, on the substrate specificity, target molecule(s) being produced, cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of an acyl-CoA oxidase in a cell is increased. Increasing the amount and/or activity of an acyl-CoA oxidase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway involving oxidative metabolism and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, the acyl-CoA oxidase activity is unchanged in a host or engineered cell or organism. In one embodiment, the amount and/or activity of a host acyl-CoA oxidase can be increased, for example, by increasing the number of copies of a nucleic acid encoding an acyl-CoA oxidase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid), by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding an acyl-CoA oxidase, or by increasing the number of copies of a nucleic acid encoding an acyl-CoA oxidase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding an acyl-CoA oxidase. In some embodiments, an acyl-CoA oxidase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of an acyl-CoA oxidase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding an acyl-CoA oxidase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding an acyl-CoA oxidase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed.



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Non-limiting examples of organisms that include, or can be used as donors for, an acyl-CoA oxidase enzyme include yeast (e.g., *Candida*, *Saccharomyces*, *Debaryomyces*, *Meyerozyma*, *Lodderomyces*, *Scheffersomyces*, *Clavispora*, *Yarrowia*, *Pichia*, *Kluyveromyces*, *Eremothecium*, *Zygosaccharomyces*, *Lachancea*, *Nakaseomyces*), animals (e.g., *Homo*, *Rattus*), bacteria (e.g., *Escherichia*, *Pseudomonas*, *Bacillus*), or plants (e.g., *Arabidopsis*, *Nictotania*, *Cuphea*). In a particular embodiment, an acyl-CoA oxidase enzyme can be a *Candida* yeast protein. Examples of *Candida viswanathii* nucleotide sequences encoding acyl-CoA oxidases are provided herein (nucleotide SEQ ID NOS: 92 and 93 and amino acid SEQ ID NOS: 36, 37, 38 and 39) and in International patent application no. PCT/US2012/045622 (publication no. WO 2013/006733) and International patent application no. PCT/US2013/076739 (publication no. WO 2014/100504). Additional examples of nucleotide sequences encoding polypeptides having acyl-CoA oxidase activity include: *Saccharomyces cerevisiae POX1* (Genbank accession no. M27515), *Candida albicans POX1-3* (Genbank accession no. XM_716636), *Candida tropicalis POX2* (Genbank accession no. XM_002548031), *Candida tropicalis POX5* (Genbank accession no. XM_002548378),*Candida tropicalis POX4* (Genbank accession nos. AB031271, AB031272), *Candida maltosa POX2* (Genbank accession no. D21228), *Yarrowia lipolytica ACO1* (Genbank accession no. AJ001299), *Yarrowia lipolytica ACO2* (Genbank accession no. A001300), *Yarrowia lipolytica ACO3* (Genbank accession no. AJ001301), *Yarrowia lipolytica ACO4* (Genbank accession no. AJ001302), *Yarrowia lipolytica ACO5* (Genbank accession no. AJ001303), *Debaryomyces fabyri* (Genbank accession no. XM_015613952).

Presence, absence or amount of acyl-CoA oxidase activity can be detected by any suitable method known in the art and/or described herein. For example, detection can be performed using enzyme activity assays (see, e.g., Shimizu et al. (1979) *Biochem. Biophys. Res. Commun.* 91:108-113; Yao et al. (2014) *J. Braz. Chem. Soc.* 25(4):777-782); Kvannes and Flatmark (1991) *J. Biochem. Biophys. Methods* 23(2):135-149). Native and/or disrupted nucleic acid sequences encoding acyl-CoA oxidase (or other polypeptide) also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), and the amount of the nucleic acids or encoded proteins can be asessed using quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cells or organisms exhibit increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.



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The promoter used for regulating transcription of a heterologous nucleic acid encoding an acyl-CoA oxidase can also be modified. For example, the amount of an acyl-CoA oxidase protein expressed in a particular cellular location may be increased by including in the heterologous nucleic acid a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequence of a *Candida viswanathii* HDE gene promoter is provided herein as are examples of additional fatty acid-inducible promoters.

Alternatively, decreasing acyl-CoA oxidase activity in a cell can be accomplished by modifying the amount of acyl-CoA oxidase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous acyl-CoA oxidase gene in an organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type acyl-CoA oxidase such that the encoded modified or substituted acyl-CoA oxidase protein has a reduced enzyme activity. Reducing or eliminating the amount and/or activity of an acyl-CoA oxidase may be particularly beneficial in embodiments in which a target molecule and/or precursor or intermediate in the production of a target molecule contains a carbon chain of a particular length. In this case, the processing of fatty acids of particular chain lengths may be decreased or eliminated by decreasing the amount and/or activity of a particular acyl-CoA oxidase having activity on fatty acids of the particular chain length in a cell. Certain aspects of the cells, microorganisms, compositions and methods provided herein include one or more modifications to reduce or eliminate an acyl-CoA oxidase. One approach to reducing or eliminating the amount and/or activity of an acyl-CoA oxidase is by disrupting or deleting nucleic acid encoding the acyl-CoA oxidase in a host cell or microorganism to reduce or eliminate the acyl-CoA oxidase activity in the host relative to a cell or microorganism in which the gene(s) have not been modified. For example, expression of a host acyl-CoA oxidase activity can be decreased or eliminated by disruption (e.g., knockout, insertion mutagenesis, the like and combinations thereof) of a host gene encoding the protein, or by decreasing the activity of the promoter (e.g., through addition of repressor sequences to the promoter or 5'UTR or replacing the promoter) that controls transcription of an acyl-CoA oxidase gene using recombinant molecular biology techniques known in the art and/or described herein.



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One method for disrupting an endogenous acyl-CoA oxidase gene is by recombinantly inserting a heterologous nucleic acid (e.g., a nucleotide sequence encoding a selectable marker such as an enzyme that restores an auxotrophic host organism to prototrophy) into the endogenous gene, thereby generating an engineered organism deficient in acyl-CoA oxidase activity. This can be done, for example, through homologous recombination in which a heterologous nucleic acid containing sequences of an endogenous acyl-CoA oxidase gene and a disrupting sequence (e.g., a knock-out gene cassette such as described herein) is introduced into a host cell or microorganism. In some embodiments, the nucleotide sequence of one or more acyl CoA oxidases (e.g., a yeast POX4, POX 5, or POX4 and POX5) can be disrupted with a URA3 nucleotide sequence encoding a selectable marker, and introduced to a host cell or microorganism, thereby generating an engineered cell or organism deficient in an acyl-CoA oxidase activity. Nucleic acids encoding an acyl-CoA oxidase can be obtained from a number of sources, including, for example, yeast cells. Genomic DNA from cell sources can be amplified using oligonucleotide primers based on the nucleotide sequence of an acyl-CoA oxidase encoding gene, including examples provided herein.

In some embodiments, of the cells, organisms, compositions and methods provided herein, the amount and/or activity of a peroxisomal acyl-CoA oxidase in a cell is modified, e.g., a POX activity of a POX polypeptide. In particular embodiments, the acyl-CoA oxidase activity to be modified is encoded by the *POX4* and/or *POX5* genes of a species of *Candida* (e.g., ATCC 20336). In certain embodiments, the amount and/or activity of an endogenous acyl-CoA oxidase can be increased. In some embodiments, the amount and/or activity of acyl-CoA oxidases in a cell or organism containing one or more acyl-CoA oxidases can be independently modified (e.g., one or more acyl-CoA oxidases can be modified). In some embodiments, the amount and/or activity of POX4 acyl-CoA oxidase and a POX5 acyl-CoA oxidase can be altered independently of each other (e.g., increase amount and/or activity of POX4 alone, POX5 alone, increase amount and/or activity of one and decrease or eliminate the amount and/or activity of the other, and the like). Increasing the amount and/or activity of one acyl-CoA oxidase, while decreasing or eliminating the amount and/or activity of another acyl-CoA oxidase, may alter the specific activity of acyl-CoA oxidase in a cell or organism with respect to carbon chain length, while maintaining or increasing overall carbon flux through the β-oxidation pathway, in certain embodiments. Disruption of nucleotide sequences encoding one or more acyl-CoA oxidases (e.g., POX4, POX 5, or POX4 and POX5) sometimes can alter pathway efficiency, specificity and/or specific activity with respect to metabolism of carbon chains of different lengths (e.g.,



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carbon chains including fatty alcohols, fatty acids, paraffins, dicarboxylic acids, aliphatic molecules of between about 1 and about 26 carbons in length).

In some embodiments of the modified cells or organisms provided herein, a β-oxidation pathway in a yeast is active and includes a genetically modified acyl-CoA oxidase. In some embodiments, an acyl-CoA oxidase is genetically modified to prevent complete oxidation of fatty acyl-CoA or diacyl-CoA substrates. Genetic modification of an acyl-CoA oxidase can increase the production yield of a desired fatty acid or fatty dicarboxylic acid product. Therefore, in some embodiments, metabolic degradation of a fatty acid of a specified chain length (e.g., the chain length of a desired or target fatty acid or fatty dicarboxylic acid product) is reduced significantly, when an acyl-CoA oxidase is genetically modified. For example, metabolic degradation of a fatty dicarboxylic acid product (e.g., dodecanedioic acid or DDDA) by beta-oxidation can be reduced significantly when an acyl-CoA oxidase is genetically modified. This can be accomplished by modifying the substrate specificity of an acyl-CoA oxidase such that the enzyme has low activity (e.g., enzymatic activity) on chain lengths equal to or less than that of a desired product.

Nucleic acids encoding a genetically modified acyl-CoA oxidase can be engineered and expressed in a suitable organism (e.g., bacteria (e.g., *E. coli*) or a yeast) to test the substrate specificity of the modified enzyme in vitro. In some embodiments, nucleic acids encoding a genetically modified acyl-CoA oxidase are engineered and expressed in a suitable yeast and the substrate specificity is tested. Yeast that express a modified acyl-CoA oxidase can be tested for production of a desired molecule, e.g., a fatty acid or fatty dicarboxylic acid product. A modified acyl-CoA oxidase can be generated in any suitable manner (e.g., random or rational mutagenesis), non-limiting examples of which are provided herein and, for example, in International patent application no. PCT/US2012/045622 (publication no. WO 2013/006733) and International patent application no. PCT/US2013/076739 (publication no. WO 2014/100504).

In some instances, a modified endogenous acyl-coA oxidase polypeptide is a modified POX4 or POX5 polypeptide from a *Candida* spp. yeast (e.g., strain ATCC 20336 or ATCC 20962). In some cases a modified POX4 polypeptide contains a modified amino acid sequence of the wild-type *Candida* strain ATCC 20336 Pox4p sequence provided herein. Sometimes the POX4 polypeptide contains an amino acid modification at one or more amino acid positions chosen from 88, 90, 96, 98, 99, 100, 102, 103, 302, 309, 310, 473, 474, 475, 476, 477, 478, 479, 480,



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481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500, 501, 502, 503, 504 and 505. A modified endogenous acyl-coA oxidase polypeptide that is not a modified POX4 polypeptide can include an amino acid modification at one or more positions corresponding to one or more of the foregoing positions in the POX4 polypeptide. In some instances a modified POX5 polypeptide contains a modified amino acid sequence of the wild-type *Candida* strain ATCC 20336 Pox5p sequence provided herein. Sometimes the POX5 polypeptide contains an amino acid modification at one or more amino acid positions chosen from 81, 82, 83, 84, 85, 86, 88, 93, 94, 95, 96, 98, 102, 284, 287, 290, 291, 292, 294, 295, 436, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462 and 463. A modified endogenous acyl-coA oxidase polypeptide that is not a modified POX5 polypeptide can include an amino acid modification at one or more positions corresponding to one or more of the foregoing positions in the POX5 polypeptide.

In some embodiments, the substrate specificity of an acyl-CoA oxidase is modified such that the enzyme has low activity for aliphatic molecules with chain lengths less than C24 (i.e., 24 carbons). In some embodiments, the substrate specificity of an acyl CoA oxidase is modified such that the enzyme has very low activity with chain lengths less than 24, 22, 20, 18, 16, 14, 12, 10, 8, 6 or 4 carbons. In some embodiments, the substrate specificity of an acyl-CoA oxidase is modified such that the enzyme has very low activity with chain lengths less than 18, 16, 14, 12, 10 or 8 carbons. In some embodiments, the substrate specificity of an acyl-CoA oxidase is modified such that the enzyme has very low activity with chain lengths less than C12. In some embodiments, the substrate specificity of an acyl-CoA oxidase is modified such that the enzyme has very low activity with chain lengths less than C10 or C8. For example, in one embodiment for producing a 6-carbon dicarboxylic acid (e.g., adipic acid), a host cell or organism can be modified to decrease or eliminate acyl-CoA oxidase activities that are active on a broad range of substrate chain lengths (e.g., Pox4p acyl-CoA oxidase of *Candida viswanathii* ATCC 20336), and, to further increase productivity, can additionally be modified to express a mutant acyl-CoA oxidase activity that is more active on substrates with chain lengths of C8 and greater with little or no activity on substrates with chain lengths less than C8 (e.g., *Candida viswanathii* ATCC 20336 Pox5p(F98G); SEQ ID NO: 37).

As described herein (and in International patent application no. PCT/US2012/045622 (publication no. WO 2013/006733) and International patent application no. PCT/US2013/076739 (publication no. WO 2014/100504)), catalytic specificity of acyl-CoA oxidases (e.g., POX4,



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POX5) can be altered by a variety of methods. Altering the binding and/or catalytic specificity of acyl-CoA oxidases may prove advantageous for generating novel acyl-CoA oxidases with altered chain length recognition, altered chain length catalytic activity, and/or generation of an acyl-CoA oxidase activity with a narrow or specific chain length specificity, thereby allowing further increases in pathway efficiency, specificity and/or specific activity with respect to metabolism of carbon chains of different lengths or metabolism of carbon chain distributions found in a particular chosen feedstock. In some embodiments the altered acyl-CoA oxidase sequences are identified and/or generated by; (i) screening naturally occurring variant populations; (ii) mutagenesis of endogenous sequences; (iii) introduction of heterologous sequences having a desired specificity; (iv) generation of chimeric sequences having a portion of the coding sequence from one polynucleotide source (e.g., gene, organism) and a portion of the coding sequence from another source and/or (v) intelligent design using nucleotide sequences and three dimensional structure analysis from an acyl-CoA oxidase having a desired specificity to remodel an endogenous acyl-CoA oxidase, thereby generating a novel specificity enzyme. In some embodiments, a chimeric acyl-CoA oxidase nucleic acid sequence can have polynucleotide sequence contributions from two or more sources. In some embodiments, a chimeric acyl-CoA oxidase nucleic acid sequence comprises a portion of the coding sequences from an endogenous polynucleotide and a portion of the coding sequence from a heterologous polynucleotide.

One method for generating modified acyl-CoA oxidase proteins having altered substrate specificity is through random mutagenesis. A library of genetically modified acyl-CoA oxidases can be generated using several methods known in the art (e.g., site-directed mutagenesis). Genetically modified acyl-CoA oxidase genes can then be transformed into a β-oxidation blocked strain of a suitable yeast strain (e.g., *Candida* spp. (e.g., *Candida viswanathii* or *Candida tropicalis*)). In some embodiments, a genetically modified acyl-CoA oxidase is expressed under the control of the *POX4* promoter or another strong constitutive or inducible promoter in a pox4Δ/ pox4Δ pox5Δ/pox5Δ (e.g., an organism that lacks some or all endogenous acyl-CoA oxidase activity) background. In some embodiments, the genetically modified acyl-CoA oxidase is expressed under the control of an endogenous promoter. In some embodiments, the genetically modified acyl-CoA oxidase is expressed under the control of a heterologous promoter. The transformants can be selected by growth in media containing a fatty acid or methyl-derivate fatty acid containing fatty acids with two more carbons than a fatty acid product of interest. For example, for an adipic acid product, the transformants can be



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grown in caprylic acid or methyl-caprylate. For example, for a dodecanedioic acid product, the transformants can be grown in tetradecanedioic acid. The group of transformants can then be moved to a medium with a carbon source of a fatty acid of interest (for example dodecanedioic acid) in the presence of an agent that kills growing cells (e.g., Nystatin) and cells that cannot metabolize the carbon source (e.g., dodecanedioic acid in this example) can be selected. The resulting modified strains can then be further characterized for acyl-CoA oxidase activity. This method can be used to select for any modified acyl CoA oxidase (e.g., those listed and/or described in International patent application no. PCT/US2012/045622 (publication no. WO 2013/006733) and International patent application no. PCT/US2013/076739 (publication no. WO 2014/100504)). In addition, this method can be used to select for any heterologous acyl-CoA oxidase expressed in a suitable organism.

Another method for generating modified acyl-CoA oxidase proteins having altered substrate specificity is through rational mutagenesis. Structural and sequence information and experimental data can be combined to determine specific mutations for testing in an acyl-CoA oxidase for altered specificity. For example, primary sequences of acyl-CoA oxidases tested can be compared and correlated with substrate specificity. Based on such an analysis, single amino-acids, small numbers of contiguous amino acids and/or domains can be proposed for providing a desired substrate specificity. Those amino acids positions can be targeted for specific or random mutations for improve specificity.

Acyl-CoA oxidase structure also can be modeled against a known tertiary structure using modeling methods known in the art. The models can be used to propose amino acids and regions pertaining to substrate selectivity. For example, biochemical, structure and sequence data suggest that the N-terminus of acyl-CoA oxidases often, in part, determines substrate specificity. Mutations or region replacements can be introduced based on such analyses and the specificity of the new acyl-CoA oxidase tested as described before. The resulting information can be used to go back to the models to postulate new potential mutations. As for random mutagenesis, any suitable acyl-CoA oxidase can be modified to alter substrate specificity (e.g., those listed in International patent application no. PCT/US2012/045622 (publication no. WO 2013/006733) and International patent application no. PCT/US2013/076739 (publication no. WO 2014/100504)).



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Examples of modified Pox5 enzymes encoded by mutated *POX5* genes from *Candida viswanathii* include Pox5p (F98G) and Pox5p(W249F) are provided herein. The design, generation and analysis of modified Pox5 enzymes encoded by mutated *POX5* genes from *Candida viswanathii* are described in the examples.

*Modification of multifunctional enzyme activities*

Next (e.g., second and third) steps of the β-oxidation pathway can be catalyzed by a multifunctional enzyme (referred to, for example, as Mfe2, Fox2 and HDE in fungi) having hydratase and dehydrogenase activities, or by separate hydratase and dehydrogenase enzymes. In these steps, a trans-2-enoyl-CoA can be converted to 3-ketoacyl-CoA via a (3*R*)-hydroxy intermediate. An enoyl-CoA hydratase enzyme (e.g., EC 4.2.1.17) can catalyze the addition of a hydroxyl group and a proton to the unsaturated β-carbon on a fatty-acyl CoA in a second step of the pathway to generate 3-hydroxyacyl-CoA. In a next (e.g., third) step, a 3-hydroxyacyl-CoA dehydrogenase enzyme (e.g., EC 1.1.1.35) can catalyze the formation of a 3-ketoacyl-CoA by removal of a hydrogen from the newly formed hydroxyl group created by the activity of an enoyl-CoA hydratase. Typically, fungi have one peroxisomal multifunctional enzyme (HDE, Mfe2 or Fox2), mammalian cells have two peroxisomal multifunctional enzymes (Mfe1 and Mfe2) and bacteria have a single multifunctional enzyme, Mfe1. In the yeast *Candida tropicalis*, the N-terminal portion of the MFE polypeptide typically contains two duplicate 3-hydroxyacyl-CoA dehydrogenase domains, referred to as the A and B domains, which have differing substrate specificities. The A domain can catalyze the reaction for substrates with medium-to-long carbon chains (e.g., C10 – C16). The catalytic activity of the B domain often is more active on substrates having shorter carbon chains (e.g., C4). The hydratase domain is generally located at the C-terminal region of the polypeptide. Thus, each Mfe2 monomer can contain a dehydrogenase heterodimer and a hydratase monomer.

Some multifunctional enzymes involved in the β-oxidation pathway have additional enzymatic activities, including, but not limited to, an isomerase (e.g., a Δ3,Δ2-enoyl-CoA isomerase) activity and/or an epimerase (e.g., 3-hydroxyacyl-CoA epimerase; EC 5.1.2.3) activity. These enzymes function as auxillary enzymes in the oxidation of polyunsaturated fatty acids. For example, 3-hydroxyacyl-CoA epimerase catalyzes the reversible conversion of *S*-3-hydroxyacyl-CoA to *R*-3-hydroxyacyl-CoA, which (unlike *S*-3-hydroxyacyl-CoA) is a substrate for 3-



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hydroxyacyl-CoA dehydrogenase contained within Mfe2-type enzymes. Therefore, β-oxidation can proceed through the third step once the *R* isomer has been generated.

In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of a multifunctional enzyme (or an enoyl-CoA hydratase or 3-hydroxyacyl-CoA dehydrogenase) in a cell is modified. In particular embodiments, the multifunctional enzyme is a peroxisomal protein. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of a multifunctional enzyme (or an enoyl-CoA hydratase or 3-hydroxyacyl-CoA dehydrogenase), may be modified to decrease the amount and/or activity of a multifunctional enzyme (or an enoyl-CoA hydratase or 3-hydroxyacyl-CoA dehydrogenase), or may be modified to alternately increase and decrease the amount and/or activity of a multifunctional enzyme (or an enoyl-CoA hydratase or 3-hydroxyacyl-CoA dehydrogenase) depending, for example, on the substrate specificity, target molecule(s) being produced, cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured. In some embodiments, the amount and/or activity of one or more of the hydratase and dehydrogenase enzymes of a multifunctional enzyme may be independently modified.

In certain aspects, the amount and/or activity of one or more of the hydratase and dehydrogenase enzymes of a multifunctional enzyme in a cell is increased. Increasing the amount and/or activity of one or more of the hydratase and dehydrogenase enzymes of a multifunctional enzyme may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway involving oxidative metabolism and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, the multifunctional enzyme (or an enoyl-CoA hydratase or 3-hydroxyacyl-CoA dehydrogenase) activity is unchanged in a host or engineered cell or organism. In one embodiment, the amount and/or activity of one or more of a hydratase and/or dehydrogenase enzyme, for example, of a multifunctional enzyme, can be increased, for example, by increasing the number of copies of a nucleic acid encoding one or more of a hydratase and/or dehydrogenase enzyme (for example, of a multifunctional enzyme) (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid), by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding one or more of a hydratase

and dehydrogenase enzyme (for example, of a multifunctional enzyme), or by increasing the number of copies of a nucleic acid encoding one or more of a hydratase and/or dehydrogenase enzyme (for example, of a multifunctional enzyme) and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding one or more of a hydratase and/or dehydrogenase enzyme (for example, of a multifunctional enzyme). In some embodiments, a multifunctional enzyme (or an enoyl-CoA hydratase and/or 3-hydroxyacyl-CoA dehydrogenase) is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of one or more of the hydratase and dehydrogenase enzymes of a multifunctional enzyme protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding one or more of the hydratase and dehydrogenase enzymes of a multifunctional enzyme into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding one or more of the hydratase and dehydrogenase enzymes of a multifunctional enzyme can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed.

Non-limiting examples of organisms that include, or can be used as donors for, one or more of the hydratase and dehydrogenase enzymes of a multifunctional enzyme include yeast (e.g., *Candida*, *Saccharomyces*, *Yarrowia*), animals (e.g., *Homo*, *Rattus*), bacteria. In a particular embodiment, one or more of the hydratase and dehydrogenase enzymes of a multifunctional enzyme can be a *Candida* yeast protein. Additional examples of nucleotide sequences encoding multifunctional enzyme polypeptides include: *Saccharomyces cerevisiae FOX2* (Genbank accession nos. NM_001179799, M86456), *Candida tropicalis* (strain PK 233) *HDE* (Genbank accession nos. X57854, M22765), *Yarrowia lipolytica MFE2* (Genbank accession no. AF198225).

Presence, absence or amount of one or more of the hydratase and dehydrogenase enzymes of a multifunctional enzyme can be detected by any suitable method known in the art and/or described herein. For example, detection can be performed using enzyme activity assays (see, e.g., Hiltunen et al. (1992) J. *Biol. Chem.* 267(10):6646-6653). Nucleic acid sequences representing native and/or modified multifunctional enzyme sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or the amounts of the nucleic acids or encoded proteins can be assessed using quantitative expression based analysis (e.g., RT-PCR, western blot analysis,



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northern blot analysis, the like and combinations thereof), where the engineered cells or organisms exhibit increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a heterologous nucleic acid encoding one or more of the hydratase and dehydrogenase enzymes (for example, of a multifunctional enzyme) can also be modified. For example, the amount of one or more of the hydratase and dehydrogenase enzymes (for example, of a multifunctional enzyme protein) expressed in a particular cellular location may be increased or decreased by including in the heterologous nucleic acid a stronger or weaker heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism.

Alternatively, decreasing the activity of one or more of the hydratase and dehydrogenase enzymes (for example, of a multifunctional enzyme) in a cell can be accomplished by modifying the amount of expression of one or more of the hydratase and dehydrogenase enzymes (for example, of a multifunctional enzyme) in the cell, for example, by replacing the wild-type promoter of an endogenous multifunctional enzyme gene (or an enoyl-CoA hydratase or 3-hydroxyacyl-CoA dehydrogenase gene) in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type multifunctional enzyme (or an enoyl-CoA hydratase or 3-hydroxyacyl-CoA dehydrogenase) such that the encoded modified or substituted protein has a reduced enzyme(s) activity.

*Modification of 3-ketoacyl-CoA thiolase activity*

In a final step of the β-oxidation pathway, 3-ketoacyl-CoA can undergo thiolytic cleavage to yield a fatty acyl-CoA shortened by 2 carbons and acetyl-CoA. The reaction can be catalyzed by 3-ketoacyl-CoA thiolase (e.g., EC 2.3.1.16; also referred to as β-ketothiolase, acetyl-CoA acyltransferase) and involves cleavage of the 3-ketoacyl-CoA by the thiol group of another molecule of CoA. The thiol is inserted between C-2 and C-3, which yields an acetyl CoA molecule and an acyl CoA molecule that is two carbons shorter.

In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of a 3-ketoacyl-CoA thiolase in a cell is modified. For example, in some



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aspects, a cell or microorganism may be modified to increase the amount and/or activity of a 3-ketoacyl-CoA thiolase, may be modified to decrease the amount and/or activity of a 3-ketoacyl-CoA thiolase, or may be modified to alternately increase and decrease the amount and/or activity of a 3-ketoacyl-CoA thiolase depending, for example, on the substrate specificity, cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of a 3-ketoacyl-CoA thiolase in a cell is increased. Increasing the amount and/or activity of a 3-ketoacyl-CoA thiolase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway involving oxidative metabolism and away from other cellular metabolic pathways not involved in target molecule production.

In certain embodiments, the 3-ketoacyl-CoA thiolase activity is unchanged in a host or engineered cell or organism. In one embodiment, the amount and/or activity of a host 3-ketoacyl-CoA thiolase can be increased, for example, by increasing the number of copies of a nucleic acid encoding a 3-ketoacyl-CoA thiolase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid), by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a 3-ketoacyl-CoA thiolase, or by increasing the number of copies of a nucleic acid encoding a 3-ketoacyl-CoA thiolase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a 3-ketoacyl-CoA thiolase. In some embodiments, a 3-ketoacyl-CoA thiolase is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of a 3-ketoacyl-CoA thiolase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding a 3-ketoacyl-CoA thiolase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding a 3-ketoacyl-CoA thiolase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed.

Non-limiting examples of organisms that include, or can be used as donors for, a 3-ketoacyl-CoA thiolase enzyme include yeast (e.g., *Candida*, *Saccharomyces*, *Debaryomyces*, *Meyerozyma*, *Lodderomyces*, *Scheffersomyces*, *Clavispora*, *Yarrowia*, *Pichia*, *Kluyveromyces*, *Eremothecium*, *Zygosaccharomyces*, *Lachancea*, *Nakaseomyces*), animals (e.g., *Homo*, *Rattus*), bacteria (e.g., *Escherichia*, *Pseudomonas*, *Bacillus*), or plants (e.g., *Arabidopsis*,



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*Nictotania*, *Cuphea*). In a particular embodiment, a 3-ketoacyl-CoA thiolase enzyme can be a *Candida* yeast protein. Examples of nucleotide sequences encoding polypeptides having 3-ketoacyl-CoA thiolase activity include, but are not limited to: *Saccharomyces cerevisiae FOX1* (Genbank accession no. NM_001179508), *Candida tenuis* (Genbank accession no. XM_006688917), *Candida tropicalis CT-T3A* (Genbank accession no. AB025647), *Candida tropicalis CT-T3B* (Genbank accession no. AB025648), *Yarrowia lipolytica POT1* (Genbank accession no. XM_504109, X69988), *Scheffersomyces stipitis POT11* (Genbank accession no. XM_001386372), *Debaryomyces fabyri* (Genbank accession no. XM_015611011), *Arabidopsis thaliana KAT2* (Genbank accession no. NM_128874), *Lillium* cultivar Belladonna (Genbank accession no. KR998331) and *Populus davidianna KAT* (Genbank accession no. KU297273).

Presence, absence or amount of 3-ketoacyl-CoA thiolase activity can be detected by any suitable method known in the art and/or described herein. For example, detection can be performed using enzyme activity assays (see, e.g., Staack et al. (1978) *J. Biol. Chem* 253: 1827-1831; Kurihara et al. (1988) *FEBS Lett.* 229(1):215-218). Nucleic acid sequences representing native and/or modified 3-ketoacyl-CoA thiolase-encoding sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or the amounts of the nucleic acids or encoded proteins can be assessed using quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cells or organisms exhibit increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a heterologous nucleic acid encoding a 3-ketoacyl-CoA thiolase can also be modified. For example, the amount of a 3-ketoacyl-CoA thiolase protein expressed in a particular cellular location may be increased by including in the heterologous nucleic acid a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequence of a *Candida viswanathii* HDE gene promoter is provided herein as are examples of additional fatty acid-inducible promoters.

Alternatively, decreasing 3-ketoacyl-CoA thiolase activity in a cell can be accomplished by modifying the amount of 3-ketoacyl-CoA thiolase protein expression in the cell, for example, by

replacing the wild-type promoter of an endogenous 3-ketoacyl-CoA thiolase gene in an organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type 3-ketoacyl-CoA thiolase such that the encoded modified or substituted 3-ketoacyl-CoA thiolase protein has a reduced enzyme activity.

*Modification of enoyl-CoA isomerase activity*

Feedstocks, such as, for example, fatty acid distillates and soapstocks, can comprise unsaturated fatty acids, for example, such as oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). In some embodiments, unsaturated fatty acids are converted to dicarboxylic acids that maintain the position and orientation of the double bonds. Unsaturated fatty acids generally are degraded through the same reactions that degrade saturated fatty acids until a Δ3-cis-acyl-CoA or Δ2-cis-acyl-CoA is formed in the process of β-oxidation. Cells can employ additional enzymes to allow the oxidation of these types of unsaturated fatty acids or diacids. In some instances, an enzyme enoyl-CoA isomerase (ECI) is required for the beta-oxidation of substrates with double bonds at odd numbered positions. In some instances, the enzyme dienoyl-CoA reductase (DCR) is required for the beta-oxidation of substrates with double bonds at even numbered positions.

Enoyl-CoA isomerase (ECI) can also be known as enoyl-CoA delta isomerase 1, dodecenoyl-CoA isomerase, 3,2 trans-enoyl-CoA isomerase, acetylene-allene isomerase, Δ3 Δ2-enoyl-CoA isomerase, dodecenoyl-CoA delta isomerase, and EC 5.3.3.8 (in human for example). Several alternatively spliced transcript variants are also known. ECI is a member of the hydratase/isomerase superfamily. ECI can be a key mitochondrial enzyme involved in beta-oxidation of unsaturated fatty acids. This enzyme can isomerize both 3-cis and 3-trans double bonds into the 2-trans form in a range of ECI enzymes from different species. ECI can catalyze the transformation of 3-cis and 3-trans-enoyl-CoA esters arising during stepwise degradation of cis-, mono-, and polyunsaturated fatty acids to the 2-trans-enoyl-CoA intermediates. ECI is present in many microorganisms and several species of yeast have at least two ECI enzymes. Nucleotide sequences (and corresponding amino acid sequences) encoding enoyl-CoA isomerase enzymes from *Candida* strain ATCC 20336 are provided herein (nucleotide SEQ ID NOS: 106 and 107 and animo acid SEQ ID NOS: 50 and 51). Examples of nucleotide sequences encoding polypeptides having enoyl-CoA isomerase activity include, but are not



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limited to: *Saccharomyces cerevisiae ECI1* (Genbank accession no. AF090442) and *Candida albicans* (Genbank accession no. XM_711189).

In some embodiments, ECI is utilized in generating a target fatty acid product through β-oxidation of an unsaturated fatty because of its activity and the normal position of double bonds in some feedstocks (e.g., soapstocks and fatty acid distillates). Many unsaturated fatty acids have a cis-Δ9 double bond. During the β -oxidation of an 18-carbon diacid with a cis-Δ9 double bond, the double bond is encountered when it has been chain shortened to 12 carbons. At this stage the 12-carbon molecule can have a cis-Δ3 double bond that is not a substrate for an acyl-CoA oxidase. ECI can convert the cis-Δ3 double bond to a trans-Δ2 double bond. In some instances, the product of the ECI reaction is a substrate for the second step in beta-oxidation (e.g., a substrate for enoyl-CoA hydratase), and ECI can effectively bypass acyl-CoA oxidase in a particular round of beta-oxidation. In some instances, even if a yeast strain lacks any acyl-CoA oxidase that is active on fatty acids of less than or equal to C12 (i.e., 12 carbons), an active ECI can effect the shortening of one more rounds of β-oxidation, which can produce a 10-carbon product for substrates with a cis-Δ9 double bond. Therefore, in some embodiments, the ECI gene is disrupted (e.g., knocked out or deleted) in a yeast (e.g., in a *Candida* strain) to prevent chain shortening past a desired chain-length (e.g., in this instance, 12 carbons). In some embodiments, disrupting the expression (e.g. knocking out the expression) of an ECI gene can result in an increase in the production of a fatty dicarboxylic acid containing 10 to 18 carbons. In some embodiments, disrupting the expression (e.g. knocking out the expression) of an ECI gene can result in an increase in the production of a fatty dicarboxylic acid containing 10, 12, 14, 16 or 18 carbons. In some embodiments, disrupting the expression of an enoyl-CoA isomerase can increase the production of fatty dicarboxylic acid containing 10, 12, 14, 16 or 18 carbons when using certain feedstocks (e.g., certain soapstocks or fatty acid distillates).

In some embodiments, an ECI knock out (i.e., eciΔ or Eci$^-$) strain is able to produce DDDA from from fatty acid feedstocks containing unsaturated fatty acids (e.g., oleic acid, linoleic acid, linolenic acid) even in the presence of acyl-CoA oxidase with activity on substrates of chain-length less than 12 carbons (but with little or nor activity on substrates having 12 carbons in the chain). This can be accomplished, for example, by discontinuation of β-oxidation after obtaining 3-dodecendioic acid (e.g., from oleic acid feedstock), 3,6-dodecenedioc acid (e.g., from linoleic acid feedstock) or 3,6,9-dodecenedioc acid (e.g., from linolenic acid feedstock) through an initial

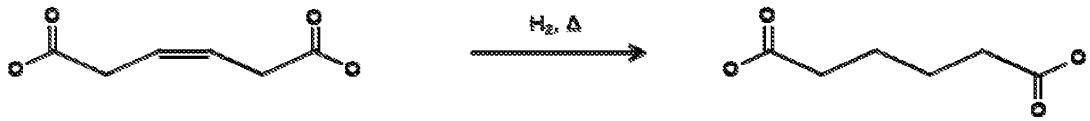


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three rounds of β-oxidation (due to the lack of enoyl-CoA isomerase activity), and then hydrogenation of the dodecendioic acids to yield a fully saturated DDDA.

Thus, in some embodiments, a 12-carbon dicarboxylic acid produced from fatty acid feedstocks containing unsaturated fatty acids can be hydrogenated to generate a the fully saturated DDDA product. An unsaturated diacid sometimes is produced from a feedstock containing an unsaturated fatty acid, and production of a fully saturated diacid in such situations can involve hydrogenation of the unsaturated diacid. For example, an unsaturated C6:1 diacid generated from one or more long chain unsaturated fatty acids in an Eci$^-$ yeast strain which also lacks acyl-CoA oxidase enzymes having activity on substrates of 6-carbon chain lengths (e.g., a pox4Δ yeast strain) can be converted to a fully saturated C6:0 diacid by reducing the double bond by a suitable method. Non-limiting examples of hydrogenation methods include the use of a metallic chemical catalyst, non-metallic chemical catalyst, enzymatic catalyst, the like or combination thereof.

A non-limiting example of a hydrogenation reaction is shown below. Sometimes source hydrogen is provided from molecular hydrogen (e.g., in the case of chemical catalysis) and sometimes source hydrogen is provided from enzymatic cofactors, non-limiting examples of which include NADH, NADPH, FADH2, the like or combination thereof (e.g., in the case of enzymatic catalysis).




In some embodiments, catalytic hydrogenation is carried out with a suitable metallic catalyst, non-limiting examples of which include platinum, palladium, rhodium, ruthenium, nickel, the like or combination thereof. Sometimes a catalyst is a homogenous catalyst and sometimes a catalyst is a heterogeneous catalyst. An elevated temperature and/or pressure can be employed to increase reaction rate. For example, an unsaturated diacid (e.g., cis, cis-muconic acid) can be hydrogenated and converted to adipic acid using a 10% Pt on carbon catalyst at 3400 kPa for 2.5 hours at ambient temperature (Niu et al., (2002) *Biotechnol.Prog.* 18:201-211). In some embodiments, catalytic hydrogenation can occur with nonmetallic catalysts such as frustrated Lewis pair compounds (Welch et al., (2006) Science 314:1124-1126).



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In certain embodiments, enzymatic hydrogenation is conducted in vivo or in vitro with a suitable native or engineered enzyme that can catalyze a redox reaction with an unsaturated diacid or fatty acid as a substrate or a product. An enzyme can be utilized in vivo in some embodiments by increasing expression of a native enzyme or expressing a non-native enzyme capable of catalyzing a desired hydrogenation reaction in an organism that produces an unsaturated diacid precursor of a saturated diacid product. A lysate of an organism containing an enzyme capable of catalyzing a desired hydrogenation reaction, or a purified or isolated enzyme preparation, sometimes is utilized in an in vitro reaction. Non-limiting examples of a suitable native or engineered enzyme include acyl-CoA dehydrogenase (EC 1.3.1.8), trans-2-enoyl-CoA reductase (EC 1.3.1.44), stearoyl-CoA 9-desaturase (EC 1.14.19.1), the like or combination thereof. In some embodiments, a desired reaction product (e.g., a saturated diacid) is produced by an enzyme operating in a forward or a reverse direction (e.g., a forward or reverse reaction).

*Modification of dienoyl-CoA reductase activity*

Dienoyl CoA reductase (DCR, e.g., EC 1.3.1.34) is a peripheral enzyme that can convert trans-2, cis-4 dienoyl-CoA substrates to trans-3-enoyl-CoA products (Gurvitz A, et al., (1997) *J.Biol. Chem.* 272:22140-22147).




The trans-3-enoyl-CoA is then converted by the enzyme enoyl-CoA isomerase (ECI) to trans-2-enoyl-CoA which is then the substrate for the second enzyme (enoyl-CoA hydratase) in β-oxidation. Complete β-oxidation of fatty acids, including diacids, with double bonds at even numbered positions (e.g., linoleic acid (C18:2) and linolenic acid (C18:3)) can be achieved by including the DCR reaction in the β-oxidation pathway. Diacids are capable of being oxidized starting from either end (diterminal β-oxidation), and sometimes the enzymes used to rearrange and degrade the double bonds are the same from either direction. This is because even-numbered diacids with double bonds at even-numbered positions maintain the even-numbered position from either end (similarly with double bonds at odd-numbered positions).



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The DCR reaction can be used for complete β-oxidation of fatty acids with double bonds at even numbered positions, such as linoleic acid and linolenic acid. Depending on the carbon chain length of a desired final diacid product, it may be useful to either amplify or reduce the activity of one or more DCR enzymes in the host cell or engineered organism. For diacid products that have a carbon chain length of eight or greater, it may be useful or desirable to reduce or eliminate one or all DCR enzymes in the host cell. For diacid products that have a carbon chain length of less than eight carbons, it may be useful or desirable to amplify the activity of one or more DCR enzymes in the host cell or engineered organism.

Table 1 is a table of diacid products that may be produced from unsaturated fatty acids using a yeast strain in which the *ECI* and/or *DCR* genes have been disrupted or deleted. A Dcr$^-$ strain that does not include mutations of other genes encoding enzyme activities of the β-oxidation pathway typically can produce exclusively a C8:3 diacid. In some embodiments, a DCR polypeptide is not decreased, such as by disrupting a Dcr-encoding polynucleotide, in a strain utilized to produce a diacid product (e.g., adipic acid sebacic acid, DDDA). In certain embodiments, Dcr polypeptide production is increased (e.g., introducing additional copy numbers of an endogenous Dcr-encoding polynucleotide; introducing one or more copies of a heterologous Dcr-encoding polynucleotide) to produce adipic acid from polyunsaturated fatty acids, such as those prevalent in soybean or corn oil.

**TABLE 1**

| | **Diacids Produced in a Pox4$^+$, Pox5$^+$ Background** | | |
|---|---|---|---|
| **Fatty Acid Carbon Source** | **Eci$^-$ Product** | **Dcr$^-$ Product** | **Eci$^-$,Dcr$^-$ Product** |
| Oleic acid (C18:1) | C6:1 (3-hexenedioic acid) | None | C6:1 (3-hexenedioic acid) |
| Linoleic acid (C18:2) | C10:2 (3,7-decenedioc acid) | C8:3 (2,4,6-octenedioic acid) | C10:3 (2,4,7-decenedioic acid) |
| Linolenic acid (C18:3) | C12:3 (3,6,9-dodecenedioc acid) | C8:3 (2,4,6-octenedioic acid) | C12:3 (3,6,9-dodecenedioic acid) |
| Eicosenoic acid (C20:1) | C6:1 (3-hexenedioic acid) | None | C6:1 (3-hexenedioic acid) |
| Erucic acid (C22:1) | C6:1 (3-hexenedioic acid) | None | C6:1 (3-hexenedioic acid) |

In yeast such as *Candida tropicalis* and *Candida viswanathii*, there are two *DCR* homologs, often referred to as *DCR1* and *DCR2*. The yeast *Saccharomyces cerevisiae* includes one Dcr enzyme, while the yeast *Yarrowia lipolytica* includes at least three *DCR* homologs, referred to herein as "*DCR1*", "*DCR2*", and "*DCR3*". Nucleotide sequences (and corresponding amino acid sequences) encoding dienoyl-CoA reductase enzymes from *Candida* strain ATCC 20336 are provided herein (nucleotide SEQ ID NOS: 108 and 109 and amino acid SEQ ID NOS: 52 and 53). Examples of nucleotide sequences encoding polypeptides having dienoyl-CoA reductase activity include, but are not limited to: *Saccharomyces cerevisiae SPS19* (Genbank accession no. NM_001183040), *Candida tropicalis SPS19* (Genbank accession no. XM_002545237) and *Yarrowia lipolytica* (Genbank accession nos. XM_501382, XM_503937, XM_502296).

Accordingly, there are multiple possible genotypes of yeast strains (having varying combinations of wild-type and mutant acyl-CoA oxidase activity specificities and functional or non-functional ECI and/or DCR genes) for the production of fatty acids and diacids of differing carbon chain lengths and degrees of saturation/unsaturation. The fatty acid or diacid produced can depend on the carbon source in the feedstock. Table 2 provides non-limiting examples of some of the yeast strain (e.g., *Candida* spp.) genotype combinations and carbon sources for the production of adipic acid, suberic acid, sebacic acid and DDDA.

| **TABLE 2** | **STRAIN GENOTYPE (with respect to *POX4/ECI/DCR*) AND CARBON SOURCE COMBINATIONS** | | |
|---|---|---|---|
| **DIACID PRODUCT** | **OLEIC ACID** | **LINOLEIC ACID** | **LINOLENIC ACID** |
| Adipic acid (C6) | *pox4Δ/ECI/DCR* (yields saturated diacid) | *pox4Δ/ECI/DCR* (yields saturated diacid) | *pox4Δ/ECI/DCR* (yields saturated diacid*) |
| | *POX4/Eci⁻/DCR* (yields 3-hexenedioic acid*) | | |
| Suberic acid (C8) | *pox4Δ/ECI/DCR* (yields saturated diacid) | *pox4Δ/ECI/DCR* (yields saturated diacid) | |
| | *pox4Δ/ Eci⁻/DCR* (yields 3-octenedioic acid*) | *POX4/ECI/Dcr⁻* (yields 2,4,6-octenedioic acid*) | *POX4/ECI/Dcr⁻* (yields 2,4,6-octenedioic acid*) |

| TABLE 2 | STRAIN GENOTYPE (with respect to *POX4/ECI/DCR*) AND CARBON SOURCE COMBINATIONS | | |
|---|---|---|---|
| **DIACID PRODUCT** | **OLEIC ACID** | **LINOLEIC ACID** | **LINOLENIC ACID** |
| | | *POX4/Eci⁻/DCR* (yields 3,6-octenedioic acid*) | |
| Sebacic acid (C10) | | *POX4/Eci⁻/DCR* or *pox4Δ/ Eci⁻/DCR* (yields 3,7-decenedioic acid*) | |
| | | *POX4/Eci⁻/ Dcr⁻* (yields 2,4,7-decenedioic acid*) | |
| Dodecanedioic acid (C12) | | | *POX4/Eci⁻/DCR* or *pox4Δ/ Eci⁻/DCR* (yields 3,6,9-dodecenedioic acid*) |

* can be hydrogenated to saturate

*Modification of dienoyl-CoA isomerase activity*

Dienoyl-CoA isomerase (DCI, e.g., EC 5.3.3, Δ3,5,Δ2,4-dienoyl-CoA isomerase, Δ3,5,Δ2,4-dienoyl-coenzyme A isomerase) is a peripheral β-oxidation enzyme that catalyzes the isomerization of a Δ3,5-dienoyl-CoA to a Δ2,4-dienoyl-CoA. This reaction is part of a minor β-oxidation pathway that occurs when the 3,2-enoyl-CoA isomerase (ECI) converts a Δ2,5-dienoyl-CoA to a Δ3,5-dienoyl-CoA. In order to fully oxidize this product DCI converts the Δ3,5-dienoyl-CoA to a Δ2,4-dienoyl-CoA, the latter of which is a substrate for the 2,4-dienoyl-CoA reductase (DCR). The product of the DCR reaction is a 3-enoyl-CoA, which is a substrate for ECI that converts it to a 2-enoyl-CoA that can be fully oxidized through β-oxidation.

In some embodiments, the amount and/or activity of a Dci enzyne in a cell or organism is decreased or increased, depending upon the chain-length of a desired target diacid product to be generated through β-oxidation of a fatty acid. For example, for adipic acid production, a DCI

activity can be increased to improve productivity of unsaturated fatty acids in a host cell or organism (e.g., DCI activity can be increased by introducing one or more copies of a polynucleotide encoding a polypeptide having DCI activity into the cell or organism (e.g., introducing one or more copies of an endogenous or exogenous polynucleotide)). In some embodiments, for production of C8 and longer diacids, the amount and/or activity of a DCI enzyme in a cell or organism can be decreased (e.g., by introducing a disruption, deletion or knockout of a polynucleotide that encodes a polypeptide having DCI activity, or replacing a promoter of a DCI gene with a weaker promoter (for example, introducing a nucleic acid containing a weak promoter operably linked to a polynucleotide that encodes a polypeptide having DCI activity into a cell in which an endogenous DCI gene has been disrupted or deleted). An example of a nucleotide sequence encoding a *Saccharomyces cerevisiae* DCI enzyme is Genbank accession no. NM_001183599.

*Modification of β-oxidation-associated activities*

There are also cellular compositions and activities that are closely associated with β-oxidation and support the core degradative functioning of the pathway. These include peroxisomal- and mitochondrial-related compositions and activities. For example, as described herein, such compositions and activities include, but are not limited to, compositions and activities involved in: generating acyl-CoA through thioesterification of fatty acids, movement of fatty acids and/or acyl-CoA into cellular sites of β-oxidation (e.g., peroxisomes), regulation of β-oxidation activities, synthesis of compositions involved in β-oxidation, and maintenance/amount of sites of β-oxidation (e.g., peroxisomes). Included in the cells, organisms, systems and methods provided herein are embodiments in which one or more of these β-oxidation-associated compositions and/or activities are modified. In some embodiments, a β-oxidation-associated composition or activity is modified to enhance β-oxidation activity.

*Modification of peroxisomal transport activity*

In order for fatty acids to undergo peroxisomal β-oxidation, they must first move into the peroxisomes. Generally, medium-chain free fatty acids present in the cytosol can traverse the peroxisomal membrane and become activated once in the peroxisome by a persoxisomal acyl-CoA synthetase to then be processed as an acyl-CoA in β-oxidation. Long-chain fatty acids that have entered a cell from the extracellular medium tend to quickly be activated by acyl-CoA

synthetases located at or near the cell membrane or in the cytosol. These acyl-CoA esters typically are not able to traverse the peroxisomal membrane and thus require a peroxisomal transporter in order to move into peroxisomes. Peroxisomal transporter proteins can be a target for modifying entry of fatty acids into peroxisomes. Free fatty acids internalized into cells, or generated within cells (e.g., by oxidation of internalized alkanes), can directly enter into and be processed in the ω-oxidation pathway without prior activation to acyl-CoA.

In some embodiments of the microorganisms, compositions and methods provided herein, the amount and/or activity of a peroxisomal transporter protein in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount of a peroxisomal transporter protein and/or a peroxisomal transporter protein activity, may be modified to decrease the amount of a peroxisomal transporter protein and/or a peroxisomal transporter protein activity, or may be modified to alternately increase and decrease the amount of a peroxisomal transporter protein and/or a peroxisomal transporter protein activity depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of a peroxisomal transporter protein in a cell is decreased. Reducing or eliminating the amount and/or activity of a peroxisomal transporter protein may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway (e.g., in peroxisomes) and away from other cellular metabolic pathways involving activated fatty acids (acyl-CoA). For example, in embodiments of the production systems in which a target molecule, or intermediate/precursor of a target molecule, is a dicarboxylic acid, it may be optimal to decrease or eliminate fatty acid entry into peroxisomes through modes other than as a dicarboxylic acid which moves freely into peroxisomes after formation though an initial ω-oxidation of a free fatty acid. Certain aspects of the cells, microorganisms, compositions and methods provided herein include one or more modifications to reduce or eliminate transport of acyl-CoA into peroxisomes. One approach to reducing or eliminating such transport is to decrease the amount and/or activity of a peroxisomal transporter protein. For example, one or more endogenous genes encoding a peroxisomal transporter protein (e.g., yeast *PXA1* and/or *PXA2*) can be disrupted or deleted in a host cell or microorganism to reduce or eliminate the amount of and/or activity of a peroxisomal transporter protein in the host relative to a cell or microorganism in which the gene(s) have not been modified.



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Methods for decreasing the amount and/or activity of a peroxisomal transporter protein in a cell include, but are not limited to, modifying the amount of peroxisomal transporter protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous a peroxisomal transporter protein gene in an organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type peroxisomal transporter protein such that the encoded modified or substituted peroxisomal transporter protein has a reduced activity. For example, expression of a host peroxisomal transporter protein activity can be decreased or eliminated by disruption (e.g., knockout, insertion mutagenesis, the like and combinations thereof) of a host gene encoding the protein, or by decreasing the activity of the promoter (e.g., through addition of repressor sequences to the promoter or 5'UTR or replacing the promoter) that controls transcription of a peroxisomal transporter protein gene using recombinant molecular biology techniques known in the art and/or described herein. In one embodiment, a diploid yeast, such as, for example, a *Candida* yeast, when used as a host microorganism can be subjected to genetic modification in which one of the two alleles of a peroxisomal transporter protein gene is disrupted or deleted. In so doing, a single allele of the gene remains for a reduced amount of peroxisomal transporter protein expression in the microorganism and a reduced amount of the protein in the cell.

One method for disrupting an endogenous peroxisomal transporter protein gene is by recombinantly inserting a heterologous nucleic acid (e.g., a nucleotide sequence encoding a selectable marker such as an enzyme that restores an auxotrophic host organism to prototrophy) into the endogenous gene, thereby generating an engineered organism deficient in a peroxisomal transporter protein activity. This can be done, for example, through homologous recombination in which a heterologous nucleic acid containing sequences of an endogenous peroxisomal transporter protein gene and a disrupting sequence (e.g., a knock-out gene cassette such as described herein) is introduced into a host cell or microorganism. Nucleic acids encoding a peroxisomal transporter protein can be obtained from a number of sources, including, for example, yeast cells. Genomic DNA from cell sources can be amplified using oligonucleotide primers based on the nucleotide sequence of a peroxisomal transporter protein encoding gene, including examples provided herein. Nucleotide sequences encoding the subunits of (and the amino acid sequences of) a *Candida viswanathii* peroxisomal transporter protein, Pxa1 and Pxa2, are provided herein (nucleotide SEQ ID NOS: 94 and 95 and amino acid SEQ ID NOS: 40 and 41). Additional non-limiting examples of nucleic acids encoding a



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peroxisomal transporter protein include *Saccharomyces cerevisiae PXA1* (Genbank accession numbers NM_001183961 and U17065), *Saccharomyces cerevisiae PXA2* (Genbank accession numbers NM_001179754 and U93584), *Schizosaccharomyces pombe PXA1* (Genbank accession number NM_001018794), *Candida albicans PXA1* (Genbank accession number XM_713564), *Yarrowia lypolytica PXA1* (Genbank accession number XM_499814), *Yarrowia lypolytica PXA2* (Genbank accession number XM_502396), *Candida orthopsilosis PXA1* (Genbank accession number XM_003865834), *Aspergillus nomius PXA1* (Genbank accession number XM_015554863), *Clavispora lusitaniae PXA1* (Genbank accession number JQ710938), *Aspergillus niger PXA1* (Genbank accession number XM_001388761) and *Arabidopsis thalina ABCD1* (Genbank accession number NM_001204043).

In other embodiments, the amount and/or activity of a host peroxisomal transporter protein can be increased, for example, by increasing the number of copies of a gene encoding a peroxisomal transporter protein (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the gene), by increasing the activity of a promoter that regulates transcription of a gene encoding a peroxisomal transporter protein, or by increasing the number of copies of a gene encoding a peroxisomal transporter protein and increasing the activity of a promoter that regulates transcription of a gene encoding a peroxisomal transporter protein. In some embodiments, a peroxisomal transporter protein is endogenous to the host cell or microorganism. In particular embodiments, the amount and/or activity of a host peroxisomal transporter protein is increased.

The presence, absence or amount of peroxisomal transporter protein can be detected by any suitable method known in the art. Non-limiting examples of suitable detection methods include nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cells or organisms exhibit increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

*Modification of peroxisome biogenesis activity*

Peroxisomes can be found in eukaryotic cells and are a cellular location for β-oxidation (i.e., the site for β-oxidation in fungi and plant cells and one of two sites (the other being mitochondria) for β-oxidation of fatty acids in animal cells). Consistent with this function, peroxisome

proliferation may occur in cells exposed to fatty acids as a sole source of carbon, and peroxisome degradation may occur in cells in the presence of glucose. Thus, the number of and volume of peroxisomes in cells can be regulated. Although most of the more than 30 peroxisomal membrane proteins, referred to as peroxins or Pex proteins, play a role in importing proteins into the peroxisomal matrix from the cytosol (e.g., Pex5, Pex7, Pex13, Pex14 Pex16, Pex17), some (e.g. Pex 11, Pex 25, Pex 27, Pex 34) are involved in peroxisome proliferation.

In some embodiments of the cells, organisms, compositions and methods provided herein, the amount and/or activity of a Pex protein in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount and/or activity of a Pex protein, to decrease the amount and/or activity of a Pex protein, or to alter the pattern of expression of a Pex protein. In particular embodiments, the Pex protein is one that is involved in peroxisome proliferation, e.g., Pex11.

In certain aspects, the amount and/or activity of a Pex protein in a cell or organism is increased. Increasing the amount and/or activity of a Pex protein may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed toward a particular target product pathway involving oxidative metabolism and away from other cellular metabolic pathways not involved in target molecule production. In some embodiments, the amount and/or activity of a Pex protein involved in peroxisome proliferation is increased in a cell or organism to provide for increased numbers of peroxisomes as sites for β-oxidation. In particular embodiments, the Pex protein is Pex11.

In certain embodiments, the Pex protein activity is unchanged in a host or engineered cell or organism. In one embodiment, the amount and/or activity of a host Pex protein can be increased, for example, by increasing the number of copies of a nucleic acid encoding a Pex protein (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid), by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a Pex protein, or by increasing the number of copies of a nucleic acid encoding a Pex protein and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding a Pex protein. In some embodiments, a Pex protein is endogenous to the host cell or microorganism. In one aspect of the cell or microbial systems and methods provided herein, the amount of a Pex protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding a Pex protein into a cell or microorganism. For example, introduction of heterologous



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nucleic acid encoding a Pex protein can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed.

Non-limiting examples of organisms that include, or can be used as donors for, a Pex protein include yeast (e.g., *Candida*, *Saccharomyces*, *Debaryomyces*, *Meyerozyma*, *Lodderomyces*, *Scheffersomyces*, *Clavispora*, *Yarrowia*, *Pichia*, *Kluyveromyces*, *Eremothecium*, *Zygosaccharomyces*, *Lachancea*, *Nakaseomyces*), animals (e.g., *Homo*, *Rattus*), or plants (e.g., *Arabidopsis*, *Nictotania*, *Cuphea*). In a particular embodiment, a Pex protein can be a *Candida* yeast protein. An example of a *Candida viswanathii* nucleotide sequence (and corresponding amino acid sequence) encoding a Pex11 protein is provided herein (nucleotide SEQ ID NO: 89 and amino acid SEQ ID NO: 33). Additional examples of nucleotide sequences encoding polypeptides having Pex protein activity include, but are not limited to: *Saccharomyces cerevisiae PEX11* (Genbank accession no. NM_001183401), *Candida albicans* (Genbank accession no. XM_707009), *Candida orthopsilosiis PEX11* (Genbank accession no. XM_003870517), *Yarrowia lipolytica PEX11* (Genbank accession nos. XM_503276, XM_501447, XM_501425), *Arabidopsis thaliana PEX11A* (Genbank accession no. NM_103668), *Neurospora crassa PEX11* (Genbank accession no. XM_011396615), *Pichia angusta PEX11* (Genbank accession no. DQ645582).

Presence, absence or amount of Pex protein or nucleic acids encoding the protein can be detected by any suitable method known in the art and/or described herein. For example, detection can be performed using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cells or organisms exhibit increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

The promoter used for regulating transcription of a heterologous nucleic acid encoding a Pex protein can also be modified. For example, the amount of a Pex protein expressed in a particular cellular location may be increased by including in the heterologous nucleic acid a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequence

of a *Candida viswanathii* HDE gene promoter is provided herein as are examples of additional fatty acid-inducible promoters.

*Modification of acetyl-CoA processing in peroxisomes*

Included in the cell-based and microbial production platform systems and components thereof provided herein are embodiments in which the processing of acetyl-CoA in organelles of a cell or microorganism is modified. In some embodiments, acetyl group carbons of organelle-generated acetyl-CoA are directed toward conversion to acetate. In particular embodiments, acetyl group carbons are directed toward conversion to acetate and away from the carnitine-carrier transport system. Accordingly, provided herein are cells, microorganisms, compositions and methods in which cellular carbon flux has been modified through the altered (e.g., increased or decreased) de novo generation of cellular acetate. In particular embodiments, cellular carbon flux has been modified to increase the production of acetate in a cell and/or a particular cellular location. In certain aspects, cells or microorganisms are modified to increase the production of acetate in peroxisomes.

*Modification of acetyl-CoA hydrolase activity*

In some embodiments of the cells, microorganisms, compositions and methods provided herein, the amount and/or activity of acetyl-CoA hydrolase in a cell is modified. Acetyl-CoA hydrolase (e.g. EC 3.1.2.1) is an enzyme that catalyzes the hydrolysis of acetyl-CoA to form acetate and CoA. For example, in some aspects, a cell or microorganism may be modified to increase acetyl-CoA hydrolase and/or acetyl-CoA hydrolase activity, may be modified to decrease acetyl-CoA hydrolase and/or acetyl-CoA hydrolase activity, or may be modified to alternately increase and decrease acetyl-CoA hydrolase and/or acetyl-CoA hydrolase activity depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In some aspects, the amount and/or activity of acetyl-CoA hydrolase in a cell and/or a particular cellular location is increased. For example, the amount and/or activity of acetyl-CoA hydrolase in peroxisomes of a cell can be increased. In some embodiments, the pattern of expression of acetyl-CoA hydrolase can be modified such that the enzyme is produced in a cellular location



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where it is not produced in an unmodified cell and/or is no longer produced in a cellular location where it is produced in an unmodified cell.

In one aspect, the amount and/or activity of a host acetyl-CoA hydrolase can be increased, for example, by increasing the number of copies of a nucleic acid encoding an acetyl-CoA hydrolase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the nucleic acid), by increasing the activity of a promoter that regulates transcription of a nucleic acid encoding an acetyl-CoA hydrolase, or by increasing the number of copies of a nucleic acid encoding an acetyl-CoA hydrolase and increasing the activity of a promoter that regulates transcription of a nucleic acid encoding an acetyl-CoA hydrolase. In some embodiments, an acetyl-CoA hydrolase is endogenous to the host cell or microorganism. In one aspect of the cell-based and microbial systems and methods provided herein, the amount of acetyl-CoA hydrolase protein expressed in a cell can be increased by introducing heterologous nucleic acid encoding acetyl-CoA hydrolase into a cell or microorganism. For example, introduction of heterologous nucleic acid encoding acetyl-CoA hydrolase can result in increased copy number of such nucleic acids and/or provide for modification of the cellular location in which the protein is expressed.

Acetyl-CoA hydrolase is typically localized to the mitochondrial compartment in eukaryotes. In one embodiment of the cells, microorganisms and methods provided herein, host cells are modified to express engineered acetyl-CoA hydrolase proteins that include targeting signals that direct the enzyme to peroxisomes, thereby introducing, or increasing the amount of, peroxisomal acetyl-CoA hydrolase in the cells. In particular embodiments, the engineered acetyl-CoA hydrolase protein has also been modified to exclude amino acids of a mitochondrial targeting sequence. One such modified acetyl-CoA hydrolase protein is a yeast Ach1p $^{\Delta mts+pts}$ which includes a heterologous peroxisomal targeting signal (pts) and excludes a mitochondrial targeting sequence (mts). In order to express engineered acetyl-CoA hydrolase in a targeted location, such as the peroxisomes, heterologous nucleic acid encoding the modified enzyme can be introduced into host cells. Acetate generated through the action of peroxisomal acetyl-CoA hydrolase can freely diffuse out of the peroxisome into the cytosol where it can be converted back to acetyl-CoA by the enzyme acetyl-CoA synthetase (e.g., EC 6.2.1.1), thereby increasing the generation and amount of cytosolic acetyl-CoA. In a particular embodiment, the acetyl-CoA hydrolase enzyme can be a *Candida* yeast protein. An example of a *Candida viswanathii* nucleotide sequence (*ACH1*$^{\Delta mts+pts}$; SEQ ID NO: 73) encoding a modified acetyl-CoA hydrolase lacking a mitochondrial-targeting sequence and including a peroxisomal-targeting

sequence (Ach1p$^{\Delta mts+pts}$; SEQ ID NO: 16) is provided herein. Additional examples of nucleotide sequences encoding acetyl-CoA hydrolase proteins include but are not limited to: *Saccharomyces cerevisiae ACH1* (Genbank accession numbers M31036, NM_001178255), *Candida tropicalis ACH1* (Genbank accession number XM_002550976), *Candida orthopsilosis ACH1* (Genbank accession number XM_003870486), *Candida albicans ACH1* (Genbank accession number XM_709496), *Aspergillus flavus ACH1* (Genbank accession number XM_002372714), *Neurospora crassa ACU8* (Genbank accession number XM_953261), *Cyberlindnera jadinii ACH1* (Genbank accession number AB641818), *Debaryomyces fabryi ACH1* (Genbank accession number XM_015614474), *Schizosaccharomyces octosporus ACH1* (Genbank accession numberXM_013163018), *Schizosaccharomyces japonicus ACH1* (Genbank accession numberXM_002173925), *Penicillium digitatum ACH1* (Genbank accession number XM_014683672), *Penicillium marneffei ACH1* (Genbank accession number XM_002152968) and *Talaromyces stipitatis ACH1* (Genbank accession number XM_002487448). Any of these, and other such acetyl-CoA hydrolase-encoding nucleic acids, can be analyzed for the presence of 5' ORF nucleotides encoding possible mitochondrial-targeting sequences of amino acids and modified to eliminate such sequences. Nucleotides encoding a peroxisomal-targeting sequence (e.g, a PTS1 sequence such as AKL or SKL) can also be added to the 3' terminus of the coding sequences of the nucleic acids.

The promoter used for regulating transcription of a heterologous nucleic acid encoding an acetyl-CoA hydrolase can also be modified. For example, the amount of an acetyl-CoA hydrolase protein expressed in a particular cellular location may be increased by including in the heterologous nucleic acid a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a fatty acid-inducible promoter that can provide for increased acetyl-CoA hydrolase expression, particularly when exposed to fatty acids as a carbon source. Such promoter elements include those that regulate expression of peroxisomal proteins and/or β-oxidation enzymes in microbes, e.g., a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequence of a *Candida viswanathii* HDE gene promoter is provided herein as are examples of additional fatty acid-inducible promoters.

The acetyl-CoA hydrolase activities of host and modified cells and microorganisms can be evaluated and monitored using methods known in the art. Examples of acetyl-CoA hydrolase activity assays include colorimetric assays (see, e.g., Connerton et al. (1992) *J. Gen. Microbiol.*

138:1797-1800; Robinson et al (1976) *Biochem. Biophys. Res. Commun.* 21:959-965) and radioactivity-based and acetylation inhibition assays (see, e.g., U.S. Patent no. 5,487,990 to Smith et al.). Nucleic acid sequences representing native and/or modified acetyl-CoA hydrolase-encoding sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or the amounts of the nucleic acids or encoded proteins can be assessed using quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cells or organisms exhibit increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

*Modification of peroxisomal carnitine acetyltransferase activity*

Also provided herein are cells, microorganisms, compositions and methods in which the amount and/or activity of peroxisomal carnitine acetyltransferase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount of peroxisomal carnitine acetyltransferase and/or peroxisomal carnitine acetyltransferase activity, may be modified to decrease the amount of peroxisomal carnitine acetyltransferase and/or peroxisomal carnitine acetyltransferase activity, or may be modified to alternately increase and decrease the amount of peroxisomal carnitine acetyltransferase and/or peroxisomal carnitine acetyltransferase activity depending, for example, on the conditions in which the modified cell or microorganism is cultured.

In some aspects, the amount and/or activity of peroxisomal carnitine acetyltransferase in a cell is decreased. Reducing or eliminating the amount and/or activity of peroxisomal carnitine acetyltransferase may be particularly beneficial in embodiments in which the flux of peroxisomal acetyl moiety carbons is directed toward generation of acetate within peroxisomes. In these embodiments, reducing or eliminating the amount and/or activity of peroxisomal carnitine acetyltransferase decreases the amount of peroxisomal acetyl group carbon atoms that are converted to acetyl-carnitine and provides increased peroxisomal acetyl-CoA availability for generation of peroxisomal acetate. Methods for decreasing peroxisomal carnitine acetyltransferase activity in a cell include, but are not limited to, modifying the amount of peroxisomal carnitine acetyltransferase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous peroxisomal carnitine acetyltransferase gene in an organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene,



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and/or replacing or modifying a gene encoding a wild-type peroxisomal carnitine acetyltransferase such that the encoded modified or substituted peroxisomal carnitine acetyltransferase protein has a reduced enzyme activity. For example, expression of a host peroxisomal carnitine acetyltransferase activity can be decreased by disruption (e.g., knockout, insertion mutagenesis, the like and combinations thereof) of a host gene encoding the protein, or by decreasing the activity of the promoter (e.g., through addition of repressor sequences to the promoter or 5'UTR or replacing the promoter) that controls transcription of a peroxisomal carnitine acetyltransferase gene using recombinant molecular biology techniques known in the art and described herein. In one embodiment, a diploid yeast, such as, for example, a *Candida* yeast, when used as a host microorganism can be subjected to genetic modification in which one of the two alleles of a peroxisomal carnitine acetyltransferase gene is disrupted or deleted. In so doing, a single allele of the gene remains for a reduced amount of peroxisomal carnitine acetyltransferase expression in the microorganism and a reduced amount of the protein in the cell.

One method for disrupting an endogenous peroxisomal carnitine acetyltransferase gene is by recombinantly inserting a heterologous nucleic acid (e.g., a nucleotide sequence encoding a selectable marker such as an enzyme that restores an auxotrophic host organism to prototrophy) into the endogenous gene, thereby generating an engineered organism deficient in peroxisomal carnitine acetyltransferase activity. This can be done, for example, through homologous recombination in which a heterologous nucleic acid containing sequences of the endogenous peroxisomal carnitine acetyltransferase gene and a disrupting sequence (e.g., a knock-out gene cassette such as described herein) is introduced into a host cell or microorganism. Nucleic acids encoding a peroxisomal carnitine acetyltransferase can be obtained from a number of sources, including, for example, yeast cells. Genomic DNA from cell sources can be amplified using oligonucleotide primers based on the nucleotide sequence of a peroxisomal carnitine acetyltransferase encoding gene, including examples provided herein.

In some instances, a host gene, e.g., certain yeast genes, encoding a peroxisomal carnitine acetyltransferase also encodes a mitochondrial carnitine acetyltransferase. In these organisms, a peroxisomal carnitine acetyltransferase is encoded by a gene that generates a protein containing mitochondrial and peroxisomal targeting sequences. Therefore, in such an instance, disruption or deletion of a gene encoding a carnitine acetyltransferase that is localized to peroxisomes will result in reducing or eliminating mitochondrial, as well as peroxisomal,



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carnitine acetyltransferase protein expression. In order to reduce or eliminate peroxisomal carnitine acetyltransferase expression in such cells without eliminating mitochondrial carnitine acetyltransferase expression, a heterologous nucleic acid encoding a mitochondria-targeted carnitine acetyltransferase can be introduced into the cell after disruption of the endogenous gene. For example, a mitochondrial-targeted enzyme that would not be expressed in peroxisomes can be produced in a cell or microorganism by introducing a heterologous nucleic acid that encodes a carnitine acetyltransferase that includes a mitochondrial targeting sequence of amino acids but lacks a peroxisomal targeting sequence of amino acids. An example of such a modified *Candida viswanathii* nucleic acid sequence (*CAT2*$^{\Delta pts}$; SEQ ID NO: 62), and the amino acid sequence encoded thereby (Cat2p$^{\Delta pts}$; SEQ ID NO: 5), are provided herein.

In another embodiment provided herein, a heterologous nucleic acid encoding a peroxisomal carnitine acetyltransferase that has a reduced carnitine acetyltransferase activity relative to the activity of the enzyme encoded by a host cell's or microorganism's endogenous peroxisomal carnitine acetyltransferase gene can be introduced into a host cell in which the endogenous peroxisomal carnitine acetyltransferase gene(s) has been disrupted or deleted. The heterologous nucleic acid encoding the less active carnitine acetyltransferase can be modified to include nucleotides encoding a peroxisomal targeting sequence for expression of the enzyme specifically in peroxisomes and not in other areas, such as mitochondria. For example, in one aspect, a heterologous nucleic acid encoding a *Candida viswanathii* cytoplasmic carnitine acetyltransferase (*YAT1*) with added nucleotides encoding a peroxisomal targeting sequence (e.g, a PTS1 sequence such as AKL or SKL, or slight variant thereof (PKL, PKF)) can be introduced into a host cell or microorganism (e.g., a *Candida viswanathii* cell). A nucleotide sequence encoding (and the amino acid sequence of) a *Candida viswanathii* YAT1$^{+pts}$p are provided herein (nucleotide SEQ ID NO: 70 and amino acid SEQ ID NO: 13). Additional non-limiting examples of nucleic acids encoding cytoplasmic carnitine acetyltransferase include *Saccharomyces cerevisiae YAT1* (Genbank accession number X74553), *Aspergillus nidulans FacC* (Genbank accession number AF023156), *Cyberlindnera jadinii YAT1* (Genbank accession number AB641829), *Candida dubliniensis YAT1* (Genbank accession number XM_002416790) and *Candida albicans* (Genbank accession number AF525683). A sequence of nucleotides encoding a peroxisomal targeting sequence can be added to the 3' end of the coding sequence of any such nucleic acid using methods as described herein.



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The peroxisomal carnitine acetyltransferase activities of host and modified cells and microorganisms can be evaluated and monitored using methods known in the art. For example, methods of isolating peroxisomal and mitochondrial components of yeast cells and of extracting carnitine acetyltransferase from subcellular fractions have been described by Ueda et al. [(1982) *Eur. J. Biochem.*124:205-210] and Kozulic et al. [(1987) *Eur. J. Biochem.*168:245-250]. Methods of measuring the enzymatic activity of carnitine acetyltransferase are also known in the art, see, e.g., Fritz and Schultz (1965) *J. Biol. Chem.* 240:2188-2192; Chase (1969) *Meth. Enzymol.*13:387-393. Nucleic acid sequences representing native and/or modified peroxisomal carnitine acetyltransferase-encoding sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or the amounts of the nucleic acids or encoded proteins can be assessed using quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cells or organisms exhibit increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

In other embodiments, the amount and/or activity of a host peroxisomal carnitine acetyltransferase can be increased, for example, by increasing the number of copies of a gene encoding a peroxisomal carnitine acetyltransferase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the gene), by increasing the activity of a promoter that regulates transcription of a gene encoding a peroxisomal carnitine acetyltransferase, or by increasing the number of copies of a gene encoding a peroxisomal carnitine acetyltransferase and increasing the activity of a promoter that regulates transcription of a gene encoding a peroxisomal carnitine acetyltransferase. In some embodiments, a peroxisomal carnitine acetyltransferase is endogenous to the host cell or microorganism.

*Modification of acetyl-CoA synthetase*

Acetyl-CoA synthetase (EC 6.2.1.1) is an enzyme that can catalyze the ligation of acetate and coenzyme A to produce acetyl-CoA. In many cells and organisms, the enzyme is encoded by one or more *ACS* genes. For example, in some yeast, acetyl-CoA synthetase is encoded by two genes, *ACS1* and *ACS2*, which may be differentially expressed in response to growth on differing carbon sources. In some cells, the proteins encoded by the two genes may also be differentially distributed within the nucleus, mitochondria, peroxisomes and cytoplasm of cells.



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Acs1p and Acs2p are expressed in the cytoplasm, but, in some cells, only Acs2p is present when cells are grown in glucose. *ACS1* expression may be repressed in some cells grown in glucose and derepressed when glucose is limited and/or in the presence of non-fermentable carbon sources, e.g., acetate and ethanol. Generally, *ACS2* is constitutively expressed in yeast cells. In some instances, the affinity of Acs1p for acetate may be higher, e.g, about 30-fold higher, than that of Acs2p.

Provided herein are cells, microorganisms, compositions and methods in which the amount and/or activity of acetyl-CoA synthetase (also referred to as ACS or acetate-CoA ligase and used interchangeably herein) in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount of acetyl-CoA synthetase and/or acetyl-CoA synthetase activity, may be modified to decrease the amount of acetyl-CoA synthetase and/or acetyl-CoA synthetase activity, or may be modified to alternately increase and decrease the amount of acetyl-CoA synthetase and/or acetyl-CoA synthetase activity depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

For example, in embodiments in which modification of cellular acetate generation yields increased amounts of cytosolic acetate, the amount and/or activity of cytosolic acetyl-CoA synthetase can also be increased to provide for increased conversion of acetate to acetyl-CoA. Heterologous nucleic acid encoding Acs1p and/or Acs2p can be introduced into a host cell to increase the amount of cytosolic acetyl-CoA synthetase. For example, the amount and/or activity of a host cytosolic acetyl-CoA synthetase can be increased by increasing the number of copies of a gene encoding a cytosolic acetyl-CoA synthetase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the gene), by increasing the activity of a promoter that regulates transcription of a gene encoding a cytosolic acetyl-CoA synthetase, or by increasing the number of copies of a gene encoding a cytosolic acetyl-CoA synthetase and increasing the activity of a promoter that regulates transcription of a gene encoding a cytosolic acetyl-CoA synthetase. In some embodiments, a cytosolic acetyl-CoA synthetase is endogenous to the host cell or microorganism. Additionally, a heterologous promoter can be used to regulate expression of a recombinant acetyl-CoA synthetase-encoding nucleic acid. An example of one such heterologous promoter is a fatty acid-inducible promoter that can provide for increased acetyl-CoA synthetase expression, particularly when exposed to fatty acids as a carbon source. Such promoter elements include those that regulate expression of peroxisomal proteins and/or β-



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oxidation enzymes in microbes, e.g., a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter.

In other aspects, the amount and/or activity of acetyl-CoA synthetase in a cell is decreased. Methods for decreasing acetyl-CoA synthetase activity in a cell include, but are not limited to, modifying the amount of acetyl-CoA synthetase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous acetyl-CoA synthetase gene in an organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type acetyl-CoA synthetase such that the encoded modified or substituted acetyl-CoA synthetase protein has a reduced enzyme activity. For example, in some instances, it may be desirable to decrease the amount and/or activity of a peroxisomal protein having acetyl-CoA synthetase activity.

In a particular embodiment, the acetyl-CoA synthetase enzyme can be a *Candida* yeast protein. Examples of *Candida viswanathii* nucleotide sequences (and corresponding amino acid sequences) encoding acetyl-CoA synthetase are provided herein (nucleotide SEQ ID NOS: 76 and 77 and amino acid SEQ ID NOS: 20 and 21). Additional examples of nucleotide sequences encoding acetyl-CoA synthetase proteins include, but are not limited to: *Saccharomyces cerevisiae ACS1* (Genbank accession number NM_001178197), *Saccharomyces cerevisiae ACS2* (Genbank accession number NM_001182040), *Candida tropicalis ACS1* (Genbank accession number XM_002547679), *Candida albicans ACS2* (Genbank accession number AF535132), *Cyberlindnera jadinii ACS1* (Genbank accession number AB641819), *Cyberlindnera jadinii ACS2* (Genbank accession number AB641820), *Kluyveromyces lactis ACS2* (Genbank accession number AF134491).

The acetyl-CoA synthetase activities of host and modified cells and microorganisms can be evaluated and monitored using methods known in the art. Examples of acetyl-CoA synthetase activity assays include a continuous coupled enzymatic assay (see, e.g., Castano-Cerezo et al. (2012) *Bio-protocol* 2(17) and Frenkel and Kitchens (1977) *J. Biol. Chem.* 252(2): 504-507). Nucleic acid sequences representing native and/or modified acetyl-CoA synthetase-encoding sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or the amounts of the nucleic acids or encoded proteins can be assessed using quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations



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thereof), where the engineered cells or organisms exhibit increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

*Modification of citrate processing*

Carbon atoms of acetyl groups in mitochondrial acetyl-CoA can also be captured from intermediates of the TCA cycle such as, for example, citrate molecules generated in the first step of the cycle through the citrate synthase-catalyzed condensation of acetyl-CoA and oxaloacetate. Under certain conditions, citrate can be transported from mitochondria into the cytoplasm via a mitochondrial inner membrane citrate transport protein (CTP). This transport protein provides for the efflux of citrate from mitochondria generally in exchange for the influx of a carboxylate molecule (e.g., malate) from the cytosol. Cytosolic citrate can be converted to isocitrate which can serve as a substrate in the NADPH-generating oxidation reaction through which it is converted to α-ketoglutarate. Some yeast, typically oleaginous yeast, express an endogenous ATP citrate lyase which can catalyze the cleavage of citrate into oxaloacetate and acetyl-CoA. Thus, in such instances, cytosolic citrate can serve as a source of acetyl carbons that can be converted to acetyl-CoA. In general, yeast ATP citrate lysate is a dimer and can be heterodimeric (e.g., Acl1p/Acl2p) or homomeric.

In another embodiment of the cells and microorganisms, target molecule production systems and methods provided herein, carbon atoms incorporated into citrate that has been transferred to the cytosol can be captured through the cleavage of citrate to oxaloacetate and acetyl-CoA by the enzyme ATP citrate lyase (i.e., ACL, used interchangeably herein; e.g., EC 2.3.3.8). The capture of metabolite carbon in this manner can divert it from use in other metabolic processes and also can result in an increase in the level cytoplasmic acetyl-CoA. In one aspect of this embodiment, the amount and/or activity of ATP citrate lyase in the cytosol of modified cells is increased relative to the unmodified host cell. The amount and/or activity of a host ATP citrate lyase can be increased, for example, by introducing and/or increasing the number of copies of a gene encoding an ATP citrate lyase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the gene), by increasing the activity of a promoter that regulates transcription of a gene encoding an ATP citrate lyase, or by increasing the number of copies of a gene encoding an ATP citrate lyase and increasing the activity of a promoter that regulates transcription of a gene encoding an ATP citrate lyase. In some embodiments, an ATP citrate lyase is endogenous to



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the host cell or microorganism. In other embodiments, a host cell or microorganism does not express an endogenous cytosolic ATP citrate lyase.

Thus, for example, heterologous nucleic acids encoding an ATP citrate lyase can be introduced into a host cell or microorganism to provide for an increased amount and/or activity of cytosolic ATP citrate lyase. In a particular embodiment, the ATP citrate lyase enzyme encoded by the heterologous nucleic acid can be an oleaginous yeast protein. An example of an oleaginous yeast ATP citrate lyase is formed by the *Yarrowia lipolytica* Acl1 and Acl2 proteins. Examples of *Y. lipolytica* Acl1p and Acl2p amino acid sequences are provided herein (SEQ ID NOS: 42 and 43). If a host cell or microorganism is a different species than the heterologous ATP citrate lyase that will be expressed in the host, it can be desirable to introduce nucleic acids encoding the ATP citrate lyase proteins that have been optimized for codons used in the host species. As a non-limiting example, the nucleotide sequences encoding *Yarrowia lipolytica* Acl1p and Acl2p that have been optimized for expression in a different yeast species (*Candida viswanathii*) are provided herein (SEQ ID NOS: 96 and 97). Additional examples of nucleotide sequences encoding ATP citrate lyase proteins include, but are not limited to: *Phaffia rhodozyma ACL1 and ACL2* (Genbank accession numbers KM503045, KM510496) and *Sordaria macrospora ACL1* and *ACL2* (Genbank accession numbers AJ224922, XM_003344949).

The promoter used for regulating transcription of a heterologous nucleic acid encoding an ATP citrate lyase can also be modified. For example, the amount of an ATP citrate lyase protein expressed in a cell may be increased by including in the heterologous nucleic acid a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a fatty acid-inducible promoter that can provide for increased ATP citrate lyase expression, particularly when exposed to fatty acids as a carbon source. Such promoter elements include those that regulate expression of peroxisomal proteins and/or β-oxidation enzymes in microbes, e.g., a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter.

ATP citrate lyase activity can be determined using assays known in the art and/or described herein. Such assays include, for example, methods described by Linn and Srere [(1979) *J. Biol. Chem.* 254:1691-1698] and Pentyala and Benjamin [(1995) *Biochemistry* 34:10961-10969]. Nucleic acid sequences representing native and/or modified ATP citrate lyase-encoding sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer



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extension, nucleic acid hybridization, the like and combinations thereof), or the amounts of the nucleic acids or encoded proteins can be assessed using quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cells or organisms exhibit increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

In some embodiments of the cells, organisms and methods provided herein involving capture of acetyl carbons from cytosolic citrate, it may be beneficial to increase the amount of citrate in the cytosol. One approach to increasing cytosolic citrate levels is by increasing efflux of citrate from mitochondria into the cytosol. One method of increasing mitochondrial citrate efflux involves increasing the amount and/or activity of citrate transporter protein (CTP) in mitochondria of the modified cells. The amount and/or activity of a host citrate transporter protein can be increased, for example, by introducing and/or increasing the number of copies of a gene encoding a mitochondrial citrate transporter (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the gene), by increasing the activity of a promoter that regulates transcription of a gene encoding a mitochondrial citrate transporter, or by increasing the number of copies of a gene encoding a mitochondrial citrate transporter and increasing the activity of a promoter that regulates transcription of a gene encoding a mitochondrial citrate transporter. In some embodiments, a mitochondrial citrate transporter is endogenous to the host cell or microorganism.

Thus, for example, heterologous nucleic acids encoding a mitochondrial citrate transporter can be introduced into a host cell or microorganism to provide for an increased amount and/or activity of a mitochondrial citrate transporter. In a particular embodiment, the mitochondrial citrate transporter encoded by the heterologous nucleic acid can be a yeast protein. If a host cell or microorganism is a different species than the heterologous mitochondrial citrate transporter that will be expressed in the host, it can be desirable to introduce nucleic acids encoding the mitochondrial citrate transporter that have been optimized for codons used in the host species. Examples of nucleotide sequences encoding a protein that may have mitochondrial citrate transporter activity include, but are not limited to: *Candida albicans CTP1* (Genbank accession number XM_019475315), *Candida orthopsilosis CTP1* (Genbank accession number XM_003868562), *Saccharomyces cerevisiae CTP1* (Genbank accession number NM_001178639) and *Candida tropicalis* (Genbank accession number XM_002548023).



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*Modification of acyl-CoA formation, hydrolysis and use*

Acyl-CoA is a molecule containing a carboxylic acid and coenzyme A joined through a thioester bond. In cells, acyl-CoA can be generated from carboxylic acids entering the cytosol from the extracellular environment, fatty acids synthesized within cells, lipid sidechains resulting from membrane turnover, products of the hydrolysis of triglyceride and sterol esters and carboxylation of acetyl-CoA. Acyl-CoA participates in multiple cellular pathways including lipid synthesis, β-oxidation, fatty acid synthesis and protein acylation. One reaction through which acyl-CoA is formed is the condensation between a thiol group of coenzyme A and a carboxy group of a carboxylic acid. This reaction between a fatty acid and coenzyme A is referred to as activation of the free fatty acid and can be catalyzed by an acyl-CoA synthetase enzyme (e.g. EC 6.2.1.3). A short-chain acyl-CoA, for example, malonyl-CoA, can also be generated through carboxylation of acetyl-CoA in a reaction catalyzed by an acetyl-CoA carboxylase (e.g., EC 6.4.1.2). Conversely, free fatty acids can be liberated from acyl-CoA through the action of a thioesterase (e.g., EC 3.1.2.20). Because acyl-CoA is a major carrier molecule of cellular carbons, its formation and hydrolysis represent certain aspects of methods of modifying carbon flux in cells.

*Modification of acyl-CoA synthetase activity*

Acyl-CoA synthetases (also referred to as fatty acid or acyl Co-A ligases and used interchangeably herein), are a family of enzymes in the enzyme classification subgroup 6.2.1 with varying substrate affinities, expression patterns and cellular localizations. In many microorganisms, there are multiple, distinct genes encoding separate acyl-CoA synthetases. Many yeast species (e.g., *Candida* spp. and *Saccharomyces* spp.) have five or six or more acyl-CoA synthetase genes encoding distinct enzymes. For example, *Saccharomyces cerevisiae* has 4 *FAA* genes (*FAA1*, *FAA2*, *FAA3* and *FAA4*) and 2 *FAT* genes (*FAT1* and *FAT2*) encoding acyl-CoA synthetase enzymes. Generally, *FAA* gene-encoded enzymes catalyze activation of acyl chains containing about 8-20 carbon atoms whereas the enzyme encoded by *FAT1* typically catalyzes activation of acyl chains containing 20 or more carbon atoms. Faa1p and Faa4p, which tend to be located in the cytosol and associated with membranes, are involved in activation of fatty acids internalized into cells from the extracellular medium and intracellular fatty acids arising from degradation of lipids, triacylglycerides and steryl esters. The Faa1p isozyme can exhibit broad substrate chain-length specificity, represents 90% of the cellular acyl-



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CoA synthetase activity, and is localized in the cytosolic and microsomal fractions. Faa4p has broad chain-length specificity and has been shown to be important in protein myristoylation. Faa2p is localized to peroxisomes, has broad chain-length specificity, and participates in activation of fatty acids occurring during β-oxidation. Fat1p is typically a dual function protein localized to the cellular membrane that has activity for both fatty acid transport and fatty acid activation. Fat2p tends to be targeted to the peroxisomal membrane for medium chain fatty acid transport and activation.

Homologs for *FAA1* and *FAT1* have been identified in *Candida* strains. Acyl-CoA synthetase has six isoforms encoded by FAA1, FAT1, ACS2A, ACS2B, ACS2C and ACS2D, respectively, in *Candida* spp. (e.g., homologous to FAA1, FAT1, and FAA2 in *S. cerevisiae*). Two of the homologs display 95% identity to one another and are most likely alleles of the same gene. Four *FAA2* homologs have been identified in *Candida* strain ATCC 20336 (also referred to in the art as acyl-CoA synthetase-encoding genes *ACS2A* through *ACS2D* in *Candida*). Examples of *Candida viswanathii* nucleotide sequences (and corresponding amino acid sequences) encoding acyl-CoA synthetases are provided herein (nucleotide SEQ ID NOS: 91 and 98 and amino acid SEQ ID NOS: 35 and 44) and in International patent application no. PCT/US2012/045615 (publication no. WO 2013/106730). Acetyl-CoA synthetase-encoding genes are also referred to as *ACS* genes, as described herein. For clarity, acyl-CoA synthetase-encoding genes are referred to as *FAA* or *FAT* herein and not as *ACS* genes (which herein refer to acetyl-CoA synthetase-encoding genes).

In some embodiments of the microorganisms, compositions and methods provided herein, the amount and/or activity of acyl-CoA synthetase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase acyl-CoA synthetase and/or acyl-CoA synthetase activity, may be modified to decrease acyl-CoA synthetase and/or acyl-CoA synthetase activity, or may be modified to alternately increase and decrease acyl-CoA synthetase and/or acyl-CoA synthetase activity depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

In certain aspects, the amount and/or activity of an acyl-CoA synthetase in a cell is decreased. Reducing or eliminating the amount and/or activity of an acyl-CoA synthetase may be particularly beneficial in embodiments in which the flux of carbons from fatty acids is directed



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toward a particular target product pathway and away from other cellular metabolic pathways involving activated fatty acids. When free internalized or cytosolic fatty acids are activated by acyl-CoA synthetase and used in cellular processes, such as lipid biosynthesis, the carbon atoms in the free fatty acids are not available for use in the cell or microorganism production of commercially important chemicals. Without being limited by theory, it is believed that reduction in the amount of fatty-acyl-CoA available for various cellular processes can increase the amount of fatty acids available for conversion into target molecules, for example, a fatty dicarboxylic acid (e.g., adipic acid, suberic acid, sebacic acid and dodecanedioic acid) by other engineered pathways in the same host cell or organism (e.g., omega oxidation pathway, beta oxidation pathway, omega oxidation pathway and beta oxidation pathway).

In some embodiments, one strategy is to control the subcellular location of acyl-CoA synthetase enzyme activity so that it is present only in the peroxisome. FAA1 and FAT1 mutants, faa1Δ and fat1Δ, of *Candida* were constructed and should have very little acyl-CoA synthetase activity targeted to the cytoplasm. In these mutant strains, exogenously supplied long-chain free fatty acids tend to accumulate in the cytoplasm since they cannot be transported into the peroxisome unless they are activated to the acyl-CoA thioester. High concentrations of free fatty acid can be toxic, so the cell acts to detoxify itself by oxidizing the free fatty acids to dicarboxylic acids that are much less toxic. Unlike long-chain fatty acids, long-chain dicarboxylic acids are able to diffuse into the peroxisomal compartment where they can then be activated to diacyl-CoA thioesters and enter into the beta-oxidation pathway. With multiple peroxisomal acyl-CoA synthetase isozymes it may be that each isozyme has different substrate specificity. In some embodiments, it is desired to retain those peroxisomal acyl-CoA synthetase enzymes with substrate specificity matching the chain-length of the fatty acid feedstock but without activity (or low activity) on diacids of chain-length ≤ 6, 8, 10, 12, 14, 16, 18 or 20 carbons. With this strategy, any long-chain dicarboxyl-CoA that is chain-shortened by beta-oxidation to 12 carbons, for example, that is subsequently hydrolyzed to a dicarboxylic acid and free CoA cannot be reactivated to a dicarboxyl-CoA for re-entry into beta-oxidation for further chain shortening. In some embodiments, in combination with controlling the substrate chain-length specificity of the peroxisomal acyl-CoA synthetase, a peroxisomal thioesterase activity is amplified with maximum activity at the desired chain-length of a target product. This strategy can control the chain-length of the dicarboxylic acid produced by beta-oxidation.



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Certain aspects of the microorganisms, compositions and methods provided herein include one or more modifications to reduce or eliminate cytosolic activation of free fatty acids into acyl-CoA. One approach to reducing or eliminating cytosolic free fatty acid activation is to decrease the amount and/or activity of an acyl-CoA synthetase. For example, endogenous microbial genes encoding one or more acyl-CoA synthetases (e.g., yeast *FAA1, FAA4* and/or *FAT1* gene) can be disrupted or deleted in a host cell or microorganism to reduce or eliminate acyl-CoA synthetase activity in the host relative to a cell or microorganism in which the gene(s) have not been modified. Methods for decreasing the amount and/or activity of one or more acyl-CoA synthetases, such as acyl-CoA synthetases involved in activation of cytosolic free fatty acids, in a cell include, but are not limited to, modifying the amount of acyl-CoA synthetase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous acyl-CoA synthetase gene in a cell or organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type acyl-CoA synthetase such that the encoded modified or substituted acyl-CoA synthetase protein has a reduced enzyme activity. For example, expression of a host acyl-CoA synthetase activity can be decreased or eliminated by disruption (e.g., knockout, insertion mutagenesis, the like and combinations thereof) of a host gene encoding the protein, or by decreasing the activity of the promoter (e.g., through addition of repressor sequences to the promoter or 5'UTR or replacing the promoter) that controls transcription of an acyl-CoA synthetase gene using recombinant molecular biology techniques known in the art and/or described herein. In one embodiment, a diploid yeast, such as, for example, a *Candida* yeast, when used as a host microorganism can be subjected to genetic modification in which one of the two alleles of an acyl-CoA synthetase gene is disrupted or deleted. In so doing, a single allele of the gene remains for a reduced amount of acyl-CoA synthetase expression in the microorganism and a reduced amount of the protein in the cell.

One method for disrupting an endogenous acyl-CoA synthetase gene is by recombinantly inserting a heterologous nucleic acid (e.g., a nucleotide sequence encoding a selectable marker such as an enzyme that restores an auxotrophic host organism to prototrophy) into the endogenous gene, thereby generating an engineered organism deficient in acyl-CoA synthetase activity. This can be done, for example, through homologous recombination in which a heterologous nucleic acid containing sequences of an endogenous acyl-CoA synthetase gene and a disrupting sequence (e.g., a knock-out gene cassette such as described herein) is introduced into a host cell or microorganism. Nucleic acids encoding an acyl-CoA synthetase



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can be obtained from a number of sources, including, for example, yeast cells. Genomic DNA from cell sources can be amplified using oligonucleotide primers based on the nucleotide sequence of an acyl-CoA synthetase encoding gene, including examples provided herein. Nucleotide sequences encoding (and the amino acid sequences of) *Candida viswanathii* acyl-CoA synthetase Faa1p and Fat1p are provided herein (nucleotide SEQ ID NOS: 91 and 98 and amino acid SEQ ID NOS: 35 and 44). Additional non-limiting examples of nucleic acids encoding acyl-CoA synthetases include *Saccharomyces cerevisiae FAA1* (Genbank accession numbers NM_001183737 and M96371), *Saccharomyces cerevisiae FAA4* (Genbank accession number NM_001182754), *Saccharomyces cerevisiae FAA2* (Genbank accession number NM_001178906), *Saccharomyces cerevisiae FAA3* (Genbank accession number NM_001179359), *Yarrowia lypolytica YAL1* (Genbank accession number XM_502959), *Yarrowia lypolytica FAT1* (Genbank accession number NC_006071), *Candida albicans FAA4* (Genbank accession number XM_714261), *Aspergillus nomius FAA4* (Genbank accession number XM_015551345), *Coccidioides immitis FAA4* (Genbank accession number XM_001240655) and *Aspergillus niger FAA4* (Genbank accession number XM_001397786).

In other embodiments, the amount and/or activity of a host acyl-CoA synthetase can be increased, for example, by increasing the number of copies of a gene encoding an acyl-CoA synthetase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the gene), by increasing the activity of a promoter that regulates transcription of a gene encoding an acyl-CoA synthetase, or by increasing the number of copies of a gene encoding an acyl-CoA synthetase and increasing the activity of a promoter that regulates transcription of a gene encoding an acyl-CoA synthetase. In some embodiments, an acyl-CoA synthetase is endogenous to the host cell or microorganism. In particular embodiments, the amount and/or activity of a host peroxisomal acyl-CoA synthetase is increased.

The presence, absence or amount of acyl-CoA synthetase activity can be detected by any suitable method known in the art. Non-limiting examples of suitable detection methods include enzymatic assays (e.g., Lageweg et al. (1991) *Anal. Biochem.* 197(2):384-388, Erland et al. (2001) *Anal. Biochem.* 295(1):38-44), PCR based assays (e.g., qPCR, RT-PCR), immunological detection methods (e.g., antibodies specific for acyl-CoA synthetase), the like and combinations thereof. Methods for determining acyl-CoA synthetase activities also include assays described by Trigatti et al. [(1992) *Biochem. Cell. Biol.* 70:76-80] and Kamiryo et al. [(1977) *Proc. Natl. Acad. Sci. USA* 74:4947-4950].



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*Modification of acetyl-CoA carboxylase activity*

Malonyl-CoA is a coenzyme A derivative of the dicarboxylic acid malonic acid that can serve as a precursor in the synthesis of numerous valuable organic molecules, including fatty acids and polyketides. In the cytosol, malonyl-CoA can be generated by carboxylation of acetyl-CoA through the addition of $CO_2$ (e.g., derived from bicarbonate) in a reaction catalyzed by the enzyme acetyl-CoA carboxylase (e.g., EC 6.4.1.2). Acetyl-CoA carboxylase sometimes is also referred to as "acetyl-CoA:carbon-dioxide ligase (ADP-forming)" and "acetyl coenzyme A carboxylase". In eukaryotes, acetyl-CoA carboxylase is a multifunctional polypeptide containing a biotin carrier protein domain, a biotin carboxylase domain and a carboxyl-transferase domain. Biotin joined to the biotin carrier protein is a co-factor in malonyl-CoA formation. It receives $CO_2$ which becomes attached to it at a biotin ring nitrogen in an ATP-dependent reaction catalyzed by the biotin carboxylase of the acetyl-CoA carboxylase. The activated $CO_2$ is then transferred from biotin to acetyl-CoA by the carboxyl-transferase domain to form malonyl-CoA. Malonyl-CoA can serve as a carbon donor in the synthesis of a fatty acid chain in repeated cycles of the addition of 2 carbon atoms per cycle to extend the chain and generate a fatty acid. The reactions of each cycle are catalyzed by fatty acid synthase (FAS) and continue until typically a 16-carbon fatty acid (palmitic acid) or 18-carbon fatty acid (stearic acid) is completed in the form of palmitoyl-CoA or stearoyl-CoA, respectively. Accordingly, a supply of malonyl-CoA, and/or precursors and enzymes (e.g., acetyl-CoA carboxylase) that generate malonyl-CoA, can be required for fatty acid synthesis.

In some embodiments of the cells, microorganisms, compositions and methods provided herein, the amount and/or activity of acetyl-CoA carboxylase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase acetyl-CoA carboxylase and/or acetyl-CoA carboxylase activity, may be modified to decrease acetyl-CoA carboxylase and/or acetyl-CoA carboxylase activity, or may be modified to alternately increase and decrease acetyl-CoA carboxylase and/or acetyl-CoA carboxylase activity depending, for example, on the target molecule(s) produced and/or on the conditions in which the modified cell or microorganism is cultured.

A modification of cellular carbon flux that increases cytosolic acetyl-CoA alone may not be optimal for enhancing fatty acid or other target molecule production in an engineered, cell-based



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or microbial system if there is not a concurrent increase in conversion of acetyl-CoA to malonyl-CoA. To maximize production efficiencies, included in the cells, microorganisms, compositions and methods provided herein are cellular carbon flux modifications that increase the amount of cytosolic malonyl-CoA. In one embodiment, the amount and/or activity of acetyl-CoA carboxylase is increased in the cytosol to direct carbon flux towards generation of malonyl-CoA. The amount and/or activity of a host acetyl-CoA carboxylase can be increased, for example, by increasing the number of copies of a gene encoding an acetyl-CoA carboxylase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the gene), by increasing the activity of a promoter that regulates transcription of a gene encoding an acetyl-CoA carboxylase, or by increasing the number of copies of a gene encoding an acetyl-CoA carboxylase and increasing the activity of a promoter that regulates transcription of a gene encoding an acetyl-CoA carboxylase. In some embodiments, an acetyl-CoA carboxylase is endogenous to the host cell or microorganism. An acetyl-CoA carboxylase activity may be amplified by over-expression of an acetyl-CoA carboxylase gene by any suitable method. Non-limiting examples of methods suitable to amplify or over express a gene include amplifying the number of acetyl-CoA carboxylase genes in yeast, for example, following transformation with a high-copy number plasmid (e.g., such as one containing a 2u origin of replication), integration of multiple copies of the gene into the host genome, over-expression of the gene directed by a strong promoter, the like or combinations thereof. An acetyl-CoA carboxylase gene may be native to *Candida tropicalis* or *Candida viswanithii*, for example, or it may be obtained from a heterologous source. Examples of a *Candida viswanathii* acetyl-CoA carboxylase polypeptide amino acid sequence (Acc1p), and nucleotide sequence encoding it (*ACC1*), are provided herein (nucleotide SEQ ID NO: 74 and amino acid SEQ ID NOS: 18 and 19). Additional non-limiting examples of nucleic acids encoding an acetyl-CoA carboxylase include *Yarrowia lypolytica ACC1* (Genbank accession NC_006069), *Saccharomyces cerevisiae ACC1* (Genbank accession NM_001183193), *Candida tropicalis ACC* (Genbank accession number XM_002546179), *Candida albicans ACC1* (Genbank accession number XM_713531), *Aspergillus nidulans ACCA* (Genbank accession number Y15996), *Aspergillus niger ACCA* (Genbank accession number XM_001395439), *Aspergillus oryzae ACC* (Genbank accession number XM_001826359), *Schizosaccharomyces pombe ACC* (Genbank accession D78169), *Neurospora crassa ACC* (Genbank accession XM_957924), *Lipomyces starkeyi ACC1* (Genbank accession KJ948118), *Debaryomyces hansenii ACC1* (Genbank accession XM_457211), *Amylomyces rouxii ACC* (Genbank accession EF397565) and *Coccidioides immitis ACC* (Genbank accession number XM_001247055).

The promoter used for regulating transcription of a heterologous nucleic acid encoding an acetyl-CoA carboxylase can also be modified. For example, the amount of an acetyl-CoA carboxylase protein expressed in a particular cellular location may be increased by including in the heterologous nucleic acid a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a fatty acid-inducible promoter that can provide for increased acetyl-CoA carboxylase expression, particularly when exposed to fatty acids as a carbon source. Such promoter elements include those that regulate expression of peroxisomal proteins and/or β-oxidation enzymes in microbes, e.g., a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene promoter. The nucleotide sequence of a *Candida viswanathii* HDE gene promoter is provided herein as are examples of additional fatty acid-inducible promoters.

The reverse activity (e.g., decarboxylation of malonyl-CoA) is carried out by a separate enzyme, malonyl-CoA decarboxylase. In some embodiments, to further increase carbon flux through a particular reaction or through a metabolic pathway, one or more reverse activities in the pathway can be altered to inhibit the back conversion of a desired product into its starting reactants. In certain embodiments, a malonyl-CoA decarboxylase activity is reduced or eliminated to further increase the carbon flux through an acetyl-CoA carboxylase activity in the direction of malonyl-CoA production.

Acetyl-CoA carboxylase is regulated by feedback inhibition of acyl-CoA (e.g., palmitoyl-CoA) and by phosphorylation. As such, increasing the copy number of acetyl-CoA carboxylase-encoding nucleic acids in a cell may not alone be sufficient in increasing the acetyl-CoA carboxylase activity in the cell. Because the dephosphorylated state is the active state of the enzyme, one approach for increasing the activity of acetyl-CoA carboxylase is to reduce or eliminate phosphorylation of the protein. Provided herein are modified acetyl-CoA carboxylase proteins (and mutant nucleic acids encoding the proteins) in which one or more phosphorylatable serine residues have been substituted with alanine residues thereby relieving the regulation by phosphorylation. In a particular embodiment, the modified acetyl-CoA carboxylase is a modified yeast enzyme. For example, as described herein, an endogenous *Candida viswanathii* acetyl-CoA carboxylase wild-type enzyme was modified to substitute alanine residues for one or more of the following serine amino acid residues: S652, S1131, S1138, S1153, S1158. The modified amino acid sequences are provided herein. Also provided



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herein are cells and microorganisms containing heterologous nucleic acid encoding a modified acetyl-CoA carboxylase protein and methods of increasing cytosolic malonyl-CoA generation and producing organic acids and other target products using the compositions provided herein. In another aspect of this embodiment, the amount and/or activity of acetyl-CoA carboxylase is increased in the cytosol of modified cells relative to an unmodified host cell by introducing multiple copies of the modified heterologous nucleic acid encoding acetyl-CoA carboxylase into a host cell to provide for increased acetyl-CoA carboxylase protein and/or by increasing the expression of the modified acetyl-CoA carboxylase in the cell through the use of a strong heterologous promoter.

In other aspects, the amount and/or activity of acetyl-CoA carboxylase in a cell is decreased. Methods for decreasing acetyl-CoA carboxylase activity in a cell include, but are not limited to, modifying the amount of acetyl-CoA carboxylase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous acetyl-CoA carboxylase gene in an organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type acetyl-CoA carboxylase such that the encoded modified or substituted acetyl-CoA carboxylase protein has a reduced enzyme activity.

The presence, absence or amount of acyl-CoA carboxylase activity can be detected by any suitable method known in the art. Non-limiting examples of suitable detection methods include radioactive $HCO_3^-$ incorporation assays and coupled enzyme assays (e.g., Diacovich et al. (2002) *J. Biol. Chem.* 277(34):31228-31236), PCR based assays (e.g., qPCR, RT-PCR), immunological detection methods (e.g., antibodies specific for acyl-CoA carboxylase), the like and combinations thereof.

*Modification of thioesterase activity*

A thioesterase is an enzyme that catalyzes the hydrolysis of a thioester bond between a carbonyl group and a sulfur atom. In cells, certain thioesterases (e.g., acyl-CoA thioesterase activity, acyl-ACP thioesterase activity) catalyze the removal of Coenzyme A or acyl carrier protein (e.g., ACP) from a fatty acid yielding a free fatty acid and unesterified carrier, e.g., Coenzyme A (CoASH). The reaction occurs in the presence of water, and Coenzyme A or acyl carrier protein is specifically removed at a thiol group. The released CoA can then be reused for other cellular activities. A non-limiting example of an enzyme with thioesterase activity is acyl-



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CoA hydrolase (e.g., EC 3.1.2.20; also referred to as acyl coenzyme A thioesterase, acyl-CoA thioesterase, acyl coenzyme A hydrolase, thioesterase B, thioesterase II, lecithinase B, lysophopholipase L1, acyl-CoA thioesterase 1, and acyl-CoA thioesterase). In eukaryotic microorganisms, acyl-CoA thioesterases are generally localized in peroxisomes but may also occur in mitochondria.

In some embodiments of the cells, microorganisms, compositions and methods provided herein, the amount and/or activity of a thioesterase in a cell is modified. For example, in some aspects, a cell or microorganism may be modified to increase the amount of thioesterase and/or thioesterase activity, may be modified to decrease thioesterase and/or thioesterase activity, or may be modified to alternately increase and decrease thioesterase and/or thioesterase activity depending, for example, on the cellular location(s) of the enzyme and/or on the conditions in which the modified cell or microorganism is cultured.

Embodiments of cells and microorganisms provided herein in which carbon flux is modified to increase acetyl-CoA carboxylase and/or cytosolic malonyl-CoA, may further benefit from also modifying the amount of medium-to-long chain fatty acids present in the cytosol in the esterified form as acyl-CoA (e.g., palmitoyl-CoA). In some of these embodiments, the increased generation of malonyl-CoA can lead to increased fatty acid synthesis in the presence of cytosolic fatty acid synthase (FAS). The end-product of cytosolic fatty acid synthesis in yeast cells is typically an acyl-CoA, e.g., palmitoyl-CoA, which can then be used in cellular metabolic pathways other than desired engineered target molecule production processes. This represents a loss of the carbon atoms in the acyl-CoA which could have been incorporated into target products. Additionally, high levels of cytosolic acyl-CoA end products of fatty acid synthesis (e.g., palmitoyl-CoA) can inhibit acetyl-CoA carboxylase. Therefore, production efficiency may be enhanced in some embodiments by decreasing the amount of fatty acids present in the cytosol in the esterified form as acyl-CoA.

Included in the cells, microorganisms, compositions and methods provided herein are cellular carbon flux modifications that decrease the amount of fatty acids present in the cytosol in the esterified form as acyl-CoA. In one embodiment, the amount and/or activity of thioesterase is increased in the cytosol of cells or microorganisms. The amount and/or activity of a thioesterase can be increased, for example, by increasing the number of copies of a gene encoding a thioesterase (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more copies of the gene),



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by increasing the activity of a promoter that regulates transcription of a gene encoding a thioesterase, or by increasing the number of copies of a gene encoding a thioesterase and increasing the activity of a promoter that regulates transcription of a gene encoding a thioesterase. In certain aspects, the amount and/or activity of a thioesterase in a cell and/or a particular cellular location is increased. For example, the amount and/or activity of a thioesterase in the cytosol of a cell can be increased. In some embodiments, the pattern of expression of a thioesterase can be modified such that the enzyme is produced in a cellular location where it is not produced in an unmodified cell and/or is no longer produced in a cellular location where it is produced in an unmodified cell.

In yeast, thioesterases are generally present in the peroxisomal compartment of the cells to ensure that free coenzyme A is available for beta-oxidation. Without being bound or limited by theory, this enzyme should not normally be present in the cytoplasm because producing fatty acyl-CoA via cytosolic fatty acid synthesis is an energy intensive process, and removing the CoA from the synthesized acyl-CoA would waste the energy put into the process. In order to provide for generation of free fatty acids in the cytoplasm of modified cells or organisms, peroxisomal thioesterase with activity on long chain fatty acids can be re-targeted to the cytoplasm. In one embodiment of the cells, microorganisms and methods provided herein, host cells are modified to express engineered thioesterase polypeptides that lack targeting signals that direct the enzyme to peroxisomes (i.e, PTS), thereby introducing, or increasing the amount of, cytosolic thioesterase in the cells. In a particular embodiment, a thioesterase lacking a PTS that has activity on medium and long chain fatty acids is heterologously expressed in the cytoplasm of modified cells. This can be accomplished by modifying nucleic acids encoding a peroxisomal thioesterase to delete the portion of the nucleic acid encoding the peroxisomal targeting signal at the C-terminus of the protein, and introducing the modified heterologous nucleic acid into host cells. An example of one such modified thioesterase protein is a yeast $Tes3p^{\Delta pts}$ which excludes a peroxisomal targeting signal (PTS). As described herein, generally, a yeast peroxisomal targeting sequence is a 3-amino acid consensus sequence (PTS1). In a particular embodiment, the thioesterase enzyme can be a *Candida* yeast protein. For example, *Candida* strain ATCC 20336 contains eight genes encoding peroxisomal thioesterases (*TES1-TES8*), each of which contains a C-terminal 3-amino acid PTS1 consensus sequence (SRL, ARL) or slight variant thereof (PKL, PKF). Nucleotide sequences encoding the 8 thioesterases, and the amino acid acid sequences of the thioesterases, are provided herein (nucleotide SEQ ID NOS: 78, 79, 80, 81, 82, 83, 84, 85, 86 and 87 and amino acid SEQ ID NOS: 22, 23, 24, 25,



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26, 27, 28, 29, 30 and 31). An example of a *Candida viswanathii* nucleotide sequence encoding a modified Tes3p lacking a peroxisomal-targeting sequence (*TES3*$^{\Delta pts}$), and the amino acid sequence of the modified Tes3p, are also provided herein (nucleotide SEQ ID NO: 88 and amino acid SEQ ID NO: 32). In some embodiments, the thioesterase polypeptide is from a different species than a host microorganism in which it is expressed.

Nucleic acid sequences encoding polypeptides conferring thioesterase activity can be obtained from a number of sources, including, for example, yeast (e.g., *Candida*, *Saccharomyces*, *Debaryomyces*, *Meyerozyma*, *Lodderomyces*, *Scheffersomyces*, *Clavispora*, *Yarrowia*, *Pichia, Kluyveromyces*, *Eremothecium*, *Zygosaccharomyces*, *Lachancea*, *Nakaseomyces*), animals (e.g., *Homo*, *Rattus*), bacteria (e.g., *Escherichia*, *Pseudomonas*, *Bacillus*), and plants (e.g., *Arabidopsis*, *Nictotania*, *Cuphea*). Examples of nucleotide sequences encoding polypeptides having thioesterase activity include, without limitation, *Saccharomyces cerevisiae PTE1* (Genbank accession no. AF124265), *Debaryomyces hansenii* (Genbank accession nos. XM_456353, XM_459767), *Aspergillus niger* (Genbank accession nos. XM_001392518, XM_011389712, XM_011395790), *Aspergillus fumigatus* (Genbank accession no. XM_742375), *Candida albicans* (Genbank accession nos. XM_705831, XM_705833), *Candida dubliniensis* (Genbank accession no. XM_002418475), *Candida orthopsilosis* (Genbank accession nos. XM_003866686, XM_003866684), *Neurospora crassa* (Genbank accession nos. XM_956915, XM_960627), *Rhodotorula toruloides* (Genbank accession no. XM_016414800), *Cryptococcus neoformans* (Genbank accession no. XM_012196078, XM_012195836), *Escherichia coli TesA* (Genbank accession no. L06182) and acyl-(ACP) thioesterase type B from *Cuphea lanceolata* (Genbank accession no. CAB60830).

The promoter used for regulating transcription of a heterologous nucleic acid encoding a thioesterase can also be modified. For example, the amount of a thioesterase protein expressed in a particular cellular location may be increased by including in the heterologous nucleic acid a strong heterologous promoter and/or a promoter that provides for a different pattern of expression in the cell or microorganism. An example of one such heterologous promoter is a fatty acid inducible promoter that can provide for increased thioesterase expression, particularly when exposed to fatty acids as a carbon source. Such promoter elements include those that regulate expression of peroxisomal proteins and/or β-oxidation enzymes in microbes, e.g., a *Candida* hydratase-dehydrogenase-epimerase (*HDE*) gene

promoter. The nucleotide sequence of a *Candida viswanathii* HDE gene promoter is provided herein as are examples of additional fatty acid-inducible promoters.

In other aspects, the amount and/or activity of a thioesterase in a cell is decreased. Methods for decreasing thioesterase activity in a cell include, but are not limited to, modifying the amount of thioesterase protein expression in the cell, for example, by replacing the wild-type promoter of an endogenous thioesterase gene in an organism with a weaker heterologous promoter, deleting or disrupting an endogenous gene, and/or replacing or modifying a gene encoding a wild-type such that the encoded modified or substituted thioesterase protein has a reduced enzyme activity.

Presence, absence or amount of thioesterase activity can be detected by any suitable method known in the art or described herein (see, e.g., Jones et al. (1999) *J. Biol. Chem.* 274(14):9216-9223 and Chemistry and Biology 9: 981-988). Nucleic acid sequences representing native and/or modified thioesterase-encoding sequences also can be detected using nucleic acid detection methods (e.g., PCR, primer extension, nucleic acid hybridization, the like and combinations thereof), or the amounts of the nucleic acids or encoded proteins can be assessed using quantitative expression based analysis (e.g., RT-PCR, western blot analysis, northern blot analysis, the like and combinations thereof), where the engineered cells or organisms exhibit increased or decreased RNA and/or polypeptide levels as compared to the host cell or organism.

*Methods of modifying cells and organisms*

Provided herein are cells and organisms (including microorganisms) that have been modified in one or more aspects relative to the unmodified cell or organism (i.e., the cell or organism prior to the modification). For example, a cell or organism can be modified by altering one or more cellular activities and/or the sum total of a cell's or organism's activities. Thus, modifications can include, for example, alteration of cellular activities, addition of cellular activities and/or elimination of cellular activities. A cell or organism may be modified, for example, by altering the amount of one or more cellular compositions, e.g, polynucleotides and/or polypeptides. In some embodiments, an activity and/or amount of a composition can be altered by genetically modifying a host cell or microorganism which yields an engineered cell or microorganism having added, increased, reduced, decreased or removed activity or composition. Genetic



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modifications can be achieved in several ways, including, for example, introducing heterologous nucleic acids into host cells or organisms using molecular biological techniques known in the art and/or described herein.

*Polynucleotides*

The term "polynucleotide" is used herein interchangeably with the term "nucleic acid" and refers to an organic polymer composed of two or more monomers including nucleotides, nucleosides or analogs thereof, including but not limited to single stranded or double stranded, sense or antisense deoxyribonucleic acid (DNA) of any length and, where appropriate, single stranded or double stranded, sense or antisense ribonucleic acid (RNA) of any length, including siRNA. The term "nucleotide" refers to any of several compounds that consist of a ribose or deoxyribose sugar joined to a purine or a pyrimidine base (nitrogenous base) and to a phosphate group, and that are the basic structural units of nucleic acids. The term "nucleoside" refers to a compound (as guanosine or adenosine) that consists of a purine or pyrimidine base (nitrogenous base) combined with deoxyribose or ribose and is found especially in nucleic acids. The term "nucleotide analog" or "nucleoside analog" refers, respectively, to a nucleotide or nucleoside in which one or more individual atoms have been replaced with a different atom or with a different functional group. Accordingly, the term polynucleotide includes nucleic acids of any length, DNA, RNA, analogs and fragments thereof. A polynucleotide of three or more nucleotides is also called nucleotidic oligomer or oligonucleotide.

A nucleic acid (e.g., a nucleic acid reagent, target nucleic acid, target nucleotide sequence, nucleic acid sequence of interest or nucleic acid region of interest) can be from any source or composition, such as DNA, cDNA, gDNA (genomic DNA), RNA, siRNA (short inhibitory RNA), RNAi, tRNA or mRNA, for example, and can be in any form (e.g., linear, circular, supercoiled, single-stranded, double-stranded, and the like). A nucleic acid can also comprise DNA or RNA analogs (e.g., containing base analogs, sugar analogs and/or a non-native backbone and the like). It is understood that the term “nucleic acid” does not refer to or infer a specific length of the polynucleotide chain, thus polynucleotides and oligonucleotides are also included in the definition. Deoxyribonucleotides include deoxyadenosine, deoxycytidine, deoxyguanosine and deoxythymidine. For RNA, the uracil base is uridine.



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The terms “endogenous polynucleotide,” “endogenous nucleic acid,” “native polynucleotide” and “native nucleic acid,” used interchangeably herein, refer to a polynucleotide of a cell or organism that exists, or is inherent, in the genetic composition of the cell or organism prior to modification.

The terms "heterologous polynucleotide," "heterologous nucleic acid," “exogenous polynucleotide,” “exogenous nucleic acid,” “foreign polynucleotide” and “foreign nucleic acid,” used interchangeably herein, refer to a polynucleotide as it relates to a particular reference cell or organism (e.g., a host cell or organism) and is one that is not present in the genetic composition of the reference cell or organism. A heterologous polynucleotide includes a polynucleotide that may be identical in nucleotide sequence to an endogenous polynucleotide present in a cell, but if introduced into the cell would alter the genetic composition of the cell by, for example, increasing the copy number of the polynucleotide in the cell, altering the position(s) of the polynucleotide in the cell genome, altering the expression of the polynucleotide in the cell, and the like. Thus, such a heterologous nucleic acid thereby genetically modifies the cell into which it is introduced. A heterologous polynucleotide in a host cell may exist in a nucleic acid autonomous of the host chromosome or may be inserted into a host chromosome. A heterologous polynucleotide can also be a polynucleotide with a different nucleotide sequence relative to any nucleic acid in a particular reference cell and can also be obtained from a different cell type or species of organism. A heterologous nucleic acid can also be generated by synthetic methods known in the art and/or described herein.

The term "expression" with respect to a nucleic acid sequence or protein refers to transcription of the nucleic acid and/or, as appropriate, translation of an mRNA transcript to a protein (protein synthesis). Thus, as will be clear from the context, expression of a protein results from transcription and translation of an open reading frame (ORF) sequence. The level of expression of a nucleic acid and/or protein in a cell may be determined, for example, on the basis of either the amount of RNA transcript of a nucleic acid that is present in the cell and/or the amount of the product encoded by the nucleic acid. For example, mRNA transcribed from a selected sequence can be quantitated by qRT-PCR or by Northern hybridization (see Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989)). Protein encoded by a nucleic acid can be quantitated by various methods, e.g., by ELISA, by assaying for the biological activity of the protein, or by employing assays that are independent of such activity, such as western blotting or radioimmunoassay, using antibodies that recognize and bind the protein (see Sambrook et al., 1989, supra).



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A nucleic acid sometimes is a plasmid, phage, autonomously replicating sequence (ARS), centromere, artificial chromosome, yeast artificial chromosome (e.g., YAC) or other nucleic acid able to replicate or be replicated in a host cell. In certain embodiments a nucleic acid can be from a library or can be obtained from enzymatically digested, sheared or sonicated genomic DNA (e.g., fragmented) from an organism of interest. Fragments can be generated by any suitable method in the art, and the average, mean or nominal length of nucleic acid fragments can be controlled by selecting an appropriate fragment-generating procedure. In some embodiments, the fragmented DNA can be size selected to obtain nucleic acid fragments of a particular size range. Nucleic acid can be fragmented by various methods known in the art, which include without limitation, physical, chemical and enzymic processes. Examples of such processes are described in U.S. Patent Application Publication No. 20050112590 (published on May 26, 2005, entitled "Fragmentation-based methods and systems for sequence variation detection and discovery," naming Van Den Boom et al.). Certain processes can be selected to generate non-specifically cleaved fragments or specifically cleaved fragments. Examples of processes that can generate non-specifically cleaved fragment sample nucleic acid include, without limitation, contacting sample nucleic acid with apparatus that expose nucleic acid to shearing force (e.g., passing nucleic acid through a syringe needle; use of a French press); exposing sample nucleic acid to irradiation (e.g., gamma, x-ray, UV irradiation; fragment sizes can be controlled by irradiation intensity); boiling nucleic acid in water (e.g., yields about 500 base pair fragments) and exposing nucleic acid to an acid and base hydrolysis process.

Nucleic acid may be specifically cleaved by contacting the nucleic acid with one or more specific cleavage agents. The term "specific cleavage agent" as used herein refers to an agent, sometimes a chemical or an enzyme that can cleave a nucleic acid at one or more specific sites. Specific cleavage agents often will cleave specifically according to a particular nucleotide sequence at a particular site. Examples of enzyme specific cleavage agents include without limitation endonucleases (e.g., DNase (e.g., DNase I, II); RNase (e.g., RNase E, F, H, P); Cleavase™ enzyme; Taq DNA polymerase; E. coli DNA polymerase I and eukaryotic structure-specific endonucleases; murine FEN-1 endonucleases; type I, II or III restriction endonucleases such as Acc I, Afl III, Alu I, Alw44 I, Apa I, Asn I, Ava I, Ava II, BamH I, Ban II, Bcl I, Bgl I. Bgl II, Bln I, Bsm I, BssH II, BstE II, Cfo I, Cla I, Dde I, Dpn I, Dra I, EclX I, EcoR I, EcoR I, EcoR II, EcoR V, Hae II, Hae II, Hind II, Hind III, Hpa I, Hpa II, Kpn I, Ksp I, Mlu I, MluN I, Msp I, Nci I, Nco I, Nde I, Nde II, Nhe I, Not I, Nru I, Nsi I, Pst I, Pvu I, Pvu II, Rsa I, Sac I, Sal I, Sau3A I,

Sca I, ScrF I, Sfi I, Sma I, Spe I, Sph I, Ssp I, Stu I, Sty I, Swa I, Taq I, Xba I, Xho I); glycosylases (e.g., uracil-DNA glycolsylase (UDG), 3-methyladenine DNA glycosylase, 3-methyladenine DNA glycosylase II, pyrimidine hydrate-DNA glycosylase, FaPy-DNA glycosylase, thymine mismatch-DNA glycosylase, hypoxanthine-DNA glycosylase, 5-Hydroxymethyluracil DNA glycosylase (HmUDG), 5-Hydroxymethylcytosine DNA glycosylase, or 1,N6-etheno-adenine DNA glycosylase); exonucleases (e.g., exonuclease III); ribozymes, and DNAzymes. Nucleic acid may be treated with a chemical agent, or synthesized using modified nucleotides, and the modified nucleic acid may be cleaved. In non-limiting examples, nucleic acid may be treated with (i) alkylating agents such as methylnitrosourea that generate several alkylated bases, including N3-methyladenine and N3-methylguanine, which are recognized and cleaved by alkyl purine DNA-glycosylase; (ii) sodium bisulfite, which causes deamination of cytosine residues in DNA to form uracil residues that can be cleaved by uracil N-glycosylase; and (iii) a chemical agent that converts guanine to its oxidized form, 8-hydroxyguanine, which can be cleaved by formamidopyrimidine DNA N-glycosylase. Examples of chemical cleavage processes include without limitation alkylation, (e.g., alkylation of phosphorothioate-modified nucleic acid); cleavage of acid lability of P3'-N5'-phosphoroamidate-containing nucleic acid; and osmium tetroxide and piperidine treatment of nucleic acid.

As used herein, the term "complementary cleavage reactions" refers to cleavage reactions that are carried out on the same nucleic acid using different cleavage reagents or by altering the cleavage specificity of the same cleavage reagent such that alternate cleavage patterns of the same target or reference nucleic acid or protein are generated. In certain embodiments, nucleic acids of interest may be treated with one or more specific cleavage agents (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more specific cleavage agents) in one or more reaction vessels (e.g., nucleic acid of interest is treated with each specific cleavage agent in a separate vessel).

A nucleic acid suitable for use in the embodiments described herein sometimes is amplified by any amplification process known in the art (e.g., PCR, RT-PCR and the like). Nucleic acid amplification may be particularly beneficial when using organisms that are typically difficult to culture (e.g., slow growing, require specialize culture conditions and the like). The terms "amplify", "amplification", "amplification reaction", or "amplifying" as used herein refer to any in vitro processes for multiplying the copies of a target sequence of nucleic acid. Amplification sometimes refers to an "exponential" increase in target nucleic acid. However, "amplifying" as used herein can also refer to linear increases in the numbers of a select target sequence of



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nucleic acid, but is different than a one-time, single primer extension step. In some embodiments, a limited amplification reaction, also known as pre-amplification, can be performed. Pre-amplification is a method in which a limited amount of amplification occurs due to a small number of cycles, for example 10 cycles, being performed. Pre-amplification can allow some amplification, but stops amplification prior to the exponential phase, and typically produces about 500 copies of the desired nucleotide sequence(s). Use of pre-amplification may also limit inaccuracies associated with depleted reactants in standard PCR reactions.

In some embodiments, a nucleic acid reagent sometimes is stably integrated into the chromosome of a host cell or organism, or a nucleic acid reagent can be a deletion of a portion of a host chromosome, in certain embodiments (e.g., genetically modified cells or organisms, where alteration of the host genome confers the ability to selectively or preferentially maintain the desired cell or organism carrying the genetic modification). Such nucleic acid reagents (e.g., nucleic acids or genetically modified cells or organisms whose altered genome confers a selectable trait to the cell or organism) can be selected for their ability to guide production of a desired protein or nucleic acid molecule. When desired, the nucleic acid reagent can be altered such that codons encode for (i) the same amino acid, using a different tRNA than that specified in the native sequence, or (ii) a different amino acid than is normal, including unconventional or unnatural amino acids (including detectably labeled amino acids).

A nucleic acid or nucleic acid reagent can comprise certain elements often selected according to the intended use of the nucleic acid. Any of the following elements can be included in or excluded from a nucleic acid reagent. A nucleic acid reagent, for example, may include one or more or all of the following nucleotide elements: one or more promoters, one or more 5' untranslated regions (5'UTRs), one or more regions into which a target nucleotide sequence may be inserted (an "insertion element"), one or more target nucleotide sequences, one or more terminator elements, one or more 3' untranslated regions (3'UTRs), and one or more selection elements. A nucleic acid reagent can be provided with one or more of such elements and other elements may be inserted into the nucleic acid before the nucleic acid is introduced into the desired cell or organism. In some embodiments, a provided nucleic acid reagent comprises a promoter, 5'UTR, optional 3'UTR and insertion element(s) by which a target nucleotide sequence is inserted (i.e., cloned) into the nucleotide acid reagent. In certain embodiments, a provided nucleic acid reagent comprises a promoter, insertion element(s) and optional 3'UTR, and a 5' UTR/target nucleotide sequence is inserted with an optional 3'UTR. The elements can



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be arranged in any order suitable for expression in the chosen expression system (e.g., expression in a chosen cell or organism, or expression in a cell free system, for example), and in some embodiments a nucleic acid reagent comprises the following elements in the 5' to 3' direction: (1) promoter, 5'UTR, and insertion element(s); (2) promoter, 5'UTR, and target nucleotide sequence; (3) promoter, 5'UTR, insertion element(s) and 3'UTR; and (4) promoter, 5'UTR, target nucleotide sequence and 3'UTR.

*Promoters*

A promoter typically is required for cellular DNA synthesis and/or RNA synthesis. A promoter often contains a region of DNA that can facilitate the transcription of a particular gene, by providing a start site for the synthesis of RNA corresponding to a gene. Promoters generally are located near the genes they regulate, are located upstream (i.e., 5') of the START codon of the structural gene, and are on the same strand of DNA as the sense strand of the gene, in some instances. Eukaryotic promoters generally include a core promoter element that may contain a TATA box, a proximal sequence and transcription enhancer sequences positioned farther upstream (referred to, e.g., with respect to yeast, as upstream activating sequences or UAS located several hundred to thousands of kilobases upstream from a transcriptional start site (TSS)). The types and combination of these elements can influence promoter strength (see, e.g., Hussain et al. (2016) *ACS Synth. Biol.* 5:213-223). As used herein, "promoter," "promoter sequence" and "promoter region" are used interchangeably to refer to nucleotide sequences that can regulate gene transcription. Such sequences can include, but are not limited to, core promoter (e.g., extending upstream from the transcription START site (TSS)) elements (e.g., TATA box, RNA polymerase binding site, CCAAT box), proximal cis-acting sequences that bind proteins and can facilitate binding of RNA polymerase to DNA, and distant cis-regulatory sequences (e.g, enhancers and silencers) that can bind transcription factors and influence (e.g., activate, increase, elevate, decrease, reduce) transcription.

In some embodiments, a promoter sequence can be isolated from a nucleic acid or cell or organism and combined in functional connection or operable linkage with a polynucleotide sequence to allow altered and/or regulated expression. A non-native promoter (e.g., promoter not normally associated with a given nucleic acid sequence) used for expression of a nucleic acid often is referred to as a heterologous promoter. In certain embodiments, a heterologous promoter and/or a 5'UTR can be combined in functional connection with a polynucleotide that



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encodes a polypeptide having a desired activity as described herein. The terms "operably linked" and "in functional connection with" as used herein with respect to promoters, refer to a relationship between a nucleic acid coding sequence and a promoter element. The promoter is operably linked or in functional connection with the coding sequence when expression from the coding sequence via transcription is regulated, or controlled by, the promoter element. The terms "operably linked" and "in functional connection with" are utilized interchangeably herein with respect to promoter elements.

A promoter often interacts with a RNA polymerase. A polymerase is an enzyme that catalyzes synthesis of nucleic acids using a preexisting nucleic acid reagent. When the template is a DNA template, an RNA molecule is transcribed before protein is synthesized. Enzymes having polymerase activity suitable for use in the present methods include any polymerase that is active in the chosen system with the chosen template to synthesize protein. In some embodiments, a promoter (e.g., a heterologous promoter), can be operably linked to a nucleotide sequence or an open reading frame (ORF). Transcription from the promoter element can catalyze the synthesis of an RNA corresponding to the nucleotide sequence or ORF sequence operably linked to the promoter, which in turn leads to synthesis of a desired peptide, polypeptide or protein.

There are generally several types of promoters, e.g., constitutive, repressible and inducible. Constitutive promoters can be considered as unregulated (i.e., regulated essentially only by RNA polymerase levels) and provide for consistent expression of a gene that is under the transcriptional control of the promoter. Repressible and inducible promoters are regulatable by various cellular conditions. A repressible promoter is one that can be silenced, or "turned off," by the binding of a repressor molecule to a particular nucleotide sequence which serves to inhibit the functional interaction of RNA polymerase with the promoter and inhibits transcription. This is referred to as negative control or regulation and is in contrast to positive control of transcription which can occur via activator molecules binding to DNA and increasing the rate of transcription. An inducible promoter is one in which transcription can be induced in the presence of an effector molecule that, for example, binds to a regulatory transcription factor and results in increased rates of transcription. As used herein, a "non-inducible" promoter is a promoter that does not exhibit increased activity, in terms of transcription activation of an operably linked nucleic acid, in response to the presence of an effector or inducing agent. A non-inducible promoter can be one that is not induced by one agent but is induced by another.

For example, the transcription-regulating activity of a non-fatty acid-inducible promoter is not detectably increased in the presence of a fatty acid, although there may be other agents that do induce the promoter and increase transcription of a nucleic acid operably linked to the promoter.

Promoters sometimes exhibit responsiveness to regulatory control. Promoters also sometimes can be regulated by a selective agent. That is, transcription from promoters sometimes can be turned on, turned off, up-regulated or down-regulated, in response to a change in environmental, nutritional or internal conditions or signals (e.g., heat inducible promoters, light regulated promoters, feedback regulated promoters, hormone influenced promoters, tissue specific promoters, oxygen and pH influenced promoters, promoters that are responsive to selective agents (e.g., kanamycin) and promoters responsive to certain carbon sources (e.g., fatty acids) and the like, for example). Promoters influenced by environmental, nutritional or internal signals frequently are influenced by a signal (direct or indirect) that binds at or near the promoter and increases or decreases expression of the target sequence under certain conditions.

The strength of a promoter sequence can be measured as the amount of transcription of a gene product initiated at the promoter relative to a reference or control. For example, a reference or control can be the amount of transcription of the same gene product (e.g., a reporter gene product) initiated from a standard or reference promoter under the same conditions. In assessing the strength of an inducible promoter, the amount of transcription of a gene product that occurs from the promoter in the absence (non-inducing conditions) and presence (inducing conditions) of an inducing factor, or environment or condition, can be compared to determine the degree of inducibility. The difference in those transcription amounts can also be compared to the difference in transcription amounts under the same non-inducing and inducing conditions of a reference or control promoter to determine relative strength and inducibility. Methods for evaluating promoter strength using quantitative techniques for measuring gene product expression include, for example, RT-qPCR, northern blot techniques, and reporter gene product expression assays (see, e.g., Teste et al. (2009) *BMC Molecular Biology* 10:99; Wang et al. (2016) *Yeast* 33:99-106; Peng et al. (2015) Microb. Cell Fact. 14:91). For example, transcription (e.g., measured in ways known in the art) can sometimes be increased by at least about the following percentages when an inducible promoter controlling transcription of a nucleic acid is subjected to inducing conditions as compared to transcription under non-inducing conditions: by at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%,



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14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 100% or more. In some instances, transcription can sometimes be increased by at least about the following fold when an inducible promoter controlling transcription of a nucleic acid is subjected to inducing conditions as compared to transcription under non-inducing conditions: at least about 1.5-fold, 2-fold, 2.5-fold, 3-fold, 3.5-fold, 4-fold, 4.5-fold, 5-fold, 5.5-fold, 6-fold, 6.5-fold, 7-fold, 7.5-fold, 8-fold, 8.5-fold, 9-fold, 9.5-fold,10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 20-fold or more.

In some instances, the use of a stronger heterologous inducible promoter to control transcription can increase the amount of induced transcription of a product-encoding nucleic acid by at least about the following percentages over the amount of induced transcription of the same nucleic acid controlled by a weaker inducible promoter: by at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, 30%, 31%, 32%, 33%, 34%, 35%, 36%, 37%, 38%, 39%, 40%, 41%, 42%, 43%, 44%, 45%, 46%, 47%, 48%, 49%, 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 100% or more. In some instances, the use of a stronger heterologous inducible promoter to control transcription can increase the amount of induced transcription of a product-encoding nucleic acid by at least about the following fold over the amount of induced transcription of the same nucleic acid controlled by a weaker inducible promoter: by at least about 1.5-fold, 2-fold, 2.5-fold, 3-fold, 3.5-fold, 4-fold, 4.5-fold, 5-fold, 5.5-fold, 6-fold, 6.5-fold, 7-fold, 7.5-fold, 8-fold, 8.5-fold, 9-fold, 9.5-fold,10-fold, 11-fold, 12-fold, 13-fold, 14-fold, 15-fold, 20-fold or more.

In some embodiments, regulation of a promoter can be used to alter (e.g., increase, add, decrease or substantially eliminate) the activity of a peptide, polypeptide or protein (e.g., enzyme activity). For example, a cell or microorganism can be engineered by genetic modification to express a nucleic acid reagent that can add a novel activity (e.g., an activity not normally found in the host cell or organism) or increase the expression of an existing activity by



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increasing transcription from a homologous or heterologous promoter operably linked to a nucleotide sequence of interest (e.g., heterologous nucleotide sequence of interest), in certain embodiments. In some embodiments, a cell or microorganism can be engineered by genetic modification to express a nucleic acid reagent that can decrease expression of an activity by decreasing or substantially eliminating transcription from a homologous or heterologous promoter operably linked to a nucleotide sequence of interest, in certain embodiments. In some embodiments, an inducible heterologous promoter can be used to regulate transcription of a protein-encoding nucleic acid that is a stronger, or more strongly inducible, promoter than an endogenous inducible promoter that regulates expression of the protein-encoding nucleic acid in its endogenous state.

In some embodiments the activity can be altered using recombinant DNA and genetic techniques known to the artisan. Methods for engineering cells and microorganisms are further described herein. Also provided herein are non-limiting examples of regulated promoters, e.g., promoters that are up-regulated by oxygen, promoters that are down-regulated by oxygen, promoters that are repressed in the presence of certain carbon sources (e.g, glucose), promoters that are de-repressed under certain carbon source conditions (e.g., limited or depleted glucose and/or non-fermentable carbon sources), promoters that are induced in the presence of certain carbon sources (e.g., fatty acids), transcriptional repressors and their associated genes, DNA binding motifs as determined using the MEME sequence analysis software. Potential regulator binding motifs can be identified using the program MEME to search intergenic regions bound by regulators for overrepresented sequences. For each regulator, the sequences of intergenic regions bound with p-values less than 0.001 are extracted to use as input for motif discovery. The MEME software can be run, for example, using the following settings: a motif width ranging from 6 to 18 bases, the "zoops" distribution model, a 6$^{th}$ order Markov background model and a discovery limit of 20 motifs. The discovered sequence motifs can be scored for significance by two criteria: an E-value calculated by MEME and a specificity score. The motif with the best score using each metric is shown for each regulator.

*Carbon source-dependent gene regulatory elements*

Many cells and organisms, including, for example, many yeast species, preferentially use glucose over other carbon sources. Often, cell and organism growth is maximal in the presence



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of glucose. However, some cells and organisms are able to use alternative carbon sources for the production of metabolic energy and cellular biomass. In doing so, cellular metabolism can undergo substantial changes as certain pathways (such as, for example, oxidative metabolism, the TCA cycle, glyoxylate cycle and gluconeogenesis) required for utilizing non-glucose carbon sources are activated. Genes encoding such pathway-specific components can be subject to carbon source regulation of transcription. When glucose is present as a carbon source, some components (e.g. enzymes) of these other pathways may not be expressed, or are less expressed, because the pathways are not essential, or are used to a lesser extent, in the presence of glucose. This is referred to as glucose repression. Thus, in contrast to unregulated constitutive promoters, transcription regulatory elements for genes such as these are repressed, derepressible and/or inducible by varying carbon sources. When glucose is depleted, genes that were subject to glucose repression are then transcribed in a process referred to as glucose derepression. For some of these genes, this increase in transcription due to derepression represents the extent to which the genes will be expressed because they are not subject to induction and further increased transcription. For others of these genes, transcription may be increased (e.g., several-fold) over the derepressed level upon induction by, for example, certain carbon sources. Examples of such carbon sources include fatty acids (e.g, oleic acid) and n-alkanes. Some genes encoding peroxisomal proteins (including enzymes involved in fatty acid catabolism) are subject to glucose repression/derepression. *Cis*-acting regulatory elements have been identified for some of these genes. For example, sequences located upstream of the TATA boxes in the *Saccharomyces cerevisiae FOX1* gene encoding an acyl-CoA oxidase and *FOX3* gene encoding 3-oxoacyl-CoA thiolase have been reported as glucose response elements (see, e.g., Wang et al. (1992) *Nucleic Acids Res*. 20:3495; Wang et al. (1994) *J. Biol. Chem*. 269:24480; and Einerhand et al. (1991) *Eur. J. Biochem*. 200:113).

As described herein, engineered alteration of carbon flux in cells and organisms can involve directing internalized carbon sources toward particular cellular processing pathways and/or away from particular pathways. Some host cell modifications made in engineered bioproduction systems described herein can depend in part on the carbon source or sources used and the target molecule being produced. For example, in some embodiments provided herein, cells or organisms are modified to enhance carbon flux through oxidative metabolism pathways (e.g., β-oxidation and/or ω-oxidation) and/or fatty acid synthesis for production of organic acid, polyketide, terpene and/or other target molecules. In particular embodiments, the modified cells or organisms are provided with non-fermentable carbon sources (e.g., fatty acids, alkanes)



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and/or limited amounts of, or no, glucose for production of target molecule production. In these and other embodiments described herein, genetic modifications may be made to the cells or organisms to, for example, modify the amount and/or activity of one or more enzymes (e.g., acetyl-CoA carboxylase, ATP citrate lyase, carnitine acetyltransferase, acyl-CoA thioesterase, acetyl-CoA hydrolase, acetyl-CoA synthetase) in carbon-processing pathways. As also described herein, in some of these genetic modifications, it may be beneficial to use heterologous transcription-regulating nucleic acid elements that are differentially responsive to certain carbon sources for controlling expression of the enzyme(s). For example, promoters and other regulatory nucleic acid elements that are repressed when glucose is present, derepressed in glucose-limiting, or depleted, conditions and/or induced in the presence of alternative carbon sources can provide for optimized and regulatable production of target molecules, such as in embodiments involving use of non-glucose carbon sources. This is particularly useful in instances where target molecules may be toxic to cells or organisms in high levels. In this example, modified cells could initially be cultured in the presence of glucose, if desired to build up cell mass during a growth phase, and then switched to an alternative carbon source for target molecule production through engineered pathways during which time expression of modified enzymes would be derepressed and/or induced.

In some embodiments of the engineered cells and organisms provided herein, carbon flux alterations may include diversion of carbon atoms (e.g., acetyl groups) away from particular cellular pathways (e.g. the TCA cycle) to minimize carbon atom loss to those pathways at the expense of target molecule-producing pathways. In these instances, it may be beneficial to modify and/or replace promoters and other transcription regulatory elements that control expression of components (e.g., mitochondrial proteins such as carnitine acetyltransferase and carnitine transporters) of pathways not involved in target molecule production such that the components are not expressed, or are expressed at reduced levels, under glucose-limiting conditions and/or in the presence of alternative carbon sources. Heterologous transcription-regulatory nucleic acid elements suitable in achieving such control include, but are not limited to, weak, constitutive promoters and promoters that are repressed when non-glucose (or non-fermentable) carbon sources are available, derepressed when glucose is present and/or induced in the presence of glucose. Examples of such transcriptional control elements include, but are not limited to, promoter sequences regulating transcription of genes encoding phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphate dehydrogenase (GPD), translation elongation factor (TEF) and glucose-6-phosphate isomerase (G6PI; also referred to



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as phosphoglucose isomerase or PGI). Nucleotide sequences for promoters of the *PGK* (SEQ ID NO: 114), *GPD* (SEQ ID NO: 119), *TEF* (SEQ ID NO: 120) and *G6PI* (SEQ ID NO: 118) genes of *Candida* strain ATCC 20336 are provided herein. Additional examples of sources of PGK, GPD, TEF and G6PI gene sequences include, but are not limited to: *Saccharomyces cerevisiae PGI1* (Genbank accession no. X13977), *Aspergillus oryzae PGK* (Genbank accession no. E04898), *Yarrowia lipolytica PGK* (Genbank accession no. M91598), *Candida albicans PGK* (Genbank accession no. U25180), *Candida maltosa C-PGK1* (Genbank accession no. D12474), *Saccharomyces cerevisiae GPD* (Genbank accession no. M13807), *Cyberlindnera jadinii GAP* (Genbank accession no. FJ664342), *Yarrowia lipolytica TEF1* (Genbank accession no. AF054508), *Debaryomyces hansenii TEF1* (Genbank accession no. AM600962).

Genes that are transcriptionally regulated by carbon source availability to cells (see, e.g., Turcotte et al. (2010) *FEMS Yeast Res.* 10:2-13; Weinhandl et al. (2014) *Microbial Cell Factories* 13(5):1-17) are possible sources of carbon source-dependent heterologous promoters for use in modification of cells and organisms as described herein. For example, genes encoding glycerol kinase and glycerol-3-phosphate dehydrogenase, such as the *GUT1* and *GUT2* genes of yeast, respectively, can be repressed in the presence of fermentable carbon sources such as glucose and expressed in the presence of non-fermentable carbon sources, e.g., glycerol or ethanol. When glucose is present, repression of the *Saccharomyces cerevisiae GUT1* and *GUT2* genes occurs in cells and is mediated by the negative regulator Opi1. The promoter region of the *Saccharomyces cerevisiae GUT1* gene contains two upstream transcription activation sequences, $UAS_{ADR1}$ and $UAS_{INO}$, that can be binding sites for Adr1p (a zinc finger transcription factor) and Ino2p/Ino4p (basic helix-loop-helix factors), respectively, which are responsible for about 90% of the *GUT1* gene expression in the presence of glycerol (see, e.g., Grauslund et al. (1999) *Nucleic Acids Res.* 27(22):4391-4398). Similarly, the promoter region of the *S. cerevisiae GUT2* gene contains a $UAS_{HAP}$ upstream sequence that can be a binding site for the Hap2/3/4/5 protein complex which activates transcription of several genes with mitochondrial functions (see, e.g., Grauslund and Ronnow (2000) *Can. J. Microbiol.* 46:1096-1100). The $UAS_{HAP}$ element is required for full expression of the *GUT2* gene in the presence of glycerol or ethanol.

Additional examples of carbon source-dependent promoters include regulatory nucleic acid sequences controlling the transcription of genes encoding some polypeptides involved in fatty



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acid metabolism, peroxisomal transport/biogenesis and/or the glyoxylate cycle. Some of these genes undergo significant induction of transcription in the presence of fatty acids (e.g., oleic acid) and/or n-alkanes. This phenomenon is referred to as fatty acid or oleic acid (or oleate) induction or alkane induction. In yeast, some of the genes subject to fatty acid induction are transcriptionally controlled by promoter regions containing an oleate response element (ORE). In *Saccharomyces cerevisiae*, for example, the ORE of fatty acid-inducible gene promoters binds the positive transcription factor of zinc cluster proteins, Pip2p-Oaf1p. The promoter region of such genes typically contains a palindrome sequence of two CGG triplets with a sequence of 15-18 nucleotides between them that includes at least one half site containing a TNA triplet (where "N" represents any nucleotide) and thus has the sequence 5'-CGGNNNTNA($N_{9-12}$)CCG-3' (see, e.g., Gurvitz and Rottensteiner (2006) *Biochim. Biophys. Acta* 1763:1392-1402). Variants of this sequence in fatty acid-inducible *S. cerevisiae* gene promoters have been identified in connection with the *ANT1* and *PEX25* genes leading to the following sequence as being considered the minimal ORE: CGGNNNTN$^{A}/_{R}$($N_{8-12}$)CCG (see, e.g., Rottensteiner et al. (2003) *Eur. J. Biochem.* 270:2013-2022). Some of the promoter regions of fatty acid-inducible *S. cerevisiae* genes also include a type 1 upstream activation sequence (UAS1) having a consensus sequence of CYCCR(A/T/G)$N_{4-36}$(T/A/C)YGGRG that binds the Adr1 transcription factor and directly regulates some *S. cerevisiae* genes including *SPS19*, *POX1*, *CTA1*, *PEX11*, *PIP2* which encode peroxisomal proteins (e.g., peroxisomal 2,4-dienoyl-CoA reductase, or SPS19p, and Pex11p) and/or proteins involved in β-oxidation (e.g., acyl-CoA oxidase or Pox1p) or involved in the regulation of genes associated with fatty acid metabolism (see, e.g., Gurvitz et al. (2000) *Mol. Cell. Biol. Res. Commun.* 4:81-89; Gurvitz et al. (2001) *J. Biol. Chem.* 276:31825-31830; Rottensteiner et al. (2003) *J. Biol. Chem.* 278:27605-276110). Additional *S. cerevisiae* gene promoter regions containing an ORE include those controlling transcription of *MDH3* (peroxisomal malate dehydrogenase), *YCAT* (peroxisomal and mitochondrial carnitine acetyltransferase), *CRC1* (mitochondrial carnitine transporter) and *TES1* (peroxisomal thioesterase) (see, e.g., Karpichev and Small (1998) *Mol. Cell. Bio.* 18:6560-6570).

There are numerous DNA-binding factors and regulatory proteins involved in transcriptional regulation associated with carbon source utilization. For example, in yeast such as *S. cerevisiae*, glucose repression is mediated by repressors such as, for example, members of the Mig family of $C_2H_2$-zinc-finger DNA-binding proteins, and some zinc cluster proteins, e.g.,Oaf3. The promoter regions of genes subject to glucose repression typically contain a GC-rich recognition site (e.g., SYGGGG) to which a repressor, e.g., Mig1, binds in the presence of high



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levels of glucose (see, e.g., Gancedo (1998) *Microbiol. Mol. Biol. Rev.* 62(2):334-361). The repressor recruits a repressor complex, e.g., Ssn6-Tup1, resulting in conformational changes in the chromatin structure that prevent transcription initiation factors (e.g., the Sip4 and Adr1 activators of genes encoding, for example, gluconeogenic and glycerol or ethanol utilization proteins) from binding to sites in the DNA. Derepression occurring when glucose is depleted can result in activation of a protein kinase, Snf1, which participates in phosphorylation and release of the repressor complex thereby allowing for the activator to bind DNA in the promoter region. A shift from glucose-repressing to derepressing conditions typically results in an increased binding of Oaf1-Pip2 to oleate-response elements in promoters of fatty acid-inducible genes; however, in the presence of inducer (e.g., oleic acid), there may be only a marginal increase in this binding. This is because under derepressed conditions, Oaf1-Pip2 may be constitutively bound to target gene promoters. Activation of Oaf1-Pip2 involves binding of oleate to Oaf1 which is hyper-phosphorylated in the presence of oleate. In the activation of some fatty acid-inducible genes, Adr1 may also be involved. For example, promoter regions of genes encoding peroxisomal proteins often include a UAS1 that binds Adr1. DNA motifs for regulator protein (e.g., Adr1p, Hap2, Mig1) binding in carbon source-dependent promoters have been identified (see e.g., Weinhandl et al. (2014) *Microbial Cell Factories* 13(5):1-17) as have entire carbon source-dependent promoter nucleic acid sequences.

In other fungi, there can be different DNA-binding factors and regulatory proteins involved in transcriptional regulation associated with carbon source utilization. For example, in the filamentous fungus *Aspergillus nidulans*, glucose repression is mediated by the CreA repressor. In order to grow on fatty acids as a sole carbon source, this fungus typically requires two $Zn_2Cys_6$ proteins, FarAp and FarBp. These proteins are transcription factors that bind to a CCTCGG motif contained in the promoter region of genes encoding proteins involved in β-oxidation, peroxisomal functions and the glyoxylate cycle in this fungus. Specifically, FarAp is required for oxidation of short- and long-chain fatty acids and FarBp is required for oxidation of short-chain fatty acids. A homolog of FarA/FarB in *Candida albicans*, referred to as Ctf1p, is typically required for growth of *C. albicans* on fatty acids and regulates expression of some of the genes encoding proteins involved in β-oxidation.

Fatty acid- and/or alkane-inducible promoters from other organisms include, but are not limited to, those regulating transcription of the following genes: *Yarrowia lipolytica POX2* (acyl-CoA oxidase; Genbank accession no. AJ001300), *Yarrowia lipolytica POT1* (3-oxo-acyl-CoA

thiolase; Genbank accession no. X69988), *Yarrowia lipolytica ICL1* (isocitrate lyase; Genbank accession no. CQ771439) and *Candida tropicalis HDE* (hydratase-dehydrogenase-epimerase; Genbank accession no. X57854), *Candida tropicalis POX4* (acyl-CoA oxidase; Genbank accession no. AB031271), *Candida tropicalis POX18* (peroxisomal 18-kDa protein; Genbank accession no. X53633), *Candida tropicalis SPS19* (2,4-dienoyl-CoA reductase; Genbank accession no. XM_002545237), *Candida albicans PEX11* (peroxisomal protein; Genbank accession no. XM_707009), *Candida tropicalis P450alk* (alkane-inducible cytochrome P450; Genbank accession no. M24894), and *Candida tropicalis CATL* (catalase; Genbank accession no. AB181391) (see, e.g., Hussain et al. (2016) *ACS Synth. Biol.* 5:213-223 and Sloots et al. (1991) *Gene* 105:129-134). Sequences of promoter elements of fatty acid-inducible genes (e.g., *HDE*, *POX4*, *PEX11*) from *Candida* strain ATCC 20336 are also provided herein (SEQ ID NOS: 113, 117 and 121).

The promoter region controlling transcription of the *Candida tropicalis* peroxisomal *HDE* gene includes a sequence similar to, but with deviations from, the *S. cerevisiae* ORE consensus sequence, and is as follows: $CGGNNNTTAN_{12}CAG$. This sequence, located in a region between nucleotides -393 and -341 (relative to the A nucleotide of the translation START codon), contains a 3' triplet of CAG in contrast to the CCG 3' triplet of the *S. cerevisiae* ORE consensus sequence. Specific nucleotides of the *C. tropicalis HDE* gene promoter ORE are CGGTTATTACGCCTGGGGGGGCAG. Similar sequences occur in the upstream promoter regions of *C. tropicalis* genes *POX4*, *POX18*, *P450alk* and *CATL* (see Sloots et al. (1991) *Gene* 105:129-134). The promoter regions for these genes (and the *HDE* gene) can also contain sequences similar to a 7-nucleotide consensus sequence ($ATTTCC_TC_T$) for regulation of the *S. cerevisiae SUC2* gene by glucose. This glucose-responsive region of the *C. tropicalis* HDE gene is located between nucleotides -526 and -393.

Alkane-assimilating organisms, such as, for example, *Candida tropicalis*, *Candida maltosa*, *Candida albicans*, *Candida bombicola*, *Candida parapsilosis*, *Yarrowia lipolytica*, *Pichia stipitis* and *Debaryomyces hansenii*, can utilize alkanes by first converting them to fatty alcohols through oxidation catalyzed by cytochrome P450. The fatty alcohols are then oxidized to fatty aldehydes which are in turn oxidized to fatty acids. Promoters for some of the genes in alkane-assimilating yeast have been shown to contain alkane-responsive elements. For example, an upstream activating sequence referred to as ARE1 and having a sequence $CTTGTGN_xCATGTG$ (where N represents any nucleotide and x refers to the number of

nucleotides) has been identified as an alkane-responsive element present in the promoter of the *Yarrowia lipolytica ALK1* gene (cytochrome P450; Genbank accession no. AB010388) (see, e.g., Sumita et al (2002) *Biochem. Biophys. Res. Commun.* 294:1071-1078). Similar ARE1-like sequences (and/or conserved repeating motif: TGTG, or the CACA complement) occur in promoters of other genes encoding enzymes involved in alkane degradation, including, for example, cytochrome P450 genes of *Candida tropicalis*, (see, e.g., Seghezzi et al. (1992) *DNA Cell Biol.* 11:767-780), *Candida maltosa* (Genbank accession no. X55881), *Debaryomces hansenii* (Genbank accession no. AF103948) and also thiolase genes such as the acetoacetyl-CoA thiolase encoded by the *Y. lipolytica PAT1* gene (Genbank accession no. AB1020846) and the peroxisomal 3-ketoacyl-CoA thiolase encoded by the *C. tropicalis CT-T3A* gene (Genbank accession no. AB025647).

Possible additional candidate fatty acid- and/or alkane-inducible promoter sequences may be identified by searching genome databases for ORE consensus sequences located within about 500-1000 bp upstream of the START codon of an ORF and operably linking identified sequences with a reporter protein-encoding nucleic acid sequence for introduction into a host cell and analysis of reporter protein expression in the presence of varying carbon sources (such as fermentable and non-fermentable carbon sources and, in particular, fatty acids). Induced reporter protein expression in the presence of fatty acids and/or alkanes is indicative of a regulable, fatty acid- and/or alkane-inducible promoter sequence being linked to the reporter protein-encoding nucleic acid. Computer-assisted bioinformatics search programs are also available for use in identifying candidate transcription regulatory elements for genes (see, e.g., Worldwide Web uniform Resource Locator (URL) yeastract.com; Worldwide Web uniform Resource Locator (URL) pepper.molgenrug.nl/; Worldwide Web uniform Resource Locator (URL) rulai.cshl.edu/SCPD/; Worldwide Web uniform Resource Locator (URL) bimas.cit.nih.gov/molbio/proscan/; Worldwide Web uniform Resource Locator (URL) bioit.dmbr.ugent.be/contrav2/index.php).

*Untranslated regions (UTR)*

Nucleic acid reagents may also contain one or more 5' UTRs, and one or more 3'UTRs. Untranslated regions of a gene are sequences that are transcribed but are not translated into protein. A 5' UTR generally extends from the transcription start initiation site up to the first nucleotide of the translation START codon. A 3' UTR generally extends from the translation



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STOP codon to the polyA tail. Untranslated sequences can play important roles in post-transcriptional gene expression, including, for example, transport of a transcript out of the nucleus, translation efficiency, subcellular localization and mRNA stability.

A 5' UTR used in a nucleic acid reagent in genetically modifying cells may include one or more elements that are associated with it in an endogenous state, e.g., in a cell from which it originates, and sometimes includes one or more exogenous elements. A 5' UTR can originate from any suitable nucleic acid, such as genomic DNA, plasmid DNA, RNA or mRNA, for example, from any suitable organism (e.g., virus, bacterium, yeast, fungi, plant, insect or mammal). Appropriate elements for the 5' UTR can be selected based upon the chosen expression system (e.g., expression in a chosen organism, or expression in a cell free system, for example). A 5' UTR sometimes contains one or more of the following elements: enhancer sequences (e.g., translational), translation regulation site, translation initiation site, translation factor binding site, accessory protein binding site, feedback regulation agent binding sites, ribosome binding site, replicon, internal ribosome entry site (IRES), silencer element and the like. In some embodiments, a promoter element may be isolated such that all 5' UTR elements necessary for proper conditional regulation are contained in the promoter element fragment, or within a functional subsequence of a promoter element fragment.

A 5 'UTR in the nucleic acid reagent can include a translational enhancer nucleotide sequence. A translational enhancer nucleotide sequence often is located between the promoter and the target nucleotide sequence in a nucleic acid reagent. A translational enhancer sequence often binds to a ribosome, sometimes is an 18S rRNA-binding ribonucleotide sequence (i.e., a 40S ribosome binding sequence) and sometimes is an internal ribosome entry sequence (IRES). An IRES generally forms an RNA scaffold with precisely placed RNA tertiary structures that contact a 40S ribosomal subunit via a number of specific intermolecular interactions. Examples of ribosomal enhancer sequences are known and can be identified by the artisan (e.g., Mignone et al. (2005) *Nucleic Acids Research 33*: D141-D146; Paulous et al. (2003) *Nucleic Acids Research 31*:722-733; Akbergenov et al. (2004) *Nucleic Acids Research 32*:239-247; Mignone et al. (2002) *Genome Biology 3*(3): reviews0004.1-0001.10; Gallie (2002) *Nucleic Acids Research 30*:3401-3411; Shaloiko et al., Worldwide Web uniform Resource Locator (URL) interscience.wiley.com, DOI: 10.1002/bit.20267; and Gallie et al. (1987) *Nucleic Acids Research 15*:3257-3273).



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A translational enhancer sequence sometimes is a eukaryotic sequence, such as a Kozak consensus sequence or other sequence (e.g., hydroid polyp sequence, GenBank accession no. U07128). A translational enhancer sequence sometimes is a prokaryotic sequence, such as a Shine-Dalgarno consensus sequence. In certain embodiments, the translational enhancer sequence is a viral nucleotide sequence. A translational enhancer sequence sometimes is from a 5' UTR of a plant virus, such as Tobacco Mosaic Virus (TMV), Alfalfa Mosaic Virus (AMV); Tobacco Etch Virus (ETV); Potato Virus Y (PVY); Turnip Mosaic (poty) Virus and Pea Seed Borne Mosaic Virus, for example. In certain embodiments, an omega sequence about 67 bases in length from TMV is included in the nucleic acid reagent as a translational enhancer sequence (e.g., devoid of guanosine nucleotides and includes a 25 nucleotide long poly (CAA) central region).

A 3' UTR used in a nucleic acid reagent in genetically modifying cells may include one or more elements that are associated with it in an endogenous state, e.g., in a cell from which it originates, and sometimes includes one or more exogenous elements. A 3' UTR may originate from any suitable nucleic acid, such as genomic DNA, plasmid DNA, RNA or mRNA, for example, from any suitable organism (e.g., a virus, bacterium, yeast, fungi, plant, insect or mammal). Appropriate elements for the 3' UTR can be selected based upon the chosen expression system (e.g., expression in a chosen organism, for example). A 3' UTR sometimes comprises one or more of the following elements: translation regulation site, translation termination site, translation initiation site, translation factor binding site, ribosome binding site, replicon, enhancer element, silencer element and polyadenosine tail. A 3' UTR often includes a polyadenosine tail and sometimes does not, and if a polyadenosine tail is present, one or more adenosine moieties may be added or deleted from it (e.g., about 5, about 10, about 15, about 20, about 25, about 30, about 35, about 40, about 45 or about 50 adenosine moieties may be added or subtracted).

In some embodiments, modification of a 5' UTR and/or a 3' UTR can be used to alter (e.g., increase, add, decrease or substantially eliminate) gene expression activity. This can in turn alter the activity of a peptide, polypeptide or protein (e.g., enzyme activity for example), by a change in transcription of the nucleotide sequence(s) of interest from an operably linked promoter element comprising the modified 5' or 3' UTR. For example, a microorganism can be engineered by genetic modification to express a nucleic acid reagent comprising a modified 5' or 3' UTR that can add a novel activity (e.g., an activity not normally found in the host organism)



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or increase the expression of an existing activity by increasing transcription from a homologous or heterologous promoter operably linked to a nucleotide sequence of interest (e.g., homologous or heterologous nucleotide sequence of interest), in certain embodiments. In some embodiments, a microorganism can be engineered by genetic modification to express a nucleic acid reagent comprising a modified 5' or 3' UTR that can decrease the expression of an activity by decreasing or substantially eliminating transcription from a homologous or heterologous promoter operably linked to a nucleotide sequence of interest, in certain embodiments.

*Nucleic acid or protein similarity*

In addition to the nucleotide and amino acid sequences provided herein, a polynucleotide or polypeptide sequence may also be one that is substantially similar to those provided herein, including, but not limited to, promoter sequences, regulatory sequences, coding polynucleotides, amino acid signal sequences and amino acid protein sequences provided herein. Similarity between two nucleic acids or polypeptides refers to the relatedness between nucleotide sequences or amino acid sequences. Similarity can be based on the degree of identity and/or homology of sequences and the residues contained therein. Methods of assessing the degree of similarity between nucleic acids or proteins are known to those of skill in the art. For example, in one method of assessing sequence similarity, two nucleotide or amino acid sequences are aligned in a manner that yields a maximal level of identity between the sequences. Identity refers to the extent to which the sequences are invariant. Alignment of amino acid sequences, and, to some extent, nucleotide sequences, also can take into account conservative differences and/or frequent substitutions in amino acids (or nucleotides). Conservative differences are those that conserve the physico-chemical properties of the residues involved. Alignments can be global (alignment of the compared sequences over the entire length of the sequences and including all residues) or local (alignment of a portion of the compared sequences e.g., a portion or portions that includes only the most similar region or regions). Homology, with reference to polynucleotide or polypeptide sequences, refers to nucleotide or amino acid sequence similarity that takes into account identical residues and residues that can substitute for one another.

Percent identity and/or homology may be determined, for example, by comparing sequence information using any of a number of computer algorithms known in the art. In one example, calculations of sequence identity can be performed as follows. Sequences are aligned for



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optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment, and non-homologous sequences can be disregarded for comparison purposes). The length of a reference sequence aligned for comparison purposes is sometimes 30% or more, 40% or more, 50% or more, often 60% or more, and more often 70% or more, 80% or more, 90% or more, or 100% of the length of the reference sequence. The nucleotides or amino acids at corresponding nucleotide or polypeptide positions, respectively, are then compared among the two sequences. When a position in the first sequence is occupied by the same nucleotide or amino acid as the corresponding position in the second sequence, the nucleotides or amino acids are deemed to be identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, introduced for optimal alignment of the two sequences. Examples of sequence alignment and analysis software that can be used to calculate sequence identity include BLAST (Worldwide Web uniform Resource Locator (URL) blast.ncbi.nlm.nih.gov/Blast.cgi), MUSCLE (Worldwide Web uniform Resource Locator (URL) ebi.ac.uk/Tools/msa/muscle/ and Worldwide Web uniform Resource Locator (URL) drive5.com/muscle/) and MAFFT (Worldwide Web uniform Resource Locator (URL) mafft.cbrc.jp/alignment/server/ and Worldwide Web uniform Resource Locator (URL) ebi.ac.uk/Tools/msa/mafft/) for comparing nucleotide sequences and SIM (Worldwide Web uniform Resource Locator (URL) web.expasy.org/sim/) and BLAST for comparison of amino acid sequences. Nucleic acid sequence identity can also be determined by hybridization assays conducted under stringent conditions. As used herein, the term "stringent conditions" refers to conditions for hybridization and washing. Stringent conditions are known to those skilled in the art and can be found, for example, in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., 6.3.1-6.3.6 (1989). Aqueous and non-aqueous methods are described in that reference and either can be used. An example of stringent hybridization conditions is hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45ºC, followed by one or more washes in 0.2X SSC, 0.1% SDS at 50ºC. Another example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45ºC, followed by one or more washes in 0.2X SSC, 0.1% SDS at 55ºC. A further example of stringent hybridization conditions is hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45ºC, followed by one or more washes in 0.2X SSC, 0.1% SDS at 60ºC. Often, stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45ºC, followed by one or more washes in 0.2X SSC, 0.1% SDS at 65ºC. More often, stringency



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conditions are 0.5M sodium phosphate, 7% SDS at 65ºC, followed by one or more washes at 0.2X SSC, 1% SDS at 65ºC.

A nucleic acid or polypeptide for use in developing cells and organisms and/or methods described herein may be, for example, a polynucleotide or amino acid sequence that is homologous or identical to a nucleotide sequence (or complement thereof) or amino acid sequence provided herein over at least about 75%, or at least about 77%, or at least about 78%, or at least about 79%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 95% or more of the provided reference sequence. For example, a polynucleotide or polypeptide can be one that is at least about 50%, or at least about 51%, or at least about 52%, or at least about 54%, or at least about 55%, or at least about 58%, or at least about 60%, or at least about 62%, or at least about 65%, or at least about 70%, or at least about 75% or at least about 80% or more homologous or identical to a nucleic acid (or complement thereof) or polypeptide provided herein over the specified extent of a nucleic acid or polypeptide provided herein. In another embodiment, a nucleic acid or polypeptide can be one that is homologous or identical to a nucleic acid (or complement thereof) or polypeptide provided herein over at least about 86%, or at least about 87%, or at least about 88%, or at least about 89%, or at least about 92%, or at least about 97% or more of the provided reference nucleic acid (or complement thereof) or polypeptide. For example, the protein can be one that is at least about 67%, or at least about 68%, or at least about 69%, or at least about 72%, or at least about 77%, or at least about 82%, or at least about 87%, or at least about 90%, or at least about 95% or at least about 96%, or at least about 97%, or at least about 98%, or at least about 99% or more homologous or identical to a reference nucleic acid (or complement thereof) or polypeptide provided herein over the specified extent of the nucleic acid (or complement thereof) or polypeptide.

In some embodiments, a nucleotide or amino acid sequence that is at least 80% or more, 81% or more, 82% or more, 83% or more, 84% or more, 85% or more, 86% or more, 87% or more, 88% or more, 89% or more, 90% or more, 91% or more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more identical to a nucleotide sequence (or complement thereof) or amino acid sequence described herein can be utilized. The term “identical” as used herein refers to two or more nucleotide or amino acid sequences having substantially the same nucleotide or amino acid sequence when compared to each other. One test for determining whether two nucleotide sequences or amino acids



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sequences are substantially identical is to determine the percent of identical nucleotide sequences or amino acid sequences shared.

*Target nucleotide sequence*

A nucleic acid reagent sometimes can comprise a target nucleotide sequence. A "target nucleotide sequence" as used herein encodes a nucleic acid, peptide, polypeptide or protein of interest, and may be a ribonucleotide sequence or a deoxyribonucleotide sequence. A target nucleic acid sometimes is an untranslated ribonucleic acid and sometimes is a translatable ribonucleic acid. An untranslated ribonucleic acid may include, but is not limited to, a small interfering ribonucleic acid (siRNA), a short hairpin ribonucleic acid (shRNA), other ribonucleic acid capable of RNA interference (RNAi), an antisense ribonucleic acid, or a ribozyme. A translatable target nucleotide sequence (e.g., a target ribonucleotide sequence) sometimes encodes a peptide, polypeptide or protein, which are sometimes referred to herein as "target peptides," "target polypeptides" or "target proteins."

Any peptides, polypeptides or proteins, or an activity catalyzed by one or more peptides, polypeptides or proteins, may be encoded by a target nucleotide sequence and may be selected by a user. Representative proteins include enzymes (e.g., acetyl-CoA carboxylase, acyl-CoA oxidase, thioesterase, monooxygenase, monooxygenase reductase, fatty alcohol oxidase, acyltransferase and the like, for example), antibodies, serum proteins (e.g., albumin), membrane bound proteins, hormones (e.g., growth hormone, erythropoietin, insulin, and the like), cytokines, and the like, and include both naturally occurring and exogenously expressed polypeptides. Representative activities (e.g., enzymes or combinations of enzymes which are functionally associated to provide an activity) include thioesterase activity, monooxygenase activity, monooxygenase reductase activity, acetyltransferase activity, omega hydroxyl fatty acid dehydrogenase activity, beta-oxidation activity, omega-oxidation activity and the like, for example. The term "enzyme" as used herein refers to a protein which can act as a catalyst to induce a chemical change in other compounds, thereby producing one or more products from one or more substrates.

Specific polypeptides (e.g., enzymes) useful for embodiments described herein are listed herein. The term "protein" as used herein refers to a molecule having a sequence of amino acids linked by peptide bonds. This term includes fusion proteins, oligopeptides, peptides,



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cyclic peptides, polypeptides and polypeptide derivatives, whether native or recombinant, and also includes fragments, derivatives, homologs, and variants thereof. A protein or polypeptide sometimes is of intracellular origin (e.g., located in the nucleus, cytosol, organelle (e.g., mitochondria or peroxisome) or interstitial space of host cells in vivo) and sometimes is a cell membrane protein in vivo. In some embodiments (described in further detail herein), a genetic modification can result in a modification (e.g., increase, substantially increase, decrease or substantially decrease) of a target activity and/or in a modification of a cellular location for a protein.

A translatable nucleotide sequence generally is located between a start codon (AUG in ribonucleic acids and ATG in deoxyribonucleic acids) and a stop codon (e.g., UAA (ochre), UAG (amber) or UGA (opal) in ribonucleic acids and TAA, TAG or TGA in deoxyribonucleic acids), and sometimes is referred to herein as an "open reading frame" (ORF). A translatable nucleotide sequence (e.g., ORF) sometimes is encoded differently in one organism (e.g., most organisms encode CTG as leucine) than in another organism (e.g., *Candida tropicalis* and *Candida viswanathii* encode CTG as serine). In some embodiments, a translatable nucleotide sequence is altered to correct alternate genetic code (e.g., codon usage) differences between a nucleotide donor organism and a nucleotide recipient organism (e.g., engineered organism). In certain embodiments, a translatable nucleotide sequence is altered to improve; (i) codon usage, (ii) transcriptional efficiency, (iii) translational efficiency, (iv) the like, and combinations thereof.

*Nucleic acid reagents and tools*

A nucleic acid reagent sometimes includes one or more ORFs. An ORF may be from any suitable source, sometimes from genomic DNA, mRNA, reverse transcribed RNA or complementary DNA (cDNA) or a nucleic acid library comprising one or more of the foregoing, and is from any organism species that contains a nucleic acid sequence of interest, protein of interest, or activity of interest. Non-limiting examples of organisms from which an ORF can be obtained include bacteria, yeast, fungi, plant, human, insect, nematode, bovine, equine, canine, feline, rat or mouse, for example.

A nucleic acid reagent sometimes contains a nucleotide sequence adjacent to an ORF that is translated in conjunction with the ORF and encodes an amino acid tag. The tag-encoding nucleotide sequence can be located 3' and/or 5' of an ORF in the nucleic acid reagent, thereby



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encoding a tag at the C-terminus or N-terminus of the protein or peptide encoded by the ORF. Any tag that does not abrogate in vitro transcription and/or translation may be utilized and may be appropriately selected by the artisan. Tags may facilitate isolation and/or purification of the desired ORF product from culture or fermentation media.

A tag sometimes specifically binds a molecule or moiety of a solid phase or a detectable label, for example, thereby having utility for isolating, purifying and/or detecting a protein or peptide encoded by the ORF. In some embodiments, a tag includes one or more of the following elements: FLAG (e.g., DYKDDDDKG), V5 (e.g., GKPIPNPLLGLDST), c-MYC (e.g., EQKLISEEDL), HSV (e.g., QPELAPEDPED), influenza hemaglutinin, HA (e.g., YPYDVPDYA), VSV-G (e.g., YTDIEMNRLGK), bacterial glutathione-S-transferase, maltose binding protein, a streptavidin- or avidin-binding tag (e.g., pcDNA™6 BioEase™ Gateway® Biotinylation System (ThermoFisher Scientific)), thioredoxin, β-galactosidase, VSV-glycoprotein, a fluorescent protein (e.g., green fluorescent protein or one of its many color variants (e.g., yellow, red, blue)), a polylysine or polyarginine sequence, a polyhistidine sequence (e.g., His6) or other sequence that chelates a metal (e.g., cobalt, zinc, copper), and/or a cysteine-rich sequence that binds to an arsenic-containing molecule. In certain embodiments, a cysteine-rich tag comprises the amino acid sequence CC-Xn-CC, where X is any amino acid and n is 1 to 3, and the cysteine-rich sequence sometimes is CCPGCC. In certain embodiments, the tag contains a cysteine-rich element and a polyhistidine element (e.g., CCPGCC and His6).

A tag often conveniently binds to a binding partner. For example, some tags bind to an antibody (e.g., FLAG) and sometimes specifically bind to a small molecule. For example, a polyhistidine tag specifically chelates a bivalent metal, such as copper, zinc and cobalt; a polylysine or polyarginine tag specifically binds to a zinc finger; a glutathione S-transferase tag binds to glutathione; and a cysteine-rich tag specifically binds to an arsenic-containing molecule. Arsenic-containing molecules include LUMIO™ agents (ThermoFisher Scientific), such as FlAsH™ (EDT2[4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein-(1,2-ethanedithiol)2]) and ReAsH reagents (e.g., U.S. Patent 5,932,474 to Tsien et al., entitled "Target Sequences for Synthetic Molecules;" U.S. Patent 6,054,271 to Tsien et al., entitled "Methods of Using Synthetic Molecules and Target Sequences;" U.S. Patents 6,451,569 and 6,008,378; published U.S. Patent Application 2003/0083373, and published PCT Patent Application WO 99/21013, all to Tsien et al. and all entitled "Synthetic Molecules that Specifically React with Target



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Sequences"). Such antibodies and small molecules sometimes are linked to a solid phase for convenient isolation of the target protein or target peptide.

A tag sometimes includes a sequence that localizes a translated protein or peptide to a component in a system, which may be referred to as a "signal sequence," "targeting sequence" or "localization signal sequence" herein. A signal sequence often is incorporated at the N-terminus of a target protein or target peptide, and sometimes is incorporated at the C-terminus. Examples of signal sequences are known to the artisan, are readily incorporated into a nucleic acid reagent, and often are selected according to the organism in which expression of the nucleic acid reagent is performed. A signal sequence in some embodiments localizes a translated protein or peptide to a cell membrane. Examples of signal sequences include, but are not limited to, a nucleus targeting signal (e.g., steroid receptor sequence and N-terminal sequence of SV40 virus large T antigen); mitochondrial targeting signal (e.g., amino acid sequence that forms an amphipathic helix); peroxisome targeting signal (e.g., C-terminal sequence in YFG from *S.cerevisiae*); and a secretion signal (e.g., N-terminal sequences from invertase, mating factor alpha, PHO5 and SUC2 in *S.cerevisiae*; multiple N-terminal sequences of *B. subtilis* proteins (e.g., Tjalsma et al., *Microbiol.Molec. Biol. Rev.* 64: 515-547 (2000)); alpha amylase signal sequence (e.g., U.S. Patent No. 6,288,302); pectate lyase signal sequence (e.g., U.S. Patent No. 5,846,818); precollagen signal sequence (e.g., U.S. Patent No. 5,712,114); OmpA signal sequence (e.g., U.S. Patent No. 5,470,719); lam beta signal sequence (e.g., U.S. Patent No. 5,389,529); *B. brevis* signal sequence (e.g., U.S. Patent No. 5,232,841); and *P. pastoris* signal sequence (e.g., U.S. Patent No. 5,268,273)).

A tag sometimes is directly adjacent to the amino acid sequence encoded by an ORF (i.e., there is no intervening sequence) and sometimes a tag is substantially adjacent to an ORF encoded amino acid sequence (e.g., an intervening sequence is present). An intervening sequence sometimes includes a recognition site for a protease, which is useful for cleaving a tag from a target protein or peptide. In some embodiments, the intervening sequence is cleaved by Factor Xa (e.g., recognition site I (E/D)GR), thrombin (e.g., recognition site LVPRGS), enterokinase (e.g., recognition site DDDDK), TEV protease (e.g., recognition site ENLYFQG) or PreScission™ protease (e.g., recognition site LEVLFQGP), for example.

An intervening sequence sometimes is referred to herein as a "linker sequence," and may be of any suitable length selected by the artisan. A linker sequence sometimes is about 1 to about 20



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amino acids in length, and sometimes about 5 to about 10 amino acids in length. The linker length can be selected to substantially preserve target protein or peptide function (e.g., a tag may reduce target protein or peptide function unless separated by a linker), to enhance disassociation of a tag from a target protein or peptide when a protease cleavage site is present (e.g., cleavage may be enhanced when a linker is present), and to enhance interaction of a tag/target protein product with a solid phase. A linker can be of any suitable amino acid content, and often comprises a higher proportion of amino acids having relatively short side chains (e.g., glycine, alanine, serine and threonine).

A nucleic acid reagent sometimes includes a stop codon between a tag element and an insertion element or ORF, which can be useful for translating an ORF with or without the tag. Mutant tRNA molecules that recognize stop codons suppress translation termination and thereby are designated "suppressor tRNAs." Suppressor tRNAs can result in the insertion of amino acids and continuation of translation past stop codons (e.g., U.S. Patent Application No. 60/587,583, filed July 14, 2004, entitled "Production of Fusion Proteins by Cell-Free Protein Synthesis,"; Eggertsson, et al., (1988) *Microbiological Review* 52(3):354-374, and Engleerg-Kukla, et al. (1996) in *Escherichia coli* and *Salmonella* Cellular and Molecular Biology, Chapter 60, pps 909-921, Neidhardt, et al. eds., ASM Press, Washington, DC). A number of suppressor tRNAs are known, including but not limited to, supE, supP, supD, supF and supZ suppressors, which suppress the termination of translation of the amber stop codon; supB, glT, supL, supN, supC and supM suppressors, which suppress the function of the ochre stop codon and glyT, trpT and Su-9 suppressors, which suppress the function of the opal stop codon. In general, suppressor tRNAs contain one or more mutations in the anti-codon loop of the tRNA that allows the tRNA to base pair with a codon that ordinarily functions as a stop codon. The mutant tRNA is charged with its cognate amino acid residue and the cognate amino acid residue is inserted into the translating polypeptide when the stop codon is encountered. Mutations that enhance the efficiency of termination suppressors (i.e., increase stop codon read-through) have been identified. These include, but are not limited to, mutations in the uar gene (also known as the prfA gene), mutations in the ups gene, mutations in the sueA, sueB and sueC genes, mutations in the rpsD (ramA) and rpsE (spcA) genes and mutations in the rplL gene.

Thus, a nucleic acid reagent containing a stop codon located between an ORF and a tag can yield a translated ORF alone when no suppressor tRNA is present in the translation system, and can yield a translated ORF-tag fusion when a suppressor tRNA is present in the system.



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Suppressor tRNA can be generated in cells transfected with a nucleic acid encoding the tRNA (e.g., a replication incompetent adenovirus containing the human tRNA-Ser suppressor gene can be transfected into cells, or a YAC containing a yeast or bacterial tRNA suppressor gene can be transfected into yeast cells, for example). Vectors for synthesizing suppressor tRNA and for translating ORFs with or without a tag are available to the artisan (e.g., Tag-On-Demand™ kit (ThermoFisher Scientific); Tag-On-Demand™ Suppressor Supernatant Instruction Manual, Version C, 31 October 2010, World Wide Web Uniform Resource Locator (URL) tools.thermofisher.com/content/sfs/manuals/tagondemand_supernatant_man.pdf; Tag-On-Demand™ Gateway® Vector Instruction Manual, Version D, 31 October 2010 World Wide Web Uniform Resource Locator (URL) tools.thermofisher.com/content/sfs/manuals/tagondemand_vectors_man.pdf; and Capone et al. (1985) Amber, ochre and opal suppressor tRNA genes derived from a human serine tRNA gene. *EMBO J.* 4:213).

Any convenient cloning strategy known in the art may be utilized to incorporate an element, such as an ORF, into a nucleic acid reagent. Known methods can be utilized to insert an element into the template independent of an insertion element, such as (1) cleaving the template at one or more existing restriction enzyme sites and ligating an element of interest and (2) adding restriction enzyme sites to the template by hybridizing oligonucleotide primers that include one or more suitable restriction enzyme sites and amplifying by polymerase chain reaction. Other cloning strategies take advantage of one or more insertion sites present or inserted into the nucleic acid reagent, such as an oligonucleotide primer hybridization site for PCR, for example, and others described herein. In some embodiments, a cloning strategy can be combined with genetic manipulation such as recombination (e.g., recombination of a nucleic acid reagent with a nucleic acid sequence of interest into the genome of the organism that is modified, as described further herein). In some embodiments, the cloned ORF(s) can produce (directly or indirectly), for example, a fatty acid or dicarboxylic acid (e.g., adipic acid, octanedioic acid, decanedioic acid, dodecanedioic acid, tetradecanedioic acid, hexadecanedioic acid, octadecanedioic acid, eicosanedioic acid), 3-hydroxyproprionic acid, triacetic acid lactone, by engineering a cell or microorganism with one or more ORFs of interest, which cell or microorganism may include one or more altered activities such as, for example, carnitine acetyltransferase activity, acetyl-CoA carboxylase activity, ATP citrate lyase activity, acetyl-CoA synthetase activity, cytochrome P450 reductase activity, acetyl-CoA hydrolase activity, 6-oxohexanoic acid dehydrogenase activity, 6-hydroxyhexanoic acid dehydrogenase activity,



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glucose-6-phosphate dehydrogenase activity, hexanoate synthase activity, lipase activity, fatty acid synthase activity, omega hydroxyl fatty acid dehydrogenase activity, acyl-CoA oxidase activity, acyltransferase activity, thioesterase activity, monooxygenase activity and monooxygenase reductase activity.

In some embodiments, a nucleic acid reagent includes one or more recombinase insertion sites. A recombinase insertion site is a recognition sequence on a nucleic acid molecule that participates in an integration/recombination reaction by recombination proteins. For example, the recombination site for Cre recombinase is loxP, which is a 34 base pair sequence comprised of two 13 base pair inverted repeats (serving as the recombinase binding sites) flanking an 8 base pair core sequence (e.g., Figure 1 of Sauer, B., *Curr. Opin. Biotech. 5*:521-527 (1994)). Other examples of recombination sites include attB, attP, attL, and attR sequences, and mutants, fragments, variants and derivatives thereof, which are recognized by the recombination protein λ Int and by the auxiliary proteins integration host factor (IHF), FIS and excisionase (Xis) (e.g., U.S. Patent Nos. 5,888,732; 6,143,557; 6,171,861; 6,270,969; 6,277,608; and 6,720,140; U.S. Patent Appln. Nos. 09/517,466, filed March 2, 2000, and 09/732,914, filed August 14, 2003, and in U.S. patent publication no. 2002-0007051-A1; Landy, Curr. Opin. Biotech. 3:699-707 (1993)).

Examples of recombinase cloning nucleic acids are in Gateway® systems (ThermoFisher Scientific), which include at least one recombination site for cloning a desired nucleic acid molecules in vivo or in vitro. In some embodiments, the system utilizes vectors that contain at least two different site-specific recombination sites, often based on the bacteriophage lambda system (e.g., att1 and att2), and are mutated from the wild-type (att0) sites. Each mutated site has a unique specificity for its cognate partner att site (i.e., its binding partner recombination site) of the same type (for example attB1 with attP1, or attL1 with attR1) and will not cross-react with recombination sites of the other mutant type or with the wild-type att0 site. Different site specificities allow directional cloning or linkage of desired molecules thus providing desired orientation of the cloned molecules. Nucleic acid fragments flanked by recombination sites are cloned and subcloned using the Gateway® system by replacing a selectable marker (for example, ccdB) flanked by att sites on the recipient plasmid molecule, sometimes termed the Destination Vector. Desired clones are then selected by transformation of a ccdB sensitive host strain and positive selection for a marker on the recipient molecule. Similar strategies for



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negative selection (e.g., use of toxic genes) can be used in other organisms such as thymidine kinase (TK) in mammals and insects.

A recombination system useful for engineering yeast is outlined briefly. The system makes use of the *URA3* gene (e.g., for *Candida tropicalis*, *Candida viswanathii*, *Saccharomyces cerevisiae* and *Candida albicans*, for example) or *URA4* and *URA5* genes (e.g., for *S. pombe*, for example) and toxicity of the nucleotide analogue 5-Fluoroorotic acid (5-FOA). The *URA3* or *URA4* and *URA5* genes encode orotidine-5'-monophosphate (OMP) dicarboxylase. Yeast with an active *URA3* or *URA4* and *URA5* gene (phenotypically Ura+) convert 5-FOA to fluorodeoxyuridine, which is toxic to yeast cells. Yeast carrying a mutation in the appropriate gene(s) or having a knock out of the appropriate gene(s) can grow in the presence of 5-FOA, if the media is also supplemented with uracil.

A nucleic acid engineering construct can be made which may contain the *URA3* gene or cassette (for *C. tropicalis*, *C. viswanathii* or *S. cerevisiae*, for example), flanked on either side by the same nucleotide sequence in the same orientation. The *URA3* cassette can include a promoter, the *URA3* gene and a functional transcription terminator. Target sequences which direct the construct to a particular nucleic acid region of interest in the cell or organism to be engineered are added such that the target sequences are adjacent to and abut the flanking sequences on either side of the *URA3* cassette. Yeast can be transformed with the engineered construct and plated on minimal media without uracil. Colonies can be screened by PCR to determine those transformants that have the engineering construct inserted in the proper location in the genome. Checking insertion location prior to selecting for recombination of the ura3 cassette may reduce the number of incorrect clones carried through to later stages of the procedure. Correctly inserted transformants can then be grown and plated on minimal media containing 5-FOA to select for recombination of the URA3 cassette out of the construct, leaving a disrupted gene and an identifiable footprint (e.g., nucleic acid sequence) that can be used to verify the presence of the disrupted gene. The technique described is useful for disrupting or "knocking out" gene function, but also can be used to insert genes or constructs into a host cell genome in a targeted, sequence specific manner.

In certain embodiments, a nucleic acid reagent includes one or more topoisomerase insertion sites. A topoisomerase insertion site is a defined nucleotide sequence recognized and bound by a site-specific topoisomerase. For example, the nucleotide sequence 5'-(C/T)CCTT-3' is a



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topoisomerase recognition site bound specifically by most poxvirus topoisomerases, including vaccinia virus DNA topoisomerase I. After binding to the recognition sequence, the topoisomerase cleaves the strand at the 3'-most thymidine of the recognition site to produce a nucleotide sequence comprising 5'-(C/T)CCTT-PO4-TOPO, a complex of the topoisomerase covalently bound to the 3' phosphate via a tyrosine in the topoisomerase (e.g., Shuman (1991) *J. Biol. Chem. 266*:11372-11379; Sekiguchi and Shuman (1994) *Nucl. Acids Res*. 22:5360-5365; U.S. Pat. No. 5,766,891; PCT/US95/16099; and PCT/US98/12372). In comparison, the nucleotide sequence 5'-GCAACTT-3' is a topoisomerase recognition site for type IA *E. coli* topoisomerase III. An element that is inserted often is combined with topoisomerase-reacted template and thereby incorporated into the nucleic acid reagent (e.g., World Wide Web Uniform Resource Locator (URL) tools.thermofisher.com/downloads/F-13512_Topo_Flyer.pdf; World Wide Web Uniform Resource Locator (URL) tools.thermofisher.com/content/sfs/brochures/topo-pcr-cloning-brochure.pdf; TOPO TA Cloning® Kit and Zero Blunt® TOPO® Cloning Kit product information).

A nucleic acid reagent sometimes contains one or more origin of replication (ORI) elements. In some embodiments, a template comprises two or more ORIs, where one functions efficiently in one organism (e.g., a bacterium) and another functions efficiently in another organism (e.g., a eukaryote, like yeast for example). In some embodiments, an ORI may function efficiently in one species (e.g., *S. cerevisiae*, for example) and another ORI may function efficiently in a different species (e.g., *S. pombe*, for example). A nucleic acid reagent also sometimes includes one or more transcription regulation sites.

A nucleic acid reagent can include one or more selection elements (e.g., elements for selection of the presence of the nucleic acid reagent, and not for activation of a promoter element which can be selectively regulated). Selection elements often are utilized using known processes to determine whether a nucleic acid reagent is included in a cell. In some embodiments, a nucleic acid reagent includes two or more selection elements, where one functions efficiently in one organism and another functions efficiently in another organism. Examples of selection elements include, but are not limited to, (1) nucleic acid segments that encode products that provide resistance against otherwise toxic compounds (e.g., antibiotics); (2) nucleic acid segments that encode products that are otherwise lacking in the recipient cell (e.g., essential products, tRNA genes, auxotrophic markers); (3) nucleic acid segments that encode products that suppress the activity of a gene product; (4) nucleic acid segments that encode products that can be readily

identified (e.g., phenotypic markers such as antibiotics (e.g., β-lactamase), β-galactosidase, green fluorescent protein (GFP), yellow fluorescent protein (YFP), red fluorescent protein (RFP), cyan fluorescent protein (CFP), and cell surface proteins); (5) nucleic acid segments that bind products that are otherwise detrimental to cell survival and/or function; (6) nucleic acid segments that otherwise inhibit the activity of any of the nucleic acid segments described in Nos. 1-5 above (e.g., antisense oligonucleotides); (7) nucleic acid segments that bind products that modify a substrate (e.g., restriction endonucleases); (8) nucleic acid segments that can be used to isolate or identify a desired molecule (e.g., specific protein binding sites); (9) nucleic acid segments that encode a specific nucleotide sequence that can be otherwise non-functional (e.g., for PCR amplification of subpopulations of molecules); (10) nucleic acid segments that, when absent, directly or indirectly confer resistance or sensitivity to particular compounds; (11) nucleic acid segments that encode products that either are toxic or convert a relatively non-toxic compound to a toxic compound (e.g., Herpes simplex thymidine kinase, cytosine deaminase) in recipient cells; (12) nucleic acid segments that inhibit replication, partition or heritability of nucleic acid molecules that contain them; and/or (13) nucleic acid segments that encode conditional replication functions, e.g., replication in certain hosts or host cell strains or under certain environmental conditions (e.g., temperature, nutritional conditions, and the like). In some embodiments, the regulatory or selective agent can be added to change the existing growth conditions to which a cell or organism is subjected (e.g., growth in liquid culture, growth in a fermenter, growth on solid nutrient plates and the like for example).

A nucleic acid reagent can sometimes be of any form useful for in vivo transcription and/or translation. A nucleic acid sometimes is a plasmid, such as a supercoiled plasmid, sometimes is a yeast artificial chromosome (e.g., YAC), sometimes is a linear nucleic acid (e.g., a linear nucleic acid produced by PCR or by restriction digest), sometimes is single-stranded and sometimes is double-stranded. A nucleic acid reagent sometimes is prepared by an amplification process, such as a polymerase chain reaction (PCR) process or transcription-mediated amplification process (TMA). In TMA, two enzymes are used in an isothermal reaction to produce amplification products detected by light emission (see, e.g., *Biochemistry* 1996 Jun 25;35(25):8429-38). Standard PCR processes are known (e.g., U. S. Patent Nos. 4,683,202; 4,683,195; 4,965,188; and 5,656,493), and generally are performed in cycles. Each cycle includes heat denaturation, in which hybrid nucleic acids dissociate; cooling, in which primer oligonucleotides hybridize; and extension of the oligonucleotides by a polymerase (i.e., Taq polymerase). An example of a PCR cyclical process is treating the sample at 95°C for 5

minutes; repeating forty-five cycles of 95°C for 1 minute, 59°C for 1 minute, 10 seconds, and 72°C for 1 minute 30 seconds; and then treating the sample at 72°C for 5 minutes. Multiple cycles frequently are performed using a commercially available thermal cycler. PCR amplification products sometimes are stored for a time at a lower temperature (e.g., at 4°C) and sometimes are frozen (e.g., at –20°C) before analysis.

In some embodiments, a nucleic acid reagent, protein reagent, protein fragment reagent or other reagent described herein is isolated or purified. The term "isolated" as used herein refers to material removed from its original environment (e.g., the natural environment if it is naturally occurring, or a host cell if expressed exogenously), and thus is altered "by the hand of man" from its original environment. The term "purified" as used herein with reference to molecules does not refer to absolute purity. Rather, "purified" refers to a substance in a composition that contains fewer substance species in the same class (e.g., nucleic acid or protein species) other than the substance of interest in comparison to the sample from which it originated. "Purified," if a nucleic acid or protein for example, refers to a substance in a composition that contains fewer nucleic acid species or protein species other than the nucleic acid or protein of interest in comparison to the sample from which it originated. Sometimes, a protein or nucleic acid is "substantially pure," indicating that the protein or nucleic acid represents at least 50% of protein or nucleic acid on a mass basis of the composition. Often, a substantially pure protein or nucleic acid is at least 75% on a mass basis of the composition, and sometimes at least 95% on a mass basis of the composition.

*Genetic engineering methods*

Methods and compositions (e.g., nucleic acid reagents) described herein can be used to generate modified or engineered cells or organisms. For example, a cell or organism can be modified by altering one or more cellular activities and/or the sum total of a cell's or organism's activities. Modifications can be, for example, any alteration of cellular activities, including addition of cellular activities and/or elimination of cellular activities. The term "altered activity" as used herein refers to an activity in an engineered cell or microorganism that is added, removed or modified in any way relative to the host cell or microorganism (e.g., added, increased, reduced, decreased, inhibited, removed or redirected activity). In some embodiments, the methods and nucleic acid reagents described herein can be used to generate genetically modified cells and organisms with altered activities in cellular carbon processing.



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For example, the methods of genetic modification can be used to alter fatty acid (e.g., oleic acid, adipic acid, sebacic acid, suberic acid, octanedioic acid, decanedioic acid, dodecanedioic acid, tetradecanedioic acid, hexadecanedioic acid, octadecanedioic acid, eicosanedioic acid) synthesis and/or catabolism. In some embodiments, an engineered cell or organism described herein may include an increased number of copies of one or more polynucleotides encoding one or more polypeptides having carnitine acetyltransferase, acetyl-CoA carboxylase, ATP citrate lyase, thioesterase, acetyl-CoA hydrolase, acetyl-CoA synthetase, acyl-CoA oxidase, cytochrome P450 reductase, monooxygenase, peroxisomal biogenesis factor, alcohol dehydrogenase, alcohol oxidase, aldehyde dehydrogenase, 3-ketoacyl-CoA thiolase, and/or multifunctional enzyme (e.g., enoyl-CoA hydratase and/or 3-hydroxyacyl-CoA dehydrogenase) activity. In certain embodiments, an engineered cell or microorganism described herein may include one or more genetic modifications that reduce one or more of the following activities: carnitine acetyltransferase (e.g., mitochondrial), acetyl-carnitine translocase (e.g., mitochondrial), acyl-CoA synthetase, acyl-CoA oxidase and peroxisomal transporter activity.

In some embodiments, the engineered cell or organism can be a prokaryote. In certain embodiments, the prokaryote can be a bacterium, e.g., *Escherichia coli*. In some embodiments, the engineered cell or organism can be a eukaryote. In some embodiments, the eukaryote may be a fungus. In certain embodiments, the eukaryote can be a yeast. In certain embodiments, the yeast can be a *Candida* yeast. In some embodiments, the *Candida* yeast may be *C. viswanathii* or *C. troplicalis*. In certain embodiments, the fungus can be a *Yarrowia* fungus. In some embodiments the *Yarrowia* fungus may be *Y. lipolytica*. In some embodiments, the fungus can be a *Blastobotrys* yeast, e.g., *B. adeninivorans*. In certain embodiments, the fungus can be an *Aspergillus* fungus. In some embodiments, the *Aspergillus* fungus may be *A. parasiticus* or A. *nidulans*.

In some embodiments, an activity and/or amount of a composition can be altered by genetically modifying a host cell or organism which yields an engineered cell or organism having added, increased, reduced, decreased, inhibited, redirected, removed and/or otherwise modified activity or composition. A cell or organism may be modified, for example, by altering the amount of one or more cellular compositions, e.g, polynucleotides and/or polypeptides. Engineered cells or organisms typically arise as a result of a genetic modification, usually introduced by one of skill in the art using readily available techniques. Such cells or organisms are referred to herein as genetically modified or genetically engineered cells, microorganisms or organisms. The term



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"genetic modification" as used herein refers to any alteration in the genetic make-up of a cell or organism, including, for example, any nucleic acid addition, removal or alteration. Genetic modifications include, without limitation, insertion of one or more nucleotides in an endogenous nucleic acid of a host cell or organism in one or more locations, deletion of one or more nucleotides in an endogenous nucleic acid of a host cell or organism in one or more locations, modification or substitution of one or more nucleotides in an endogenous nucleic acid of a host cell or organism in one or more locations. In some embodiments, a portion of a host genome can be replaced with a heterologous nucleic acid. A genetic modification can also be insertion of a nucleic acid into a host cell organism that is distinct from the host endogenous genome (e.g., insertion of an autonomously replicating vector), and removal of a nucleic acid that is distinct from the endogenous host genome (e.g., removal of a vector).

Non-limiting examples of methods useful for genetically modifying a cell or organism include, introducing a heterologous polynucleotide (e.g., nucleic acid or gene integration, also referred to as "knock in"), removing an endogenous polynucleotide, altering the sequence of an existing endogenous nucleic acid sequence ( e.g., site-directed mutagenesis), disruption of an existing endogenous nucleic acid sequence (e.g., knock outs and transposon or insertion element mediated mutagenesis), selection for an altered activity where the selection causes a change in a naturally occurring activity that can be stably inherited (e.g., causes a change in a nucleic acid sequence in the genome of the cell or organism or in an epigenetic nucleic acid that is replicated and passed on to daughter cells), PCR-based mutagenesis, and the like. The terms "mutant" and "mutagenesis" as used herein refer to any modification to a nucleic acid (e.g., nucleic acid reagent or host chromosome) and/or polypeptide which results in an altered nucleic acid and/or polypeptide. Non-limiting examples of mutagenesis include, deletion, insertion, substitution, rearrangement, point mutations, suppressor mutations and the like of a single or multiple residues in a polynucleotide. Mutagenesis methods are known in the art and are readily available to the artisan. Non-limiting examples of mutagenesis methods are described herein and can also be found in Maniatis, T., E. F. Fritsch and J. Sambrook (1982) *Molecular Cloning: a Laboratory Manual*; Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. Another non-limiting example of mutagenesis can be conducted using an Agilent (Santa Clara, CA) "QuickChange" kit according to the manufacturer's instructions.



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*Decreasing an amount of a composition and/or activity in a cell*

An altered activity or composition sometimes is an activity or composition detectable in a host cell or organism and that is reduced, decreased, inhibited or removed (i.e., not detectable) in an engineered cell or organism. For example, a genetic modification that disrupts cellular synthesis of a composition (e.g., acyl-CoA synthetase protein) and/or or disrupts an activity, such as activation of fatty acids, or disrupts a polynucleotide that encodes a polypeptide that carries out a forward reaction in the activity (e.g., acyl-CoA synthetase activity), may render the composition (e.g., acyl-CoA synthetase protein) or activity, such as fatty acid activation, undetectable. The term "undetectable" as used herein refers to an amount of an analyte (including an activity) that is below the limits of detection, using know detection methods or assays (e.g., described herein). In certain embodiments, the genetic modification may partially reduce or decrease a composition or an activity. The term "reduces" or "decreases" with reference to a composition or an activity as used herein refers to a level of the composition or activity in an engineered cell or organism that is lower than the level of the composition or activity found in the host or starting cell or organism. A "lower" level can be a level that is detectable or undetectable. The term "partially reduces" or "partially decreases" with reference to a composition or an activity as used herein refers to a level of the composition or activity in an engineered cell or organism that is lower than the level of the composition or activity found in the host or starting cell or organism but that is still detectable. Thus, an activity or composition can be reduced to undetectable levels in some embodiments, or detectable levels in certain embodiments. An activity or composition can be decreased to any suitable level for production of a target molecule product (e.g., an organic acid), including but not limited to less than 2-fold (e.g., about 10% decrease to about 99% decrease; about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% decrease), 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, of 10-fold decrease, or greater than about 10-fold decrease.

The term "level", as used herein, often refers to an amount (e.g., a quantitative or relative amount) of a nucleic acid (e.g. an RNA (e.g. an mRNA) or DNA), polypeptide or activty.

In some embodiments, an activity or composition may be reduced or removed by decreasing the number of copies of a polynucleotide that encodes a composition polypeptide or polypeptide having a target activity. In some embodiments, an activity or composition can be reduced or removed by (i) inserting a polynucleotide within a polynucleotide that encodes a protein having



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the target activity or the target composition (disruptive insertion), and/or (ii) removing a portion of or all of a polynucleotide that encodes a polypeptide having the target activity or the target composition (deletion or knock out, respectively). In certain embodiments, an activity or composition can be reduced or removed by inserting into a host cell or microorganism a heterologous polynucleotide that is (i) operably linked to another polynucleotide that encodes a polypeptide having the target activity or target composition, and (ii) down regulates production of the polypeptide. Thus, an activity or composition can be reduced or removed by inserting or modifying a regulatory polynucleotide operably linked to another polynucleotide that encodes a polypeptide having the target activity or target composition.

An activity or composition also can be reduced or removed by (i) inhibiting a polynucleotide that encodes a polypeptide having the activity or the targeted composition or (ii) inhibiting a polynucleotide operably linked to another polynucleotide that encodes a polypeptide having the activity or targeted composition. A polynucleotide can be inhibited by a suitable technique known in the art, such as by contacting an RNA encoded by the polynucleotide with a specific inhibitory RNA (e.g., RNAi, siRNA, ribozyme). An activity also can be reduced or removed by contacting a polypeptide having the activity with a molecule that specifically inhibits the activity (e.g., enzyme inhibitor, antibody). In certain embodiments, an activity or composition can be reduced or removed by subjecting a host cell or organism to a selective environment and screening for cells or organisms that have a reduced level or removal of the activity or composition.

In some embodiments, an untranslated ribonucleic acid or a cDNA can be used to reduce the expression of a particular activity or enzyme. For example, a host cell or organism can be engineered by genetic modification to express a nucleic acid reagent that reduces the expression of an activity by producing an RNA molecule that is partially or substantially homologous to a nucleic acid sequence of interest which encodes the activity of interest. The RNA molecule can bind to the nucleic acid sequence of interest and inhibit the nucleic acid sequence from performing its natural function, in certain embodiments. In some embodiments, the RNA may alter the nucleic acid sequence of interest which encodes the activity of interest in a manner that the nucleic acid sequence of interest is no longer capable of performing its natural function (e.g., the action of a ribozyme for example).



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In some embodiments, an activity and/or composition may be reduced in, or removed from, a host cell or organism by increasing or adding a separate activity or composition in the host cell or organism. For example, an activity and/or composition that inhibits a targeted activity or composition in a host cell or organism can be increased or added thereby reducing or eliminating the targeted activity or composition by adding or increasing an inhibiting activity or composition. Methods of increasing or adding an activity or composition in a cell or organism are described herein.

*Increasing an amount of a composition and/or activity in a cell*

An altered activity and/or composition in an engineered cell or organism is sometimes an added composition or activity that is not detectable in a host cell or organism. An altered activity or composition can also be an increased or elevated activity or amount of a composition in an engineered cell or organism. An increased or elevated activity or composition generally is an activity or an amount of the composition that is greater than the activity or composition amount detectable in a host cell or organism. However, an increased or elevated activity or amount of a composition in an engineered cell or organism can also be a detectable activity or detectable composition that is not detectable in a host cell or organism. An activity or amount of a composition can be increased to any suitable level for example, for production of a target molecule product (e.g., an organic acid), including but not limited to less than 2-fold (e.g., about 10% increase to about 99% increase; about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% increase), 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, of 10-fold increase, or greater than about 10-fold increase.

In some embodiments, an activity and/or composition may be added to or increased in a host cell or organism by increasing the number of copies of a polynucleotide that encodes a polypeptide composition or polypeptide having the activity. In some embodiments, the activity and/or amount of a native or endogenous polypeptide can be increased by introducing heterologous nucleic acid into a host cell or organism that includes copies of a polynucleotide that encodes the polypeptide, for example, introducing 1 to about 100 additional heterologous copies of the polynucleotide (e.g., introducing 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, 18 or more, 19 or more, 20 or more, 22 or more, 24 or more, 25 or more, 26 or more, 28 or more, 30 or more additional copies of the



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polynucleotide). In certain embodiments, an activity and/or composition can be added or increased by inserting into a host cell or organism a polynucleotide that encodes a heterologous polypeptide from a different species having the added activity or composition, or encodes a heterologous polypeptide that is a modified version of an endogenous polypeptide. In such embodiments, 1 to about 100 copies of the polynucleotide can be introduced (e.g., introducing 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more, 17 or more, 18 or more, 19 or more, 20 or more, 22 or more, 24 or more, 25 or more, 26 or more, 28 or more, 30 or more copies). A heterologous polypeptide that is a "modified endogenous polypeptide" often has an activity different than an activity of a native polypeptide counterpart (e.g., different catalytic activity and/or different substrate specificity), and often is active (e.g., an activity (e.g., substrate turnover) is detectable). A heterologous polypeptide that is a "modified endogenous polypeptide" also often includes or lacks a cell location-targeting amino acid sequence that a native polypeptide counterpart has or doesn't have (e.g., in order to modify the cellular location of the expressed polypeptide). In certain embodiments, an activity or composition can be added or increased by inserting into a host cell or organism a heterologous polynucleotide that is (i) operably linked to another polynucleotide that encodes a polypeptide composition or a polypeptide having the added activity, and (ii) up regulates production of the polypeptide. Thus, a composition or an activity can be added or increased by inserting or modifying a regulatory polynucleotide operably linked to another polynucleotide that encodes a composition polypeptide or polypeptide having the targeted activity. In certain embodiments, an activity or composition can be added or increased by subjecting a host cell or organism to a selective environment and screening for cells or organisms that have a detectable level of the activity or composition. Examples of a selective environment include, without limitation, a medium containing a substrate that a host cell or organism can process and a medium lacking a substrate that a host cell or organism can process.

In some embodiments, an activity and/or composition may be added to or increased in a host cell or organism by decreasing or removing a separate activity or composition in a host cell or organism. For example, an activity and/or composition in a host cell or organism that inhibits a desired target activity or composition can be decreased or removed thereby reducing or eliminating the inhibition of the desired activity or composition and adding or increasing the desired activity. Methods of decreasing or removing an activity or composition in a cell or organism are described herein.



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*Nucleic acid manipulation*

In certain embodiments, nucleotide sequences sometimes are added to, modified or removed from one or more of the nucleic acid reagent elements, such as the promoter, 5' UTR, target sequence, or 3' UTR elements, to enhance, potentially enhance, reduce, or potentially reduce transcription and/or translation before or after such elements are incorporated in a nucleic acid reagent. In some embodiments, one or more of the following sequences may be modified or removed if they are present in a 5' UTR: a sequence that forms a stable secondary structure (e.g., quadruplex structure or stem loop stem structure (e.g., EMBL sequences X12949, AF274954, AF139980, AF152961, S95936, U194144, AF116649 or substantially identical sequences that form such stem loop stem structures)); a translation initiation codon upstream of the nucleotide sequence start codon; a stop codon upstream of the nucleotide sequence translation initiation codon; an ORF upstream of the nucleotide sequence translation initiation codon; an iron responsive element (IRE) or like sequence; and a 5' terminal oligopyrimidine tract (TOP, e.g., consisting of 5-15 pyrimidines adjacent to the cap). Computer-assisted software programs are available for nucleic acid sequence evaluation to optimize untranslated region sequences (see, e.g., World Wide Web Uniform Resource Locator (URL) bioinformatics.ua.pt/software/mrna-optimiser/). A translational enhancer sequence and/or an internal ribosome entry site (IRES) sometimes is inserted into a 5'UTR (e.g., EMBL nucleotide sequences J04513, X87949, M95825, M12783, AF025841, AF013263, AF006822, M17169, M13440, M22427, D14838 and M17446 and substantially identical nucleotide sequences).

An AU-rich element (ARE, e.g., AUUUA repeats) and/or splicing junction that follows a non-sense codon sometimes is removed from or modified in a 3' UTR. A polyadenosine tail sometimes is inserted into a 3' UTR if none is present, sometimes is removed if it is present, and adenosine moieties sometimes are added to or removed from a polyadenosine tail present in a 3' UTR. Thus, some embodiments are directed to a process that includes: determining whether any nucleotide sequences that increase, potentially increase, reduce or potentially reduce translation efficiency are present in the elements, and adding, removing or modifying one or more of such sequences if they are identified. Certain embodiments are directed to a process that includes: determining whether any nucleotide sequences that increase or potentially increase translation efficiency are not present in the elements, and incorporating such sequences into the nucleic acid reagent.



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In some embodiments, an activity and/or composition can be altered by modifying the nucleotide sequence of an ORF. An ORF sometimes is mutated or modified (for example, by point mutation, deletion mutation, insertion mutation, PCR based mutagenesis and the like) to alter, enhance or increase, reduce, substantially reduce or eliminate the activity of the encoded protein or peptide. The protein or peptide encoded by a modified ORF sometimes is produced in a lower amount or may not be produced at detectable levels, and in other embodiments, the product or protein encoded by the modified ORF is produced at a higher level (e.g., codons sometimes are modified so they are compatible with tRNA's preferentially used in the host or engineered cell or organism). To determine the relative activity, the activity from the product of the mutated ORF (or cell containing it) can be compared to the activity of the product or protein encoded by the unmodified ORF (or cell containing it).

In some embodiments, an ORF nucleotide sequence sometimes is mutated or modified to alter the triplet nucleotide sequences used to encode amino acids (e.g., amino acid codon triplets, for example). Modification of the nucleotide sequence of an ORF to alter codon triplets sometimes is used to change the codon found in the original sequence to better match the preferred codon usage of the organism in which the ORF or nucleic acid reagent will be expressed. The codon usage, and therefore the codon triplets encoded by a nucleic acid sequence, in bacteria may be different from the preferred codon usage in eukaryotes, like yeast or plants for example. Preferred codon usage also may be different between bacterial species. In certain embodiments, an ORF nucleotide sequence sometimes is modified to eliminate codon pairs and/or eliminate mRNA secondary structures that can cause pauses during translation of the mRNA encoded by the ORF nucleotide sequence. Translational pausing sometimes occurs when nucleic acid secondary structures exist in an mRNA, and sometimes occurs due to the presence of codon pairs that slow the rate of translation by causing ribosomes to pause. In some embodiments, the use of lower abundance codon triplets can reduce translational pausing due to a decrease in the pause time needed to load a charged tRNA into the ribosome translation machinery. Therefore, to increase transcriptional and translational efficiency in bacteria (e.g., where transcription and translation are concurrent, for example) or to increase translational efficiency in eukaryotes (e.g., where transcription and translation are functionally separated), the nucleotide sequence of a nucleotide sequence of interest can be altered to better suit the transcription and/or translational machinery of the host and/or genetically modified cell or organism. In certain embodiments, slowing the rate of translation by the use of



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lower abundance codons, which slow or pause the ribosome, can lead to higher yields of the desired product due to an increase in correctly folded proteins and a reduction in the formation of inclusion bodies.

Codons can be altered and optimized according to the preferred usage by a given organism by determining the codon distribution of the nucleotide sequence donor organism and comparing the distribution of codons to the distribution of codons in the recipient or host organism. Techniques described herein (e.g., site directed mutagenesis and the like) can then be used to alter the codons accordingly. Comparisons of codon usage can be done by hand, or using nucleic acid analysis software commercially available to the artisan (see, e.g., World Wide Web Uniform Resource Locator (URL) kazusa.or.jp/codon/, World Wide Web Uniform Resource Locator (URL) jcat.de, World Wide Web Uniform Resource Locator (URL) idtdna.com/CodonOpt).

Modification of the nucleotide sequence of an ORF also can be used to correct codon triplet sequences that have diverged in different organisms. For example, certain yeast (e.g., *Candida tropicalis*, *Candida viswanathii* and *Candida maltosa*) use the amino acid triplet CUG (e.g., CTG in the DNA sequence) to encode serine. CUG typically encodes leucine in most organisms. In order to maintain the correct amino acid in the resultant polypeptide or protein, the CUG codon must be altered to reflect the organism in which the nucleic acid reagent will be expressed. Thus, if an ORF from a bacterial donor is expressed in such a *Candida* yeast strain mentioned above, the heterologous nucleotide sequence must first be altered or modified to the appropriate leucine codon. Therefore, in some embodiments, the nucleotide sequence of an ORF sometimes is altered or modified to correct for differences that have occurred in the evolution of the amino acid codon triplets between different organisms. In some embodiments, the nucleotide sequence can be left unchanged at a particular amino acid codon, if the amino acid encoded is a conservative or neutral change in amino acid when compared to the originally encoded amino acid.

In some embodiments, an activity can be altered by modifying translational regulation signals, like a stop codon for example. A stop codon at the end of an ORF sometimes is modified to another stop codon, such as an amber stop codon. In some embodiments, a stop codon is introduced within an ORF, sometimes by insertion or mutation of an existing codon. An ORF comprising a modified terminal stop codon and/or internal stop codon often is translated in a



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system comprising a suppressor tRNA that recognizes the stop codon. An ORF comprising a stop codon sometimes is translated in a system comprising a suppressor tRNA that incorporates an unnatural amino acid during translation of the target protein or target peptide. Methods for incorporating unnatural amino acids into a target protein or peptide are known, which include, for example, processes utilizing a heterologous tRNA/synthetase pair, where the tRNA recognizes an amber stop codon and is loaded with an unnatural amino acid (e.g., World Wide Web Uniform Resource Locator (URL) iupac.org/news/prize/2003/wang.pdf).

Depending on the portion of a nucleic acid reagent (e.g., promoter, 5' or 3' UTR, ORI, ORF, and the like) chosen for alteration (e.g., by mutagenesis, introduction or deletion, for example), the modifications described above can alter a given activity by (i) increasing or decreasing feedback inhibition mechanisms, (ii) increasing or decreasing promoter initiation, (iii) increasing or decreasing translation initiation, (iv) increasing or decreasing translational efficiency, (v) modifying localization of peptides or products expressed from nucleic acid reagents described herein, (vi) increasing or decreasing the copy number of a nucleotide sequence of interest, or (vii) expression of an anti-sense RNA, RNAi, siRNA, ribozyme and the like. In some embodiments, alteration of a nucleic acid reagent or nucleotide sequence can alter a region involved in feedback inhibition (e.g., 5' UTR, promoter and the like). A modification sometimes is made that can add or enhance binding of a feedback regulator and sometimes a modification is made that can reduce, inhibit or eliminate binding of a feedback regulator.

In certain embodiments, alteration of a nucleic acid reagent or nucleotide sequence can alter sequences involved in transcription initiation (e.g., promoters, 5' UTR, and the like). A modification sometimes can be made that can enhance or increase initiation from an endogenous or heterologous promoter element. A modification sometimes can be made that removes or disrupts sequences that increase or enhance transcription initiation, resulting in a decrease or elimination of transcription from an endogenous or heterologous promoter element.

In some embodiments, alteration of a nucleic acid reagent or nucleotide sequence can alter sequences involved in translational initiation or translational efficiency (e.g., 5' UTR, 3' UTR, codon triplets of higher or lower abundance, translational terminator sequences and the like, for example). A modification sometimes can be made that can increase or decrease translational initiation, modifying a ribosome binding site for example. A modification sometimes can be made that can increase or decrease translational efficiency. Removing or adding sequences



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that form hairpins and changing codon triplets to a more or less preferred codon are non-limiting examples of genetic modifications that can be made to alter translation initiation and translation efficiency.

In certain embodiments, alteration of a nucleic acid reagent or nucleotide sequence can alter sequences involved in localization of peptides, proteins or other desired products (e.g., an organic acid, for example). A modification sometimes can be made that can alter, add or remove sequences responsible for targeting a polypeptide, protein or product to an intracellular organelle, the periplasm, cellular membranes, or extracellularly. Transport of a heterologous product to a different intracellular space or extracellularly sometimes can reduce or eliminate the formation of inclusion bodies (e.g., insoluble aggregates of the desired product).

In some embodiments, alteration of a nucleic acid reagent or nucleotide sequence can alter sequences involved in increasing or decreasing the copy number of a nucleotide sequence of interest. A modification sometimes can be made that increases or decreases the number of copies of an ORF stably integrated into the genome of an organism or on an epigenetic nucleic acid reagent. Non-limiting examples of alterations that can increase the number of copies of a sequence of interest include, adding copies of the sequence of interest by duplication of regions in the genome (e.g., adding additional copies by recombination or by causing gene amplification of the host genome, for example), cloning additional copies of a sequence onto a nucleic acid reagent, or altering an ORI to increase the number of copies of an epigenetic nucleic acid reagent. Non-limiting examples of alterations that can decrease the number of copies of a sequence of interest include, removing copies of the sequence of interest by deletion or disruption of regions in the genome, removing additional copies of the sequence from epigenetic nucleic acid reagents, or altering an ORI to decrease the number of copies of an epigenetic nucleic acid reagent.

In certain embodiments, increasing or decreasing the expression of a nucleotide sequence of interest can also be accomplished by altering, adding or removing sequences involved in the expression of an anti-sense RNA, RNAi, siRNA, ribozyme and the like. The methods described herein can be used to modify expression of anti-sense RNA, RNAi, siRNA, ribozyme and the like.



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Nucleic acid sequences of interest can be genetically modified using methods known in the art. Mutagenesis techniques are particularly useful for small scale (e.g., 1, 2, 5, 10 or more nucleotides) or large scale (e.g., 50, 100, 150, 200, 500, or more nucleotides) genetic modification. Mutagenesis allows the artisan to alter the genetic information of a cell or organism in a stable manner, either naturally (e.g., isolation using selection and screening) or experimentally by the use of chemicals, radiation or inaccurate DNA replication (e.g., PCR mutagenesis). In some embodiments, genetic modification can be performed by whole scale synthetic synthesis of nucleic acids, using a native nucleotide sequence as the reference sequence, and modifying nucleotides that can result in the desired alteration of activity. Mutagenesis methods sometimes are specific or targeted to specific regions or nucleotides (e.g., site-directed mutagenesis, PCR-based site-directed mutagenesis, and in vitro mutagenesis techniques such as transplacement and in vivo oligonucleotide site-directed mutagenesis, for example). Mutagenesis methods sometimes are non-specific or random with respect to the placement of genetic modifications (e.g., chemical mutagenesis, insertion element (e.g., insertion or transposon elements) and inaccurate PCR based methods, for example).

Site directed mutagenesis is a procedure in which a specific nucleotide or specific nucleotides in a nucleic acid molecule are mutated or altered. Site directed mutagenesis typically is performed using a nucleic acid sequence of interest cloned into a circular plasmid vector. Site-directed mutagenesis requires that the wild type sequence be known and used a platform for the genetic alteration. Site-directed mutagenesis sometimes is referred to as oligonucleotide-directed mutagenesis because the technique can be performed using oligonucleotides which have the desired genetic modification incorporated into the complement of a nucleotide sequence of interest. The wild type sequence and the altered nucleotide are allowed to hybridize and the hybridized nucleic acids are extended and replicated using a DNA polymerase. The double stranded nucleic acids are introduced into a host (e.g., *E. coli*, for example) and further rounds of replication are carried out in vivo. The transformed cells carrying the mutated nucleic acid sequence are then selected and/or screened for those cells carrying the correctly mutagenized sequence. Cassette mutagenesis and PCR-based site-directed mutagenesis are further modifications of the site-directed mutagenesis technique. Site-directed mutagenesis can also be performed in vivo (e.g., transplacement "pop-in pop-out", in vivo site-directed mutagenesis with synthetic oligonucleotides and the like, for example).



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PCR-based mutagenesis can be performed using PCR with oligonucleotide primers that contain the desired mutation or mutations. The technique functions in a manner similar to standard site-directed mutagenesis, with the exception that a thermocycler and PCR conditions are used to replace replication and selection of the clones in a microorganism host. As PCR-based mutagenesis also uses a circular plasmid vector, the amplified fragment (e.g., linear nucleic acid molecule) containing the incorporated genetic modifications can be separated from the plasmid containing the template sequence after a sufficient number of rounds of thermocycler amplification, using standard electrophoretic procedures. A modification of this method uses linear amplification methods and a pair of mutagenic primers that amplify the entire plasmid. The procedure can take advantage of the *E. coli* Dam methylase system which causes DNA replicated in vivo to be sensitive to the restriction endonucleases DpnI. PCR synthesized DNA is not methylated and is therefore resistant to DpnI. This approach allows digestion of the template plasmid, leaving the genetically modified, PCR synthesized plasmids for isolating and transforming into a host bacteria for DNA repair and replication, thereby facilitating subsequent cloning and identification steps. A certain amount of randomness can be added to PCR-based sited directed mutagenesis by using partially degenerate primers.

DNA shuffling is a method which uses DNA fragments from members of a mutant library and reshuffles the fragments randomly to generate new mutant sequence combinations. The fragments are typically generated using DNaseI, followed by random annealing and re-joining using self priming PCR. The DNA overhanging ends, from annealing of random fragments, provide "primer" sequences for the PCR process. Shuffling can be applied to libraries generated by any of the above mutagenesis methods.

Error prone PCR and its derivative rolling circle error prone PCR uses increased magnesium and manganese concentrations in conjunction with limiting amounts of one or two nucleotides to reduce the fidelity of the Taq polymerase. The error rate can be as high as 2% under appropriate conditions, when the resultant mutant sequence is compared to the wild type starting sequence. After amplification, the library of mutant coding sequences must be cloned into a suitable plasmid. Although point mutations are the most common types of mutation in error prone PCR, deletions and frameshift mutations are also possible. There are a number of commercial error-prone PCR kits available, including those from Agilent and Takara Bio, U.S.A. (e.g., World Wide Web Uniform Resource Locator (URL) agilent.com and World Wide Web Uniform Resource Locator (URL) clontech.com, respectively, for example). Rolling circle error-



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prone PCR is a variant of error-prone PCR in which wild-type sequence is first cloned into a plasmid, then the whole plasmid is amplified under error-prone conditions.

In contrast to site-directed or specific mutagenesis, random mutagenesis does not require any sequence information and can be accomplished by a number of widely different methods. Random mutagenesis often is used to generate mutant libraries that can be used to screen for the desired genotype or phenotype. Non-limiting examples of random mutagenesis include; chemical mutagenesis, UV-induced mutagenesis, insertion element or transposon-mediated mutagenesis, DNA shuffling, error-prone PCR mutagenesis, and the like.

Chemical mutagenesis often involves chemicals like ethyl methanesulfonate (EMS), nitrous acid, mitomycin C, N-methyl-N-nitrosourea (MNU), diepoxybutane (DEB), 1, 2, 7, 8-diepoxyoctane (DEO), methyl methane sulfonate (MMS), N-methyl- N'-nitro-N-nitrosoguanidine (MNNG), 4-nitroquinoline 1-oxide (4-NQO), 2-methyloxy-6-chloro-9(3-[ethyl-2-chloroethyl]-aminopropylamino)-acridinedihydrochloride (ICR-170), 2-amino purine (2AP), and hydroxylamine (HA), provided herein as non-limiting examples. These chemicals can cause base-pair substitutions, frameshift mutations, deletions, transversion mutations, transition mutations, incorrect replication, and the like. In some embodiments, the mutagenesis can be carried out in vivo. Sometimes the mutagenic process involves the use of the host organism's DNA replication and repair mechanisms to incorporate and replicate the mutagenized base or bases.

Another type of chemical mutagenesis involves the use of base-analogs. The use of base-analogs causes incorrect base pairing which in the following round of replication is corrected to a mismatched nucleotide when compared to the starting sequence. Base analog mutagenesis introduces a small amount of non-randomness to random mutagenesis, because specific base analogs can be chosen which can be incorporated at certain nucleotides in the starting sequence. Correction of the mispairing typically yields a known substitution. For example, bromo-deoxyuridine (BrdU) can be incorporated into DNA and replaces T in the sequence. The host DNA repair and replication machinery can sometime correct the defect, but sometimes will mispair the BrdU with a G. The next round of replication then causes a G-C transversion from the original A-T in the native sequence.



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Ultra violet (UV) induced mutagenesis is caused by the formation of thymidine dimers when UV light irradiates chemical bonds between two adjacent thymine residues. Excision repair mechanism of the host organism correct the lesion in the DNA, but occasionally the lesion is incorrectly repaired typically resulting in a C to T transition.

In some embodiments, an altered activity can be found by screening cells or an organism under conditions that select for the desired change in activity. For example, certain microorganisms can be adapted to increase or decrease an activity by selecting or screening the organism in question on a media containing substances that are poorly metabolized or even toxic. An increase in the ability of an organism to grow a substance that is normally poorly metabolized may result in an increase in the growth rate on that substance, for example. A decrease in the sensitivity to a toxic substance might be manifested by growth on higher concentrations of the toxic substance, for example. Modifications obtained in this manner are not limited to alterations in promoter sequences. That is, screening microorganisms by selective pressure, as described above, can yield genetic alterations that can occur in non-promoter sequences, and sometimes also can occur in sequences that are not in the nucleotide sequence of interest, but in a related nucleotide sequences (e.g., a gene involved in a different step of the same pathway, a transport gene, and the like). Such mutants sometimes can be found by isolating variants from unique environments.

Cells or organisms with altered activities can also be isolated using genetic selection and screening of cells or organisms challenged on selective media or by identifying naturally occurring variants from unique environments. For example, 2-deoxy-D-glucose is a toxic glucose analog. Growth of yeast on this substance yields mutants that are glucose-deregulated. A number of mutants have been isolated using 2-deoxy-D-glucose including transport mutants, and mutants that ferment glucose and galactose simultaneously instead of glucose first then galactose when glucose is depleted. Similar techniques have been used to isolate mutant microorganisms that can metabolize plastics (e.g., from landfills), petrochemicals (e.g., from oil spills), and the like, either in a laboratory setting or from unique environments.

Similar methods can be used to isolate cells or organisms having existing mutations in a desired activity when the activity exists at a relatively low or nearly undetectable level in the cell or organism of choice, in some embodiments. The method generally consists of growing the cell or organism to a specific density in liquid culture, concentrating the cells, and plating the cells on



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various concentrations of the substance to which an increase in metabolic activity is desired. The cells are incubated at a moderate growth temperature, for 5 to 10 days. To enhance the selection process, the plates can be stored for another 5 to 10 days at a low temperature. The low temperature sometimes can allow strains that have gained or increased an activity to continue growing while other strains are inhibited for growth at the low temperature. Following the initial selection and secondary growth at low temperature, the plates can be replica plated on higher or lower concentrations of the selection substance to further select for the desired activity.

Insertion element or transposon-mediated mutagenesis makes use of naturally occurring or modified naturally occurring mobile genetic elements. Transposons often encode accessory activities in addition to the activities necessary for transposition (e.g., movement using a transposase activity, for example). In many examples, transposon accessory activities are antibiotic resistance markers (e.g., Tn903 $kan^r$). Insertion elements typically only encode the activities necessary for movement of the nucleic acid sequence. Insertion element and transposon mediated mutagenesis often can occur randomly, however specific target sequences are known for some transposons. Mobile genetic elements like IS elements or Transposons (Tn) often have inverted repeats, direct repeats or both inverted and direct repeats flanking the region coding for the transposition genes. Recombination events catalyzed by the transposase cause the element to remove itself from the genome and move to a new location, leaving behind a portion of an inverted or direct repeat. Classic examples of transposons are the "mobile genetic elements" discovered in maize. Transposon mutagenesis kits are commercially available which are designed to leave behind a 5 codon insert (e.g., Mutation Generation System kit, ThermoFisher Scientific, World Wide Web Uniform Resource Locator (URL) thermofisher.com/, for example). This allows the artisan to identify the insertion site, without fully disrupting the function of most genes.

*Introduction of nucleic acids into cells*

Engineered cells and organisms can be prepared by altering, introducing and/or removing nucleotide sequences in the host genome or in stably maintained epigenetic nucleic acid reagents, as described herein. The nucleic acid reagents used to alter, introduce or remove nucleotide sequences in the host genome or epigenetic nucleic acids can be prepared using the methods described herein and/or available to the artisan.



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Nucleic acid sequences having a desired activity can be isolated from cells of a suitable organism using lysis and nucleic acid purification procedures described in a known reference manual (e.g., Maniatis, T., E. F. Fritsch and J. Sambrook (1982) *Molecular Cloning: a Laboratory Manual*; Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) or using commercially available cell lysis and DNA purification reagents and kits. In some embodiments, nucleic acids used to engineer cells or microorganisms can be provided for conducting methods described herein after processing of the organism containing the nucleic acid. For example, the nucleic acid of interest may be extracted, isolated, purified or amplified from a sample (e.g., from a cell(s) or organism of interest or culture containing a plurality of cells or organisms of interest, like yeast or bacteria for example). The term "isolated" as used herein refers to nucleic acid removed from its original environment (e.g., the natural environment if it is naturally occurring, or a host cell if expressed exogenously), and thus is altered "by the hand of man" from its original environment. An isolated nucleic acid generally is provided with fewer non-nucleic acid components (e.g., protein, lipid) than the amount of components present in a source sample. A composition containing isolated sample nucleic acid can be substantially isolated (e.g., about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or greater than 99% free of non-nucleic acid components). The term "purified" as used herein refers to sample nucleic acid provided that contains fewer nucleic acid species than in the sample source from which the sample nucleic acid is derived. A composition containing sample nucleic acid may be substantially purified (e.g., about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or greater than 99% free of other nucleic acid species). The term "amplified" as used herein refers to subjecting nucleic acid of a cell, organism or sample to a process that linearly or exponentially generates amplicon nucleic acids having the same or substantially the same nucleotide sequence as the nucleotide sequence of the nucleic acid in the sample, or portion thereof. As noted herein, the nucleic acids used to prepare nucleic acid reagents as described herein can be subjected to fragmentation or cleavage.

Amplification of nucleic acids is sometimes necessary when dealing with cells or organisms that are difficult to culture. Where amplification may be desired, any suitable amplification technique can be utilized. Non-limiting examples of methods for amplification of polynucleotides include, polymerase chain reaction (PCR); ligation amplification (or ligase chain reaction (LCR)); amplification methods based on the use of Q-beta replicase or template-dependent polymerase (see US Patent Publication Number US20050287592); helicase-dependent isothermal



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amplification (Vincent et al., "Helicase-dependent isothermal DNA amplification". EMBO reports 5 (8): 795–800 (2004)); strand displacement amplification (SDA); thermophilic SDA nucleic acid sequence based amplification (3SR or NASBA) and transcription-associated amplification (TAA). Non-limiting examples of PCR amplification methods include standard PCR, AFLP-PCR, Allele-specific PCR, Alu-PCR, Asymmetric PCR, Colony PCR, Hot start PCR, Inverse PCR (IPCR), In situ PCR (ISH), Intersequence-specific PCR (ISSR-PCR), Long PCR, Multiplex PCR, Nested PCR, Quantitative PCR, Reverse Transcriptase PCR (RT-PCR), Real Time PCR, Single cell PCR, Solid phase PCR, combinations thereof, and the like. Reagents and hardware for conducting PCR are commercially available.

Protocols for conducting the various type of PCR listed above are readily available to the artisan. PCR conditions can be dependent upon primer sequences, target abundance, and the desired amount of amplification, and therefore, one of skill in the art may choose from a number of PCR protocols available (see, e.g., U.S. Pat. Nos. 4,683,195 and 4,683,202; and PCR Protocols: A Guide to Methods and Applications, Innis et al., eds, 1990). PCR often is carried out as an automated process with a thermostable enzyme. In this process, the temperature of the reaction mixture is cycled through a denaturing region, a primer-annealing region, and an extension reaction region automatically. Machines specifically adapted for this purpose are commercially available. A non-limiting example of a PCR protocol that may be suitable for embodiments described herein is, treating the sample at 95ºC for 5 minutes; repeating forty-five cycles of 95ºC for 1 minute, 59ºC for 1 minute, 10 seconds, and 72ºC for 1 minute 30 seconds; and then treating the sample at 72ºC for 5 minutes. Multiple cycles frequently are performed using a commercially available thermal cycler. Suitable isothermal amplification processes known and selected by the person of ordinary skill in the art also may be applied, in certain embodiments. In some embodiments, nucleic acids encoding polypeptides with a desired activity can be isolated by amplifying the desired sequence from a cell or organism having the desired activity using oligonucleotides or primers designed based on sequences described herein.

Synthetic nucleic acids, e.g., codon-optimized sequences, can be generated using a variety of methods. For example, whole-scale synthetic chemistry can be used to generate an entire sequence. Other methods include use of chemically-generated oligonucleotides in amplification methods, e.g., recursive PCR, that build an entire nucleotide sequence (see, e.g., Prodromou and Pearl (1992) *Protein Engineering* 5(8):827-829; Yehezkel et al. (2013) *Gene Synthesis:*



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*Methods and Protocols* in Methods in Molecular Biology 852:35-47, Jean Piccoud (ed.) Springer Science and Business Media LLC).

Amplified, isolated and/or purified nucleic acids can be cloned into the recombinant DNA vectors described herein or into suitable commercially available recombinant DNA vectors. Cloning of nucleic acid sequences of interest into recombinant DNA vectors can facilitate further manipulations of the nucleic acids for preparation of nucleic acid reagents, (e.g., alteration of nucleotide sequences by mutagenesis, homologous recombination, amplification and the like, for example). Standard cloning procedures (e.g., enzymatic digestion, ligation, and the like) are known (e.g., described in Maniatis, T., E. F. Fritsch and J. Sambrook (1982) *Molecular Cloning: a Laboratory Manual*; Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

In some embodiments, nucleic acid sequences prepared by isolation or amplification can be used, without any further modification, to add an activity to a cell or microorganism and thereby create a genetically modified or engineered cell or microorganism. In certain embodiments, nucleic acid sequences prepared by isolation or amplification can be genetically modified to alter (e.g., increase or decrease, for example) a desired activity. In some embodiments, nucleic acids, used to add an activity or composition to a cell or organism, sometimes are genetically modified to optimize the heterologous polynucleotide sequence encoding the desired activity (e.g., polypeptide or protein, for example). The term "optimize" as used herein can refer to alteration to increase or enhance expression by preferred codon usage. The term optimize can also refer to modifications to the amino acid sequence to increase the activity of a polypeptide or protein, such that the activity exhibits a higher catalytic activity as compared to the "natural" version of the polypeptide or protein.

A heterologous, recombinant, or mutagenized polynucleotide can be introduced into a nucleic acid reagent for introduction into a host cell or organism, thereby generating an engineered cell or microorganism. Standard recombinant DNA techniques (restriction enzyme digests, ligation, and the like) can be used by the artisan to combine a nucleic acid of interest into a suitable nucleic acid reagent capable of (i) being stably maintained by selection in the host cell or organism, or (ii) being integrated into the genome of the host cell or organism. Sometimes nucleic acid reagents include two replication origins to allow manipulation of the same nucleic acid reagent in bacteria before final introduction of the final product into the host cell or organism (e.g., yeast or fungus for example). Standard molecular biology and recombinant



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DNA methods are known (e.g., described in Maniatis, T., E. F. Fritsch and J. Sambrook (1982) *Molecular Cloning: a Laboratory Manual*; Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

Nucleic acid reagents can be introduced into cells or microorganisms using various techniques. Non-limiting examples of methods used to introduce heterologous nucleic acids into various cells or organisms include; transformation, transfection, transduction, electroporation, ultrasound-mediated transformation, particle bombardment and the like. In some instances the addition of carrier molecules (e.g., bis-benzimdazolyl compounds, for example, see US Patent 5595899) can increase the uptake of DNA in cells that may be difficult to transform by conventional methods. Conventional methods of transformation are known (e.g., described in Maniatis, T., E. F. Fritsch and J. Sambrook (1982) *Molecular Cloning: a Laboratory Manual*; Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

Linear DNA transformed into a host cell can be integrated into the genome by homologous recombination. The localization of genomic integration is determined by the homologous sequence at the ends of the transformed linear DNA. Fig. 1 is a diagrammatic representation of a cassette for the addition of a gene of interest (GOI) into a host non-functional ura3 locus using the single crossover integration method. The core of the cassette contains the GOI gene with a promoter ($P_{XXX}$) and terminator ($T_{XXX}$) for controlling transcription of the GOI gene. These DNA cassettes are typically generated by overlap extension PCR assembly of the cassette elements or by PCR amplification from circular plasmids containing the entire cassette. Additionally, circular plasmids containing cassette elements may be cut within (e.g., the middle of) the *URA3* ORF to generate a linear DNA fragment used in transforming cells. A circular DNA vector containing the cassette core and an intact *URA3* gene can be linearized by endonuclease-mediated cutting the vector such that it splits the *URA3* selectable marker within (e.g., the middle of) the ORF. The resulting linear DNA contains the expression cassette of the gene of interest (GOI) positioned between the *URA3* promoter ($P_{URA3}$) and terminator ($T_{URA3}$). Parts A and B of Fig. 1 show the results of integration of one copy (A) and two copies (B) of the cassette into a $Ura^-$ auxotrophic mutant strain. Integration of one cassette generates an added, functional GOI expression unit and may or may not provide for expression of a functional Ura3p, depending on the nature of ura3 locus and the location of the split in the *URA3* selectable marker. Integration of two or more copies of the cassette in tandem arrays generates a complete, functional *URA3* sequence by combining the 5' end of *URA3* from one copy of the



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cassette and the 3' end of *URA3* from the second copy of the cassette. Additional copies may also be integrated. Transformants can be selected by growth on uracil-free media. This integration method thus favors selection of transformants containing multiple copies of the GOI.

In some embodiments, other auxotrophic or dominant selection markers can be used in place of URA3 (e.g., an auxotrophic selectable marker), with the appropriate change in selection media and selection agents. Auxotrophic selectable markers are used in strains deficient for synthesis of a required biological molecule (e.g., amino acid or nucleoside, for example). Non-limiting examples of additional auxotrophic markers include; HIS3, TRP1, LEU2, LEU2-d, and LYS2. Certain auxotrophic markers (e.g., URA3 and LYS2) allow counter selection to select for the second recombination event that pops out all but one of the direct repeats of the recombination construct. HIS3 encodes an activity involved in histidine synthesis. TRP1 encodes an activity involved in tryptophan synthesis. LEU2 encodes an activity involved in leucine synthesis. LEU2-d is a low expression version of LEU2 that selects for increased copy number (e.g., gene or plasmid copy number, for example) to allow survival on minimal media without leucine. LYS2 encodes an activity involved in lysine synthesis, and allows counter selection for recombination out of the LYS2 gene using alpha-aminoadipate ($\alpha$-aminoadipate).

Dominant selectable markers are useful because they also allow use of industrial and/or prototrophic strains for genetic manipulations. Additionally, dominant selectable markers provide the advantage that rich medium can be used for plating and culture growth, and thus growth rates are markedly increased. Non-limiting examples of dominant selectable markers include; Tn903 $kan^r$, $Cm^r$, $Hyg^r$, CUP1, and DHFR. Tn903 $kan^r$ encodes an activity involved in kanamycin antibiotic resistance (e.g., typically neomycin phosphotransferase II or NPTII, for example). $Cm^r$ encodes an activity involved in chloramphenicol antibiotic resistance (e.g., typically chloramphenicol acetyl transferase, for example). $Hyg^r$ encodes an activity involved in hygromycin resistance by phosphorylation of hygromycin B (e.g., hygromycin phosphotransferase, or HPT). CUP1 encodes an activity involved in resistance to heavy metal (e.g., copper, for example) toxicity. DHFR encodes a dihydrofolate reductase activity which confers resistance to methotrexate and sulfanilamde compounds.

Homologous recombination can also be used as a tool for mutagenesis. Homologous recombination can be used to specifically target regions of known sequence for insertion of heterologous nucleotide sequences using the host cell's natural DNA replication and repair



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enzymes. Homologous recombination methods sometimes are referred to as mutagenesis, transplacement, knock-out mutagenesis or knock-in mutagenesis. Integration of a nucleic acid sequence into a host genome by a double crossover homologous recombination event inserts the entire nucleic acid reagent at the targeted location. A second homologous recombination event driven by direct repeat DNA sequences contained in the integrated nucleic acid cassette excises (e.g., "pop out" or "loop out") all but a portion of the nucleic acid reagent, leaving behind a heterologous sequence, often referred to as a "footprint" or "scar". Mutagenesis by insertion (e.g., knock in) or by leaving behind a disrupting heterologous nucleic acid (e.g., knock out) serves to disrupt or "knock out" the function of the gene or nucleic acid sequence in which insertion occurs. By combining selectable markers and/or auxotrophic markers with nucleic acid reagents designed to provide the appropriate nucleic acid target sequences, the artisan can target a selectable nucleic acid reagent to a specific genomic region, and then select for recombination events that "pop out" a portion of the inserted nucleic acid reagent.

Such methods take advantage of nucleic acid reagents that have been specifically designed with known target nucleic acid sequences at or near a nucleic acid or genomic region of interest. Popping out typically leaves a "foot print" of left over sequences that remain after the recombination event. The left over sequence can disrupt a gene and thereby reduce or eliminate expression of that gene. In some embodiments, the method can be used to insert sequences, upstream or downstream of genes that can result in an enhancement or reduction in expression of the gene. In certain embodiments, new genes can be introduced into the genome of a host cell or organism using similar homologous recombination methods. An example of a yeast recombination system using the *URA3* gene and 5-FOA is described herein.

One method for genetic modification is described by Alani et al. ("A method for gene disruption that allows repeated use of URA3 selection in the construction of multiply disrupted yeast strains", *Genetics* 116(4):541-545 August 1987). The original method uses a *URA3* gene cassette with 1000 base pairs (bp) of the same nucleotide sequence cloned in the same orientation on either side of the *URA3* cassette. Targeting sequences of about 50 bp are added to each side of the construct. The double-stranded targeting sequences are complementary to sequences in the genome of the host cell or organism. The targeting sequences allow site-specific recombination in a region of interest. A modification of the original technique replaces the two 1000 bp sequence direct repeats with two 200 bp direct repeats. The modified method also uses 50 bp targeting sequences. The modification reduces or eliminates recombination of



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a second knock out into the 1000 bp repeat left behind in a first mutagenesis, therefore allowing multiply knocked out yeast. Additionally, the 200 bp sequences used in the method are uniquely designed, self-assembling sequences that leave behind identifiable footprints. The technique used to design the sequences incorporate design features such as low identity to the yeast genome, and low identity to each other. Therefore, a library of the self-assembling sequences can be generated to allow multiple knockouts in the same organism, while reducing or eliminating the potential for integration into a previous knockout.

Fig. 2 is a diagrammatic illustration of an exemplary gene knock out cassette. In this example, two slightly different cassettes are shown for use in separately disrupting each of the two *FAT1* alleles in a diploid yeast such as *Candida viswanathii*. The two cassette-containing nucleic acid segments are referred to as "Deletion 1" and "Deletion 2," respectively, in the figure. Each cassette contains a *URA3* gene including a URA3 promoter ($P_{URA3}$) and terminator ($T_{URA3}$). The complete *URA3* expression cassette provides for expression of orotidine-5'-monophosphate (OMP) dicarboxylase in a $Ura^-$ host cell into which the cassette has integrated, and yields a prototrophic transformant that can be selected for by growth in uracil-free media. Integration into the *FAT1* locus by a first crossover event is provided for by the presence of sequences located on either side of the cassette that are homologous to sequences in the target locus (e.g., *FAT1*). Additionally, each cassette contains a repeat of the $P_{URA3}$ sequence immediately downstream of the terminator sequence. This repeat sequence can be used in a second recombination event that results in the looping out of the *URA3* gene sequence which is facilitated by growth of the prototrophic transformants in the presence of 5-FOA yielding a $ura^-$ auxotroph. All or a portion of the $P_{URA3}$ sequence repeat remains in the genome and disrupts the *FAT1* gene allele such that it no longer yields a functional gene product. The heterozygous transformant can then be transformed with the second cassette (e.g., Deletion 2) and undergo the same two crossover events to yield a homozygous $Ura^-$ cell. The two separate deletion cassette-containing fragments differ in the sequences of the target gene that they contain on each side of the *URA3* cassette which results in integration into different positions in the target gene.

The URA3 cassette makes use of the toxicity of 5-FOA in yeast carrying a functional URA3 gene. Uracil synthesis-deficient host yeast are transformed with the modified URA3 cassette, using standard yeast transformation protocols, and the transformed cells are plated on minimal media minus uracil. In some embodiments, PCR can be used to verify correct insertion into the



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region of interest in the host genome, and in certain embodiments the PCR step can be omitted. Inclusion of the PCR step can reduce the number of transformants that are counter selected to "pop out" the URA3 cassette. The transformants (e.g., all or the ones determined as correct by PCR, for example) can then be counter-selected on media containing 5-FOA, which will select for recombination events looping out the URA3 cassette, thus rendering the yeast Ura$^-$ again, and resistant to 5-FOA toxicity. Targeting sequences used to direct recombination events to specific regions are presented herein. A modification of the method described above can be used to integrate genes into the chromosome in which, following recombination, a functional gene is left in the chromosome next to a, e.g., 200-bp, footprint. Such methods provide for addition of a desired nucleic acid into the host genome in combination with disruption of an endogenous nucleic acid.

Fig. 3A, Fig. 3B, and Fig 3C show diagrammatic illustrations of a knock-in gene disruption method which disrupts one target gene ("GOI1") and also adds a desired gene of interest ("GOI2") at the disrupted locus. As shown in Fig. 3A, the basic URA3 disruption cassette can be the same as that described in Fig. 2; however, there is an additional expression cassette immediately downstream of the second $P_{URA3}$ repeat sequence. This expression cassette contains the gene of interest, GOI2, for adding to the endogenous GOI1 locus and includes a promoter ($P_{XXX}$) and terminator ($T_{XXX}$) for controlling transcription of GOI2. Immediately upstream of the first $P_{URA3}$ sequence is a sequence of nucleotides of the GOI1 gene, and immediately downstream of the terminator ($T_{XXX}$) for GOI2 is another sequence of the GOI1 gene. These sequences are for use in integration of the cassettes into the GOI1 locus. Fig. 3B shows the locus after the first crossover event. These transformants are selected for growth in uracil-free media. In order to remove the *URA3* gene and regenerate an auxotrophic cell that can be further modified using the *URA3* marker method, the transformants are grown in the presence of 5-FOA to facilitate the second crossover event. The result of that event is shown in Fig. 3C which depicts the $P_{URA3}$ sequence that remains followed by a functional GOI2 cassette.

*Protein engineering methods*

As described herein, one method of altering carbon flux in cells and organisms is to modify one or more activities involved in carbon processing in cells. These activities can be modified by altering one or more elements directly and/or indirectly involved in the activities. Such elements include, but are not limited to, nucleic acids (e.g., transcription regulatory elements, addition



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and/or deletion of nucleic acids), peptides (e.g., signal peptides regulating protein localization in cells) and polypeptides (e.g., enzymes regulating reactions in metabolic pathways). Peptides and polypeptides can be modified in multiple ways, including, for example, alteration of the primary structure (i.e., amino acid sequence), secondary structure, post-translational chemical modification (e.g., phosphorylation, acylation, glycosylation) and processing (e.g., proteolytic cleavage). Many protein modifications can be achieved through alteration of the nucleic acid encoding the protein in a cell. Alteration of the nucleic acid coding sequence can result in alteration of the amino acid sequence which in turn can modify the intra- and inter-polypeptide interactions of the encoded protein. Such alterations can thus result in modification of the activity of the polypeptide and the activity of any metabolic processes in which it may participate.

In some embodiments of the cells, organisms, compositions and methods provided herein, a modified polypeptide can be expressed in a cell or organism by introducing a modification into nucleic acid encoding the polypeptide in the cell or organism. Modified polypeptides often have an activity different than the activity of an unmodified counterpart. A modified activity sometimes is a different transport activity, a different catalytic activity, a different substrate specificity, or a different catalytic activity and a different substrate specificity. A different activity sometimes is an activity that is higher (e.g., increased activity) or lower (e.g., decreased activity) than the activity of an unmodified counterpart polypeptide. In some embodiments, the catalytic activity of a modified polypeptide is higher or lower than the catalytic activity of the unmodified counterpart for a particular substrate. In certain embodiments, the specificity of a modified polypeptide for a particular substrate is higher or lower than the specificity of the unmodified counterpart for a particular substrate. A modified polypeptide often is active and an activity of a modified polypeptide often can be detected (e.g., substrate turnover can be detected). An activity for a particular polypeptide that is modified sometimes is referred to as a "target activity." As described herein, target activities include, but are not limited to, activities of ω-oxidation, β-oxidation, acetyl-CoA processing, carnitine/acetylcarnitine shuttle, membrane transport, fatty acid biosynthesis, acyl-CoA formation/degradation. Non-limiting examples of particular target activities include carnitine acetyltransferase, carnitine translocase, acetyl-CoA carboxylase, ATP citrate lyase, acetyl-CoA hydrolase, acetyl-CoA synthetase, thioesterase, acyl-CoA synthetase, monooxygenase, cytochrome P450 reductase, alcohol dehydrogenase, alcohol oxidase, aldehyde dehydrogenase, acyl-CoA oxidase, 3-ketoacyl-CoA thiolase, peroxisomal transporter, peroxisome biogenesis factor and multifunctional enzyme (e.g., enoyl-CoA hydratase and/or 3-



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hydroxyacyl-CoA dehydrogenase) activities. In some of the embodiments provided herein, these and other activities can be modified in a cell or organism.

One or more particular modifications can be selected to generate a modified polypeptide having a target activity. Modifications often are amino acid modifications (e.g., deletion, insertion of one or more amino acids). Amino acid modifications sometimes are amino acid substitutions. Amino acid substitutions sometimes are conservative, non-limiting examples of which include substitution of an amino acid containing an acidic moiety for another amino acid containing an acidic moiety (e.g., D, E), substitution of an amino acid containing a basic moiety for another amino acid containing a basic moiety (e.g., H, K, R), substitution of an amino acid containing an aliphatic chain moiety for another amino acid containing an aliphatic chain moiety (e.g., V, L, I, A), substitution of an amino acid containing a cyclic moiety for another amino acid containing a cyclic moiety (e.g., W, F, Y), and substitution of an amino acid containing a polar moiety for another amino acid containing a polar moiety (e.g., S, T). Amino acid substitutions sometimes are non-conservative, non-limiting examples of which include substitution of an amino acid containing an acidic moiety for an amino acid containing a basic moiety, substitution of an amino acid containing a basic moiety for an amino acid containing an acidic moiety, substitution of an amino acid containing relatively small moiety (e.g., G, A) for another amino acid containing a relatively large moiety (e.g., Y, W, F, I, L), and substitution of an amino acid containing a relatively large moiety for another amino acid containing an relatively small moiety.

Particular modifications can be selected using any suitable method known in the art. In certain embodiments, a reference structure is known for a related polypeptide with a known activity, and modifications to a target polypeptide can be guided by alignment of the target polypeptide structure to the reference structure. A reference structure sometimes is a primary structure (e.g., polynucleotide or polypeptide sequence) and the primary structure of a target can be aligned to the reference structure using an alignment method known in the art. Particular amino acids in the target that align with (e.g., are identical to or homologous to) or do not align with (e.g., are not identical to or not homologous to) particular amino acids in the reference can be selected for modification. Selections can be made by inspection of an alignment or by software known in the art that identifies, scores and/or ranks amino acids for modification based on an alignment. A reference structure sometimes is a secondary structure, tertiary structure or quaternary structure, each of which are three dimensional structures pertaining to a polypeptide. A primary structure of a target polypeptide can be modeled to a secondary, tertiary or



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quaternary reference structure using three-dimensional modeling software known in the art. A secondary, tertiary or quaternary structure of a target polypeptide can be compared to a secondary, tertiary or quaternary reference structure using three-dimensional comparative software known in the art. Particular structures (e.g., a particular individual amino acid; a particular group of contiguous or non-contiguous amino acids) in the target that align with or map to, or do not align with or map to, particular structures in the reference can be selected for modification. Also, particular structures in the target that are in proximity to a substrate or co-factor can be selected for modification. Selections can be made by inspection of an alignment or map or by software known in the art that identifies, scores and/or ranks amino acids and/or structures for modification based on an alignment and map. After particular amino acids and/or structures are selected for modification in a first polypeptide, amino acids and structures in a second polypeptide that align with the selected amino acids and structures in the first polypeptide may be identified.

For example, a structural model of a protein can be created based on the crystal structure of the protein using SWISS-MODEL, which has been described by Arnold *et al.* ((2006) *Bioinformatics* 22: 195-201), Guex *et al.* ((2009) *Electrophoresis* 30 Supplement 1: S162-S173) and Kiefer *et al.* ((2009) *Nucleic Acids Res.* 37 (Database issue): D387-D392). As described herein, the resulting structural model can be analyzed to identify sites in the protein that potentially participate in determining an activity of the protein. HotSpot Wizard is an example of a tool for identifying sites for engineering of substrate specificity and/or activity of enzymes using a combination of structural, functional and sequence analysis and has been described by Pavelka *et al.* ((2009) *Nucleic Acids Res.* 37 (Web Server issue): W376-W383) (see also: HotSpot Wizard 1.7 World Wide Web Uniform Resource Locator (URL) loschmidt.chemi.muni.cz/hotspotwizard/index.jsp). Identification of such sites facilitates a determination of possible amino acids to target for mutagenesis in modifying the activity of a protein (e.g., enzyme). Part of the HotSpot Wizard analysis is the identification of homologs by a BLAST search (see, e.g., Johnson *et al.* (2008) *Nucleic Acids Res.* 36 (Web Server issue): W5-W9) and their alignment using MUSCLE as described, for example, by Edgar ((2004) *BMC Bioinformatics* 5: 113 and *Nucleic Acids Res.* 32: 1792-1797). The multiple sequence alignment reveals the variety of amino acids found at each position and their relative frequency amongst all the sequences. This information can be useful in determining possible amino acid substitutions that may be made at identified sites in the protein.



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In a non-limiting example, particular amino acid substitutions for a *Candida* spp. Pox5 acyl-CoA oxidase polypeptide are provided herein. For example, some substitutions were designed to modify a substrate specificity of an acyl-CoA oxidase polypeptide. As described herein, in embodiments in which the target product molecule is a six-carbon fatty acid (e.g., adipic acid) produced by β-oxidation of a longer-chain fatty acid, it is optimal to modify the activity of acyl-CoA oxidases (which can catalyze the first step in β-oxidation) in host cells or organisms such that there is little to no activity on substrates with chain lengths less than 8 carbons. Deletion of nucleic acids encoding acyl-CoA oxidases (e.g., Pox4 in *Candida viswanathii*) with relatively broad carbon-chain length specificity that are active on short-chain length substrates prevents generation of fatty acid products with fewer than eight carbon atoms (i.e., chain length shorter than C8) by peroxisomal β-oxidation. This is because the remaining acyl-CoA oxidase activity (e.g., Pox5) is specific for longer chain substrates and has low activity on substrates with carbon chain lengths less than 10. In order to increase the activity of Pox5 on substrates with a chain length of 8 carbons and thereby increase the amount of 6-carbon fatty acid target molecule products relative to 8-carbon fatty acid molecules, the Pox5 protein was subjected to engineering as described herein. Modified Pox5 proteins obtained by amino acid substitutions of the wild-type *Candida viswanathii* Pox5 protein (made via corresponding nucleotide sequence changes in the nucleic acid encoding the protein) resulted in an increased ratio of 6-carbon to 8-carbon fatty acid products in *Candida* cells expressing the modified enzymes as compared to cells expressing wild-type Pox5p (as shown by experimental results presented in the Examples herein). Using the teachings described herein, a primary structure of another acyl-CoA oxidase polypeptide can be aligned with the amino acid sequence or modeled structure of a Pox5 polypeptide and some or all amino acids of the other polypeptide that align with those selected for modification in the Pox5 polypeptide also can be selected for modification.

Additional nonlimiting examples of protein modifications that can be made in altering the carbon flux in a cell or organism include modifications to alter a substrate specificity of an acyl-CoA dehydrogenase polypeptide produced in the cell and that is involved in β-oxidation. An acyl-CoA dehydrogenase enzyme can require an NAD cofactor in carrying out a catalytic function. Sometimes a co-factor specificity of an acyl-CoA dehydrogenase is modified, and in some embodiments the modified polypeptide prefers to utilize oxygen as a co-factor.

In another non-limiting example of a protein modification designed to modify an enzyme activity, amino acid substitutions can be made to enhance or reduce regulation of the enzyme. For

example, enzymes can be regulated in a number of ways, including, for example, covalent modification of an enzyme such as phosphorylation/dephosphorylation and acetylation/deacetylation. The activity of an enzyme can be modified by altering its ability to be activated or inhibited within a cell. In one embodiment, regulation of an enzyme by phosphorylation can be decreased or eliminated by modifying a nucleic acid encoding the enzyme to substitute codons for phosphorylatable amino acid residues (e.g., serine) with codons for non-phosphorylatable residues (e.g, alanine). Computer-assisted software programs are available for identifying potential phosphorylatable amino acid residues (see, e.g., NetPhos (World Wide Web Uniform Resource Locator (URL) cbs.dtu.dk/services/NetPhos/), NetPhosYeast (World Wide Web Uniform Resource Locator (URL) cbs.dtu.dk/services/NetPhosYeast/)). In an example described herein, an acetyl-CoA carboxylase protein (e.g., Acc1 of *Candida viswanathii*) is modified to reduce regulation of the enzyme by phosphorylation. Because the dephosphorylated state is the active state of the enzyme, the protein (and nucleic acid encoding it) was modified to eliminate one or more phosphorylatable serine residues by substituting them with alanine residues, thereby relieving the regulation by phosphorylation. For example, as described herein, a *Candida viswanathii* acetyl-CoA carboxylase endogenous, wild-type enzyme was modified to substitute alanine residues for one or more of the following serine amino acid residues: S652, S1131, S1138, S1153, S1158.

One or more activities of a modified polypeptide can be characterized using any suitable assay known in the art. A modified polypeptide can be expressed in a cell or organism other than a target organism in which a target product will be produced, for assaying activity. For example, a modified polypeptide can be expressed in a bacterium (e.g., *E. coli*), assayed and then introduced into a yeast (e.g., *Candida* spp. yeast) for production of a target molecule product.

*Engineered carbon flux pathways for efficient production of target molecules*

Provided herein are multiple compositions for, and methods of, modifying cells and organisms to alter carbon flux. Also provided are the modified cells and organisms generated by the methods. The modification methods can be combined in a number of ways as described herein to engineer cell- or organism-based systems for enhanced, efficient production of target molecules. Also provided herein are methods of producing target molecules, including, for



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example, organic acids, polyketides and terpenes, using the modified cells or organisms provided herein.

Included in the cells, organisms, compositions and methods provided herein are modified cells and organisms in which carbon processing activities have been engineered to enhance carbon flow through cellular oxidative metabolism pathways. One advantage of such modified bioproduction systems is that they are well suited for use with lower cost, alternative carbon sources, including, for example, non-carbohydrate and non-fermentable carbon sources such as aliphatic compounds and hydrocarbons (e.g, alkanes, fatty acids and fatty alcohols). Use of such carbon sources is not only more cost-effective but can also have the added advantage of reducing the environmental impact of harmful wastes (e.g., agro-industrial by-products, waste cooking oil and waste motor oil) that can be used as feedstocks in target molecule production instead of being discarded. As also described herein, embodiments of the cell- and microbial-based systems in which carbon processing activities have been engineered to direct carbon flow through oxidative metabolism can be controlled to provide for maximal, coordinated and highly efficient target molecule production based on, for example, use of carbon source-dependent transcription regulation.

Figs. 5-11 schematically illustrate non-limiting embodiments of engineered carbon flux pathways of modified cells and organisms that can be used to produce a target molecule (e.g., adipic acid, malonyl-CoA, 3-hydroxypropionic acid, polyketide, triacetic acid lactone, terpene) from various starting carbon sources or feedstocks.

*Engineered carbon recycling loop pathways for a platform target molecule production system*

In order to minimize carbon loss and increase process efficiency of biological cell-based production systems, provided herein are cells and organisms (e.g., microorganisms) that have been modified to reduce, inhibit, slow and/or delay carbon flow into one or more growth and/or energy production metabolic pathways so that it is available for use in other inherent and/or engineered production processes. In doing so, carbon that would be lost to metabolic pathways uninvolved in target molecule production in an unmodified cell or organism are rescued or captured for use in target molecule production processes. As such, modified cells and organisms provided herein in some embodiments are useful as platform systems (as well as



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production systems) that can be used as the basis for further engineering for enhanced production of many different desired target molecules either singly or multiply in co-production cell- and microbial-based systems.

*Engineered pathways for capturing carbon atoms expelled from the β-oxidation pathway*

Figs. 5 and 6 depict possible cellular modifications in exemplary embodiments of a eukaryotic (i.e., yeast in this example) platform system designed to capture carbon atoms in the cytosol by enhancing carbon flow through cellular oxidative metabolism pathways (ω-oxidation and peroxisomal β-oxidation) and reducing flow of carbon into mitochondria, and the endoplasmic reticulum and lipid particles (in the form of acyl-CoA). Although multiple, possible, cellular modifications are illustrated in Figs. 5 and 6, as described herein, some of the modifications depicted in the figures are optional enhancements of exemplary engineered systems and may or may not be included in a modified cell or organism depending on, for example, the intended use of the system (e.g., development of a particular single, or multiple, target molecule(s) production system) and the selection of variable features (e.g., host cell or organism, carbon source, regulatory controls (such as transcription control elements), culture conditions and the like) of the system. Thus, it is understood that any optional modifications set forth in the exemplary systems shown in Figs. 5 and 6 are non-limiting and may or may not be included in a particular engineered system and, if included, may be in utilized in different combinations than illustrated in the figures.

Acetyl-CoA is a central molecule in the biochemical network of a cell that can be utilized for the biosynthesis of many useful chemicals. The β-oxidation pathway produces acetyl-CoA through the oxidation of fatty acids. In yeast, β-oxidation can be localized to the peroxisomal compartment which thus can be a primary location of fatty acid-derived acetyl-CoA. Generally, the peroxisomal acetyl-CoA would be converted to acetyl-carnitine by carnitine acetyltransferase (Cat2p). The acetyl-carnitine, being smaller, can diffuse out of the peroxisome and be transported across the mitochondrial inner membrane by Crc1p (a translocase protein). Once inside the mitochondria, the acetyl-carnitine is converted back into acetyl-CoA by mitochondrial Cat2p and can be used in the TCA cycle for energy generation or the synthesis of other biomolecules (Fig. 4). To take advantage of the acetyl-CoA generated by peroxisomal β-oxidation, genetic engineering strategies may be employed to reroute the carbon

in acetyl-CoA destined for the mitochondria to the cytosol instead, thereby making it available for use in biosynthetic pathways making desired chemical products. Thus, the amount of carbon that is lost to the TCA and lipid-generating (e.g., glycerol-3-phosphate (G3P) and/or dihydroxyacetone phosphate (DHAP)) pathways at the expense of target molecule production is reduced in this platform system. In embodiments of a platform system such as, for example, systems shown in Figs. 5 and 6, carbon processing activities can be engineered to enhance carbon flow through cellular oxidative metabolism pathways, e.g., ω-oxidation and peroxisomal β-oxidation, and decrease carbon flow to mitochondria and other organelles. Such platform systems can include an acetyl group carbon recycle loop that diverts acetyl moieties generated in the breakdown of fatty acids in peroxisomal β-oxidation into cytosolic fatty acid synthesis to regenerate a fatty acid that can be subjected to another cycle of peroxisomal β-oxidation. The recycle loop is depicted in Figs. 5 and 6 by the dark, solid reaction arrows beginning with extracellular fatty acid internalization in the upper left corner of the figure, extending through ω-oxidation and into the peroxisome for β-oxidation which yields acetyl-CoA that is transported out of the peroxisome and into the cytosol (initially in the form of either acetyl-carnitine or acetate), utilized in fatty acid synthesis to generate acyl-CoA which is then hydrolyzed to free fatty acid for re-entry into the loop at the starting point of ω-oxidation.

As shown in Figs. 5 and 6, carbon flux through the ω-oxidation and β-oxidation (peroxisomal) pathways can be enhanced through one or more of multiple modifications introduced via genetic manipulation of the cell. The enhancements can begin with the cellular internalization of external carbon. A non-fermentable or alternative carbon source (e.g., fatty acids, alkanes) enters the modified cell through the plasma membrane from the extracellular medium (shown in the upper left corner of Figs. 5 and 6). In an unmodified cell, a long-chain fatty acid (either as the carbon source or generated from processing of a carbon source, e.g., alkane) would be activated (through thioesterifcation with CoA) to acyl-CoA upon cell entry by acyl-CoA synthetase (encoded, e.g., by *FAA1* and/or *FAT1*). However, in some embodiments, a gene(s) encoding cytosolic and/or membrane-bound acyl-CoA synthetase can be disrupted or deleted resulting in a decrease or elimination of cytosolic and/or membrane-bound acyl-CoA synthetase and, thus, cytosolic and/or membrane-bound acyl-CoA synthetase activity in the cytosol. Cytosolic activation of fatty acids can thus also be decreased or eliminated in such modified cells (indicated in Figs. 5 and 6 as a lightly shaded dotted line reaction arrow labeled as "faa1Δ" and "fat1Δ" in blackened ovals). Most fatty acid metabolic pathways, including lipid (e.g, triacylglycerides (TAG) and phospholipids (PL)) biosynthesis and protein acylation, require that



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a free fatty acid be activated to acyl-CoA (or to acyl-ACP) prior to being metabolized. Therefore, in embodiments that include an enhancement such as a reduced or abolished acyl-CoA synthetase activity, the decreased cytosolic fatty acid activation can result in fewer internalized fatty acid carbons being lost to such pathways at the expense of target molecule-producing processes.

Free fatty acids that have entered the cell (shown as "FA" in Figs. 5 and 6), or generated from metabolism of an alkane carbon source, can then undergo oxidation to dicarboxylic acids (DCA) through ω-oxidation ("ω-ox" in Figs. 5 and 6). The availability of this oxidative process in the cell presents multiple advantages in these platform systems for target molecule production. For example, long-chain fatty acids that have not been activated to acyl-CoA do not readily cross the peroxisomal membrane; however, long-chain dicarboxylic acids are able to enter peroxisomes. Therefore, conversion of free fatty acids to DCA through ω-oxidation can be a further enhancement of carbon flow toward peroxisomal β-oxidation, particularly because there are no or limited other pathways in the cell for processing of free dicarboxylic acids. The availability of ω-oxidation-processing of free fatty acids in the modified cell is also beneficial to engineered production systems in which the target molecule (or an intermediate in target molecule production) is a dicarboxylic acid (e.g., adipic acid, suberic acid, sebacic acid, dodecanedioic acid, tetradecanedioic acid). For example, as shown in Figs. 5 and 6, under certain conditions, a dicarboxylic acid processed in β-oxidation can be converted into a shorter chain diacid which can be secreted from the cell as a target molecule upon removal of the coenzyme A carrier via hydrolysis catalyzed by peroxisomal thioesterase. Thus, the ω-oxidation pathway in a modified cell can serve as a cellular gateway for funneling internalized fatty acids into oxidative metabolism and target molecule production and away from cytosolic activation. Modification of ω-oxidation activity is another potential enhancement of these embodiments. For example, if a host cell or organism (e.g., *Candida* spp, *Yarrowia* spp, *Bacillus* spp, *Blastobotrys* spp) expresses an endogenous ω-oxidation pathway, one or more enzymes (e.g., monooxygenase, cytochrome P450 reductase, such as CPRB, and others) of the pathway can be modified (e.g., as described herein) to increase catalytic activity and/or alter substrate specificity in order to increase fatty acid processing in the pathway and/or target specific fatty acids for processing into dicarboxylic acids. If a host cell or organism does not express an endogenous ω-oxidation pathway, it can be genetically modified to express heterologous enzymes to engineer an ω-oxidation pathway in the cell or organism.



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In an oxidative metabolism-enhanced platform system, dicarboxylic acids, such as those generated by ω-oxidation, can traverse the peroxisomal membrane and move into peroxisomes where they can be activated via thioesterification to a dicarboxylic acid ester (shown as DCA-CoA in Figs. 5 and 6) and enter β-oxidation ("β -ox" in Figs. 5 and 6). In each cycle of β-oxidation, fatty acids are degraded through removal of two carbons from the carbon chain which are released as acetyl-CoA. The remaining fatty acid carbon chain can reenter another cycle of oxidation as an acyl-CoA shortened by two carbons atoms. Through successive cycles, a monocarboxylic fatty acid can be completely degraded such that only acetyl-CoA (for fatty acids with an even number of carbon atoms in the chain) or propionyl-CoA (for fatty acids with an odd number of carbon atoms in the chain) remains. Through successive cycles of β-oxidation of a dicarboxylic acid, the molecule can be completely degraded such that only succinyl-CoA (for fatty diacids with an even number of carbon atoms in the chain) or malonyl-CoA (for fatty diacids with an odd number of carbon atoms in the chain) remains. Thus, in these platform systems, the enhanced movement of fatty acids toward degradation via β-oxidation can yield acetyl-CoA (which can be used in target molecule production), and, in certain instances as described herein, shorter chain diacids at the completion of the oxidative process. A short-chain diacid thus produced can be a final target molecule (or a precursor or intermediate in the production of a target molecule).

The oxidative metabolism aspect of some platform systems, such as those shown in Figs. 5 and 6, can be further enhanced through modification of β-oxidation activity. For example, one or more enzymes (e.g., acyl-CoA oxidase, ketoacyl-CoA thiolase, multifunctional enzyme hydratase and/or dehydrogenase, and others) of the pathway can be modified (e.g., as described herein) to increase catalytic activity and/or alter substrate specificity in order to increase fatty acid processing in the pathway and/or select for specific fatty diacids for processing into target dicarboxylic acids. One example of a modification of β-oxidation activity, as described herein, is alteration of the substrate specificity of one or more acyl-CoA oxidase enzymes in the pathway, such as Pox4 and/or Pox5 of *Candida* yeast strains. In so doing, the process can be optimized for the production of fatty diacids of particular carbon chain lengths. For example, by genetically modifying a host cell or microorganism to decrease or eliminate Pox4 expression and/or activity in the host (e.g., *Candida*), the amount of shorter-chain (e.g., having less than about 8-10 carbons) fatty acids or diacids resulting from β-oxidation of longer chain fatty acids can be increased. Production of fatty acids or diacids of particular lengths can also be enhanced by genetically modifying (e.g., mutagenesis of the gene coding sequence to



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alter the encoded amino acid sequence) the activity of another acyl-CoA oxidase, such as Pox5, to alter the substrate specificity. For example, as described herein, some alterations of a *Candida* Pox5 amino acid sequence increase activity of the enzyme on C8 substrates and provide for a relative increase in the amount of C6 diacid (adipic acid) produced and decrease in the amount of C8 and longer diacids resulting from β-oxidation of a longer chain fatty acid. Thus, the platform system shown in Figs. 5 and 6 can also serve as a production system for diacids of particular carbon chain lengths. Additional optional modifications that can provide for enhanced carbon flux through β-oxidation (and enhanced target molecule production) in these systems include, but are not limited to, modification of β-oxidation-associated activities, such as peroxisome biogenesis and proliferation activities. For example, as described herein, the abundance and/or volume of peroxisomes in which β-oxidation occurs can be increased in host cells through genetic modification. An example of such a modification is increasing the transcription of, and/or number of copies of, one or more peroxin-encoding nucleic acids (e.g., *PEX11*) in a host cell. Amplification of such peroxin-encoding nucleic acids and/or activities can lead to an overall increased β-oxidation capacity.

One feature of the carbon recycle loops of the platform systems shown in Figs. 5 and 6 is the management and capture of acetyl-CoA generated during β-oxidation. Peroxisomal acetyl-CoA generally has two main fates: (i) conversion to acetyl-carnitine for transfer to mitochondria for use in the TCA cycle and (ii) the generation of malate in the glyoxylate cycle ("GlyOx" in Figs. 5 and 6) which is then used in gluconeogenesis or moves into mitochondria. In unmodified cells, these uses of acetyl-CoA generated in β-oxidation represent loss of carbon atoms that could be used in target molecule production. Through modifications that are a part of the platform systems shown in Figs. 5 and 6, acetyl groups can be captured either (1) as they move through the cytosol toward the mitochondria in the form of acetyl-carnitine or (2) in the form of acetate generated in peroxisomes. In capturing these acetyl group carbons, they thus can be diverted from the TCA cycle.

*Capture of carbon from acetyl-carnitine*

In the example platform system depicted in Fig. 5, modifications in the host cell that enhance the capture and diversion of acetyl group carbon include, but are not limited to, modification of acetyl-carnitine entry into mitochondria, and modification of conversion of cytosolic acetyl-carnitine to acetyl-CoA. As described herein, unmodified cells may contain a cytosolic carnitine



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acetyltransferase activity for conversion of cytosolic acetyl-carnitine to acetyl-CoA. However, in some instances, it may not be as catalytically active and/or abundant as it is in organelles, e.g., peroxisomes and mitochondria. In a platform system provided herein and depicted in Fig. 5, one modification that can be made to enhance capture of acetyl groups in the cytosol is to increase the amount and/or activity of cytosolic carnitine acetyltransferase. As described herein, methods of achieving this include increasing the copy number of nucleic acids encoding cytosolic carnitine acetyltransferase in the cell, increasing the transcription of such nucleic acids and/or introducing nucleic acid encoding a more active cytosolic carnitine acetyltransferase enzyme into the cell (e.g., modifying an endogenous cytosolic enzyme activity by replacing it with, or adding to it, a heterologous enzyme activity). For example, in one embodiment described herein, a *Candida* mitochondrial/peroxisomal carnitine acetyltransferase (e.g, Cat2) with greater catalytic activity than an endogenous *Candida* cytoplasmic carnitine acetyltransferase (e.g., Yat1) can be recombinantly expressed cytosolically in a host cell by engineering a nucleic acid encoding the more active enzyme such that the encoded enzyme lacks a mitochondrial (and a peroxisomal) targeting sequence of amino acids (shown, as *CAT2*$^{cyt}$ in Fig. 5; see also, e.g., amino acid SEQ ID NO: 4 and a nucleotide sequence (SEQ ID NO: 61) encoding the amino acid sequence). Once acetyl-carnitine in transit from the peroxisomes to the mitochondria has been converted to acetyl-CoA in the cytosol by carnitine acetyltransferase activity in the cytosol (e.g., *CAT2*$^{cyt}$), it cannot cross the mitochondrial inner membrane and is diverted from the TCA cycle. This acetyl-CoA is now available for use in target molecule production. The amount of carnitine acetyltransferase activity in the cytosol of such a modified cell or organism can be further increased by using a strong and/or fatty acid-inducible heterologous promoter (e.g., a yeast *HDE* gene promoter) to regulate transcription of the engineered nucleic acid encoding a carnitine acetyltransferase activity.

Another modification that can enhance cytosolic capture and diversion of acetyl moieties in cells is an alteration of acetyl-carnitine uptake into mitochondria from the cytosol (shown as faded, dotted reaction arrow lines into and in the mitochondrial compartment in Fig. 5). One method of modifying mitochondrial acetyl-carnitine uptake is by altering the processing of acetyl-carnitine that occurs in the mitochondria to convert it to acetyl-CoA for use in the TCA cycle. For example, by decreasing the amount and/or activity level of the enzyme that catalyzes this processing, i.e., mitochondrial carnitine acetyltransferase, there can be a corresponding decrease in conversion of acetyl-carnitine to acetyl-CoA in the mitochondria. Without being limited or bound by theory, this can introduce a bottleneck in acetyl-carnitine processing in the



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mitochondria which slows acetyl-CoA entry into the TCA cycle. If the mitochondrial carnitine acetyltransferase activity is not sufficient to handle the acetyl carbon flux coming from the peroxisome, then the cytoplasmic acetyl-carnitine concentration should build up and, in effect, acetyl-carnitine is diverted from the TCA cycle. The increased concentration of cytoplasmic acetyl-carnitine can thus be a source of substrate for carnitine acetyltransferase activity in the cytosol which converts the substrate to cytosolic acetyl-CoA for use in target molecule production. The amount and/or activity of mitochondrial carnitine acetyltransferase can be decreased in a number of ways, as described herein. For example, the number of copies of nucleic acid encoding the enzyme in a host cell can be reduced (e.g, an endogenous gene encoding the enzyme can be disrupted or deleted), the transcription of such nucleic acid can be decreased and/or nucleic acid encoding a less active mitochondrial carnitine acetyltransferase enzyme can be introduced into the cell (i.e., replacing the endogenous mitochondrial enzyme with a heterologous enzyme). For example, in one embodiment described herein, a *Candida* cytoplasmic carnitine acetyltransferase (e.g, Yat1) which is less active than an endogenous *Candida* mitochondrial carnitine acetyltransferase (e.g., Cat2) can be recombinantly expressed in a host cell mitochondria by engineering a nucleic acid encoding the less active enzyme such that the encoded enzyme includes a mitochondrial targeting sequence of amino acids (shown as "*CAT2*" in a diagonal line-hatched background in the mitochondria in Fig. 5; see also, e.g., amino acid SEQ ID NOS: 10, 11 and 12 and nucleotide SEQ ID NOS: 67, 68 and 69 encoding such amino acid sequences). The modified nucleic acid can be introduced into a host cell in which the endogenous mitochondrial carnitine acetyltransferase gene has been disrupted or deleted. Although not specifically indicated in Fig. 5, in some cells and organisms (e.g., *Candida* spp), an endogenous gene encoding a mitochondrial carnitine acetyltransferase may also encode the cell's peroxisomal carnitine acetyltransferase. For example, such a gene can encode an enzyme that includes mitochondrial and peroxisomal targeting sequences for localization to each of these areas of the cell. If the gene encoding an endogenous mitochondrial carnitine acetyltransferase in such a cell is disrupted or deleted, it may be optimal (e.g., for cell viability and/or efficient processing of peroxisomal acetyl-CoA) to introduce a heterologous nucleic acid encoding carnitine acetyltransferase that includes a peroxisomal targeting sequence into the cell.

Another method of modifying mitochondrial acetyl-carnitine uptake is by altering a transport mechanism that moves acetyl-carnitine into the mitochondrial matrix, e.g., an acetyl-carnitine translocase. A mitochondrial inner-membrane transport protein (e.g., Crc1p) may function as an

acetyl-carnitine transporter providing for transport of acetyl-carnitine into the mitochondrial matrix. By decreasing the amount and/or activity level of the transport protein, movement of acetyl-carnitine from the cytosol into mitochondria can be reduced thereby increasing the concentration of acetyl-carnitine in the cytosol that can be converted to acetyl-CoA by cytoplasmic carnitine acetyltransferase. The amount and/or activity of mitochondrial acetyl-carnitine transport protein can be decreased in a number of ways, as described herein. For example, the number of copies of nucleic acid encoding a mitochondrial acetyl-carnitine transport protein in a host cell can be reduced (e.g, an endogenous gene encoding the protein can be disrupted or deleted), the transcription of such nucleic acid can be decreased and/or nucleic acid encoding a less active transport protein can be introduced into the cell (e.g., replacing the endogenous mitochondrial transport protein with a heterologous protein). For example, in one embodiment described herein, the transcription of nucleic acid encoding a *Candida* acetyl-carnitine translocase (shown as "CRC1" in a diagonal line-hatched background in Fig. 5) can be reduced in a host cell by introducing such nucleic acid, which is operably linked to a heterologous promoter (e.g., a yeast glucose-6-phosphate isomerase gene promoter such as, for example, SEQ ID NO: 118) that provides for less transcription and/or a reduced transcription rate, and/or that can be regulated to provide for alternately weak and stronger transcription, into host cells in which the endogenous gene has been disrupted. The resulting reduction in transcription of the nucleic acid results in decreased amounts of the transporter protein in the mitochondrial membrane of modified cells. Thus, modification of mitochondrial acetyl-carnitine transporter expression and/or mitochondrial carnitine acetyltransferase activity, as shown in Fig. 5, can serve to divert acetyl-carnitine from use in the TCA cycle and increase the concentration of acetyl-carnitine in the cytosol of cells including one or more of these modifications. This, combined with increased amounts and/or activity of cytosolic carnitine acetyltransferase can result in increased amounts of acetyl-CoA available in the cytosol for use in target molecule production. Although all three of these modifications, i.e., alteration of mitochondrial carnitine acetyltransferase activity (e.g., Cat2), alteration of mitochondrial acetyl-carnitine transporter protein activity (e.g., Crc1) and modification of cytosolic carnitine acetyltransferase activity (e.g., Yat1), are shown in the depiction of an example platform system in Fig. 5, each can be used singly or in any combination in modifying carbon processing in cells or microorganisms for the production of target molecules. For example, modifications of a host cell in generating a platform and/or production system with respect to these three modifications include, but are not limited to: (1) a decreased mitochondrial carnitine acetyltransferase expression and/or activity and an increased cytosolic carnitine acetyltransferase expression



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and/or activity, (2) a decreased mitochondrial acetyl-carnitine transporter protein expression and/or activity and an increased cytosolic carnitine acetyltransferase expression and/or activity, (3) a decreased mitochondrial carnitine acetyltransferase expression and/or activity, a decreased mitochondrial acetyl-carnitine transporter protein expression and/or activity, and an increased cytosolic carnitine acetyltransferase expression and/or activity, (4) a decreased mitochondrial carnitine acetyltransferase expression and/or activity, and a decreased mitochondrial acetyl-carnitine transporter protein expression and/or activity, (5) a decreased mitochondrial carnitine acetyltransferase expression and/or activity, (6) a decreased mitochondrial acetyl-carnitine transporter protein expression and/or activity and (7) an increased cytosolic carnitine acetyltransferase expression and/or activity.

*Capture of carbon from acetate*

In the platform system depicted in Fig. 6, another example of a modification of a host cell that can enhance the capture and diversion of acetyl group carbons that have been generated in β-oxidation is modification of acetyl-CoA processing in peroxisomes. In a cell that does not include such a modification, acetyl-CoA generated during the degradation of fatty acids in β-oxidation typically is converted by peroxisomal carnitine acetyltransferase into acetyl-carnitine for transport into the cytosol and eventually to the mitochondria. The engineered carbon recycling loop of the embodiment of the platform system depicted in Fig. 6 can capture the acetyl group carbons through conversion of peroxisomal acetyl-CoA into acetate. The acetate readily traverses the peroxisomal membrane and can move into the cytosol where it can be reconverted to acetyl-CoA and thus diverted from entry into, and loss to, mitochondrial metabolism.

Modification of peroxisomal acetyl-CoA processing in a host cell can be accomplished, for example, as described herein. Peroxisomal acetyl-CoA can be converted to acetate through hydrolysis catalyzed by acetyl-CoA hydrolase which also liberates coenzyme A for reuse in β-oxidation. A cell or microorganism may be modified to increase (or introduce) acetyl-CoA hydrolase and/or acetyl-CoA hydrolase activity in the peroxisomes. For example, the copy number of nucleic acids encoding a peroxisomal acetyl-CoA hydrolase in the cell can be increased, transcription of such nucleic acids can be increased and/or, if a cell expresses an endogenous peroxisomal acetyl-CoA hydrolase, a nucleic acid encoding a more active hydrolase enzyme can be introduced into the cell (e.g., modifying an endogenous peroxisomal



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hydrolase enzyme activity by replacing it with, or adding to it, a heterologous enzyme activity). For example, in one embodiment described herein, a *Candida* acetyl-CoA hydrolase (e.g, Ach) that is expressed in mitochondria of unmodified cells can be recombinantly expressed in host cell peroxisomes by engineering a nucleic acid encoding the enzyme such that the encoded protein lacks a mitochondrial targeting sequence of amino acids and includes a peroxisomal targeting sequence (shown as *ACH* in the peroxisomal compartment in Fig. 6; see e.g, amino acid SEQ ID NO: 16 and a nucleotide sequence (SEQ ID NO: 73) encoding the amino acid sequence). The amount of acetyl-CoA hydrolase activity in peroxisomes of such a modified cell or organism can be further increased by using a strong and/or fatty acid-inducible heterologous promoter (e.g., a yeast *HDE* gene promoter) to regulate transcription of the engineered nucleic acid encoding an acetyl-CoA hydrolase activity. In order to reduce or eliminate peroxisomal conversion of acetyl-CoA to acetyl-carnitine (shown as "Ac-Carn" in the peroxisome compartment of Fig. 6) so that a maximal amount of the acetyl-CoA is converted to acetate, the host cell can also be modified to decrease or eliminate (e.g., by disrupting or deleting a gene encoding peroxisomal carnitine acetyltransferase) carnitine acetyltransferase activity in the peroxisomes (shown in Fig. 6 as "*cat2Δ*" in a black oval).

Once in the cytoplasm, the acetate can be converted to acetyl-CoA by acetyl-CoA synthetase (shown as "*ACS*" in Fig. 6) which catalyzes the ligation of acetate and coenzyme A to produce acetyl-CoA. To provide optimal processing of the increased cytosolic acetate generated in this embodiment into cytosolic acetyl-CoA, the amount and/or activity of cytosolic acetyl-CoA synthetase can be increased in modified host cells or organisms. For example, the copy number of nucleic acids encoding a cytosolic acetyl-CoA synthetase in the cell can be increased, transcription of such nucleic acids can be increased (e.g., using a heterologous strong and/or fatty acid-inducible promotor, for example, a yeast *HDE* gene promoter) and/or a nucleic acid encoding a more active acetyl-CoA synthetase enzyme can be introduced into the cell (e.g., modifying an endogenous cytosolic acetyl-CoA synthetase enzyme activity by replacing it with, or adding to it, a heterologous enzyme activity).

As described herein, in some host cells or organisms, e.g., some yeast species, such as *Candida*, peroxisomal and mitochondrial carnitine acetyltransferase may be encoded by the same gene which can contain two in-frame start codons. Carbon source-dependent alternate transcription initiation can result in expression of a carnitine acetyltransferase initiated from the first start codon or a shorter carnitine acetyltransferase initiated from the second start codon.



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The longer version of carnitine acetyltransferase encodes an N-terminal mitochondrial targeting signal wherease the shorter version does not. Therefore, in an embodiment in which a peroxisomal carnitine acetyltransferase activity is decreased by disruption or deletion of the corresponding gene in such cells, the mitochondrial carnitine acetyltransferase activity may also be decreased or eliminated. Therefore, because cell survival may require a minimal amount of energy generated through mitochondrial metabolism which in turn requires a supply of acetyl-CoA, a host cell or microorganism for the platform system shown in Fig. 6 can also be modified to express a mitochondrial carnitine acetyl transferase. This can be accomplished by introducing a recombinant nucleic acid encoding a carnitine acetyltransferase that includes a mitochondrial targeting sequence of amino acids into the host cell or microorganism. Because a system such as that shown in Fig. 6 is designed to direct most of the acetyl group carbons from acetyl-CoA generated in β-oxidation to the next segment of the carbon recycling loop (i.e., cytosolic fatty acid synthesis), the recombinant mitochondrial carnitine acetyltransferase can be one with reduced catalytic activity relative to the endogenous mitochondrial carnitine acetyltransferase (e.g., a Yat1 enzyme instead of a Cat2 enzyme). This can serve to minimize the rate at which any acetyl-carnitine that does enter the mitochondria is converted to acetyl-CoA which, in turn, could minimize the amount of carbon loss to mitochondrial metabolism at the expense of the recycling mechanism in this system. Loss of carbon to mitochondrial metabolism could also be minimized (in addition to, or as an alternative to, introducing nucleic acid encoding a less active mitochondrial carnitine acetyltransferase) by decreasing the amount and/or activity level of a mitochondrial acetyl-carnitine translocase (e.g., Crc1) into the system shown in Fig. 6. This can result in slowing of acetyl-carnitine transport into mitochondria and serve to divert some of the cytoplasmic acetyl-carnitine from use in the TCA cycle. Although both of these modifications, i.e., alteration of mitochondrial carnitine acetyltransferase activity (e.g., Cat2) and alteration of mitochondrial acetyl-carnitine transporter protein activity (e.g., Crc1), are shown in the depiction of an example platform system in Fig. 6, each can be used singly or in combination in modifying carbon processing in cells or microorganisms for the production of target molecules.

Additionally, because the only cytoplasmic acetyl-carnitine being generated in the system shown in Fig. 6 is through the carnitine acetyltransferase activity present in the cytosol (e.g., endogenous Yat1p in a *Candida* cell), the amount of acetyl-carnitine available to mitochondria is limited due to competition between the cytosolic carnitine acetyltransferase and acetyl-CoA carboxylase (shown as "ACC1" in Fig. 6) for the acetyl-CoA substrate. In some instances, it

may be optimal to increase the amount and/or activity of carnitine acetyltransferase in the cytosol in a system such as that depicted in Fig. 6 in order to insure sufficient generation of acetylcarnitine for any minimal amount of acetylcarnitine that may be needed for mitochondrial metabolism. This can be accomplished, for example, by increasing the copy number of nucleic acids encoding cytosolic carnitine acetyltransferase in the cell, increasing the transcription of such nucleic acids and/or introducing nucleic acid encoding a more active cytosolic carnitine acetyltransferase enzyme into the cell (e.g., modifying an endogenous cytosolic enzyme activity by replacing it with, or adding to it, a heterologous enzyme activity (e.g., Cat2p)).

*Redirecting carbon flow toward fatty acid biosynthesis*

At this point in an acetyl group carbon recycling loop, such as that illustrated in the systems depicted in Figs. 5 and 6, when acetyl moieties are accumulated in the cytosol as acetyl-CoA, they are primarily directed into the cytosolic fatty acid synthesis pathway to regenerate fatty acids. Cytoplasmic acetyl-CoA can be converted into malonyl-CoA, which can be a carbon donor in the synthesis of a fatty acid chain in repeated cycles of the addition of 2 carbon atoms per cycle to extend the chain and generate a fatty acid. The reactions of each cycle are typically catalyzed by fatty acid synthase (FAS) and generally continue until a 16- or 18-carbon fatty acid (palmitic acid or stearic acid) is completed in the form of palmitoyl-CoA or stearoyl-CoA. To enhance flow of the accumulated cytosolic acetyl-CoA into the fatty acid biosynthesis pathway, the process of converting acetyl-CoA into malonyl-CoA can optionally be modified in host cells or microorganisms. For example, the amount and/or activity of an enzyme that can catalyze the reaction, acetyl-CoA carboxylase, can be modified, e.g., increased, in host cells or microorganisms using genetic engineering methods as described herein (e.g., increasing the copy number and/or transcription of nucleic acid encoding acetyl-CoA carboxylase, increasing the activity of the enzyme by introducing nucleic acid encoding a modified amino acid sequence of the enzyme into host cells). In one embodiment described herein, the activity of a yeast (e.g., *Candida*) cytosolic acetyl-CoA carboxylase (shown as "*ACC1*" in a diagonal line-hatched background oval in Figs. 5 and 6) can be increased through substitution of phosphorylatable serine residues with alternate (e.g., alanine) residues to reduce inhibition of the enzyme by phosphorylation (see, e.g., SEQ ID NO: 19). Heterologous nucleic acid encoding the modified protein can be introduced into a host cell for expression of the enzyme therein. Increasing the amount and/or activity of acetyl-CoA carboxylase can reduce or prevent any limitation on flow of accumulated acetyl-CoA into fatty acid biosynthesis due to insufficient enzyme activity. The



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amount of cytosolic acetyl-CoA carboxylase activity in such a modified cell or organism can also be increased by using a strong and/or fatty acid-inducible heterologous promoter (e.g., a yeast *HDE* gene promoter) to regulate transcription of the engineered nucleic acid encoding an acetyl-CoA carboxylase activity.

Additional optional modifications of cells or organisms to enhance the platform systems shown in Figs. 5 and 6 include increasing the amount, activity, and/or altering the specificity, of enzymes in the fatty acid synthesis (FAS) enzyme complex, shown as "FAS" in Figs. 5 and 6 (e.g., the enzyme activities of the FAS1 and FAS2 subunits of yeast). A fatty acid synthase (e.g., FAS) activity can catalyze a series of decarboxylative Claisen condensation reactions from acetyl-CoA and malonyl-CoA. Without being limited or bound by any theory, it is believed that following each round of elongation the beta keto group is reduced to the fully saturated carbon chain by the sequential action of a ketoreductase activity, a dehydratase activity, and an enol reductase activity. In the case of Type I FAS enzymes, the growing fatty acid chain typically is carried between these active sites while attached covalently to the phosphopantetheine prosthetic group of an acyl carrier protein (ACP), and can be released by the action of a thioesterase (TE) upon reaching a carbon chain length of, for example, 16 (e.g., palmitic acid). In some instances, the collection of activities is found in a multifunctional, multi-subunit protein complex (e.g., Type I FAS activity). A fatty acid synthase enzyme (FAS) can be coded by fatty acid synthase subunit alpha (FAS2) and fatty acid synthase subunit beta (FAS1) genes. Thus, a fatty acid synthase activity usually includes a collection of activities (e.g., an enzymatic system) that perform functions associated with the synthesis of fatty acids. Therefore, the terms "fatty acid synthase activity", "fatty acid synthase", "FAS", and "FAS activity", as used herein refer to a collection of activities, or an enzymatic system, that perform functions associated with the synthesis of fatty acids. Fatty acid synthase activity may be amplified by over-expression of the FAS2 and/or FAS1 genes by any suitable method. Non-limiting examples of methods suitable to amplify or over express FAS2 and FAS1 genes include amplifying the number of FAS2 and/or FAS1 genes in a host cell following transformation with a high-copy number plasmid (e.g., such as one containing a 2u origin of replication), integration of multiple copies of FAS2 and/or FAS1 genes into the host genome, over-expression of the FAS2 and/or FAS1 genes directed by a strong and/or fatty acid-inducible promoter, the like or combinations thereof. Examples of polynucleotides from *Candida* strain ATCC 20336 that encode fatty acid synthase molecules (FAS1, FAS2) are provided herein (SEQ ID NOS: 102



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and 103) and are also described in International patent application no. PCT/US2012/056562 (publication no. WO 2012/056562).

*Redirecting synthesized fatty acids toward ω- and β-oxidation*

A typical product of cytosolic fatty acid synthesis is an acyl-CoA (e.g., palmitoyl- or stearoyl-CoA), which is shown as "FA-CoA" in Figs. 5 and 6. Because this is an activated (i.e., thioester) form of a fatty acid, it can be used in cellular metabolic pathways (e.g., synthesis of triacylglycerides and phospholipids) other than desired engineered target molecule production processes. This represents a loss of the carbon atoms in the acyl-CoA which could have been incorporated into target products. To reduce loss of the cytosolic acetyl group carbons (now in the form of an acyl-CoA) captured in the recycling loop of the exemplary platform systems shown in Figs. 5 and 6, the final segment of the loop often includes an engineered cytosolic thioesterase enzyme to, in effect, "deactivate" the fatty acid-CoA through hydrolysis and removal of coenzyme A. This can divert the carbons in the acyl-CoA from use in cellular processes not involved in target molecule production and/or generation of cytosolic acetyl-CoA and can complete the recycling loop by generating a cytosolic free fatty acid that can then begin the loop pathway at the initial point of ω-oxidation. Many cells (e.g., some eukaryotic cells, such as yeast) do not have a cytosolic thioesterase activity that is active on long-chain acyl-CoA substrates. Host cells that express an endogenous cytosolic thioesterase activity may also benefit from increasing the activity to enhance the flow of the acyl-CoA carbons through the final segment of the recycling loop. In the embodiment of the platform system shown in Fig.5, a host cell can be modified to increase (in this case by introducing) a thioesterase activity in the cytosol in order to direct acyl-CoA carbon flux toward oxidative metabolism pathways (ω- and β-oxidation). This can be achieved using genetic engineering methods as described herein (e.g., increasing the copy number and/or transcription of nucleic acid encoding a thioesterase, increasing the activity of the enzyme by introducing nucleic acid encoding a modified amino acid sequence of the enzyme into host cells). For example, in one embodiment described herein, a *Candida* peroxisomal thioesterase (e.g, Tes) can be recombinantly expressed cytosolically in a host cell by engineering a nucleic acid encoding the enzyme such that the encoded enzyme lacks a peroxisomal targeting sequence of amino acids (shown, as $TES3^{cyt}$ in a diagonal line-hatched background oval in Figs. 5 and 6; see also, e.g, nucleotide SEQ ID NO: 88 and encoded amino acid SEQ ID NO: 32). Some organisms, such as some yeast (e.g., *Candida*), for example, may express several distinct thioesterases (e.g., *Candida viswanathii* has 8

peroxisomal thioesterase genes) having varied activities. The activities of thioesterases encoded by different genes can be evaluated, using methods known in the art and/or described herein, to compare the enzymes and select the type and level of activity that is optimal for achieving conversion of cytosolic acyl-CoA to free fatty acid in a recycle loop such as the one depicted in Figs. 5 and 6.

Another modification that can optionally be included in platform systems, such as those depicted in Figs. 5 and 6, is a decrease in the amount and/or activity of, or elimination of, mechanisms for the transport of acyl-CoA across the peroxisomal membrane and into peroxisomes. This modification can be beneficial in embodiments in which a target molecule (or precursor or intermediate in the production of a target molecule) is a dicarboxylic acid. For example, the platform systems shown in Figs. 5 and 6 can be used as a modified cell or microorganism for the enhanced production of dicarboxylic acids via ω- and β-oxidation of a fatty acid or alkane carbon source. Feedstock fatty acid (or alkane) carbon atoms that would have been lost as acetyl-CoA formed during β-oxidation degradation of a long-chain diacid (that had been generated by ω-oxidation) in an unmodified cell are captured and used in the generation of additional target diacid molecules (e.g., adipic acid) through the engineered recycling loops in these diacid production systems. Thus, a recycling loop such as the one depicted in Fig. 5 or Fig. 6 can provide for enhanced, highly efficient fatty acid production by significantly reducing "waste" of feedstock carbons in other cellular processes not involved in target molecule production. When the target molecule is a diacid, the carbon atoms recycled through the cytosolic fatty acid synthesis segment of the loop can optimally be directed through the final loop segment of conversion of the synthesized acyl-CoA into free fatty acid so that the carbon atoms can be used again in generating more diacid target through ω- and β-oxidation. Therefore, it can be beneficial to reduce or eliminate any transport of the synthesized acyl-CoA across the peroxisomal membrane and into peroxisomes where it would directly enter into β-oxidation without first being converted to a diacid through ω-oxidation. This can be achieved through disruption or deletion of genes encoding peroxisomal acyl-CoA transporters. In one embodiment described herein, a yeast (e.g., *Candida*) peroxisomal transport protein (e.g, Pxa1) activity is decreased or eliminated by disrupting the gene encoding the protein in the host cell (e.g., shown as "pxa1Δ" in a black background oval in Figs. 5 and 6).



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*Engineered pathways for capturing carbon atoms from the TCA cycle*

Fig. 7 depicts possible cellular modifications in an exemplary embodiment of a eukaryotic (i.e., yeast in this example) platform system designed to capture carbon atoms in the cytosol by enhancing carbon flow through cellular oxidative metabolism pathways (ω-oxidation and peroxisomal β-oxidation) and reducing flow of carbon into the endoplasmic reticulum and lipid particles (in the form of acyl-CoA). Thus, the amount of carbon that is lost to lipid-generating (e.g., glycerol-3-phosphate (G3P) and/or dihydroxyacetone phosphate (DHAP)) pathways at the expense of target molecule production can be reduced in this platform system. Although multiple, possible, cellular modifications are illustrated in Fig. 7, as described herein, some of the modifications depicted in the figure are optional enhancements of an exemplary engineered system and may or may not be included in a modified cell or organism depending on, for example, the intended use of the system (e.g., development of a particular single, or multiple, target molecule(s) production system) and the selection of variable features (e.g., host cell or organism, carbon source, regulatory controls (such as transcription control elements), culture conditions and the like) of the system. Thus, it is understood that any optional modifications set forth in the exemplary system shown in Fig. 7 are non-limiting and may or may not be included in a particular engineered system and, if included, may be in utilized in different combinations than illustrated in the figure.

In the system shown in Fig. 7, as in the embodiments of the platform systems depicted in Figs. 5 and 6, carbon processing activities can be engineered to enhance carbon flow through cellular oxidative metabolism pathways, e.g., ω-oxidation and peroxisomal β-oxidation. However, unlike the embodiments depicted in Figs. 5 and 6, carbon flow from the peroxisomes to the mitochondria is usually not decreased in the platform system shown in Fig. 7. Instead, a portion of the acetyl carbon that is allowed to be utilized in mitochondrial metabolism in this embodiment can be captured from citrate that moves out of the mitochondria and accumulates in the cytosol, particularly in conditions of low nitrogen and high carbon. In the cytosol, the citrate can be converted to oxaloacetate in a reaction which releases acetyl-CoA that, as in the embodiments depicted in Figs. 5 and 6, can be directed into cytosolic fatty acid synthesis. Thus, this platform system can also include a form of an acetyl group carbon recycling loop that can capture acetyl moieties in the cytosol after they have participated in the TCA cycle in mitochondria instead of before they enter mitochondria as acetyl-carnitine. The carbon recycle loop is depicted in Fig. 7 by the dark, solid reaction arrows beginning with extracellular fatty acid



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internalization in the upper left corner of the figure. Starting with carbon internalization, the initial segments of the loop (i.e., carbon processing in ω-oxidation and β-oxidation yielding acetyl-CoA that moves out of the peroxisome and into the cytosol as acetyl-carnitine) are the same as those of the recycling loop shown in Fig. 5 until the point of the processing of cytosolic acetyl-carnitine. In the platform system shown in Fig. 7, acetyl group carbon can continue to flow unimpeded into mitochondria, typically in the form of acetyl-carnitine, and can be converted to acetyl-CoA, utilized in the TCA cycle, and then can be regenerated as acetyl-CoA in the cytosol from a TCA cycle intermediate, citrate ("Cit" in Fig. 7). From that point on, the processing of carbon in the recycling loop is typically similar to that of cytosolic acetyl-CoA in the systems shown in Figs. 5 and 6. The acetyl-CoA can enter the final segments of the loop with the acetyl carbons being utilized in fatty acid synthesis to generate acyl-CoA which is then hydrolyzed to free fatty acid for re-entry into another cycle of the loop at the starting point of ω-oxidation.

Many of the exemplary host cell or organism modifications in the platform system shown in Fig. 7 are the same as those in the platform system shown in Fig. 5, e.g., decreased or eliminated cytosolic acyl-CoA synthetase activity, increased amount and/or activity of cytosolic acetyl-CoA carboxylase and cytosolic thioesterase. Additionally, modifications to enhance ω- and β-oxidation pathway activities and peroxisome proliferation, as well as to reduce transport of acyl-CoA into peroxisomes, can optionally be included in the engineering of a platform system shown in Fig. 7 in a host cell or organism. If a host cell or organism being modified to create a system shown in Fig. 7 does not express an endogenous cytosolic ATP citrate lyase activity (shown as "ACL1/2" in Fig. 7) to catalyze the conversion of citrate to oxaloacetate thereby releasing acetyl-CoA, it can be geneticially modified to generate the enzyme activity. For example, one or more copies of a heterologous nucleic acid encoding an ATP citrate lyase operably linked to a suitable promoter element (e.g., a strong and/or fatty acid-inducible promoter such as a yeast *HDE* gene promoter) can be introduced into the cell or organism for recombinant expression of the activity. If a host cell or organism (for example, an oleaginous yeast) does express an endogenous cytosolic ATP citrate lyase activity, the activity can be increased using genetic engineering methods as described herein (e.g., increasing the copy number and/or transcription of nucleic acid encoding an ATP citrate lyase, increasing the activity of the enzyme by introducing nucleic acid encoding a modified amino acid sequence of the enzyme into host cells).



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Capturing a portion of the acetyl-carbon that is utilized in mitochondrial metabolism in the form of citrate that moves out of the mitochondria and accumulates in the cytosol is a modification that can also be incorporated into the platform systems shown in Figs. 5 and 6. For example, one or more copies of a heterologous nucleic acid encoding an ATP citrate lyase operably linked to a suitable promoter element can be introduced into the cell or organism modified as shown in Fig. 5 or Fig. 6 for expression of ATP citrate lyase activity in the cytosol and conversion of cytosolic citrate to oxaloacetate with concommitant release of acetyl-CoA. The released acetyl-CoA can then be utilized in the cytosolic fatty acid synthesis segment of the carbon recycle loop along with acetyl-CoA generated from acetate (as shown in Fig. 6) or from acetyl-carnitine (as shown in Fig. 5).

*Engineered pathways for producing fatty acids*

An example of a target molecule that can be produced using the modified cells or organisms and methods provided herein is a fatty acid. Examples of enhanced fatty acid-producing cell- or microbial-based systems provided herein include the systems depicted in Figs. 5, 6 and 7. The fatty acid target molecule generation pathways in these examples center on a modified oxidative metabolism (ω- and β-oxidation) pathway through which a longer-chain hydrocarbon feedstock carbon source (e.g., fatty acids and/or alkanes) can be degraded to yield a shorter-chain target fatty acid molecule. In a typical unmodified cell or organism, the β-oxidation cycle releases two carbon atoms in the form of acetyl-CoA generated during chain shortening which, in eukaryotic cells, can then be used in other metabolic processes, including, for example, the TCA cycle after moving from the peroxisome to the mitochondria. In engineered cell- or organism-based production systems such as shown in Figs. 5, 6 and 7, acetyl group carbons released during β-oxidation can be captured in the cytoplasm (e.g., as acetyl-carnitine, acetate and/or citrate) and converted back to acetyl-CoA so that they can be used for the synthesis of longer-chain fatty acids in the cytoplasm and eventually be incorporated into shorter-chain fatty acid target molecule product through, for example, modified oxidative metabolism. These high-efficiency systems can enhance fatty acid target molecule production by including an engineered carbon recycling loop that can increase the efficiency of fatty acid production and can provide greater target molecule yields by decreasing loss of feedstock carbons to cellular energy-generating and/or growth processes. This can be accomplished through rerouting carbon that would have been lost as acetyl-CoA during β-oxidation degradation back into the target molecule fatty acid product.



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For example, one target fatty acid molecule that can be produced using cells or organisms modified to include a carbon-capturing recycling loop such as those shown in Figs. 5, 6 and 7, is adipic acid, which is a 6-carbon dicarboxylic acid. A cell or microorganism that has been modified as shown in any of Figs. 5, 6 or 7, when provided with a fatty acid carbon source (e.g., a typical 18-carbon vegetable-oil fatty acid), can metabolize the fatty acid first through ω-oxidation, in which it can be converted to a diacid containing the same number of carbons as the fatty acid carbon source, e.g., 18 carbons, and can then metabolize the diacid through β-oxidation. The products of the first cycle of β-oxidation typically are a 16-carbon diacid and a 2-carbon acetyl-CoA molecule. The products of a second cycle of β-oxidation starting with the 16-carbon diacid are typically a 14-carbon diacid and another 2-carbon acetyl-CoA molecule. After four more cycles of β-oxidation, the 6-carbon adipic acid target molecule can be produced, in which case 6 molecules of acetyl-CoA (a total of 12 carbon atoms) will have been released into the peroxisome. In a cell- or microbial-based production system that does not include an engineered carbon recycling pathway such as those shown in Figs. 5, 6 and 7, the 12 carbon atoms released during β-oxidation are usually not used in generating 2 more 6-carbon adipic acid target molecule products but would be "lost" to cellular metabolism pathways involved in energy generation and growth. Thus, only one-third of the source carbons would be used in product generation whereas two-thirds of the source carbons would be lost to processes that do not require all of the lost carbon in order for the cell or microbe to survive. However, cells and microbes modified as described herein to alter cellular carbon flux can capture more of the source carbon atoms and use them in generating more product. Accordingly, the modified cells and microorganisms provided herein as target molecule production systems can be significantly more efficient and can provide a greater product yield from a given amount of feedstock than cell- or microbial-based systems that have not been modified for enhanced production.

*Engineered malonyl-CoA-producing pathways in a platform target molecule production system*

Figure 8 depicts possible cellular modifications in some embodiments of a eukaryotic (e.g., yeast in this example) platform system for the enhanced production of malonyl-CoA. Malonyl-CoA is a versatile precursor molecule in the synthesis of many industrially valuable molecules. Accordingly, a system such as that depicted in Fig. 8 can be used as a platform for incorporating pathways for target molecule production. Although multiple, possible, cellular



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modifications are illustrated in Fig. 8, as described herein, some of the modifications depicted in the figure are optional enhancements of an exemplary engineered system and may or may not be included in a modified cell or organism depending on, for example, the intended use of the system (e.g., development of a particular single, or multiple, target molecule(s) production system) and the selection of variable features (e.g., host cell or organism, carbon source, regulatory controls (such as transcription control elements), culture conditions and the like) of the system. Thus, it is understood that any optional modifications set forth in the exemplary system shown in Fig. 8 are non-limiting and may or may not be included in a particular engineered system and, if included, may be in utilized in different combinations than illustrated in the figure.

The cell-based platform system shown in Fig. 8 incorporates carbon flux modifications designed to capture carbon atoms as acetyl-CoA, the precursor to malonyl-CoA, in the cytosol by enhancing carbon flow through cellular oxidative metabolism pathways (ω-oxidation and peroxisomal β-oxidation) and reducing flow of carbon into mitochondria. Thus, the amount of carbon that is lost to the TCA cycle at the expense of malonyl-CoA production can be reduced in this platform system. At the core of this platform system are acetyl group diversion elements that impede the flow of acetyl group carbons into mitochondria and enhance flow of the carbons into generation of malonyl-CoA. These elements can optionally be combined with a carbon recycle loop that can capture any cytosolic acetyl moieties that are not funneled into the target molecule production pathway. Such cytosolic acetyl moieties can be captured in the fatty acid biosynthesis segment of the carbon recycle loop and can be used to regenerate an acyl-CoA that can be diverted from lipid synthesis and converted into a fatty acid that can be subjected to another cycle of peroxisomal β-oxidation (depicted as a dashed line extending from malonyl-CoA to FA-CoA in Fig. 8).

As set forth in connection with the description of the platform system shown in Fig. 5, carbon flux alteration in the system shown in Fig. 8 can begin with modifications, e.g., decreased acyl-CoA synthetase (Faa1 and/or Fat1) activity, that enhance carbon flow usually in the form of a fatty acid (from, for example, a fatty acid carbon source or derived from an alkane source) into ω- and β-oxidation pathways in the cell or organism. The oxidative metabolism aspect of the platform system can be further enhanced through optional modification of ω- and/or β-oxidation activity as described herein. For example, with respect to the ω-oxidation pathway, one or more enzymes (e.g., monooxygenase, cytochrome P450 reductase, such as CPRB, and others) of



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the pathway can be modified (e.g., as described herein) to increase catalytic activity and/or alter substrate specificity in order to increase fatty acid processing in the pathway and/or to target specific fatty acids for processing into dicarboxylic acids. If a host cell or organism does not express an endogenous ω-oxidation pathway, it can be genetically modified to express heterologous enzymes to engineer an ω-oxidation pathway in the cell or organism. Optional enhancements of the β-oxidation activity can include, for example, modification of one or more enzymes (e.g., acyl-CoA oxidase, ketoacyl-CoA thiolase, multifunctional enzyme hydratase and/or dehydrogenase, and others) of the pathway (e.g., as described herein) to increase catalytic activity and/or alter substrate specificity in order to increase fatty acid processing in the pathway and/or to target specific fatty diacids for processing into target dicarboxylic acids (in an instance in which the system shown in Fig. 8 could be used as a multiple target molecule production system, i.e., a "co-production" system). One example of a modification of β-oxidation activity, as described herein, is alteration of the substrate specificity of one or more acyl-CoA oxidase enzymes in the pathway, such as Pox4 and/or Pox5 of *Candida* yeast strains. Additional modifications that can provide for enhanced carbon flux through β-oxidation (and enhanced target molecule production) in these systems include, but are not limited to, modification of β-oxidation-associated activities, such as peroxisome biogenesis and proliferation activities. For example, as described herein, the abundance and/or volume of peroxisomes in which β-oxidation occurs can be increased in host cells through genetic modification. An example of such a modification is increasing the transcription of, and/or number of copies of, one or more peroxin-encoding nucleic acids (e.g., *PEX11*) in a host cell. Amplification of such peroxin-encoding nucleic acids and/or activities leads to an overall increased β-oxidation capacity.

Carbon capture modifications of this platform system, as in the system depicted in Fig. 5, occurring in the processing of the acetyl-CoA generated during β-oxidation can include diversion elements that can impede the flow of acetyl group carbons into mitochondria (e.g., decreasing mitochondrial carnitine transporters and carnitine acetyltransferase activities) and enhance flow of the carbons into generation of malonyl-CoA (e.g., increasing cytosolic carnitine acetyltransferase and acetyl-CoA carboxylase activities). Modifications in the host cell or organism that can enhance the capture and diversion of acetyl-CoA in the cytosol include, but are not limited to, modification of acetyl-carnitine entry into mitochondria and modification of conversion of cytosolic acetyl-carnitine to acetyl-CoA. As described herein, one modification that can be made to enhance capture of acetyl groups in the cytosol is to increase the amount



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and/or activity of cytosolic carnitine acetyltransferase which converts acetyl-carnitine into acetyl-CoA, a thioester form that cannot move into the mitochondrial matrix. Methods of achieving this include increasing the copy number of nucleic acids encoding cytosolic carnitine acetyltransferase in the cell, increasing the transcription of such nucleic acids and/or introducing nucleic acid encoding a more active cytosolic carnitine acetyltransferase enzyme into the cell (e.g., modifying an endogenous cytosolic enzyme activity by replacing it with, or adding to it, a heterologous enzyme activity; see, e.g., "*CAT2*$^{cyt}$" in Fig. 8). Another modification that can enhance cytosolic capture and diversion of acetyl moieties in cells is an alteration of acetyl-carnitine uptake into mitochondria from the cytosol (depicted as faded, dotted reaction arrow lines into and in the mitochondrial compartment in Fig. 8). One method of modifying mitochondrial acetyl-carnitine uptake can be altering the processing of acetyl-carnitine that occurs in the mitochondria to convert it to acetyl-CoA for use in the TCA cycle. For example, by decreasing the activity level of an enzyme that catalyzes this processing, e.g., mitochondrial carnitine acetyltransferase, there can be a corresponding decrease in conversion of acetyl-carnitine to acetyl-CoA in the mitochondria. Without being limited or bound by theory, if the mitochondrial carnitine acetyltransferase activity is not sufficient to efficiently process the acetyl carbon flux coming from the peroxisome, then the cytoplasmic acetyl-carnitine concentration should build up and, in effect, acetyl-carnitine can be diverted from the TCA cycle. The increased concentration of cytoplasmic acetyl-carnitine thus can be a source of substrate for carnitine acetyltransferase activity in the cytosol which can convert the substrate to cytosolic acetyl-CoA for use in target molecule production. The amount and/or activity of mitochondrial carnitine acetyltransferase can be decreased in a number of ways, as described herein. For example, the number of copies of nucleic acid encoding the enzyme in a host cell can be reduced, the transcription of such nucleic acid can be decreased and/or nucleic acid encoding a less active mitochondrial carnitine acetyltransferase enzyme can be introduced into the cell (e.g., replacing the endogenous mitochondrial enzyme with a heterologous enzyme; see, e.g., "*CAT2*" in a diagonal line-hatched background in the mitochondria in Fig. 8).

As also described with reference to Fig. 5, another method of modifying mitochondrial acetyl-carnitine uptake can be altering the transport mechanism that moves acetyl-carnitine into the mitochondrial matrix, e.g., an acetyl-carnitine translocase. By decreasing the amount and/or activity level of the transport protein, movement of acetyl-carnitine from the cytosol into mitochondria can be slowed and/or reduced thereby increasing the concentration of acetyl-carnitine in the cytosol that can be converted to acetyl-CoA by cytoplasmic carnitine



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acetyltransferase. The amount and/or activity of mitochondrial acetyl-carnitine transport protein can be decreased, for example, by reducing the number of copies of nucleic acid encoding a mitochondrial acetyl-carnitine transport protein in a host cell, reducing the transcription of such nucleic acid and/or introducing nucleic acid encoding a less active transport protein into the cell (e.g., replacing the endogenous mitochondrial transport protein with a heterologous protein). For example, in one embodiment described herein, the transcription of nucleic acid encoding a *Candida* acetyl-carnitine translocase (shown as "CRC1" in a diagonal line-hatched background in Fig. 8) can be reduced in a host cell by introducing such nucleic acid, which is operably linked to a heterologous promoter that provides for less transcription and/or a reduced transcription rate, and/or that can be regulated to provide for alternately weak and stronger transcription, into host cells in which the endogenous gene has been disrupted. Thus, modification of mitochondrial acetyl-carnitine transporter expression and/or mitochondrial carnitine acetyltransferase activity, as shown in Fig. 8, can serve to divert acetyl-carnitine from use in the TCA cycle and increase the concentration of acetyl-carnitine in the cytosol of cells that include one or more these modifications. This, combined with increased amounts and/or activity of cytosolic carnitine acetyltransferase, can result in increased amounts of acetyl-CoA available in the cytosol for use in target molecule production. As also described with reference to Fig. 5, although all three of these modifications, i.e., alteration of mitochondrial carnitine acetyltransferase activity (e.g., Cat2), alteration of mitochondrial acetyl-carnitine transporter protein activity (e.g., Crc1) and modification of cytosolic carnitine acetyltransferase activity (e.g., Yat1), are shown in the depiction of a platform system in Fig. 8, each can be used singly or in any combination in modifying carbon processing in cells or microorganisms for the production of malonyl-CoA and/or other target molecules.

At this point in the platform pathway system depicted in Fig. 8, when acetyl moieties are accumulated in the cytosol as acetyl-CoA, they can be directed into generation of malonyl-CoA. To enhance flow of the accumulated cytosolic acetyl-CoA toward malonyl-CoA generation, the process of converting acetyl-CoA into malonyl-CoA can be modified in host cells or microorganisms. For example, the amount and/or activity of an enzyme that catalyzes the reaction, acetyl-CoA carboxylase, can be modified, e.g., increased, in host cells or organisms using genetic engineering methods as described herein (e.g., increasing the copy number and/or transcription of nucleic acid encoding acetyl-CoA carboxylase, increasing the activity of the enzyme by introducing nucleic acid encoding a modified amino acid sequence of the enzyme into host cells). In one embodiment described herein, the activity of a yeast (e.g.,



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*Candida*) cytosolic acetyl-CoA carboxylase (shown as "*ACC1*" in a diagonal line-hatched background oval in Fig. 8) can be increased through substitution of phosphorylatable serine residues with alternate (e.g., alanine) residues to reduce inhibition of the enzyme by phosphorylation. Heterologous nucleic acid encoding the modified protein can be introduced into a host cell for expression of the enzyme therein. Increasing the amount and/or activity of acetyl-CoA carboxylase can reduce or prevent any limitation on flow of accumulated acetyl-CoA into malonyl-CoA generation.

The system depicted in Fig. 8 serves as a platform that can be used in multiple ways. For example, as shown, the pathway can serve as an enhanced, high-efficiency malonyl-CoA production system that can be further modified for use in generating a target molecule in an engineered pathway that initiates with a malonyl-CoA precursor. Examples of this are provided herein in which cells or organisms modified to contain the platform system are genetically modified to express pathways for the production of 3-hydroxypropionic acid or triacetic acid lactone from malonyl-CoA. Additionally, the system depicted in Fig. 8 can be used in the co-production of a fatty dicarboxlic acid, e.g., adipic acid, and a target molecule generated using malonyl-CoA as a precursor in the synthesis pathway.

Cells or microorganisms that have been modified to incorporate the biological platform system depicted in Fig. 8 can be further modified depending on the purpose(s) for which the system is being used. For example, if the system is being used solely for the production of a target molecule synthesized from a malonyl-CoA precursor, then it may be beneficial to modify the cell or microorganism to decrease cytosolic fatty acid synthesis. A committed step in fatty acid biosynthesis is the conversion of cytoplasmic acetyl-CoA into malonyl-CoA. Malonyl-CoA can serve as a carbon donor in the synthesis of a fatty acid chain in repeated cycles of the addition of 2 carbon atoms per cycle to extend the chain and generate a fatty acid. The fatty acid synthesis (FAS) pathway in host cells or organisms can compete with any pathways for target molecule synthesis for the malonyl-CoA substrate produced in this platform system. Therefore, to enhance malonyl-CoA carbon atom flux toward target molecule synthesis and direct carbon flux away from fatty acid biosynthesis, a host cell or organism can be modified to alter FAS enzyme activities. Non-limiting examples of methods suitable to decrease the amount and/or activity of enzymes involved in fatty acid synthesis, e.g., FAS2 and FAS1 enzymes, include decreasing the number of nucleic acids encoding fatty acid synthase enzymes, such as FAS2 and/or FAS1, in a host cell (e.g., disruption or deletion of one or more genes) and reducing the



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transcription of nucleic acids encoding fatty acid synthase enzymes, e.g., FAS2 and/or FAS1 genes, by replacing an endogenous promoter of one or more genes with a weak and/or constitutive promoter, the like or combinations thereof. Examples of polynucleotides from *Candida* strain ATCC 20336 that encode fatty acid synthase molecules (FAS1, FAS2) are provided herein and are also described in International patent application no. PCT/US2012/056562 (publication no. WO 2012/056562).

If the platform system depicted in Fig. 8 is being used for co-production of a fatty dicarboxylic acid and a target molecule derived from malonyl-CoA, then it may be beneficial to either make no modifications of the FAS pathway activities or modify the host cell or organism to increase the amount, activity, and/or alter the specificity, of enzymes in the fatty acid synthesis (FAS) enzyme complex, shown as "FAS" in Fig. 8 (e.g., the enzyme activities of the FAS1 and FAS2 subunits of yeast). Examples of methods of altering fatty acid synthesis through modification of the amount and/or activity of fatty acid synthase enzymes (e.g., increasing cellular copy number of nucleic acids encoding one or more enzymes, increasing transcription of nucleic acids encoding one or more enzymes) are described herein. A system such as that shown in Fig. 8 being used for co-production of a fatty dicarboxylic acid and a target molecule derived from malonyl-CoA, could include modifications, such as those described herein, of one or more activities of the ω- and/or β-oxidation pathways to enhance production of a desired dicarboxylic acid as described herein (e.g., modification of acyl-CoA oxidases, such as Pox4 and Pox5, monooxygenase, cytochrome P450 reductase). Such a co-production system would be depicted in Fig. 8 as including another arrow extending from a dicarboxylic acid (DCA-CoA) in the peroxisome to an acyl-CoA and then to a chain-shortened diacid (as a result of β-oxidation, "β-Ox") as is shown in the systems depicted in Figs. 5 and 6.

Additionally, the efficiency of the system for co-production of a dicarboxylic acid may optionally be enhanced by modifying the host cell or organism to introduce the final segment of the carbon recycling loop as described with reference to Fig. 5 (and shown at the top of Fig. 8 as a solid reaction arrow extending from "FA-CoA" to "FA" and including the "*TES*$^{cyt}$" thioesterase enzyme as catalyst). In platform systems depicted in Fig. 8 that include a functional FAS pathway, the final product of the cytosolic fatty acid synthesis is typically an acyl-CoA (e.g., palmitoyl-CoA), which is shown as "FA-CoA" in Fig. 8. To avoid loss of the carbon atoms in the acyl-CoA lipid synthesis pathways, and complete the carbon recycling loop, the host cell or organism can be modified to include an engineered cytosolic thioesterase enzyme to, in effect, "deactivate" the



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fatty acid-CoA through hydrolysis and removal of coenzyme A. This can divert the carbons in the acyl-CoA from use in cellular processes not involved in target molecule production and/or generation of cytosolic acetyl-CoA and can complete the recycling loop by generating a cytosolic free fatty acid that can then begin a new cycle of the loop pathway at the initial point of ω-oxidation. A host cell can be modified to increase (or introduce) a thioesterase activity in the cytosol in order to direct acyl-CoA carbon flux toward oxidative metabolism pathways (ω- and β-oxidation). This can be achieved using genetic engineering methods as described herein (e.g., increasing the copy number and/or transcription of nucleic acid encoding a thioesterase, increasing the activity of the enzyme by introducing nucleic acid encoding a modified amino acid sequence of the enzyme into host cells). For example, in one embodiment described herein, a *Candida* peroxisomal thioesterase (e.g, Tes) can be recombinantly expressed cytosolically in a host cell (shown as $TES3^{cyt}$ in a diagonal line-hatched background oval in Fig. 8).

Another modification that can optionally be included in, but that is not required for, platform systems, such as those depicted in Fig. 8, is a decrease in the amount and/or activity of, or elimination of, mechanisms for the transport of acyl-CoA across the peroxisomal membrane and into peroxisomes. This modification can be beneficial in embodiments in which one of the target molecules (or precursor or intermediate in the production of a target molecule) is a dicarboxylic acid. This is because if an acyl-CoA generated from fatty acid synthesis can move across the peroxisomal membrane and into peroxisomes, it could directly enter into β-oxidation without first being converted to a diacid through ω-oxidation. A reduction in the transfer of acyl-CoA into peroxisomes can be achieved through disruption or deletion of genes encoding peroxisomal acyl-CoA transporters. In one embodiment described herein, a yeast (e.g., *Candida*) peroxisomal transport protein (e.g, Pxa1) activity can be decreased or eliminated by disrupting the gene encoding the protein in the host cell (e.g., shown as "pxa1Δ" in a black background oval in Fig. 8).

*Engineered pathways for producing 3-hydroxypropionic acid*

Modified cells, organisms, compositions and methods provided herein can also be used for enhanced production of other organic acids, such as, for example, 3-hydroxypropionic acid (3HP). An example of an engineered production pathway for cell- or microbial-based synthesis of 3HP is shown in Fig. 9. This biochemical pathway is based in a synthetic method using malonyl-CoA as a precursor molecule and incorporates elements as described for the platform



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system depicted in Fig. 8. As shown in Fig. 9, 3HP can be produced from malonyl-CoA in a 2-step reduction process. In the first step, malonyl-CoA ("Mal-CoA" in Fig. 9) can be reduced to malonate semialdehyde (MSA) in a reaction catalyzed by malonyl-CoA reductase (e.g., EC 1.2.1.75 ; "MCR" in Fig. 9). Malonate semialdehyde can be further reduced to 3HP in a reaction catalyzed by 3-hydroxy-propionate-dehydrogenase (e.g., EC 1.1.1.59; "HPD1" in Fig. 9). If a host cell or organism being modified to create a system shown in Fig. 9 does not express an endogenous cytosolic malonyl-CoA reductase or 3-hydroxy-propionate-dehydrogenase activity, it can be geneticially modified to generate one or both of the enzyme activities. For example, one or more copies of a heterologous nucleic acid encoding an Mcr (e.g., nucleotide SEQ ID NO: 323 or any nucleotide sequence encoding amino acid SEQ ID NO: 322) or Hpd1 (e.g., nucleotide SEQ ID NO: 104 or any nucleotide sequence encoding amino acid SEQ ID NO: 48) operably linked to a suitable promoter element (e.g., a strong and/or fatty acid-inducible promoter such as a yeast *HDE* gene promoter) can be introduced into the cell or organism for recombinant expression of the activity. If a host cell or organism does express an endogenous cytosolic malonyl-CoA reductase and/or 3-hydroxy-propionate-dehydrogenase activity, the activity may be increased using genetic engineering methods as described herein (e.g., increasing the copy number and/or transcription of nucleic acid encoding an Mcr or Hpd1, increasing the activity of the enzyme by introducing nucleic acid encoding a modified amino acid sequence of the enzyme into host cells). Nucleic acid encoding an MCR activity can be obtained, for example, from bacteria, including, but not limited to, *Sulfolobus islandicus* (see, e.g, nucleic acid SEQ ID NO: 323 and encoded amino acid SEQ ID NO:322) *Sulfolobus tokodaii* (nucleotide sequence: EMBL-EBI accession no. BAB67276.2) strain 7 (DSMZ 16693; available from the Leibniz Institute DSMZ-German Collection of Microorganisms and cell lines). An example of a *Candida viswanathii* nucleotide sequence (SEQ ID NO:104) encoding an Hpd1 protein (amino acid SEQ ID NO:48) is provided herein. Nucleic acid encoding Hpd1 can be obtained from additional sources, for example, yeast strains such as *Candida albicans* (nucleotide sequence: Genbank accession no. XM_714034), e.g., strain SC5314 (ATCC No. MYA-2876). Methods for detecting the presence and/or activity of a malonyl Co-A reductase include spectrophotometric assays such as described by Alber et al. ((2006) *J. Bacteriol.* 188:8551-8559). Methods for detecting the presence and/or activity of a malonyl 3-hydroxy-propionate-dehydrogenase include assays such as described by Otzen et al. ((2014) *J. Biol. Chem.* 289(12):8151-8169).



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Some cells and microbial hosts, e.g., yeast spp., may express an endogenous semialdehyde dehydrogenase (e.g., EC 1.2.1.18; Ald6p) enzyme that catalyzes the oxidation of malonate semialdehyde to acetyl-CoA and $CO_2$ which would result in loss of the MSA intermediate in the synthesis of 3HP. Therefore, a gene encoding a semialdehyde dehydrogenase in any such host cell or organism can be disrupted or deleted, for example, using gene disruption techniques known in the art and/or described herein, to decrease or eliminate the enzyme activity. An example of a *Candida viswanathii* nucleotide sequence (SEQ ID NO:105) encoding an Ald6 protein (amino acid SEQ ID NO:49) is provided herein. Additional nucleotide sequences encoding Ald6 include, but are not limited to, *Candida albicans* strain SC5314 *ALD6* (Genbank accession no. XM-705897), *Saccharomyces cerevisiae ALD6* (Genbank accession no. NM_001183875). Methods for detecting the presence and/or activity of a semialdehyde dehydrogenase include assays such as described by Otzen et al. ((2014) *J. Biol. Chem.* 289(12):8151-8169) and Banerjee et al. ((1970) *J. Biol. Chem.* 245:1828-1835).

As shown in Fig. 9, a cellular platform pathway enhanced for production of malonyl-CoA, such as that depicted in Fig. 8, can provide a highly compatible system for use in the efficient production of 3HP. The platform is designed to enable maximal use of a lower cost carbon source (e.g., fatty acid or alkane) through modifications of cellular carbon flux that can enhance flow of source carbons to the generation of the malonyl-CoA precursor in the reaction scheme for production of 3HP. The presence and/or amount of 3HP in a sample can be determined, for example, using analytical methods such as HPLC (see, e.g., Raj et al. (2008) *Process Biochem.* 43:1440-1446 and International patent application no. PCT/US2016/023243 (publication no. WO 2016/154046)).

Cells or organisms that have been modified to incorporate a 3HP production system such as that depicted in Fig. 9 can be further modified depending on the purpose(s) for which the system is being used. For example, if the system is being used solely for the production of 3HP, then it may be beneficial to modify the cell or organism to decrease cytosolic fatty acid synthesis (e.g., as described with respect to the sytem shown in Fig. 8). If the system depicted in Fig. 9 is being used for co-production of a fatty dicarboxylic acid and 3HP, then it may be beneficial to either make no modifications of the FAS pathway activities or modify the host cell or organism to increase the amount, activity, and/or alter the specificity, of enzymes in the fatty acid synthesis (FAS) enzyme complex, shown as "FAS" in Fig. 9 (and as described, for example, with respect to the system shown in Fig. 8). A system such as that shown in Fig. 9 being used for co-

production of a fatty dicarboxylic acid and 3HP could also optionally include modifications, such as those described herein, of one or more activities of the ω- and/or β-oxidation pathways to enhance production of a desired dicarboxylic acid as described herein (e.g., modification of acyl-CoA oxidases, such as Pox4 and Pox5, monooxygenase, cytochrome P450 reductase). Such a co-production system would be depicted in Fig. 9 as including another arrow extending from a dicarboxylic acid (DCA-CoA) in the peroxisome to an acyl-CoA and then to a chain-shortened diacid (as a result of β-oxidation, "β-Ox") as is shown in the systems depicted in Figs. 5 and 6.

Although multiple, possible, cellular modifications are illustrated in Fig. 9, as described herein, some of the modifications depicted in the figure are optional enhancements of an exemplary engineered system and may or may not be included in a modified cell or organism depending on, for example, the intended use of the system (e.g., development of a particular single, or multiple, target molecule(s) production system) and the selection of variable features (e.g., host cell or organism, carbon source, regulatory controls (such as transcription control elements), culture conditions and the like) of the system. Thus, it is understood that any optional modifications set forth in the exemplary system shown in Fig. 9 are non-limiting and may or may not be included in a particular engineered system and, if included, may be in utilized in different combinations than illustrated in the figure.

*Engineered pathways for producing polyketides*

Modified cells, organisms, compositions and methods provided herein can also be used for enhanced production of polyketides, such as, for example, triacetic acid lactone (TAL). An example of an engineered production pathway for cell- or microbial-based synthesis of TAL is shown in Fig. 10. This biochemical pathway is based in a synthetic method using malonyl-CoA as a precursor molecule. As shown in Fig. 10, TAL can be produced from malonyl-CoA in two condensation reactions with acetyl-CoA catalyzed by 2-pyrone synthase (EC 2.3.1; "2PS" in Fig. 10). A malonyl-CoA-producing cell or organism can be genetically modified to express a 2-pyrone synthase enzymatic activity by introducing heterologous nucleic acid encoding the enzyme into a host cell or organism. For example, one or more copies of a heterologous nucleic acid encoding 2PS operably linked to a suitable promoter element (e.g., a strong and/or fatty acid-inducible promoter such as a yeast *HDE* gene promoter) can be introduced into the cell or organism for recombinant expression of the activity. Nucleic acid encoding a 2-pyrone



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synthase can be obtained, for example, from plant species such as *Gerbera hybrida* (e.g., nucleotide SEQ ID NO: 325 encoding amino acid SEQ ID NO: 324, and Genbank nucleotide sequence accession no. Z38097). The enzymatic activity of 2-pyrone synthase can be determined using a TLC-based radiometric assay as described by Jez et al. ((2000) *Chemistry and Biology* 7(12):919-930).

As shown in Fig. 10, a cellular platform pathway enhanced for production of malonyl-CoA, such as that depicted in Fig. 8, can provide a highly compatible system for use in the efficient production of TAL. The platform is designed to enable maximal use of a lower cost carbon source (e.g., fatty acid or alkane) through modifications of cellular carbon flux that can enhance flow of source carbons to the generation of the malonyl-CoA precursor in the reaction scheme for production of TAL. TAL concentrations can be determined using reversed-phase HPLC as described by Xie et al. ((2006) *Biotechnol Bioengineering* 93(4):727-736). Cells or organisms that have been modified to incorporate the TAL production system depicted in Fig. 10 can be further modified depending on the purpose(s) for which the system is being used. For example, if the system is being used solely for the production of TAL, then it may be beneficial to modify the cell or organism to decrease cytosolic fatty acid synthesis (e.g., as described with respect to the sytem shown in Fig. 8). If the system depicted in Fig. 10 is being used for co-production of a fatty dicarboxylic acid and TAL, then it may be beneficial to either make no modifications of the FAS pathway activities or modify the host cell or organism to increase the amount, activity, and/or alter the specificity, of enzymes in the fatty acid synthesis (FAS) enzyme complex, shown as "FAS" in Fig. 10 (and as described, for example, with respect to the system shown in Fig. 8). A system such as that shown in Fig. 10 being used for co-production of a fatty dicarboxylic acid and TAL could also optionally include modifications, such as those described herein, of one or more activities of the ω- and/or β-oxidation pathways to enhance production of a desired dicarboxylic acid as described herein (e.g., modification of acyl-CoA oxidases, such as Pox4 and Pox5, monooxygenase, cytochrome P450 reductase). Such a co-production system would be depicted in Fig. 10 as including another arrow extending from a dicarboxylic acid (DCA-CoA) in the peroxisome to an acyl-CoA and then to a chain-shortened diacid (as a result of β-oxidation, "β-Ox") as is shown in the systems depicted in Figs. 5 and 6.

Although multiple, possible, cellular modifications are illustrated in Fig. 10, as described herein, some of the modifications depicted in the figure are optional enhancements of an exemplary engineered system and may or may not be included in a modified cell or organism depending



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on, for example, the intended use of the system (e.g., development of a particular single, or multiple, target molecule(s) production system) and the selection of variable features (e.g., host cell or organism, carbon source, regulatory controls (such as transcription control elements), culture conditions and the like) of the system. Thus, it is understood that any optional modifications set forth in the exemplary system shown in Fig. 10 are non-limiting and may or may not be included in a particular engineered system and, if included, may be in utilized in different combinations than illustrated in the figure.

*Engineered acetyl-CoA-generating pathways in a platform target molecule production system*

Figure 11 depicts possible cellular modifications in some embodiments of a eukaryotic (i.e., yeast in this example) platform system for the enhanced generation of acetyl-CoA. Acetyl-CoA can be a versatile precursor molecule in the synthesis of many industrially valuable molecules. Accordingly, a system such as that depicted in Fig. 11 can be used as a platform for incorporating particular pathways for the production of a diverse array of target molecules. The cell-based platform system shown in Fig. 11 can incorporate carbon-flux modifications shown in Fig. 8 that are designed to capture carbon atoms as acetyl-CoA in the cytosol by enhancing carbon flow through cellular oxidative metabolism pathways (ω-oxidation and peroxisomal β-oxidation) and reducing flow of carbon into mitochondria. Fig. 11 differs from Fig. 8 in that it shows an embodiment of the platform system in which target molecule production pathways extend from acetyl-CoA, instead of malonyl-CoA, as a precursor molecule. The core of this platform system, as it is for the system shown in Fig. 8, centers on acetyl group diversion elements that can impede the flow of acetyl group carbons into mitochondria and can enhance flow of the carbons into generation of acetyl-CoA. In systems designed for production of target molecules using acetyl-CoA as a precursor, these elements can optionally be combined with a carbon recycle loop that captures any cytosolic acetyl moieties that are not funneled into the target molecule production pathway into cytosolic fatty acid synthesis to regenerate an acyl-CoA that can be diverted from lipid synthesis and converted into a fatty acid that can be subjected to another cycle of peroxisomal β-oxidation.

Figure 11 shows an embodiment of a platform system for the enhanced generation of acetyl-CoA in connection with terpene target molecule production as an example of the use of acetyl-CoA as a precursor molecule. As shown in the figure, terpenes can be generated, for example,

from isopentenyl diphosphate produced through mevalonate pathways in cells. Through a series of reactions in the mevalonate pathway present in eukaryotes and some bacteria, acetyl-CoA can be converted to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) which are precursors in the production of polyisoprenoids (e.g., dolichol) and sterols (e.g., ergosterol, cholesterol). Terpenes, e.g., valencene, lycopene, carotenes and the like, can be produced from IPP through further reactions involving enzymes such as, for example, valencene synthase, and enzymes encoded by carotenoid biosynthesis *CRT* genes (e.g., geranylgeranyl pyrophosphate synthase (CrtE), phytoene synthase (CrtB), phytoene desaturase (CrtI), beta-carotene ketolase (CrtW), beta-carotene hydroxylase (CrtZ)). Cells and organisms engineered for production of terpenes as shown in Fig. 11 can be modified to enhance flow of carbon through the terpene production pathways. For example, such engineered cells can be genetically modified to express, or increase expression of, any of the mevalonate pathway enzymes and/or enzymes involved in terpene synthesis pathways.

As set forth in connection with the description of the platform systems shown in Figs. 5 and 8, carbon flux alteration in the system shown in Fig. 11 can begin with modifications (e.g., decreased acyl-CoA synthetase (Faa1 and/or Fat1) activity) that can enhance carbon flow in the form of a fatty acid (from, for example, a fatty acid carbon source or derived from an alkane source) into ω- and β-oxidation pathways in the cell or organism. The oxidative metabolism aspect of the platform system can be further enhanced through modification of ω- and/or β-oxidation activity as described herein. Carbon capture modifications of this platform system, as in the system depicted in Figs. 5 and 8, can occur in the processing of the acetyl-CoA generated during β-oxidation and can include diversion elements that can impede the flow of acetyl group carbons into mitochondria (e.g., decreasing mitochondrial carnitine transporters and carnitine acetyltransferase activities) and enhance flow of the carbons into generation of acetyl-CoA (e.g., increasing cytosolic carnitine acetyltransferase activity). At this point in the platform pathway system depicted in Fig. 11, when acetyl moieties are accumulated in the cytosol as acetyl-CoA, there primarily are two pathways in which they can be used: (1) fatty acid synthesis (FAS) through initial conversion into malonyl-CoA and (2) target molecule synthesis pathways that can be engineered into the host cell or organism. Thus, target molecule synthesis can be limited by competition for the acetyl-CoA substrate by the FAS pathway. To minimize this competition, in this platform system, unlike the systems shown in Figs. 5 and 8, the host cell or organism is not modified to increase acetyl-CoA carboxylase activity, which is an enzyme that catalyzes conversion of acetyl-CoA into malonyl-CoA and can commit it to fatty acid synthesis.



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However, if an acetyl-CoA carboxylase activity is present in the cytosol of this cellular system, and the system includes a functional FAS pathway, any acetyl-CoA molecules that are lost to the fatty acid synthesis pathway can be recaptured for possible use in target molecule synthesis by including an optional carbon recycling loop (such as that described in reference to Fig. 5) in the system. The final product of cytosolic fatty acid synthesis is typically an acyl-CoA (e.g., palmitoyl-CoA), which is shown as "FA-CoA" in Fig. 11. To avoid loss of the carbon atoms in the acyl-CoA lipid synthesis pathways, and complete the carbon recycling loop, the host cell or organism can optionally be modified to include a cytosolic thioesterase enzyme to, in effect, "deactivate" the fatty acid-CoA through hydrolysis and removal of coenzyme A. This can divert the carbons in the acyl-CoA from use in cellular processes not involved in target molecule production and/or generation of cytosolic acetyl-CoA and can complete the recycling loop by generating a cytosolic free fatty acid that can then begin a new cycle of the loop pathway at the initial point of ω-oxidation. As described herein, a host cell can be modified to increase (or introduce) a thioesterase activity (shown as *TES3*$^{cyt}$ in a diagonal line-hatched background oval in Fig. 11) in the cytosol in order to direct acyl-CoA carbon flux toward oxidative metabolism pathways (ω- and β-oxidation).

The system depicted in Fig. 11 can serve as a platform that can be used in multiple ways. For example, as shown, the pathway can serve as an enhanced, high-efficiency cytosolic acetyl-CoA-generating system that can be further modified for use in generating a target molecule (e.g., terpenes) in an engineered pathway that initiates with an acetyl-CoA precursor. Additionally, the system depicted in Fig. 11 can be used in the co-production of a fatty dicarboxylic acid, e.g., adipic acid, and a target molecule generated using acetyl-CoA as a precursor in the synthesis pathway. In embodiments in which the system is used for co-production of a fatty diacid and another target molecule, the host cell or organism can optionally also be modified as described herein to decrease the amount and/or activity of, or eliminate, mechanisms for the transport of acyl-CoA across the peroxisomal membrane and into peroxisomes (e.g., shown as "pxa1Δ" in a black background oval in Fig. 11). This modification avoids the by-passing of ω-oxidation by acyl-CoA generated from fatty acid synthesis which would allow the formation of monocarboxlic acids.

Cells or organisms that have been modified to incorporate a biological platform system such as that depicted in Fig. 11 can be further modified depending on the purpose(s) for which the

system is being used. For example, if the system is being used solely for the production of one or more target molecules synthesized from an acetyl-CoA precursor (i.e., not being used as a fatty acid co-production system), then it may be beneficial to modify the cell or organism to decrease or slow cytosolic fatty acid synthesis. The fatty acid synthesis (FAS) pathway in host cells or organisms can compete with any pathways for target molecule synthesis for the cytosolic acetyl-CoA produced in this platform system. Therefore, to enhance acetyl-CoA carbon atom flux toward target molecule synthesis and direct carbon flux away from fatty acid biosynthesis, a host cell or organism can optionally be modified to decrease or attenuate activity of one or more enzymes involved in fatty acid synthesis. Flux of acetyl-CoA carbon into the mevalonate pathway may also optionally be enhanced by increasing one or more activities of the mevalonate pathway. For example, a mevalonate pathway activity may be enhanced by genetically modifying an enzyme that catalyzes a reaction in the pathway (e.g., increasing the copy number of nucleic acids encoding a mevalonate pathway enzyme in the cell, increasing the transcription of such nucleic acids and/or introducing nucleic acid encoding a more active enzyme into the cell).

If a platform system such as that depicted in Fig. 11 is being used for co-production of a fatty dicarboxylic acid and a target product derived from another pathway using acetyl-CoA precursors (e.g., the mevalonate pathway), then it may be beneficial to either make no modifications of the FAS pathway activities or modify the host cell or organism to increase the amount, activity, and/or alter the specificity, of enzymes in the fatty acid synthesis (FAS) enzyme complex, shown as "FAS" in Fig. 11 (e.g., the enzyme activities of the FAS1 and FAS2 subunits of yeast). A system such as that shown in Fig. 11 being used for co-production of a fatty dicarboxylic acid and other acetyl-CoA-dervied product could also optionally include modifications, such as those described herein, of one or more activities of the ω- and/or β-oxidation pathways to enhance production of a desired dicarboxylic acid as described herein (e.g., modification of acyl-CoA oxidases, such as Pox4 and Pox5, monooxygenase, cytochrome P450 reductase). Such a co-production system would be depicted in Fig. 11 as including another arrow extending from a dicarboxylic acid (DCA-CoA) in the peroxisome to an acyl-CoA and then to a chain-shortened diacid (as a result of β-oxidation, "β-Ox") as is shown in the systems depicted in Figs 5 and 6.

Although multiple, possible, cellular modifications are illustrated in Fig. 11, as described herein, some of the modifications depicted in the figure are optional enhancements of an exemplary



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engineered system and may or may not be included in a modified cell or organism depending on, for example, the intended use of the system (e.g., development of a particular single, or multiple, target molecule(s) production system) and the selection of variable features (e.g., host cell or organism, carbon source, regulatory controls (such as transcription control elements), culture conditions and the like) of the system. Thus, it is understood that any optional modifications set forth in the exemplary system shown in Fig. 11 are non-limiting and may or may not be included in a particular engineered system and, if included, may be in utilized in different combinations than illustrated in the figure.

*Methods for producing target molecules*

Provided herein are methods for contacting an engineered cell, microorganism or organism with a carbon source (e.g., a feedstock containing fatty acids and/or alkanes) under conditions whereby one or more target molecules is produced. Biological methods provided herein for producing a target molecule can incorporate cells or organisms, such as those provided herein, that have been modified to enhance production efficiency by maximizing use and minimizing the costs of raw starting materials input into the process. The methods are designed to provide flexibility in culture conditions, particularly carbon source utilization, and coordinated regulatory mechanisms to enable efficient production of a variety of carbon-containing target molecules. In some embodiments of the methods, the cell or organism used for target molecule production is one that is able to assimilate a variety of carbon sources, including one or more non-fermentable (e.g., alkanes, fatty acids, alcohols) as well as fermentable carbon sources. In particular embodiments, the cell or organism is one that is able to survive under conditions in which the sole carbon source is a non-fermentable carbon source. The use of such cells and organisms in some embodiments of the production methods can contribute to the cost-effectiveness and environmental soundness of the methods.

In certain embodiments, a cell or organism used in the production methods has been modified to alter cellular carbon flux, including, for example, cells and organisms provided herein. Such carbon flux alterations include those that can increase the amount of the source carbon that is available for use in target molecule generation pathways. For example, one or more activities in one or more metabolic pathways of the cell or organism can be engineered to increase carbon flux through the pathways to produce a desired product. The engineered activities can be chosen such that there is an increased production of metabolic intermediates that can be



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utilized in one or more pathways to achieve increased production of a desired product relative to the unmodified host cell or organism. The engineered activities also can be chosen such that there is a decreased activity of enzymes that reduce production of a desired intermediate or end product (e.g., reverse activities).

This cellular carbon flux management can be optimized, for any chosen feedstock used in culturing the cells or organisms, by engineering the appropriate activities in the appropriate pathways. Non-limiting examples are given herein using pure alkanes (e.g., single chain length alkanes, dodecane for example), mixed chain-length alkanes, long-chain alkanes, pure fatty acids (e.g., single chain length fatty acids, capric acid or oleic acid for example) and mixed chain length fatty acids as a carbon source in a feedstock. The process of carbon flux management through engineered pathways can be used to produce a target molecule (e.g., an organic acid, a fatty acid, dicarboxylic acid, polyketide, terpene) at a level and/or rate closer to the calculated maximum theoretical yield for any given feedstock, than does a production process that has not been enhanced or modified using methods described herein. The terms "theoretical yield" or "maximum theoretical yield" as used herein refer to the yield of product of a chemical or biological reaction that can be formed if the reaction went to completion. Theoretical yield is based on the stoichiometry of the reaction and ideal conditions in which starting material is completely consumed, undesired side reactions do not occur, the reverse reaction does not occur, and there are no losses in the work-up procedure.

*Growth conditions and fermentation*

Large-scale cell- or microbial-based target molecule production is generally conducted by culturing the cells or organisms in a fermentor. The culture conditions can vary depending on the cell or organism and the target molecule being produced. In some embodiments of the production processes provided herein, the modified cell or organism is eukaryotic, such as, for example, a yeast. In particular embodiments, the yeast is one that is able to assimilate fatty acids and/or alkanes. For example, in some emodiments, the yeast is a species of *Candida*, e.g., *C. tropicalis* or C. *viswanathii* (e.g., ATCC 20336, ATCC 20913, ATCC 20962) or *Yarrowia*, e.g., *Y. lipolytica* (e.g., ATCC 20228). In some embodiments, the yeast is a thermotolerant yeast, e.g., a species of *Blastobotrys*, such as *Blastobotrys adeninivorans*. In some embdodiments, the yeast is a non-oleaginous yeast. Engineered organisms often are cultured under conditions that optimize yield of a target molecule. For example, culture conditions can

be selected to balance the levels of one or more of the following activities in order to optimize target molecule yield: carnitine acetyltransferase, acetyl-carnitine translocase, acetyl-CoA carboxylase, ATP citrate lyase, acetyl-CoA hydrolase, acetyl-CoA synthetase, thioesterase, acyl-CoA synthetase, monooxygenase, cytochrome P450 reductase, alcohol dehydrogenase, alcohol oxidase, aldehyde dehydrogenase, acyl-CoA oxidase, 3-ketoacyl-CoA thiolase, peroxisomal transporter, peroxisome biogenesis factor, fatty acid synthase activity and multifunctional enzyme (e.g., enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase) activities. In general, non-limiting examples of conditions that may be optimized include the type and amount of carbon source, the type and amount of nitrogen source, the carbon-to-nitrogen ratio, the oxygen level, growth temperature, pH, length of the biomass production phase, length of target product accumulation phase, and time of cell harvest.

A suitable pH range for fermentation often is between about pH 4.0 to about pH 8.0, where a pH in the range of about pH 5.5 to about pH 7.0 sometimes is utilized for initial culture conditions. Depending on the host cell or organism, culturing may be conducted under aerobic or anaerobic conditions, where microaerobic conditions sometimes are maintained. In embodiments in which a *Candida* yeast (e.g., *C. tropicalis* or *C. viswanathii*) is used as the host microorganism, aerobic conditions can be optimal. A two-stage process may be utilized, where one stage promotes organism proliferation and another stage promotes production of target molecule. Suitable temperatures for culturing microorganisms generally are in the range of 28°C to 35°C. However, some organisms are able to survive and grow in more extreme temperatures. Thermotolerant and/or osmotolerant organisms can be well suited for use in industrial production systems operating at elevated temperatures and/or osmotic pressures that would impair growth and/or metabolism and/or completely inactivate organisms that are not thermotolerant and/or osmotolerant. In some instances, production efficiency can be improved and production costs reduced in using such organisms due to decreases in losses and avoidance of implementation of cooling processes. Nitrogen may be supplied from an inorganic (e.g., $(NH_4)_2SO_4$) or organic source (e.g., urea or glutamate). In addition to appropriate carbon and nitrogen sources, culture media also can contain suitable minerals, salts, cofactors, buffers, vitamins, metal ions (e.g., $Mn^{+2}$, $Co^{+2}$, $Zn^{+2}$, $Mg^{+2}$) and other components suitable for culture of microorganisms.

Engineered microorganisms sometimes are cultured in complex media (e.g., yeast extract-peptone-dextrose broth (YPD)). In some embodiments, engineered microorganisms are



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cultured in a defined minimal media that lacks a component necessary for growth and thereby forces selection of a desired expression cassette (e.g., Yeast Nitrogen Base (DIFCO Laboratories, Detroit, Mich.)). Culture media in some embodiments are common commercially prepared media, such as Yeast Nitrogen Base (DIFCO Laboratories, Detroit, Mich.). Other defined or synthetic growth media may also be used. In specific embodiments, yeast are cultured in YPD media (10 g/L Bacto Yeast Extract, 20 g/L Bacto Peptone, and 20 g/L Dextrose). Filamentous fungi, in particular embodiments, can be grown in CM (Complete Medium) containing 10 g/L Dextrose, 2 g/L Bacto Peptone, 1g/L Bacto Yeast Extract, 1 g/L Casamino acids, 50 mL /L 20X Nitrate Salts (120 g/L $NaNO_3$, 10.4 g/L KCl, 10.4 g/L $MgSO_4 \cdot 7 H_2O$ ), 1 mL/L 1000X Trace Elements (22 g/L $ZnSO_4 \cdot 7 H_2O$, 11 g/L $H_3BO_3$, 5 g/L $MnCl_2 \cdot 7 H_2O$, 5 g/L $FeSO_4 \cdot 7 H_2O$, 1.7 g/L $CoCl_2 \cdot 6 H_2O$, 1.6 g/L $CuSO_4 \cdot 5 H_2O$, 1.5 g/L $Na_2MoO_4 \cdot 2 H_2O$, and 50 g/L $Na_4EDTA$), and 1 mL/L Vitamin Solution (100 mg each of Biotin, pyridoxine, thiamine, riboflavin, p-aminobenzoic acid, and nicotinic acid in 100 mL water).

A variety of fermentation processes may be applied for commercial biological production of a target product. In some embodiments, commercial production of a target product from a recombinant microbial host is conducted using a batch, fed-batch or continuous fermentation process, for example. A batch fermentation process often is a closed system where the media composition is fixed at the beginning of the process and not subject to further additions beyond those required for maintenance of pH and oxygen level during the process. At the beginning of the culturing process the media is inoculated with the desired organism and growth or metabolic activity is permitted to occur without adding additional sources (i.e., carbon and nitrogen sources) to the medium. In batch processes the metabolite and biomass compositions of the system change constantly up to the time the culture is terminated. In a typical batch process, cells proceed through a static lag phase to a high-growth log phase and finally to a stationary phase, where the growth rate is diminished or halted. Left untreated, cells in the stationary phase will eventually die.

A variation of the standard batch process is the fed-batch process, where the carbon source is continually added to the fermenter over the course of the fermentation process. Fed-batch processes are useful when catabolite repression is apt to inhibit the metabolism of the cells or where it is desirable to have limited amounts of carbon source in the media at any one time. Measurement of the carbon source concentration in fed-batch systems may be estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen and the partial



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pressure of waste gases (e.g., $CO_2$). Batch and fed-batch culturing methods are known in the art. Examples of such methods may be found in Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, 2.sup.nd ed., (1989) Sinauer Associates Sunderland, Mass. and Deshpande, Mukund V., *Appl. Biochem. Biotechnol. 36*:227 (1992).

In a continuous fermentation process, a defined media often is continuously added to a bioreactor while an equal amount of culture volume is removed simultaneously for product recovery. Continuous cultures generally maintain cells in the log phase of growth at a constant cell density. Continuous or semi-continuous culture methods permit the modulation of one factor or any number of factors that affect cell growth or end product concentration. For example, an approach may limit the carbon source and allow all other parameters to moderate metabolism. In some systems, a number of factors affecting growth may be altered continuously while the cell concentration, measured by media turbidity, is kept constant. Continuous systems often maintain steady state growth and thus the cell growth rate often is balanced against cell loss due to media being drawn off the culture. Methods of modulating nutrients and growth factors for continuous culture processes, as well as techniques for maximizing the rate of product formation, are known and a variety of methods are detailed by Brock, supra.

A non-limiting exemplary fermentation protocol, which includes an initial batch growth phase followed by a fed-batch production, or conversion, phase, that can be used for production of target molecules using modified yeast strains, such as some of those provided herein, is as follows. Fermentation medium of composition 14.0 g/L ammonium sulfate, 10.2 g/L potassium phosphate monobasic, 1.0 g/L magnesium sulfate, 0.2 g/L calcium chloride, 120 mg/L citric acid, 46 mg/L ferric chloride, 0.4 mg/L biotin, 54 g/L glucose and 2X trace metals mix is filter sterilized and transferred to a sterile fermentation vessel. Growth of a yeast strain, e.g., *Candida viswanathii*, is initiated with a 5% inoculum (initial OD600 nm = 1.0) and growth conditions of 35°C, 1000 rpm, 1 vvm, pH 5.8 and initial volume of 1.0 L. Growth continues for approximately 15 hours before exhaustion of the initial carbon source. The temperature control is changed to 30°C and the conversion phase is initiated by the addition of oleic acid to 5 g/L. At the same time as the oleic acid bolus, a continuous feed of oleic acid is initiated at a rate of 1.5 g/L-h. Fermentation conditions are maintained at 30°C, 1000 rpm, 1 vvm, and pH 5.8 for 24 hours at which point the pH set-point is changed to 3.5. The fermentation is carried out for a total of about 135 hours. Samples are collected for GC analysis every 24 hours after initiating



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the conversion phase. In this protocol, cells are allowed to grow on glucose as a carbon source in the initial phase (i.e., growth phase) until the glucose is depleted. At this point, a different carbon source, e.g., a fatty acid such as oleic acid, is introduced into the fermentor (i.e., the conversion phase). This new carbon source is continuously fed into the fermentor to initiate and maintain target molecule production (i.e., production phase) that involves oxidative metabolic pathways (e.g., ω-oxidation and β-oxidation) and fatty acid-induced enzyme expression.

*Feedstocks, media, supplements and additives*

Culture media generally contain a suitable carbon source. Carbon sources useful for culturing cells, microorganisms and/or fermentation processes sometimes are referred to as feedstocks. The term "feedstock" as used herein refers to a composition containing a carbon source that is provided to a cell or organism, which is used by the cell or organism to produce energy and metabolic products useful for growth. A feedstock may be a natural substance, a "man-made substance," a purified or isolated substance, a mixture of purified substances, a mixture of unpurified substances or combinations thereof. A feedstock often is prepared by and/or provided to a cell or organism by a person, and a feedstock often is formulated prior to administration to the cell or organism. A carbon source may include, but is not limited to including, one or more of the following substances: monosaccharides (e.g., also referred to as "saccharides," which include 6-carbon sugars (e.g., glucose, fructose), 5-carbon sugars (e.g., xylose and other pentoses) and the like), disaccharides (e.g., lactose, sucrose), oligosaccharides (e.g., glycans, homopolymers of a monosaccharide), polysaccharides (e.g., starch, cellulose, heteropolymers of monosaccharides or mixtures thereof), sugar alcohols (e.g., glycerol), and renewable feedstocks (e.g., cheese whey permeate, cornsteep liquor, sugar beet molasses, barley malt).

A carbon source also may include a metabolic product that can be used directly as a metabolic substrate in an engineered pathway described herein, or indirectly via conversion to a different molecule using engineered or native biosynthetic pathways in an engineered cell or microorganism. In some embodiments, a carbon source may include glycerol backbones generated by the action of an engineered pathway including at least a lipase activity. In certain embodiments, metabolic pathways can be preferentially biased towards production of a desired product by increasing the levels of one or more activities in one or more metabolic pathways having and/or generating at least one common metabolic and/or synthetic substrate. In some



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embodiments, a metabolic byproduct (e.g., glycerol) of an engineered activity (e.g., lipase activity) can be used in one or more metabolic pathways such as gluconeogenesis, pentose phosphate pathway, glycolysis, fatty acid synthesis, beta oxidation, and omega oxidation, to generate a carbon source that can be converted to a target molecule, e.g., adipic acid.

In some embodiments, a feedstock includes a mixture of carbon sources, where each carbon source in the feedstock is selected based on the genotype of the cultured cell or organism. In certain embodiments, a mixed carbon source feedstock includes one or more carbon sources selected from sugars, cellulose, fatty acids, triacylglycerides, paraffins, the like and combinations thereof.

In some embodiments a feedstock is selected according to the genotype and/or phenotype of the cell or organism that is cultured. For example, as described herein, for the production of certain target molecules (e.g., dicarboxylic acids of a particular carbon chain length) the activities of oxidative processes, such as β-oxidation, can be altered through genetic modification of a host cell or organism. In some instances, the catalytic activities and/or substrate specificities of, for example, one or more acyl-CoA oxidases of the host cell β-oxidation pathway can be modified in order to ensure that carbon sources of a particular chain length are or are not subject to degradation. The feedstock used in target molecule production by such modified cells or organisms can be selected to enhance the production process. For example, a feedstock rich in 12-carbon fatty acids, 12-carbon dicarboxylic acids or 12-carbon paraffins, or a mixture of 10, 12 and 14-carbon compounds can be useful for culturing yeast strains harboring an alteration that partially blocks beta oxidation by disrupting POX4 activity, as described herein. Non-limiting examples of carbon sources having 10 to 14 carbons include fats (e.g., coconut oil, palm kernel oil), paraffins (e.g., alkanes, alkenes, or alkynes) having 10 to 14 carbons, (e.g., dodecane (also referred to as adakane12, bihexyl, dihexyl and duodecane); tetradecane), alkene and alkyne derivatives), fatty acids (decanoic, dodecanoic acid, tetradecanoic acid), fatty alcohols (decanol, dodecanol, tetradecanol), the like, non-toxic substituted derivatives or combinations thereof.

In certain embodiments involving genetically modified cells or organisms having partially blocked beta-oxidation pathways, feedstocks suitable for use include, but are not limited to, fatty acid distillates or soapstocks of renewable oils (palm oil fatty acid distillate, soybean oil soapstock, coconut oil soapstock), renewable oils (coconut oil, palm oil, palm kernel oil,



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soybean oil, corn oil, and the like), fatty acids of chain length equal to or greater than C10 (in substantially single form (e.g., in substantially pure form) or in mixture form, alkanes of chain length equal to or greater than C10 in substantially single form (e.g., substantially pure form) or in mixture form. Any suitable alkane, fatty acid, fatty alcohol, plant based oil, seed based oil, non-petroleum derived soap stock or the like can be used as the feedstock for the cell or organism (e.g., dodecane, methyl laurate, lauric acid, carbon sources having 10 or greater carbons (e.g. for sebacic acid production) or carbon sources having 12 or greater carbons (e.g. for dodecanedioic acid production)). In some embodiments, carbon sources with greater than 12 carbons can be metabolized using naturally occurring and/or engineered pathways to yield molecules that can be further metabolized using the beta oxidation pathway.

In some embodiments, one acyl-CoA oxidase activity of the beta-oxidation pathway of a host cell or organism is engineered such that it is enhanced, and in certain embodiments, another acyl-CoA oxidase activity in the beta-oxidation pathway is altered to reduce or eliminate the activity, thereby optimizing the production of a diacid of a desired chain-length or diacids with a distribution of desired chain lengths. In some embodiments, an acyl-CoA oxidase is selected and/or engineered to alter the substrate specificity of the enzyme. In certain embodiments, the substrate specificity of a heterologous and/or engineered acyl-CoA oxidase is for carbon chain lengths of between about 12 carbons and about 18 carbons, and in some embodiments a heterologous and/or engineered acyl-CoA oxidase exhibits no activity on substrates below 12 carbons in length. In certain embodiments, a heterologous acyl-CoA oxidase with a desired chain length specificity can be isolated from any suitable organism. In certain embodiments, a carbon source starting material (e.g., alkane, fatty acid, fatty alcohol, dicarboxylic acid) of intermediate or long chain length (e.g., between about 10 carbons and 22 carbons) is converted into an acyl-CoA derivative for entry into the beta-oxidation pathway. A fatty acid can be processed using omega oxidation to yield a dicarboxylic acid (e.g., dodecanedioic acid).

*Examples of carbon sources*

A carbon source may include, but is not limited to including, one or more of the following substances: alkanes, alkenes, mono-carboxylic acids, di-carboxylic acids, monosaccharides (e.g., also referred to as "saccharides," which include 6-carbon sugars (e.g., glucose, fructose), 5-carbon sugars (e.g., xylose and other pentoses) and the like), disaccharides (e.g., lactose, sucrose), oligosaccharides (e.g., glycans, homopolymers of a monosaccharide),

polysaccharides (e.g., starch, cellulose, heteropolymers of monosaccharides or mixtures thereof), sugar alcohols (e.g., glycerol), and renewable feedstocks (e.g., cheese whey permeate, cornsteep liquor, sugar beet molasses, barley malt).

Carbon sources also can be selected from one or more of the following non-limiting examples: paraffin (e.g., saturated paraffin, unsaturated paraffin, substituted paraffin, linear paraffin, branched paraffin, or combinations thereof); alkanes (e.g., hexane, dodecane), alkenes or alkynes, each of which may be linear, branched, saturated, unsaturated, substituted or combinations thereof (described in greater herein); linear or branched alcohols (e.g., hexanol, dodecanol); saturated or unsaturated fatty acids (e.g., each fatty acid is about 1 carbon to about 60 carbons with 0 to 10 unsaturations, including free fatty acids, mixed fatty acids, single fatty acid, purified fatty acids (e.g., single fatty acid or mixture of fatty acids), fatty acid distillates, soap stocks, the like and combinations thereof); esters of fatty acids; salts of fatty acids, monoglycerides; diglycerides; triglycerides, phospholipids. Non-limiting commercial sources of products for preparing feedstocks include plants, plant oils or plant products (e.g., vegetable oils (e.g., almond oil, canola oil, cocoa butter, coconut oil, corn oil, cottonseed oil, flaxseed oil, grape seed oil, illipe, jatropha oil, olive oil, palm oil, palm olein, palm kernel oil, rapeseed oil, safflower oil, peanut oil, soybean oil, sesame oil, shea nut oil, sunflower oil, walnut oil, the like and combinations thereof) and vegetable oil products), purified fatty acids (e.g., myristoleic acid, palmitoleic acid, sapienic acid, oleic acid, elaidic acid, vaccenic acid, linoleic acid, linoelaidic acid, α-linolenic acid, arachidonic acid, eicosapentaenoic acid, erucic acid and docosahexaenoic acid) and animal fats (e.g., beef tallow, butterfat, lard, cod liver oil). A carbon source may include a petroleum product and/or a petroleum distillate (e.g., diesel, fuel oils, gasoline, kerosene, paraffin wax, paraffin oil, petrochemicals). In some embodiments, a feedstock comprises petroleum distillate. A carbon source can be a fatty acid distillate (e.g., a palm oil distillate or corn oil distillate). Fatty acid distillates can be by-products from the refining of crude plant oils. In some embodiments, a feedstock comprises a fatty acid distillate.

In some embodiments, a feedstock comprises a soapstock (i.e. soap stock). A widely practiced method for purifying crude vegetable oils for edible use is the alkali or caustic refining method. This process employs a dilute aqueous solution of caustic soda to react with the free fatty acids present which results in the formation of soaps. The soaps together with hydrated phosphatides, gums and prooxidant metals are typically separated from the refined oil as the heavy phase discharge from the refining centrifuge and are typically known as soapstock.

A carbon source also may include a metabolic product that can be used directly as a metabolic substrate in an engineered pathway described herein, or indirectly via conversion to a different molecule using engineered or native biosynthetic pathways in an engineered cell or microorganism. In some embodiments, a carbon source may include glycerol backbones generated by the action of an engineered pathway including at least a lipase activity. In certain embodiments, metabolic pathways can be preferentially biased towards production of a desired product by increasing the levels of one or more activities in one or more metabolic pathways having and/or generating at least one common metabolic and/or synthetic substrate. In some embodiments, a metabolic byproduct (e.g., fatty acid, glycerol) of an engineered activity (e.g., ω- oxidation activity, lipase activity) can be used in one or more metabolic pathways, such as gluconeogenesis, pentose phosphate pathway, glycolysis, fatty acid synthesis, beta oxidation, and omega oxidation, to generate a carbon source that can be converted to a fatty dicarboxylic acid (e.g., adipic acid, octanedioic acid, decanedioic acid, dodecanedioic acid, tetradecanedioic acid, hexadecanedioic acid, octadecanedioic acid, eicosanedioic acid) or other target molecule.

A carbon source can be an organic acid, including, but not limited to, fatty acids, diacids and β-hydroxy acids (e.g., hydroxyalkanoate monomers). As used herein, "organic acid" and "fatty acid" encompass the free-acid forms thereof and salts or esters thereof. Fatty acids are aliphatic acids of varying carbon chain lengths. Fatty acids generally have a formula that includes: R1-COOR2. In some embodiments, R1 can be an aliphatic group, and can include 1 to 30 carbon atoms, or 6 to 24 carbon atoms, and R2 can be hydrogen, methyl, ethyl, propyl or butyl. For example, R1 can include about 1 carbon atom, about 2 carbon atoms, about 3 carbon atoms, about 4 carbon atoms, about 5 carbon atoms, about 6 carbon atoms, about 7 carbon atoms, about 8 carbon atoms, about 9 carbon atoms, about 10 carbon atoms, about 12 carbon atoms, about 14 carbon atoms, about 16 carbon atoms, about 18 carbon atoms, about 20 carbon atoms, about 22 carbon atoms, about 24 carbon atoms, about 26 carbon atoms, about 28 carbon atoms or about 30 carbon atoms. Naturally occurring fatty acids in biological systems generally contain an even number of carbon atoms, typically between about 12 to about 24, or about 14 to about 24, and most commonly, 16 or 18 carbon atoms. Based on the number of carbons in a fatty acid carbon chain, it can be categorized as a short-, medium- or long-chain fatty acid. Generally, short-chain fatty acids have a chain length of about 2 to about 6 carbon atoms, medium-chain fatty acids have a chain length of about 8 to about 10 carbon atoms, long-chain fatty acids have a chain length of about 12 to about 20 carbon atoms and



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very long-chain length fatty acids have a chain length of about 22 or about 24 or more carbon atoms. The carbon atom bonds in the alkyl chain may all be single bonds (i.e., a saturated fatty acid) or may contain one or more double bonds (i.e., an unsaturated fatty acid). Unsaturated fatty acids having one double bond are also referred to as monoenoic; unsaturated fatty acids having two or more double bonds in the carbon chain are also referred to as polyenoic and polyunsaturated (PUFA). The carbon chain in a fatty acid may also be substituted with hydroxyl, methyl, or other groups in place of a hydrogen. Carboxylic acids, such as fatty acids, can partially dissociate in aqueous media and exist as undissociated, uncharged molecules and as a dissociated, anionic form.

Fatty acids containing one carboxyl group can also be referred to as monocarboxylic fatty acids. A fatty acid containing two carboxyl groups (e.g., α,ω-dicarboxylic acids) is a fatty dicarboxylic acid, also referred to herein as a diacid. Fatty dicarboxylic acids generally have a formula that includes: R1OOC-R-COOR2. In some embodiments, R can be an aliphatic group, and can include 1 to 30 carbon atoms, or 4 to 24 carbon atoms, and R1 and R2 can be hydrogen, methyl, ethyl, propyl or butyl. For example, R can include about 1 carbon atom, about 2 carbon atoms, about 3 carbon atoms, about 4 carbon atoms, about 5 carbon atoms, about 6 carbon atoms, about 7 carbon atoms, about 8 carbon atoms, about 9 carbon atoms, about 10 carbon atoms, about 12 carbon atoms, about 14 carbon atoms, about 16 carbon atoms, about 18 carbon atoms, about 20 carbon atoms, about 22 carbon atoms, about 24 carbon atoms, about 26 carbon atoms, about 28 carbon atoms or about 30 carbon atoms. An example of a diacid is adipic acid (hexanedioic acid) which contains six carbon atoms. A diacid sometimes is a C4 to a C24 diacid (i.e., a diacid containing 4 carbons to 24 carbons) and sometimes is a C8, C10, C12, C14, C16, C18, or C20 diacid. A hydrocarbon portion of a diacid sometimes is fully saturated and sometimes a diacid includes one or more unsaturations (e.g., double bonds). In some embodiments, genetically modified cells and organisms and processes provided herein are capable of producing a diacid.

Non-limiting examples of diacids include octadecanedioic acid, decanedioic acid, dodecanedioic acid, tetradecanedioic acid, hexadecanedioic acid, octadecanedioic acid, eicosanedioic acid and other organic intermediates. Non-limiting examples of fatty dicarboxylic acids include adipic acid (hexanedioic acid, 1,4-butanedicarboxylic acid), suberic acid (i.e., octanedioic acid, 1,8-octanedioic acid, octanedioic acid, octane-1,8-dioic acid, 1,6-hexanedicarboxylic acid, capryllic diacids), sebacic acid (i.e., 1,10-decanedioic acid, decanedioic acid, decane-1,10-dioic acid,

1,8-octanedicarboxylic acid, capric diacid), azelaic acid, undecanedioc acid, dodecanedioic acid (i.e., DDDA, 1,12-dodecanedioic acid, dodecanedioic acid, dodecane-1,12-dioic acid, 1,10-decanedicarboxylic acid, decamethylenedicaboxylic acid, 1,10-dicarboxydecane, lauric diacid), tetradecanedioic acid (i.e., TDDA, 1,14-tetradecanedioic acid, tetradecanedioic acid, tetradecane-1,14-dioic acid, 1,12-dodecanedicarboxylic acid, myristic diacid), thapsic acid (i.e., hexadecanedioic acid, 1,16-hexadecanedioic acid, hexadecanedioic acid, hexadecane-1,16-dioic acid, 1,14-tetradecanedicarboxylic acid, palmitic diacid), cis-9-hexadecenedioic acid (i.e., palmitoleic diacids), octanedioic acid (i.e., 1,18-octadecanedioic acid, octadecanedioic acid, octadecane-1,18-dioic acid, 1,16-hexadecanedicarboxylic acid, stearic diacid), cis-9-octadecenedioic acid (i.e., oleic diacids), cis-9,12-octadecenedioic acid (i.e., linoleic diacids), cis-9,12,15-octadecenedioic acid (i.e., linolenic diacids), arachidic diacid (i.e., eicosanoic diacid, icosanoic diacid), 11-eicosenoic diacid (i.e., cis-11-eicosenedioic acid), 13-eicosenoic diacids (i.e., cis-13-eicosenedioic acid), arachidonic diacid (i.e., cis-5,8,11,14-eicosatetraenedioic acid) and salts and esters of fatty acids, including, for example, any of the foregoing diacids.

The term "paraffin" as used herein refers to the common name for alkane hydrocarbons, independent of the source (e.g., plant derived, petroleum derived, chemically synthesized, fermented by a microorganism), or carbon chain length. A carbon source sometimes comprises a paraffin, and in some embodiments, a paraffin is predominant in a carbon source (e.g., about 75%, 80%, 85%, 90% or 95% paraffin). A paraffin sometimes is saturated (e.g., fully saturated), sometimes includes one or more unsaturations (e.g., about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 unsaturations) and sometimes is substituted with one or more non-hydrogen substituents. Non-limiting examples of non-hydrogen substituents include halo, acetyl, =O, =N-CN, =N-OR, =NR, OR, $NR_2$, SR, $SO_2R$, $SO_2NR_2$, $NRSO_2R$, $NRCONR_2$, NRCOOR, NRCOR, CN, COOR, $CONR_2$, OOCR, COR, and $NO_2$, where each R is independently H, C1-C8 alkyl, C2-C8 heteroalkyl, C1-C8 acyl, C2-C8 heteroacyl, C2-C8 alkenyl, C2-C8 heteroalkenyl, C2-C8 alkynyl, C2-C8 heteroalkynyl, C6-C10 aryl, or C5-C10 heteroaryl, and each R is optionally substituted with halo, =O, =N-CN, =N-OR', =NR', OR', $NR'_2$, SR', $SO_2R'$, $SO_2NR'_2$, $NR'SO_2R'$, $NR'CONR'_2$, NR'COOR', NR'COR', CN, COOR', $CONR'_2$, OOCR', COR', and $NO_2$, where each R' is independently H, C1-C8 alkyl, C2-C8 heteroalkyl, C1-C8 acyl, C2-C8 heteroacyl, C6-C10 aryl or C5-C10 heteroaryl.

A carbon source sometimes comprises an alkyl, alkenyl or alkynyl compound or molecule (e.g., a compound that includes an alkyl, alkenyl or alkynyl moiety (e.g., alkane, alkene, alkyne)). In



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certain embodiments, an alkyl, alkenyl or alkynyl molecule, or combination thereof, is predominant in a carbon source (e.g., about 75%, 80%, 85%, 90% or 95% of such molecules). As used herein, the terms “alkyl,” “alkenyl” and “alkynyl” include straight-chain (referred to herein as "linear"), branched-chain (referred to herein as "non-linear"), cyclic monovalent hydrocarbyl radicals, and combinations of these, which contain only C and H atoms when they are unsubstituted. Non-limiting examples of alkyl moieties include methyl, ethyl, isobutyl, cyclohexyl, cyclopentylethyl, 2-propenyl, 3-butynyl, and the like. An alkyl that contains only C and H atoms and is unsubstituted sometimes is referred to as "saturated." An alkenyl or alkynyl generally is "unsaturated" as it contains one or more double bonds or triple bonds, respectively. An alkenyl can include any number of double bonds, such as 1, 2, 3, 4 or 5 double bonds, for example. An alkynyl can include any number of triple bonds, such as 1, 2, 3, 4 or 5 triple bonds, for example. Alkyl, alkenyl and alkynyl molecules sometimes contain between about 2 to about 60 carbon atoms (C). For example, an alkyl, alkenyl and alkynyl molecule can include about 1 carbon atom, about 2 carbon atoms, about 3 carbon atoms, about 4 carbon atoms, about 5 carbon atoms, about 6 carbon atoms, about 7 carbon atoms, about 8 carbon atoms, about 9 carbon atoms, about 10 carbon atoms, about 12 carbon atoms, about 14 carbon atoms, about 16 carbon atoms, about 18 carbon atoms, about 20 carbon atoms, about 22 carbon atoms, about 24 carbon atoms, about 26 carbon atoms, about 28 carbon atoms, about 30 carbon atoms, about 32 carbon atoms, about 34 carbon atoms, about 36 carbon atoms, about 38 carbon atoms, about 40 carbon atoms, about 42 carbon atoms, about 44 carbon atoms, about 46 carbon atoms, about 48 carbon atoms, about 50 carbon atoms, about 52 carbon atoms, about 54 carbon atoms, about 56 carbon atoms, about 58 carbon atoms or about 60 carbon atoms. In some embodiments, paraffins can have a mean number of carbon atoms of between about 8 to about 18 carbon atoms (e.g., about 8 carbon atoms, about 9 carbon atoms, about 10 carbon atoms, about 11 carbon atoms, about 12 carbon atoms, about 13 carbon atoms, about 14 carbon atoms, about 15 carbon atoms, about 16 carbon atoms, about 17 carbon atoms and about 18 carbon atoms). A single group can include more than one type of multiple bond, or more than one multiple bond. Such groups are included within the definition of the term “alkenyl” when they contain at least one carbon-carbon double bond, and are included within the term “alkynyl” when they contain at least one carbon-carbon triple bond. Alkyl, alkenyl and alkynyl molecules include molecules that comprise an alkyl, alkenyl and/or alkynyl moiety, and include molecules that consist of an alkyl, alkenyl or alkynyl moiety (i.e., alkane, alkene and alkyne molecules).

Alkyl, alkenyl and alkynyl substituents sometimes contain 1-20C (alkyl) or 2-20C (alkenyl or alkynyl). They can contain about 8-14C or about 10-14C in some embodiments. A single group can include more than one type of multiple bond, or more than one multiple bond. Such groups are included within the definition of the term "alkenyl" when they contain at least one carbon-carbon double bond, and are included within the term "alkynyl" when they contain at least one carbon-carbon triple bond.

Alkyl, alkenyl and alkynyl groups or compounds sometimes are substituted to the extent that such substitution can be synthesized and can exist. Typical substituents include, but are not limited to, halo, acetyl, =O, =N-CN, =N-OR, =NR, OR, $NR_2$, SR, $SO_2R$, $SO_2NR_2$, $NRSO_2R$, $NRCONR_2$, NRCOOR, NRCOR, CN, COOR, $CONR_2$, OOCR, COR, and $NO_2$, where each R is independently H, C1-C8 alkyl, C2-C8 heteroalkyl, C1-C8 acyl, C2-C8 heteroacyl, C2-C8 alkenyl, C2-C8 heteroalkenyl, C2-C8 alkynyl, C2-C8 heteroalkynyl, C6-C11 aryl, or C5-C11 heteroaryl, and each R is optionally substituted with halo, =O, =N-CN, =N-OR', =NR', OR', $NR'_2$, SR', $SO_2R'$, $SO_2NR'_2$, $NR'SO_2R'$, $NR'CONR'_2$, NR'COOR', NR'COR', CN, COOR', $CONR'_2$, OOCR', COR', and $NO_2$, where each R' is independently H, C1-C8 alkyl, C2-C8 heteroalkyl, C1-C8 acyl, C2-C8 heteroacyl, C6-C10 aryl or C5-C10 heteroaryl. Alkyl, alkenyl and alkynyl groups can also be substituted by C1-C8 acyl, C2-C8 heteroacyl, C6-C10 aryl or C5-C10 heteroaryl, each of which can be substituted by the substituents that are appropriate for the particular group.

"Acetylene" substituents are 2-10C alkynyl groups that are optionally substituted, and are of the formula -C≡C-Ri, where Ri is H or C1-C8 alkyl, C2-C8 heteroalkyl, C2-C8 alkenyl, C2-C8 heteroalkenyl, C2-C8 alkynyl, C2-C8 heteroalkynyl, C1-C8 acyl, C2-C8 heteroacyl, C6-C10 aryl, C5-C10 heteroaryl, C7-C12 arylalkyl, or C6-C12 heteroarylalkyl, and each Ri group is optionally substituted with one or more substituents selected from halo, =O, =N-CN, =N-OR', =NR', OR', NR'2, SR', $SO_2R'$, $SO_2NR'_2$, $NR'SO_2R'$, $NR'CONR'_2$, NR'COOR', NR'COR', CN, COOR', $CONR'_2$, OOCR', COR', and $NO_2$, where each R' is independently H, C1-C6 alkyl, C2-C6 heteroalkyl, C1-C6 acyl, C2-C6 heteroacyl, C6-C10 aryl, C5-C10 heteroaryl, C7-12 arylalkyl, or C6-12 heteroarylalkyl, each of which is optionally substituted with one or more groups selected from halo, C1-C4 alkyl, C1-C4 heteroalkyl, C1-C6 acyl, C1-C6 heteroacyl, hydroxy, amino, and =O; and where two R' can be linked to form a 3-7 membered ring optionally containing up to three heteroatoms selected from N, O and S. In some embodiments, Ri of -C≡C-Ri is H or Me.



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A carbon source sometimes comprises a heteroalkyl, heteroalkenyl and/or heteroalkynyl molecule or compound (e.g., comprises heteroalkyl, heteroalkenyl and/or heteroalkynyl moiety (e.g., heteroalkane, heteroalkene or heteroalkyne)). "Heteroalkyl", "heteroalkenyl", and "heteroalkynyl" and the like are defined similarly to the corresponding hydrocarbyl (alkyl, alkenyl and alkynyl) groups, but the 'hetero' terms refer to groups that contain one to three O, S or N heteroatoms or combinations thereof within the backbone; thus at least one carbon atom of a corresponding alkyl, alkenyl, or alkynyl group is replaced by one of the specified heteroatoms to form a heteroalkyl, heteroalkenyl, or heteroalkynyl group. The typical and sizes for heteroforms of alkyl, alkenyl and alkynyl groups are generally the same as for the corresponding hydrocarbyl groups, and the substituents that may be present on the heteroforms are the same as those described above for the hydrocarbyl groups. For reasons of chemical stability, it is also understood that, unless otherwise specified, such groups do not include more than two contiguous heteroatoms except where an oxo group is present on N or S as in a nitro or sulfonyl group.

The term "alkyl" as used herein includes cycloalkyl and cycloalkylalkyl groups and compounds, the term "cycloalkyl" may be used herein to describe a carbocyclic non-aromatic compound or group that is connected via a ring carbon atom, and "cycloalkylalkyl" may be used to describe a carbocyclic non-aromatic compound or group that is connected to a molecule through an alkyl linker. Similarly, "heterocyclyl" may be used to describe a non-aromatic cyclic group that contains at least one heteroatom as a ring member and that is connected to the molecule via a ring atom, which may be C or N; and "heterocyclylalkyl" may be used to describe such a group that is connected to another molecule through a linker. The sizes and substituents that are suitable for the cycloalkyl, cycloalkylalkyl, heterocyclyl, and heterocyclylalkyl groups are the same as those described above for alkyl groups. As used herein, these terms also include rings that contain a double bond or two, as long as the ring is not aromatic.

A carbon source sometimes comprises an acyl compound or moiety (e.g., compound comprising an acyl moiety). As used herein, "acyl" encompasses groups comprising an alkyl, alkenyl, alkynyl, aryl or arylalkyl radical attached at one of the two available valence positions of a carbonyl carbon atom, and heteroacyl refers to the corresponding groups where at least one carbon other than the carbonyl carbon has been replaced by a heteroatom chosen from N, O and S. Thus heteroacyl includes, for example, -C(=O)OR and $-C(=O)NR_2$ as well as –C(=O)-heteroaryl.



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Acyl and heteroacyl groups are bonded to any group or molecule to which they are attached through the open valence of the carbonyl carbon atom. Typically, they are C1-C8 acyl groups, which include formyl, acetyl, pivaloyl, and benzoyl, and C2-C8 heteroacyl groups, which include methoxyacetyl, ethoxycarbonyl, and 4-pyridinoyl. The hydrocarbyl groups, aryl groups, and heteroforms of such groups that comprise an acyl or heteroacyl group can be substituted with the substituents described herein as generally suitable substituents for each of the corresponding component of the acyl or heteroacyl group.

A carbon source sometimes comprises one or more aromatic moieties and/or heteroaromatic moieties. "Aromatic" moiety or "aryl" moiety refers to a monocyclic or fused bicyclic moiety having the well-known characteristics of aromaticity; examples include phenyl and naphthyl. Similarly, "heteroaromatic" and "heteroaryl" refer to such monocyclic or fused bicyclic ring systems which contain as ring members one or more heteroatoms selected from O, S and N. The inclusion of a heteroatom permits aromaticity in 5 membered rings as well as 6 membered rings. Typical heteroaromatic systems include monocyclic C5-C6 aromatic groups such as pyridyl, pyrimidyl, pyrazinyl, thienyl, furanyl, pyrrolyl, pyrazolyl, thiazolyl, oxazolyl, and imidazolyl and the fused bicyclic moieties formed by fusing one of these monocyclic groups with a phenyl ring or with any of the heteroaromatic monocyclic groups to form a C8-C10 bicyclic group such as indolyl, benzimidazolyl, indazolyl, benzotriazolyl, isoquinolyl, quinolyl, benzothiazolyl, benzofuranyl, pyrazolopyridyl, quinazolinyl, quinoxalinyl, cinnolinyl, and the like. Any monocyclic or fused ring bicyclic system which has the characteristics of aromaticity in terms of electron distribution throughout the ring system is included in this definition. It also includes bicyclic groups where at least the ring which is directly attached to the remainder of the molecule has the characteristics of aromaticity. Typically, the ring systems contain 5-12 ring member atoms. The monocyclic heteroaryls sometimes contain 5-6 ring members, and the bicyclic heteroaryls sometimes contain 8-10 ring members.

Aryl and heteroaryl moieties may be substituted with a variety of substituents including C1-C8 alkyl, C2-C8 alkenyl, C2-C8 alkynyl, C5-C12 aryl, C1-C8 acyl, and heteroforms of these, each of which can itself be further substituted; other substituents for aryl and heteroaryl moieties include halo, OR, $NR_2$, SR, $SO_2R$, $SO_2NR_2$, $NRSO_2R$, $NRCONR_2$, NRCOOR, NRCOR, CN, COOR, $CONR_2$, OOCR, COR, and $NO_2$, where each R is independently H, C1-C8 alkyl, C2-C8 heteroalkyl, C2-C8 alkenyl, C2-C8 heteroalkenyl, C2-C8 alkynyl, C2-C8 heteroalkynyl, C6-C10

aryl, C5-C10 heteroaryl, C7-C12 arylalkyl, or C6-C12 heteroarylalkyl, and each R is optionally substituted as described above for alkyl groups. The substituent groups on an aryl or heteroaryl group may be further substituted with the groups described herein as suitable for each type of such substituents or for each component of the substituent. Thus, for example, an arylalkyl substituent may be substituted on the aryl portion with substituents typical for aryl groups, and it may be further substituted on the alkyl portion with substituents as typical or suitable for alkyl groups.

Similarly, "arylalkyl" and "heteroarylalkyl" refer to aromatic and heteroaromatic ring systems, which are stand-alone molecules (e.g., benzene or substituted benzene, pyridine or substituted pyridine), or which are bonded to an attachment point through a linking group such as an alkylene, including substituted or unsubstituted, saturated or unsaturated, cyclic or acyclic linkers. A linker often is C1-C8 alkyl or a hetero form thereof. These linkers also may include a carbonyl group, thus making them able to provide substituents as an acyl or heteroacyl moiety. An aryl or heteroaryl ring in an arylalkyl or heteroarylalkyl group may be substituted with the same substituents described above for aryl groups. An arylalkyl group sometimes includes a phenyl ring optionally substituted with the groups defined above for aryl groups and a C1-C4 alkylene that is unsubstituted or is substituted with one or two C1-C4 alkyl groups or heteroalkyl groups, where the alkyl or heteroalkyl groups can optionally cyclize to form a ring such as cyclopropane, dioxolane, or oxacyclopentane. Similarly, a heteroarylalkyl group often includes a C5-C6 monocyclic heteroaryl group optionally substituted with one or more of the groups described above as substituents typical on aryl groups and a C1-C4 alkylene that is unsubstituted. A heteroarylalkyl group sometimes is substituted with one or two C1-C4 alkyl groups or heteroalkyl groups, or includes an optionally substituted phenyl ring or C5-C6 monocyclic heteroaryl and a C1-C4 heteroalkylene that is unsubstituted or is substituted with one or two C1-C4 alkyl or heteroalkyl groups, where the alkyl or heteroalkyl groups can optionally cyclize to form a ring such as cyclopropane, dioxolane, or oxacyclopentane.

Where an arylalkyl or heteroarylalkyl group is described as optionally substituted, the substituents may be on the alkyl or heteroalkyl portion or on the aryl or heteroaryl portion of the group. The substituents optionally present on the alkyl or heteroalkyl portion sometimes are the same as those described above for alkyl groups, and the substituents optionally present on the aryl or heteroaryl portion often are the same as those described above for aryl groups generally.



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"Arylalkyl" groups as used herein are hydrocarbyl groups if they are unsubstituted, and are described by the total number of carbon atoms in the ring and alkylene or similar linker. Thus a benzyl group is a C7-arylalkyl group, and phenylethyl is a C8-arylalkyl.

"Heteroarylalkyl" as described above refers to a moiety comprising an aryl group that is attached through a linking group, and differs from "arylalkyl" in that at least one ring atom of the aryl moiety or one atom in the linking group is a heteroatom selected from N, O and S. The heteroarylalkyl groups are described herein according to the total number of atoms in the ring and linker combined, and they include aryl groups linked through a heteroalkyl linker; heteroaryl groups linked through a hydrocarbyl linker such as an alkylene; and heteroaryl groups linked through a heteroalkyl linker. Thus, for example, C7-heteroarylalkyl includes pyridylmethyl, phenoxy, and N-pyrrolylmethoxy.

"Alkylene" as used herein refers to a divalent hydrocarbyl group. Because an alkylene is divalent, it can link two other groups together. An alkylene often is referred to as $-(CH_2)_n-$ where n can be 1-20, 1-10, 1-8, or 1-4, though where specified, an alkylene can also be substituted by other groups, and can be of other lengths, and the open valences need not be at opposite ends of a chain. Thus –CH(Me)- and $-C(Me)_2-$ may also be referred to as alkylenes, as can a cyclic group such as cyclopropan-1,1-diyl. Where an alkylene group is substituted, the substituents include those typically present on alkyl groups as described herein.

In certain embodiments, the feedstock contains a mixed set of aliphatic molecules from which diacids may be produced. In some embodiments, an aliphatic molecule in the feedstock is the predominant aliphatic species and sometimes a particular fatty acid produced from that aliphatic molecule is the predominant fatty acid species produced. A predominant species generally is 51% or more by weight of aliphatic molecule species in a feedstock or 51% or more by weight of diacid species in a product (e.g., about 55% or more, 60% or more, 65% or more, 70% or more, 75% or more, 80% or more, 85% or more, 90% or more or 95% or more).

*Target production, isolation and yield*

Provided herein are methods for producing a target molecule or one or more target molecules. For example, in some embodiments, a method for producing one or more target molecules includes culturing a modified cell, microorganism or organism such as any of the modified cells



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and organisms described herein, under conditions in which the cell, organism or microorganism produces one or more target molecules. In some embodiments, a method for producing one or more target molecules includes contacting an engineered cell, organism, or microorganism with a carbon source (e.g., a feedstock, including a feedstock containing a fatty acid or alkane) under conditions whereby one or more target molecules is produced. In various embodiments of the methods provided herein, a target molecule, e.g., a fatty acid, including, for example, a dicarboxylic acid (e.g., adipic acid), and salts and/or esters thereof, is isolated or purified from the culture media or extracted from the engineered cells or organisms. Target molecule yield may be expressed as percent (%) theoretical yield, percent (%) maximum theoretical yield, units of target molecule produced per unit of feedstock added (e.g., grams of target molecule produced per gram of feedstock added), units of target molecule per volume of culture (e.g., grams of target molecule per liter of culture), units of target molecule per volume of cells (e.g., grams of target molecule per liter of cells), units of target molecule per weight of cells (e.g., grams of target molecule per dry cell weight (DCW) of cells), units of target molecule per volume of culture per unit of time (e.g., grams of target molecule per liter of culture per hour), and/or fold change (increase or decrease) when comparing target molecule production by a modified cell to production by an unmodified cell.

In some embodiments, fermentation of feedstocks by methods described herein can produce a target molecule product at a level of about 10% to about 100% of theoretical yield (e.g., about 15%, about 20%, about 25% or more of theoretical yield (e.g., 25% or more, 26% or more, 27% or more, 28% or more, 29% or more, 30% or more, 31% or more, 32% or more, 33% or more, 34% or more, 35% or more, 36% or more, 37% or more, 38% or more, 39% or more, 40% or more, 41% or more, 42% or more, 43% or more, 44% or more, 45% or more, 46% or more, 47% or more, 48% or more, 49% or more, 50% or more, 51% or more, 52% or more, 53% or more, 54% or more, 55% or more, 56% or more, 57% or more, 58% or more, 59% or more, 60% or more, 61% or more, 62% or more, 63% or more, 64% or more, 65% or more, 66% or more, 67% or more, 68% or more, 69% or more, 70% or more, 71% or more, 72% or more, 73% or more, 74% or more, 75% or more, 76% or more, 77% or more, 78% or more, 79% or more, 80% or more, 81% or more, 82% or more, 83% or more, 84% or more, 85% or more, 86% or more, 87% or more, 88% or more, 89% or more, 90% or more, 91% or more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more of theoretical yield). The term "theoretical yield" as used herein refers to the amount of product that could be made from a starting material if the reaction is 100% complete. Theoretical yield

is based on the stoichiometry of a reaction and ideal conditions in which starting material is completely consumed, undesired side reactions do not occur, the reverse reaction does not occur, and there are no losses in the work-up procedure. Culture media may be tested for target product concentration and drawn off when the concentration reaches a predetermined level. Detection methods are known in the art, including but not limited to chromatographic methods (e.g., gas chromatography) or combined chromatographic/mass spectrometry (e.g., GC-MS) methods. Target product may be present at a range of levels as described herein.

A target product sometimes is retained within an engineered cell or organism after a culture process is completed, and in certain embodiments, the target product is secreted out of the cell or organism into the culture medium. For example, in some embodiments in which a target molecule is secreted, culture media may be drawn from the culture system and fresh medium may be supplemented, and/or target product may be extracted from the culture media during or after the culture process is completed. Engineered cells or organisms may be cultured on or in solid, semi-solid or liquid media. In some embodiments media is drained from cells adhering to a plate. In certain embodiments, a liquid-cell mixture is centrifuged at a speed sufficient to pellet the cells but not disrupt the cells and allow extraction of the media, as known in the art. The cells may then be resuspended in fresh media. Target product may be purified from culture media according to methods known in the art.

Provided herein are non-limiting examples of methods useful for recovering target product from fermentation broth and/or isolating/partially purifying a target product from non-target products when utilizing mixed chain length feedstocks. Recovery of a fatty dicarboxylic acid (e.g., adipic acid, sebacic acid, suberic acid, octanedioic acid, decanedioic acid, dodecanedioic acid, tetradecanedioic acid, hexadecanedioic acid, octadecanedioic acid, eicosanedioic acid) from fermentation broth can be accomplished using a variety of methods. Generally, for example, the cells are filtered away from the media, and the target molecule is extracted with a water-imiscible solvent appropriate for target chemical. Optionally, a centrifugation step can first be employed to separate cell mass and a fatty dicarboxylic acid from the aqueous phase.

A fatty dicarboxylic acid has limited solubility in water under fermentation conditions, and can have a density similar to that of water. In some embodiments, upon centrifugation, the majority of fatty dicarboxylic acid may be pulled away from the water stream, and be concentrated in the cell mass stream. The concentrated fatty dicarboxylic acid stream can then be further



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concentrated via filtration steps (e.g., solid dodecanedioic acid will be retained on a filter, allowing water to pass through, concentrating the product). Once the fatty dicarboxylic acid is concentrated to the desired level, the temperature can be increased to above its melting point of 130°C. After the fatty dicarboxylic acid is melted, the remaining impurities can be removed via filtration; the final product may be recovered by decreasing the temperature, allowing the fatty dicarboxylic acid to solidify, and collecting the solid product.

Alternatively, a fatty dicarboxylic acid can be recovered from fermentation broth by first extracting the broth with an organic solvent in which a fatty dicarboxylic acid is soluble. The organic solvent phase can then be filtered through various membranes to further purify the fatty dicarboxylic acid. Subsequent extractions with the same or a different organic solvent can then be performed and each round of extraction can be followed by membrane filtration to further concentrate the fatty dicarboxylic acid. The organic solvent can be evaporated, leaving the fatty dicarboxylic acid behind as a residue and the residue can be dried to provide the fatty dicarboxylic acid in solid form.

In certain embodiments, target product is extracted from the cultured engineered cells or organisms. The cells may be concentrated through centrifugation at a speed sufficient to shear the cell membranes. In some embodiments, the cells may be physically disrupted (e.g., shear force, sonication) or chemically disrupted (e.g., contacted with detergent or other lysing agent). The phases may be separated by centrifugation or other method known in the art and target product may be isolated according to known methods.

Commercial grade target product sometimes is provided in substantially pure form (e.g., 90% pure or greater, 95% pure or greater, 99% pure or greater or 99.5% pure or greater). In some embodiments, target product may be modified into any one of a number of downstream products. For example, a fatty dicarboxylic acid (e.g., octanedioic acid, decanedioic acid, dodecanedioic acid, tetradecanedioic acid, hexadecanedioic acid, octadecanedioic acid, eicosanedioic acid) may be polycondensed with hexamethylenediamine to produce nylon. Nylon may be further processed into fibers for applications in carpeting, automobile tire cord and clothing. A fatty dicarboxylic acid can also be used for manufacturing plasticizers, lubricant components and polyester polyols for polyurethane systems. Various esters of food grade fatty dicarboxylic acids can be used as components in fragrance manufacture, gelling aids, flavorings, acidulant, leavening and buffering agent. A fatty dicarboxylic acid has two carboxylic

acid (-COOH) groups, which can yield two kinds of salts. Its derivatives, acyl halides, anhydrides, esters, amides and nitriles, are used in making a variety of downstream products through further reactions of substitution, catalytic reduction, metal hydride reduction, diborane reduction, keto formation with organometallic reagents, electrophile bonding at oxygen, and condensation.

Target product may be provided within cultured cells and organisms containing target product, and cultured cells may be supplied fresh or frozen in a liquid media or dried. Fresh or frozen cells or organisms may be contained in appropriate moisture-proof containers that may also be temperature controlled as necessary. Target product sometimes is provided in culture medium that is substantially cell-free. In some embodiments target product or modified target product purified from cells or organisms is provided, and target product sometimes is provided in substantially pure form. In certain embodiments crystallized or powdered target product is provided. For example, dodecanedioic acid (1,12 dodecanedioic acid; DDDA) is a white powder or crystal with a melting point of between 260°F and 266°F. Sebacic acid (1,8 ocatanedicarboxylic acid) is also a white powder or crystal with a melting point of between 268°F and 274°F. A crystallized or powdered fatty dicarboxylic acid may be transported in a variety of containers including one ton cartons, drums, 50 pound bags and the like.

In certain embodiments, a target product is produced with a yield of about 0.50 grams of target product per gram of feedstock or carbon source added, or greater; 0.51 grams of target product per gram of feedstock or carbon source added, or greater; 0.52 grams of target product per gram of feedstock or carbon source added, or greater; 0.53 grams of target product per gram of feedstock or carbon source added, or greater; 0.54 grams of target product per gram of feedstock or carbon source added, or greater; 0.55 grams of target product per gram of feedstock or carbon source added, or greater; 0.56 grams of target product per gram of feedstock or carbon source added, or greater; 0.57 grams of target product per gram of feedstock or carbon source added, or greater; 0.58 grams of target product per gram of feedstock or carbon source added, or greater; 0.59 grams of target product per gram of feedstock or carbon source added, or greater; 0.60 grams of target product per gram of feedstock or carbon source added, or greater; 0.61 grams of target product per gram of feedstock or carbon source added, or greater; 0.62 grams of target product per gram of feedstock or carbon source added, or greater; 0.63 grams of target product per gram of feedstock or carbon source added, or greater; 0.64 grams of target product per gram of

feedstock or carbon source added, or greater; 0.65 grams of target product per gram of feedstock or carbon source added, or greater; 0.66 grams of target product per gram of feedstock or carbon source added, or greater; 0.67 grams of target product per gram of feedstock or carbon source added, or greater; 0.68 grams of target product per gram of feedstock or carbon source added, or greater; 0.69 grams of target product per gram of feedstock or carbon source added, or greater; 0.70 grams of target product per gram of feedstock or carbon source added or greater; 0.71 grams of target product per gram of feedstock or carbon source added, or greater; 0.72 grams of target product per gram of feedstock or carbon source added, or greater; 0.73 grams of target product per gram of feedstock or carbon source added, or greater; 0.74 grams of target product per gram of feedstock or carbon source added, or greater; 0.75 grams of target product per gram of feedstock or carbon source added, or greater; 0.76 grams of target product per gram of feedstock or carbon source added, or greater; 0.77 grams of target product per gram of feedstock or carbon source added, or greater; 0.78 grams of target product per gram of feedstock or carbon source added, or greater; 0.79 grams of target product per gram of feedstock or carbon source added, or greater; 0.80 grams of target product per gram of feedstock or carbon source added, or greater; 0.81 grams of target product per gram of feedstock or carbon source added, or greater; 0.82 grams of target product per gram of feedstock or carbon source added, or greater; 0.83 grams of target product per gram of feedstock or carbon source added, or greater; 0.84 grams of target product per gram of feedstock or carbon source added, or greater; 0.85 grams of target product per gram of feedstock or carbon source added, or greater; 0.86 grams of target product per gram of feedstock or carbon source added, or greater; 0.87 grams of target product per gram of feedstock or carbon source added, or greater; 0.88 grams of target product per gram of feedstock or carbon source added, or greater; 0.89 grams of target product per gram of feedstock or carbon source added, or greater; 0.90 grams of target product per gram of feedstock or carbon source added, or greater; 0.91 grams of target product per gram of feedstock or carbon source added, or greater; 0.92 grams of target product per gram of feedstock or carbon source added, or greater; 0.93 grams of target product per gram of feedstock or carbon source added, or greater; 0.94 grams of target product per gram of feedstock or carbon source added, or greater; 0.95 grams of target product per gram of feedstock or carbon source added, or greater; 0.96 grams of target product per gram of feedstock or carbon source added, or greater; 0.97 grams of target product per gram of feedstock or carbon source added, or greater; 0.98 grams of target product per gram of

feedstock or carbon source added, or greater; 0.99 grams of target product per gram of feedstock or carbon source added, or greater; 1.0 grams of target product per gram of feedstock or carbon source added, or greater; 1.1 grams of target product per gram of feedstock or carbon source added, or greater; 1.2 grams of target product per gram of feedstock or carbon source added, or greater; 1.3 grams of target product per gram of feedstock or carbon source added, or greater; 1.4 grams of target product per gram of feedstock or carbon source added, or greater; 1.5 grams of target product per gram of feedstock or carbon source added, or greater; 1.6 grams of target product per gram of feedstock or carbon source added, or greater; 1.7 grams of target product per gram of feedstock or carbon source added, or greater; 1.8 grams of target product per gram of feedstock or carbon source added, or greater; 1.9 grams of target product per gram of feedstock or carbon source added, or greater; or about 2.0 grams of target product per gram of feedstock or carbon source added, or greater.

$Y_{max}$ is maximum theoretical yield. It is the amount of product that can be produced for a given biochemical pathway given a certain amount of consumed feedstock (e.g., grams adipic acid/grams oleic acid). Programs are available to calculate $Y_{max}$ values that can include algorithms used to calculate flux balance (see, e.g., COBRA; Becker et al. (2007) *Nature Protocols 2*:727-738). In some embodiments, the maximum theoretical yield ($Y_{max}$) of adipic acid in an engineered cell or organism is about 0.92 grams of adipic acid produced per gram of oleic acid added. In some embodiments, the maximum theoretical yield ($Y_{max}$) of suberic acid in an engineered cell or organism is about 0.96 grams of suberic acid produced per gram of oleic acid added. In some embodiments, the maximum theoretical yield ($Y_{max}$) of sebacic acid in an engineered cell or organism is about 0.99 grams of sebacic acid produced per gram of oleic acid added. In some embodiments, the maximum theoretical yield ($Y_{max}$) of docecanedioic acid in an engineered cell or organism is about 1.02 grams of dodecanedioic acid produced per gram of oleic acid added. In some embodiments, the maximum theoretical yield ($Y_{max}$) of 3-hydroxy-propionic acid in an engineered cell or organism is about 1.91 grams of 3-hydroxy-propionic acid produced per gram of oleic acid added. In some embodiments, the maximum theoretical yield ($Y_{max}$) of triacetic acid lactone in an engineered cell or organism is about 1.00 grams of triacetic acid lactone produced per gram of oleic acid added. In some embodiments, the maximum theoretical yield ($Y_{max}$) of lycopene in an engineered cell or organism is about 1.20 grams of lycopene produced per gram of oleic acid added. In some embodiments of the engineered cells and organisms and target production methods provided herein in which a carbon recycle loop is included in the engineered cell or organism, the $Y_{max}$ for production of a

particular target molecule using a particular carbon source(s) is greater than it is for the same cell or organism that does not include an engineered carbon recycle loop. In some embodiments, for example, the $Y_{max}$ may be at least about 5% to about 100% greater for a modified cell or organism engineered to include a carbon recycle loop than for the same cell or organism that does not contain an engineered recycle loop. For example, in some embodiments, the $Y_{max}$ can be at least about 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% greater for a modified cell or organism engineered to include a carbon recycle loop than for the same cell or organism that does not contain an engineered recycle loop.

The percentage of $Y_{max}$ for the engineered cell or organism under conditions in which a target molecule is produced is calculated as $(\%Y_{max}) = Y_{p/s} / Y_{max} * 100$, where $(Y_{p/s})$ = [target molecule (g/L)] * final volume of culture in flask (L)] / [feedstock added to flask (g)]. In some embodiments, the engineered cell or organism produces target molecule at about 10% to about 100% of maximum theoretical yield. In some embodiments, the engineered cell or organism produces target molecule at about 10% or greater of maximum theoretical yield, 15% or greater of maximum theoretical yield, 20% or greater of maximum theoretical yield, 25% or greater of maximum theoretical yield, 30% or greater of maximum theoretical yield, 35% or greater of maximum theoretical yield, 40% or greater of maximum theoretical yield, 45% or greater of maximum theoretical yield, 50% or greater of maximum theoretical yield, 55% or greater of maximum theoretical yield, 60% or greater of maximum theoretical yield, 65% or greater of maximum theoretical yield, 70% or greater of maximum theoretical yield, 75% or greater of maximum theoretical yield, 80% or greater of maximum theoretical yield, 85% or greater of maximum theoretical yield, 90% or greater of maximum theoretical yield, 95% or greater of maximum theoretical yield, or 100% of maximum theoretical yield.

In certain embodiments, a target molecule product (e.g., adipic acid, suberic acid, sebacic acid, dodecanedioic acid, 3-hydroxy-propionic acid, triacetic acid lactone, terpene) is produced with a yield of greater than about 0.15 grams per gram of the feedstock or carbon source (e.g., dodecane, mixed chain length alkanes, lauric acid, mixed chain length fatty acids, oil, the like or combinations of the foregoing). In some embodiments, a target molecule product is produced at between about 10% and about 100% of maximum theoretical yield of any introduced feedstock or carbon source ((e.g., about 15%, about 20%, about 25% or more of theoretical yield (e.g.,

25% or more, 26% or more, 27% or more, 28% or more, 29% or more, 30% or more, 31% or more, 32% or more, 33% or more, 34% or more, 35% or more, 36% or more, 37% or more, 38% or more, 39% or more, 40% or more, 41% or more, 42% or more, 43% or more, 44% or more, 45% or more, 46% or more, 47% or more, 48% or more, 49% or more, 50% or more, 51% or more, 52% or more, 53% or more, 54% or more, 55% or more, 56% or more, 57% or more, 58% or more, 59% or more, 60% or more, 61% or more, 62% or more, 63% or more, 64% or more, 65% or more, 66% or more, 67% or more, 68% or more, 69% or more, 70% or more, 71% or more, 72% or more, 73% or more, 74% or more, 75% or more, 76% or more, 77% or more, 78% or more, 79% or more, 80% or more, 81% or more, 82% or more, 83% or more, 84% or more, 85% or more, 86% or more, 87% or more, 88% or more, 89% or more, 90% or more, 91% or more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more of theoretical maximum yield).

In certain embodiments, a target molecule product is produced in a concentration range of between about 50 g/L to about 1000g/L of culture media (e.g., about 50 g/L, about 55 g/L, about 60 g/L, about 65 g/L, about 70 g/L, about 75 g/L, about 80 g/L, about 85 g/L, about 90 g/L, about 95 g/L, about 100 g/L, about 110 g/L, about 120 g/L, about 130 g/L, about 140 g/L, about 150 g/L, about 160 g/L, about 170 g/L, about 180 g/L, about 190 g/L, about 200 g/L, about 225 g/L, about 250 g/L, about 275 g/L, about 300 g/L, about 325 g/L, about 350 g/L, about 375 g/L, about 400 g/L, about 425 g/L, about 450 g/L, about 475 g/L, about 500 g/L, about 550 g/L, about 600 g/L, about 650 g/L, about 700 g/L, about 750 g/L, about 800 g/L, about 850 g/L, about 900 g/L, about 950 g/L, or about 1000 g/L).

In some embodiments, a target molecule product is produced at a rate of between about 0.5 g/L/hour to about 5 g/L/hour (e.g., about 0.5 g/L/hour, about 0.6 g/L/hour, about 0.7 g/L/hour, about 0.8 g/L/hour, about 0.9 g/L/hour, about 1.0 g/L/hour, about 1.1 g/L/hour, about 1.2 g/L/hour, about 1.3 g/L/hour, about 1.4 g/L/hour, about 1.5 g/L/hour, about 1.6 g/L/hour, about 1.7 g/L/hour, about 1.8 g/L/hour, about 1.9 g/L/hour, about 2.0 g/L/hour, about 2.25 g/L/hour, about 2.5 g/L/hour, about 2.75 g/L/hour, about 3.0 g/L/hour, about 3.25 g/L/hour, about 3.5 g/L/hour, about 3.75 g/L/hour, about 4.0 g/L/hour, about 4.25 g/L/hour, about 4.5 g/L/hour, about 4.75 g/L/hour, or about 5.0 g/L/hour.)

In certain embodiments, the engineered cell or organism produces between about 5-fold to about 500-fold more target molecule (a particular fatty acid, dicarboxylic acid, or other target

molecule) compared to the amount produced by a wild-type or partially engineered cell or organism of the same strain, under identical fermentation conditions (e.g., about a 5-fold increase, about a 10-fold increase, about a 15-fold increase, about a 20-fold increase, about a 25-fold increase, about a 30-fold increase, about a 35-fold increase, about a 40-fold increase, about a 45-fold increase, about a 50-fold increase, about a 55-fold increase, about a 60-fold increase, about a 65-fold increase, about a 70-fold increase, about a 75-fold increase, about a 80-fold increase, about a 85-fold increase, about a 90-fold increase, about a 95-fold increase, about a 100-fold increase, about a 125-fold increase, about a 150-fold increase, about a 175-fold increase, about a 200-fold increase, about a 250-fold increase, about a 300-fold increase, about a 350-fold increase, about a 400-fold increase, about a 450-fold increase, or about a 500-fold increase).

In some embodiments, the engineered cell or organism produces a yield of target molecule (e.g., a particular fatty acid, dicarboxylic acid, or other target molecule), in terms of the percentage of the maximum theoretical yield ($Y_{max}$) that the yield is, that is greater than the yield (as a percentage of maximum theoretical yield) produced by a wild-type or partially engineered cell or organism of the same strain, under identical culture conditions. For example, the yield of a target molecule produced by an engineered or modified cell or organism provided herein can be a percent of maximum theoretical yield that is at least about 1 unit or more greater, 2 units or more greater, 3 units or more greater, 4 units or more greater, 5 units or more greater, 6 units or more greater, 7 units or more greater, 8 units or more greater, 9 units or more greater, 10 units or more greater, 11% units or more greater, 12 units or more greater, 13% units or more greater, 14 units or more greater, 15 units or more greater, 16 units or more greater, 17 units or more greater, 18 units or more greater, 19 units or more greater, 20 units or more greater, 21 units or more greater, 22 units or more greater, 23 units or more greater, 24 units or more greater, 25 units or more greater, 26 units or more greater, 27 units or more greater, 28 units or more greater, 29 units or more greater, 30 units or more greater, 31 units or more greater, 32 units or more greater, 33 units or more greater, 34 units or more greater, 35 units or more greater, 36 units or more greater, 37 units or more greater, 38 units or more greater, 39 units or more greater, 40 units or more greater, 41 units or more greater, 42 units or more greater, 43 units or more greater, 44 units or more greater, 45 units or more greater, 46 units or more greater, 47 units or more greater, 48 units or more greater, 49 units or more greater, 50 units or more greater, or more than 50 units greater than the yield (as a percentage of maximum theoretical yield) of the target molecule produced by a wild-type or partially engineered cell or



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organism of the same strain (i.e., a reference cell or organism) under the same culture conditions, wherein a unit is defined as 1% of the maximum theoretical yield. As an illustration, if the yield of a target molecule produced by an unmodified reference cell or organism is, for example, 80% of the maximum theoretical yield (Ymax), then a target molecule yield of an engineered cell or organism that is 1 unit greater would be 81% of the maximum theoretical yield, and a target molecule yield of an engineered cell or organism that is 5 units greater would be 85% of the maximum theoretical yield, and a target molecule yield of an engineered cell or organism that is 10 units greater would be 90% of the maximum theoretical yield, and so on.

Examples

The examples set forth below illustrate certain embodiments and do not limit the technology. Certain examples set forth below utilize standard recombinant DNA and other biotechnology protocols known in the art. Many such techniques are described in detail in Maniatis, T., E. F. Fritsch and J. Sambrook (1982) *Molecular Cloning: a Laboratory Manual*; Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. DNA mutagenesis can be accomplished using the Stratagene (San Diego, CA) "QuickChange" kit according to the manufacturer's instructions.

Non-limiting examples of recombinant DNA techniques and genetic manipulation of microorganisms are described herein. In some embodiments, strains of engineered organisms described herein can be mated to combine genetic backgrounds to further enhance carbon flux management through native and/or engineered pathways described herein, for the production of a desired target product (e.g., sebacic or dodecanedioic acid).

*EXAMPLE 1: Construction of shuttle vectors and template expression vectors*

Nucleotide sequences of nucleic acids and amino acid sequences of polypeptides referred to in the Examples that follow are provided in Examples 24 - 27.

Construction of the shuttle vector pAA061

Vector pAA061 was constructed from a pUC19 backbone to harbor the selectable marker *URA3* (e.g., nucleotide SEQ ID NO: 54 and amino acid SEQ ID NO: 1) from *Candida* strain ATCC 20336 as well as modifications to allow insertion of *Candida* strain ATCC 20336 promoters and

terminators. A 1,507-bp DNA fragment containing the promoter (e.g., SEQ ID NO: 55), ORF (e.g., SEQ ID NO: 54), and terminator (e.g., SEQ ID NO: 56) of *URA3* from *Candida* strain ATCC 20336 was amplified using primers oAA0124 and oAA0125. The *URA3* PCR product was cut with NdeI/MluI and ligated into the 2,505-bp fragment of pUC19 cut with NdeI/BsmBI (an MluI compatible overhang was produced by BsmBI). In order to replace the *lac* promoter with a short 21-bp linker sequence, the resulting plasmid was opened with SphI/SapI and filled in with a linker produced by annealing oligos oAA0173 and oAA0174. The resulting plasmid was named pAA061 (Fig. 12).

Cloning of promoter and terminator pairs into vector pAA061

The multiple cloning site in pAA061 allows the insertion of promoter and terminator sequences from *Candida* strain ATCC 20336 that can subsequently be used to control the expression of an ORF (Fig. 12). The following example describes the insertion of two different promoter/terminator pairs into pAA061. This cloning method is general and can be applied to the insertion of other promoter and/or terminator sequences into pAA061.

Construction of vector pAA105

Vector pAA105 was constructed from base vector pAA061 to include the phosphoglycerate kinase (*PGK*) promoter (e.g., SEQ ID NO: 114) and terminator (e.g., SEQ ID NO: 115) regions from *Candida* strain ATCC 20336 with an intervening multiple cloning site for insertion of ORFs. The *PGK* promoter region was amplified by PCR from genomic DNA using primers oAA0347 and oAA0348. The 1,029-bp DNA fragment containing the *PGK* promoter was then cut with restriction enzymes PstI/XbaI. The *PGK* terminator region was amplified by PCR from genomic DNA using primers oAA0351 and oAA0352. The 396-bp DNA fragment containing the *PGK* terminator was then cut with restriction enzymes XbaI/NdeI. The 3,522-bp PstI/NdeI DNA fragment from pAA061 was used in a three-piece ligation reaction with the *PGK* promoter and terminator regions to produce plasmid pAA105 (Fig. 12 and Fig. 13). The sequence between the *PGK* promoter and terminator contains restriction sites for incorporating ORFs to be controlled by the constitutive *PGK* promoter. One example, plasmid pAA219, was constructed for expression of cytochrome P450 reductase (*CPRB*). The *CPRB* gene (e.g., coding nucleotide SEQ ID NO: 90) was amplified by PCR from *Candida* strain ATCC 20336 genomic



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DNA with primers oAA0570 and oAA0571. The resulting 2,071-bp amplicon was cut with BspQI and ligated into BspQI-cut plasmid pAA105 resulting in the expression vector pAA219 (Fig. 14).

Construction of vector pAA073

Vector pAA073 was constructed from base vector pAA061 to include the acyl-CoA oxidase (*POX4*) promoter (e.g., SEQ ID NO: 117) and terminator (e.g., SEQ ID NO 116) regions from *Candida* strain ATCC 20336 with an intervening multiple cloning site for insertion of ORFs (Fig. 15). The *POX4* promoter region was amplified by PCR from genomic DNA using primers oAA0208 and oAA0209. The 568-bp DNA fragment containing the *POX4* promoter was then cut with restriction enzymes PstI/XmaI. The *POX4* terminator region was amplified by PCR from genomic DNA using primers oAA0216 and oAA0217. The 214-bp DNA fragment containing the *POX4* terminator was then cut with restriction enzymes XmaI/NdeI. The 3,522-bp PstI/NdeI DNA fragment from pAA061 was used in a three-piece ligation reaction with the *POX4* promoter and terminator regions to produce plasmid pAA073 (Fig. 16 and SEQ ID NO: 110). The sequence between the *POX4* promoter and terminator contains restriction sites (including SapI and BspQI) for incorporating ORFs to be controlled by the fatty acid-inducible *POX4* promoter. One example, plasmid pAA153, was constructed for expression of cytochrome P450 monooxygenase A14 (*CYP52A14*). The *CYP52A14* gene (see, e.g., international patent application no. PCT/US2012/045615 published as WO2013/006730) was amplified by PCR from *Candida* strain ATCC 20336 genomic DNA with primers oAA0519 and oAA0520. The resulting 1,613-bp amplicon was cut with BspQI and ligated into BspQI-cut plasmid pAA073 resulting in the expression vector pAA153 (Fig. 17). In another example, plasmid pAA218, was constructed for expression of cytochrome P450 reductase (*CPRB*). The *CPRB* gene was amplified by PCR from *Candida* strain ATCC 20336 genomic DNA with primers oAA0572 and oAA0573. The resulting amplicon was cut with BspQI and ligated into BspQI-cut plasmid pAA073 resulting in the expression vector pAA218.

Table 3 lists the names of plasmids with promoter and terminator combinations that were cloned into the shuttle vector pAA061. All promoters and terminators listed were cloned from *Candida* strain ATCC 20336.



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| Table 3: *Candida* strain ATCC 20336 gene promoter/terminator combinations cloned into shuttle vector pAA061 | | |
|---|---|---|
| **Plasmid** | **Promoter** | **Terminator** |
| pAA072 | *FAO1* | *FAO1* |
| pAA073 | *POX4* | *POX4* |
| pAA074 | *POX5* | *POX5* |
| pAA105 | *PGK* | *PGK* |
| pAA332 | *TEF1* | *TEF1* |
| pAA334 | *GPD* | *GPD* |
| pAA335 | *PEX11* | *PEX11* |
| pAA1617 | *HDE* | *PEX11* |

*EXAMPLE 2: Genetic engineering methods*

Genomic DNA preparation

Genomic DNA from yeast strains such as *Candida* strain ATCC 20336 can be prepared as follows. About 1.5 ml of an overnight culture of cells is centrifuged and the pellet is resuspended in about 200 µl of a solution containing 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 MM Tris pH 8.0, and 1 mM EDTA. About 200 µl of acid washed glass beads are added with about 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1) at a pH of about 8.0. The sample is vortexed for about 2 minutes after which about 200 µl of water is added. The sample is then centrifuged at 13,000 rpm for about 10 minutes. The aqueous layer is transferred to a new microcentrifuge tube and an equal volume of chloroform:isoamyl alcohol (24:1) solution is added. This sample is vortexed for 10 seconds and then centrifuged at 13,000 rpm for about 2 minutes. The aqueous layer is transferred to a new microfuge tube and 1 ml of ethanol is added. The tube is then placed at -80 °C for about 15 minutes and then spun at 13,000 rpm for 15 minutes to pellet the DNA. The DNA is washed with 70% ethanol and air-dried. The DNA is then resuspended in about 500 µl of water.

To calculate gene copy number, a qPCR method can be used as described by Jin et a.l (*Appl. Environ. Microbiol.* January 2003 vol. 69, no. 1, 495-503). qPCR is performed according to the manufacturer's instructions using either the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies, Englewood, CO USA) or the QuantiTect Multiplex PCR NoROX Kit

(Qiagen). Genomic DNA from *Candida* strain ATCC 20336 or plasmid DNA containing the actin gene from ATCC 20336 and a gene of interest can be used as standards.

Primers and probes can be made via standard DNA synthesis techniques and be obtained, for example, from Integrated DNA Technologies (Coralville, IA, USA).

Amplification of gene copy number by single-crossover integration of linearized vectors

Linear DNA transformed into yeast can be integrated into the genome by homologous recombination. The localization of genomic integration is determined by the homologous sequence at the ends of the transformed DNA. In the case of vectors without a yeast autonomous replication sequence (ARS), integration may occur by so-called single-crossover integration of multiple tandem arrays. For example, as shown in Fig. 1, a nucleic acid, or gene, of interest (GOI) can be added to a host cell genome by recombination between homologous sequences in the transforming linear DNA and the host genome (sequences of the host cell nonfunctional *ura3* gene in the example shown in Fig. 1). A Ura$^-$ auxotrophic mutant containing a non-functional gene for orotidine 5'-phosphate decarboxylase (*ura3*) may be "rescued" to the Ura$^+$ phenotype by the genomic integration of a functional *URA3* gene. A plasmid containing a nucleic acid of interest (GOI) to be added to the host genome can be linearized by cutting in the middle of a functional *URA3* gene in the plasmid and may integrate into the genome in multiple tandem arrays to generate a functional *URA3* gene. Integration of only one copy of such a plasmid by single-crossover integration may not always result in a functional *URA3* gene (Fig. 1, part A), however integration of tandem arrays ensures that a functional *URA3* gene is formed (Fig. 1, part B). Vector pAA153 linearized by cutting in the middle of the *URA3* ORF with *ClaI* is an example of a single-crossover vector (Fig. 18).

Construction of antibiotic-free single-crossover cassettes

When a vector is linearized for single-crossover integration, all elements of the vector are integrated into the genome of the transformed organism, including any genes for antibiotic resistance used for maintaining the plasmid in *E. coli*. To avoid the integration of genes encoding antibiotic resistance into yeast production strains, plasmids were designed to allow the PCR amplification of linear DNA cassettes for single-crossover integration that did not contain antibiotic resistance genes. One example of this is the rearrangement of plasmid pAA153 (Fig.

17) to produce the antibiotic-free cassette plasmid pAA367 (Fig. 19). Plasmid pAA153 was used as the template for PCR amplification with primer pairs oAA2206/oAA2207 and oAA2208/oAA2209 producing amplicons of 1,022 bp and 2,822 bp, respectively. The two amplicons contained overlapping sequence allowing overlap extension PCR with primer pairs oAA2206/oAA2209 producing a 3,804-bp amplicon that was cloned into pCR-BluntII-TOPO (Thermo Fisher Scientific) resulting in plasmid pAA367 (Fig. 19). An antibiotic-free linear DNA cassette was amplified by PCR using pAA367 as template with primer pair oAA2206/oAA2209 and gel purified before transformation to remove the plasmid template. The 3,804-bp antibiotic-free cassette (Fig. 20) contains all of the critical DNA elements of plasmid pAA153 without the antibiotic resistance gene. Other antibiotic-free single-crossover cassettes were generated using the same general method of splitting the *URA3* selectable marker on either side of the desired promoter, gene of interest, and terminator sequences.

Generation of a Ura$^-$ mutant of *Candida* strain ATCC 20962

*Candida* strain ATCC 20962 (*pox5::ura3/pox5::ura3 pox4a::ura3/pox4b:: URA3* is a beta-oxidation blocked (*pox4Δ, pox5Δ*) and Ura$^+$ derivative of *Candida* strain ATCC 20336. To reutilize the *URA3* marker for subsequent engineering, a Ura$^-$ derivative was generated and isolated via the following method. A single colony having the Ura$^+$ phenotype was inoculated into 3 mL YPD media (10 g/L Bacto Yeast Extract, 20 g/L Bacto Peptone, and 20 g/L dextrose) and grown overnight at 30°C with shaking. The overnight culture was then harvested by centrifugation and resuspended in 1 mL YNB+YE (6.7 g/L Yeast Nitrogen Broth, 3g/L Yeast Extract). The resuspension was then serially diluted in YNB+YE and 100 uL aliquots plated on YPD plates (incubation overnight at 30°C) to determine titer of the original suspension. Additionally, triplicate 100 uL aliquots of the undiluted suspension were plated on SC Dextrose (Bacto Agar 20g/L, Uracil 0.3 g/L, Dextrose 20 g/L, Yeast Nitrogen Broth 6.7 g/L, Amino Acid Dropout Mix 2.14 g/L) and 5-FOA at 3 different concentrations (0.5, 0.75, and 1 mg/mL). Plates were incubated for at least 5 days at 30°C. Colonies arising on the SC Dextrose + 5-FOA plates were picked into 50 uL sterile, distilled water and 5 uL struck out to YPD and SC–URA (SC Dextrose medium without Uracil). Colonies growing only on YPD and not on SC–URA plates were then inoculated into 3 mL YPD and grown overnight at 30°C with shaking. The overnight culture was then harvested by centrifugation and resuspended in 1.5 mL YNB (6.7 g/L Yeast Nitrogen Broth). The resuspension was then serially diluted in YNB and 100 uL aliquots plated on YPD plates (incubation overnight at 30°C) to determine initial titer. Also, for each



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undiluted suspension, 1 mL was plated on SC–URA and incubated for up to 7 days at 30°C. Colonies on the SC-URA plates were revertants, and the isolate with the lowest reversion frequency (< $10^{-7}$) was designated sAA103 (*ura3*/*ura3 pox4*::*ura3*/*pox4*::*ura3 pox5*::*ura3*/*pox5*::*ura3*) and used for subsequent strain engineering.

Construction of double-crossover gene knock-out cassettes with recyclable *URA3* marker

Linear DNA transformed into yeast can also be integrated into the genome by double-crossover homologous recombination. Constructs for knocking out the function of a gene of interest (GOI) in *Candida* strain ATCC 20336 (or its descendants) were designed by placing a *URA3* selectable marker between 5' and 3' homologous sequences for the GOI (Fig. 21A). The *URA3* selectable marker was designed to have DNA sequence direct repeats at the beginning and at the end of the gene sequence that provide a mechanism for removing the *URA3* selectable marker from the genome by a second crossover homologous recombination event after integration of the linear DNA while retaining some of the linear DNA sequence for disruption of the GOI. This enables the transformed cell to subsequently receive additional transforming DNA using *URA3* as a selectable marker and is thus referred to as "recycling" of the *URA3* marker. The direct repeat can be the *URA3* promoter, the *URA3* terminator, or any other DNA sequence. After transformation of a double-crossover gene knock-out cassette into an $Ura^-$ mutant, the *URA3* marker allows selection on SC-URA plates for colonies that have integrated the construct (Fig. 21B) disrupting the GOI and generating an $Ura^+$ phenotype. The $Ura^+$ transformant can be isolated and subsequently treated to remove the functional *URA3* gene so that the *URA3* marker can be reused for another round of gene knock-out. The Ura+ transformant (Fig. 21B) plated on media containing 5-fluoroorotic acid (5-FOA) will only grow by rendering the *URA3* gene non-functional since cells that have an intact *URA3* gene convert, through a series of reactions, 5-FOA to fluorodeoxyuridylate, which is toxic to the cells. The DNA sequence direct repeats provide a convenient means for the cell to "loop out" the *URA3* gene by homologous recombination (Fig. 21C), resulting in a $Ura^-$ phenotype with the DNA sequence direct repeat as a "scar" left behind at the gene knock out site. The *URA3* selection marker may now be used again for further genetic modifications such as deletion of the second allele of a GOI (Fig. 22).

Construction of double-crossover gene knock-in cassettes with recyclable *URA3* marker

As described in the previous example, linear DNA cassettes may integrate into the yeast genome by double crossover homologous recombination. The DNA cassettes from the previous example can be modified to knock out a first gene of interest, GOI1, while simultaneously integrating, or knocking in, a second gene of interest, GOI2 (Fig. 3A). The GOI2 may be placed under the control of any promoter and terminator desired, except for the URA3 promoter and terminator. After transformation of a $Ura^-$ mutant, GOI1 is knocked out while GOI2 is knocked in and the *URA3* marker confers a $Ura^+$ phenotype (Fig. 3B). The *URA3* marker can be recycled as described previously by selection on media containing 5-FOA converting the phenotype back to $Ura^-$ (Fig. 3C).

*EXAMPLE 3: Modification of nucleic acids encoding carnitine acetyltransferase*

In order to generate new cells and organisms with altered carbon flux for enhanced production of target molecules, nucleic acid constructs were developed to introduce desired genetic modifications into host cells. The following Examples describe the construction and use of exemplary nucleic acid constructs in making genetic modifications of cells and organisms. Carnitine O-acetyltransferase (e.g., EC 2.3.1.7), also referred to herein as carnitine acetyltransferase, is an enzyme that reversibly links acetyl units from acetyl-CoA to the carrier molecule carnitine. In some organisms, such as, for example, certain yeast species, the enzyme is dually targeted to mitochondria and peroxisomes by N-terminal and C-terminal targeting signals, respectively (see, e.g., Elgersma *et al.* (1995) *EMBO J.* 14: 3472-3479 and Kawachi *et al.* (1996) *Eur. J. Biochem.* 238: 845-852). An N-terminal sequence is referred to as the mitochondrial targeting signal (mts) and a C-terminal sequence is referred to as the peroxisomal targeting sequence (pts). Peroxisomal acetyl-CoA not destined for the glyoxylate cycle can be converted to acetyl-carnitine by carnitine O-acetyltransferase. Due to its smaller size compared to acetyl-CoA, acetyl-carnitine is able to diffuse through pores in the peroxisomal membrane, across the cytoplasm to mitochondria where it is converted back to acetyl-CoA by mitochondrial carnitine O-acetyltransferase (see, e.g., Strijbis *et al.* (2008) *Eukaryotic Cell* 7: 610-618 and Strijbis *et al.* (2010) *J. Biol. Chem.* 285: 24335-24346).

One method for increasing the amount of cytosolic acetyl-CoA in an organism is to modify the expression of carnitine O-acetyltransferase to provide increased carnitine O-acetyltransferase



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activity in the cytoplasm. In one embodiment, increased activity can be obtained by increasing the amount of carnitine O-acetyltransferase enzyme in the cytosol. For example, engineered carnitine O-acetyltransferase proteins lacking amino acid sequence targeting signals that direct the enzyme to one or more cellular locations (e.g., peroxisomes and mitochondria) other than the cytosol can be expressed in host cells thereby increasing the amount of carnitine O-acetyltransferase in the cytosol. Such engineered proteins will remain in the cytoplasm after being produced by the organism. A modified carnitine O-acetyltransferase protein ($Cat2p^{\Delta mts\Delta pts}$) lacking a mitochondrial targeting signal (mts) and a peroxisomal targeting signal (pts) converts acetyl-carnitine in transit from the peroxisome to the mitochondria into acetyl-CoA in the cytoplasm. Modified carnitine O-acetyltransferases can be produced upon expression of heterologous nucleic acids encoding the proteins that have been introduced into host cells.

Cloning of DNA encoding Cat2p from *Candida* strain ATCC 20336

DNA encoding carnitine O-acetyltransferase (Cat2p) from *Candida* strain ATCC 20336 was amplified by PCR from genomic DNA using primers oAA2586 and oAA2587. The PCR product containing the DNA encoding Cat2p was purified and ligated into pCR-BluntII-TOPO vector (Thermo Fisher Scientific), transformed into competent TOP10 *E. coli* cells (Thermo Fisher Scientific) and clones containing PCR inserts were sequenced to confirm the correct nucleic acid sequence. The resulting sequence-confirmed plasmid was named pAA426.

Construction of *CAT2* knock out cassettes

Two *CAT2* knock-out cassettes were constructed for disruption of the two *CAT2* gene alleles in *Candida* strain ATCC 20336 using homologous recombination methods known in the art and described herein. Each cassette contained a 5' *CAT2* DNA fragment and a 3' *CAT2* DNA fragment (referred to as gene homology fragments) to provide sequence used in a first single cross over homologous recombination event that results in the insertion of the entire cassette into an endogenous *CAT2* gene. Each cassette also contained a *URA3* selectable marker gene positioned between the homology regions in the cassette. The selectable marker included a direct repeat of sequence of the *URA3* gene promoter at the 3' end of the gene that facilitates a second cross over homologous recombination event that results in excision of most of the *URA3* selectable marker gene from the allele. However, a portion of the cassette remains in the *CAT2* gene allele, which thereby disrupts and effectively knocks out the function of the endogenous



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gene. The cassettes differed in the size of the *CAT2* gene homology regions and the orientation of the *URA3* selectable marker between the homology regions. Each deletion cassette was generated by combining three DNA fragments: a 5' *CAT2* DNA fragment (i.e., gene homology fragment), a 3' *CAT2* DNA fragment (i.e., gene homology fragment) and a *URA3* gene fragment originally cloned from *Candida* strain ATCC 20336 for use as a selectable marker. In constructing the first *CAT2* deletion cassette, a *CAT2* 5' homology DNA fragment was generated by amplifying a 5' region of the *CAT2* gene from plasmid pAA426 using primers oAA2372 and oAA2373. A *CAT2* 3' homology DNA fragment was generated by amplifying a 3' region of the *CAT2* gene from plasmid pAA426 using primers oAA2376 and oAA2377. A *URA3* selectable marker DNA fragment ($P_{URA3}URA3T_{URA3}P_{URA3}$; see, e.g., SEQ ID NO: 57) was amplified by PCR from plasmid pAA298 (Fig. 23) using primers oAA2374 and oAA2375. The three PCR products were purified and combined in the same reaction to generate the first full-length deletion cassette which was assembled by overlap extension PCR. The cassette was then ligated into pCR-BluntII-TOPO generating plasmid pAA1519 (Fig. 24). The linear *CAT2* deletion cassette could then be amplified from plasmid pAA1519 with primers oAA2372 and oAA2377.

In constructing the second *CAT2* deletion cassette, a *CAT2* 5' homology DNA fragment was generated by amplifying a 5' region of the *CAT2* gene from plasmid pAA426 using primers oAA2372 and oAA3312. A *CAT2* 3' homology DNA fragment was generated by amplifying a 3' region of the *CAT2* gene from plasmid pAA426 using primers oAA3315 and oAA2377. A *URA3* selectable marker DNA fragment was amplified from plasmid pAA298 using primers oAA3313 and oAA3314. The three PCR products were purified, and combined in the same reaction to generate the second full-length deletion cassette which was assembled by overlap extension PCR. The cassette was then ligated into pCR-Blunt II-TOPO generating plasmid pAA1520. The linear *CAT2* deletion cassette could then be amplified from plasmid pAA1520 (Fig. 24) with primers oAA2372/oAA2377.

Generation of nucleic acid constructs encoding modified carnitine acetyltransferase proteins

Engineered nucleic acids encoding carnitine O-acetyltransferase lacking targeting signals were generated as follows. A portion of the *CAT2* gene from *Candida* strain ATCC 20336 was PCR amplified from pAA426 using primers oAA4719 and oAA4720, generating a DNA fragment



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(*CAT2*$^{\Delta mts}$; SEQ ID NO: 60) encoding a truncated protein lacking the 18 N-terminal amino acids normally present in the wild-type enzyme (Cat2p$^{\Delta mts}$; Fig. 25A and Fig. 25B and SEQ ID NO: 3). A plasmid backbone was then amplified from plasmid pAA1164 (Fig. 26; SEQ ID NO: 112) using primers oAA4722 and oAA4723. This DNA fragment contained all the elements of the pCR-BluntII-TOPO vector, a split *URA3* selectable marker (i.e., a *URA3* DNA fragment that has been cleaved to yield a 5' URA3 fragment and a 3' *URA3* fragment (referred to as homology regions)) originally cloned from *Candida* strain ATCC 20336, and the *HDE1* gene promoter (SEQ ID NO: 113) and *POX4* gene terminator (SEQ ID NO: 116), both from *Candida* strain ATCC 20336. The nucleic acid products amplified from pAA426 and pAA1164 contained overlapping sequence at their termini allowing directional ligation of the products to generate plasmid pAA1610. Plasmid pAA1610 contains the nucleic acid encoding the modified Cat2p$^{\Delta mts}$ protein under the control of the *HDE1* gene promoter and *POX4* gene terminator positioned between the two separate fragments of the *URA3* gene marker.

The nucleic acid segment encoding the three C-terminal residues of the modified Cat2p$^{\Delta mts}$ protein was deleted from the CAT2$^{\Delta mts}$ DNA fragment by site-directed mutagenesis in order to remove the peroxisomal targeting sequence from the encoded protein. Plasmid pAA1610 was used as template and PCR amplified using primers oAA5319 and oAA5320 containing the desired mutation eliminating the C-terminal-encoding nucleic acids. The amplification reaction was digested with *Dpn*I to eliminate the template plasmid before transformation into *E. coli* DH5α cells. One sequence verified construct was saved and named plasmid pAA1667. Plasmid pAA1667 is identical to pAA1610, other than the removal of the codons for the last 3 amino acids (the peroxisomal targeting sequence) of the Cat2p$^{\Delta mts}$ enzyme, resulting in the formation of a construct containing a modified DNA encoding Cat2p$^{\Delta mts\Delta pts}$ (Fig. 25A and Fig. 25B; SEQ ID NO: 4). The modified nucleic acid encoding Cat2p$^{\Delta mts\Delta pts}$ was referred to as *CAT2*$^{\Delta mts\Delta pts}$ (SEQ ID NO: 61).

In order to generate a DNA fragment encoding a modified carnitine O-acetyltransferase protein containing a mitochondrial targeting sequence but lacking the peroxisomal targeting sequence (Cat2p$^{\Delta pts}$), genomic DNA from strain ATCC 20336 was PCR amplified using primers oAA6472 and oAA6473 to generate DNA fragment *CAT2*$^{\Delta pts}$ (see, e.g., SEQ ID NO: 62), which is a truncated *CAT2* DNA fragment missing codons for the peroxisomal targeting sequence (PTS). A plasmid backbone containing all the elements of the pCR-BluntII-TOPO vector, a split URA3 (*Candida* strain ATCC 20336) selectable marker, and the *HDE1* gene promoter and *POX4* gene



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terminator (both from *Candida* strain ATCC20336) was then amplified from plasmid pAA1164 (Fig. 26) using primers oAA6367 and oAA6368. The nucleic acid products amplified from the genomic DNA and pAA1164 contained overlapping sequence at their termini allowing directional ligation of the products to generate plasmid pAA1876. Plasmid pAA1876 contains the nucleic acid encoding the modified Cat2p$^{\Delta pts}$ protein under the control of the *HDE1* gene promoter and *POX4* gene terminator positioned between the two separate fragments of the *URA3* gene marker.

Cloning and modification of DNA encoding Yat1p from Candida strain ATCC 20336

Another method of increasing the amount or concentration of cytosolic acetyl-CoA in an organism is to modify the expression of carnitine acetyltransferase in the organism such that there is decreased carnitine acetyltransferase activity in cellular organelles. For example, by decreasing the activity level of carnitine acetyltransferase in the mitochondria, there is a corresponding decrease in mitochondrial conversion of acetyl-carnitine to acetyl-CoA. This can introduce a bottleneck in acetyl-carnitine processing in the mitochondria which can have the effect of diverting acetyl-carnitine from entering the mitochondria from the cytoplasm. Methods of decreasing carnitine acetyltransferase activity in cellular compartments, such as the mitochondria, of a *Candida* yeast strain include replacing the wild-type promoter of an endogenous *CAT2* gene in the yeast with a weaker heterologous promoter and/or replacing or modifying a gene encoding a wild-type carnitine acetyltransferase such that the encoded modified or substituted carnitine acetyltransferase protein has a reduced enzyme activity.

The *YAT1* gene from *Candida* strain ATCC 20336 encodes a cytoplasmic-targeted enzyme with a reduced carnitine acetyltransferase activity relative to the activity of the enzyme encoded by the endogenous CAT2 gene. DNA encoding the YAT1p protein (SEQ ID NO: 6) was amplified from genomic DNA using primers oAA9946 and oAA9947 and the PCR product was purified and the sequence was verified. A plasmid backbone containing all the elements of the pCR-BluntII-TOPO vector, a split *URA3* (from *Candida* strain ATCC 20336) selectable marker, and the *HDE1* gene promoter and *POX4* gene terminator (both from *Candida* strain ATCC20336) was then amplified from plasmid pAA1164 (Fig. 26) using primers oAA4722 and oAA4723. The nucleic acid products amplified from *Candida* strain ATCC 20336 genomic DNA and pAA1164 contained overlapping sequence at their termini allowing directional ligation of the products to generate plasmid pAA1817. Plasmid pAA1817 contains the nucleic acid (see, e.g., SEQ ID NO:



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63) encoding the YAT1 protein under the control of the *HDE1* gene promoter and *POX4* gene terminator positioned between the two separate fragments of the *URA3* gene marker.

The Yat1p enzyme with carnitine acetyl transferase activity is normally targeted to the cytoplasm in *Candida* strain ATCC 20336. Modification of Yat1p to target the enzyme to the mitochondrial compartment of a yeast cell involved the addition of amino acids at the N-terminus of the wild-type protein to act as a mitochondrial targeting signal (MTS). The DNA sequences encoding the MTS for three genes encoding mitochondrial-targeted enzymes in *Candida* strain ATCC 20336 were cloned separately and added to the 5' end of the *YAT1* gene coding sequence providing DNA encoding the required N-terminal amino acids for a functional MTS. DNA encoding the predicted MTS from the Cox4p (SEQ ID NO: 7), Cit1p (SEQ ID NO: 8) and Cat2p (SEQ ID NO: 9) proteins was added to the 5' end of the coding sequence of the *YAT1* gene such that the resulting encoded protein contained the heterologous MTS in place of the native Yat1p initiating methionine (Fig. 27). Plasmid pAA1817 containing the DNA encoding the YAT1 protein was PCR amplified with primers oAA6771 and oAA6669 to yield an amplification product of the entire plasmid backbone and *YAT1* gene without the codon for the initiating methionine. The DNA sequences encoding the Cox4p MTS (SEQ ID NO: 64), Cit1p MTS (SEQ ID NO: 65) and Cat2p MTS (SEQ ID NO: 66) sequences were amplified from genomic DNA of *Candida* strain ATCC 20336 using primer pairs oAA6772/oAA6773, oAA6774/oAA6775, and oAA6776/oAA6777, respectively. The PCR products containing the modified YAT1p-encoding DNA with plasmid backbone, and the MTS-encoding sequences were designed with overlapping sequence at their termini allowing directional ligation, generating plasmids pAA1967 (containing $YAT1^{+COX4mts}$; SEQ ID NO: 67), pAA1968 (containing $YAT1^{+CIT1mts}$; SEQ ID NO: 68) and pAA1969 (containing $YAT1^{+CAT2mts}$; SEQ ID NO: 69). The modified YAT1p+mts-encoding DNA of each of plasmids pAA1967, pAA1968 and pAA1969 is under the control of the *HDE1* gene promoter and *POX4* gene terminator positioned between the two separate fragments of the *URA3* gene marker.

*EXAMPLE 4: Modification of nucleic acids encoding thioesterase proteins*

One approach to altering cellular carbon flux to recapture cytoplasmic acetyl-CoA that would have been lost to growth and energy generation processes and divert it to use in target molecule production is through an engineered acetyl group carbon recycle loop as described herein. In an exemplary recycle loop, acetyl moieties generated in the breakdown of fatty acids

in peroxisomal β-oxidation are diverted from their usual mitochondrial destination and to cytosolic fatty acid synthesis to regenerate a fatty acid that can be subjected to another cycle of peroxisomal β-oxidation. The cytosolic fatty acid that is synthesized is in the form of an acyl-CoA that can be utilized in non-target molecule processes, such as lipid synthesis, by the cell. To divert the synthesized acyl-CoA away from such processes and toward ω- and β-oxidation, it can be converted to a free fatty acid through the action of a thioesterase (e.g., EC 3.1.2.20) enzyme which catalyzes the release of coenzyme A from long-chain acyl-CoA. In yeast, thioesterases (TES) are present in the peroxisomal compartment of the cells to ensure that free coenzyme A is available for β-oxidation. In one method of producing free fatty acids in the cytoplasm of yeast, a peroxisomal thioesterase with activity on long chain fatty acids is heterologously expressed in the cytoplasm. In one embodiment, this can be accomplished by modifying nucleic acids encoding a yeast thioesterase to delete the portion of the nucleic acid encoding the peroxisomal targeting signal at the C-terminus of the protein.

Cloning DNA encoding Tesp proteins from *Candida* strain ATCC 20336

*Candida* strain ATCC 20336 contains eight genes encoding peroxisomal thioesterases. DNA encoding proteins encoded by each gene was amplified by PCR from genomic DNA using the primers indicated in Table 4. The PCR products were purified and cloned into pCR-BluntII-TOPO. Sequence confirmed plasmids were named as listed in Table 4.

**Table 4: Primers and plasmids used in amplification cloning of DNA encoding *Candida* thioesterase proteins***

| Thioesterase | Primers | Plasmid |
|---|---|---|
| TES1-1 (nucleotide SEQ ID NO: 78; amino acid SEQ ID NO: 22) | oAA3582<br>oAA3583 | pAA895 |
| TES2-1 (nucleotide SEQ ID NO: 79; amino acid SEQ ID NO: 23) | oAA3584<br>oAA3585 | pAA896 |
| TES2-2 (nucleotide SEQ ID NO: 80; amino acid SEQ ID NO: 24) | oAA3584<br>oAA3585 | pAA897 |
| TES3-1 (nucleotide SEQ ID NO: 81; amino acid SEQ ID NO: 25) | oAA3586<br>oAA3587 | pAA898 |
| TES4-1 (nucleotide SEQ | oAA3588 | pAA899 |

**Table 4: Primers and plasmids used in amplification cloning of DNA encoding *Candida* thioesterase proteins***

| Thioesterase | Primers | Plasmid |
|---|---|---|
| ID NO: 82; amino acid SEQ ID NO: 26) | oAA3589 | |
| TES5-1 (nucleotide SEQ ID NO: 83; amino acid SEQ ID NO: 27) | oAA3590<br>oAA3591 | pAA900 |
| TES6-1 (nucleotide SEQ ID NO: 84; amino acid SEQ ID NO: 28) | oAA3592<br>oAA3593 | pAA902 |
| TES7-1 (nucleotide SEQ ID NO: 85; amino acid SEQ ID NO: 29) | oAA3594<br>oAA3595 | pAA903 |
| TES7-2 (nucleotide SEQ ID NO: 86; amino acid SEQ ID NO: 30) | oAA3594<br>oAA3595 | pAA908 |
| TES8-1 (nucleotide SEQ ID NO: 87; amino acid SEQ ID NO: 31) | oAA3596<br>oAA3597 | pAA904 |

*the "-1" and "-2" designations for the thioesterases indicate which member of the sequence-confirmed allelic pair is contained in the plasmid

Construction of a *TES3*$^{\Delta pts}$ mutant

A mutant of Tes3p lacking a peroxisomal targeting sequence was constructed to retarget the Tes3p enzyme to the cytoplasm. DNA encoding Tes3p lacking the C-terminal 3 residues (SEQ ID NO: 88) was amplified from plasmid pAA898 with primers oAA6449 and oAA6450. A plasmid backbone containing all the elements of the pCR-BluntII-TOPO vector, a split URA3 selectable marker, and the *HD1E* gene promoter and *POX4* gene terminator was then amplified from plasmid pAA1164 (Fig. 26) using primers oAA6367 and oAA6368. The nucleic acid products amplified from plasmids pAA898 and pAA1164 contained overlapping sequence at their termini allowing directional ligation of the products to generate plasmid pAA1609. Plasmid pAA1609 contains a nucleic acid encoding the Tes3p$^{\Delta pts}$ protein (SEQ ID NO: 32) under the control of the *HDE1* gene promoter and *POX4* gene terminator situated between the two separate fragments of the *URA3* gene marker.

Modified forms of the proteins encoded by the other TES genes lacking a peroxisomal targeting sequence can be constructed as described for the construction of the DNA encoding Tes3p$^{\Delta pts}$.



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First, DNA encoding thioesterases lacking the C-terminal 3 residues is PCR amplified from each of pAA895, pAA896, pAA897, pAA899, pAA900, pAA902, pAA908 and pAA904. Then, to obtain a plasmid backbone containing all the elements of the pCR-BluntII-TOPO vector, a split URA3 selectable marker, the *HD1E* gene promoter and the *POX4* gene terminator, plasmid pAA1164 is amplified using primers oAA6367 and oAA6368. The final plasmid for each Tes protein-encoding DNA can then be obtained by ligating the amplicons with the backbone amplified plasmid pAA1164 through directional ligation.

*EXAMPLE 5: Modification of nucleic acids encoding acetyl-CoA carboxylase proteins*

Cytosolic acetyl-CoA can be converted to malonyl-CoA by the enzyme acetyl-CoA carboxylase (EC 6.4.1.2). This biochemical step represents a committed step in fatty acid biosynthesis and can be highly regulated. One approach to increasing fatty acid biosynthesis in an organism is to increase the acetyl-CoA carboxylase (ACC1p) activity in the cytosol. Generally, ACC1p is regulated by feedback inhibition of acyl-CoA and also by phosphorylation. Because the dephosphorylated state is the active state of the enzyme, one approach for increasing ACC1p activity is to reduce or eliminate phosphorylation of the protein. In one embodiment, the endogenous *ACC1* coding sequence is modified to encode an enzyme in which the phosphorylatable serine residues have been substituted with alanine residues thereby relieving the regulation by phosphorylation and dephosphorylation.

Cloning DNA encoding Acc1p from *Candida* strain ATCC 20336

Primers oAA0784 and oAA0785 were used to amplify DNA encoding Acc1p from genomic DNA of *Candida* strain ATCC 20336, and the resulting 6869-bp amplicon was cloned into pCR-BluntII-TOPO. The sequence-verified plasmid was named pAA245 (Fig. 28). The DNA sequence cloned into plasmid pAA245 included the entire open reading frame of the *ACC1* gene and the partial sequence for an intron at the 5' end of the *ACC1* coding sequence.

Mutation of *ACC1* to replace serine-encoding with alanine-encoding sequences

An *ACC1* gene fragment and 5' partial intron (see, e.g., SEQ ID NO: 75) were restriction cloned from pAA245 by cutting with BspQI and ligated into BspQI-cut plasmid pAA105 (Fig. 13) to put the gene under the control of the *PGK* promoter and terminator (SEQ ID NOS: 114 and 115,



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respectively) from *Candida* strain ATCC 20336. The resulting sequence-confirmed plasmid was named pAA326 (Fig. 29). A truncated version of the *ACC1* gene encoding R643 to the stop codon was generated by cutting pAA326 with SpeI/XbaI and was cloned into plasmid pAA061 (Fig. 12) cut with the same enzymes. The resulting plasmid was named pAA1634 (Fig. 30) and provided a smaller plasmid template for performing site-directed mutagenesis. Site-directed mutagenesis was performed on pAA1634 using the primers shown in Table 5 to introduce the mutations listed in the Table and resulting in plasmids pAA1647, pAA1648, pAA1649, pAA1650, and pAA1900.

In order to generate mutant *ACC1* gene fragments containing the entire protein coding sequence of nucleic acids, PCR products containing the *HDE1* promoter from *Candida* strain ATCC 20336 and the 5'-end 1,926 bp of the *ACC1* gene encoding M1 to S642 were cloned into each of the plasmids containing a truncated mutant *ACC1* fragment resulting in plasmids pAA1906-pAA1909, and pAA1915. Additionally, plasmid pAA1910 was generated in the same manner, although the *ACC1* gene in that plasmid did not contain any mutations. The S652A mutation was introduced into all of plasmids pAA1906-pAA1910 and pAA1915 by site-directed mutagenesis with primers oAA6691 and oAA6692 generating plasmids pAA1957-pAA1962.

**Table 5: Primers used in generating nucleic acid sequences encoding ACC1p mutant proteins and plasmids containing the nucleic acids**

| Truncated Plasmid | Mutation(s) | Primers | Full-Length Plasmid | IGR5 Integration Plasmid |
|---|---|---|---|---|
| pAA1634 | None | None | pAA1910 | pAA2249 |
| pAA1647 | S1138A | oAA5668<br>oAA5669 | pAA1906 | pAA2245 |
| pAA1648 | S1153A | oAA5670<br>oAA5671 | pAA1907 | pAA2246 |
| pAA1649 | S1158A | oAA5672<br>oAA5673 | pAA1908 | pAA2247 |
| pAA1650 | S1153A,<br>S1158A | oAA5690<br>oAA5691 | pAA1909 | pAA2248 |
| pAA1900 | S1138A,<br>S1153A,<br>S1158A | oAA6145<br>oAA6146 | pAA1915 | pAA2250 |
| | S652A,<br>S1138A | oAA6691<br>oAA6692 | pAA1957 | pAA2251 |
| | S652A, | oAA6691 | pAA1958 | pAA2252 |

**Table 5: Primers used in generating nucleic acid sequences encoding ACC1p mutant proteins and plasmids containing the nucleic acids**

| Truncated Plasmid | Mutation(s) | Primers | Full-Length Plasmid | IGR5 Integration Plasmid |
|---|---|---|---|---|
| | S1153A | oAA6692 | | |
| | S652A, S1158A | oAA6691 oAA6692 | pAA1959 | pAA2253 |
| | S652A, S1153A, S1158A | oAA6691 oAA6692 | pAA1960 | pAA2254 |
| | S652A | oAA6691 oAA6692 | pAA1961 | pAA2255 |
| | S652A, S1138A, S1153A, S1158A | oAA6691 oAA6692 | pAA1962 | pAA2256 |

Construction of double crossover integration cassettes for *ACC1* mutants

The *ACC1* mutant genes under the control of the *HDE1* promoter and *PGK* terminator ($P_{HDE}$-*ACC1*-$T_{PGK}$) were PCR amplified from plasmids pAA1906-pAA1909, pAA1915, and pAA1957-pAA1962, as well as the unmodified *ACC1* gene from pAA1910, with primers oAA7257 and oAA7258. The PCR products were cut with SbfI/MluI and ligated into plasmid pAA2153 cut with the same restriction enzymes. Plasmid pAA2153 contains DNA encoding a *URA3* selectable marker with a direct repeat of the URA3 terminator sequence ($T_{URA3}$) sequence, i.e., $T_{URA3}$-$P_{URA3}$-*URA3*-$T_{URA3}$ (see, e.g., SEQ ID NO: 58). The *URA3* selectable marker in pAA2153 is placed between genomic DNA sequence elements from *Candida* strain ATCC 20336 which are named IGR5. The IGR5-5' (SEQ ID NO: 125; 446 bp) and IGR5-3' (SEQ ID NO: 126; 500 bp) homology regions target the integration of the intervening DNA into genomic DNA by homologous recombination. Plasmids constructed for the integration of *ACC1* mutants at IGR5 were named pAA2245 – pAA2256 (see, e.g., Fig. 31). Linear DNA used for transformation was generated by PCR with primers oAA7259 and oAA7260 using plasmids pAA2245 – pAA2256 as template DNA.



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*EXAMPLE 6: Modification of nucleic acids encoding acetyl-carnitine transporter proteins*

Another approach to increasing the amount of cytosolic acetyl-CoA in an organism is to retain acetyl-carnitine within the cytoplasm that would otherwise be transported out of the cytoplasm and into organelles, such as the mitochondria. One method for increasing the amount of acetyl-carnitine within the cytoplasm is to reduce the transport of acetyl-carnitine into the mitochondria or other organelles. Mitochondria possess two membranes with the outer membrane generally allowing free diffusion of metabolites and the inner membrane controlling metabolite transport with multiple membrane transport proteins. One mitochondrial inner-membrane transport protein (Crc1p) may function as an acetyl-carnitine transporter providing for transport of acetyl-carnitine into the mitochondria (see, e.g., van Roermund *et al.* (1999) *EMBO J.* 18: 5843-5852 and Palmieri *et al.* (1999) *FEBS Lett.* 462: 472-476). Without being bound or limited by theory, reducing the amount of this acetyl-carnitine transporter in the mitochondrial membrane can create a bottleneck in the transport of acetyl-carnitine into the mitochondria resulting in accumulation of acetyl-carnitine in the cytoplasm. The cytoplasmic acetyl-carnitine can then be converted to acetyl-CoA by a cytoplasmic carnitine O-acetyltransferase (e.g.,Cat2p$^{\Delta mts\Delta pts}$) as described herein. Reduction in the amount of Crc1p can be achieved, for example, by modifying the promoter controlling expression of DNA encoding Crc1p in a cell through introduction of heterologous nucleic acids encoding the protein into host cells that do not express a functional Crc1p transporter.

Cloning DNA encoding Crc1p from *Candida* strain ATCC 20336

The amount of CRC1p transporter expressed in the mitochondrial membrane in *Candida* host cells can be reduced by disrupting or deleting the endogenous *CRC1* gene and replacing it with a heterologous transporter-encoding construct under the control of a promoter that is weaker than the native *CRC1* promoter as described herein. DNA encoding a mitochondrial inner membrane acetyl-carnitine transport protein Crc1p (amino acid SEQ ID NO: 14; nucleotide SEQ ID NO: 71) was amplified from genomic DNA of *Candida* strain ATCC 20336 with primers oAA5511 and oAA5512. The amplified DNA was gel purified and cloned into the pCR-Blunt II-TOPO vector. Plasmids isolated from transformants were sequenced and one sequence-verified plasmid was saved as plasmid pAA1564.



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Construction of *CRC1* knock-out cassettes

*CRC1* gene knock-out cassettes were constructed for use in generating host cells that could then be transformed with heterologous nucleic acids for modified expression of Crc1p. Two *CRC1* knock-out cassettes were constructed for disruption of the two endogenous *CRC1* gene alleles in *Candida* strain ATCC 20336 using homologous recombination methods known in the art and described herein. Each cassette contained a 5' *CRC1* DNA fragment and a 3' *CRC1* DNA fragment (referred to as gene homology fragments) to provide sequence used in a first crossover homologous recombination event that results in integration of the entire cassette into an endogenous *CRC1* gene. Each cassette also contained a *URA3* selectable marker gene positioned between the homology regions in the cassette. The selectable marker included a direct repeat of sequence of the *URA3* gene promoter at the 3' end of the gene that facilitates a second cross over homologous recombination event that results in excision of most of the URA3 gene from the allele. However, a portion of the cassette remains in the *CRC1* gene which thereby disrupts and effectively knocks out the function of the endogenous gene. The cassettes differed in the sizes of the *CRC1* gene homology regions and the orientation of the *URA3* selectable marker between the homology regions. Each deletion cassette was generated by combining three DNA fragments: a 5' *CRC1* DNA fragment (i.e., gene homology fragment), a 3' *CRC1* DNA fragment (i.e., gene homology fragment) and a *URA3* selectable marker.

In constructing the first *CRC1* deletion cassette, a *CRC1* 5' homology DNA fragment was generated by amplifying a 5' region of the *CRC1* gene from plasmid pAA1564 using primers oAA5511 and oAA5553. A *CRC1* 3' homology DNA fragment was generated by amplifying a 3' region of the *CRC1* gene from plasmid pAA1564 using primers oAA5512 and oAA5554. A *URA3* selectable marker DNA fragment ($P_{URA3}URA3T_{URA3}P_{URA3}$) was amplified by PCR from plasmid pAA298 (Fig. 23) using primers oAA5555 and oAA5556. The three PCR products were purified and combined in the same reaction to generate the first full-length deletion cassette which was assembled by overlap extension PCR. The cassette was then ligated into pCR-BluntII-TOPO generating plasmid pAA1613 (Fig. 32). The linear *CRC1* deletion cassette could then be amplified from plasmid pAA1613 with primers oAA5511 and oAA5512.

A second *CRC1* deletion cassette was constructed by inserting the *URA3* selectable marker ($P_{URA3}URA3T_{URA3}P_{URA3}$) at a different location and in the opposite orientation as was done for plasmid pAA1613. A first PCR product was generated using plasmid pAA1564 as template and



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primers oAA5698 and oAA5699. This product contained 5' *CRC1* and 3' *CRC1* DNA fragments and all the elements of the pCR-Blunt II-TOPO vector. A second PCR product containing a *URA3* selectable marker ($P_{URA3}URA3T_{URA3}P_{URA3}$) was generated using plasmid pAA298 as template and primers oAA5700 and oAA5701. The two PCR products contained overlapping sequence at their termini allowing directional ligation, generating plasmid pAA1701 (Fig. 32). The linear *CRC1* deletion cassette could then be amplified from plasmid pAA1701 with primers oAA5511 and oAA5512.

Construction of modified *CRC1* genes with a heterologous promoter

The promoter for the glucose-6-phosphate isomerase gene (*G6PI*) from *Candida* strain ATCC 20336 drives low level expression of the *G6PI* gene. The *G6PI* promoter (SEQ ID NO: 118) was amplified from genomic DNA with primers oAA7409 and oAA7410. The 745-bp amplified DNA fragment was gel purified and cloned into the pCR-Blunt II-TOPO vector. Plasmids isolated from transformants were sequenced, and one sequence-verified plasmid was saved as plasmid pAA2218.

A DNA fragment containing the *G6PI* promoter, *CRC1* coding region, and *POX4* terminator ($P_{G6PI}$-*CRC1*-$T_{POX4}$) from *Candida* strain ATCC 20336 was constructed by overlap extension PCR. The *G6PI* promoter was amplified from plasmid pAA2218 with primers oAA7403 and oAA7404, the *CRC1* gene was amplified from plasmid pAA1564 using primers oAA7381 and oAA7382, and the *POX4* terminator was amplified from plasmid pAA073 using primers oAA7379 and oAA7380. The three amplicons were used in a subsequent overlap extension PCR reaction with primers oAA7379 and oAA7403 to build the desired DNA fragment, $P_{G6PI}$-*CRC1*-$T_{POX4}$. A plasmid backbone containing the IGR5 homology regions and *URA3* selectable marker (with $T_{URA3}$ repeat) was amplified from plasmid pAA2247 (Fig. 31) using primers oAA7377 and oAA7378. The resulting 6,223-bp amplified plasmid backbone and the 1,816-bp DNA fragment $P_{G6PI}$-*CRC1*-$T_{POX4}$ encoded overlapping sequence allowing directional ligation, resulting in plasmid pAA2214 SEQ ID NO: 122)6. A linear double crossover DNA cassette for genomic integration could then be amplified from plasmid pAA2214 using primers oAA7259 and oAA7260 (Fig. 33).

A plasmid containing an antibiotic-free single-crossover cassette encoding the *CRC1* gene driven by the low expression *G6PI* promoter was also constructed. A DNA fragment encoding



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the elements $P_{G6PI}$-*CRC1* was amplified from plasmid pAA2214 using primers oAA7624 and oAA7625. A plasmid backbone encoding all of the elements of the pCR-BluntII-TOPO, a split *URA3* marker, and *POX4* terminator was then amplified using primers oAA7265 and oAA7512 from plasmid pAA1164 (Fig. 26; SEQ ID NO: 112). In certain instances, a plasmid backbone was amplified from plasmid pAA1116 (SEQ ID NO: 123). The two PCR products encoded overlapping sequence at their termini allowing directional ligation placing the *CRC1* gene under the control of the *G6PI* promoter and *POX4* terminator, generating plasmid pAA2311 (Fig. 34; SEQ ID NO: 124). A 3,307-bp antibiotic-free linear DNA cassette was amplified by PCR using pAA2311 as template with primer pair oAA2206/oAA2209 and gel purified before transformation to remove the plasmid template.

*EXAMPLE 7: Modification of nucleic acids encoding acetyl-CoA hydrolase proteins*

Another approach to increasing the amount of cytosolic acetyl-CoA in an organism is to facilitate the generation and transport of acetate from acetyl-CoA-containing organelles, such as peroxisomes, into the cytoplasm. Acetyl-CoA produced via beta-oxidation in the peroxisome may be converted to acetate by the action of acetyl-CoA hydrolase (e.g. EC 3.1.2.1). This enzyme is typically not localized to the peroxisomal compartment. In order to introduce acetyl-CoA hydrolase into peroxisomes, a heterologous nucleic acid construct was developed in which the enzyme coding sequence was modified to include DNA encoding a peroxisomal targeting signal at the C-terminus of the hydrolase and exclude a mitochondrial targeting sequence at the N-terminus. The acetate produced freely diffuses out of the peroxisome into the cytoplasm where it can be converted back to acetyl-CoA by the enzyme acetyl-CoA synthetase. The expression of acetyl-CoA synthetase can also be amplified by increasing the genomic copy number and by promoter replacement. The acetyl-CoA generated from acetate in the cytosol is not able to enter the mitochondria, and thus is not subject to loss due to use in mitochondrial metabolic pathways such as the TCA cycle, unlike acetyl groups in acetyl-carnitine which can be taken up by mitochondria. To decrease peroxisomal generation of acetyl-carnitine, the carnitine acetyltransferase gene (e.g., *CAT2* in *Candida*) can be disrupted in cells that have been modified to produce peroxisomal acetate using, for example, *CAT2* knock-out cassettes as described herein. To provide mitochondria in such *CAT2*-disrupted cells with acetyl-CoA for use in cellular energy production for cell viability, the cell can be transformed with nucleic acid encoding a carnitine acetyltransferase (e.g., a reduced activity version of the enzyme) targeted



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to mitochondria (e.g., *YAT1*$^{+mts}$) to restore a minimum amount of acetyl-CoA generation in the mitochondria.

The enzyme encoded by the *ACH1* gene has acetyl-CoA hydrolase activity and is targeted to the mitochondria in *Candida* strain ATCC 20336. DNA encoding Ach1p (amino acid SEQ ID NO: 15 and nucleotide SEQ ID NO: 72) was amplified from genomic DNA of *Candida* strain ATCC 20336 by PCR using primers oAA6369 and oAA6370. The primers were designed to amplify a portion of the *ACH1* gene such that the amplification product, *ACH1*$^{\Delta mts+pts}$, (1) lacked nucleic acid sequence encoding the N-terminal 11 amino acids that form the mitochondrial targeting sequence, and (2) included a nucleic acid sequence encoding a peroxisomal targeting sequence (Gly-Arg-Arg-Ala-Lys-Leu) at the C-terminus. A plasmid backbone containing all the elements of the pCR-BluntII-TOPO vector, a 5' *URA3* homology region and a 3' *URA3* homology region selectable marker, and the *HDE* gene promoter and *POX4* gene terminator was then amplified from plasmid pAA1164 (Fig. 26) using primers oAA6367 and oAA6368. The nucleic acid products amplified from *Candida* strain ATCC 20336 and pAA1164 contained overlapping sequence at their termini allowing directional ligation of the products to generate plasmid pAA1846. Plasmid pAA1846 contains the nucleic acid encoding the ACH1$^{\Delta mts+pts}$ protein (amino acid SEQ ID NO: 16 and nucleotide SEQ ID NO: 73) under the control of the *HDE1* gene promoter and *POX4* gene terminator situated between the two separate fragments of the *URA3* gene marker.

*EXAMPLE 8: Modification of nucleic acids encoding acetyl-CoA synthetase proteins*

Another approach to increasing the amount of cytosolic acetyl-CoA in an organism is to increase the acetyl-CoA synthetase activity in the cytoplasm. Acetyl-CoA synthetase (e.g., EC 6.2.1.1; also referred to as acetate-CoA ligase) can catalyze the conversion of acetate to acetyl-CoA. Thus, for example, acetate that diffuses into the cytoplasm after being produced in the peroxisome by the action of heterologous peroxisomal acetyl-CoA hydrolase, as described herein, can be converted to acetyl-CoA by the enzyme acetyl-CoA synthetase. One method for increasing the acetyl-CoA synthetase activity in the cytoplasm is to increase the expression of acetyl-CoA synthetase by, for example, increasing the genomic copy number of acetyl-CoA synthetase-encoding DNA (e.g., *ACS*1 and *ACS*2 genes) and/or by replacing the endogenous *ACS*1 and/or *ACS*2 promoter(s) with a stronger, high-expression heterologous promoter (e.g.,

the fatty acid-inducible hydratase-dehydrogenase-epimerase (*HDE*) gene promoter from *Candida*).

Cloning DNA encoding Acs1p from *Candida* strain ATCC 20336

DNA encoding an acetyl-CoA synthetase (acetate-CoA ligase; Acs1p) from *Candida* strain ATCC 20336 was amplified from genomic DNA using primers oAA6371 and oAA6372. A plasmid backbone containing all the elements of the pCR-BluntII-TOPO vector, a 5' *URA3* and a 3' *URA3* homology region selectable marker, and the *HDE* gene promoter and *POX4* gene terminator from *Candida* strain ATCC 20336 was then amplified from plasmid pAA1164 (Fig. 26) using primers oAA6367 and oAA6368. The nucleic acid products amplified from *Candida* strain ATCC 20336 and pAA1164 contained overlapping sequence at their termini allowing directional ligation of the products to generate plasmid pAA1847. Plasmid pAA1847 contains a nucleic acid encoding the ACS1 protein (amino acid SEQ ID NO: 20 and nucleotide SEQ ID NO: 76) under the control of the *HDE1* gene promoter and *POX4* gene terminator situated between the two separate fragments of the *URA3* gene marker.

Cloning DNA encoding Acs2p from *Candida* strain ATCC 20336

DNA encoding an acetyl-CoA synthetase (acetate-CoA ligase; Acs2p) from *Candida* strain ATCC 20336 was amplified from genomic DNA using primers oAA6470 and oAA6471. A plasmid backbone containing all the elements of the pCR-BluntII-TOPO vector, a 5' *URA3* and a 3' *URA3* homology region selectable marker, and the *HDE1* gene promoter and *POX4* gene terminator from *Candida* strain ATCC20336 was then amplified from plasmid pAA1164 (Fig. 26) using primers oAA6367 and oAA6368. The nucleic acid products amplified from *Candida* strain ATCC 20336 and pAA1164 contained overlapping sequence at their termini allowing directional ligation of the products to generate plasmid pAA1875. Plasmid pAA1875 contains a nucleic acid encoding the ACS2 protein (amino acid SEQ ID NO: 21 and nucleotide SEQ ID NO: 77) under the control of the *HDE1* gene promoter and *POX4* gene terminator situated between the two separate fragments of the *URA3* gene marker.



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*EXAMPLE 9: Modification of nucleic acids encoding ATP citrate lyase proteins*

Another approach to increasing cytosolic acetyl-CoA is to increase the activity of ATP citrate lyase (e.g., EC 2.3.3.8) in the cytoplasm of host cells. This enzyme can catalyze the conversion of citrate to acetyl-CoA and oxaloacetate. For example, in oleaginous yeast, ATP citrate lyase typically is a heterodimeric enzyme (Acl1p/Acl2p) that converts citric acid from the TCA cycle that overflows into the cytoplasm into acetyl-CoA and oxaloacetate at the expense of an ATP. The acetyl-CoA can then used for the production of fatty acids and eventually triacylglycerides as a carbon-storage mechanism. One method of increasing ATP citrate lyase activity in the cytosol of host cells is to increase the amount of the enzyme in the cytosol. This can be accomplished in a number of ways. For example, heterologous DNA encoding Acl1p and Acl2p can be introduced into host cells and/or the regulation of expression of Acl1p and Acl2p in a cell can be modified.

Construction of synthetic nucleic acids encoding ATP citrate lyase subunits

The amino acid sequences from NCBI accession numbers CAG80394 and XP_503231 for Acl1p (SEQ ID NO: 42) and Acl2p (SEQ ID NO: 43), respectively, from *Yarrowia lipolytica* CLIB122 were used to design DNA sequences (SEQ ID NOS: 96 and 97) encoding each enzyme subunit that were codon optimized for expression in *Candida* strain ATCC 20336. DNA fragments 500 bp in length with 50 bp overlaps (synthetic Gene blocks from Integrated DNA Technologies) were amplified in a PCR reaction without primers: 95°C for 3 minutes for 1 cycle, 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds for a total of 15 cycles, and final extension at 72°C for 2 minutes. 5µL of the PCR product was used as template for TouchDown PCR reaction (see, for example, Don *et al*. (1991) *Nucleic Acids Res.* 19:4008) including the primers: 95°C for 3 minutes for 1 cycle, 95°C for 30 seconds, 68°C for 30 seconds, decreasing by 0.4°C per cycle, 72°C for 30 seconds for a total of 30 cycles, and final extension at 72°C for 2 minutes. The primers used, oAA3051 and oAA3052 for *ACL1* and oAA3053 and oAA3052 for *ACL2*, incorporated *Bsp*QI restriction sites outside of the coding sequence. The PCR products were purified and cloned into pCR-BluntII-TOPO. Sequence-confirmed plasmids were named pAA709 (*ACL1*) and pAA710 (*ACL2*).



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Construction of expression cassettes for synthetic ACL-encoding nucleic acids

Synthetic DNA expression constructs encoding Acl1p and Acl2p were generated using different promoter and terminator elements contained in pAA073 (Fig. 16) and pAA1617. Plasmid pAA073 contains the *POX4* promoter and terminator from *Candida* strain ATCC 20336. Plasmid pAA1617 was constructed from pAA061 (Fig. 12) as described herein, in the same manner that pAA073 was constructed from pAA061 except that it contains the *HDE1* promoter and *PEX11* terminator from *Candida* strain ATCC 20336 in place of the *POX4* promoter and terminator as shown for pAA073.

Plasmids pAA709 and pAA710 were cut with *Bsp*QI and the DNA fragments containing coding sequences were purified and cloned into *Bsp*QI-cut plasmid pAA073, placing the genes under the control of the *POX4* promoter and terminator. Sequence-confirmed plasmids were named pAA731 (*ACL1*) and pAA732 (*ACL2*). Similarly, the genes were PCR amplified with primers oAA6658 and oAA6659 for *ACL1* and oAA6660 and oAA6661 for *ACL2*, cut with *Bsp*QI and cloned into *Bsp*QI-cut plasmid pAA1617 placing them under the control of the *HDE1* promoter and *PEX11* terminator. Sequence-confirmed plasmids were named pAA1980 (*ACL1*) and pAA1981 (*ACL2*).

*EXAMPLE 10: Modification of nucleic acids encoding acyl-CoA synthetase and cytochrome P450 reductase proteins*

Acyl-CoA synthetases (e.g., EC 6.2.1.3) are enzymes that can catalyze the activation of free fatty acids in the cytoplasm into CoA esters (fatty acyl-CoA) which can be involved in several metabolic pathways. When free fatty acids are activated and then used in other cellular processes, they generally are not available for the fatty acid and acetyl-CoA generation that can occur through β-oxidation in peroxisomes. Therefore, because acyl-CoA synthetases can reduce the amount of free fatty acids in the cytoplasm of an organism, such as a yeast, one approach to enhancing fatty acid and acetyl-CoA generation in an organism involves reduction or elimination of the activity of acyl-CoA synthetase activity in the cytoplasm. When acyl-CoA synthetase activity is decreased or eliminated, free fatty acids that accumulate in the cytoplasm can then enter the ω- and β-oxidation pathways.



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A cytochrome P450 reductase (e.g., EC 1.6.2.4) is an enzyme that can catalyze the reduction of the heme thiolate moiety of cytochrome P450 by transferring an electron to the cytochrome P450. This activity recycles cytochrome P450 and makes it available for further use in catalyzing reactions that occur in ω-oxidation of fatty acids. This, in turn, can provide dicarboxylic acids that are able to undergo β-oxidation in peroxisomes which can result in generation of shorter-chain dicarboxylic acids and acetyl-CoA. Another approach to enhancing dicarboxylic acid and acetyl-CoA production is to increase the activity of cytochrome P450 reductase in an organism. This can be accomplished in a number of ways aimed at increasing the amount of cytochrome P450 reductase protein in the cells. For example, a method for simultaneously decreasing or eliminating acyl-CoA synthetase activity and increasing cytochrome P450 reductase activity in an organism is to disrupt a gene encoding an acyl-CoA synthetase and replace it with nucleic acid encoding a cytochrome P450 reductase.

Cloning nucleic acid encoding the FAA1p from ATCC 20336

The *Candida* strain ATCC 20336 *FAA1* and *CPRB* genes encode an acyl-CoA synthetase (amino acid SEQ ID NO: 35 and nucleotide SEQ ID NO: 91) and cytochrome P450 reductase (amino acid SEQ ID NO: 34 and nucleotide SEQ ID NO: 90), respectively. A double-crossover knock-in cassette was constructed for use in disrupting a *Candida* host strain *FAA*1 gene by integrating into the host genome at the *FAA1* locus a DNA encoding a CPRB protein under the control of the *Candida* strain ATCC 20336 *POX4* promoter and terminator. A DNA fragment encoding the Faa1p acyl-CoA synthetase was amplified from genomic DNA of *Candida* strain ATCC 20336 using primers oAA951 and oAA952. The PCR product was gel purified and ligated into pCR-Blunt II-TOPO (Thermo Fisher Scientific), transformed into competent TOP10 *E. coli* cells (Thermo Fisher Scientific) and clones containing PCR inserts were sequenced to confirm the DNA sequence. One such plasmid was designated pAA275.

Construction of a cassette for disruption of *FAA1* with DNA encoding CPRBp

A DNA fragment from a 5' region of the *FAA*1 gene was amplified from plasmid pAA275 using primers oAA3557 and oAA3558, and a DNA fragment from a 3' region of the *FAA1* gene was amplified from plasmid pAA275 using primers oAA3563 and oAA3564. The *URA3* selectable marker ($P_{URA3}URA3T_{URA3}P_{URA3}$) was amplified from plasmid pAA298 (Fig. 23; SEQ ID NO: 111) using primers oAA3559 and oAA3560. A DNA fragment containing an ORF of a *Candida* strain



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ATCC 20336 *CPRB* gene surrounded by the *POX4* promoter and terminator was amplified from plasmid pAA218 using primers oAA3561 and oAA3562. The two PCR products from amplification of pAA275, and the PCR products from amplification of pAA298 and pAA218 were purified, assembled by overlap extension PCR with primers oAA3557 and oAA3564 and ligated into pCR-BluntII-TOPO generating plasmid pAA879 (Fig. 35). The linear *FAA1* deletion cassette could then be amplified from plasmid pAA879 with primers oAA3557 and oAA3564.

*EXAMPLE 11: Modification of nucleic acids encoding cytochrome P450 monooxygenase proteins*

A cytochrome P450 enzyme (e.g., monooxygenase activity, EC 1.14.14.1) often catalyzes the insertion of one atom of oxygen bound to the heme group in cytochrome P450 into an organic substrate (RH) while the other oxygen atom is reduced to water. This activity can occur in the initial step in the ω-oxidation pathway in which a fatty acid can be converted to a corresponding fatty alcohol. Dicarboxylic acids that are ultimately produced after completion of all steps in the ω-oxidation pathway generally are able to undergo β-oxidation in peroxisomes which results in generation of shorter-chain dicarboxylic acids and acetyl-CoA. Another approach to enhancing dicarboxylic acid and acetyl-CoA production is to increase the activity of cytochrome P450 monooxygenase in a cell. One method for increasing the cytochrome P450 monooxygenase activity in a cell is to increase the expression of cytochrome P450 monooxygenase by, for example, increasing the genomic copy number of cytochrome P450 monooxygenase-encoding DNA (e.g., *CYP52A17* gene) and/or by replacing an endogenous cytochrome P450 monooygenase promoter with a stronger, high-expression heterologous promoter (e.g., the fatty acid-inducible hydratase-dehydrogenase-epimerase (*HDE*) gene promoter from *Candida*).

DNA encoding cytochrome P450 monooxygenase A17 (*CYP52A17*; amino acid SEQ ID NO: 45 and nucleotide SEQ ID NO: 99) from *Candida* strain ATCC 20336 was amplified from genomic DNA using primers oAA5770 and oAA5771. A plasmid backbone containing all the elements of the pCR-BluntII-TOPO vector, a split *URA3* marker and the *HDE* gene promoter and *POX4* gene terminator was amplified from plasmid pAA1164 (Fig. 26) using primers oAA5768 and oAA5769. The nucleic acid products amplified from *Candida* strain ATCC 20336 and pAA1164 contained overlapping sequence at their termini allowing directional ligation of the products to generate plasmid pAA1712. Plasmid pAA1712 contains nucleic acid encoding the CYP52A17



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protein under the control of the *HDE1* gene promoter and *POX4* gene terminator situated between the two separate fragments of the *URA3* selectable marker.

*EXAMPLE 12: Modification of nucleic acids encoding acyl-CoA oxidase and peroxisomal biogenesis proteins*

Acyl-CoA oxidases can be involved in the β-oxidation pathway that occurs in the peroxisomes of many cells and organisms in which long-chain acyl-CoA molecules can be broken down into acetyl-CoA molecules and shorter chain acyl-CoA molecules. The β-oxidation pathway generally includes four main reaction steps resulting in an acyl-CoA that is shortened by two carbon atoms which are released as acetyl-CoA. The shortened acyl-CoA molecule can re-enter the pathway after each cycle and be subjected to another removal of two carbons from the acyl carbon chain. As such, each cycle of the β-oxidation pathway generates a shorter-chain fatty acid and acetyl-CoA, and is a major source of acetyl-CoA in cells. There are multiple acyl-CoA oxidases expressed in yeast and other cells and organisms. These acyl-CoA oxidases can have differing substrate specificities. For example, the peroxisomal acyl-CoA oxidases encoded by the *POX4* and *POX5* genes of *Candida* strain ATCC 20336 exhibit differing activities. The Pox4p enzyme has broad chain length fatty acid specificity whereas the Pox5p enzyme is not very active on fatty acids with short chain lengths. The Pox5p enzyme displays optimal activity on fatty acid substrates of 12 to 18 carbons and a decreased activity on substrates below C10 with a low activity on C6 and C8 substrates.

The catabolism of fatty acids through β-oxidation can be manipulated to serve, in effect, as a process for the production of shorter-chain fatty acids from long-chain acyl-CoA molecules. This can be accomplished by modifying enzymatic activities of the β-oxidation pathway to reduce or eliminate activities that break down fatty acids once they reach a certain short-chain length. For example, one approach to targeting production of short chain dicarboxylic acids (e.g., adipic acid) through the β-oxidation pathway is to delete or disrupt the *POX4* gene in a cell or organism, such as a yeast, e.g., *Candida*. Elimination or reduction of Pox4p acyl-CoA oxidase activity prevents breakdown of long-chain dicarboxylic acids to dicarboxylic acid products with fewer than eight carbon atoms (i.e., chain length shorter than C8) by peroxisomal β-oxidation. One method of disrupting the *POX4* gene and simultaneously introducing a desired nucleic acid for enhancing generation of shorter-chain dicarboxylic acids and acetyl-CoA through modification of β-oxidation is to replace the *POX4* gene with nucleic acid encoding a



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peroxisomal membrane protein that promotes peroxisome biogenesis, such as the protein encoded by a yeast *PEX11* (peroxisomal biogenesis factor 11) gene from *Candida* strain ATCC 20336.

Cloning DNA encoding Pex11p from *Candida* strain ATCC 20336

DNA encoding a Pex11p (amino acid SEQ ID NO: 33 and nucleotide SEQ ID NO: 89) from *Candida* strain ATCC 20336 was PCR amplified from genomic DNA using primers oAA2127 and oAA2128. The PCR product was purified, cut with *Bsp*QI, and ligated into *Bsp*QI-cut plasmid pAA073 to operably position the *PEX11* gene fragment under the control of the *POX4* promoter and terminator. The resulting sequence-confirmed plasmid was named pAA336.

Construction of a cassette for disruption of *POX4* by insertion of nucleic acid encoding *P*ex11p

A double-crossover knock-in cassette was constructed to integrate into a host cell genome at the *POX4* locus, DNA encoding *Candida* strain ATCC 20336 Pex11p under the control of the *POX4* promoter and terminator. A DNA fragment containing a 5' homology region of the *Candida* strain ATCC 20336 *POX4* gene and a *Candida* strain ATCC 20336 *URA3* gene selectable marker ($P_{URA3}URA3T_{URA3}P_{URA3}$) was amplified by PCR from plasmid pAA208 (Fig. 36; SEQ ID NO: 127) with primers oAA3156 and oAA3354. A DNA fragment containing a 3' homology region of the *Candida* strain ATCC 20336 *POX4* gene was amplified by PCR from plasmid pAA208 with primers oAA3159 and oAA3356. A DNA fragment containing the coding sequence of the *PEX11* gene under the control of the *POX4* promoter and terminator was amplified by PCR from plasmid pAA336 with primers oAA3157 and oAA3158. All three DNA fragments were assembled by overlap PCR and primers oAA3355 and oAA3357. The 5,190-bp PCR product was then cloned into pCR-BluntII-TOPO and the resulting plasmid was named plasmid pAA850 (Fig. 37).

Modification of DNA encoding Pox5p

In a cell, such as a *Candida* yeast cell, in which Pox5p is the primary or only functional acyl-CoA oxidase, long chain fatty acyl-CoA or diacyl-CoA substrates can be shortened to about 8 carbons and do not typically enter another cycle of β-oxidation for further shortening of the



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carbon chain length. The shorter substrates (e.g., a C8 fatty dicarboxylic acid) are not typically recognized as a substrate by Pox5p. In such cells, shorter chain fatty-acyl-CoA molecules are then acted on by peroxisomal thioesterases resulting in removal of Co-A, and the released fatty dicarboxylic acid (e.g., an $\alpha,\omega$ - dicarboxylic acid) product is secreted from the cell. In this instance, the acyl-CoA oxidase chain-length substrate specificity effectively controls the chain length of the fatty acid, e.g., a diacid, being produced.

Protein engineering of Pox5p was performed to identify mutants correlated with increased adipic acid production in yeast cells expressing the modified Pox5p enzymes during the fermentation process. A structural model of a Pox5p was created using SWISS-MODEL to identify amino acid positions to mutate. The SWISS-MODEL system has been described, for example, by Arnold *et al.* ((2006) *Bioinformatics* 22: 195-201), Guex *et al.* ((2009) *Electrophoresis* 30 Supplement 1: S162-S173) and Kiefer *et al.* ((2009) *Nucleic Acids Res.* 37 (Database issue): D387-D392). The crystal structure of rat peroxisomal acyl-CoA oxidase II (PDB ID: 1IS2, chain A) (see, for example, Nakajima *et al.* (2002) *J. Biochem.* 131: 376-374) was used as the template to model Pox5p. The resulting structural model was analyzed using the HotSpot Wizard 1.7 program (Worldwide web Uniform Resource Locator (URL) loschmidt.chemi.muni.cz/hotspotwizard/index.jsp) to determine amino acids to target for mutagenesis. HotSpot Wizard is a tool for identifying sites for engineering of substrate specificity and/or activity of enzymes using a combination of structural, functional and sequence analysis and has been described by Pavelka *et al.* ((2009) *Nucleic Acids Res.* 37 (Web Server issue): W376-W383). HotSpot Wizard identified several amino acid positions, or "hotspots," of Pox5p to mutate, with each position given a score from 1 (cold) to 9 (hot) (Fig. 38). The residues identified as hotspots (a score of 6 to 9) are generally found in the portions of the protein that form the walls of the catalytic site pocket and tunnels for entry or exit of the substrate. Part of the HotSpot Wizard analysis is the identification of homologs by a BLAST search (see, e.g., Johnson *et al.* (2008) *Nucleic Acids Res.* 36 (Web Server issue): W5-W9) and their alignment using MUSCLE as described, for example, by Edgar ((2004) *BMC Bioinformatics* 5: 113 and *Nucleic Acids Res.* 32: 1792-1797). The multiple sequence alignment revealed the variety of amino acids found at each position and their relative frequency amongst all the sequences. In the structural model of Pox5p, positions 98 and 429 are located near the back of the catalytic site pocket and are near the catalytic glutamate at position 436. These residues were identified as hotspots and were selected for substitution. Amino acid residue 98, phenylalanine, was identified as a hotspot (with a score of 9) and the residue most frequently



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found at this position amongst other homologs is glycine. In Pox5p of *Candida* ATCC 20336, the residue at position 98 is phenylalanine. Therefore, the phenylalanine codon at position 98 was changed to a glycine codon in one modified Pox5p-encoding DNA construct that was generated. The most common residue at amino acid position 429 (with a score of 6) in the analyzed homologs was a tryptophan, which is also the amino acid residue at position 429 in Pox5p of *Candida* ATCC 20336. Therefore, the tryptophan codon at position 429 was changed to the second most common residue, phenylalanine, in another modified Pox5p-encoding DNA construct that was generated. The HotSpot scores for residues 98 and 429 and the amino acids found at that position in the multiple sequence alignment are shown in Table 6.

**Table 6: HotSpot Scores and comparison of amino acids at residues 98 and 429 in *Candida* Pox5p**

| Amino Acid Position | HotSpot Score | Amino Acid in Pox5p of *Candida* ATCC 20336 | Residues at the Position from the Alignment |
|---|---|---|---|
| 98 | 9 | Phenylalanine | 12xG,8xF,5xA,4xY,3xP,3xS,3xW,2xD,2xQ,2xR,1xE,1xH, 1xL,1xN,1Xv |
| 429 | 6 | Tryptophan | 23xW,7xF,7xY,4xA,4xV,1xC,1xI,1xL,1xN,1xQ |

Site-directed mutagenesis of *POX5*

The *POX5* mutants F98G and W429F were constructed by overlap extension PCR of the template pAA166 containing DNA encoding a wild-type Pox5p. To construct pAA166, oligonucleotides oAA540 and oAA541 were used to amplify DNA encoding Pox5p (amino acid SEQ ID NO: 36 and nucleotide SEQ ID NO: 92) from genomic DNA of *Candida* strain ATCC 20336. The resulting PCR fragment was digested with the restriction enzyme *Bsp*QI and ligated into *Bsp*QI-cut pAA073. The resulting plasmid pAA166 contained DNA encoding Pox5p positioned between the *POX4* promoter and terminator from *Candida* strain ATCC 20336 and also contained a *Candida* strain ATCC 20336 *URA3* gene selectable marker. The nucleic acids encoding *POX5* mutants F98G and W429F were constructed by overlap extension PCR (Fig. 39) of pAA166 using the primers shown in the table shown in Fig. 39 as follows: PCR #1, using oligos A and B, and PCR #2, using oligos C and D were used to amplify the *POX5* coding sequence. Oligos B and C contain the desired point mutation(s). The resulting PCR fragments from PCR #1 and #2 were then used as template and primer for each other to form the full-length coding sequence of the *POX5* mutant in an overlap extension PCR. The resulting overlap extension PCR products were cloned into plasmid pAA073 via the BspQI restriction



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sites encoded by oligos A and D. The sequence-confirmed plasmids containing the Pox5p(F98G) (amino acid SEQ ID NO: 37) and Pox5p(W429F) (amino acid SEQ ID NO: 38) mutants were named pAA1055 and pAA1129, respectively. Each plasmid contained DNA encoding a Pox5p mutant (F98G or W429F) positioned between the *POX4* promoter and terminator from *Candida* strain ATCC 20336 and also contained a *Candida* strain ATCC 20336 *URA3* gene selectable marker. Plasmid pAA1164 (Fig. 26), containing DNA encoding a Pox5p mutant (F98G) positioned between, and operably linked to, the *HDE1* promoter and *POX4* terminator from *Candida* strain ATCC 20336 and also containing a *Candida* strain ATCC 20336 *URA3* gene selectable marker, was also generated for expression of Pox5p(F98G).

*EXAMPLE 13: Modification of nucleic acids encoding peroxisomal transport proteins*

Another approach to enhancing the production of fatty acids, such as dicarboxylic acids, and increasing the amount of cytosolic acetyl-CoA is to reduce or eliminate the transport of fatty acyl-CoA from the cytoplasm of cells directly to peroxisomes. For example, fatty acyl-CoA generated in the cytoplasm can move into peroxisomes by ATP-binding cassette transporters responsible for transporting long-chain fatty acyl-CoA from the cytoplasm across the peroxisomal membrane and into the peroxisomal matrix. Exemplary peroxisomal transport proteins are encoded by yeast *PXA*1 and *PXA*2 (peroxisomal ATP-binding cassette transporter complex) genes. One method of reducing or eliminating the transport of fatty acyl-CoA from the cytoplasm into the peroxisome is to decrease the activity of Pxa1p and Pxa2p transport proteins. This can be accomplished, for example, through deletion or disruption of the *PXA1* and/or *PXA2* genes in an organism. By blocking import of activated acyl-CoA into the peroxisome in, for example, *Candida* host cells, the fatty acyl-CoA generated in the cytosol can be converted to free fatty acid available to undergo ω-oxidation to generate additional dicarboxylic acids for entry into the peroxisome and generation of shorter-chain fatty acids and acetyl-CoA through peroxisomal β-oxidation.

Cloning DNA encoding Pxa1p from *Candida* strain ATCC 20336

DNA encoding a Pxa1p (amino acid SEQ ID NO: 40 and nucleotide SEQ ID NO: 94) from *Candida* strain ATCC 20336 was PCR amplified from genomic DNA using primers oAA2125 and oAA2126. The PCR product, containing DNA encoding a Pxa1p, was purified and cloned into pCR-BluntII-TOPO vector. The resulting sequence-confirmed plasmid was named pAA353.



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Construction of *PXA1* deletion cassettes

Two *PXA1* deletion cassettes for use in disrupting host cell *PXA*1 genes were constructed that differed in the size of the *PXA*1 homology sequences contained in the cassettes and the orientation of the *URA3* selectable marker DNA positioned between the homology sequences. A first deletion cassette was constructed by amplifying 5' and 3' DNA fragments of *PXA1* from plasmid pAA353 using primers oAA2679 and oAA2680 for amplifying a 5' DNA fragment and oAA2683 and oAA2684 for amplifying a 3' DNA fragment. A *URA3* selectable marker ($P_{URA3}URA3T_{URA3}P_{URA3}$) was amplified from plasmid pAA298 using primers oAA2681 and oAA2682. The three PCR products were purified, assembled by overlap extension PCR and ligated into pCR-BluntII-TOPO generating plasmid pAA1117 (Fig. 40). A linear *PXA1* deletion cassette could then be amplified from plasmid pAA1117 with primers oAA2679 and oAA2684.

A second deletion cassette was constructed by amplifying 5' and 3' DNA fragments of *PXA1* from plasmid pAA353 using primers oAA2914 and oAA2915 for amplifying a 5' DNA fragment and oAA2918 and oAA2919 for amplifying a 3' DNA fragment. A *URA3* selectable marker was amplified from plasmid pAA298 using primers oAA2916 and oAA2917. The three PCR products were purified, assembled by overlap extension PCR and ligated into pCR-Blunt II-TOPO generating plasmid pAA1155 (Fig. 40). A linear *PXA1* deletion cassette could then be amplified from plasmid pAA1155 with primers oAA2914 and oAA2919.

Cloning DNA encoding Pxa2p from *Candida* strain ATCC 20336

DNA encoding Pxa2p (amino acid SEQ ID NO: 41 and nucleotide SEQ ID NO: 95) from *Candida* strain ATCC 20336 was PCR amplified from genomic DNA using primers oAA2159 and oAA2162. The PCR product containing DNA encoding Pxa2p was purified and cloned into pCR-BluntII-TOPO vector. The resulting sequence-confirmed plasmid was named pAA354.

*PXA2* deletion cassettes for use in disrupting host *PXA2* genes can be constructed as described for the construction of *PXA1* genes.



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*EXAMPLE 14: Generation of host strains for use in development of genetically modified organisms*

In some embodiments of exemplary methods for the development of genetically modified cells and organisms, host strains for transformation can carry beneficial mutations and/or amplifications. Examples include auxotrophic mutations (e.g., *ura3*) that facilitate selection of transformants, and mutations and/or amplifications yielding host cell alterations that serve to work in conjunction with subsequent engineered genetic modifications to enhance generation of cytosolic acetyl-CoA and/or production of fatty acids in the transformants. Alterations in an acyl-CoA oxidase (Pox4p, Pox5p), acyl-CoA synthetase (Faa1p, Fat1p), peroxisomal biogenesis factor 11 (Pex11p) and/or cytochrome P450 reductase (CPRBp) are non-limiting examples of host cell mutations and/or amplifications that can augment the subsequent genetic modifications introduced in the development of organisms for enhanced generation of cytosolic acetyl-CoA and/or production of fatty acids.

For example, in some embodiments, the activity of β- and/or ω-oxidation enzymes in host cells was amplified by placing the genes encoding them under the control of strong and/or inducible promoters and integrating them in additional copies in the genome. To allow the recycling of the *URA3* selectable marker for additional genetic modification, some of these amplified genes were integrated by double-crossover integration at genetic loci providing a simultaneous deletion of an unwanted enzyme activity. For example, in some host cells, the *PEX11* gene was integrated under the control of the *POX4* promoter at the *POX4* locus. This simultaneously provided the deletion of an unwanted activity (Pox4p, acyl-CoA oxidase with undesired substrate specificity) and amplification of a desired activity (Pex11p, peroxisome biogenesis factor). Examples of some possible host strains for use in developing genetically modified cells and organisms for enhanced production of cytosolic acetyl-CoA and/or production of fatty acids are described herein. Fig. 41 provides a flow diagram showing the parent-child relationship for some of the exemplary engineered yeast strains.

Cell transformation methods

Development of genetically modified cells and organisms can involve introduction of heterologous nucleic acids into cells using cell transformation methods described herein and/or known in the art. For example, generally, for the transformation of *Candida* yeast cells, 5 mL



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YPD start cultures are inoculated with a single colony of *Candida* cells and incubated overnight at 30°C, with shaking at about 200 rpm. The following day, fresh 25 mL YPD cultures, containing 0.05% Antifoam B, are inoculated to an initial $OD_{600nm}$ of 0.4 and the culture incubated at 30°C, with shaking at about 200 rpm until an $OD_{600nm}$ of 1.0-2.0 is reached. Cells are pelleted by centrifugation at 1,000 x *g*, 4°C for 10 minutes. Cells are washed by resuspending in 10 mL sterile water, pelleted, resuspended in 1 mL sterile water and transferred to a 1.5 mL microcentrifuge tube. The cells are then washed in 1 mL sterile TE/LiOAC solution, pH 7.5, pelleted, resuspended in 0.25 mL TE/LiOAC solution and incubated with shaking at 30°C for 30 minutes.

The cell solution is divided into 50 µL aliquots in 1.5 mL tubes to which is added 5-8 µg of linearized transforming DNA and 5 µL of carrier DNA (boiled and cooled salmon sperm DNA, 10 mg/mL). Sterile PEG solution (300 µL of 40% PEG 3500, 1X TE, 1X LiOAC) is added, mixed thoroughly and incubated at 30°C for 60 minutes with gentle mixing every 15 minutes. DMSO (40 µL) is added, mixed thoroughly and the cell solution is incubated at 42°C for 15 minutes. Cells are then pelleted by centrifugation at 1,000 x *g* 30 seconds, resuspended in 500 µL of YPD media and incubated at 30°C with shaking at about 200 rpm for 2 hours. Cells are then pelleted by centrifugation and resuspended in 1 mL 1X TE, cells are pelleted again, resuspended in 0.2 mL 1X TE and plated on selective media. Plates are incubated at 30°C for growth of transformants. Transformation methods using standard electroporation techniques are also described in U.S. Patent Nos. 5,648,247 and 5,204,252.

Strain sAA886 (*ura3/ura3 pox4a::ura3/pox4b::ura3 faa1::$P_{URA3}$/faa1::$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$*)

Table 7 summarizes the genotypes and steps in the construction of *Candida* strain sAA886. Functional *POX5* alleles were restored in *Candida* strain ATCC 20962 (also referred to as sAA003; *ura3/ura3 pox4a::ura3/pox4b::URA3/pox5::ura3/pox5::ura3*; see U.S. Patent No. 5,254,466) by transformation of sAA003 with *POX5* linear DNA to replace the URA3-disrupted loci with a functional allele. The *POX5* DNA was obtained from pAA049 which was constructed by amplifying the entire sequence of NCBI accession number M12161 for the YSAPOX5 locus from genomic DNA prepared from *Candida* strain ATCC 20336 using primers oAA0179 and oAA0182. The 2,624-bp PCR product was cloned into the vector, pCR-BluntII-TOPO, sequenced and designated pAA049. A 2,624-bp DNA fragment containing the *POX5* ORF as



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well as 456 bp upstream and 179 bp downstream of the ORF was amplified by PCR of plasmid pAA049 using primers oAA0179 and oAA0182. The purified PCR product was used to transform competent sAA003 cells which were plated on YNB agar plates supplemented with dodecane vapor as the carbon source (e.g., by placing a filter paper soaked with dodecane in the lid of an inverted petri dish) and incubated at 30°C for 4-5 days. Colonies growing on dodecane as the sole carbon source were restreaked onto YPD-agar and incubated at 30°C. Single colonies were grown in YPD cultures and used for the preparation of genomic DNA. PCR analysis of the genomic DNA prepared from the transformants was performed with oligos oAA0179 and oAA0182. A ura3-disrupted *POX5* would produce a PCR product of 4,784 bp while a functional *POX5* would produce a PCR product of 2,624 bp. In a resulting strain, sAA235 (see also U.S. Patent No. 8,343,752), a PCR product 2,624 bp was amplified indicating that both *POX5* alleles had been functionally restored.

**Table 7: Yeast strain genotypes and steps in constructing sAA886 and sAA2428**

| Strain | Genotype | Construction |
|---|---|---|
| sAA003 | *ura3/ura3 pox4a::ura3/pox4b::URA3 pox5::ura3/pox5::ura3* | ATCC 20962 |
| sAA235 | *ura3/ura3 pox4a::ura3/pox4b::URA3* ***POX5/POX5*** | Transformation of sAA003 with PCR product using pAA049 as template and growth on YNB-dodecane |
| sAA329 | *ura3/ura3 pox4a::ura3/pox4b::**ura3** POX5/POX5* | Growth of sAA235 on 5-FOA |
| sAA722 | *ura3/ura3 pox4a::ura3/pox4b::ura3 POX5/POX5* ***faa1::URA3****/FAA1* | Transformation of sAA239 with BamHI/XhoI fragment of pAA276 |
| sAA741 | *ura3/ura3 pox4a::ura3/pox4b::ura3 POX5/POX5* ***faa1::*** $\mathbf{P_{URA3}}$*/FAA1* | Growth of sAA722 on 5-FOA |
| sAA776 | *ura3/ura3 pox4a::ura3/pox4b::ura3 POX5/POX5 faa1::* $P_{URA3}$***/faa1::URA3*** | Transformation of sAA741 with BamHI/XhoI fragment of pAA282 |
| sAA779 | *ura3/ura3 pox4a::ura3/pox4b::ura3 POX5/POX5 faa1::* $P_{URA3}$***/faa1::*** $\mathbf{P_{URA3}}$ | Growth of sAA776 on 5-FOA |
| sAA865 | *ura3/ura3 pox4a::ura3/pox4b::ura3 POX5/POX5 faa1::* $P_{URA3}$*/faa1::*$P_{URA3}$ ***fat1-Δ1::URA3****/FAT1* | Transformation of sAA779 with deletion cassette generated by OE-PCR |
| sAA869 | *ura3/ura3 pox4a::ura3/pox4b::ura3 POX5/POX5 faa1::* $P_{URA3}$*/faa1::*$P_{URA3}$ ***fat1-Δ1::*** $\mathbf{P_{URA3}}$*/FAT1* | Growth of sAA865 on 5-FOA |

**Table 7: Yeast strain genotypes and steps in constructing sAA886 and sAA2428**

| Strain | Genotype | Construction |
|---|---|---|
| sAA875 | *ura3/ura3 pox4a::ura3/pox4b::ura3 POX5/POX5 faa1::* $P_{URA3}$*/faa1::*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$***/fat1-Δ2::URA3*** | Transformation of sAA869 with deletion cassette generated by OE-PCR |
| sAA886 | *ura3/ura3 pox4a::ura3/pox4b::ura3 POX5/POX5 faa1::* $P_{URA3}$*/faa1::*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$***/fat1-Δ2::**$P_{URA3}$ | Growth of sAA875 on 5-FOA |
| sAA2291 | *ura3/ura3 pox4a::ura3/pox4b::ura3* ***pox5::URA3****/POX5 faa1::*$P_{URA3}$*/faa1::*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::*$P_{URA3}$ | Transformation of sAA886 with a *Pac*I-digested fragment of pAA918 (SEQ ID NO: 128) |
| sAA2310 | *ura3/ura3 pox4a::ura3/pox4b::ura3* ***pox5Δ::**$T_{URA3}$ */POX5 faa1::*$P_{URA3}$*/faa1::*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::*$P_{URA3}$ | Growth of sAA2291 on 5-FOA |
| sAA2399 | *ura3/ura3 pox4a::ura3/pox4b::ura3 pox5Δ::*$T_{URA3}$*/* ***pox5::URA3*** *faa1::*$P_{URA3}$*/faa1::*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::*$P_{URA3}$ | Transformation of sAA2310 with a *Pac*I-digested fragment of pAA918 |
| sAA2428 | *ura3/ura3 pox4a::ura3/pox4b::ura3 pox5Δ::*$T_{URA3}$*/* ***pox5Δ::**$T_{URA3}$ *faa1::* $P_{URA3}$*/faa1::*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::*$P_{URA3}$ | Growth of sAA2399 on 5-FOA |

In order to delete the *FAA1* genes in the *POX5*-restored strain, sAA329 (generated by growing sAA235 on 5-FOA and selecting for $Ura^-$ cells) was transformed with a fragment of pAA276 (Fig. 42) containing DNA from an *FAA1* gene. *FAA1* DNA was PCR amplified from *Candida* ATCC 20336 genomic DNA using primers oAA951 and oAA952. The PCR product was gel purified and ligated into pCR-BluntII-TOPO, transformed into competent TOP10 *E. coli* cells and clones containing PCR inserts were sequenced to confirm the DNA sequence. One such plasmid was designated pAA275. Plasmid pAA280 was digested with BamHI to release a 2.0 kb $P_{URA3}$*URA3*$T_{URA3}P_{URA3}$ cassette. Plasmid pAA275 was digested with *Bgl*II and gel purified. The DNA cassette and the *Bgl*II fragment of pAA275 were ligated together to generate plasmids pAA276 and pAA282. Plasmids pAA276 and pAA282 have the $P_{URA3}$*URA3*$T_{URA3}P_{URA3}$ cassette inserted between the 5' (N-terminal-encoding) and 3' (C-terminal-encoding) fragments of the *FAA1* gene in opposite orientations.

Plasmid pAA276 was digested with *Bam*HI/*Xho*I and column purified. Strain sAA329 (*ura3/ura3 pox4a::ura3/pox4b::ura3 POX5/POX5*) was transformed with the linearized DNA and plated on SCD-URA plate. Several colonies were checked for *FAA1* disruption. One such strain was designated sAA722. Strain sAA722 was grown in YPD media overnight and plated on 5-FOA plate. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of

the *URA3* gene leaving behind only the *URA3* promoter ($P_{URA3}$) in the *FAA1* site. Out of 30 colonies analyzed, only one strain showed the correct genetic modification. The strain was designated sAA741. Plasmid pAA282 was digested with *Bam*HI/*Xho*I and column purified. Strain sAA741 was transformed with the linearized DNA and plated on SCD-ura plate. Several colonies were checked for double *FAA1* knockout by insertional inactivation. One such strain was designated sAA776. Strain sAA776 was grown in YPD media overnight and plated on 5-FOA plate. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of the URA3 gene leaving behind only the *URA3* promoter (PURA3) in both *FAA1* gene alleles. One such strain was named sAA779.

Deletion of each *FAT1* allele was achieved by transforming sAA779 cells with linear DNA cassettes constructed by overlap extension PCR (OE-PCR). The deletion cassette for the first *FAT1* allele in sAA779 was created from three DNA fragments: one containing *FAT1* 5' DNA, one containing *FAT1* 3' DNA and one containing *URA3* marker DNA. The first two DNA fragments containing the *FAT1* DNA were obtained from DNA encoding Fat1p. The full-length coding sequence of the *FAT1* gene was amplified from *Candida* strain ATCC 20336 genomic DNA using primers oAA1023 and oAA1024. The 2,086-bp PCR product was gel purified and ligated into pCR-Blunt II-TOPO, transformed into competent TOP10 E. coli cells and clones containing PCR inserts were sequenced to confirm correct DNA sequence. One such plasmid was named pAA296.

The first DNA fragment (*FAT1* 5' homology) was amplified from plasmid pAA296 using primers oAA2055 and oAA2056. The second DNA fragment (FAT1 3' homology) was amplified from plasmid pAA296 using primers oAA2069 and oAA2060. The third DNA fragment ($P_{URA3}$*URA3*$T_{URA3}P_{URA3}$) was amplified from plasmid pAA298 using primers oAA2057 and oAA2068. A map of plasmid pAA298 is set forth in Fig. 23. All three DNA fragments were combined in the same reaction to generate the full-length deletion cassette by overlap extension PCR using primers oAA2055 and oAA2060. Strain sAA779 was transformed with the full-length deletion cassette and plated on SCD-URA plate. Several colonies were screened by PCR for integration of the deletion cassette at the first *FAT1* allele. One such strain was named sAA865. Strain sAA865 was grown in YPD media overnight and plated on 5-FOA plate. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of the *URA3* gene leaving behind only the URA3 promoter (PURA3) in the first *FAT1* allele. One such strain was named sAA869. The deletion of the second *FAT1* allele in sAA869 was performed by



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transformation with a deletion cassette created by overlap extension PCR. The deletion cassette for the second *FAT1* allele was constructed from three DNA fragments. The first DNA fragment (FAT1 5' homology) was amplified from plasmid pAA296 using primers oAA2070 and oAA2075. The second DNA fragment (FAT1 3' homology) was amplified from plasmid pAA296 using primers oAA2074 and oAA2075. The third DNA fragment ($P_{URA3}$*URA3*$T_{URA3}P_{URA3}$) was amplified from plasmid pAA298 using primers oAA2072 and oAA2073. All three DNA fragments were combined in the same reaction to create a full-length deletion cassette by overlap extension PCR using primers oAA2070 and oAA2071. Strain sAA869 was transformed with the full-length deletion cassette and plated on SCD-URA plate. Several colonies were screened by PCR for integration of the deletion cassette at the second *FAT1* allele. One such strain was named sAA875. Strain sAA875 was grown overnight in YPD media and then streaked on to 5-FOA containing plates. Single colonies were tested for *URA3* reversion frequency, and the isolate with least reversion frequency was named sAA886.

Strain sAA2428 (*ura3/ura3 pox4a::ura3/pox4b::ura3 pox5Δ::*$T_{URA3}$*/pox5Δ::*$T_{URA3}$ *faa1::*$P_{URA3}$*/faa1::*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::*$P_{URA3}$)

Strain sAA2428 was generated by knocking out both alleles of *POX5* from the genome of the starting strain sAA886 (*ura3/ura3 pox4a::ura3/pox4b::ura3 POX5/POX5 faa1::* $P_{URA3}$*/faa1::* $P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::*$P_{URA3}$) in a series of homologous recombination events as outlined in Table 7 above and described herein. Both *POX5* alleles were disrupted by transforming the strain with the double-crossover knock out cassette from plasmid pAA918.

Knock out plasmid pAA918 (SEQ ID NO: 128) was constructed by combining DNA fragments containing *POX5* 5', *POX5* 3' and a *URA3* marker gene. Approximately 600 bp of the 5' flanking region of the *Candida* strain ATCC 20336 *POX5* gene was amplified from genomic DNA using oligos oAA2656 and oAA2657. This fragment was gel purified and cloned into a pCR-Blunt II-TOPO vector to create plasmid pAA494. Approximately 500 bp of the 3' flanking region of the *POX5* gene was amplified with primers oAA2658 and oAA2659 using genomic DNA of *Candida* strain ATCC 20336 and this fragment was cloned into a pCR-Blunt II-TOPO vector to create plasmid pAA495. *EcoRI*/*BamHI*-digested fragment of pAA494, *HindIII*/*BamHI* fragment of pAA495 and *HindIII*/*EcoRI* fragment of pUC19 were ligated together to construct plasmid pAA496. Subsequently, a DNA fragment containing the $T_{URA3}P_{URA3}$*URA3*$T_{URA3}$ cassette flanked by *NotI* restriction sites was cloned into the *NotI* site of pAA496 to create the *POX5* knockout

construct plasmid pAA918 (Fig. 43). Strain sAA886 was transformed with *PacI*-digested fragment of pAA918 and plated on SCD-URA plate. Several colonies were screened by PCR for integration of the deletion cassette in the first *POX5* allele. A positive colony was named sAA2291. Strain sAA2291 was grown in YPD media overnight and plated on a 5-FOA plate. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of the *URA3* gene to leave behind only the *URA3* terminator ($T_{URA3}$) in the first *POX5* allele. This strain was named sAA2310. The deletion of the second *POX5* allele was performed by transformation of *PacI*-digested fragment of pAA918 into sAA2310 and plating on a SCD-URA plate. Colonies that grew on the SCD-URA plate were PCR screened for integration of the knockout cassette in the second *POX5* allele. A positive colony was named sAA2399. Strain sAA2399 was grown in YPD media overnight and plated on a 5-FOA plate. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of the *URA3* gene to leave behind only the *URA3* terminator ($T_{URA3}$) in the second *POX5* allele. This strain was named sAA2428.

Strains sAA2570 and sAA2571

Host strains containing DNA encoding modified Pox5p enzymes were generated by transformation of strain sAA2428. Strains sAA2570 and sAA2571 were generated by transformation of sAA2428 with DNA encoding a modified Pox5p enzyme (Pox5p F98G) in which the codon for amino acid at position 98 in the protein is a glycine codon instead of the phenylalanine codon at position 98 in the wild-type Pox5p enzyme. Plasmid pAA1055, containing DNA encoding Pox5p (F98G) flanked by the *POX4* promoter and terminator, and also containing a *URA3* selectable marker, was digested with BstZ17I. The resulting linear DNA fragment was transformed into sAA2428. Transformants were selected by growth on SCD-URA plates. Clones that grew well on minimal media containing 0.1% Tween 80 and 0.1% oleic acid as the sole carbon source were then grown in shake flask fermentations and analyzed for dicarboxylic acid production. Two of the selected strains were sAA2570 and sAA2571.

Strains sAA2782, sAA2783 and sAA2784

Strains sAA2782, sAA2783 and sAA2784 were generated by transformation of sAA2428 with DNA encoding a modified Pox5p enzyme (Pox5p W429F) in which the codon for amino acid at position 429 in the protein is a phenylalanine codon instead of the tryptophan codon at position 429 in the wild-type Pox5p enzyme. Plasmid pAA1129, containing DNA encoding Pox5p



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(W429F) flanked by the *POX4* promoter and terminator, and also containing a *URA3* selectable marker, was digested with BstZ17I. The resulting linear DNA fragment was transformed into sAA2428. Transformants were selected by growth on SCD-URA plates. Clones that grew well on minimal media containing 0.1% Tween 80 and 0.1% oleic acid as the sole carbon source were then grown in shake flask fermentations and analyzed for fatty acid production. Three of the selected strains were sAA2782, sAA2783 and sAA2784.

Analysis of adipic acid production by strains sAA2570, sAA2571, sAA2782, sAA2783 and sAA2784

Host strains containing DNA encoding modified Pox5p enzymes were grown in shake flask fermentations on oleic acid and analyzed for production of fatty acids. Cultures (5 ml) of each strain that had been grown overnight in YPD medium to an initial $OD_{600nm}$ of 0.4 were used to inoculate 250-mL glass flasks containing 50mL of SP92 media (6.7g/L Difco yeast nitrogen base, 3.0g/L Difco yeast extract, 3.0g/L ammonium sulfate, 1.0g/L potassium phosphate monobasic, 1.0g/L potassium phosphate dibasic, 75g/L glycerol). After 24 h incubation at 30°C with shaking at 250 rpm (2" throw incubator) the cells were centrifuged and the cell pellet resuspended in 15 mL of HiP-TAB media (yeast nitrogen base without amino acids and without ammonium sulfate, 1.7 g/L; yeast extract, 3.0 g/L; potassium phosphate monobasic, 10.0 g/L; potassium phosphate dibasic, 10.0 g/L). The cultures were transferred to fresh 250-mL glass bottom-baffled flasks and 2% v/v oleic acid was added. Cultures were incubated at 30°C with shaking at 300 rpm to start fatty acid production. Samples of culture broth were taken every 24 hours for gas chromatographic (GC) analysis to determine the diacid composition of the culture. The results of analyses of the host strains containing DNA encoding modified Pox5p enzymes were compared to those of a strain containing DNA encoding wild-type Pox5p (sAA875: *pox4a::ura3/pox4b::ura3 POX5/POX5 faa1::* $P_{URA3}$*/faa1::* $P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::URA3*).

The results of the gas chromatographic determinations of fatty acid content of the culture broth of shake flask fermentations comparing strains containing DNA encoding wild-type Pox5p to strains containing DNA encoding mutant Pox5p enzymes are provided in Table 8 and Table 9. The strains containing DNA encoding Pox5p mutants exhibited higher adipic acid (C6 diacid) selectivity, productivity, and yield.

**Table 8: Comparison of Diacids in Culture Broth from 72-hour Shake Flask Cultures of Strains Expressing Wild-Type Pox5p and an F98G Mutant**

| Strain name | Pox5p Acyl CoA-Oxidase | g/L C8 diacid | g/L C6 diacid (yield as %Ymax*) |
|---|---|---|---|
| sAA875 | Wild-type Pox5p | 3.93 | 10.66 (54) |
| sAA2570 | Pox5p F98G | 0.00 | 17.17 (78) |
| sAA2571 | Pox5p F98G | 0.00 | 15.50 (79) |

*Ymax is maximum theoretical yield. It is the amount of product that can be produced for a given biochemical pathway given a certain amount of consumed feedstock (i.e., grams adipic acid/grams oleic acid). The empirical yield is calculated by: mass of product produced divided by the mass of substrate consumed.

**Table 9: Comparison of Diacids in Culture Broth from 72-hour Shake Flask Cultures of Strains Expressing Wild-Type Pox5p and a W429F Mutant**

| Strain name | Pox5p Acyl CoA-Oxidase | g/L C8 diacid | g/L C6 diacid (yield as %Ymax) |
|---|---|---|---|
| sAA875 | Wild-type Pox5p | 5.75 | 8.80 (48) |
| sAA2782 | Pox5p W429F | 0.41 | 12.46 (67) |
| sAA2783 | Pox5p W429F | 0.62 | 13.24 (72) |
| sAA2784 | Pox5p W429F | 0.77 | 12.90 (71) |

Strain sAA2323 (*ura3/ura3 pox4a::PEX11-$P_{URA3}$/pox4b::PEX11-$P_{URA3}$ faa1::$P_{URA3}$/faa1::$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$*)

Strain sAA2323 was generated by integration of a double-crossover knock-in expression cassette containing nucleic acid encoding *Candida* strain ATCC 20336 Pex11p into both alleles of *pox4* in the genome of the starting strain sAA886 in a series of successive double-crossover homologous recombination events as outlined in Fig. 41 and described herein. Integration of the expression cassette into each *pox4* allele was accomplished by transforming the strain with a double-crossover knock-in cassette from plasmid pAA850.

A linear nucleic acid fragment containing a *POX4* promoter-*PEX11* ORF-*POX4* terminator expression cassette, a *URA3* selectable marker ($P_{URA3}URA3T_{URA3}P_{URA3}$) and a POX4 5' sequence on one end of the fragment and *POX4* 3' sequence on the other end of the fragment was amplified from plasmid pAA850 (FIG. 37) using primers oAA3355 and oAA3357. The linear nucleic acid fragment was used to chemically transform strain sAA886, and transformants were



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plated onto SCD-URA plates. The resultant colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of the knock-in cassette and one isolate was saved as strain sAA2046 (*ura3/ura3 pox4a::PEX11-URA3/pox4b::ura3 faa1::$P_{URA3}$/faa1::$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$*).

Strain sAA2046 was grown overnight in YPD media and plated on 5-FOA plates. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of the *URA3* gene leaving behind the *URA3* promoter ($P_{URA3}$) and *PEX11* gene in the first *pox4* allele. The resultant strain was saved as strain sAA2122 (*ura3/ura3 pox4a::PEX11- $P_{URA3}$/pox4b::ura3 faa1::$P_{URA3}$/faa1::$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$*).

A linear nucleic acid fragment amplified from plasmid pAA850 (Fig. 37) using primers oAA3355 and oAA3357 was also used to integrate the *POX4* promoter-*PEX11* ORF-*POX4* terminator expression cassette into the second *pox4* allele. Strain sAA2122 was chemically transformed with the linear nucleic acid, and transformants were plated onto SCD-URA plates. The resultant colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of the knock-in cassette at both alleles and one isolate was saved as strain sAA2235 (*ura3/ura3 pox4a::PEX11- $P_{URA3}$/pox4b:: PEX11-URA3 faa1::$P_{URA3}$/faa1::$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$*).

Strain sAA2235 was grown overnight in YPD media and plated on 5-FOA plates. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of the *URA3* gene leaving behind the *URA3* promoter ($P_{URA3}$) and *PEX11* gene in the second *pox4* allele. The resultant strain was saved as strain sAA2323 (*ura3/ura3 pox4a::PEX11- $P_{URA3}$/pox4b::PEX11-$P_{URA3}$ faa1::$P_{URA3}$/faa1::$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$*).

Strain sAA2697 (*ura3/ura3 pox4a::PEX11-$P_{URA3}$/pox4b::PEX11-$P_{URA3}$ faa1::CPRB-$P_{URA3}$/faa1::CPRB-$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$*)

Strain sAA2697 was generated by integration of a double-crossover knock-in expression cassette containing nucleic acid encoding *Candida* ATCC 20336 strain CPRBp into both alleles of *faa1* in the genome of the starting strain sAA2323 in a series of successive double-crossover homologous recombination events as outlined in Fig. 41 and described herein. Integration of the expression cassete into each *faa1* allele was accomplished by transforming the strain with the plasmid construct pAA879.

A linear nucleic acid fragment containing a *POX4* promoter-*CPRB* ORF-*POX4* terminator expression cassette, a *URA3* selectable marker ($P_{URA3}URA3T_{URA3}P_{URA3}$) and an *FAA1* 5' sequence on one end of the fragment and *FAA1* 3' sequence on the other end of the fragment was amplified from plasmid pAA879 (Fig. 35) using primers oAA3557 and oAA3564. The linear nucleic acid fragment was used to chemically transform strain sAA2323, and transformants were plated onto SCD-URA plates. The resultant colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of the knock-in cassette and one isolate was saved as strain sAA2404 (*ura3/ura3 pox4a::PEX11-*$P_{URA3}$*/pox4b::PEX11-*$P_{URA3}$ *faa1::CPRB-URA3/faa1::*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::*$P_{URA3}$). Strain sAA2404 was grown overnight in YPD media and plated on 5-FOA plates. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of the *URA3* gene leaving behind the *URA3* promoter ($P_{URA3}$) and *CPRB* gene in the first *FAA1* allele. The resultant strain was saved as strain sAA2539 (*ura3/ura3 pox4a::PEX11-*$P_{URA3}$*/pox4b::PEX11-*$P_{URA3}$ *faa1::CPRB-*$P_{URA3}$*/faa1::*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::*$P_{URA3}$).

A linear nucleic acid fragment amplified from plasmid pAA879 (Fig. 35) using primers oAA3557 and oAA3564 was also used to integrate the *POX4* promoter-*CPRB* ORF-*POX4* terminator expression cassette into the second *faa1* allele. Strain sAA2539 was chemically transformed with the linear nucleic acid, and transformants were plated onto SCD-URA plates. The resultant colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of the knock-in cassette at both alleles and one isolate was saved as strain sAA2622 (*ura3/ura3 pox4a::PEX11-* $P_{URA3}$*/pox4b::PEX11-*$P_{URA3}$ *faa1::CPRB-*$P_{URA3}$*/faa1::CPRB-URA3 fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::*$P_{URA3}$).

Strain sAA2622 was grown overnight in YPD media and plated on 5-FOA plates. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of the *URA3* gene leaving behind the *URA3* promoter ($P_{URA3}$) and *CPRB* gene in the second *FAA1* allele. The resultant strain was saved as strain sAA2697 (*ura3/ura3 pox4a::PEX11-*$P_{URA3}$*/pox4b::PEX11-*$P_{URA3}$ *faa1::CPRB-*$P_{URA3}$*/faa1::CPRB-*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::*$P_{URA3}$).



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Strain sAA2800 (*ura3/ura3 pox4a::PEX11-$P_{URA3}$/pox4b::PEX11-$P_{URA3}$ faa1::CPRB-$P_{URA3}$/faa1::CPRB-$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$ pox5Δ::$T_{URA3}$/pox5Δ::$T_{URA3}$*)

Strain sAA2800 was generated by knocking out both alleles of *POX5* from the genome of the starting strain sAA2697 in a series of successive double-crossover homologous recombination events as outlined in Fig. 41 and described herein. Both *POX5* alleles were disrupted by transforming the strain with the double-crossover knock-out cassette from plasmid pAA918.

A linear nucleic acid fragment containing a *URA3* selectable marker ($T_{URA3}P_{URA3}$*URA3*$T_{URA3}$) and a *POX5* 5' sequence on one end of the fragment and *POX5* 3' sequence on the other end of the fragment was obtained by cutting plasmid pAA918 (Fig. 43) with PacI and gel purifying the 2,893-bp DNA cassette. The linear nucleic acid fragment was used to chemically transform strain sAA2697, and transformants were plated onto SCD-URA plates. The resultant colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of the knock-out cassette and one isolate was saved as strain sAA2704 (*ura3/ura3 pox4a::PEX11-$P_{URA3}$/pox4b::PEX11-$P_{URA3}$ faa1::CPRB-$P_{URA3}$/faa1::CPRB-$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$ pox5Δ::URA3/ POX5*).

Strain sAA2704 was grown overnight in YPD media and plated on 5-FOA plates. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of the *URA3* gene leaving behind the *URA3* terminator ($T_{URA3}$) in the first *POX5* allele. The resultant strain was saved as strain sAA2739 (*ura3/ura3 pox4a::PEX11-$P_{URA3}$/pox4b::PEX11-$P_{URA3}$ faa1::CPRB-$P_{URA3}$/faa1::CPRB-$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$ pox5Δ::$T_{URA3}$/ POX5*).

The knock-out of the second *POX5* allele was performed by chemical transformation of strain sAA2739 with a double-crossover knock-out cassette generated by cutting plasmid pAA918 (Fig. 43) with PacI and gel purifying the 2,893-bp DNA cassette. The transformation reaction was plated onto SCD-URA plates. The resultant colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of the knock-out cassette at both alleles and one isolate was saved as strain sAA2748 (*ura3/ura3 pox4a::PEX11-$P_{URA3}$/pox4b::PEX11-$P_{URA3}$ faa1::CPRB-$P_{URA3}$/faa1::CPRB-$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$ pox5Δ::$T_{URA3}$/pox5Δ::URA3*).



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Strain sAA2748 was grown overnight in YPD media and plated on 5-FOA plates. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of the *URA3* gene leaving behind the *URA3* terminator ($T_{URA3}$) in the second *POX5* allele. The resultant strain was saved as strain sAA2800 (*ura3/ura3 pox4a::PEX11-*$P_{URA3}$*/pox4b::PEX11-*$P_{URA3}$ *faa1::CPRB-*$P_{URA3}$*/faa1::CPRB-*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::*$P_{URA3}$ *pox5Δ::*$T_{URA3}$*/pox5Δ::*$T_{URA3}$).

Strain sAA3656 (*ura3/ura3 pox4a::PEX11-*$P_{URA3}$*/pox4b::PEX11-*$P_{URA3}$ *faa1::CPRB-*$P_{URA3}$*/faa1::CPRB-*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::*$P_{URA3}$ *pox5Δ::*$T_{URA3}$*/pox5Δ::*$T_{URA3}$ *pxa1-Δ1::*$P_{URA3}$*/pxa1-Δ2::*$P_{URA3}$)

Strain sAA3656 was generated by disrupting both alleles of *PXA1* in the genome of the starting strain sAA2800 (*ura3/ura3 pox4a::PEX11-*$P_{URA3}$*/pox4b::PEX11-*$P_{URA3}$ *faa1::CPRB-*$P_{URA3}$*/faa1::CPRB-*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::*$P_{URA3}$ *pox5Δ::*$T_{URA3}$*/pox5Δ::*$T_{URA3}$) in a series of successive double-crossover homologous recombination events as outlined in Fig. 41 and described herein. Both *PXA1* alleles were disrupted by transforming the strain with the double-crossover knock-out cassettes from plasmids pAA1117 and pAA1155.

A linear nucleic acid fragment containing a *URA3* selectable marker ($P_{URA3}URA3T_{URA3}P_{URA3}$) and a PXA1 5' sequence on one end of the fragment and *PXA1* 3' sequence on the other end of the fragment was amplified from plasmid pAA1117 (Fig. 40) using primers oAA2679 and oAA2684. The linear nucleic acid fragment was used to chemically transform strain sAA2800, and transformants were plated onto SCD-URA plates. The resultant colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of the knock-out cassette and one isolate was saved as strain sAA3467 (*ura3/ura3 pox4a::PEX11-*$P_{URA3}$*/pox4b::PEX11-*$P_{URA3}$ *faa1::CPRB-*$P_{URA3}$*/faa1::CPRB-*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::*$P_{URA3}$ *pox5Δ::*$T_{URA3}$*/pox5Δ::*$T_{URA3}$ *pxa1-Δ1::URA3/PXA1*).

Strain sAA3467 was grown overnight in YPD media and plated on 5-FOA plates. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of the *URA3* gene leaving behind the *URA3* promoter ($P_{URA3}$) in the first *PXA1* allele. The resultant strain was saved as strain sAA3522 (*ura3/ura3 pox4a::PEX11-*$P_{URA3}$*/pox4b::PEX11-*$P_{URA3}$ *faa1::CPRB-*$P_{URA3}$*/faa1::CPRB-*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::*$P_{URA3}$ *pox5Δ::*$T_{URA3}$*/pox5Δ::*$T_{URA3}$ *pxa1-Δ1::*$P_{URA3}$*/PXA1*).



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The knock-out of the second *PXA1* allele was performed by chemical transformation of strain sAA3522 with a double-crossover knock-out cassette generated by PCR with primers oAA2914 and oAA2919 and plasmid pAA1155 (Fig. 40) as template. The transformation reaction was plated onto SCD-URA plates. The resultant colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of the knock-out cassette at both alleles and one isolate was saved as strain sAA3584 (*ura3/ura3 pox4a::PEX11-$P_{URA3}$/pox4b::PEX11-$P_{URA3}$ faa1::CPRB-$P_{URA3}$/faa1::CPRB-$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$ pox5Δ::$T_{URA3}$/pox5Δ::$T_{URA3}$ pxa1-Δ1::$P_{URA3}$/pxa1-Δ2::URA3*).

Strain sAA3584 was grown overnight in YPD media and plated on 5-FOA plates. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of the *URA3* gene leaving behind the *URA3* promoter ($P_{URA3}$) in the second *PXA1* allele. The resultant strain was saved as strain sAA3656 (*ura3/ura3 pox4a::PEX11-$P_{URA3}$/pox4b::PEX11-$P_{URA3}$ faa1::CPRB-$P_{URA3}$/faa1::CPRB-$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$ pox5Δ::$T_{URA3}$/pox5Δ::$T_{URA3}$ pxa1-Δ1::$P_{URA3}$/pxa1-Δ2:: $P_{URA3}$*).

*EXAMPLE 15: Analysis of the growth of cells and organisms*

As described herein, modified organisms developed using compositions and methods provided herein were analyzed for growth in different media. The analysis method was generally carried out as follows. Overnight cultures of engineered transformant strains, as well as control strains, were grown in YPD medium at 30°C with shaking at 250 rpm. The $OD_{600nm}$ of the overnight cultures was measured and the cultures were normalized to an $OD_{600nm}$ of 0.1 by dilution with sterile water in sterile 1.5mL tubes. A serial dilution of the normalized cultures was prepared in 96-well microtiter plates by transferring 250 microliters of normalized cultures into a column 1 well and 200 microliters of water into wells of columns 2 through 6. Using a multichannel pipet, 50 microliters of normalized culture from a column 1 well was transferred to a column 2 well and pipetted up and down to mix. Then 50 microliters of the suspension in a column 2 well was transferred to a column 3 well and pipetted up and down to mix. This process was repeated for successive columns ending at column 6. Finally, 50 microliters of suspension from the column 6 well was removed and discarded, leaving 200 microliters in all wells. A sterile stamp tool formatted for use with 96 well plates was dipped into the 96 well plate containing the culture serial dilution and the adhering hanging drops were transferred to agar plates containing growth medium. The stamping was repeated for all growth medium plates desired. After allowing the



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transferred liquid to soak into the plates, they were incubated at 30°C for 1 to 5 days and inspected for growth.

*EXAMPLE 16: Generation of cells and organisms expressing modified carnitine acetyltransferase*

In one embodiment, increased carnitine O-acetyltransferase activity can be obtained by increasing the amount of carnitine O-acetyltransferase enzyme in the cytosol. For example, engineered carnitine O-acetyltransferase proteins lacking amino acid sequence targeting signals that direct the enzyme to one or more cellular locations (e.g., peroxisomes and mitochondria) other than the cytoplasm can be expressed in host cells in one method of increasing the amount of carnitine O-acetyltransferase in the cytoplasm. Such modified carnitine O-acetyltransferases can be produced upon expression of heterologous nucleic acids encoding the proteins that have been introduced into host cells. In some embodiments, carnitine O-acetyltransferase activity can be decreased in particular cellular locations. For example, host cells that have been modified to knock out endogenous carnitine acetyltransferase activity in mitochondria and peroxisomes can be transformed with nucleic acid encoding carnitine O-acetyltransferases that are less enzymatically active than the endogenous enzyme that target the protein to a particular cellular location(s) (e.g., mitochondria) other than the cytoplasm.

Generation of *CAT2* knock-out strains

Generation of cells in which endogenous *CAT2* genes are disrupted can be accomplished, for example, by successive double-crossover homologous recombination events using gene disruption methods known in the art and/or described herein. In one embodiment, the host is a strain of the yeast *Candida* that contains two alleles of the *CAT2* gene. The effective deletion via disruption of the first *CAT2* allele was performed using a double-crossover knock-out cassette that both deleted a portion of the *CAT2* coding region and disrupted the gene with a *URA3* selectable marker. A double-crossover knock-out cassette was obtained by PCR amplification of plasmid pAA1519 (Fig. 24) with primers oAA2372 and oAA2377. The PCR product was purified and chemically transformed into *Candida* strain ATCC 20913 (a $Ura^-$ version of *Candida* strain ATCC 20336) and the cells were grown on plates containing synthetic complete media supplemented with dextrose and lacking uracil (SCD-URA) to select for cells



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that incorporated the *URA3*-containing deletion cassette. The resultant colonies were streaked onto SCD-URA for isolation and characterization. PCR of genomic DNA was performed to confirm the presence of the deletion cassette, and two isolates (*ura3/ura3 cat2-Δ1::URA3/CAT2*) were saved as sAA4477 and sAA4478.

In order to generate a uracil auxotrophic version of strain sAA4477 that could be used as a host to disrupt the second *CAT2* gene allele using uracil-based selection techniques, sAA4477 was grown overnight in YPD media and plated on plates containing 5-fluoroorotic acid (5-FOA). 5-FOA is a compound that is metabolized by *URA3* cells into a chemical that is toxic to the cells and causes cell death. Growth of cells containing the $P_{URA3}URA3T_{URA3}P_{URA3}$ cassette from pAA1519, such as sAA4477 cells, on 5-FOA promotes recombination between the homologous $P_{URA3}$ sequences of the cassette resulting in "looping out" of the functional *URA3* gene and cells that are able to survive in the presence of 5-FOA. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of the *URA3* gene leaving behind only the *URA3* promoter ($P_{URA3}$) in the first *CAT2* allele. Two isolates with the correct construction (*ura3/ura3 cat2-Δ1::*$P_{URA3}$*/CAT2*) were saved as sAA4551 and sAA4552.

The deletion of the second *CAT2* allele was performed using a double-crossover knock-out cassette that both deleted a portion of the *CAT2* coding region and disrupted the gene with a *URA3* selectable marker. Plasmid pAA1520 (Fig. 24) was PCR amplified with primers oAA2372 and oAA2377. The PCR product was purified and chemically transformed into strain sAA4551 and the cells were plated onto SCD-URA plates. The resultant colonies were streaked onto SCD-URA for isolation and characterization. PCR of genomic DNA was performed to confirm the presence of the deletion cassette and two isolates (*ura3/ura3 cat2-Δ1::*$P_{URA3}$*/cat2-Δ2::URA3*) were saved as sAA4594 and sAA4595.

In order to generate a Ura$^-$ version of strain sAA4594 that could be used as a host to disrupt and/or introduce additional genes using uracil-based selection techniques, strain sAA4594 was grown overnight in YPD media and plated on 5-FOA plates. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of the *URA3* gene leaving behind only the *URA3* promoter ($P_{URA3}$) in the second *CAT2* allele. Two isolates with the correct construction (*ura3/ura3 cat2-Δ1::*$P_{URA3}$*/cat2-Δ2::*$P_{URA3}$) were saved as sAA4625 and sAA4626. Strains sAA4625 and sAA4626 have both *CAT2* alleles effectively deleted due to their disruption by the presence of the *URA3* promoter ($P_{URA3}$) at different positions of each *CAT2* allele.

Generation of strains containing nucleic acids encoding modified Cat2p

Yeast strains containing nucleic acids encoding modified Cat2 proteins lacking peroxisomal and/or mitochondrial targeting sequences were generated by transformation of a Cat2$^-$ strain sAA4625 with nucleic acid encoding Cat2p$^{\Delta mts}$, Cat2p$^{\Delta pts}$ or nucleic acid encoding Cat2p$^{\Delta mts\Delta pts}$. Plasmid pAA1610 containing nucleic acid encoding the modified Cat2p$^{\Delta mts}$ protein under the control of the *HDE1* gene promoter and *POX4* gene terminator positioned between two separate fragments of the *URA3* gene selectable marker was PCR amplified with primers oAA2206 and oAA2209. The PCR product was purified and chemically transformed into strain sAA4625 and the cells were grown on SC-URA plates. The resultant colonies were restreaked onto YPD for isolation and characterization. PCR of genomic DNA was performed to confirm the presence of the cassette, and an isolate was analyzed for growth as described herein.

Plasmid pAA1876 containing nucleic acid encoding the modified Cat2p$^{\Delta pts}$ protein under the control of the *HDE1* gene promoter and *POX4* gene terminator positioned between the two separate fragments of the *URA3* gene selectable marker was PCR amplified with primers oAA2206 and oAA2209. The PCR product was purified and chemically transformed into strain sAA4625 and the cells were grown on SC-URA plates. The resultant colonies were restreaked onto YPD for isolation and characterization. PCR of genomic DNA was performed to confirm the presence of the cassette, and an isolate was analyzed for growth as described herein.

*EXAMPLE 17: Generation of cells and organisms expressing a modified mitochondrial carnitine acetyltransferase*

As described in the following Example of spot-growth assays of yeast expressing modified carnitine acetyltransferase proteins, transformation of a Cat2$^-$ strain of *Candida* with nucleic acid encoding Cat2p$^{\Delta mts}$, which lacks a mitochondrial targeting sequence (MTS) but retains an intact peroxisomal targeting sequence (PTS) for targeting the enzyme to the peroxisome, was not sufficient to rescue the growth deficiency of the Cat2$^-$ strain when cultured using oleic acid as the sole carbon source. However, growth of the Cat2$^-$ strain on oleic acid could be restored through expression of a mitochondrial-targeted enzyme with carnitine acetyltransferase activity in conjunction with expression of the Cat2$^{\Delta mts}$ mutant. The mitochondrial carnitine acetyltransferase enzyme could be engineered to have a low activity level to restore minimal



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growth on fatty acids and provide a bottleneck resulting in increased cytoplasmic acetyl-carnitine concentrations. In order to generate yeast strains having a reduced carnitine acetyltransferase activity in the mitochondria, Cat2$^-$ strains were transformed with DNA encoding modified Yat1p enzymes. In wild-type *Candida* strain ATCC 20336, Yat1p, which has lower enzymatic activity than Cat2p, is localized to the cytoplasm. Therefore, to express the lower-activity carnitine acetyltransferase activity in the mitochondria, the Yat1p-encoding nucleic acid used in the transformation was modified to include nucleic acid encoding mitochondrial targeting sequences (MTS). The transforming DNA encoded the predicted MTS from the Cox4p, Cit1p or Cat2p proteins of *Candida* strain ATCC 20336 which was added to the 5' end of the DNA coding sequence of the *YAT1* gene such that the resulting encoded proteins contained the heterologous MTS in place of the native Yat1p initiating methionine.

Plasmid pAA1967 containing nucleic acid encoding the modified YAT1p$^{+COX4mts}$ protein under the control of the *HDE1* gene promoter and *POX4* gene terminator positioned between the two separate fragments of the *URA3* gene marker was PCR amplified with primers oAA2206 and oAA2209. The PCR product was purified and chemically transformed into Ura$^-$,Cat2$^-$ strain sAA4625 and the cells were grown on SC-URA plates. The resultant colonies were restreaked onto YPD for isolation and characterization. PCR of genomic DNA was performed to confirm the presence of the cassette, and an isolate was analyzed for growth as described herein. Plasmid pAA1968 containing nucleic acid encoding the modified YAT1p$^{+CIT1mts}$ protein under the control of the *HDE1* gene promoter and *POX4* gene terminator positioned between the two separate fragments of the *URA3* gene marker was PCR amplified with primers oAA2206 and oAA2209. The PCR product was purified and chemically transformed into strain sAA4625 and the cells were grown on SC-URA plates. The resultant colonies were restreaked onto YPD for isolation and characterization. PCR of genomic DNA was performed to confirm the presence of the cassette, and an isolate was analyzed for growth as described herein.

Plasmid pAA1969 containing nucleic acid encoding the modified YAT1p$^{+CAT2mts}$ protein under the control of the *HDE1* gene promoter and *POX4* gene terminator positioned between the two separate fragments of the *URA3* gene marker was PCR amplified with primers oAA2206 and oAA2209. The PCR product was purified and chemically transformed into strain sAA4625 and the cells were grown on SC-URA plates. The resultant colonies were restreaked onto YPD for isolation and characterization. PCR of genomic DNA was performed to confirm the presence of the cassette, and an isolate was analyzed for growth as described herein.



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*EXAMPLE 18: Analysis of organisms in which carnitine acetyltransferase expression has been modified*

Analysis of strains via growth assays

Transformant strains engineered for modified carnitine acetyltransferase expression were cultured in control media and in media containing oleic acid and inspected for growth using methods described herein in a previous Example. As shown in Fig. 44, the wild-type strain ATCC 20336 containing two complete *CAT2* genes was able to grow on the control plate containing synthetic complete media with dextrose minus uracil (SCD–URA) and on the fatty acid plate containing yeast nitrogen base without amino acids, plus phosphate and 2% oleic acid (YNBP + 2% oleic acid). A $Cat2^{-}$,$Ura^{+}$ strain (*cat2-Δ1::*$P_{URA3}$*/cat2-Δ2::URA3*) sAA4594 was able to grow only on the control plate. The deletion of the *CAT2* genes resulted in a mutant strain unable to grow on oleic acid as the sole carbon source. Spot growth assays of serial dilutions were used to evaluate the ability of a genetic construct encoding a Cat2p mutant to rescue the fatty acid growth deficiency of a $Cat2^{-}$ strain. Transformation of a $Ura3^{-}$,$Cat2^{-}$ strain sAA4625 with a genetic construct containing nucleic acid encoding $Cat2p^{\Delta mts}$ and *URA3* selectable marker (amplicon from PCR amplification of pAA1610 with primers oAA2206 and oAA2209) resulted in a strain able to grow on only the control plate. Transformation of a $Ura3^{-}$,$Cat2^{-}$ strain sAA4625 with a genetic construct containing nucleic acid encoding $Cat2p^{\Delta pts}$ and *URA3* selectable marker (amplicon from PCR amplification of pAA1876 with primers oAA2206/oAA2209) resulted in strains (three different transformants designated as *cat2-Δ1::*$P_{URA3}$*/cat2-Δ2::* $P_{URA3}$ + pAA1876 in Fig. 44) able to grow on both the control and oleic acid plates. Removal of the N-terminal MTS from the Cat2p prevented targeting of the enzyme to the mitochondrial compartment thereby preventing conversion of acetyl-carnitine to acetyl-CoA in the mitochondria and preventing growth on oleic acid. However, removal of the type 1 PTS from the C-terminus of Cat2p did not affect the ability of the enzyme to restore growth on fatty acids to a $Cat2^{-}$ strain. Without being limited or bound by theory, it is believed that the $Cat2p^{\Delta pts}$ is still targeted to the peroxisomal compartment via another mechanism and, with the intact MTS targeting it to the mitochondria, the $Cat2p^{\Delta pts}$ enzyme is able to restore growth on fatty acids.



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Although expression of *CAT2*$^{\Delta mts}$, which includes nucleic acid encoding a peroxisomal targeting sequence, should result in a Cat2p$^{\Delta mts}$ that is targeted to peroxisomes, expression of *CAT2*$^{\Delta mts}$ alone was not sufficient to rescue the oleic acid growth deficiency in strains in which both *CAT2* genes were disrupted. *Candida* Cat2$^{-}$ deletion strains transformed with DNA encoding additional modified mitochondrial carnitine acetyltransferase enzymes were also tested for growth on oleic acid to further investigate whether growth on fatty acids is restored by a mitochondrial targeted enzyme in conjunction with Cat2p$^{\Delta mts}$. Growth of the Cat2$^{-}$ strains on oleic acid was restored when a carnitine acetyl transferase enzyme (i.e., Yat1p) targeted to the mitochondria was expressed in cells in conjunction with expression of a peroxisomal-targeted Cat2p (encoded by *CAT2*$^{\Delta mts}$). A Ura3$^{-}$,Cat2$^{-}$ mutant sAA4625 was transformed with two separate DNA constructs: one containing a *URA3* selectable marker and *CAT2*$^{\Delta mts}$ (an amplicon from PCR amplification of pAA1610 with primers oAA2206 and oAA2209) and another containing DNA encoding a Yat1p with the MTS of one of three different mitochondrial enzymes (Cox4p, Cit1p or Cat2p) fused to its N-terminus (three separate amplicons from PCR amplification of plasmids pAA1967, pAA1968 and pAA1969 with primers oAA2206 and oAA2209). Because the wild-type endogenous Yat1p is a cytoplasmic enzyme, DNA constructs pAA1967, pAA1968 and pAA1969 were engineered as described herein to encode Yat1p with an MTS from the Cox4p, Cit1p and Cat2p proteins, respectively, added to the N-terminus for mitochondrial targeting. As shown in Fig. 45, the co-transformation of a Cat2$^{-}$ strain with DNA encoding Cat2p$^{\Delta mts}$ and a Yat1p$^{+mts}$ resulted in growth on both the control and oleic acid plates demonstrating a rescue of the fatty acid growth deficiency of a Cat2$^{-}$ deletion strain. Some of the transformants displayed slower growth which may be related to copy number of the transformed genetic constructs that integrated into the host cell genome and/or to the lower activity level of Yat1p. As shown in these results, a mitochondrial-targeted carnitine acetyltransferase enzyme (a Yat1p$^{+mts}$), which has a decreased activity level compared to wild-type, endogenous Cat2p enzymes, could restore minimal growth on oleic acid to a Cat2$^{-}$ *Candida* strain.

In vitro carnitine acetyltransferase enzyme activity assays

Wild-type control and transformant strains engineered for modified carnitine acetyltransferase expression can also be analyzed for total cellular carnitine acetyltransferase activity. Cells are grown for 48 hours in HiP-TAB medium (yeast nitrogen base without amino acids and without ammonium sulfate, 1.7 g/L; yeast extract, 3.0 g/L; potassium phosphate monobasic, 10.0 g/L;

potassium phosphate dibasic, 10.0 g/L) with 2% oleic acid. Approximately 100 mg of cells are harvested and washed with water then resuspended in 100 mM Tris-HCl, pH 8.0. Soluble cell extracts (in 100 mM Tris-HCl pH 8.0) are prepared by 3 rounds of bead beating cells using 1-minute pulses with 1-minute ice rests followed by pelleting insoluble debris at 13,000 x *g* for 10 min at 4°C. The assay reaction mixture contains 0.18 mM coenzyme A, 5.6 mM acetyl-DL-carnitine, and 92 mM Tris-HCl pH 8.0 buffer. Reactions are carried out at 25°C and enzyme activity is determined by monitoring an increase of absorption at 233 nm. One unit of activity represents the conversion of 1.0 µmole of acetyl-L-carnitine and CoA to L-carnitine and acetyl-coA per minute at pH 8.0 at 25°C.

*EXAMPLE 19: Generation of cells and organisms expressing a modified acetyl Co-A hydrolase, acetyl-CoA synthetase and/or carnitine acetyltransferase*

Peroxisomal beta-oxidation, which results in generation of acetyl Co-A and fatty acids, generally requires a supply of free coenzyme-A which, in a wild-type cell, can be supplied by peroxisomal Cat2p enzyme transferring acetyl units from acetyl-CoA to carnitine. Another enzyme for liberating coenzyme-A from acetyl-CoA is acetyl-CoA hydrolase (Ach1p). This enzyme can catalyze the conversion of acetyl-CoA to acetate. Because the Ach1p enzyme is targeted to the mitochondrial compartment in wild-type cells, engineering of Ach1p to reroute the enzyme to the peroxisome can involve removing the N-terminal mitochondrial targeting sequence (MTS) and adding a C-terminal peroxisomal targeting sequence (PTS) as described herein. The acetate produced in the peroxisomal compartment via the Ach1p reaction can diffuse out of the peroxisome into the cytoplasm where it can be converted back to acetyl-CoA by cytosolic acetyl-CoA synthetases (Acs1p, Acs2p), the expression of which can be amplified by increasing the copy number of ACS1/2p-encoding nucleic acids in cells and/or by replacing the promoter controlling transcription of the nucleic acids. Cytoplasmic acetyl-CoA can then be used in generating cytosolic malonyl-CoA and in fatty acid synthesis as described herein and/or converted to acetyl-carnitine by cytoplasmic Yat1p, transported into the mitochondria and converted back to acetyl-CoA by endogenous mitochondrial Cat2p or an engineered Yat1p with an added MTS expressed in mitochondria as also described herein.

A Ura3$^-$,Cat2$^-$ mutant strain sAA4625 of *Candida* was transformed with DNA constructs containing a *URA3* selectable marker, *ACH1*$^{\Delta mts+pts}$ encoding an Ach1p lacking an MTS and containing an added PTS, *ACS1*, *ACS2*, and *YAT1*$^{+mts}$ encoding a Yat1p containing an added

MTS from either Cox4p, Cit1p or Cat2p fused to the N-terminus as follows. All transformation cassettes were generated by PCR amplification from their respective plasmids (plasmids pAA1846, pAA1847, pAA1875, pAA1967, pAA1968 and pAA1969) using primers oAA2206 and oAA2209 then purified before transformation. Transformation reactions were plated on SC-URA to select for transformants that had integrated the DNA cassettes containing the URA3 selectable marker(s). Individual colonies were restreaked onto YPD plates for isolation prior to further use.

*EXAMPLE 20: Analysis of organisms in which acetyl Co-A hydrolase expression has been modified*

To assess the growth capabilities of strains transformed with DNA encoding a modified Ach1p, the strains were analyzed in spot growth assays (using assay methods described herein in a previous Example) and the results were compared to the growth of control strains. A Ura3$^-$,Cat2$^-$ *Candida* strain sAA4625 was transformed with DNA constructs containing a *URA3* selectable marker, *ACH1*$^{\Delta mts+pts}$ encoding an Ach1p lacking an MTS and containing an added PTS, *ACS1*, *ACS2*, and *YAT1*$^{+mts}$ encoding a Yat1p containing an added MTS from either Cox4p, Cit1p or Cat2p fused to the N-terminus. As shown in Fig. 46, although the control Cat2$^-$ deletion strain was unable to grow on oleic acid, the strain co-transformed with *ACH1*$^{\Delta mts+pts}$, *ACS1* and/or *ACS2*, and *YAT1*$^{+mts}$ resulted in growth on both the control (SCD-URA) and oleic acid plates demonstrating a rescue of the fatty acid growth deficiency of the Cat2$^-$ mutant. These results demonstrated that carbon from peroxisomal beta-oxidation could be rerouted to the cytoplasmic compartment in modified host cells.

*EXAMPLE 21: Generation of cells and organisms expressing heterologous nucleic acids for modified expression of acetyl-carnitine transport proteins, thioesterase, carnitine acetyltransferase and acetyl-CoA carboxylase*

Recombinant microorganisms were developed for modified expression of acetyl-carnitine transport proteins, thioesterase, carnitine acetyltransferase and acetyl-CoA carboxylase through transformation with heterologous nucleic acids as described herein. The cells used in the development of these microorganisms were uracil auxotrophs in which multiple genes (e.g., *CRC1*, *POX4*, *FAA1*, *FAT1*, *PXA1*) had been disrupted and the expression of multiple proteins (e.g., Pox5p, Pex11p, CPRBp, P450A17p) had been amplified and/or altered for increased



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activity of the proteins (e.g., enzymes). In one example, *Candida* host yeast cells in which the endogenous *CRC1* genes had been disrupted were used in the development of the modified recombinant microorganisms.

Generation of *CRC1* knock-out strains

Generation of cells in which endogenous *CRC1* genes are disrupted can be accomplished, for example, by double-crossover homologous recombination events using gene disruption methods known in the art and/or described herein. In one embodiment, the host is a strain of the yeast *Candida* that contains two alleles of the *CRC1* gene.

Generation of *CRC1* deletion strain sAA9426

The effective deletion via disruption of the first *CRC1* allele was performed using a cassette that both deleted a portion of the *CRC1* coding region and disrupted the gene with a *URA3* selectable marker containing a $P_{URA3}$ repeat ($P_{URA3}URA3T_{URA3}P_{URA3}$). The linear DNA construct for transformation was PCR amplified from plasmid pAA1613 (Fig. 32) with primers oAA5511 and oAA5512. The PCR product was purified and chemically transformed into *Candida* strain ATCC 20913 (a Ura$^-$ derivative of *Candida* strain ATCC 20336) and the cells were plated onto SCD-URA plates. The resultant colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of the deletion cassette and one isolate was saved as strain sAA4368 (*ura3/ura3 crc1-Δ1::URA3/CRC1)*.

Strain sAA4368 was grown overnight in YPD media and plated on 5-FOA plates. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of the *URA3* gene leaving behind only the *URA3* promoter ($P_{URA3}$) in the first *CRC1* allele. The resultant strain was saved as strain sAA4388 (*ura3/ura3 crc1-Δ1::*$P_{URA3}$*/CRC1)*.

The deletion of the second *CRC1* allele was performed using a cassette that deleted a shorter portion of the *CRC1* coding region than the first allele disruption cassette and disrupted the gene with a *URA3* selectable marker containing a $P_{URA3}$ repeat ($P_{URA3}URA3T_{URA3}P_{URA3}$). The linear DNA construct for transformation was PCR amplified from plasmid pAA1701 (Fig. 32) with primers oAA5511 and oAA5512. The PCR product was purified and chemically transformed into strain sAA4388 and the cells were plated onto SCD-URA plates. The resultant colonies



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were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of the deletion cassette and one isolate was saved as strain sAA9398 (*ura3/ura3 crc1-Δ1::$P_{URA3}$/crc1-Δ2::URA3)*.

Strain sAA9398 was grown overnight in YPD media and plated on 5-FOA plates. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of the *URA3* gene leaving behind only the *URA3* promoter ($P_{URA3}$) in the second *CRC1* allele. The resultant strain was saved as strain sAA9426 (*ura3/ura3 crc1-Δ1::$P_{URA3}$/ crc1-Δ2::$P_{URA3}$)*. Table 10 summarizes the steps, intermediate strains and genotypes in the development of strain sAA9426.

**Table 10: Yeast strain genotypes and steps in constructing sAA9426**

| Name | Ura3 +/- | Plasmid | Genotype |
|---|---|---|---|
| ATCC 20913 | - | NA | *ura3/ura3* |
| sAA4368 | + | pAA1613 | *ura3/ura3* ***crc1-Δ1::URA3****/CRC1* |
| sAA4388 | - | NA | *ura3/ura3* ***crc1-Δ1::$P_{URA3}$****/CRC1* |
| sAA9398 | + | pAA1701 | *ura3/ura3 crc1-Δ1::$P_{URA3}$/****crc1-Δ2::URA3*** |
| sAA9426 | - | NA | *ura3/ura3 crc1-Δ1::$P_{URA3}$/****crc1-Δ2::$P_{URA3}$*** |

Generation of *CRC1* deletion strain sAA4377

The effective deletion via disruption of the first *CRC1* allele was performed using a cassette that both deleted a portion of the *CRC1* coding region and disrupted the gene with a *URA3* selectable marker containing a $P_{URA3}$ repeat ($P_{URA3}URA3T_{URA3}P_{URA3}$). The linear DNA construct for transformation was PCR amplified from plasmid pAA1613 (Fig. 32) with primers oAA5511 and oAA5512. The PCR product was purified and chemically transformed into strain sAA886 (*ura3/ura3 pox4a::ura3/pox4b::ura3 faa1::*$P_{URA3}$*/faa1::*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::*$P_{URA3}$), which was developed through a series of homologous recombination steps from *Candida* strain ATCC 20962 (sAA003) as described herein, and the cells were plated onto SCD-URA plates. The resultant colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of the deletion cassette and one isolate was saved as strain sAA4057 (*ura3/ura3 pox4a::ura3/pox4b::ura3 faa1::*$P_{URA3}$*/faa1::*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::*$P_{URA3}$ *crc1-Δ1::URA3/CRC1)*.

Strain sAA4057 was grown overnight in YPD media and plated on 5-FOA plates. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of the *URA3* gene leaving behind only the *URA3* promoter ($P_{URA3}$) in the first *CRC1* allele. The resultant strain was saved as strain sAA4096 (*ura3/ura3 pox4a::ura3/pox4b::ura3 faa1::*$P_{URA3}$*/faa1::*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::*$P_{URA3}$ *crc1-Δ1::*$P_{URA3}$*/CRC1)*.

The deletion of the second *CRC1* allele was performed using a cassette that deleted a shorter portion of the *CRC1* coding region than the first allele disruption cassette and disrupted the gene with a *URA3* selectable marker containing a $P_{URA3}$ repeat ($P_{URA3}URA3T_{URA3}P_{URA3}$). The linear DNA construct for transformation was PCR amplified from plasmid pAA1701 (Fig. 32) with primers oAA5511 and oAA5512. The PCR product was purified and chemically transformed into strain sAA4096 and the cells were plated onto SCD-URA plates. The resultant colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of the deletion cassette and one isolate was saved as strain sAA4281 (*ura3/ura3 pox4a::ura3/pox4b::ura3 faa1::*$P_{URA3}$*/faa1::*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::*$P_{URA3}$ *crc1-Δ1::*$P_{URA3}$*/crc1-Δ2::URA3)*.

Strain sAA4281 was grown overnight in YPD media and plated on 5-FOA plates. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of the *URA3* gene



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leaving behind only the *URA3* promoter ($P_{URA3}$) in the second *CRC1* allele. The resultant strain was saved as strain sAA4377 (*ura3/ura3 pox4a::ura3/pox4b::ura3 faa1::$P_{URA3}$/faa1::$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$ crc1-Δ1::$P_{URA3}$/ crc1-Δ2::$P_{URA3}$)*. This *CRC1* deletion strain contains mutations in the *FAA1*, *FAT1*, *POX4* and *URA3* genes that inactivate cytoplasmic acyl-CoA synthetases (Faa1p and Fat1p), which prevents free fatty acids in the cytoplasm from being reactivated to the acyl-CoA form, a peroxisomal acyl-CoA oxidase (Pox4p), which blocks break down of short-chain acyl-CoA in β-oxidation, and orotidine-5-phosphate decarboxylase, which results in uracil auxotrophy of the strain. Table 11 summarizes the steps, intermediate strains and genotypes in the development of strain sAA4377.

**Table 11: Yeast strain genotypes and steps in constructing sAA4377**

| Name | Ura3 +/- | Plasmid | Genotype |
|---|---|---|---|
| sAA886 | - | NA | *ura3/ura3 pox4a::ura3/pox4b::ura3 faa1::$P_{URA3}$/faa1::$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$* |
| sAA4057 | + | pAA1613 | *ura3/ura3 pox4a::ura3/pox4b::ura3 faa1::$P_{URA3}$/faa1::$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$* ***crc1-Δ1::URA3****/CRC1* |
| sAA4096 | - | NA | *ura3/ura3 pox4a::ura3/pox4b::ura3 faa1::$P_{URA3}$/faa1::$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$* ***crc1-Δ1::$P_{URA3}$****/CRC1* |
| sAA4281 | + | pAA1701 | *ura3/ura3 pox4a::ura3/pox4b::ura3 faa1::$P_{URA3}$/faa1::$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$ crc1-Δ1::$P_{URA3}$/**crc1-Δ2::URA3*** |
| sAA4377 | - | NA | *ura3/ura3 pox4a::ura3/pox4b::ura3 faa1::$P_{URA3}$/faa1::$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$ crc1-Δ1::$P_{URA3}$/**crc1-Δ2::$P_{URA3}$*** |

Generation of *CRC1* deletion strain sAA6270

A *CRC1* deletion strain was constructed in host strain sAA3656 (described in a previous Example) that had been modified for enhanced ω-oxidation and altered β-oxidation. The effective deletion via disruption of the first *CRC1* allele was performed using a cassette that both deleted a portion of the *CRC1* coding region and disrupted the gene with a *URA3* selectable marker containing a $P_{URA3}$ repeat ($P_{URA3}URA3T_{URA3}P_{URA3}$). The linear DNA construct for transformation was PCR amplified from plasmid pAA1613 (Fig. 32) with primers oAA5511 and oAA5512. The PCR product was purified and chemically transformed into strain sAA3656 and the cells were plated onto SCD-URA plates. The resultant colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of the deletion cassette and one isolate was saved as strain sAA6087 (*ura3/ura3 pox4a::PEX11-*

*$P_{URA3}$/pox4b::PEX11-$P_{URA3}$ faa1::CPRB-$P_{URA3}$/faa1::CPRB-$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$ pox5Δ::$T_{URA3}$/pox5Δ::$T_{URA3}$ pxa1-Δ1::$P_{URA3}$/pxa1-Δ2::$P_{URA3}$ crc1-Δ1::URA3/CRC1*).

Strain sAA6087 was grown overnight in YPD media and plated on 5-FOA plates. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of the *URA3* gene leaving behind only the *URA3* promoter ($P_{URA3}$) in the first *CRC1* allele. The resultant strain was saved as strain sAA6151 (*ura3/ura3 pox4a::PEX11-$P_{URA3}$/pox4b::PEX11-$P_{URA3}$ faa1::CPRB-$P_{URA3}$/faa1::CPRB-$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$ pox5Δ::$T_{URA3}$/pox5Δ::$T_{URA3}$ pxa1-Δ1::$P_{URA3}$/pxa1-Δ2::$P_{URA3}$ crc1-Δ1::$P_{URA3}$/CRC1*).

The deletion of the second *CRC1* allele was performed using a cassette that deleted a shorter portion of the *CRC1* coding region than the first allele disruption cassette and disrupted the gene with a *URA3* selectable marker containing a $P_{URA3}$ repeat (*$P_{URA3}$URA3$T_{URA3}$$P_{URA3}$*). The linear DNA construct for transformation was PCR amplified from plasmid pAA1701 (Fig. 32) with primers oAA5511 and oAA5512. The PCR product was purified and chemically transformed into strain sAA6151 and the cells were plated onto SCD-URA plates. The resultant colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of the deletion cassette and one isolate was saved as strain sAA6192 (*ura3/ura3 pox4a::PEX11-$P_{URA3}$/pox4b::PEX11-$P_{URA3}$ faa1::CPRB-$P_{URA3}$/faa1::CPRB-$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$ pox5Δ::$T_{URA3}$/pox5Δ::$T_{URA3}$ pxa1-Δ1::$P_{URA3}$/pxa1-Δ2::$P_{URA3}$ crc1-Δ1::$P_{URA3}$/crc1-Δ2::URA3*).

Strain sAA6192 was grown overnight in YPD media and plated on 5-FOA plates. Colonies that grew in the presence of 5-FOA were PCR screened for the looping out of the *URA3* gene leaving behind only the *URA3* promoter ($P_{URA3}$) in the second *CRC1* allele. The resultant strain was saved as strain sAA6270 (*ura3/ura3 pox4a::PEX11-$P_{URA3}$/pox4b::PEX11-$P_{URA3}$ faa1::CPRB-$P_{URA3}$/faa1::CPRB-$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$ pox5Δ::$T_{URA3}$/pox5Δ::$T_{URA3}$ pxa1-Δ1::$P_{URA3}$/pxa1-Δ2::$P_{URA3}$ crc1-Δ1::$P_{URA3}$/crc1-Δ2::$P_{URA3}$*). This *CRC1* deletion strain contains mutations in the *FAA1*, *FAT1*, *POX4, POX5, PXA1* and *URA3* genes that inactivate cytoplasmic acyl-CoA synthetases (thereby preventing free fatty acids in the cytoplasm from being reactivated to the acyl-CoA form), peroxisomal acyl-CoA oxidases (thereby blocking break down of acyl-CoA in β-oxidation), an ATP-binding cassette transporter (thereby blocking import of activated acyl-CoA into the peroxisomes) and orotidine-5-phosphate decarboxylase (resulting



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in uracil auxotrophy of the strain). Table 12 summarizes the steps, intermediate strains and genotypes in the development of strain sAA6270.

**Table 12: Yeast strain genotypes and steps in constructing sAA6270**

| Name | Ura3 +/- | Plasmid | Genotype |
|---|---|---|---|
| sAA3656 | - | NA | *ura3/ura3 pox4a::PEX11-$P_{URA3}$/pox4b::PEX11-$P_{URA3}$ faa1::CPRB-$P_{URA3}$/faa1::CPRB-$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$ pox5Δ::$T_{URA3}$/pox5Δ::$T_{URA3}$ pxa1-Δ1::$P_{URA3}$/**pxa1-Δ2::$P_{URA3}$*** |
| sAA6087 | + | pAA1613 | *ura3/ura3 pox4a::PEX11-$P_{URA3}$/pox4b::PEX11-$P_{URA3}$ faa1::CPRB-$P_{URA3}$/faa1::CPRB-$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$ pox5Δ::$T_{URA3}$/pox5Δ::$T_{URA3}$ pxa1-Δ1::$P_{URA3}$/pxa1-Δ2::$P_{URA3}$ **crc1-Δ1::URA3**/CRC1* |
| sAA6151 | - | NA | *ura3/ura3 pox4a::PEX11-$P_{URA3}$/pox4b::PEX11-$P_{URA3}$ faa1::CPRB-$P_{URA3}$/faa1::CPRB-$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$ pox5Δ::$T_{URA3}$/pox5Δ::$T_{URA3}$ pxa1-Δ1::$P_{URA3}$/pxa1-Δ2::$P_{URA3}$ **crc1-Δ1::$P_{URA3}$**/CRC1* |
| sAA6192 | + | pAA1701 | *ura3/ura3 pox4a::PEX11-$P_{URA3}$/pox4b::PEX11-$P_{URA3}$ faa1::CPRB-$P_{URA3}$/faa1::CPRB-$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$ pox5Δ::$T_{URA3}$/pox5Δ::$T_{URA3}$ pxa1-Δ1::$P_{URA3}$/pxa1-Δ2::$P_{URA3}$ crc1-Δ1::$P_{URA3}$/**crc1-Δ2::URA3*** |
| sAA6270 | - | NA | *ura3/ura3 pox4a::PEX11-$P_{URA3}$/pox4b::PEX11-$P_{URA3}$ faa1::CPRB-$P_{URA3}$/faa1::CPRB-$P_{URA3}$ fat1-Δ1::$P_{URA3}$/fat1-Δ2::$P_{URA3}$ pox5Δ::$T_{URA3}$/pox5Δ::$T_{URA3}$ pxa1-Δ1::$P_{URA3}$/pxa1-Δ2::$P_{URA3}$ crc1-Δ1::$P_{URA3}$/**crc1-Δ2::$P_{URA3}$*** |

Analysis of *CRC1* deletion strains

Growth assays of *CRC1* deletion strains

Transformant strains engineered for modified Crc1p expression were cultured in control media and in media containing oleic acid and inspected for growth using methods described herein in a previous Example. As shown in Fig. 47, yeast strains encoding at least one functional allele of *CRC1* are able to grow on both control media (SCD-URA) and on the fatty acid media (YNBP + 2% oleic acid). Yeast strains with both *CRC1* alleles knocked out are able to grow on control media but not on fatty acid media, demonstrating the key function of the Crc1p inner mitochondrial acetyl-carnitine transporter in fatty acid metabolism. The effect of the double *CRC1* knockout is demonstrated in two different genetic backgrounds. The single (sAA4368) and double (sAA9398) *CRC1* knockouts were generated from *Candida* strain ATCC 20913 (a

Ura- derivative of *Candida* strain ATCC20336). The single (sAA4057) and double (sAA4281) *CRC1* knockouts were generated from strain sAA875, which is a partially beta-oxidation blocked strain described in a previous Example. Included in the growth assay are yeast strains (sAA5916, sAA5917 and sAA5918) generated by transformation of a double-crossover integration cassette into sAA4377 (the Ura- derivative of sAA4281) that was targeted to IGR5 and contained a *URA3* selectable marker and a *CRC1* gene linked to a weak promoter. The weak promoters used were from the genes encoding glucose-6-phosphate isomerase (*G6PI* in sAA5916), mitochondrial copper and phosphate carrier (*PIC2* in sAA5917), or high-affinity sulfate permease (*SUL2* in sAA5918). The transformants with *CRC1* driven by a weak promoter each contained one integrated copy of the double-crossover cassette. None of the weak promoters were sufficient, with one genomic copy, to drive the expression of enough *CRC1* to rescue the growth defect of the parental strain on fatty acid media.

Generation of strains sAA6317 and sAA6424-sAA6428 expressing heterologous nucleic acids for modified expression of Crc1p, Tes3p, Cat2p and Acc1p

*CRC1* deletion strain sAA6270 was used as a host strain in the development of recombinant yeast strains expressing heterologous nucleic acids for modified expression of acetyl-carnitine transport proteins (Crc1p), thioesterase (Tes3p), carnitine acetyltransferase (Cat2p) and acetyl-CoA carboxylase (Acc1p). The final recombinant strains contain DNA encoding: (1) Crc1p under the control of the glucose-6-phosphate isomerase (G6PI) promoter for reduced expression of Crc1p, (2) $Tes3p^{\Delta pts}$ under the control of the hydratase-dehydrogenase-epimerase (*HDE*) promoter to provide for thioesterase enzyme activity in the cytoplasm, (3) $Cat2p^{\Delta mts\Delta pts}$ under the control of the HDE promoter to provide for increased carnitine O-acetyltransferase activity in the cytoplasm, and (4) modified Acc1p (S1153A) under the control of the HDE promoter to provide for increased acetyl-CoA carboxylase activity in the cytoplasm. These heterologous nucleic acids are expressed in the genetic background of a host cell that has been modified to express heterologous DNA encoding a protein (Pox5p(F98G)) for increased selective peroxisomal acyl-CoA oxidase activity as well as proteins for increased cytochrome P450 reductase (CPRBp) and monooxygenase (P450A17p) activities and peroxisomal biogenesis (Pex11p) activity. The host cell has also been modified to decrease or eliminate expression of cytosolic acyl-CoA synthetase, Pox4p acyl-CoA oxidase and peroxisomal Pxa1p acyl-CoA transport protein.



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Linear DNA cassettes were generated from plasmids pAA1667 ($P_{HDE1}CAT2^{\Delta mts\Delta pts}T_{POX4}$), pAA1164 ($P_{HDE}POX5(F98G)T_{POX4}$), pAA1609 ($P_{HDE1}TES3^{\Delta pts}T_{POX4}$), pAA1712 ($P_{HDE1}P450A17T_{POX4}$), and pAA2311 ($P_{G6PI}CRC1T_{POX4}$) by PCR amplification using primers oAA2206 and oAA2209. A linear DNA cassette was also generated from plasmid pAA1907 ($P_{HDE}$-*ACC1(S1153A)*-$T_{PGK}$) by digestion with *Spe*I. The linear DNA cassettes were designed for homologous recombination into the host *ura3* locus. The DNA cassettes were purified and chemically transformed together into strain sAA6270, which was then spread onto plates lacking uracil (SC-URA). The resultant colonies were streaked onto SC-URA plates for isolation and colony PCR was used to verify the presence of the transformed genes. Growth on YNBP+oleic acid plates was used to confirm integration of DNA encoding Crc1p and Pox5p(F98G) into the host genome. Strains confirmed to be $URA^+$ and containing DNA encoding the recycling loop enzymes (Crc1p, $Tes3p^{\Delta pts}$, $Cat2p^{\Delta mts\Delta pts}$ and Acc1(S1153A)p) were selected and stocked as sAA6317, sAA6424, sAA6425, sAA6426, sAA6427, and sAA6428.

Diacid production by strains sAA6317 and sAA6424-sAA6428 from oleic acid in shake flasks

Transformant strains were grown in shake flask fermentations of oleic acid and analyzed for production of fatty acids. Cultures (5 ml) of each strain were grown overnight in YPD medium and used to inoculate 250-mL glass flasks containing 50mL of SP92 media to an initial $OD_{600nm}$ of 0.4 (SP92 media: 6.7 g/L Difco yeast nitrogen base, 3.0 g/L Difco yeast extract, 3.0 g/L ammonium sulfate, 1.0 g/L potassium phosphate monobasic, 1.0 g/L potassium phosphate dibasic, 75 g/L glycerol). After 24 h incubation at 30°C with shaking at 250 rpm (2" throw incubator) the cells were centrifuged and the cell pellet resuspended in 15 mL of HiP-TAB media (yeast nitrogen base without amino acids and without ammonium sulfate, 1.7 g/L; yeast extract, 3.0 g/L; potassium phosphate monobasic, 10.0 g/L; potassium phosphate dibasic, 10.0 g/L). The cultures were transferred to fresh 250-mL glass bottom-baffled flasks and 2% v/v oleic acid was added. Cultures were incubated at 30°C with shaking at 300 rpm to start fatty acid production. Samples of culture broth were taken every 24 hours for gas chromatographic (GC) analysis to determine the diacid composition of the culture broth. The results of analyses of the transformant strains were compared to those of control strain sAA5082. Strain sAA5082 was generated by transforming strain sAA3656 with antibiotic-free DNA cassettes amplified by PCR with primers oAA2206 and oAA2209 from plasmids pAA1164, pAA1712, pAA573, pAA1672 and pAA1763. Respectively, these plasmids contain DNA encoding Pox5p(F98G),



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P450A17, Zwf1p (a *Candida* strain ATCC 20336 glucose-6-phosphate dehydrogenase), and Idp2p and Idp1p$^{\Delta mts}$ (both a *Candida* strain ATCC 20336 isocitrate dehydrogenase). Nucleic acids encoding Pox5p(F98G) and P450A17 are under the control of the *HDE1* promoter and *POX4* terminator. Nucleic acids encoding Idp2p and Idp1p$^{\Delta mts}$ are under the control of the promoter and terminator of *Candida* strain ATCC 20336 *SPS19* gene (encoding peroxisomal 2,4-dienoyl-CoA reductase). DNA encoding Zwf1p is under the control of the *Candida* strain ATCC 20336 *POX4* promoter and terminator. Strain sAA5082 does not contain genetic modifications for rerouting acetyl-CoA (i.e., DNA encoding the recycling loop enzymes Crc1p, Tes3p$^{\Delta pts}$, Cat2p$^{\Delta mts\Delta pts}$ and Acc1(S1153A)p); however, it does contain modified genes for the expression of enzymes producing cytoplasmic NADPH.

The dicarboxylic acid content of the culture broth of shake flask fermentations comparing transformant and control strains are provided in Table 13. As shown in the Table, the transformant strains containing DNA encoding Tes3p$^{\Delta pts}$, Cat2p$^{\Delta mts\Delta pts}$, Acc1(S1153A)p and Crc1p (under the control of the G6PI promoter), generally exhibit higher adipic acid (C6 diacid) production than a control strain that does not contain these transforming nucleic acids.

**Table 13: Diacid content of culture broth after 48-hour shake flask growth of strains**

| Strain | g/L C8 diacid | g/L C6 diacid (yield as %Ymax*) |
|---|---|---|
| sAA5082 | 0.2 | 15.6 (83) |
| sAA6317 | 0.4 | 15.6 (84) |
| sAA6424 | 0.2 | 16.6 (90) |
| sAA6425 | 0.6 | 17.2 (92) |
| sAA6426 | 0.3 | 16.8 (93) |
| sAA6427 | 1.3 | 16.5 (93) |
| sAA6428 | 1.3 | 15.6 (95) |

*Ymax is maximum theoretical yield. It is the amount of product that can be produced for a given biochemical pathway given a certain amount of consumed feedstock (i.e., grams adipic acid/grams oleic acid). The empirical yield is calculated by: mass of product produced divided by the mass of substrate consumed.

Diacid production by strains sAA875, sAA6317 and sAA6425 from oleic acid in shake flasks

Transformant strains sAA6317 and sAA6425 were also grown in additional shake flask fermentations of oleic acid (using the same growth and fermentation methods as described in the preceding example for shake flask fermentation and production of diacids by strains sAA6317 and sAA6424-sAA6428) and analyzed for production of fatty acids. Samples of culture broth were taken every 24 hours for gas chromatographic (GC) analysis to determine the diacid composition of the culture broth. The results of analyses of the transformant strains were compared to those of control strain sAA875 (*ura3/ura3 pox4a::ura3/pox4b::ura3 POX5/POX5 faa1::* $P_{URA3}$*/faa1::*$P_{URA3}$ *fat1-Δ1::*$P_{URA3}$*/fat1-Δ2::URA3*). Strain sAA875 does not contain genetic modifications for rerouting acetyl-CoA (i.e., DNA encoding the recycling loop enzymes Crc1p, $Tes3p^{\Delta pts}$, $Cat2p^{\Delta mts\Delta pts}$ and Acc1(S1153A)p).

The dicarboxylic acid content of the culture broth of shake flask fermentations comparing transformant and control strains are provided in Table 14. As shown in the Table, the transformant strains containing DNA encoding $Tes3p^{\Delta pts}$, $Cat2p^{\Delta mts\Delta pts}$, Acc1(S1153A)p and Crc1p (under the control of the G6PI promoter), generally exhibit higher adipic acid (C6 diacid) production than a control strain that does not contain these transforming nucleic acids.

**Table 14: Diacid content of culture broth after 48-hour shake flask growth of strains**

| Strain | g/L C8 diacid | g/L C6 diacid (yield as %Ymax*) |
|---|---|---|
| sAA875 | 6.4 | 8.5 (79) |
| sAA6317 | 0.7 | 18.7 (93) |
| sAA6425 | 1.3 | 17.5 (92) |

*Ymax is maximum theoretical yield. It is the amount of product that can be produced for a given biochemical pathway given a certain amount of consumed feedstock (i.e., grams adipic acid/grams oleic acid). The empirical yield is calculated by: mass of product produced divided by the mass of substrate consumed.

Diacid production by strains sAA6317, sAA6425, and sAA6428 from palm fatty acid distillate (PFAD) in a fermentor

Fermentation medium of composition 0.810 g/L phosphoric acid, 1.22 g/L potassium phosphate dibasic, 1.536 g/L magnesium sulfate heptahydrate, 0.058 g/L calcium sulfate dihydrate, 90



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mg/L citric acid anhydrous, 3.0 mg/L biotin, 54 g/L glucose, 1 mL of iron solution (1000X stock solution: 60 g/L iron(II)sulfate heptahydrate, 10% sulfuric acid) and 1 mL trace metals mix (1,000X stock solution: 2.5 g/L citric acid, 0.165 g/L cupric sulfate pehtahydrate, 1.209 g/L manganese sulfate monohydrate, 0.54 g/L sodium molybdate, 1.08 g/L zinc sulfate heptahydrate) was filter sterilized and transferred to a sterile fermentation vessel (New Brunswick Bioflo 310 system using 2.5 L vessels). Growth of all *Candida* strains was initiated with a 5% inoculum (initial $OD_{600nm}$=1.0) and growth conditions of 35°C, 1000 rpm, 1 vvm, pH 5.8 and initial volume of 1.0 L. The pH was maintained with a 7% solution of $NH_4OH$. Growth continued for approximately 15 hours before exhaustion of the initial carbon source. When the initial carbon source was exhausted as noted by a marked increase in DO%, the temperature control was changed to 30°C, the pH control was changed to 4.5, and the conversion phase was initiated by turning on a continuous feed of ethylated PFAD at a rate of 1.04 g/L-h. PFAD is a fatty acid-containing residue resulting from the process of refining palm oil. Fermentation conditions were maintained at 30°C, 1000 rpm, 1 vvm, and pH 4.5 for 12 hours at which point the continuous feed rate of ethylated PFAD was changed to 2.25 g/L-h. The fermentation was carried out for a total of 130 hours. Samples were collected for gas chromatographic analysis every 24 hours after initiating the conversion phase.

The dicarboxylic acid content of the final fermentation broth is provided in Table 15. As shown in the Table, the transformant strains sAA6317, sAA6425, and sAA6428 containing DNA encoding $Tes3p^{\Delta pts}$, $Cat2p^{\Delta mts\Delta pts}$, Acc1(S1153A)p and Crc1p (under the control of the G6PI promoter), generally exhibit higher adipic acid (C6 diacid) titer and yield than a control strain (sAA5082) that does not contain these transforming nucleic acids.

**Table 15: Diacid content of fermentation broth after 130-hour fermentation of strains**

| Strain | g/L C8 diacid | g/L C6 diacid (yield as %Ymax*) |
|---|---|---|
| sAA5082 | 0.9 | 59.0 (81.5) |
| sAA6317 | 2.7 | 60.8 (90.0) |
| sAA6425 | 3.1 | 63.9 (92.4) |
| sAA6428 | 3.9 | 69.3 (96.2) |

*Ymax is maximum theoretical yield. It is the amount of product that can be produced for a given biochemical pathway given a certain amount of consumed feedstock (i.e., grams adipic acid/grams oleic acid). The empirical yield is calculated by: mass of product produced divided by the mass of substrate consumed.

*EXAMPLE 22: Generation of organisms expressing modified ACL1 and ACL2 genes*

Heterolgous nucleic acid constructs containing DNA encoding *Yarrowia lipolytica* CLIB122 ATP citrate lyase subunits (Acl1p and Acl2p) that was codon-optimized for expression in *Candida* strain ATCC 20336 were used to transform yeast host cells. Plasmids pAA1980 and pAA1981 contained the Acl1p and Acl2p subunit-encoding nucleic acids, respectively, operably linked to an *HDE1* promoter and *PEX11* terminator. The heterologous nucleic acids were used to transform strain sAA886 lacking functional *POX4*, *FAA1* and *FAT1* genes.

For transformation of host cells with DNA contained within pAA1980 and pAA1981, the plasmids were digested with BSTZ17I and the linear DNA was introduced into the cells for integration at the *ura3* alleles. Transformation reactions were plated on SC-URA to select for transformants that had integrated the DNA cassettes containing the *URA3* selectable marker. Individual colonies were restreaked onto YPD plates for isolation prior to further use. Four isolates were verified by PCR to have integrated both pAA1980 and pAA1981 and were saved as strains sAA5244, sAA5245, sAA5246, and sAA5247.

*EXAMPLE 23: Analysis of organisms in which ATP citrate lyase expression has been modified*

Strains transformed with heterologous nucleic acids encoding Acl1p and Acl2p were analyzed for dicarboxylic acid production during growth in shake flasks. Glass flasks (250 mL) containing 25 mL of SP92 media were inoculated with a 5 mL YPD overnight culture to an initial $OD_{600nm}$ of 0.2. After 24 h incubation at 30°C with shaking at 250 rpm (2" throw incubator), the cells were centrifuged and the cell pellet resuspended in 12.5 mL of HiP-TAB media (yeast nitrogen base without amino acids and without ammonium sulfate, 1.7 g/L; yeast extract, 3.0 g/L; potassium phosphate monobasic, 10.0 g/L; potassium phosphate dibasic, 10.0 g/L). The cultures were transferred to fresh 250-mL glass bottom-baffled flasks and 2% v/v oleic acid was added. Cultures were incubated at 30°C with shaking at 300 rpm to start adipic acid production. Samples of culture broth were taken every 24 hours for gas chromatographic (GC) analysis. Table 16 shows the diacid profile in the culture broth of the shake flasks 48 hours after addition of the oleic acid.

The results of analyses of the transformed strains containing heterologous DNA encoding Acl1p and Acl2p were compared to those of the *Candida* strain sAA875 that was not transformed with

the heterologous DNA and does not express an endogenous ATP citrate lyase. As shown in Table 16, the transformants containing heterologous ACL enzyme display an increased titer of adipic acid (C6 diacid) compared to the control strain. Additionally, the transformants selectively produce C6 diacid with very little C8 diacid produced.

**Table 16: Diacid content of culture broth after 48-hour shake flask growth of strains transformed with DNA encoding ATP citrate lyase**

| Strain name | Heterologous ATP-Citrate Lyase | g/L C8 diacid | g/L C6 diacid |
|---|---|---|---|
| sAA875 | None | 3.51 | 7.60 |
| sAA5244 | *Y. lipolytica* ACL1 & ACL2 | 0.01 | 8.76 |
| sAA5245 | *Y. lipolytica* ACL1 & ACL2 | 0.01 | 8.31 |
| sAA5246 | *Y. lipolytica* ACL1 & ACL2 | 0.01 | 8.43 |
| sAA5247 | *Y. lipolytica* ACL1 & ACL2 | 0.01 | 7.87 |

*EXAMPLE 24: Analysis of organisms in which thioesterase expression has been modified*

Plasmid pAA1609 harboring the nucleic acid encoding the Tes3p$^{\Delta pts}$ protein under the control of the *HDE1* gene promoter and *POX4* gene terminator was transformed into strain sAA2800 (*ura3/ura3 pox4a::PEX11-P$_{URA3}$/pox4b::PEX11-P$_{URA3}$ faa1::CPRB-P$_{URA3}$/faa1::CPRB-P$_{URA3}$ fat1-Δ1::P$_{URA3}$/fat1-Δ2::P$_{URA3}$ pox5Δ::T$_{URA3}$/pox5Δ::T$_{URA3}$*). Two transformants with verified integration of plasmid pAA1609 were compared to the Ura+ parent of strain sAA2800 (strain sAA2748). The control strain (sAA2748) and the two transformants have both alleles of *POX4* and *POX5* disrupted and are completely beta-oxidation blocked. They are still able to grow on glucose but cannot metabolize fatty acids. Cultures (5 ml) of each strain were grown overnight in YPD medium and used to inoculate 250-mL glass flasks containing 50mL of SP92 media to an initial OD600nm of 0.4 (SP92 media: 6.7 g/L Difco yeast nitrogen base, 3.0 g/L Difco yeast extract, 3.0 g/L ammonium sulfate, 1.0 g/L potassium phosphate monobasic, 1.0 g/L potassium phosphate dibasic, 75 g/L glucose). After 24 h incubation at 30°C with shaking at 250 rpm (2" throw incubator) samples of culture broth were taken for gas chromatographic (GC) analysis to determine the diacid composition of the culture broth.

As shown in Table 17, transformants with the nucleic acid encoding the Tes3p$^{\Delta pts}$ protein localized to the cytoplasm displayed increased concentrations of long chain (C16 and C18)

diacids compared to the control strain that does not contain the Tes3p$^{\Delta pts}$-encoding nucleic acid. These long chain diacids may arrive from the activity of the cytoplasmic Tes3p$^{\Delta pts}$ protein converting long chain acyl-CoA products of fatty acid synthesis into free fatty acids. The free fatty acids can then be converted to diacids by the omega oxidation pathway.

5

**Table 17: Diacid content of culture broth after 24-hour shake flask growth of strains**

| Strain | C16 Diacid (g/L) | C18 Diacid (g/L) | Total long chain Diacid (g/L) |
|---|---|---|---|
| sAA2748 | 0.31 | 0.37 | 0.68 |
| sAA2800 + pAA1609 clone 1 | 0.88 | 1.41 | 2.28 |
| sAA2800 + pAA1609 clone 2 | 0.93 | 1.51 | 2.44 |

*EXAMPLE 25: Amino acid sequences referenced herein*

**Table 18**

| Gene (Organism) | Amino acid sequence | SEQ ID NO |
|---|---|---|
| *URA3* (*Candida viswanathii* ATCC 20336) | MVSTKTYTERASAHPSKVAQRLFRLMESKKTNLCASIDVTTTAEFLSLIDKLGPHICLVKTHIDIISDFSYEGTIEPLLVL AERHGFLIFEDRKFADIGNTVMLQYTSGVYRIAAWSDITNAHGVTGKGVVEGLKRGAEGVEKERGVLMLAELSSKGS LAHGEYTRETIEIAKSDREFVIGFIAQRDMGGREEGFDWIIMTPGVGLDDKGDALGQQYRTVDEVVLTGTDVIIVGRG LFGKGRDPEVEGKRYRDAGWKAYLKRTGQLE | 1 |
| *CAT2* (*Candida viswanathii* ATCC 20336) | MFNFKLSQQVLKNSTKSIMPILKKPFSTSHAKGDLFKYQSQLPKLPVPTLEETASKYLKTVEPFLNQEQLESTKAKVA EFVRPGGAGEALQARLNNFAADKDNWLAEFWDDYAYMSYRDPVVPYVSYFFSHKDVKNIIGQDQLLKATLIAYYTIE FQEKVLDESLDPEVIKGNPFCMNAFKYMFNNSRVPAEGSDITQHYNGEENQFFVVIYKNNFYKVPTHKNGQRLTKG EIYSYLQEIKNDATPKGLGLGALTSLNRDEWLSAYNNLLKSPINEASLGSIFASSFVIALDSNNPVTIEEKSKNCWHGD GQNRFFDKPLEFFVSANGNSGFLGEHSRMDATPTVQLNNTIYKQILETNPNDLIVEIGSSAPRFGNAEILPFDINPTTR ANIKDAIAKFDATIAAHDEEIFQHYGYGKGLIKKFKVSPDAYVQLLMQLAYFKYTGKIRPTYESAATRKFLKGRTETGR TVSNESKKFVETWSDPNASSADKVATFQAAAKQHVAYLSAAADGKGVDRHLFGLKQMIQPGEPIPEIFTDPIFSYSQ TWYISSSQVPSEFFQSWGWSQVIDDGFGLAYLINNDWIHVHISCKRGNGLQSDHLKWYLVDSANEMKDVLTKGLLT DAKPKL | 2 |
| *CAT2*$^{\Delta mts}$ contained in pAA1610 | MPILKKPFSTSHAKGDLFKYQSQLPKLPVPTLEETASKYLKTVEPFLNQEQLESTKAKVAEFVRPGGAGEALQARLN NFAADKDNWLAEFWDDYAYMSYRDPVVPYVSYFFSHKDVKNIIGQDQLLKATLIAYYTIEFQEKVLDESLDPEVIKGN PFCMNAFKYMFNNSRVPAEGSDITQHYNGEENQFFVVIYKNNFYKVPTHKNGQRLTKGEIYSYLQEIKNDATPKGLG LGALTSLNRDEWLSAYNNLLKSPINEASLGSIFASSFVIALDSNNPVTIEEKSKNCWHGDGQNRFFDKPLEFFVSANG NSGFLGEHSRMDATPTVQLNNTIYKQILETNPNDLIVEIGSSAPRFGNAEILPFDINPTTRANIKDAIAKFDATIAAHDEE IFQHYGYGKGLIKKFKVSPDAYVQLLMQLAYFKYTGKIRPTYESAATRKFLKGRTETGRTVSNESKKFVETWSDPNA SSADKVATFQAAAKQHVAYLSAAADGKGVDRHLFGLKQMIQPGEPIPEIFTDPIFSYSQTWYISSSQVPSEFFQSWG WSQVIDDGFGLAYLINNDWIHVHISCKRGNGLQSDHLKWYLVDSANEMKDVLTKGLLTDAKPKL | 3 |
| *CAT2*$^{\Delta mts\Delta pts}$ contained in pAA1667 | MPILKKPFSTSHAKGDLFKYQSQLPKLPVPTLEETASKYLKTVEPFLNQEQLESTKAKVAEFVRPGGAGEALQARLN NFAADKDNWLAEFWDDYAYMSYRDPVVPYVSYFFSHKDVKNIIGQDQLLKATLIAYYTIEFQEKVLDESLDPEVIKGN PFCMNAFKYMFNNSRVPAEGSDITQHYNGEENQFFVVIYKNNFYKVPTHKNGQRLTKGEIYSYLQEIKNDATPKGLG LGALTSLNRDEWLSAYNNLLKSPINEASLGSIFASSFVIALDSNNPVTIEEKSKNCWHGDGQNRFFDKPLEFFVSANG NSGFLGEHSRMDATPTVQLNNTIYKQILETNPNDLIVEIGSSAPRFGNAEILPFDINPTTRANIKDAIAKFDATIAAHDEE IFQHYGYGKGLIKKFKVSPDAYVQLLMQLAYFKYTGKIRPTYESAATRKFLKGRTETGRTVSNESKKFVETWSDPNA SSADKVATFQAAAKQHVAYLSAAADGKGVDRHLFGLKQMIQPGEPIPEIFTDPIFSYSQTWYISSSQVPSEFFQSWG WSQVIDDGFGLAYLINNDWIHVHISCKRGNGLQSDHLKWYLVDSANEMKDVLTKGLLTDAK | 4 |



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| Table 18 | | |
|---|---|---|
| **Gene (Organism)** | **Amino acid sequence** | **SEQ ID NO** |
| *CAT2*$^{\Delta pts}$ contained in pAA1876 | Mfnfklsqqvlknstksimpilkkpfstshakgdlfkyqsqlpklpvptleetaskylktvepflnqeqlestkakvaefvrpggagealqarlnnfaadkdnwlaefwd dyaymsyrdpvvpyvsyffshkdvkniigqdqllkatliayytiefqekvldesldpevikgnpfcmnafkymfnnsrvpaegsditqhyngeenqffvviyknnfyk vpthkngqrltkgeiysylqeiknd atpkglglgaltslnrdewlsaynnllkspineaslgsifassfvialdsnnpvtieeksknc whgdgqnrffdkpleffvsangns gflgehsrmdatptvqlnntiykqiletnpndliveigssaprfgnaeilpfdinpttranikdaiakfdatiaahdeeifqhygygkglikkfkvspdayvqllmqlayfkyt gkirptyesaatrkflkgrtetgrtvsneskkfvetwsdpnassadkvatfqaaakqhvaylsaaadgkgvdrhlfglkqmiqpgepipeiftdpifsysqtwyisssqv pseffqswgwsqviddgfglaylinndwihvhisckrgnglqsdhlkwylvdsanemkdvltkglltdak | 5 |
| *YAT1* (*Candida viswanathii* ATCC20336) | Mstyqfqetleklpipdlnqtcanylnvlrplqteqehiktktavenflkngtgqyldaalreyaqtrpsyieqfwydaylnydspvvlnlnpfflleddpftnesssvnpqv kratslvmsslkfiqalknetlsvdtlkggkplcmyqytklfgasripsedgcvmqsdpasnhivvmsksqlywfdvldsknnlilseaelnvnfqsiihdslrtpsdeia kssfgvlttenrriwanvrhnlmsttnnkvnhevlsiidsalfvlcfddivindlselsknmlcglsildngiqvgtctnrwydklqiivtknakaginfehtgvdghtvlrfvsd iytdsilsfansinsnapslwntttnykeldgedlitvprklewe ltpdlslalrfgetrlsdlinqnefrhlefknygstqikkmkfspdafvqmafqatyyalygkvectyep amtkqfyhgrteairtvsqesnlfvrkffdstvsiqkkleyltqactkhsqqtrmssagqgvdrhlyalfciwkrylhdaeddddd askdqpsstelsrddtiiaqtngdst ddddsttvvghganhhhnlksadllktipeifadngwdklnntiistsncgnpslrlfgfgpvsangfgigyilkddsisicasskhrqtqrflvtlnsylleiqniwkqaqk meklkaelakavedakpkgqksgeaegiiepkpnnlstllggygyfdmgdediksrgqspeppflhragsgfsireigkklrlsey | 6 |
| *COX4* mitochondrial targeting sequence (*Candida viswanathii* ATCC20336) | Mlsrttlrvarqqtrllstsrilfnsktdq | 7 |
| *CIT1* mitochondrial targeting sequence (*Candida viswanathii* ATCC20336) | Msalrsfqrssnvakstlknsvrty | 8 |



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| Table 18 | | |
|---|---|---|
| **Gene (Organism)** | **Amino acid sequence** | **SEQ ID NO** |
| *CAT2* mitochondrial targeting sequence (*Candida viswanathii* ATCC20336) | Mfnfklsqqvlknstksimpilkkpfstshakg | 9 |
| *YAT1*$^{+COX4mts}$ contained in pAA1967 | MlsrttlrvarqqtrllstsrilfnsktdqstyqfqetlekIpipdlnqtcanylnvlrplqteqehiktktavenflkngtgqyldaalreyaqtrpsyieqfwydaylnydspvvl nlnpfflleddpftnesssvnpqvkratslvmsslkfiqalknetlsvdtlkggkplcmyqytklfgasripsedgcvmqsdpasnhivvmsksqlywfdvldsknnlils eaelnvnfqsiihdslrtpsdeiakssfgvlttenrriwanvrhnlmsttnnkvnhevlsiidsalfvlcfddivindlselsknmlcglsildngiqvgtctnrwydklqiivtk nakaginfehtgvdghtvlrfvsdiytdsilsfansinsnapslwntttnykeldgedlitvprkleweltpdlslalrfgetrlsdlinqnefrhlefknygstqikkmkfspda fvqmafqatyyalygkvectyepamtkqfyhgrteairtvsqesnlfvrkffdstvsiqkkleyltqactkhsqqtrmssagqgvdrhlyalfciwkrylhdaeddddda skdqpsstelsrddtiiaqtngdstddddsttvvghganhhhnlksadllktipeifadngwdklnntiistsncgnpslrlfgfgpvsangfgigyilkddsisicasskhr qtqrflvtlnsylleiqniwkqaqkmeklkaelakavedakpkgqksgeaegiiepkpnnlstllggygyfdmgdediksrgqspeppflhragsgfsireigkklrlse y | 10 |
| *YAT1*$^{+CIT1mts}$ contained in pAA1968 | MsalrsfqrssnvakstlknsvrtystyqfqetlekIpipdlnqtcanylnvlrplqteqehiktktavenflkngtgqyldaalreyaqtrpsyieqfwydaylnydspvvl nlnpfflleddpftnesssvnpqvkratslvmsslkfiqalknetlsvdtlkggkplcmyqytklfgasripsedgcvmqsdpasnhivvmsksqlywfdvldsknnlils eaelnvnfqsiihdslrtpsdeiakssfgvlttenrriwanvrhnlmsttnnkvnhevlsiidsalfvlcfddivindlselsknmlcglsildngiqvgtctnrwydklqiivtk nakaginfehtgvdghtvlrfvsdiytdsilsfansinsnapslwntttnykeldgedlitvprkleweltpdlslalrfgetrlsdlinqnefrhlefknygstqikkmkfspda fvqmafqatyyalygkvectyepamtkqfyhgrteairtvsqesnlfvrkffdstvsiqkkleyltqactkhsqqtrmssagqgvdrhlyalfciwkrylhdaeddddda skdqpsstelsrddtiiaqtngdstddddsttvvghganhhhnlksadllktipeifadngwdklnntiistsncgnpslrlfgfgpvsangfgigyilkddsisicasskhr qtqrflvtlnsylleiqniwkqaqkmeklkaelakavedakpkgqksgeaegiiepkpnnlstllggygyfdmgdediksrgqspeppflhragsgfsireigkklrlse y | 11 |



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| Table 18 | | |
|---|---|---|
| **Gene (Organism)** | **Amino acid sequence** | **SEQ ID NO** |
| *YAT1*$^{+CAT2mts}$ contained in pAA1969 | Mfnfklsqqvlknstksimpilkkpfstshakgstyqfqetleklpipdlnqtcanylnvlrplqteqehiktktavenflkngtgqyldaalreyaqtrpsyieqfwydayln ydspvvlnlnpfflleddpftnesssvnpqvkratslvmsslkfiqalknetlsvdtlkggkplcmyqytklfgasripsedgcvmqsdpasnhivvmsksqlywfdvl dsknnlilseaelnvnfqsiihdslrtpsdeiakssfgvlttenrriwanvrhnlmsttnnkvnhevlsiidsalfvlcfddivindlselsknmlcglsildngiqvgtctnrwy dklqiivtknakaginfehtgvdghtvlrfvsdiytdsilsfansinsnapslwntttnykeldgedlitvprklewеltpdlslalrfgetrlsdlinqnefrhlefknygstqikk mkfspdafvqmafqatyyalygkvectyepamtkqfyhgrteairtvsqesnlfvrkffdstvsiqkkleyltqactkhsqqtrmssagqgvdrhlyalfciwkrylhda eddddddaskdqpsstelsrddtiiaqtngdstddddsttvvghganhhhnlksadllktipeifadngwdklnntiistsncgnpslrlfgfgpvsangfgigyilkddsis icasskhrqtqrflvtlnsylleiqniwkqaqkmeklkaelakavedakpkgqksgeaegiiepkpnnlstllggygyfdmgdediksrgqspeppflhragsgfsirei gkklrlsey | 12 |
| *YAT1*$^{+pts}$ | Mstyqfqetleklpipdlnqtcanylnvlrplqteqehiktktavenflkngtgqyldaalreyaqtrpsyieqfwydaylnydspvvlnlnpfflleddpftnesssvnpqv kratslvmsslkfiqalknetlsvdtlkggkplcmyqytklfgasripsedgcvmqsdpasnhivvmsksqlywfdvldsknnlilseaelnvnfqsiihdslrtpsdeia kssfgvlttenrriwanvrhnlmsttnnkvnhevlsiidsalfvlcfddivindlselsknmlcglsildngiqvgtctnrwydklqiivtknakaginfehtgvdghtvlrfvsd iytdsilsfansinsnapslwntttnykeldgedlitvprklewеltpdlslalrfgetrlsdlinqnefrhlefknygstqikkmkfspdafvqmafqatyyalygkvectyep amtkqfyhgrteairtvsqesnlfvrkffdstvsiqkkleyltqactkhsqqtrmssagqgvdrhlyalfciwkrylhdaeddddddaskdqpsstelsrddtiiaqtngdst ddddsttvvghganhhhnlksadllktipeifadngwdklnntiistsncgnpslrlfgfgpvsangfgigyilkddsisicasskhrqtqrflvtlnsylleiqniwkqaqk meklkaelakavedakpkgqksgeaegiiepkpnnlstllggygyfdmgdediksrgqspeppflhragsgfsireigkklrlseypkl | 13 |
| *CRC1* (*Candida viswanathii* ATCC20336) | mddvdsaladnvksfaaggfggicavltghpfdlvkvrlqtglykssvqcvketiakdglfglyrgvlppllgvtpmfavsfwgydvgkklvssftgksvdkfeikdista gfisaipttlvaapfervkvmmqiqegaksksmgavvaemyrtggirsifkgtvatlardgpgsalyfatyewvkkeltapgedlslfaittaggfagiamwlgvfpidti kstqqssnvkvsivqatkniyakggikaffpgvgpalarafpanaatflgvelarkfldkvi | 14 |
| *ACH1* (*Candida viswanathii* ATCC20336) | Msailkqrvryapylkklrtaeqcvdlfkngqylgwsgftgvgapkavpaalvdhveknklqgqmgfhlfvgasagpeesrwaennmllsrsphqvgkpiaaain dgrtqffdkhlsmfpqdltygfytknkpngsnldytiieataitedgaiipgpavgaspemlsvsdniiievntktpsfegihdidlpvnppfrqpyphttadyrigrtaipv dpekviaivettegdkvppntpsdaqsqaiaghlieflenevkqgrlpenlhplqsgignianavveglaqsnfknltwtevlqdsfldffesgsldfatatsirlteegfk kfyenwdvyskklclrsqvvsnspeiirrlgvlafntpvevdiyahanstnvmgsrmlnglggsgdflrnaklsvmhtpsarptkvdptgvscivpmathvdqtehdl dvvvteqgladlrglspkeraqviinncahpdyqaqlqdyfdravfyatkkkmlhephmlneafamhlnlqengtmklnk | 15 |



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**Table 18**

| Gene (Organism) | Amino acid sequence | SEQ ID NO |
|---|---|---|
| *ACH1*$^{\Delta mts+pts}$ contained in pAA1846 | Mapylkklrtaeqcvdlfkngqylgwsgftgvgapkavpaalvdhveknklqgqmgfhlfvgasagpeesrwaennmllsrsphqvgkpiaaaindgrtqffdkhl smfpqdltygfytknkpngsnldytiieataitedgaiipgpavgaspemlsvsdniiievntktpsfegihdidlpvnppfrqpyphttadyrigrtaipvdpekviaivet tegdkvppntpsdaqsqaiaghlieflenevkqgrlpenlhplqsgignianavveglaqsnfknltvwtevlqdsfldffesgsldfatatsirlteegfkkfyenwdvys kklclrsqvvsnspeiirrlgvlafntpvevdiyahanstnvmgsrmlnglggsgdflrnaklsvmhtpsarptkvdptgvscivpmathvdqtehdldvvvteqgladl rglspkeraqviinncahpdyqaqlqdyfdravfyatkkkmlhephmlneafamhlnlqengtmklnkgrrakl | 16 |
| *ACC1* contained in pAA245 including 5' intron sequence (*Candida viswanathii* ATCC20336) | mrcqvsqpsrftnllvhrlprtllnypvvntlfiprrhyslnfsfknllkkmtdlspsptdslnytqlhsslpshflggnsvltaepsavtdfvkthqghtvitkvlianngigavk eirsvrkwayetfgderaiqfvamatpedmeanaeyirmadqfvevpggtnnnnyanvdliveiaertdvhavwagwghasenpllperlaaspkkivfigppgs amrslgdkisstivaqhakvpcipwsgtgveevhvdpetklvsvddhvyakgcctspedglekakrigfpvmvkaseggggkgirkvdhekdfislynqaaneip gspifimklagdarhlevqlfadqygtnislfgrdcsvqrrhqkiieeapvtiankdtfvemekaavrlgklvgyvsagtveylysyaedkfyflelnprlqvehpttemvs gvnlpaaqlqiamglpmhrirdirllygvdphsateidfefkspnslitqrkpapkghctacritsedpgegfkpsggtlhelnfrsssnvwgyfsvanqssihsfadsqf ghifafgenrqasrkhmivalkelsirgdfrttveyliklletpdfadntittgwldelitkkltaerpdpivavvcgavtkahiqaeedkkeyieslekgqvpnksllktifpvef iyegerykftatkssedkytlflngsrcvigarslsdgglicaldgkshsvywkeeaaatrlsvdgktcllevendptqlrtpspgklvkylvesgehvdagqsyaevev mkmcmpliaqengtvqllkqpgstlnagdilailalddpskvkhakpyegtlpemgdptvtgskpahlfqhydtilknilagydnqvilnstlknmmeilknkelpyse wrlqisalhsrippkldealtsliertesrgaefparqilklvnktlgepgnellgdvvaplvsianryqnglveheydyfaslvneycnvehffsgenvreedvilrlrdenk sdlkkvisiclshsrvsaknnlilaileayepllqsnsstavairdslkkivqldsracakvglkarelliqcslpsikersdqlehilrsavvetsygevfakhrepkleiiqevv eskhvvfdvlsqflvhqdcwvaiaaaevyvrrsyraydlgkidyhihdrlpivewkfklaqiagsrynavqsasvgddsttmkhaasvsdlsfvvdsksestsrtgvlv parhlddvdeilsaaleyfqpsdalsfqakgerpellnvlnivitdidgysdedeclkriheilneyeddlvfagvrrvtfvfahqvgsypkyytftgpvyeenkvirhiepal afqlelgrlanfdikpiftnnrnihvyeaigknapsdkrfftrgiirggvlkdeislteyliaesnrlisdildtlevidtsnsdlnhifinfsnvfnvqpadveaafasflerfgrrlwr lrvtgaeirivctdpqgnsfplraiinnvsgyvvkselylevknpkgdwvfksighpgsmhlqpistpypvkeslqpkryrahnmgttfvydfpelfrqatisqwkkhgk kapkdvftslelitdendalvaverdpgankigmvgfkvtaktpeyprgrsfiivandithkigsfgpdedeyfnkctdlarklgvpriylsansgarigvaeeliplyqva wneegnpdkgfrylylnpdakealekdgkgdtivterivedgqerhvikaiigaenglgveclkgsgliagatsrayrdiftitlvtcrsvgigaylvrlgqraiqiegqpiiltg apainkllgrevyssnlqlggtqimynngvshltasddlagvekimewlsyvpakrgmpvpilesedtwdrdidyyppkqeafdirwmiegkqvegeefesglfdk gsfqetlsgwakgvvvgrarlggipigvigvetrtienmipadpanpsstealiqeagqvwypnsafktaqaindfnngeqlplmilanwrgfsggqrdmynevlky gsfivdalvdfkqpiftyippngelrggswvvvdptinsdmmemyadvdsragvlepegmvgikyrrdkllatmqrldptyaqlkeklndsslspeehaqvstkivkr ekallpiyaqisvqfadlhdrsgrmmakgvirkeikwvdarrfffwrlrrrlneeyvlkligeqvknanklekvarlkswmptvdydddqavstwieenhaklqkrveel rqeknksdivkllqedpsnaasvmrdfvdrlsdeekekflksln | 17 |
| *ACC1* contained in pAA1910 (*Candida viswanathii* ATCC20336) | mtdlspsptdslnytqlhsslpshflggnsvltaepsavtdfvkthqghtvitkvlianngigavkeirsvrkwayetfgderaiqfvamatpedmeanaeyirmadqf vevpggtnnnnyanvdliveiaertdvhavwagwghasenpllperlaaspkkivfigppgsamrslgdkisstivaqhakvpcipwsgtgveevhvdpetklvsv ddhvyakgcctspedglekakrigfpvmvkasegggkgirkvdhekdfislynqaaneipgspifimklagdarhlevqlfadqygtnislfgrdcsvqrrhqkiiee apvtiankdtfvemekaavrlgklvgyvsagtveylysyaedkfyflelnprlqvehpttemvsgvnlpaaqlqiamglpmhrirdirllygvdphsateidfefkspnsl itqrkpapkghctacritsedpgegfkpsggtlhelnfrsssnvwgyfsvanqssihsfadsqfghifafgenrqasrkhmivalkelsirgdfrttveyliklletpdfadnt ittgwldelitkkltaerpdpivavvcgavtkahiqaeedkkeyieslekgqvpnksllktifpvefiyegerykftatkssedkytlflngsrcvigarslsdgglicaldgks hsvywkeeaaatrlsvdgktcllevendptqlrtpspgklvkylvesgehvdagqsyaevevmkmcmpliaqengtvqllkqpgstlnagdilailalddpskvkha | 18 |



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| Table 18 | | |
|---|---|---|
| **Gene (Organism)** | **Amino acid sequence** | **SEQ ID NO** |
| | kpyegtlpemgdptvtgskpahlfqhydtilknilagydnqvilnstlknmmeilknkelpysewrlqisalhsrippkldealtsliertesrgaefparqilklvnktlgep<br>gnellgdvvaplvsianryqnglveheydyfaslvneycnvehffsgenvreedvilrlrdenksdlkkvisiclshsrvsaknnlilaileayepllqsnsstavairdslk<br>kivqldsracakvglkarelliqcslpsikersdqlehilrsavvetsygevfakhrepkleiiqevveskhvvfdvlsqflvhqdcwvaiaaaevyvrrsyraydlgkidy<br>hihdrlpivewkfklaqiagsrynavqsasvgddsttmkhaasvsdlsfvvdsksestsrtgvlvparhlddvdeilsaaleyfqpsdalsfqakgerpellnvlnivitdi<br>dgysdedeclkriheilneyeddlvfagvrrvtfvfahqvgsypkyytftgpvyeenkvirhiepalafqlelgrlanfdikpiftnnrnihvyeaigknapsdkrfftrgiirg<br>gvlkdeislteyliaesnrlisdildtlevidtsnsdlnhifinfsnvfnvqpadveaafasflerfgrrlwrlrvtgaeirivctdpqgnsfplraiinnvsgyvvkselylevknp<br>kgdwvfksighpgsmhlqpistpypvkeslqpkryrahnmgttfvydfpelfrqatisqwkkhgkkapkdvftslelitdendalvaverdpgankigmvgfkvtakt<br>peyprgrsfiivandithkigsfgpdedeyfnkctdlarklgvpriylsansgarigvaeeliplyqvawneegnpdkgfrylylnpdakealekdgkgdtivterivedg<br>qerhvikaiigaenglgveclkgsgliagatsrayrdiftitlvtcrsvgigaylvrlgqraiqiegqpiiltgapainkllgrevyssnlqlggtqimynngvshltasddlagv<br>ekimewlsyvpakrgmpvpilesedtwdrdidyyppkqeafdirwmiegkqvegeefesglfdkgsfqetlsgwakgvvvgrarlggipigvigvetrtienmipad<br>panpsstealiqeagqvwypnsafktaqaindfnngeqlplmilanwrgfsggqrdmynevlkygsfivdalvdfkqpiftyippngelrggswvvvdptinsdmm<br>emyadvdsragvlepegmvgikyrrdkllatmqrldptyaqlkeklndsslspeehaqvstkivkrekallpiyaqisvqfadlhdrsgrmmakgvirkeikwvdarr<br>fffwrlrrrlneeyvlkligeqvknanklekvarlkswmptvdydddqavstwieenhaklqkrveelrqeknksdivkllqedpsnaasvmrdfvdrlsdeekekflk<br>sln | |
| *ACC1* contained in pAA1907 (S1153A) | mtdlspsptdslnytqlhsslpshflggnsvltaepsavtdfvkthqghtvitkvlianngigavkeirsvrkwayetfgderaiqfvamatpedmeanaeyirmadqf<br>vevpggtnnnnyanvdliveiaertdvhavwagwghasenpllperlaaspkkivfigppgsamrslgdkisstivaqhakvpcipwsgtgveevhvdpetklvsv<br>ddhvyakgcctspedglekakrigfpvmvkaseggggkgirkvdhekdfislynqaaneipgspifimklagdarhlevqlfadqygtnislfgrdcsvqrrhqkiiee<br>apvtiankdtfvemekaavrlgklvgyvsagtveylysyaedkfyflelnprlqvehpttemvsgvnlpaaqlqiamglpmhrirdirllygvdphsateidfefkspnsl<br>itqrkpapkghctacritsedpgegfkpsggtlhelnfrsssnvwgyfsvanqssihsfadsqfghifafgenrqasrkhmivalkelsirgdfrttveyliklletpdfadnt<br>ittgwldelitkkltaerpdpivavvcgavtkahiqaeedkkeyieslekgqvpnksllktifpvefiyegerykftatkssedkytlflngsrcvigarslsdgglicaldgks<br>hsvywkeeaaatrlsvdgktcllevendptqlrtpspgklvkylvesgehvdagqsyaevevmkmcmpliaqengtvqllkqpgstlnagdilailalddpskvkha<br>kpyegtlpemgdptvtgskpahlfqhydtilknilagydnqvilnstlknmmeilknkelpysewrlqisalhsrippkldealtsliertesrgaefparqilklvnktlgep<br>gnellgdvvaplvsianryqnglveheydyfaslvneycnvehffsgenvreedvilrlrdenksdlkkvisiclshsrvsaknnlilaileayepllqsnsstavairdslk<br>kivqldsracakvglkarelliqcslpsikersdqlehilrsavvetsygevfakhrepkleiiqevveskhvvfdvlsqflvhqdcwvaiaaaevyvrrsyraydlgkidy<br>hihdrlpivewkfklaqiagsrynavqsasvgddsttmkhaaavsdlsfvvdsksestsrtgvlvparhlddvdeilsaaleyfqpsdalsfqakgerpellnvlnivitdi<br>dgysdedeclkriheilneyeddlvfagvrrvtfvfahqvgsypkyytftgpvyeenkvirhiepalafqlelgrlanfdikpiftnnrnihvyeaigknapsdkrfftrgiirg<br>gvlkdeislteyliaesnrlisdildtlevidtsnsdlnhifinfsnvfnvqpadveaafasflerfgrrlwrlrvtgaeirivctdpqgnsfplraiinnvsgyvvkselylevknp<br>kgdwvfksighpgsmhlqpistpypvkeslqpkryrahnmgttfvydfpelfrqatisqwkkhgkkapkdvftslelitdendalvaverdpgankigmvgfkvtakt<br>peyprgrsfiivandithkigsfgpdedeyfnkctdlarklgvpriylsansgarigvaeeliplyqvawneegnpdkgfrylylnpdakealekdgkgdtivterivedg<br>qerhvikaiigaenglgveclkgsgliagatsrayrdiftitlvtcrsvgigaylvrlgqraiqiegqpiiltgapainkllgrevyssnlqlggtqimynngvshltasddlagv<br>ekimewlsyvpakrgmpvpilesedtwdrdidyyppkqeafdirwmiegkqvegeefesglfdkgsfqetlsgwakgvvvgrarlggipigvigvetrtienmipad<br>panpsstealiqeagqvwypnsafktaqaindfnngeqlplmilanwrgfsggqrdmynevlkygsfivdalvdfkqpiftyippngelrggswvvvdptinsdmm<br>emyadvdsragvlepegmvgikyrrdkllatmqrldptyaqlkeklndsslspeehaqvstkivkrekallpiyaqisvqfadlhdrsgrmmakgvirkeikwvdarr<br>fffwrlrrrlneeyvlkligeqvknanklekvarlkswmptvdydddqavstwieenhaklqkrveelrqeknksdivkllqedpsnaasvmrdfvdrlsdeekekflk | 19 |



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| Table 18 | | |
|---|---|---|
| **Gene (Organism)** | **Amino acid sequence** | **SEQ ID NO** |
| | sln | |
| *ACS1* (*Candida viswanathii* ATCC20336) | Mpestqqshisldhekmqqpptgfterstakpnladfdtykklykqsvenpnefftеqanknldwfkpfdlarfpvdpkddfkngdlpawfingqlnasynavdrw aiknpdkpaiiyegdepdsgriitygellkevsklaqtltklglvkkgdsvavylpmipeaivtllaivrigavhsvvfagfssaslrdrildadsrivitadeskrggktietkki vddalkecpkvrnvivfkrtgnshvpfsagrdlwwheemakygpyfppvpvnsedplfllytsgstgkpkgvqhntagyllgailttkytfdvheedvlftagdvgwitg htycvygpllcgattvvfegtpaypnysryweivdqykvnqfyvaptalrllkragtkyvekydlsslrvlgsvgepiaaevwhwyndnigrgkahivdtywqtesgsh lltplagvtptkpgsaslpffgvvpkildpttgeelegndvegvlaiksawpsitrgiyndynrfietylapyhdhyfsgdgaardndgfywilgrvddvvnvsghrlstaei eaaliehplvaesavvgyaddltgqavaayvslkkdkvigddmeavkkeliltvrkeigpfaapklillvddlpktrsgkimrrilrkvlageedqlgdistlsnpgvvqqii eivhackk | 20 |
| *ACS2* (*Candida viswanathii* ATCC20336) | Mttdsnthkvvheangvklrqtpkeffqrqpnkghiqdteeykklyeqsikdpqgffgplakellswdsdfhtvksgtlkngdaawflggklnasyncvarhalanpn kpaiiyeadeerdsyiltygdllrevskvagvlhswgirkgdtvavylpmnaqaliamlaiarlgaahsvifagfssgsikdrvndasckalitcdegrrggrttnikklcd ealkqcptvkhvlvhrrtgnpeiklegrdyywdeetakfsgyfppvpvdsedplfllytsgstgtpkgvvhttagyllgaalstkyifdvhpedvfftagdvgwitghtyal ygpllgvpsvvfegtpaypdygrfwqivekhkathfyvaptalrllrkageqevgkydlsslrtlgsvgepispdiwewynefvgkdqchisdtywqtesgshliapla gaiankpgsasypffgieaalidpvtgveikgndvegvlvvkdhwpsmartvfnnhvkymdtymnpypgyyftgdgaardndgyywirgrvddvvnvsghrlst aeiesaliedprvgesavvginddltgqaviayvalksgdvadedalrkelillvrkeigpfaapksvilvqdlpktrsgkimrrilrkvssneadqlgdittlqnpgsveg misafgaqfgrk | 21 |
| *TES1-1* (*Candida viswanathii* ATCC20336) | Mltltsgpnplpdfeealrvikvddthyvgahslrlpvkggrgvygghmiaqsllvgiestrddktnkvfipdsyhsyfigagnakipmnytveklyddenvskrfiiaeq kgrhrltclvtlrrpgtkpfhdsdnldisipvpkiqlkhpdpdklhqvqhtdfirnafgkelmdyrecpeenelyaaerwltvftgirnqpkpgasletvveelpdaqgqm htveksilrpkdsqsfkdpiynfvgladlsdsafilttmarilhipwapsieiddtydpardatyimrstlnaahifhynamsldhhiyfhnedytsddgsgfdickdwlaft yqmkrlsnnrtlvrgfllfnekhkciatvvqeqltimqngvgrtadksrl | 22 |
| *TES2-1* (*Candida viswanathii* ATCC20336) | Mienisgngnypqnhevdlekefgvekiginlyrgkspipkpdrrsrgayggylagqallvamkstppeyrphsfhsyfikavndketlewrveetsngrnyanrslq afqagnlvytanvsltkknsakkaeeatgvkpfefqgkphewfekhkrddlplatpssslliyhkffpevvsleaskeeeskpaadrelswyfkwginneeghhqpl vnlnsdyqyvgmaaltdavylnrllrilrvedadhtqlvhyfsvsldhtmyfhdddfdvtkwmgftfkvtrfshnralcqgevyndkgvhvctivqeglmmlngleqga kl | 23 |
| *TES2-2* (*Candida viswanathii* ATCC20336) | Miesisengnypqnhevdlekefgvekiginlyrgkspipkpdrrsrgayggylagqallvamkstppdyrphsfhsyfikavndkvtlewrveetsngrnyanralq afqagdlvytanvsltkknsakkteeatgvkpfefqgkphewfdkhkiddlplatpssslliyhkffpevvsleaskeeeskpaadrelswyfkwginneeghhqplv nlnsdyqyvgmaaltdavylnrllrilrvqdadhtqlvhyfsvsldhtmyfhdddfdvtkwmgftfkvtrfshnralcqgevyndkgvhvctivqeglmmlngleegakl | 24 |



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| Table 18 | | |
|---|---|---|
| **Gene (Organism)** | **Amino acid sequence** | **SEQ ID NO** |
| *TES3-1* (*Candida viswanathii* ATCC20336) | Mshptpeevygvtkvaenkyvgnrplnkptpktrgvyggnfcaqailvaiesapegftphsihsnfirggdpevpvewevevisngksfanrivkgvqhgivvyvatvsltnknsttrnesftydtppdetvktygnaeldtyyqgwlylevknypkqlhshqisysvkwgpendawkdasqtyqfvglaaisdvldlgqilrnldihlstpkfnvsldhsvyfhgadfdvtrwstttirmtklahgraliegemysdqgrhiasivqerlyiaespkl | 25 |
| *TES4-1* (*Candida viswanathii* ATCC20336) | Mtsiapatdipavdistiydvkqidanryrgvrplqkqarefrgvfggnlvaqsvvvalrsvpagfhpnsvhayyvravtdetpieweveetttgrtfanrsirglqnkkvvftasisltrknsnaeviqktghpslqfqrdtepyyeqmrarpgeckvlyinanthltvrqfpevssrdvfsflvrfgndgrerivgmtpeyqyaalaalsdwvrlrfyfdnmgvdvqtsfdvsldhsiyfhddgfdateylvfsvkvsrishsrvlydgqifndrgahvasirqerlyvvsnkpkf | 26 |
| *TES5-1* (*Candida viswanathii* ATCC20336) | Mptfnykdgetidvqkefgvvetapnkyvgvkplvkpmphvkgvfggnlagqallvamksvgpdfsphslhsyfiragsdqtpvewtvqaisdgnsfcnrfikgvqngqviyianvsltkrnsaadamkkyeeyhaqirqkgkdgdadeededdddeddnapakpfgfqtpshkwikdrdldklpvsdmesnlllyyklppefvslkssteeslpvserrmgalakwgieneqgfnqpltnldksfqyvglanitdglylgtlnrilriddltlderatnyfsvsldhviyfhdddfdvtkwmgftfrcsryshnrvifegeiysdkgvqvasiiqeglvrfkdgylknakl | 27 |
| *TES6-1* (*Candida viswanathii* ATCC20336) | Msttgiynakpaaailetktkvklvytegpklvyeglhpvetiketvrgtyggdfiaqgvnaawesignrmdfqphslhayyiksgsqesvlrwevfkvsdsrsfanrmltayqthtnqlvftmqisftkdnneeikraeyekllasggkirsipfvikkapnekyfklrdrinelhyseytngiiamaspldfheyatemnydtlgnkeygtfmkildnyslgkdyskqsflglsflsdgvwvfcftralglplgsyekeffrvsldhtvyfhdanfdssewlfldfrfvnmknnrfltvlnyytlqgkliatvlqevytslhqgiidksqeiavksgnkkqvttpkl | 28 |
| *TES7-1* (*Candida viswanathii* ATCC20336) | Mdklqaevyeaeppvakleaktaaklissdgakltyegvypvelvrkglrgtyggdfiaqginvawesignktdfqphslhayfvkagsdssvlrwevlkvsdsrnfanrlmlayqthtnelvftmqisftkdnneeikraeykqllqsggkirsipfaikkppnekyfklkdkvddlpyfehtngnmataippdfleyatemnhdtvgnkefgifmkvlddyslgknyerqsflglaflsdavwlssftpalglplgtlerkffrvsldhtmyfhdanfdssewifvdfrfvnlnnnrllgvvnfytlqgklvatviqeaymflhqaiidksqeiaeksghkkqvitpkl | 29 |
| *TES7-2* (*Candida viswanathii* ATCC20336) | Mdklqaevydaeppvakleaktaaklisadgakliyegvypvelvrkglrgtyggdfiaqginvawesignktdfqphslhayfvkagsdqsvlrwevlkvsdsrsfanrlmlayqthtnqlvftmqisftkdnneeikraeykqllesggkirsipfaikkppnekyfklkdkvddlpyfehtngnmataippdfleyatemnhdtvgnkefgifmkvlddysldknyerqsflglaflsdavwlssftpalglplgtferkffrvsldhtmyfhdanfdssewifvdfrfvnlnnnrllgvvnfytlqgklvatviqeaymflhqgiidqsqeiaeksghkkqvitpkl | 30 |



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| Table 18 | | |
|---|---|---|
| **Gene (Organism)** | **Amino acid sequence** | **SEQ ID NO** |
| *TES8-1* (*Candida viswanathii* ATCC20336) | Merlqaevydakptvakletkttvklvaqdgkrlvyeaiypvehikegipgayggdtlaqgmnaawdslgdkkdfqphsvhsyfvkpatnksilrwevikvsdgrsf anrfvsgyqthnnalvftmqisftkyndevvkiaeynkllesggkirsipfvikkapnekyfkfkdnlgdlryiehtngniatamsedlfeyatemnhdtlgnqefgifmk vldnyslgsdytkqsylglaflsdaiwmsvcsralglpfgsyhrqffgvsmdhsmyfhdanfdstewifldfrfvnlkndrllgvanfytlegklistliqeaylflhpgiiaks qeiavksgnkrqvkmpkl | 31 |
| *TES3-1Δpts Contained in pAA1609* | Mshptpeevygvtkvaenkyvgnrplnkptpktrgvyggnfcaqailvaiesapegftphsihsnfirggdpevpvewevevisngksfanrivkgvqhgivvyvat vsltnknsttrnesftydtppdetvktygnaeldtyyqgwlylevknypkqlhshqisysvkwgpendawkdasqtyqfvglaaisdvldlgqilrnldihlstpkfnvsl dhsvyfhgadfdvtrwstttirmtklahgraliegemysdqgrhiasivqerlyiaes | 32 |
| *PEX11* (*Candida viswanathii* ATCC20336) | mvadslvyhptvsklvkfldttpkrekvfrllsylsrflgyyayrkgysketialfanlkgnftfirkamrflkpinhlqlaskaydnklldpvlqittiirnlayagyltidgviffkllgl idakkfpnlatyasrfwligliaglinslriiyslkdyehqegdkeketdakaihtklyaakrklvwdlldtfialnsldilhftegdvgfagtitsllgledlwkat | 33 |
| *CPRB* (*Candida viswanathii* ATCC20336) | MALDKLDLYVIITLVVAVAAYFAKNQFLDQPQDTGFLNTDSGSNSRDVLSTLKKNNKNTLLLFGSQTGTAEDYANKLS RELHSRFGLKTMVADFADYDWDNFGDITEDILVFFIVATYGEGEPTDNADEFHTWLTEEADTLSTLRYTVFGLGNSTY EFFNAIGRKFDRLLSEKGGDRFAEYAEGDDGTGTLDEDFMAWKDNVFDALKNDLNFEEKELKYEPNVKLTERDDLS AADSQVSLGEPNKKYINSEGIDLTKGPFDHTHPYLARITETRELFSSKERHCIHVEFDISESNLKYTTGDHLAIWPSNS DENIKQFAKCFGLEDKLDTVIELKALDSTYTIPFPTPITYGAVIRHHLEISGPVSRQFFLSIAGFAPDEETKKTFTRLGGD KQEFATKVTRRKFNIADALLYSSNNTPWSDVPFEFLIENIQHLTPRYYSISSSSLSEKQLINVTAVVEAEEEADGRPVT GVVTNLLKNIEIAQNKTGEKPLVHYDLSGPRGKFNKFKLPVHVRRSNFKLPKNSTTPVILIGPGTGVAPLRGFVRERV QQVKNGVNVGKTLLFYGCRNSNEDFLYKQEWAEYASVLGENFEMFNAFSRQDPSKKVYVQDKILENSQLVHELLTE GAIIYVCGDASRMARDVQTTISKIVAKSREISEDKAAELVKSWKVQNRYQEDVW | 34 |
| *FAA1* (*Candida viswanathii* ATCC20336) | MGAPLTVAVGEAKPGETAPRRKAAQKMASVERPTDSKATTLPDFIEECFARNGTRDAMAWRDLVEIHVETKQVTKII DGEQKKVDKDWIYYEMGPYNYISYPKLLTLVKNYSKGLLELGLAPDQESKLMIFASTSHKWMQTFLASSFQGIPVVT AYDTLGESGLTHSLVQTESDAVFTDNQLLSSLIRPLEKATSVKYVIHGEKIDPNDKRQGGKIYQDAEKAKEKILQIRPDI KFISFDEVVALGEQSSKELHFPKPEDPICIMYTSGSTGAPKGVVITNANIVAAVGGISTNATRDLVRTVDRVIAFLPLAHI FELAFELVTFWWGAPLGYANVKTLTEASCRNCQPDLIEFKPTIMVGVAAVWESVRKGVLSKLKQASPIQQKIFWAAF NAKSTLNRYGLPGGGLFDAVFKKVKAATGGQLRYVLNGGSPISVDAQVFISTLLAPMLLGYGLTETCANTTIVEHTRF QIGTLGTLVGSVTAKLVDVADAGYYAKNNQGEIWLKGGPVVKEYYKNEEETKAAFTEDGWFKTGDIGEWTADGGLN IIDRKKNLVKTLNGEYIALEKLESIYRSNHLILNLCVYADQTKVKPIAIVLPIEANLKSMLKDEKIIPDADSQELSSLVHNKK VAQAVLRHLLQTGKQQGLKGIELLQNVVLLDDEWTPQNGFVTSAQKLQRKKILESCKKEVEEAYKSS | 35 |



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| Table 18 | | |
|---|---|---|
| **Gene (Organism)** | **Amino acid sequence** | **SEQ ID NO** |
| *POX5* (*Candida viswanathii* ATCC20336) | MPTELQKERELTKFNPKELNYFLEGSQERSEIISNMVEQMQKDPILKVDASYYNLTKDQQREVTAKKIARLSRYFEHE YPDQQAQRLSILGVFDPQVFTRIGVNLGLFVSCVRGNGTNSQFFYWTINKGIDKLRGIYGCFGMTELAHGSNVQGIE TTATFDEDTDEFVINTPHIGATKWWIGGAAHSATHCSVYARLKVKGKDYGVKTFVVPLRDSNHDLEPGVTVGDIGAK MGRDGIDNGWIQFSNVRIPRFFMLQKYCKVSRLGEVTMPPSEQLSYSALIGGRVTMMMDSYRMTSRFITIALRYAIH RRQFKKKDTDTIETKLIDYPLHQKRLFPFLAAAYLFSQGALYLEQTMNATNDKLDEAVSAGEKEAIDAAIVESKKLFVA SGCLKSTCTWLTAEAIDEARQACGGHGYSSYNGFGKAYSDWVVQCTWEGDNNILAMNVAKPMVRDLLKEPEQKG LVLSSVADLDDPAKLVKAFDHALSGLARDIGAVAEDKGFDITGPSLVLVSKLNAHRFLIDGFFKRITPEWSEVLRPLGF LYADWILTNFGATFLQYGIITPDVSRKISSEHFPALCAKVRPNVVGLTDGFNLTDMMTNAAIGRYDGNVYEHYFETVK ALNPPENTKAPYSKALEDMLNRPDLEVRERGEKSEEAAEILSS | 36 |
| *POX5*(F98G) (*Candida viswanathii* ATCC20336 mutant) | MPTELQKERELTKFNPKELNYFLEGSQERSEIISNMVEQMQKDPILKVDASYYNLTKDQQREVTAKKIARLSRYFEHE YPDQQAQRLSILGVFDPQVGTRIGVNLGLFVSCVRGNGTNSQFFYWTINKGIDKLRGIYGCFGMTELAHGSNVQGIE TTATFDEDTDEFVINTPHIGATKWWIGGAAHSATHCSVYARLKVKGKDYGVKTFVVPLRDSNHDLEPGVTVGDIGAK MGRDGIDNGWIQFSNVRIPRFFMLQKYCKVSRLGEVTMPPSEQLSYSALIGGRVTMMMDSYRMTSRFITIALRYAIH RRQFKKKDTDTIETKLIDYPLHQKRLFPFLAAAYLFSQGALYLEQTMNATNDKLDEAVSAGEKEAIDAAIVESKKLFVA SGCLKSTCTWLTAEAIDEARQACGGHGYSSYNGFGKAYSDFVVQCTWEGDNNILAMNVAKPMVRDLLKEPEQKGL VLSSVADLDDPAKLVKAFDHALSGLARDIGAVAEDKGFDITGPSLVLVSKLNAHRFLIDGFFKRITPEWSEVLRPLGFL YADWILTNFGATFLQYGIITPDVSRKISSEHFPALCAKVRPNVVGLTDGFNLTDMMTNAAIGRYDGNVYEHYFETVKA LNPPENTKAPYSKALEDMLNRPDLEVRERGEKSEEAAEILSS | 37 |
| *POX5*(W429F) (*Candida viswanathii* ATCC20336 mutant) | MPTELQKERELTKFNPKELNYFLEGSQERSEIISNMVEQMQKDPILKVDASYYNLTKDQQREVTAKKIARLSRYFEHE YPDQQAQRLSILGVFDPQVFTRIGVNLGLFVSCVRGNGTNSQFFYWTINKGIDKLRGIYGCFGMTELAHGSNVQGIE TTATFDEDTDEFVINTPHIGATKWWIGGAAHSATHCSVYARLKVKGKDYGVKTFVVPLRDSNHDLEPGVTVGDIGAK MGRDGIDNGWIQFSNVRIPRFFMLQKYCKVSRLGEVTMPPSEQLSYSALIGGRVTMMMDSYRMTSRFITIALRYAIH RRQFKKKDTDTIETKLIDYPLHQKRLFPFLAAAYLFSQGALYLEQTMNATNDKLDEAVSAGEKEAIDAAIVESKKLFVA SGCLKSTCTWLTAEAIDEARQACGGHGYSSYNGFGKAYSDWVVQCTWEGDNNILAMNVAKPMVRDLLKEPEQKG LVLSSVADLDDPAKLVKAFDHALSGLARDIGAVAEDKGFDITGPSLVLVSKLNAHRFLIDGFFKRITPEWSEVLRPLGF LYADWILTNFGATFLQYGIITPDVSRKISSEHFPALCAKVRPNVVGLTDGFNLTDMMTNAAIGRYDGNVYEHYFETVK ALNPPENTKAPYSKALEDMLNRPDLEVRERGEKSEEAAEILSS | 38 |
| *POX4* (*Candida viswanathii* ATCC20336) | MTFTKKNVSVSQGPDPRSSIQKERDSSKWNPQQMNYFLEGSVERSELMKALAQQMERDPILFTDGSYYDLTKDQQ RELTAVKINRIARYREQESIDTFNKRLSLIGIFDPQVGTRIGVNLGLFLSCIRGNGTTSQLNYWANEKETADVKGIYGCF GMTELAHGSNVAGLETTATFDKESDEFVINTPHIGATKWWIGGAAHSATHCSVYARLIVDGQDYGVKTFVVPLRDSN HDLMPGVTVGDIGAKMGRDGIDNGWIQFSNVRIPRFFMLQKFCKVSAEGEVTLPPLEQLSYSALLGGRVMMVLDSY RMLARMSTIALRYAIGRRQFKGDNVDPKDPNALETQLIDYPLHQKRLFPYLAAAYVISAGALKVEDTIHNTLAELDAAV EKNDTKAIFKSIDDMKSLFVDSGSLKSTATWLGAEAIDQCRQACGGHGYSSYNGFGKAYNDWVVQCTWEGDNNVL | 39 |



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| Table 18 | | |
|---|---|---|
| **Gene (Organism)** | **Amino acid sequence** | **SEQ ID NO** |
| | AMSVGKPIVKQVISIEDAGKTVRGSTAFLNQLKDYTGSNSSKVVLNTVADLDDIKTVIKAIEVAIIRLSQEAASIVKKESF DYVGAELVQLSKLKAHHYLLTEYIRRIDTFDQKDLVPYLITLGKLYAATIVLDRFAGVFLTFNVASTEAITALASVQIPKL CAEVRPNVVAYTDSFQQSDMIVNSAIGRYDGDIYENYFDLVKLQNPPSKTKAPYSDALEAMLNRPTLDERERFEKSD ETAAILSK | |
| *PXA1* (*Candida viswanathii* ATCC20336) | Mvniskltgynkqdirnvvlllqefvktykdnkiklnyssrpvilflstlvatagigvfftlrsivtkyneyllnkrlrrpslirqssnilkngsreifiqkgngkvtriiipkanndqya adkylykdfarneqilqqqkgrlfnsrflnqltiiwkilipkfycqntslllsqcfflifrtwlslliakldgqivknliaadgrkfardliyflliafpasytnaaikylelrlalgfrtnltryih dmyldktmsyykvglngadiqnidqyitedvtkfcmslcslfssmgkpfidliffsvylrdnlgtgaiigifanyfataimlkkatprfgklaakrthlegvyfnqqlnimtns eeigfykgskiekskiaenfdklmghvsreinlsssyaaledyvlkytwsawgyifsglpvfldvlfpkedpssghiadidddd hahghghtgeetssttenmktfvtn krllsladagsrlmvslkevttltgitnrvfnmltqlhrvhdpkfdygdkyglpdihgtyqlnydglrlehvpitvptaegsystplipdltfdikgknllfvgpngsgktsvarvl aglwplyaglvskpsdlffnpqksyfttgslrdqvvypnrsenttndqifhilhcvhldhivkryglnqnldfaktlsggekqrlsfarvlfnrpsivilddstsalspdmeelm yqvlqdhkinyvtlsnrpslskfhdkvfei | 40 |
| *PXA2* (*Candida viswanathii* ATCC20336) | Mtvenaklqknslavsllkvyksnrslllntsyiiliiaaftgatntgrgtssrssakvetdeeqsvkkkhpklsresfhrlrkailptffdrtivyffanltllvvrallttlrvatldgqlv galvsrrirvfakyllywmllgipaaltnallnwtksnlsksirmnlnnnimeeylpdnldpnyyslihltdnkirdpnqrittdtsrlsdalaslpghilkptldiilcaqqlsksg vgngegtlalgilahfstmiirffsppfaklaaeranlegqlrsahskivanseeiafllgghdreldhidhcyytlerfskgeywkraiheitqtfivkyfwgvaglvlcsapvf iakylgepedknvagnfitnrrllmsasdsldrliysrryllqvvghatrvsdfldtlheveekkkritsnvqfnndeitfdhvrlmtptevtlipdlnfsikpgdhllivgpngsg kssllfrmlgglwpvrfgtiripntenmfylpqkaylvegsfreqiiyphnvtqqkktdqqlkeilkvlkledysgqldevkkwseelsigaqqrlamarlyyhepkfavlde ctsavspdmeqlmyqhaqglgitllsvahrpalwhfhkyllefdgkgsyyfgtldekhkmkleeeerlkkenekksvakk | 41 |
| *ACL1* (*Yarrowia lipolytica* CLIB122) | msanenisrfdapvgkehpayelfhnhtrsfvyglqpracqgmldfdfickrenpsvagviypfggqfvtkmywgtketllpvyqqvekaaakhpevdvvvnfass rsvysstmelleypqfrtiaiiaegvperrareilhkaqkkgvtiigpatvggikpgcfkvgntggmmdnivasklyrpgsvayvsksggmsnelnniishttdgvyegi aiggdrypgttfidhilryeadpkckiivllgevggveeyrvieavkngqikkpivawaigtcasmfktevqfghagsmansdletakaknaamksagfyvpdtfed mpevlaelyekmvakgelsrisepevpkipidyswaqelglirkpaafistisddrgqellyagmpisevfkedigiggvmsllwfrrrlpdyaskflemvlmltadhgp avsgamntiittragkdlisslvaglltigtrfggaldgaatefttaydkglsprqfvdtmrkqnklipgighrvksrnnpdfrvelvkdfvkknfpstqlldyalaveevttskk dnlilnvdgaiavsfvdlmrscgaftveetedylkngvlnglfvlgrsigliahhldqkrlktglyrhpwddtitylvgqeaiqkkrveisagdvskaktrs | 42 |
| *ACL2* (*Yarrowia lipolytica* CLIB122) | Msaksiheadgkallahflskapvwaeqqpintfemgtpklasltfedgvapeqifaaaektypwllesgakfvakpdqlikrrgkaglllvlnksweeckpwiaeraa kpinvegidgvlrtflvepfvphdqkheyyinihsvregdwilfyheggvdvgdvdakaakilipvdieneypsnatltkellahvpedqhqtlldfinrlyavyvdlqftyl einplvviptaqgvevhyldlagkldqtaefecgpkwaaarspaalgqvvtidagstkvsidagpamvfpapfgrelskeeayiaeldsktgaslkltvlnakgriwtlv agggasvvyadaiasagfadelanygeysgapnetqtyeyaktvldlmtrgdahpegkvlfigggianftqvgstfkgiirafrdyqsslhnhkvkiyvrrggpnwqe glrliksagdelnlpmeiygpdmhvsgivplallgkrpknvkpfgtgpsteastplgv | 43 |
| *FAT1* (*Candida viswanathii* ATCC20336) | MSGLEIAAAAILGSQLLEAKYLIADDVSLAKTVAVNALPYLWKASRGKASYWYFFEQSVFKNPNNKALAFPRPRKNAP TPKTDAEGFQIYDDQFDLEEYTYKELYDMVLKYSYILKNEYGVTANDTIGVSCMNKPLFIVLWLALWNIGALPAFLNFN TKDKPLIHCLKIVNASQVFVDPDCDSPIRDTEAQIREELPHVQINYIDEFALFDRLRLKSTPKHRAEDKTRRPTDTDSS ACALIYTSGTTGLPKAGIMSWRKAFMASVFFGHIMKIDSKSNVLTAMPLYHSTAAMLGLCPTLIVGGCVSVSQKFSAT | 44 |



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**Table 18**

| Gene (Organism) | Amino acid sequence | SEQ ID NO |
|---|---|---|
| | SFWTQARLCGATHVQYVGEVCRYLLNSKPHPDQDRHNVRIAYGNGLRPDIWSEFKRRFHIEGIGEFYAATESPIATT NLQYGEYGVGACRKYGSLISLLLSTQQKLAKMDPEDESEIYKDPKTGFCTEAAYNEPGELLMRILNPNDVQKSFQGY YGNKSATNSKILTNVFKKGDAWYRSGDLLKMDEDKLLYFVDRLGDTFRWKSENVSATEVENELMGSKALKQSVVVG VKVPNHEGRACFAVCEAKDELSHEEILKLIHSHVTKSLPVYAQPAFIKIGTIEASHNHKVPKNQFKNQKLPKGEDGKDL IYWLNGDKYQELTEDDWSLICTGKAKL | |
| CYP52A17 (*Candida viswanathii* ATCC20336) FROM pAA1712 | Mieqlleywyvvvpvlyiikqllaytktrvlmkklgaapvtnklydnafgivngwkalqfkkegraqeyndykfdhsknpsvgtyvsilfgtrivvtkdpenikailatqfgd fslgkrhtlfkpllgdgiftldgegwkhsramlrpqfareqvahvtslephfqllkkhilkhkgeyfdiqelffrftvdsateflfgesvhslkdesiginqddidfagrkdfaesf nkaqeylairtlvqtfywlvnnkefrdctksvhkftnyyvqkaldaspeelekqsgyvflyelvkqtrdpnvlrdqslnillagrdttagllsfavfelarhpeiwaklreeieq qfglgedsrveeitfeslkrceylkaflnetlriypsvpmfriatknttlprgggsdgtspiliqkgeavsyginsthldpvyygpdaaefrperwfepstkklgwaylpfng gpriclgqqfalteagyvlvrlvqefshvrsdpdevyppkrltnltmclqdgaivkfd | 45 |
| *ADH1-2* alcohol dehydrogenase (*Candida viswanathii* ATCC20336) | MHALFSKSVFLKYVSSPTTSAIPHSLEFIVSRSSYLRRRIPPYLPRCSHFPSFYYSSSSVYTKKSFHTMSANIPKTQKA VVFEKNGGELKYKDIPVPTPKANELLINVKYSGVCHTDLHAWKGDWPLDTKLPLVGGHEGAGVVVGMGENVKGWKI GDFAGIKWLNGSCMSCEFCQQGAEPNCGEADLSGYTHDGSFEQYATADAVQAARIPAGTDLAEVAPILCAGVTVYK ALKTADLAAGQWVAISGAGGGLGSLAVQYAVAMGLRVVAIDGGDEKGDFVKSLGAEAYIDFLKEKGIVAAVKKATDG GPHGAINVSVSEKAIDQSVEYVRPLGKVVLVGLPAGSKVTAGVFEAVVKSIEIKGSYVGNRKDTAEAVDFFSRGLIKC PIKIVGLSELPQVFKLMEEGKILGRYVLDTSK | 46 |
| *FAO1* fatty alcohol oxidase (*Candida viswanathii* ATCC20336) | MAPFLPDQVDYKHVDTLMLLCDGIIHETTVDQIKDVIAPDFPADKYEEYVRTFTKPSETPGFRETVYNTVNANTTDAIH QFIILTNVLASRVLAPALTNSLTPIKDMSLEDREKLLASWRDSPIAAKRKLFRLVSTLTLVTFTRLANELHLKAIHYPGRE DREKAYETQEIDPFKYQFLEKPKFYGAELYLPDIDVIIIGSGAGAGVVAHTLANDGFKSLVLEKGKYFSNSELNFDDKD GVQELYQSGGTLTTVNQQLFVLAGSTFGGGTTVNWSACLKTPFKVRKEWYDEFGVDFAADEAYDKAQDYVWQQM GASTEGITHSLANEIIIEGGKKLGYKAKVLDQNSGGHPQHRCGFCYLGCKHGIKQGSVNNWFRDAAAHGSQFMQQV RVLQILNKKGIAYGILCEDVVTGAKFTITGPKKFVVAAGALNTPSVLVNSGFKNKNIGKNLTLHPVSVVFGDFGKDVQA DHFHNSIMTALCSEAADLDGKGHGCRIETILNAPFIQASFLPWRGSNEARRDLLRYNNMVAMLLLSRDTTSGSVSAH PTKPEALVVEYDVNKFDRNSILQALLVTADLLYIQGAKRILSPQAWVPIFESDKPKDKRSIKDEDYVEWRAKVAKIPFD TYGSPYGSAHQMSSCRMSGKGPKYGAVDTDGRLFECSNVYVADASLLPTASGANPMVTTMTLARHVALGLADSLK TKAKL | 47 |
| *HPD1* 3-hydroxy-propionate-dehydrogenase (*Candida* | MIrssvrtfstqsrvlanygfvglglmgqhmarhvynqlqpadklyvhdvnpqhttqfvtdvttqkpqnatqltplsslkefttepesqldfivtmvpegkhvkavvsel vdhynasgkydpskkltfvdsstidiptsrevhqlvadklqgatfidapvsggvagarngtlsfmvsrdtkedvdpnlvtllnymgsnifpcggthgtglaaklannylla itniavadsfqlansfglnlqnyaklvststgkswasvdncpipgvypeknltcdngykggfvtkltrkdvvlatesakannqflmlgevgrywydkacedekyanrdl svlfeflgdlkk | 48 |



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| Table 18 | | |
|---|---|---|
| **Gene (Organism)** | **Amino acid sequence** | **SEQ ID NO** |
| *viswanathii* ATCC20336) | | |
| *ALD6* malonate semialdehyde dehydrogenase (*Candida viswanathii* ATCC20336) | Mlsrvlfktkprvptksitamairnksivtlssttstyptdhttpstepyitpsfvnnefiksdsntwfdvhdpatnyvvskvpqstpeeleeaiasahaafpkwrdtsiikrq giafkfvqllrenmdriasvivleqgktfvdaqgdvtrglqvaeaacnitndlkgeslevstdmetkmireplgvvgsicpfnfpamvplwslplvlvtgntavikpserv pgasmiicelaakagvppgvlnivhgkhdtvnkliedprikaltfvggdkagkyiyekgsslgkrvqanlgaknhlvvlpdahkqsfvnavngaafgaagqrcmais vlvtvgktkewvqdvikdakllntgsgfdpksdlgpvinpesltraeeiiadsvangavleldgrgyrpedarfakgnflgptiltnvkpglraydeeifapvlsvvnvdtid eaielinnnkygngvslftssggsaqyftkridvgqvginvpipvplpmfsftgsrgsflgdlnfygkagitfltkpktitsawktnliddeilkpstsmpvqq | 49 |
| *ECI1* enoyl-CoA isomerase (*Candida viswanathii* ATCC20336) | MSDEESDILYEVRDRTAIITLNIPKRLNALNGAQYLKLGKFLERANNEEDTVLTLIQASGRFFSAGANFADNDMAKVEM SKLFSHEYWLERFVARNIWLTNLFNDHKKILAAAVNGPVIGLSTGLLLLVDLVYVHDLNKFYLLAPFANLGLVAEGASS ATLFNRLGWSKASEALLLAKPIGGQDCYNAGFINKHYDGKFSSTEEFNEHVYKELTEAFENLHDDSILQNKQLLKLSR DQAIN | 50 |
| *ECI2* enoyl-CoA isomerase (*Candida viswanathii* ATCC20336) | MSDDLITYEVKDRAAVITLNNPKKLNALSIPQYDTICKLLERANAEEDTVITLLQSTGRVFSAGANADSIVGQDAELET WLNMSVAKQTFLVQTFLAHKKILAVALNGPVIGLSAAFVALCDLVYVHNAAKTFFLTPFANIGILAEGGTSATLPMRVG WSRAAEALLLSKRISGDDLQRAGFFNKDYKGQFKSAEEFNEVVLKELLDATENLHEDSIIQNKELLKAIFKPKISEVNS QEVSRGVYKWTSGVPMDRFKKLLNGELKHKL | 51 |
| *DCR1* dienoyl-CoA reductase (*Candida viswanathii* ATCC20336) | MPNTLDHNYLKKSVWKSDIFAGKVIFITGGAGTICRVQAEAMVLLGANAAIIGRNVEKTEEAAKEIASLRPGAKVIGIGA VDVRKIQTIKDAVDRTVAELGRIDYVIAGAAGNFLCDFNHLSANAFKSVIDIDLLGSFNTVKVTFDQLRKNKGAVLFVS ATLHYTGVPMQSHVGAAKSGVDALSNALAVELGPLGIRFNCIAPGAIAGTEGMSRLAPPTDTPLETKIPLQRQGTTED IADATVFLFSPASSYITGDVLVVDGAMWQTGGGILNDFYPDIIIHQNADPEGKL | 52 |
| *DCR2* dienoyl-CoA reductase (*Candida viswanathii* ATCC20336) | MPNTLTEAYKQESSWKPDLFKGKVVFITGGAGSICRVQAEAMVLLGANAAIIGRNVEKTESAAKEIASLRSGAKVLGI GGIDVRKVDSLKSAVDKTVAELGRIDFVIAGAAGNFLCDFNHLSSNAFKSIIDIDLLGSFNTVKVTFDQLRKNKGAILFV SATLHYYGVPFQIGVGAAKAGVDALSNALAVELGPLGIRSNCIAPGPIDGTEGVERLVRASKAEAAKKVPLQRLGTTQ DIADGTVYLFSPAASFVTGDVLVVDGASWQISSGVGATNYPVSILNAIDAPKGGKL | 53 |



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| Table 18 | | |
|---|---|---|
| **Gene (Organism)** | **Amino acid sequence** | **SEQ ID NO** |
| *MCR* malonyl-CoA reductase (*Sulfolobus islandicus*) | Mrrtlkaailgstglvgieyvrmlanhpyikvgylagkgsvgkpyeevvrwqtvgqvpkeiadmevkptdpklmddvdiifsplpqgaagpveeefakhgfpvisn spdhrfdpdvpllipeinphtislidkqrerhdwrgfivttplctaqgaaiplapiymnfridsslittiqslsgagypgipsldvvdnvlplgdnydnktvkeisrilsetkrmv nddndlslgatthristihghyeviyvtfredvsvekiretldsfrgepqklklptapdkpilltnqdarpqvyfdrwagdppgmsvvvgrlsqinrrtirlvsvvhntvrgaa gggilaaellvekgyidkr | 322 |
| *2PS* 2-pyrone synthase (*Gerbera hybrida*) | Mgsyssddvevireagraqglatilaigtatppncvaqadyadyyfrvtksehmvdlkekfkricektaikkrylaltedylqenptmcefmapslnarqdlvvtgvp mlgkeaavkaidewglpkskithlifcttagvdmpgadyqlvkllglspsvkrymlyqqgcaaggtvlrlakdlaennkgsrvlivcseitailfhgpnenhldslvaqalf gdgaaalivgsgphlaverpifeivstdqtilpdtekamklhlreggltfqlhrdvplmvaknienaaekalsplgitdwnsvfwmvhpggraildqverklnlkedklra srhvlseygnlisacvlfiidevrkrsmaegksttgegldcgvlfgfgpgmtvetvvlrsvrvtaavangn | 324 |

*EXAMPLE 26: Nucleic acid sequences of genes referenced herein*

| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| *URA3* (*Candida viswanathii* ATCC 20336) | Atggttagcacaaaaacatacacagaaagggcatcagcacacccctccaaggttgcccaacgtttattccgcttaatggagtccaaaaagaccaacctctgcgcc tcgatcgacgtgaccacaaccgccgagttcctttcgctcatcgacaagctcggtccccacatctgtctcgtgaagacgcacatcgatatcatctcagacttcagctac gagggcacgattgagccgttgcttgtgcttgcagagcgccacgggttcttgatattcgaggacaggaagtttgctgatatcggaaacaccgtgatgttgcagtacacc tcgggggtataccggatcgcggcgtggagtgacatcacgaacgcgcacggagtgactgggaagggcgtcgttgaagggttgaaacgcggtgcggaggggggta gaaaaggaaaggggcgtgttgatgttggcggagttgtcgagtaaaggctcgttggcgcatggtgaatatacccgtgagacgatcgagattgcgaagagtgatcgg gagttcgtgattgggttcatcgcgcagcgggacatgggggtagagaagaagggtttgattggatcatcatgacgcctggtgtgggggttggatgataaaggcgatgc gttgggccagcagtataggactgttgatgaggtggttctgactggtaccgatgtgattattgtcgggagagggttgtttggaaaaggaagagaccctgaggtggagg gaaagagatacagggatgctggatggaaggcatacttgaagagaactggtcagttagaataa | 54 |
| $P_{URA3}$ (*URA3* promoter from *Candida viswanathii* ATCC 20336) | cgacgggtacaacgagaattgtattgaattgatcaagaacatgatcttggtgttacagaacatcaagttcttggaccagactgagaatgcacagatatacaaggcgt catgtgataaaatggatgagatttatccacaattgaagaaagagtttatggaaagtggtcaaccagaagctaaacaggaagaagcaaacgaagaggtgaaaca agaagaagaaggtaaataagtattttgtattatataacaaacaaagtaaggaatacagatttatacaataaattgccatactagtcacgtgagatatctcatccattcc ccaactcccaagaaaaaaaaaaagtgaaaaaaaaaatcaaacccaaagatcaacctccccatcatcatcgtcatcaaacccccagctcaattcgca | 55 |



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**Table 19**

| Gene (Organism) | Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted) | SEQ ID NO |
|---|---|---|
| $T_{URA3}$ (*URA3* terminator from *Candida viswanathii* ATCC 20336) | Taaatattgtaataaataggtctatatacatacactaagcttctaggacgtcattgtagtcttcgaagttgtctgctagtttagttctcatgatttcgaaaaccaataacgca atggatgtagcagggatggtggttagtgcgttcctgacaaacccagagtacgccgcctcaaaccacgtcacattcgccctttgcttcatccgcatcacttgcttgaagg tatccacgtacgagttgtaatacaccttgaagaacggcttcgtct | 56 |
| $P_{URA3}$-*URA3*-$T_{URA3}$-$P_{URA3}$ (sequence of each element from *Candida viswanathii* ATCC 20336) | ttaaaggtatctacggttgtttcggtatgacccccggggatctgacgggtacaacgagaattgtattgaattgatcaagaacatgatcttggtgttacagaacatcaagt tcttggaccagactgagaatgcacagatatacaaggcgtcatgtgataaaatggatgagatttatccacaattgaagaaagagtttatggaaagtggtcaaccaga agctaaacaggaagaagcaaacgaagaggtgaaacaagaagaagaaggtaaataagtattttgtattatataacaaacaaagtaaggaatacagatttataca ataaattgccatactagtcacgtgagatatctcatccattccccaactcccaagaaaaaaaaaaagtgaaaaaaaaaatcaaacccaaagatcaacctccccatc atcatcgtcatcaaacccccagctcaattcgcaatggttagcacaaaaacatacacagaaagggcatcagcacacccctccaaggttgcccaacgtttattccgctt aatggagtccaaaaagaccaacctctgcgcctcgatcgacgtgaccacaaccgccgagttcctttcgctcatcgacaagctcggtccccacatctgtctcgtgaag acgcacatcgatatcatctcagacttcagctacgagggcacgattgagccgttgcttgtgcttgcagagcgccacgggttcttgatattcgaggacaggaagtttgctg atatcggaaacaccgtgatgttgcagtacacctcgggggtataccggatcgcggcgtggagtgacatcacgaacgcgcacggagtgactgggaagggcgtcgtt gaagggttgaaacgcggtgcggaggggtagaaaaggaaaggggcgtgttgatgttggcggagttgtcgagtaaaggctcgttggcgcatggtgaatatacccg tgagacgatcgagattgcgaagagtgatcgggagttcgtgattgggttcatcgcgcagcgggacatgggggtagagaagaagggtttgattggatcatcatgacg cctggtgtggggttggatgataaaggcgatgcgttgggccagcagtataggactgttgatgaggtggttctgactggtaccgatgtgattattgtcgggagagggttgtt tggaaaaggaagagaccctgaggtggagggaaagagatacagggatgctggatggaaggcatacttgaagagaactggtcagttagaataaatattgtaataa ataggtctatatacatacactaagcttctaggacgtcattgtagtcttcgaagttgtctgctagtttagttctcatgatttcgaaaaccaataacgcaatggatgtagcagg gatggtggttagtgcgttcctgacaaacccagagtacgccgcctcaaaccacgtcacattcgccctttgcttcatccgcatcacttgcttgaaggtatccacgtacgagt tgtaatacaccttgaagaacggcttcgtctacggtcgacgacgggtacaacgagaattgtattgaattgatcaagaacatgatcttggtgttacagaacatcaagttctt ggaccagactgagaatgcacagatatacaaggcgtcatgtgataaaatggatgagatttatccacaattgaagaaagagtttatggaaagtggtcaaccagaagc taaacaggaagaagcaaacgaagaggtgaaacaagaagaagaaggtaaataagtattttgtattatataacaaacaaagtaaggaatacagatttatacaata aattgccatactagtcacgtgagatatctcatccattccccaactcccaagaaaaaaaaaaagtgaaaaaaaaaatcaaacccaaagatcaacctccccatcatc atcgtcatcaaacccccagctcaattcgcagagctcggtaccc | 57 |
| $T_{URA3}$-$P_{URA3}$-*URA3*-$T_{URA3}$ (sequence of each element from *Candida viswanathii* ATCC 20336) | taaatattgtaataaataggtctatatacatacactaagcttctaggacgtcattgtagtcttcgaagttgtctgctagtttagttctcatgatttcgaaaaccaataacgcaa tggatgtagcagggatggtggttagtgcgttcctgacaaacccagagtacgccgcctcaaaccacgtcacattcgccctttgcttcatccgcatcacttgcttgaaggt atccacgtacgagttgtaatacaccttgaagaacggcttcgtctcgacgggtacaacgagaattgtattgaattgatcaagaacatgatcttggtgttacagaacatca agttcttggaccagactgagaatgcacagatatacaaggcgtcatgtgataaaatggatgagatttatccacaattgaagaaagagtttatggaaagtggtcaacca gaagctaaacaggaagaagcaaacgaagaggtgaaacaagaagaagaaggtaaataagtattttgtattatataacaaacaaagtaaggaatacagatttata caataaattgccatactagtcacgtgagatatctcatccattccccaactcccaagaaaaaaaaaaagtgaaaaaaaaaatcaaacccaaagatcaacctcccc atcatcatcgtcatcaaacccccagctcaattcgcaatggttagcacaaaaacatacacagaaagggcatcagcacacccctccaaggttgcccaacgtttattcc gcttaatggagtccaaaaagaccaacctctgcgcctcgatcgacgtgaccacaaccgccgagttcctttcgctcatcgacaagctcggtccccacatctgtctcgtg aagacgcacatcgatatcatctcagacttcagctacgagggcacgattgagccgttgcttgtgcttgcagagcgccacgggttcttgatattcgaggacaggaagttt | 58 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| | gctgatatcggaaacaccgtgatgttgcagtacacctcgggggtataccggatcgcggcgtggagtgacatcacgaacgcgcacggagtgactgggaagggcgt<br>cgttgaagggttgaaacgcggtgcggaggggggtagaaaaggaaaggggcgtgttgatgttggcggagttgtcgagtaaaggctcgttggcgcatggtgaatatac<br>ccgtgagacgatcgagattgcgaagagtgatcgggagttcgtgattgggttcatcgcgcagcgggacatgggggggtagagaagaagggtttgattggatcatcatg<br>acgcctggtgtggggttggatgataaaggcgatgcgttgggccagcagtataggactgttgatgaggtggttctgactggtaccgatgtgattattgtcgggagagggt<br>tgtttggaaaaggaagagaccctgaggtggagggaaagagatacagggatgctggatggaaggcatacttgaagagaactggtcagttagaataaatattgtaat<br>aaataggtctatatacatacactaagcttctaggacgtcattgtagtcttcgaagttgtctgctagtttagttctcatgatttcgaaaaccaataacgcaatggatgtagca<br>gggatggtggttagtgcgttcctgacaaacccagagtacgccgcctcaaaccacgtcacattcgcccctttgcttcatccgcatcacttgcttgaaggtatccacgtacg<br>agttgtaatacaca | |
| *CAT2* (*Candida viswanathii* ATCC 20336) | atgtttaactttaagttgtcgcaacaagtattaaagaattccaccaaatccattatgccaattttgaaaaaaccattctccaccagccacgcaaagggtgacttgttcaa<br>ataccagtcacaattacccaagttgcctgttcctactttggaagaaaccgcatccaagtacctcaagaccgttgagccattcttgaaccaagagcaattggaatccac<br>caaggccaaagtcgctgagtttgttagaccaggtggtgccggtgaagccttgcaagccagattgaacaactttgccgccgacaaggacaactggttggctgaatttt<br>gggacgactatgcatacatgtcttatagagatcctgttgttccatatgtttcttacttttcagtcacaaggatgtcaagaacatcattggccaagaccaattgttgaaggc<br>cactttgattgcttactacactattgagttccaagaaaaggttttggacgaaagtttggacccagaagtcatcaagggtaacccattctgtatgaacgccttcaagtaca<br>tgttcaacaactcgagagttccagctgaaggctccgacatcacccaacactacaacggtgaagaaaaccaatttttcgttgtcatctacaagaacaacttctacaag<br>gttccaacccacaagaacggccaaagattgaccaagggtgaaatctacagctacttgcaagaaatcaagaacgatgccactccaaagggtctcggtttgggtgct<br>ttgacctcattgaacagagacgaatggttgagtgcctacaacaacttgttgaagtccccaatcaacgaagcttccttgggatccatctttgcttccagctttgtcattgcctt<br>ggactccaacaacccagtcaccattgaagaaaaatccaagaactgctggcacggggacggtcaaaacagattctttgacaagcctttggaattcttcgtcagtgct<br>aacggtaactctggtttccttggtgaacactccagaatggacgctaccccaaccgtgcaattgaacaacaccatctacaagcaaatcttggaaaccaatccaaacg<br>acttgattgttgaaattggttcttctgctccaagattcggcaatgctgaaatcttgcctttcgacatcaacccaaccaccagagccaacatcaaagacgctattgccaag<br>tttgacgccaccattgctgcccacgacgaagaaatcttccaacactacggttacggtaagggattgatcaagaagttcaaggtctccccagatgcctacgtgcaattg<br>ttgatgcaattggcatacttcaagtacaccggcaagatcagaccaacttatgaatccgccgccaccagaaagttcttgaagggtagaaccgaaaccggtagaact<br>gtttccaacgaatccaagaagtttgttgagacctggtccgatccaaacgctagcagcgccgacaaggttgccactttccaagctgccgctaagcaacacgttgctta<br>cttgtctgctgccgccgatggtaagggtgtcgaccgtcacttgtttggtttgaagcagatgattcaaccaggcgaaccaatccctgaaatcttcactgacccaatctttag<br>ctattctcaaacctggtacatttcttcttcccaagtcccatctgaattcttccaatcttggggttggtcgcaagtcattgacgacggtttcggtttggcttacttgatcaacaac<br>gactggatccacgttcacatttcttgtaagagaggtaacggcttgcaatccgaccacttgaaatggtacttggttgatagtgctaacgaaatgaaggatgtcttgactaa<br>gggattattgactgatgctaagcctaagttgtaa | 59 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| Engineered DNA fragment *CAT2*$^{\Delta mts}$ | atgccaattttgaaaaaaccattctccaccagccacgcaaagggtgacttgttcaaataccagtcacaattacccaagttgcctgttcctactttggaagaaaccgcat ccaagtacctcaagaccgttgagccattcttgaaccaagagcaattggaatccaccaaggccaaagtcgctgagtttgttagaccaggtggtgccggtgaagcctt gcaagccagattgaacaactttgccgccgacaaggacaactggttggctgaattttgggacgactatgcatacatgtcttatagagatcctgttgttccatatgtttcttac tttttcagtcacaaggatgtcaagaacatcattggccaagaccaattgttgaaggccactttgattgcttactacactattgagttccaagaaaaggttttggacgaaagt ttggacccagaagtcatcaagggtaacccattctgtatgaacgccttcaagtacatgttcaacaactcgagagttccagctgaaggctccgacatcacccaacacta caacggtgaagaaaaccaatttttcgttgtcatctacaagaacaacttctacaaggttccaacccacaagaacggccaaagattgaccaagggtgaaatctacag ctacttgcaagaaatcaagaacgatgccactccaaagggtctcggtttgggtgctttgacctcattgaacagagacgaatggttgagtgcctacaacaacttgttgaa gtccccaatcaacgaagcttccttgggatccatctttgcttccagctttgtcattgccttggactccaacaacccagtcaccattgaagaaaaatccaagaactgctggc acggggacggtcaaaacagattctttgacaagcctttggaattcttcgtcagtgctaacggtaactctggtttccttggtgaacactccagaatggacgctaccccaac cgtgcaattgaacaacaccatctacaagcaaatcttggaaaccaatccaaacgacttgattgttgaaattggttcttctgctccaagattcggcaatgctgaaatcttgc ctttcgacatcaacccaaccaccagagccaacatcaaagacgctattgccaagtttgacgccaccattgctgcccacgacgaagaaatcttccaacactacggtta cggtaagggattgatcaagaagttcaaggtctccccagatgcctacgtgcaattgttgatgcaattggcatacttcaagtacaccggcaagatcagaccaacttatga atccgccgccaccagaaagttcttgaagggtagaaccgaaaccggtagaactgtttccaacgaatccaagaagtttgttgagacctggtccgatccaaacgctag cagcgccgacaaggttgccactttccaagctgccgctaagcaacacgttgcttacttgtctgctgccgccgatggtaagggtgtcgaccgtcacttgtttggtttgaagc agatgattcaaccaggcgaaccaatccctgaaatcttcactgacccaatctttagctattctcaaacctggtacatttcttcttcccaagtcccatctgaattcttccaatctt ggggttggtcgcaagtcattgacgacggtttcggtttggcttacttgatcaacaacgactggatccacgttcacatttcttgtaagagaggtaacggcttgcaatccgac cacttgaaatggtacttggttgatagtgctaacgaaatgaaggatgtcttgactaagggattattgactgatgctaagcctaagttgtaa | 60 |



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**Table 19**

| Gene (Organism) | Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted) | SEQ ID NO |
|---|---|---|
| Engineered DNA fragment *CAT2*$^{\Delta mts\ \Delta pts}$ | atgccaattttgaaaaaaccattctccaccagccacgcaaagggtgacttgttcaaataccagtcacaattacccaagttgcctgttcctactttggaagaaaccgcat ccaagtacctcaagaccgttgagccattcttgaaccaagagcaattggaatccaccaaggccaaagtcgctgagtttgttagaccaggtggtgccggtgaagcctt gcaagccagattgaacaactttgccgccgacaaggacaactggttggctgaattttgggacgactatgcatacatgtcttatagagatcctgttgttccatatgtttcttac tttttcagtcacaaggatgtcaagaacatcattggccaagaccaattgttgaaggccactttgattgcttactacactattgagttccaagaaaaggttttggacgaaagt ttggacccagaagtcatcaagggtaacccattctgtatgaacgccttcaagtacatgttcaacaactcgagagttccagctgaaggctccgacatcacccaacacta caacggtgaagaaaaccaatttttcgttgtcatctacaagaacaacttctacaaggttccaacccacaagaacggccaaagattgaccaagggtgaaatctacag ctacttgcaagaaatcaagaacgatgccactccaaagggtctcggtttgggtgctttgacctcattgaacagagacgaatggttgagtgcctacaacaacttgttgaa gtccccaatcaacgaagcttccttgggatccatctttgcttccagctttgtcattgccttggactccaacaacccagtcaccattgaagaaaaatccaagaactgctggc acggggacggtcaaaacagattctttgacaagcctttggaattcttcgtcagtgctaacggtaactctggtttccttggtgaacactccagaatggacgctaccccaac cgtgcaattgaacaacaccatctacaagcaaatcttggaaaccaatccaaacgacttgattgttgaaattggttcttctgctccaagattcggcaatgctgaaatcttgc ctttcgacatcaacccaaccaccagagccaacatcaaagacgctattgccaagtttgacgccaccattgctgcccacgacgaagaaatcttccaacactacggtta cggtaagggattgatcaagaagttcaaggtctccccagatgcctacgtgcaattgttgatgcaattggcatacttcaagtacaccggcaagatcagaccaacttatga atccgccgccaccagaaagttcttgaagggtagaaccgaaaccggtagaactgtttccaacgaatccaagaagtttgttgagacctggtccgatccaaacgctag cagcgccgacaaggttgccactttccaagctgccgctaagcaacacgttgcttacttgtctgctgccgccgatggtaagggtgtcgaccgtcacttgtttggtttgaagc agatgattcaaccaggcgaaccaatccctgaaatcttcactgacccaatctttagctattctcaaacctggtacatttcttcttcccaagtcccatctgaattcttccaatctt ggggttggtcgcaagtcattgacgacggtttcggtttggcttacttgatcaacaacgactggatccacgttcacatttcttgtaagagaggtaacggcttgcaatccgac cacttgaaatggtacttggttgatagtgctaacgaaatgaaggatgtcttgactaagggattattgactgatgctaagtaa | 61 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| Engineered DNA fragment *CAT2*$^{\Delta pts}$ | atgtttaactttaagttgtcgcaacaagtattaaagaattccaccaaatccattatgccaattttgaaaaaaccattctccaccagccacgcaaagggtgacttgttcaa<br>ataccagtcacaattacccaagttgcctgttcctactttggaagaaaccgcatccaagtacctcaagaccgttgagccattcttgaaccaagagcaattggaatccac<br>caaggccaaagtcgctgagtttgttagaccaggtggtgccggtgaagccttgcaagccagattgaacaactttgccgccgacaaggacaactggttggctgaatttt<br>gggacgactatgcatacatgtcttatagagatcctgttgttccatatgtttcttacttttcagtcacaaggatgtcaagaacatcattggccaagaccaattgttgaaggc<br>cactttgattgcttactacactattgagttccaagaaaaggttttggacgaaagtttggacccagaagtcatcaagggtaacccattctgtatgaacgccttcaagtaca<br>tgttcaacaactcgagagttccagctgaaggctccgacatcacccaacactacaacggtgaagaaaaccaatttttcgttgtcatctacaagaacaacttctacaag<br>gttccaacccacaagaacggccaaagattgaccaagggtgaaatctacagctacttgcaagaaatcaagaacgatgccactccaaagggtctcggtttgggtgct<br>ttgacctcattgaacagagacgaatggttgagtgcctacaacaacttgttgaagtccccaatcaacgaagcttccttgggatccatctttgcttccagctttgtcattgcctt<br>ggactccaacaacccagtcaccattgaagaaaaatccaagaactgctggcacggggacggtcaaaacagattctttgacaagcctttggaattcttcgtcagtgct<br>aacggtaactctggtttccttggtgaacactccagaatggacgctaccccaaccgtgcaattgaacaacaccatctacaagcaaatcttggaaaccaatccaaacg<br>acttgattgttgaaattggttcttctgctccaagattcggcaatgctgaaatcttgcctttcgacatcaacccaaccaccagagccaacatcaaagacgctattgccaag<br>tttgacgccaccattgctgcccacgacgaagaaatcttccaacactacggttacggtaagggattgatcaagaagttcaaggtctccccagatgcctacgtgcaattg<br>ttgatgcaattggcatacttcaagtacaccggcaagatcagaccaacttatgaatccgccgccaccagaaagttcttgaagggtagaaccgaaaccggtagaact<br>gtttccaacgaatccaagaagtttgttgagacctggtccgatccaaacgctagcagccgccgacaaggttgccactttccaagctgccgctaagcaacacgttgctta<br>cttgtctgctgccgccgatggtaagggtgtcgaccgtcacttgtttggtttgaagcagatgattcaaccaggcgaaccaatccctgaaatcttcactgacccaatctttag<br>ctattctcaaacctggtacatttcttcttcccaagtcccatctgaattcttccaatcttggggttggtcgcaagtcattgacgacggtttcggtttggcttacttgatcaacaac<br>gactggatccacgttcacatttcttgtaagagaggtaacggcttgcaatccgaccacttgaaatggtacttggttgatagtgctaacgaaatgaaggatgtcttgactaa<br>gggattattgactgatgctaagtaa | 62 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| *YAT1* (*Candida viswanathii* ATCC20336) | ATGTCAACTTACCAGTTCCAAGAAACTTTAGAAAAGCTTCCAATCCCAGACTTAAACCAAACATGCGCTAACTAC<br>CTCAACGTCTTGCGTCCGCTCCAAACAGAGCAAGAACACATCAAGACCAAGACTGCCGTTGAGAATTTCCTCAA<br>GAATGGCACCGGCCAGTACCTCGACGCTGCCTTGCGCGAATACGCCCAGACCCGCCCCAGCTACATTGAACA<br>GTTCTGGTACGACGCATACTTAAACTACGACTCCCCCGTTGTTTTAAACTTGAATCCGTTTTTCTTACTCGAAGA<br>CGACCCATTCACCAACGAGTCCAGCTCCGTCAATCCCCAGGTTAAGCGTGCCACCAGCTTGGTGATGTCCTCG<br>TTGAAGTTCATCCAGGCGTTGAAGAACGAGACGTTGAGCGTCGACACCTTGAAAGGAGGCAAGCCGTTGTGCA<br>TGTACCAGTACACCAAATTGTTTGGGGCGCTGCGTATCCCCTCCGAGGACGGGTGTGTGATGCAGAGCGACCC<br>GGCAAGTAACCACATTGTCGTGATGTCTAAGTCCCAGTTGTATTGGTTTGACGTGTTGGATAGCAAGAACAACT<br>TGATTTTGAGCGAAGCGGAGTTGAATGTGAACTTCCAGTCGATCATTCATGACTCTTTGAGAACTCCCTCGGAT<br>GAGATTGCAAAGAGCTCGTTTGGGGTGTTGACGACGGAGAATAGAAGAATTTGGGCTAATGTTAGACATAATTT<br>GATGTCGACTACCAATAATAAGGTTAACCATGAGGTGTTGTCGATTATTGACTCAGCGTTGTTTGTGTTATGCTT<br>TGATGATATCGTGATTAATGACTTGTCGGAGTTGTCCAAGAATATGCTTTGTGGGTTGTCCATTTTGGATAATGG<br>TATCCAAGTGGGTACTTGTACAAACAGATGGTATGACAAGTTGCAGATTATCGTTACCAAGAATGCCAAAGCCG<br>GTATTAATTTCGAACATACTGGTGTTGACGGTCATACGGTGTTGAGATTTGTCAGTGATATCTACACCGACTCCA<br>TCTTGTCCTTTGCCAACTCCATCAATAGCAACGCTCCATCTTTATGGAACACCACTACAAACTACAAAGAACTTG<br>ATGGGGAAGATTTAATCACCGTGCCACGTAAGTTGGAATGGGAGTTGACCCCTGACTTATCCTTGGCTTTGAGA<br>TTTGGAGAAACCAGATTGTCGGACTTGATCAACCAGAACGAGTTCCGCCACTTGGAGTTCAAGAACTATGGGTC<br>TACACAAATCAAGAAGATGAAGTTCTCACCAGATGCGTTTGTTCAAATGGCGTTCCAAGCAACCTACTATGCGC<br>TTTACGGTAAGGTTGAATGTACTTATGAGCCAGCCATGACGAAGCAGTTCTACCACGGAAGAACAGAAGCTATA<br>AGAACCGTCTCGCAAGAATCGAACTTGTTTGTGCGTAAGTTCTTTGACTCCACTGTGTCCATCCAGAAGAAGTT<br>AGAGTACTTGACCCAAGCTTGCACCAAGCACTCCCAGCAAACCAGAATGAGTTCTGCCGGTCAAGGTGTCGAT<br>CGTCACTTGTATGCTTTATTCTGTATCTGGAAGAGATACTTACACGATGCCGAAGACGACGATGACGATGCCTC<br>CAAAGACCAACCATCATCGACGGAATTATCCCGTGATGACACCATCATTGCTCAAACCAACGGTGACAGCACCG<br>ATGATGACGACTCCACCACCGTTGTCGGACACGGCGCCAACCACCATCACAACTTGAAAAGCGCCGATTTGTT<br>GAAAACCATCCCTGAGATATTCGCAGACAATGGCTGGGACAAGTTGAACAACACGATCATATCCACCTCCAACT<br>GTGGTAATCCTTCTTTGAGATTGTTTGGATTTGGTCCTGTTTCAGCTAACGGGTTTGGTATCGGGTATATCTTGA<br>AGGACGACTCGATCTCGATCTGTGCTTCGTCGAAGCATCGTCAGACGCAGAGATTCTTGGTCACTTTGAACTCG<br>TACTTGTTGGAGATCCAGAACATCTGGAAACAAGCGCAAAAGATGGAGAAGTTGAAGGCTGAGTTGGCTAAAG<br>CCGTCGAGGATGCAAAACCTAAGGGACAGAAATCAGGTGAAGCAGAGGGGATCATCGAGCCTAAGCCTAATAA<br>CTTGAGCACTCTTTTGGGAGGTTATGGTTATTTCGATATGGGCGATGAGGACATCAAGAGCAGAGGACAATCTC<br>CAGAACCTCCGTTCTTGCATCGTGCTGGATCCGGTTTCAGCATTCGTGAAATAGGCAAGAAGTTGAGATTAAGC<br>GAGTATTAG | 63 |



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**Table 19**

| Gene (Organism) | Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted) | SEQ ID NO |
|---|---|---|
| *COX4* first 90 nucleic acids encoding mitochondrial targeting sequence (*Candida viswanathii* ATCC20336) | Atgctttcccgtaccactttaagagttgccagacaacaaaccagattattgtctacttccagaatcttgttcaacagcaagaccgaccaa | 64 |
| *CIT1* first 75 nucleic acids encoding mitochondrial targeting sequence (*Candida viswanathii* ATCC20336) | Atgtctgcattaagatcattccaacgctcttctaacgttgctaagtccaccttaaagaactcagtcagaacctat | 65 |
| *CAT2* first 99 nucleic acids encoding mitochondrial targeting sequence (*Candida viswanathii* ATCC20336) | Atgtttaactttaagttgtcgcaacaagtattaaagaattccaccaaatccattatgccaattttgaaaaaaccattctccaccagccacgcaaagggt | 66 |
| Engineered DNA fragment *YAT1*$^{+COX4mts}$ | atgctttcccgtaccactttaagagttgccagacaacaaaccagattattgtctacttccagaatcttgttcaacagcaagaccgaccaatcaacttaccagttccaag aaactttagaaaagcttccaatcccagacttaaaccaaacatgcgctaactacctcaacgtcttgcgtccgctccaaacagagcaagaacacatcaagaccaag actgccgttgagaatttcctcaagaatggcaccggccagtacctcgacgctgccttgcgcgaatacgcccagacccgccccagctacattgaacagttctggtacg acgcatacttaaactacgactcccccgttgttttaaacttgaatccgtttttcttactcgaagacgacccattcaccaacgagtccagctccgtcaatccccaggttaagc gtgccaccagcttggtgatgtcctcgttgaagttcatccaggcgttgaagaacgagacgttgagcgtcgacaccttgaaaggaggcaagccgttgtgcatgtaccag tacaccaaattgtttggggcgctgcgtatcccctccgaggacgggtgtgtgatgcagagcgacccggcaagtaaccacattgtcgtgatgtctaagtcccagttgtatt | 67 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| | ggtttgacgtgttggatagcaagaacaacttgattttgagcgaagcggagttgaatgtgaacttccagtcgatcattcatgactctttgagaactccctcggatgagattg<br>caaagagctcgtttggggtgttgacgacggagaatagaagaatttgggctaatgttagacataatttgatgtcgactaccaataataaggttaaccatgaggtgttgtc<br>gattattgactcagcgttgtttgtgttatgctttgatgatatcgtgattaatgacttgtcggagttgtccaagaatatgctttgtgggttgtccattttggataatggtatccaagtg<br>ggtacttgtacaaacagatggtatgacaagttgcagattatcgttaccaagaatgccaaagccggtattaatttcgaacatactggtgttgacggtcatacggtgttga<br>gatttgtcagtgatatctacaccgactccatcttgtcctttgccaactccatcaatagcaacgctccatctttatggaacaccactacaaactacaaagaacttgatggg<br>gaagatttaatcaccgtgccacgtaagttggaatgggagttgacccctgacttatccttggctttgagatttggagaaaccagattgtcggacttgatcaaccagaacg<br>agttccgccacttggagttcaagaactatgggtctacacaaatcaagaagatgaagttctcaccagatgcgtttgttcaaatggcgttccaagcaacctactatgcgct<br>ttacggtaaggttgaatgtacttatgagccagccatgacgaagcagttctaccacggaagaacagaagctataagaaccgtctcgcaagaatcgaacttgtttgtgc<br>gtaagttctttgactccactgtgtccatccagaagaagttagagtacttgacccaagcttgcaccaagcactcccagcaaaccagaatgagttctgccggtcaaggtg<br>tcgatcgtcacttgtatgctttattctgtatctggaagagatacttacacgatgccgaagacgacgatgacgatgcctccaaagaccaaccatcatcgacggaattatc<br>ccgtgatgacaccatcattgctcaaaccaacggtgacagcaccgatgatgacgactccaccaccgttgtcggacacggcgccaaccaccatcacaacttgaaaa<br>gcgccgatttgttgaaaaccatccctgagatattcgcagacaatggctgggacaagttgaacaacacgatcatatccacctccaactgtggtaatccttctttgagatt<br>gtttggatttggtcctgtttcagctaacgggtttggtatcgggtatatcttgaaggacgactcgatctcgatctgtgcttcgtcgaagcatcgtcagacgcagagattcttgg<br>tcactttgaactcgtacttgttggagatccagaacatctggaaacaagcgcaaaagatggagaagttgaaggctgagttggctaaagccgtcgaggatgcaaaac<br>ctaagggacagaaatcaggtgaagcagaggggatcatcgagcctaagcctaataacttgagcactcttttgggaggttatggttatttcgatatgggcgatgaggac<br>atcaagagcagaggacaatctccagaacctccgttcttgcatcgtgctggatccggtttcagcattcgtgaaataggcaagaagttgagattaagcgagtattag | |



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**Table 19**

| Gene (Organism) | Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted) | SEQ ID NO |
|---|---|---|
| Engineered DNA fragment *YAT1*$^{+CIT1mts}$ | atgtctgcattaagatcattccaacgctcttctaacgttgctaagtccaccttaaagaactcagtcagaacctattcaacttaccagttccaagaaactttagaaaagctt<br>ccaatcccagacttaaaccaaacatgcgctaactacctcaacgtcttgcgtccgctccaaacagagcaagaacacatcaagaccaagactgccgttgagaatttc<br>ctcaagaatggcaccggccagtacctcgacgctgccttgcgcgaatacgcccagacccgccccagctacattgaacagttctggtacgacgcatacttaaactac<br>gactcccccgttgttttaaacttgaatccgtttttcttactcgaagacgacccattcaccaacgagtccagctccgtcaatccccaggttaagcgtgccaccagcttggtg<br>atgtcctcgttgaagttcatccaggcgttgaagaacgagacgttgagcgtcgacaccttgaaaggaggcaagccgttgtgcatgtaccagtacaccaaattgtttgg<br>ggcgctgcgtatcccctccgaggacgggtgtgtgatgcagagcgacccggcaagtaaccacattgtcgtgatgtctaagtcccagttgtattggtttgacgtgttggat<br>agcaagaacaacttgattttgagcgaagcggagttgaatgtgaacttccagtcgatcattcatgactctttgagaactccctcggatgagattgcaaagagctcgtttg<br>gggtgttgacgacggagaatagaagaatttgggctaatgttagacataatttgatgtcgactaccaataataaggttaaccatgaggtgttgtcgattattgactcagcg<br>ttgtttgtgttatgctttgatgatatcgtgattaatgacttgtcggagttgtccaagaatatgctttgtgggttgtccattttggataatggtatccaagtgggtacttgtacaaac<br>agatggtatgacaagttgcagattatcgttaccaagaatgccaaagccggtattaatttcgaacatactggtgttgacggtcatacggtgttgagatttgtcagtgatatc<br>tacaccgactccatcttgtcctttgccaactccatcaatagcaacgctccatctttatggaacaccactacaaactacaaagaacttgatggggaagatttaatcaccg<br>tgccacgtaagttggaatgggagttgacccctgacttatccttggctttgagatttggagaaaccagattgtcggacttgatcaaccagaacgagttccgccacttgga<br>gttcaagaactatgggtctacacaaatcaagaagatgaagttctcaccagatgcgtttgttcaaatggcgttccaagcaacctactatgcgctttacggtaaggttgaa<br>tgtacttatgagccagccatgacgaagcagttctaccacggaagaacagaagctataagaaccgtctcgcaagaatcgaacttgtttgtgcgtaagttctttgactcc<br>actgtgtccatccagaagaagttagagtacttgacccaagcttgcaccaagcactcccagcaaaccagaatgagttctgccggtcaaggtgtcgatcgtcacttgtat<br>gctttattctgtatctggaagagatacttacacgatgccgaagacgacgatgacgatgcctccaaagaccaaccatcatcgacggaattatcccgtgatgacaccat<br>cattgctcaaaccaacggtgacagcaccgatgatgacgactccaccaccgttgtcggacacggcgccaaccaccatcacaacttgaaaagcgccgatttgttgaa<br>aaccatccctgagatattcgcagacaatggctgggacaagttgaacaacacgatcatatccacctccaactgtggtaatccttctttgagattgtttggatttggtcctgtt<br>tcagctaacgggtttggtatcgggtatatcttgaaggacgactcgatctcgatctgtgcttcgtcgaagcatcgtcagacgcagagattcttggtcactttgaactcgtact<br>tgttggagatccagaacatctggaaacaagcgcaaaagatggagaagttgaaggctgagttggctaaagccgtcgaggatgcaaaacctaagggacagaaat<br>caggtgaagcagaggggatcatcgagcctaagcctaataacttgagcactcttttgggaggttatggttatttcgatatgggcgatgaggacatcaagagcagagg<br>acaatctccagaacctccgttcttgcatcgtgctggatccggtttcagcattcgtgaaataggcaagaagttgagattaagcgagtattag | 68 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| Engineered DNA fragment *YAT1*$^{+CAT2mts}$ | atgtttaactttaagttgtcgcaacaagtattaaagaattccaccaaatccattatgccaattttgaaaaaaccattctccaccagccacgcaaagggttcaacttacca gttccaagaaacttttagaaaagcttccaatcccagacttaaaccaaacatgcgctaactacctcaacgtcttgcgtccgctccaaacagagcaagaacacatcaa gaccaagactgccgttgagaatttcctcaagaatggcaccggccagtacctcgacgctgccttgcgcgaatacgcccagacccgccccagctacattgaacagtt ctggtacgacgcatacttaaactacgactcccccgttgttttaaacttgaatccgtttttcttactcgaagacgacccattcaccaacgagtccagctccgtcaatcccca ggttaagcgtgccaccagcttggtgatgtcctcgttgaagttcatccaggcgttgaagaacgagacgttgagcgtcgacaccttgaaaggaggcaagccgttgtgca tgtaccagtacaccaaattgtttggggcgctgcgtatcccctccgaggacgggtgtgtgatgcagagcgacccggcaagtaaccacattgtcgtgatgtctaagtccc agttgtattggtttgacgtgttggatagcaagaacaacttgattttgagcgaagcggagttgaatgtgaacttccagtcgatcattcatgactctttgagaactccctcgga tgagattgcaaagagctcgtttggggtgttgacgacggagaatagaagaatttgggctaatgttagacataatttgatgtcgactaccaataataaggttaaccatga ggtgttgtcgattattgactcagcgttgtttgtgttatgctttgatgatatcgtgattaatgacttgtcggagttgtccaagaatatgctttgtgggttgtccattttggataatggt atccaagtgggtacttgtacaaacagatggtatgacaagttgcagattatcgttaccaagaatgccaaagccggtattaatttcgaacatactggtgttgacggtcata cggtgttgagatttgtcagtgatatctacaccgactccatcttgtcctttgccaactccatcaatagcaacgctccatctttatggaacaccactacaaactacaaagaa cttgatggggaagatttaatcaccgtgccacgtaagttggaatgggagttgacccctgacttatccttggctttgagatttggagaaaccagattgtcggacttgatcaa ccagaacgagttccgccacttggagttcaagaactatgggtctacacaaatcaagaagatgaagttctcaccagatgcgtttgttcaaatggcgttccaagcaacct actatgcgctttacggtaaggttgaatgtacttatgagccagccatgacgaagcagttctaccacggaagaacagaagctataagaaccgtctcgcaagaatcga acttgtttgtgcgtaagttctttgactccactgtgtccatccagaagaagttagagtacttgacccaagcttgcaccaagcactcccagcaaaccagaatgagttctgcc ggtcaaggtgtcgatcgtcacttgtatgctttattctgtatctggaagagatacttacacgatgccgaagacgacgatgacgatgcctccaaagaccaaccatcatcg acggaattatcccgtgatgacaccatcattgctcaaaccaacggtgacagcaccgatgatgacgactccaccaccgttgtcggacacggcgccaaccaccatca caacttgaaaagcgccgatttgttgaaaaccatccctgagatattcgcagacaatggctgggacaagttgaacaacacgatcatatccacctccaactgtggtaatc cttctttgagattgtttggatttggtcctgtttcagctaacgggtttggtatcgggtatatcttgaaggacgactcgatctcgatctgtgcttcgtcgaagcatcgtcagacgc agagattcttggtcactttgaactcgtacttgttggagatccagaacatctggaaacaagcgcaaaagatggagaagttgaaggctgagttggctaaagccgtcga ggatgcaaaacctaagggacagaaatcaggtgaagcagaggggatcatcgagcctaagcctaataacttgagcactcttttgggaggttatggttatttcgatatgg gcgatgaggacatcaagagcagaggacaatctccagaacctccgttcttgcatcgtgctggatccggtttcagcattcgtgaaataggcaagaagttgagattaag cgagtattag | 69 |



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**Table 19**

| Gene (Organism) | Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted) | SEQ ID NO |
|---|---|---|
| Engineered DNA fragment *YAT1*$^{+pts}$ | ATGTCAACTTACCAGTTCCAAGAAACTTTAGAAAAGCTTCCAATCCCAGACTTAAACCAAACATGCGCTAACTAC<br>CTCAACGTCTTGCGTCCGCTCCAAACAGAGCAAGAACACATCAAGACCAAGACTGCCGTTGAGAATTTCCTCAA<br>GAATGGCACCGGCCAGTACCTCGACGCTGCCTTGCGCGAATACGCCCAGACCCGCCCCAGCTACATTGAACA<br>GTTCTGGTACGACGCATACTTAAACTACGACTCCCCCGTTGTTTTAAACTTGAATCCGTTTTTCTTACTCGAAGA<br>CGACCCATTCACCAACGAGTCCAGCTCCGTCAATCCCCAGGTTAAGCGTGCCACCAGCTTGGTGATGTCCTCG<br>TTGAAGTTCATCCAGGCGTTGAAGAACGAGACGTTGAGCGTCGACACCTTGAAAGGAGGCAAGCCGTTGTGCA<br>TGTACCAGTACACCAAATTGTTTGGGGCGCTGCGTATCCCCTCCGAGGACGGGTGTGTGATGCAGAGCGACCC<br>GGCAAGTAACCACATTGTCGTGATGTCTAAGTCCCAGTTGTATTGGTTTGACGTGTTGGATAGCAAGAACAACT<br>TGATTTTGAGCGAAGCGGAGTTGAATGTGAACTTCCAGTCGATCATTCATGACTCTTTGAGAACTCCCTCGGAT<br>GAGATTGCAAAGAGCTCGTTTGGGGTGTTGACGACGGAGAATAGAAGAATTTGGGCTAATGTTAGACATAATTT<br>GATGTCGACTACCAATAATAAGGTTAACCATGAGGTGTTGTCGATTATTGACTCAGCGTTGTTTGTGTTATGCTT<br>TGATGATATCGTGATTAATGACTTGTCGGAGTTGTCCAAGAATATGCTTTGTGGGTTGTCCATTTTGGATAATGG<br>TATCCAAGTGGGTACTTGTACAAACAGATGGTATGACAAGTTGCAGATTATCGTTACCAAGAATGCCAAAGCCG<br>GTATTAATTTCGAACATACTGGTGTTGACGGTCATACGGTGTTGAGATTTGTCAGTGATATCTACACCGACTCCA<br>TCTTGTCCTTTGCCAACTCCATCAATAGCAACGCTCCATCTTTATGGAACACCACTACAAACTACAAAGAACTTG<br>ATGGGGAAGATTTAATCACCGTGCCACGTAAGTTGGAATGGGAGTTGACCCCTGACTTATCCTTGGCTTTGAGA<br>TTTGGAGAAACCAGATTGTCGGACTTGATCAACCAGAACGAGTTCCGCCACTTGGAGTTCAAGAACTATGGGTC<br>TACACAAATCAAGAAGATGAAGTTCTCACCAGATGCGTTTGTTCAAATGGCGTTCCAAGCAACCTACTATGCGC<br>TTTACGGTAAGGTTGAATGTACTTATGAGCCAGCCATGACGAAGCAGTTCTACCACGGAAGAACAGAAGCTATA<br>AGAACCGTCTCGCAAGAATCGAACTTGTTTGTGCGTAAGTTCTTTGACTCCACTGTGTCCATCCAGAAGAAGTT<br>AGAGTACTTGACCCAAGCTTGCACCAAGCACTCCCAGCAAACCAGAATGAGTTCTGCCGGTCAAGGTGTCGAT<br>CGTCACTTGTATGCTTTATTCTGTATCTGGAAGAGATACTTACACGATGCCGAAGACGACGATGACGATGCCTC<br>CAAAGACCAACCATCATCGACGGAATTATCCCGTGATGACACCATCATTGCTCAAACCAACGGTGACAGCACCG<br>ATGATGACGACTCCACCACCGTTGTCGGACACGGCGCCAACCACCATCACAACTTGAAAAGCGCCGATTTGTT<br>GAAAACCATCCCTGAGATATTCGCAGACAATGGCTGGGACAAGTTGAACAACACGATCATATCCACCTCCAACT<br>GTGGTAATCCTTCTTTGAGATTGTTTGGATTTGGTCCTGTTTCAGCTAACGGGTTTGGTATCGGGTATATCTTGA<br>AGGACGACTCGATCTCGATCTGTGCTTCGTCGAAGCATCGTCAGACGCAGAGATTCTTGGTCACTTTGAACTCG<br>TACTTGTTGGAGATCCAGAACATCTGGAAACAAGCGCAAAAGATGGAGAAGTTGAAGGCTGAGTTGGCTAAAG<br>CCGTCGAGGATGCAAAACCTAAGGGACAGAAATCAGGTGAAGCAGAGGGGATCATCGAGCCTAAGCCTAATAA<br>CTTGAGCACTCTTTTGGGAGGTTATGGTTATTTCGATATGGGCGATGAGGACATCAAGAGCAGAGGACAATCTC<br>CAGAACCTCCGTTCTTGCATCGTGCTGGATCCGGTTTCAGCATTCGTGAAATAGGCAAGAAGTTGAGATTAAGC<br>GAGTATCCTAAGTTGTAG | 70 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| *CRC1* (*Candida viswanathii* ATCC20336) | atggacgacgttgattctgctttagccgataatgttaaatccttcgctgccggtggttttggtggtatttgtgccgtgttgaccggtcatccattcgacttggtcaaggtcaga<br>ttgcaaactgggttgtacaaatcgtcagtgcaatgtgtcaaagaaacaatagccaaagacgggttgtttgggttatacagaggtgtcttgcctccattgttgggtgtcac<br>cccaatgtttgctgtttccttctggggttacgacgttggtaagaaattggtttcttccttcactggcaaatcagtcgacaagtttgaaatcaaagacatttcgactgccggttt<br>catcagtgccatcccaaccaccttggttgccgctccattcgaaagagtcaaggtcatgatgcagatccaagaaggtgccaagtccaagtccatgggtgccgttgttg<br>ctgaaatgtacagaaccggtggtatcagatccatcttcaagggtactgtggctactttggccagagatggtccaggttctgccttgtatttcgctacttatgaatgggtca<br>agaaggaattgactgcccctggtgaagacttgtccttgtttgctattaccactgctggtggtttcgctggtattgccatgtggttgggtgttttcccaattgacaccatcaagt<br>ctacccagcagtcttccaacgtcaaggtctccattgtgcaggccactaagaacatctacgctaaaggtggtatcaaggctttcttccctggtgttggtcctgccttggcc<br>agagctttcccagccaacgctgctaccttcctaggggttgaattggccagaaagttcttggacaaagttatataa | 71 |
| *ACH1* (*Candida viswanathii* ATCC20336) | atgtctgccattttgaaacaaagagttagatatgctccatacctcaaaaaattgagaacagcagaacaatgtgtcgacttgttcaagaacggccaatacttgggttggt<br>cgggtttcaccggtgtcggtgccccaaaggctgtcccagccgctttggttgaccacgttgaaaagaacaaattgcagggccaaatgggcttccacttgtttgtcggtgc<br>ttctgccggtccagaagaaagcagatgggctgaaaacaacatgcttttatctagatctccacatcaagtcggtaagccaattgctgctgccatcaatgacggcagaa<br>ctcagttcttcgacaagcacttgtccatgttcccacaagatttgacctacggcttctacaccaagaacaagccaaacggctccaacttggattacaccatcattgaagc<br>taccgccataacagaggatggtgccattatcccaggtcctgccgtcggtgcctccccagaaatgctttcggtttccgacaacatcatcattgaagtcaacacaaaaa<br>caccatccttcgaaggtatacacgacattgacttgccagtcaacccaccattcagacaaccatacccacacaccactgccgactacagaattggcagaaccgcca<br>tcccagtcgacccagaaaaggttatcgccattgtcgaaaccacagagggtgacaaggttccaccaaacaccccaagtgacgctcaatcccaagccattgctggtc<br>acttgattgaattcttggaaaacgaagtcaaacaaggtcgtctcccagaaaacttgcacccattacaatccggtatcggtaacattgccaacgctgtcgttgaaggttt<br>ggcccaatccaacttcaagaacttgactgtctggaccgaagtcttgcaagattccttcttggacttcttcgaatccggaagtttggactttgcaaccgccacctccatca<br>gattgaccgaggaaggtttcaagaagttctacgaaaactgggacgtctactccaagaagttgtgcttgagatcgcaagttgtctccaactcgccagaaatcatcaga<br>agattgggtgtcttggctttcaacaccccagttgaagttgacatctacgcccacgccaactccaccaacgtcatgggttccagaatgttgaacggtttgggaggctcgg<br>gtgatttcttgagaaacgccaagttgtccgtcatgcacaccccatctgctcgtccaaccaaggttgacccaaccggtgtctcttgtattgtcccaatggccacccacgtt<br>gatcaaaccgaacacgatctcgatgttgtcgttaccgaacaaggtttggctgatctcagaggtctcagtccaaaggaaagagctcaagttataatcaacaactgtgc<br>ccacccagactaccaggctcaattgcaagactactttgacagagctgttttctacgccaccaagaagaagatgttgcacgaaccacacatgttgaacgaagctttcg<br>ctatgcacttgaacttgcaagaaaatggtaccatgaagttgaacaaatag | 72 |



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**Table 19**

| Gene (Organism) | Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted) | SEQ ID NO |
|---|---|---|
| Engineered DNA *ACH1*$^{\Delta mts+pts}$ | atggctccatacctcaaaaaattgagaacagcagaacaatgtgtcgacttgttcaagaacggccaatacttgggttggtcgggtttcaccggtgtcggtgccccaaa ggctgtcccagccgctttggttgaccacgttgaaaagaacaaattgcagggccaaatgggcttccacttgtttgtcggtgcttctgccggtccagaagaaagcagatg ggctgaaaacaacatgcttttatctagatctccacatcaagtcggtaagccaattgctgctgccatcaatgacggcagaactcagttcttcgacaagcacttgtccatgt tcccacaagatttgacctacggcttctacaccaagaacaagccaaacggctccaacttggattacaccatcattgaagctaccgccataacagaggatggtgccat tatcccaggtcctgccgtcggtgcctccccagaaatgctttcggtttccgacaacatcatcattgaagtcaacacaaaaacaccatccttcgaaggtatacacgacatt gacttgccagtcaacccaccattcagacaaccatacccacacaccactgccgactacagaattggcagaaccgccatcccagtcgacccagaaaaggttatcgc cattgtcgaaaccacagagggtgacaaggttccaccaaacacccccaagtgacgctcaatcccaagccattgctggtcacttgattgaattcttggaaaacgaagtc aaacaaggtcgtctcccagaaaacttgcacccattacaatccggtatcggtaacattgccaacgctgtcgttgaaggtttggcccaatccaacttcaagaacttgact gtctggaccgaagtcttgcaagattccttcttggacttcttcgaatccggaagtttggacttgcaaccgccacctccatcagattgaccgaggaaggtttcaagaagtt ctacgaaaactgggacgtctactccaagaagttgtgcttgagatcgcaagttgtctccaactcgccagaaatcatcagaagattgggtgtcttggctttcaacacccc agttgaagttgacatctacgcccacgccaactccaccaacgtcatgggttccagaatgttgaacggtttgggaggctcgggtgatttcttgagaaacgccaagttgtc cgtcatgcacaccccatctgctcgtccaaccaaggttgacccaaccggtgtctcttgtattgtcccaatggccacccacgttgatcaaaccgaacacgatctcgatgtt gtcgttaccgaacaaggtttggctgatctcagaggtctcagtccaaaggaaagagctcaagttataatcaacaactgtgcccacccagactaccggctcaattgc aagactactttgacagagctgttttctacgccaccaagaagaagatgttgcacgaaccacacatgttgaacgaagctttcgctatgcacttgaacttgcaagaaaatg gtaccatgaagttgaacaaaggtagacgggccaagttgtag | 73 |
| *ACC1* (*Candida viswanathii* ATCC20336) contained in pAA1910 | atgacagatctttccccaagtccaacagactcccttaattacacacagttgcactcatccttgccatcacatttcttaggtgggaactcggtgctcaccgctgagccttct gccgtgacagatttcgtcaaaacacaccaaggtcacactgttatcaccaaagtcttgattgccaacaacggtattggtgccgtcaaagaaataagatccgtcagaa aatgggcctacgaaacttttggtgacgaaagagctatacagtttgtcgccatggccactcccgaagatatggaggctaacgccgagtacattcgaatggccgacca gtttgtcgaggtcccaggtggtaccaataacaacaactacgcgaatgttgacttgattgtcgaaatcgctgaaagaaccgatgtccacgccgtttgggctggttgggg tcatgcctccgaaaaccctttgttgccagraaggttggcagcttcccctaagaagatcgtgtttattggtcctccagggtctgccatgagatctttgggtgacaagatttctt ccaccattgttgcacaacacgccaaagtgccatgtatcccatggtctggtactggtgtcgaagaggtccacgtcgacccagaaaccaagttggtgtctgttgacgac cacgtctacgccaaaggttgctgtacctcgccagaagacggtttggaaaaagccaaacgtatcggattcccagttatggttaaggcatccgaaggtggtggtggta aaggtatcagaaaagtcgaccacgaaaaggacttcatcagtttgtacaaccaggcggctaacgaaataccagggtcaccaattttcatcatgaagttggccggtg acgccagacacttggaagtgcaattgtttgccgatcagtacggtaccaacatttcgcttttcggtagagattgttctgtgcaaagaagacatcaaaagatcattgaaga agctccagtcacaattgccaacaaagacacttttgttgagatggagaaagctgccgtcagattgggtaagttggttggttacgtgtctgccggtaccgttgaatacctttt actcctacgccgaagacaagttctacttttttggaattgaacccaagattgcaagttgaacatccaactaccgaaatggtttccggtgtcaacttaccagccgctcagttg caaattgctatgggtctcccaatgcacagaatcagagacatcagattgttgtacggtgttgatccacactctgccactgagattgatttcgagttcaagtccccaaactc attgatcacgcaaagaaagccagctccaaagggtcactgtaccgcttgtcgtatcacttctgaagatccaggtgaagggttcaagccaagtggtggtactcttcacg agttgaacttccgttcttcgtccaatgtctggggttacttctcggtcgccaaccaatcttctatccactcctttgctgattcccagtttggtcacattttcgcctttggtgaaaatc gtcaagcctctagaaagcacatgattgttgccttgaaggaattgagtatcagaggtgactttagaaccactgttgaatacttgatcaagttgttggagactccagatttcg ccgacaacaccatcactaccggttggttggatgagttgatcaccaagaagttgactgccgaaagaccagatcctatcgttgctgttgtctgtggtgccgtcaccaaag cccacatccaagccgaagaagacaagaaggagtacattgagtctttggaaaagggtcaagttccaaacaagtccttgttgaaaactatcttcccagttgagtttatct acgaaggtgaaagatacaagtttactgccaccaagtcctccgaagacaagtacactttgttcctcaacggttctagatgtgtcattggtgctcgctcattgtctgatggtg gcttgttgtgtgctttggacggtaagtcccactctgtctactggaaggaagaagcagcggccactagattgtctgttgacggtaagacttgcttgttggaagttgaaaac | 74 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| | gacccaacccaattgagaactccgtctccaggtaagttggtcaagtacttggttgagagtggtgaacacgttgatgccggccaatcttatgccgaagttgaagtcatg<br>aagatgtgtatgcctttgattgcacaagaaaacggtactgttcaattgctcaaacaaccaggttccactcttaacgctggtgacatcttggcaatcttggcattggacgat<br>ccatctaaagttaaacacgccaagccatatgaaggcactttgccagagatgggtgatccaactgttaccggttccaaaccagctcacttgttccaacattacgacac<br>catcttgaagaacatcttggctggttacgataaccaagtcattttgaactccactttgaagaacatgatggagatcttgaagaacaaggagttgccttattctgaatgga<br>gattgcaaatctccgccttgcattcaagaatcccaccaaagttggatgaggctttgacgtccttgattgaaagaaccgaaagcagaggcgccgaattcccagctcgt<br>cagattttgaagctcgtcaacaagactcttggtgaaccaggcaacgaattgttgggcgatgttgttgctcctcttgtctccattgccaaccgctaccagaacggcttggtt<br>gaacacgagtacgactactttgcttcattggttaacgagtactgcaatgttgaacacttctttagtggtgaaaacgtgagagaagaagatgttatcttgagattgagaga<br>cgagaacaagtctgatttgaagaaggttatcagcatttgcttgtcccactcccgtgtcagtgctaagaacaacttgattttggccatcttggaagcttatgaaccattgttg<br>caatccaactcttcaactgccgttgccattagagattctttgaagaagatagtccagttggattctcgtgcttgtgccaaggttggtttgaaagctagagaacttttgattca<br>atgttctttgccatccatcaaggaaagatctgaccaattggaacacattttgagaagtgcagtcgttgagacttcttatggtgaagttttcgccaagcacagagaaccta<br>aattggaaatcatccaagaagttgtcgaatccaagcacgttgttttcgatgtcttgtcgcaatttttggtccaccaagactgctgggttgccattgctgctgccgaagtctat<br>gttagacgttcctacagagcttatgatttgggtaagatcgattaccacattcatgacagattgccaattgttgaatggaagttcaagttggctcaaatcgcaggttccaga<br>tacaacgccgtccaatctgccagtgttggtgacgactcgaccactatgaagcatgctgcatctgtttctgacttgtcgtttgttgttgattccaagagcgaatccacttcca<br>gaactggtgttttggttccagctagacatttggacgatgttgatgagattctttctgctgcattggagtacttccaaccatctgatgcactctctttccaagctaagggagaa<br>agaccagagttgttgaatgttttgaacattgtcatcaccgacattgacggttactctgacgaagatgaatgcttgaagagaattcatgaaatcttgaacgagtacgaag<br>acgatttggtctttgctggtgttcgtcgtgttacttttgttttcgcccaccaagttggttcttatccaaagtactacaccttcactggtccagtgtatgaagaaaacaaggttatc<br>agacacatcgaaccagctttggctttccaattggaattgggaagattagccaactttgacatcaagccaattttcaccaataacagaaacattcatgtttacgaagctat<br>tggtaagaatgctccttcggataagagattcttcactagaggtattattagaggtggtgtcctcaaagatgaaatcagtcttactgagtacttgattgctgaatcgaacag<br>attgatcagtgntatcttggataccttggaagttattgacacttccaactccgatttgaaccacattttcatcaacttctccaacgttttcaacgtccaaccagctgatgttga<br>agctgcttttgcttcattcttggaaagatttggtagaagattgtggagattgagagttactggtgctgaaatcagaattgtctgtaccgacccacagggcaactcattccc<br>attgcgtgccattatcaataacgtttcaggttatgttgtcaagtcggaattgtacttggaagtgaagaaccctaagggtgattgggtcttcaaatccattggccaccctgg<br>ctcaatgcacttgcaaccaatctcgactccatacccagtcaaggaatccttgcagccaaaacgttacagagctcacaacatgggaaccacttttgtttacgatttccca<br>gagttgttccgtcaagccaccatttcccaatggaagaagcacggcaagaaggctcctaaagatgtcttcacttctttggagttgatcaccgatgaaaacgatgctttgg<br>ttgccgttgaaagagatccaggtgccaacaagattggtatggttggtttcaaggtcactgccaagacccctgaatacccacgcggacgttcattcatcattgttgccaa<br>tgatatcacccacaagattggttcctttggtccagatgaagatgaatacttcaacaagtgtaccgacttggccagaaagttgggtgttccaagaatttacctttctgccaa<br>ctccggtgccagaattggtgttgctgaagagttgattccattgtaccaagttgcttggaacgaagaaggtaacccagataaaggtttcagatacttgtacttgaaccca<br>gacgccaaagaagctttggaaaaagacggcaagggtgacactattgttactgaacgtattgtcgaagatggtcaagaacgtcacgttatcaaggccattattggtgc<br>tgagaacggcttgggtgttgaatgtttgaaaggttccggtttgattgctggtgccacttcaagagcctacagagacatcttcaccattaccttggtcacttgtagatctgttg<br>gtattggtgcctatttggtcagattgggtcaaagagctatccaaattgaaggtcaaccaatcattttgactggtgcaccagctatcaacaagttgttgggtagagaagttt<br>actcgtcgaacttgcaattgggtggtacccagatcatgtacaacaatggtgtttcccacttaactgccagtgacgatttggctggtgttgagaagatcatggaatggttgt<br>cctacgttccagctaagcgtggtatgccagtaccaatcttggaaagtgaagatacctgggacagagacattgactactacccaccaaagcaagaagctttcgacat<br>cagatggatgatcgaaggtaagcaagttgaaggtgaagagtttgaatctggtttgtttgacaaaggttcattccaggaaactttatcaggatgggctaaaggtgttgtc<br>gttggtagagctcgtctcggtggtatcccaattggtgtcattggtgttgagaccagaactattgaaaacatgatcccagctgacccagccaacccaagttccactgaa<br>gccttgatccaagaagccggtcaagtctggtatccaaactctgcgttcaagaccgcacaagccattaacgacttcaacaacggtgaacaattgccattgatgatcttg<br>gccaactggagaggtttctctggtggtcagagagatatgtacaacgaggtcttgaagtacggttccttcattgttgacgctttagttgatttcaagcagccaatcttcactt | |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| | acatcccaccaaatggtgaattgagaggtggctcttgggtcgttgttgatccaaccatcaactccgacatgatggaaatgtatgccgacgttgactccagagctggtgt<br>tttggaaccagaaggtatggttggtatcaaatacagacgggacaagttgttggctaccatgcaaagattggatccaacttatgcccaattgaaggagaagttgaacg<br>actcgagcttgtcgccagaagaacatgcccaagtcagcaccaagattgtcaagcgtgaaaaggcattgttgccaatctatgcccaaatttctgtccagtttgccgactt<br>gcacgacagatccggacgtatgatggctaaaggtgtcattagaaaagaaatcaagtgggttgacgccagacgtttcttcttctggagattgagaagaagattgaac<br>gaagagtacgttttgaagttgattggtgaacaggtcaagaatgccaacaagttggaaaaggttgccaggttgaagagttggatgccaactgttgactacgacgatga<br>ccaagctgtcagtacttggattgaagagaaccacgccaaattgcaaaagagagttgaagaattgagacaggagaagaacaagtccgacattgtcaaattgttgc<br>aagaagacccatcaaacgctgcctctgttatgagggatttcgttgatagattgtccgatgaagaaaaggaaaagttccttaaatcattgaactag | |
| *ACC1* (*Candida viswanathii* ATCC20336) contained in pAA245 (includes 5' intron sequence) | atgagatgccaagtatctcaaccatcacgatttactaacttgcttgtacatagactcccacgaacactacttaattatccagttgtaaatacccta tttattcccttaatttttcattcaagaacctactaaagaaaatgacagatctttccccaagtccaacagactcccttaattacacacagttgcactcatccttgccatcacatt<br>tcttaggtgggaactcggtgctcaccgctgagccttctgccgtgacagatttcgtcaaaacacaccaaggtcacactgttatcaccaaagtcttgattgccaacaacg<br>gtattggtgccgtcaaagaaataagatccgtcagaaaatgggcctacgaaactttggtgacgaaagagctatacagtttgtcgccatggccactcccgaagatatg<br>gaggctaacgccgagtacattcgaatggccgaccagtttgtcgaggtcccaggtggtaccaataacaacaactacgcgaatgttgacttgattgtcgaaatcgctga<br>aagaaccgatgtccacgccgtttgggctggttggggtcatgcctccgaaaacccttgttgccagaaaggttggcagcttccctaagaagatcgtgtttattggtcctc<br>cagggtctgccatgagatctttgggtgacaagatttcttccaccattgttgcacaacacgccaaagtgccatgtatcccatggtctggtactggtgtcgaagaggtcca<br>cgtcgacccagaaaccaagttggtgtctgttgacgaccacgtctacgccaaaggttgctgtacctcgccagaagacggtttggaaaaagccaaacgtatcggattc<br>ccagttatggttaaggcatccgaaggtggtggtggtaaaggtatcagaaaagtcgaccacgaaaaggacttcatcagtttgtacaaccaggcggctaacgaaata<br>ccagggtcaccaattttcatcatgaagttggccggtgacgccagacacttggaagtgcaattgtttgccgatcagtacggtaccaacatttcgcttttcggtagagattgt<br>tctgtgcaaagaagacatcaaaagatcattgaagaagctccagtcacaattgccaacaaagacacttttgttgagatggagaaagctgccgtcagattgggtaagtt<br>ggttggttacgtgtctgccggtaccgttgaataccttactcctacgccgaagacaagttctacttttggaattgaacccaagattgcaagttgaacatccaactaccga<br>aatggtttccggtgtcaacttaccagccgctcagttgcaaattgctatgggtctcccaatgcacagaatcagagacatcagattgttgtacggtgttgatccacactctg<br>ccactgagattgatttcgagttcaagtccccaaactcattgatcacgcaaagaaagccagctccaaagggtcactgtaccgcttgtcgtatcacttctgaagatccag<br>gtgaagggttcaagccaagtggtggtactcttcacgagttgaacttccgttcttcgtccaatgtctggggttacttctcggtcgccaaccaatcttctatccactcctttgctg<br>attcccagtttggtcacattttcgcctttggtgaaaatcgtcaagcctctagaaagcacatgattgttgccttgaaggaattgagtatcagaggtgactttagaaccactgt<br>tgaatacttgatcaagttgttggagactccagatttcgccgacaacaccatcactaccggttggttggatgagttgatcaccaagaagttgactgccgaaagaccaga<br>tcctatcgttgctgttgtctgtggtgccgtcaccaaagcccacatccaagccgaagaagacaagaaggagtacattgagtctttggaaaagggtcaagttccaaaca<br>agtccttgttgaaaactatcttcccagttgagtttatctacgaaggtgaaagatacaagtttactgccaccaagtcctccgaagacaagtacacttttgttcctcaacggtt<br>ctagatgtgtcattggtgctcgctcattgtctgatggtggcttgttgtgtgctttggacggtaagtcccactctgtctactggaaggaagaagcagcggccactagattgtc<br>tgttgacggtaagacttgcttgttggaagttgaaaacgacccaacccaattgagaactccgtctccaggtaagttggtcaagtacttggttgagagtggtgaacacgtt<br>gatgccggccaatcttatgccgaagttgaagtcatgaagatgtgtatgcctttgattgcacaagaaaacggtactgttcaattgctcaaacaaccaggttccactcttaa<br>cgctggtgacatcttggcaatcttggcattggacgatccatctaaagttaaacacgccaagccatatgaaggcactttgccagagatgggtgatccaactgttaccgg<br>ttccaaaccagctcacttgttccaacattacgacaccatcttgaagaacatcttggctggttacgataaccaagtcattttgaactccacttttgaagaacatgatggaga<br>tcttgaagaacaaggagttgccttattctgaatggagattgcaaatctccgccttgcattcaagaatcccaccaaagttggatgaggctttgacgtccttgattgaaaga<br>accgaaagcagaggcgccgaattcccagctcgtcagattttgaagctcgtcaacaagactcttggtgaaccaggcaacgaattgttgggcgatgttgttgctcctctt<br>gtctccattgccaaccgctaccagaacggcttggttgaacacgagtacgactactttgcttcattggttaacgagtactgcaatgttgaacacttctttagtggtgaaaac | 75 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| | gtgagagaagaagatgttatcttgagattgagagacgagaacaagtctgatttgaagaaggttatcagcatttgcttgtcccactcccgtgtcagtgctaagaacaac<br>ttgattttggccatcttggaagcttatgaaccattgttgcaatccaactcttcaactgccgttgccattagagattctttgaagaagatagtccagttggattctcgtgcttgtg<br>ccaaggttggtttgaaagctagagaacttttgattcaatgttctttgccatccatcaaggaaagatctgaccaattggaacacattttgagaagtgcagtcgttgagactt<br>cttatggtgaagttttcgccaagcacagagaacctaaattggaaatcatccaagaagttgtcgaatccaagcacgttgttttcgatgtcttgtcgcaatttttggtccacca<br>agactgctgggttgccattgctgctgccgaagtctatgttagacgttcctacagagcttatgatttgggtaagatcgattaccacattcatgacagattgccaattgttgaa<br>tggaagttcaagttggctcaaatcgcaggttccagatacaacgccgtccaatctgccagtgttggtgacgactcgaccactatgaagcatgctgcatctgtttctgactt<br>gtcgtttgttgttgattccaagagcgaatccacttccagaactggtgttttggttccagctagacatttggacgatgttgatgagattctttctgctgcattggagtacttccaa<br>ccatctgatgcactctctttccaagctaagggagaaagaccagagttgttgaatgttttgaacattgtcatcaccgacattgacggttactctgacgaagatgaatgctt<br>gaagagaattcatgaaatcttgaacgagtacgaagacgatttggtctttgctggtgttcgtcgtgttacttttgttttcgcccaccaagttggttcttatccaaagtactacac<br>cttcactggtccagtgtatgaagaaaacaaggttatcagacacatcgaaccagctttggctttccaattggaattgggaagattagccaactttgacatcaagccaatt<br>ttcaccaataacagaaacattcatgtttacgaagctattggtaagaatgctccttcggataagagattcttcactagaggtattattagaggtggtgtcctcaaagatga<br>aatcagtcttactgagtacttgattgctgaatcgaacagattgatcagtgatatcttggatacctggaagttattgacacttccaactccgatttgaaccacattttcatca<br>acttctccaacgttttcaacgtccaaccagctgatgttgaagctgcttttgcttcattcttggaaagatttggtagaagattgtggagattgagagttactggtgctgaaatc<br>agaattgtctgtaccgacccacagggcaactcattcccattgcgtgccattatcaataacgtttcaggttatgttgtcaagtcggaattgtacttggaagtgaagaaccct<br>aagggtgattgggtcttcaaatccattggccaccctggctcaatgcacttgcaaccaatctcgactccatacccagtcaaggaatccttgcagccaaaacgttacag<br>agctcacaacatgggaaccacttttgtttacgatttcccagagttgttccgtcaagccaccatttcccaatggaagaagcacggcaagaaggctcctaaagatgtcttc<br>acttctttggagttgatcaccgatgaaaacgatgctttggttgccgttgaaagagatccaggtgccaacaagattggtatggttggtttcaaggtcactgccaagaccc<br>ctgaatacccacgcggacgttcattcatcattgttgccaatgatatcacccacaagattggttcctttggtccagatgaagatgaatacttcaacaagtgtaccgacttg<br>gccagaaagttgggtgttccaagaatttacctttctgccaactccggtgccagaattggtgttgctgaagagttgattccattgtaccaagttgcttggaacgaagaagg<br>taacccagataaaggtttcagatacttgtacttgaacccagacgccaaagaagctttggaaaaagacggcaagggtgacactattgttactgaacgtattgtcgaag<br>atggtcaagaacgtcacgttatcaaggccattattggtgctgagaacggcttgggtgttgaatgtttgaaaggttccggtttgattgctggtgccacttcaagagcctaca<br>gagacatcttcaccattaccttggtcacttgtagatctgttggtattggtgcctatttggtcagattgggtcaaagagctatccaaattgaaggtcaaccaatcattttgact<br>ggtgcaccagctatcaacaagttgttgggtagagaagtttactcgtcgaacttgcaattgggtggtacccagatcatgtacaacaatggtgtttcccacttaactgccag<br>tgacgatttggctggtgttgagaagatcatggaatggttgtcctacgttccagctaagcgtggtatgccagtaccaatcttggaaagtgaagatacctgggacagaga<br>cattgactactacccaccaaagcaagaagctttcgacatcagatggatgatcgaaggtaagcaagttgaaggtgaagagtttgaatctggtttgtttgacaaaggttc<br>attccaggaaactttatcaggatgggctaaaggtgttgtcgttggtagagctcgtctcggtggtatcccaattggtgtcattggtgttgagaccagaactattgaaaacat<br>gatcccagctgacccagccaacccaagttccactgaagccttgatccaagaagccggtcaagtctggtatccaaactctgcgttcaagaccgcacaagccattaa<br>cgacttcaacaacggtgaacaattgccattgatgatcttggccaactggagaggtttctctggtggtcagagagatatgtacaacgaggtcttgaagtacggttccttc<br>attgttgacgctttagttgatttcaagcagccaatcttcacttacatcccaccaaatggtgaattgagaggtggctcttgggtcgttgttgatccaaccatcaactccgaca<br>tgatggaaatgtatgccgacgttgactccagagctggtgttttggaaccagaaggtatggttggtatcaaatacagacgggacaagttgttggctaccatgcaaagat<br>tggatccaacttatgcccaattgaaggagaagttgaacgactcgagcttgtcgccagaagaacatgcccaagtcagcaccaagattgtcaagcgtgaaaaggcat<br>tgttgccaatctatgcccaaatttctgtccagtttgccgacttgcacgacagatccggacgtatgatggctaaaggtgtcattagaaaagaaatcaagtgggttgacgc<br>cagacgtttcttcttctggagattgagaagaagattgaacgaagagtacgttttgaagttgattggtgaacaggtcaagaatgccaacaagttggaaaaggttgccag<br>gttgaagagttggatgccaactgttgactacgacgatgaccaagctgtcagtacttggattgaagagaaccacgccaaattgcaaaagagagttgaagaattgag<br>acaggagaagaacaagtccgacattgtcaaattgttgcaagaagacccatcaaacgctgcctctgttatgagggatttcgttgatagattgtccgatgaagaaaagg | |



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**Table 19**

| Gene (Organism) | Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted) | SEQ ID NO |
|---|---|---|
| | aaaagttccttaaatcattgaactag | |
| *ACS1* (*Candida viswanathii* ATCC20336) | atgccagaatcaactcaacaatctcacatttctttggaccatgaaaagatgcagcaacctccaactggcttcaccgaacgctccaccgcaaagccaaacttggccg<br>actttgacacctacaagaagttgtacaagcagtcggtagaaaacccaaacgagtttttcacagaacaagcaaacaaaaacttggactggttcaaacctttcgacttg<br>gccagattccccgtcgatccaaaggacgacttcaagaatggcgacttgcctgcctggttcatcaacggtcagttgaacgcttcttataacgcggttgatagatgggcc<br>atcaagaacccagacaagccagctatcatttatgaaggtgacgaaccagacctgggcagaatcatcacttatggcgaattgttgaaagaagtcagtaagttggcc<br>cagactttgaccaagttgggtgtcaaaaaaggtgactctgttgctgtctacttgccaatgatccctgaagctatcgtcaccttgttggccattgtcagaatcggtgccgtg<br>cactcggttgtctttgctggtttctcctccgcttccttgagagacagaatcttggatgctgactccagaatcgtcatcactgccgacgagtccaagagaggtggtaagac<br>cattgaaaccaagaagattgtcgatgacgccttgaaggaatgtcctaaggttagaaacgttattgttttcaagagaaccggcaactcccacgttcctttctctgctggta<br>gagacttgtggtggcacgaagaaatggccaaatacggtccatacttcccaccagtgccagtcaactccgaagatccattgttcttgttgtacacttctggttctactggt<br>aagccaaagggtgtccaacacaacaccgccggttacttgttgggcgctatcttgaccaccaagtacacttttgatgtccacgaagaagacgtcttgttcaccgctggt<br>gacgtcggttggatcacgggtcacacctactgtgtctacggcccattgttgtgtggtgccaccaccgttgtctttgaaggtaccccagcttatccaaactactccagata<br>ctgggagattgtcgaccaatacaaggtcaaccaattctacgttgccccaaccgctttgagattgttgaagagagccggtaccaagtacgttgaaaaatacgacttgtc<br>ctccttgagagtcttgggctctgtcggtgaaccaattgcggccgaggtctggcactggtacaatgacaacattggtagaggcaaggcccacattgtcgatacctactg<br>gcaaaccgaatctggttcccacttgttgaccccattggccggtgtcactccaaccaagccaggttccgcttccttgccattctttggtgttgttccaaagatcttggaccca<br>accaccggtgaagaattagaaggtaacgatgttgaaggtgtccttgccattaaatccgcttggccatccattactagaggtatttacaacgattacaatagatttatcga<br>aacctacttggccccataccacgaccactacttttccggtgatggtgctgctagagataacgacggcttctactggatcttgggtagagtcgatgatgtcgtcaatgtttc<br>cggtcacagattgtcgactgctgaaattgaagctgccttgattgagcacccattggttgctgaatccgctgttgttggttatgctgacgacttgactggtcaagccgtcgct<br>gcctatgtgtccttgaagaaagataaagttattggtgacgacatggaggcagtcaagaaggagttgatcttgactgtcagaaaagaaattggtccatttgctgcacca<br>aagttgatcttgttggttgacgacttgccaaagaccagatccggtaagatcatgagaagaatcttgagaaaggtcttggctggtgaagaagatcaattgggtgatatct<br>ccaccttgtccaacccaggcgttgtccaacaaatcattgaaattgtccatgcctgcaagaaataa | 76 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| *ACS2* (*Candida viswanathii* ATCC20336) | atgactacagactcaaacacccacaaggttgttcacgaagccaatggcgtcaagttgagacaaaccccaaaggaattcttccaaaggcaaccaaacaaggga<br>cacatccaagacacggaagagtacaagaaattgtatgagcaatccataaaagacccccaaggtttctttggcccattggctaaagaattgttgtcctgggacagcg<br>atttccacaccgtcaagtcaggtaccttaagaacggtgacgctgcctggttcttgggcggtaagttgaacgcttcttacaactgtgttgccagacacgctttggccaac<br>ccaaacaagccagccatcatctacgaagcagatgaagaaagggattcttacatcttgacctacggtgatttgttgagagaagtttccaaagtcgctggtgtcttgcact<br>cctggggcatcagaaagggtgacaccgttgctgtttacttaccaatgaatgcccaagccctcattgccatgttggccattgctagattgggtgctgcccactccgtcatc<br>tttgctgggttctcctccggttctatcaaagacagagtcaacgacgccagctgtaaagcgcttattacctgtgatgaaggtagaagaggtggcagaaccaccaacat<br>caagaagttgtgcgacgaagctttgaagcaatgcccaacggtcaagcacgtcttggtccacagaagaaccggcaacccagaaatcaaattgaccgaaggcag<br>agattactactgggacgaagaaaccgccaagttttccggatacttcccacccgtgccagttgactctgaagacccattgttcttgttgtacacttctggttccactggtact<br>ccaaagggtgttgtccacaccaccgccggttacttgttgggtgctgctctttccaccaaatacatctttgacgtccacccagaagatgtcttcttcactgctggtgatgtcg<br>gttggatcactggtcacacctacgctttatatggtccattgttgttgggtgttccttctgttgttttcgaaggtactccagcttacccagactacggaagattctggcaaattgtt<br>gaaaaacacaaggctacccacttctacgttgctccaactgctttgagattgttgcgtaaagctggtgaacaagaagttggcaagtacgacttgagttcattgagaacc<br>ttgggttctgttggtgaaccaatctccccagatatttgggaatggtacaatgaatttgtcggtaaggaccaatgtcatatctcggatacctactggcaaaccgaatccgg<br>ttctcacttgattgctccattggctggtgccattgccaacaagccaggttctgcttcttacccattctttggtattgaagctgctttgatcgacccagtcactggtgttgaaatc<br>aagggcaacgatgttgaaggtgtgttggttgtcaaggaccactggccatccatggcaagaactgttttcaacaaccacgtcaagtacatggatacctacatgaaccc<br>atacccaggctactatttcactggtgatggtgctgccagagataacgacggttactactggatcagaggtagagttgatgatgtcgtcaatgtttctggccacagattgt<br>ccaccgccgaaattgaatctgcattgattgaagacccaagagttggtgaatccgctgttgttggtatcaacgatgaccttactggtcaagctgttattgcttacgttgcctt<br>gaaatccggcgacgttgctgatgaagacgctttgagaaaggaattgatcttgttggttagaaaagaaattggtccatttgcggctccaaaatctgtcatcttggttcaag<br>acttgcctaagaccagatctggtaagatcatgagaagaatcttgagaaaagtcagttctaacgaagccgatcaattgggtgacatcaccactttgcaaaacccagg<br>ttctgttgaaggtatgatttctgccttcggtgctcaattcggtagaaagtaa | 77 |

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**Table 19**

| Gene (Organism) | Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted) | SEQ ID NO |
|---|---|---|
| *TES1-1* (*Candida viswanathii* ATCC20336) | atgcttacactaacttctggtcccaacccctttgcctgatttcgaagaagccttgagagtcatcaaagtggacgatacacactatgttggtgcccattcgttgagattgcct gtgaaaggaggacgaggcgtttatggtggccatatgattgcccagtctttgctagtgggtattgaatcaacaagagatgacaagactaataaagtgttcattcctgact cgtaccacctgtactttattggagccggaaatgccaaaatccccatgaactacacggtagagaaactatacgacgatgaaaacgtcagcaagcgtttcatcattgc ggaacaaaagggaagacatcgtttgacttgtttggttacattgaggagacccggtaccaagcctttccacgacagcgacaacttggacatcagcatccccgtgccc aagatccagttaaaacaccctgatcccgacaagttgcaccaagttcagcacacggactttattaggaatgcattcggtaaagagcttatggattacagagagtgccc tgaagagaacgaactttatgccgcagaaagatggttgactgtgttcaccggtattcgaaaccagcctaaaccgggcgccagtttggaaacggtcgtcgaagaattg ccagacgcccagggccagatgcacacagtggagaagagtattttaagacccaaagattcccagtcgttcaaggaccctatctacaactttgtgggtttagccgactt gtctgattcggcattcctcaccaccatggcgagaatcttgcacatcccatgggcgccttccatcgaaatcgacgacacatacgatccagcaagagatgcaacgtat atcatgcgttcaactttgaatgcagcccatatcttccattacaatgcgatgtcattggatcaccatatttatttccacaatgaagattacacttccgacgacggttcaggttt cgacatttgcaaagattggttggctttcacttatcagatgaaaagactctctaataaccgtaccctcgtccgtgggttcttgttcaacgaaaaacacaaatgtatcgcca cagtggtgcaagaagggttgactattatgcaaaatggagttggtcgaactgctgataaatcacgtctatag | 78 |
| *TES2-1* (*Candida viswanathii* ATCC20336) | Atgattgagaacatttcaggaaacggaaactacccacagaatcacgaggttgacttggagaaggaattcggagttgaaaaaataggtatcaacttgtaccgtggc aagagcccaataccgaagccagacagacgttccagaggtgcttacggtggttacttggccggtcaagctttgttagttgccatgaagtctaccccgccagagtaca gaccacacctgttccattcctactttatcaaagctgtcaatgacaaggagacattggagtggagagttgaagaaacatccaacggcagaaactacgccaacagat ccttgcaagccttccaagccggtaacttggtctacacagccaacgtctccttgaccaagaaaaactctgccaagaaagctgaggaagcaacgggtgtcaagccct ttgagttccagggaaaaccacacgaatggtttgaaaaacacaagagagatgaccttcctcttgctacaccttcttcatccctttgatctaccacaaattcttcccagag gttgtctcgttggaagcttccaaagaagaagaatcaaagccagcagccgacagagaattgagctggtacttcaaatggggtatcaacaacgaagagggccacc accagccattagtcaacttgaactccgattaccaatatgttggaatggctgcgttgaccgatgctgtctacttgaacagattattgagaattttgagagttgaagacgctg accacacccagcttgtccactacttttccgtctccttggaccatactatgtacttccatgacgacgactttgacgtaaccaaatggatgggattcactttcaaggttacca gattcagtcacaaccgtgcattgtgccagggtgaagtttataacgacaaggggtacacgtgtgtacaattgtccaggaagggctcatgatgcttaacggtcttgaac aaggtgctaaattataa | 79 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| *TES2-2* (*Candida viswanathii* ATCC20336) | Atgattgagagcatttcagaaaacggaaactacccacagaaccacgaagttgacttggagaaggaattcggagttgaaaaaataggtatcaacttgtaccgtggc aagagcccaataccgaagccagacagacgttccagaggtgcttacggtggttacttggctggtcaagctttgttagttgccatgaaatctaccccgccagattacag accacatctgttccattcctactttatcaaagctgtcaatgacaaggtgacattggagtggagagttgaagaaacatccaacggccgaaactacgccaacagagcc ttgcaagccttccaagccggtgacttggtctacacagctaacgtctccttgaccaagaaaaactccgccaagaaaactgaggaagcaacgggtgtcaagccctttg agttccaggggaagccacacgaatggtttgataagcacaagatagatgaccttcctcttgctacgccttcttcatccctttgatctaccacaaattcttcccagaggttgt gtcgttggaagcttccaaagaagaggaatcaaagccagcagctgacagagaattgagctggtatttcaaatggggtatcaacaacgaagagggccaccaccag ccattagtcaacttgaactctgattaccaatatgttggtatggctgcgttgaccgatgccgtctacttgaacagattattgagaatcttgagagttcaagacgctgaccac acccaacttgtccactacttttccgtctccttggaccacaccatgtacttccatgacgacgactttgatgttaccaaatggatgggattcactttcaaggttaccagattca gtcacaaccgtgcattgtgccaaggtgaagtttacaacgacaaggggtacacgtgtgtacaattgttcaagaaggactcatgatgcttaacggtcttgaagaaggt gctaaattataa | 80 |
| *TES3-1* (*Candida viswanathii* ATCC20336) | Atgagtcacccaacgccagaagaagtctacggagtcaccaaagttgccgaaaacaaatacgtggggaacagacccttgaacaagcccacgccgaaaacac gcggagtgtatggcggaaacttctgtgcccaggccatcttggtagccatcgagtcagccccggaagggttcacaccacactccatccacagcaacttcattcgtggc ggcgaccccgaagtccctgttgagtgggaggtcgaggtcatctcgaacggcaagtctttcgccaaccgcatagtcaagggcgtgcagcacggcatcgtggtctac gtcgccacggtctcgttgaccaacaagaactcaaccacccgcaacgagagcttcacgtacgacacgcccccggacgagaccgtcaagacgtacggcaacgcc gagctagacacatactaccagggctggctctacttggaggtcaagaactaccccaagcagttgcactcgcaccagatctcgtactccgtcaagtgggggccggag aacgacgcgtggaaggacgccagccagacgtaccagtttgttgggctcgctgcgatctccgacgtgcttgacctcggccagatcttgcgcaacttggacatccactt gagcacgcccaagttcaacgtgtcgctcgaccacagtgtgtacttccacggcgcggactttgacgtgaccaggtggtcgacgaccacgatccgcatgacgaagct cgcgcacggccgcgcgttgatagagggcgagatgtactcggaccagggcaggcacatcgccagcatagtgcaagagaggttgtacatagctgagtcgccgaa gctctga | 81 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| *TES4-1* (*Candida viswanathii* ATCC20336) | atgacgctgattgcacccgccacagacatacccgccgtggacatctccaccatatacgacgtcaaacagatcgacgccaaccgctaccgcggcgttcggccgct ccagaaacaggcacgcgagttccgcggagtctttggaggcaacttggttgcccagtccgtggtggtggccttgcggtcggtgcccgcaggattccaccccaactcc gtccacgcctactacgtgcgtgccgtgaccgacgagacgccaattgagtgggaggtcgaagagacaaccacgggccgcaccttcgccaaccggctgatacgcg gattgcagaacaagaaggtggtgttcacggcgagcatctcgttgaccaggaagaactccaatgcggaagtgatccaaaagaccgggcacccgtcgttgcagttc cagcgggacaccgagccatactacgagcagatgcgagcccgccctggcgagtgcaaggtgctctacattaacgccaacacgcacctcacggtcagacagttcc ctgaagtcagctcccgcgacgtgttctcgttcttggtgcggtttggcaacgacgggcgggagaggatcgtggggatgactcccgagtaccagtacgccgcgttggct gcgttgtcggactgggtgaggctccgtttctactttgacaatatgggggtcgacgtgcagacgtcgttcgacgtgtcgttggaccacagcatctacttccacgacgacg ggttcgacgctaccgagtacttggttttctccgtcaaggtgtcgcggatcagccacagccgggtgctttacgacgggcagatcttcaacgaccgtggagcccacgttg ctagcatcagacaggagcgcttatatgtcgtcagcaacaagccaaagttttag | 82 |
| *TES5-1* (*Candida viswanathii* ATCC20336) | atgcccacattcaactacaaggacggcgaaacaatagacgtgcagaaagagtttggcgtggtcgagacagcgcccaacaagtacgtcggcgtgaagccactc gtcaagccgatgcctcatgtaaagggagtctttggcggtaaccttgcaggacaagcattgctagtcgccatgaagtcggttggcccagactttagcccacactccttg cattcgtactttatacgcgccggcagcgaccagacacccgtcgagtggactgtgcaggcgatatctgatggtaactcgttttgtaacaggtttatcaagggagtccaa aacggacaggtcatatacattgcgaatgtgtcgttgactaaacgcaactctgctgctgatgcgatgaagaagtacgaggagtaccatgcgcagatccgccagaag ggcaaggacggcgatgctgatgaagaggacgaggatgacgatgacgaggatgataatgccccagctaagccatttgggttccagactccgagccataagtgga tcaaggaccgcgatctcgacaagttgccagtttccgacatggagtcgaatcttttgttgtactacaagttgcctcccgagttcgtgagcttgaagtcttcaacagaggaa gagctgttgccagtctcagagcgcaggatgggtgctttggcgaagtggggtattgagaacgagcaagggttcaaccagcctttgactaatctcgacaagtcgttcca gtatgttggtttggccaatatcactgatgggttgtacttgggcacattgaataggatattgcgcattgatgacttgacacttgatgagcgggcgactaactactttagcgta tcattggaccacgtcatctatttccacgatgatgattttgatgtgaccaagtggatgggctttacgttcaggtgttcgagatattcacataatcgggttatttttgaaggtgag atctacagtgacaagggagtccaggttgcatctattattcaagagggcttagttagatttaaagacggctacttgaaaaacgccaagttgtga | 83 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| *TES6-1* (*Candida viswanathii* ATCC20336) | Atgtcaaccaccggaatctacaacgccaaacccgcggctgccatattggaaacaaaaaccaaggtcaagttagtttatactgaaggaccgaaattggtctacgaaggattgcaccccgttgagaccataaaagaaactgttagaggtacttatggtggtgattttattgctcaaggggtgaacgcagcttgggaatcaattggcaacaggatggattttcaacctcattcattgcacgcatactatataaaaagcggttcccaggagtcagtattacgctgggaagtcttcaaggtgagtgatagcagaagttttgccaaccggatgttaactgcataccagacgcacaccaaccaattggtgttcacgatgcagatatcgttcaccaaagacaacaacgaggagatcaagcgtgcggaatacgaaaagttgttggccagcggtggcaaaatcagaagcatcccatttgtcatcaaaaaggctccgaatgaaaagtatttcaagttacgagacagaatcaatgagcttcattattctgagtataccaatgggatcattgccatggcttcccccttgacttccatgagtatgccactgagatgaactatgacaccttgggaaacaaagagtacgggacgtttatgaaaatcttggataactattccttaggcaaggattattctaaacaatccttcttggggttgtcattcttatccgatggtgtttgggtattttgctttactagagctttggggttgccacttggtagctacgaaaaggagttctttcgtgtcagtttggaccacacggtatatttccacgacgctaatttcgactccagtgagtggctcttcttagattttaggtttgtgaacatgaagaacaacagattcttgactgtgttgaactactatactttgcaaggcaaacttattgcgactgtgctccaagaagtctacacttctttacatcagggtatcattgacaagtcgcaagaaatagctgtcaaaagcggaaataagaagcaggtcacaactccaaagttgtag | 84 |
| *TES7-1* (*Candida viswanathii* ATCC20336) | atggacaaactacaagctgaagtatacgaggccgaacctcccgtggccaaattggaagccaaaacagcagccaaattgatatcttctgatggggcaaaattgacctacgaaggagtgtaccctgtggagctagtcagaaaaggtctcaggggaacctacgggggagatttcatagcacagggaatcaatgttgcctgggagtcaattggcaataagactgactttcaaccccattcattacatgcatattttgtcaaggcaggatccgacctgtctgtcctacgttgggaagtgctcaaggtgagtgatagcagaaatttgccaaccgtctaatgttagcgtaccagactcacaccaacgaattggtgttcacaatgcagatatcgttcactaaggataacaacgaggagatcaagcgtgccgagtacaagcaattattgcagagtggcggtaaaataagaagtatcccgtttgcaatcaagaaaccacccaatgaaaagtactttaaattgaaagacaaggtggacgatcttccatattttgagcacaccaatgggaatatggcgactgcgattccaccagacttccttgaatacgccacagagatgaatcacgacacggtggggaacaaagagtttggtattttcatgaaagtattggatgattactccttgggcaaaaactatgagagacagtcgttcttggggttggccttcttgtctgatgccgtgtggttatcgagtttcacccc agccttagggttgccattgggcactttagaaaggaaattctttcgtgttagcttagaccacaccatgtatttccatgacgccaactttgattctagtgagtggatctttgtgg atttcaggtttgtgaatttgaacaacaacaggctcttgggtgttgtgaacttttacacgttacagggtaagcttgttgctactgtgattcaagaagcgtacatgtttttgcacc aggcgatcatagacaaatcacaagagatagctgaaaaaagcgggcataagaagcaggtgatcaccccgaaattatag | 85 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| *TES7-2* (*Candida viswanathii* ATCC20336) | atggacaaattacaagctgaagtatacgacgccgaacctcctgtggccaaattggaagccaaaacagcggccaaattgatatctgctgatggggcgaaattgatc tacgaaggagtgtaccctgtggagctagtcagaaaaggtcttaggggaacctacgggggagatttcatagcacaaggaatcaatgttgcctgggagtcgattggc aataagactgactttcaacccattcattacatgcatattttgtcaaggcagggtccgaccagtctgttttacgttgggaagtgctcaaggtgagtgatagcagaagcttt gccaaccgtctaatgttagcatatcagactcacaccaaccaattggtcttcacgatgcagatatcgttcactaaggataacaacgaagaaattaaacgtgctgagta caagcagctattggagagtggcggtaaaataagaagtatcccgtttgcaatcaagaaaccacccaatgagaagtacttcaaattgaaagacaaagtggacgacc ttccatattttgaacataccaatgggaatatggcaactgcaataccacctgatttccttgaatacgccacagagatgaatcacgacacggtggggaacaaagagttt ggtattttcatgaaagtattagatgattactccttggataaaaactatgagagacagtcgttcttggggttggccttcttgtctgacgccgtgtggttgtcgagtttcacgcca gccttagggttgccattgggcacttttgaaaggaaattctttcgtgttagtttagaccacaccatgtatttccatgacgccaactttgattctagtgagtggatctttgtggattt caggtttgtgaatttgaacaacaacaggctcttgggtgtggtgaacttttacactttacagggtaagcttgttgctactgtgattcaagaagcgtacatgtttttgcaccagg ggatcatagaccaatcacaagagatagctgaaaagagcgggcataagaagcaggtgatcaccccgaaattatag | 86 |
| *TES8-1* (*Candida viswanathii* ATCC20336) | Atggagagattacaggccgaagtctacgacgctaaaccaacagttgctaaattggaaaccaagactaccgtcaaattagttgctcaggacggaaagagattggtt tatgaagctatttatccagttgaacatatcaaagaagggattccaggagcttatggaggtgataccttggcccaagggatgaatgctgcgtgggactcccttggcgac aagaaagatttccagcctcattcagttcattcgtactttgttaagcctgcaacaaacaaatcaattttgagatgggaagtgatcaaggtcagtgatggcagaagttttgct aatcgtttcgtgtctggctatcaaacccacaacaatgcgttggtcttcactatgcagatctcctttaccaagtacaacgatgaagtggtcaagattgctgaatacaacaa gttgttggaaagtggtggcaaaatcagaagtattccatttgtcatcaagaaagctccgaatgagaagtacttcaaatttaaggacaatcttggagatctcagatacatt gagcacaccaatggcaacattgccacagccatgctggaagatttgtttgaatacgccactgagatgaaccatgatactctcggaaatcaagagtttggtattttcatg aaggtcttggataattactcgttaggtagtgactacactaaacaatcttacttggggttggcattcttatcggatgctatttggatgtctgtttgttcaagagccttggggttgc catttggtagctaccatagacaatttttggagttagcatggaccactcgatgtatttccacgacgctaatttcgactccaccgagtggatctttttagactttaggtttgtcaa tttgaaaaatgacaggctcttgggcgttgctaatttttacactttagagggtaagcttatttccactttgattcaagaagcttacctcttttacacccaggaatcattgccaa gtcccaagagatagctgtcaagagtggaaacaagaggcaagttaaaatgccaaagttgtag | 87 |



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**Table 19**

| Gene (Organism) | Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted) | SEQ ID NO |
|---|---|---|
| Engineered DNA *TES3-1$^{\Delta pts}$* | atgagtcacccaacgccagaagaagtctacggagtcaccaaagttgccgaaaacaaatacgtggggaacagacccttgaacaagcccacgccgaaaacacg cggagtgtatggcggaaacttctgtgcccaggccatcttggtagccatcgagtcagccccggaagggttcacaccacactccatccacagcaacttcattcgtggcg gcgaccccgaagtccctgttgagtgggaggtcgaggtcatctcgaacggcaagtctttcgccaaccgcatagtcaagggcgtgcagcacggcatcgtggtctacgt cgccacggtctcgttgaccaacaagaactcaaccacccgcaacgagagcttcacgtacgacacgccccggacgagaccgtcaagacgtacggcaacgccg agctagacacatactaccagggctggctctacttggaggtcaagaactaccccaagcagttgcactcgcaccagatctcgtactccgtcaagtgggggccggaga acgacgcgtggaaggacgccagccagacgtaccagtttgttgggctcgctgcgatctccgacgtgcttgacctcggccagatcttgcgcaacttggacatccacttg agcacgcccaagttcaacgtgtcgctcgaccacagtgtgtacttccacggcgcggactttgacgtgaccaggtggtcgacgaccacgatccgcatgacgaagctc gcgcacggccgcgcgttgatagagggcgagatgtactcggaccagggcaggcacatcgccagcatagtgcaagagaggttgtacatagctgagtcgtaa | 88 |
| *PEX11* (*Candida viswanathii* ATCC20336) | Atggtcgccgattctttagtctaccacccaaccgtctccaaattagtcaagttcttggacacaaccccaaagagggaaaaggtcttcagattattgtcctacttgtccag attcttgggctactacgcctacagaaagggctactccaaggaaaccatcgccctttttcgccaacttgaaaggaaacttcacattcatcagaaaggccatgagattctt gaagccaataaatcacttgcaattggcctccaaggcatacgacaacaagttgttggacccagtcttgcagatcaccaccatcatcagaaacttggcctacgccggc tacttgaccatcgacggtgtcatattcttcaagttgttgggtctcattgacgccaagaagttccctaacttggctacatacgcctccagattctggttgatcgggttgattgc cggtttgatcaactccttgagaatcatctactccttgaaggactacgagcaccaggagggcgacaaggagaaggagaccgacgctaaggctatccacactaagtt gtacgccgctaagagaaaattggtctgggacttgttggatacttttattgctttgaactccttggacatcttgcatttcaccgagggtgacgtcgggttcgctggtactatca cctccctcttgggattggaagacttgtggaaggccacttaa | 89 |
| *CPRB* (*Candida viswanathii* ATCC20336) | atggctttagacaagttagatttgtatgtcatcataacattggtggtcgctgtggccgcctattttgctaagaaccagttccttgatcagccccaggacaccgggttcctca acacggacagcggaagcaactccagagacgtcttgctgacattgaagaagaataataaaaacacgttgttgttgtttgggtcccagaccggtacggcagaagatt acgccaacaaattgtcaagagaattgcactccagatttggcttgaaaaccatggttgcagatttcgctgattacgattgggataacttcggagatatcaccgaagatat cttggtgtttttcatcgttgccacctacggtgagggtgaacctaccgacaatgccgacgagttccacacctggttgactgaagaagctgacactttgagtactttgagat ataccgtgttcgggttgggtaactccacctacgagttcttcaatgctattggtagaaagtttgacagattgttgagtgagaaaggtggtgacagatttgctgaatatgctg aaggtgacgacggcactggcaccttggacgaagatttcatggcctggaaggataatgtctttgacgccttgaagaatgacttgaacttgaagaaaaggaattgaa gtacgaaccaaacgtgaaattgactgagagagatgacttgtctgctgccgactcccaagtttccttgggtgagccaaacaagaagtacatcaactccgagggcatc gacttgaccaagggtccattcgaccacacccacccatacttggccaggatcaccgagaccagagagttgttcagctccaaggaaagacactgtattcacgttgaat ttgacatttctgaatcgaacttgaaatacaccaccggtgaccatctagccatctggccatccaactccgacgaaaacatcaagcaatttgccaagtgtttcggattgga agataaactcgacactgttattgaattgaaggcattggactccacttacaccattccattcccaactccaattacttacggtgctgtcattagacaccatttagaaatctcc ggtccagtctcgagacaattcttttgtcgattgctgggtttgctcctgatgaagaaacaaagaagactttcaccagacttggtggtgacaaacaagaattcgccacca aggttacccgcagaaagttcaacattgccgatgccttgttatattcctccaacaacactccatggtccgatgttccttttgagttccttattgaaaacatccaacacttgact ccacgttactactccatttcttcttcgtcgttgagtgaaaaacaactcatcaatgttactgcagtcgttgaggccgaagaagaagccgatggcagaccagtcactggtg ttgttaccaacttgttgaagaacattgaaattgcgcaaaacaagactggcgaaaagccacttgttcactacgatttgagcggcccaagaggcaagttcaacaagttc aagttgccagtgcacgtgagaagatccaactttaagttgccaaagaactccaccaccccagttatcttgattggtccaggtactggtgttgccccattgagaggtttcgt tagagaaagagttcaacaagtcaagaatggtgtcaatgttggcaagactttgttgttttatggttgcagaaactccaacgaggacttttgtacaagcaagaatgggcc | 90 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| | gagtacgcttctgttttgggtgaaaactttgagatgttcaatgccttctctagacaagacccatccaagaaggtttacgtccaggataagattttagaaaacagccaact tgtgcacgaattgttgaccgaaggtgccattatctacgtctgtggtgacgccagtagaatggccagagacgtccagaccacgatctccaagattgttgccaaaagca gagaaatcagtgaagacaaggccgctgaattggtcaagtcctggaaagtccaaaatagataccaagaagatgtttggtag | |
| *FAA1* (*Candida viswanathii* ATCC20336) | ATGGGTGCCCCTTTAACAGTCGCCGTTGGCGAAGCAAAACCAGGCGAAACCGCTCCAAGAAGAAAAGCCGCTC AAAAAATGGCCTCTGTCGAACGCCCAACAGACTCAAAGGCAACCACTTTGCCAGACTTCATTGAAGAGTGTTTT GCCAGAAACGGCACCAGAGATGCCATGGCCTGGAGAGACTTGGTCGAAATCCACGTCGAAACCAAACAGGTTA CCAAAATCATTGACGGCGAACAGAAAAAGGTCGATAAGGACTGGATCTACTACGAAATGGGTCCTTACAACTAC ATATCCTACCCCAAGTTGTTGACGTTGGTCAAGAACTACTCCAAGGGTTTGTTGGAGTTGGGCTTGGCCCCAGA TCAAGAATCCAAGTTGATGATCTTTGCCAGTACCTCCCACAAGTGGATGCAGACCTTCTTAGCCTCCAGTTTCCA AGGTATCCCCGTTGTCACCGCCTACGACACCTTGGGTGAGTCGGGCTTGACCCACTCCTTGGTGCAAACCGAA TCCGATGCCGTGTTCACCGACAACCAATTGTTGTCCTCCTTGATTCGTCCTTTGGAGAAGGCCACCTCCGTCAA GTATGTCATCCACGGGGAAAAGATTGACCCTAACGACAAGAGACAGGGCGGCAAAATCTACCAGGATGCGGAA AAGGCCAAGGAGAAGATTTTACAAATTAGACCAGATATTAAATTTATTTCTTTCGACGAGGTTGTTGCATTGGGT GAACAATCGTCCAAAGAATTGCATTTCCCAAAACCAGAAGACCCAATCTGTATCATGTACACCTCGGGTTCCAC CGGTGCTCCAAAGGGTGTGGTTATCACCAATGCCAACATTGTTGCCGCCGTGGGTGGTATCTCCACCAATGCT ACTAGAGACTTGGTTAGAACTGTCGACAGAGTGATTGCATTTTTGCCATTGGCCCACATTTTCGAGTTGGCCTTT GAGTTGGTTACCTTCTGGTGGGGGGCTCCATTGGGTTACGCCAATGTCAAGACTTTGACCGAAGCCTCCTGCA GAAACTGTCAGCCAGACTTGATTGAATTCAAACCAACCATCATGGTTGGTGTTGCTGCCGTTTGGGAATCGGTC AGAAAGGGTGTCTTGTCTAAATTGAAACAGGCTTCTCCAATCCAACAAAAGATCTTCTGGGCTGCATTCAATGC CAAGTCTACTTTGAACCGTTATGGCTTGCCAGGCGGTGGGTTGTTTGACGCTGTCTTCAAGAAGGTTAAAGCCG CCACTGGTGGCCAATTGCGTTATGTGTTGAATGGTGGGTCCCCAATCTCTGTTGATGCCCAAGTGTTTATCTCC ACCTTGCTTGCGCCAATGTTGTTGGGTTACGGTTTGACTGAAACCTGTGCCAATACCACCATTGTCGAACACAC GCGCTTCCAGATTGGTACTTTGGGTACCTTGGTTGGATCTGTCACTGCCAAGTTGGTTGATGTTGCTGATGCTG GATACTACGCCAAGAACAACCAGGGTGAAATCTGGTTGAAAGGCGGTCCAGTTGTCAAGGAATACTACAAGAA CGAAGAAGAAACCAAGGCTGCATTCACCGAAGATGGCTGGTTCAAGACTGGTGATATTGGTGAATGGACCGCC GACGGTGGTTTGAACATCATTGACCGTAAGAAGAACTTGGTCAAGACTTTGAATGGTGAATACATTGCTTTGGA GAAATTGGAAAGTATTTACAGATCCAACCACTTGATTTTGAACTTGTGTGTTTACGCTGACCAAACCAAGGTCAA GCCAATTGCTATTGTCTTGCCAATTGAAGCCAACTTGAAGTCTATGTTGAAGGACGAAAAGATTATCCCAGATGC TGATTCACAAGAATTGAGCAGCTTGGTTCACAACAAGAAGGTTGCCCAAGCTGTCTTGAGACACTTGCTCCAAA CCGGTAAACAACAAGGTTTGAAAGGTATTGAATTGTTGCAGAATGTTGTCTTGTTGGATGACGAGTGGACCCCA CAGAATGGTTTTGTTACTTCTGCCCAAAAGTTGCAGAGAAAGAAGATTTTAGAAAGTTGTAAAAAAGAAGTTGAA GAGGCATACAAGTCGTCTTAG | 91 |



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**Table 19**

| Gene (Organism) | Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted) | SEQ ID NO |
|---|---|---|
| *POX5* (*Candida viswanathii* ATCC20336) | atgcctaccgaacttcaaaaagaaagagaactcaccaagttcaacccaaaggagttgaactacttcttggaaggttcccaagaaagatccgagatcatcagcaa<br>catggtcgaacaaatgcaaaaagaccctatcttgaaggtcgacgcttcatactacaacttgaccaaagaccaacaaagagaagtcaccgccaagaagattgcc<br>agactctccagatactttgagcacgagtacccagaccaacaggcccagagattgtcgatcctcggtgtctttgacccacaagtcttcaccagaatcggtgtcaacttg<br>ggtttgtttgtttcctgtgtccgtggtaacggtaccaactcccagttcttctactggaccataaataagggtatcgacaagttgagaggtatctatggttgttttggtatgactg<br>agttggcccacggttccaacgtccaaggtattgaaaccaccgccacttttgacgaagacactgacgagtttgtcatcaacaccccacacattggtgccaccaagtg<br>gtggatcggtggtgctgcgcactccgccacccactgctccgtctacgccagattgaaggtcaaaggaaaggactacggtgtcaagaccttgttgtcccattgagag<br>actccaaccacgacctcgagccaggtgtgactgttggtgacattggtgccaagatgggtagagacggtatcgataacggttggatccagttctccaacgtcagaatc<br>ccaagattctttatgttgcaaaagtactgtaaggtttcccgtctgggtgaagtcaccatgccaccatctgaacaattgtcttactcggctttgattggtggtagagtcaccat<br>gatgatggactcctacagaatgaccagtagattcatcaccattgccttgagatacgccatccacagaagacaattcaagaagaaggacaccgataccattgaaac<br>caagttgattgactacccattgcatcaaaagagattgttcccattcttggctgccgcttacttgttctcccaaggtgccttgtacttagaacaaaccatgaacgcaaccaa<br>cgacaagttggacgaagctgtcagtgctggtgaaaaggaagccattgacgctgccattgtcgaatccaagaaattgttcgtcgcttccggttgtttgaagtccacctgt<br>acctggttgactgctgaagccattgacgaagctcgtcaagcttgtggtggtcacggttactcgtcttacaacggtttcggtaaagcctactccgactgggttgtccaatgt<br>acctgggaaggtgacaacaacatcttggccatgaacgttgccaagccaatggttagagacttgttgaaggagccagaacaaaagggattggttctctccagcgttg<br>ccgacttggacgacccagccaagttggttaaggctttcgaccacgccctttccggcttggccagagacattggtgctgttgctgaagacaagggtttcgacattaccg<br>gtccaagtttggttttggtttccaagttgaacgctcacagattcttgattgacggtttcttcaagcgtatcaccccagaatggtctgaagtcttgagacctttgggtttcttgtat<br>gccgactggatcttgaccaactttggtgccaccttcttgcagtacggtatcattaccccagatgtcagcagaaagatttcctccgagcacttcccagccttgtgtgccaa<br>ggttagaccaaacgttgttggtttgactgatggtttcaacttgactgacatgatgaccaatgctgctattggtagatatgatggtaacgtctacgaacactacttcgaaact<br>gtcaaggctttgaacccaccagaaaacaccaaggctccatactccaaggctttggaagacatgttgaaccgtccagaccttgaagtcagagaaagaggtgaaaa<br>gtccgaagaagctgctgaaatcttgtccagttaa | 92 |
| *POX4* (*Candida viswanathii* ATCC20336) | atgacttttacaaagaaaaacgttagtgtatcacaaggtcctgaccctagatcatccatccaaaaggaaagagacagctccaaatggaaccctcaacaaatgaac<br>tacttcttggaaggctccgtcgaaagaagtgagttgatgaaggctttggcccaacaaatggaaagagacccaatcttgttcacagacggctcctactacgacttgac<br>caaggaccaacaaagagaattgaccgccgtcaagatcaacagaatcgccagatacagagaacaagaatccatcgacactttcaacaagagattgtccttgattg<br>gtatctttgacccacaggtcggtaccagaattggtgtcaacctcggtttgttcctttcttgtatcagaggtaacggtaccacttcccaattgaactactgggctaacgaaa<br>aggaaaccgctgacgttaaaggtatctacggttgtttcggtatgaccgaattggcccacggttccaacgttgctggtttggaaaccaccgccacatttgacaaggaat<br>ctgacgagtttgtcatcaacaccccacacattggtgccaccaagtggtggattggtggtgctgctcactccgccacccactgttctgtctacgccagattgattgttgac<br>ggtcaagattacggtgtcaagacttttgttgtcccattgagagactccaaccacgacctcatgccaggtgtcactgttggtgacattggtgccaagatgggtagagatg<br>gtatcgataacggttggatccaattctccaacgtcagaatcccaagattctttatgttgcaaaagttctgtaaggtttctgctgaaggtgaagtcaccttgccacctttgga<br>acaattgtcttactccgccttgttgggtggtagagtcatgatggttttggactcctacagaatgttggctagaatgtccaccattgccttgagatacgccattggtagaaga<br>caattcaagggtgacaatgtcgatccaaaagatccaaacgctttggaaacccaattgatagattacccattgcaccaaaagagattgttcccatacttggctgctgcc<br>tacgtcatctccgctggtgccctcaaggttgaagacaccatccataacaccttggctgaattggacgctgccgttgaaaagaacgacaccaaggctatctttaagtct<br>attgacgacatgaagtcattgtttgttgactctggttccttgaagtccactgccacttggttgggtgctgaagccattgaccaatgtagacaagcctgtggtggtcacggtt<br>actcgtcctacaacggcttcggtaaagcctacaacgattgggttgtccaatgtacttgggaaggtgacaacaatgtcttggccatgagtgttggtaagccaattgtcaa<br>gcaagttatcagcattgaagatgccggcaagaccgtcagaggttccaccgctttcttgaaccaattgaaggactacactggttccaacagctccaaggttgttttgaa<br>cactgttgctgacttggacgacatcaagactgtcatcaaggctattgaagttgccatcatcagattgtcccaagaagctgcttctattgtcaagaaggaatctttcgacta | 93 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| | tgtcggcgctgaattggttcaactctccaagttgaaggctcaccactacttgttgactgaatacatcagaagaattgacacctttgaccaaaaggacttggttccatactt gatcaccctcggtaagttgtacgctgccactattgtcttggacagatttgccggtgtcttcttgactttcaacgttgcctccaccgaagccatcactgctttggcctctgtgca aattccaaagttgtgtgctgaagtcagaccaaacgttgttgcttacaccgactccttccaacaatccgacatgattgtcaattctgctattggtagatacgatggtgacat ctatgagaactactttgacttggtcaagttgcagaacccaccatccaagaccaaggctccttactctgatgctttggaagccatgttgaacagaccaaccttggacga aagagaaagatttgaaaagtctgatgaaaccgctgctatcttgtccaagtaa | |
| *PXA1* (*Candida viswanathii* ATCC20336) | atggtcaacatatcgaaattgacgggttataacaagcaggacatcaggaatgtggtgctattgctacaggagtttgtcaagacctacaaagacaacaagatcaaac tcaactacctgagtagacctgtcatcttgttcttgagtaccttggttgcaactgccggtattggggtgtttttcaccttgagaagcatcgtcactaagtacaacgagtaccta ctcaacaagagattgagacgcccaagtcttatcagacaatcctccaatatcttgaagaacggatcccgtgagatctttatccagaagggcaacggcaaagtaaca agaatcatcatcccaaaagcaaacaacgaccagtatgccgccgacaagtatttgtataaagattttgcccgcaacgagcaaatattgcaacagcaaaagggaag gctcttcaattccagattcttgaaccagttgaccattatctggaagatcttgattccaaagttctactgccaaaacacttccttgttgttatcgcagtgcttctttttgattttcag aacatggttgtccttgttgattgccaagctagatggtcagattgtcaagaacttgattgctgcagacggtaggaagtttgcccgtgacttgatttacttttgttgattgccttc cctgcttcgtacaccaacgccgctatcaaatacttggaattgagattggcgttaggattcagaactaatcttaccagatacatccatgacatgtacttggacaaaacca tgtcgtactacaaagtgggattgaacggcgccgatatccaaaacatagaccagtacatcaccgaagatgtcaccaaattctgtatgtcgttgtgttcgttgttttcctcca tgggtaagccattcattgacttgatctttttcagtgtttatttgagagacaatttgggtactggtgccattattggcatttttgccaactattttgctaccgccatcatgttgaaaa aggcaacaccaagattcggtaagttggctgccaaaagaacccacttggaaggtgtttatttcaaccaacagttgaacataatgaccaacagtgaagagattgggtt ctacaaaggatcgaagattgagaagtccaagcttgcggagaactttgacaagttgatgggtcacgtatcgagagaaatcaatttatcgtccagctatgccgctctag aagactacgtgcttaaatacacgtggctggcctggggttacatcttttctggtctacctgtgtttttggatgtgcttttccctaaagaagacccaagtagtggccatattgctg atatagatgacgatgaccatgcccatggacacgggcacaccgggggaagagaccagctcaacaactgaaaacatgaagaccttcgtcaccaacaagcgattatt gttgagtcttgccgatgctggttccagattgatggttagtttgaaagaggtcaccacgttgacaggtataacgaatagagtcttcaacatgttgactcagctccaccgtgt ccatgatcctaaatttgactacggtgacaagtatggtttgcctgatattcacgggacttatcaattgaactacgatggtttgagattggaacatgttccaattactgtgcca actgccgagggttcttactccacaccattgatcccagacctcacttttgacatcaagggcaagaatttgttatttgttggtccaaacggttcgggcaaaacttctgttgcca gggttcttgcaggtctttggcccttgtatgccgggttagtgctgaaaccactggatttgttctttaacccacaaaagagttatttcaccaccggaagtttgcgtgaccaagtt gtttaccctaatagatccgaaaacaccaccaacgatcaaattttccacatcttacactgtgtacacttagaccatattgttaaacggtacggattgaaccagaacttgg atttcgctaaaacattgagtggaggtgagaagcaaagattgagtttcgccagagtgttgtttaacagaccaagtattgtcattcttgatgattcgacgtcggcgttgtccc cagatatggaagagttgatgtaccaggtgttgcaagatcacaagatcaattacgtcacactttcaaatcgtccctctctcagtaagttccatgataaagtatttgaaatat aa | 94 |



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**Table 19**

| Gene (Organism) | Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted) | SEQ ID NO |
|---|---|---|
| *PXA2* (*Candida viswanathii* ATCC20336) | atgacagtggagaatgcaaaactacagaagaactcgttggcggttctgctcttgaaggtgtacaaatccaacagatcattattgttaaacacctcatacatcatattaa<br>tcattgctgccttcactggcgcaacgaataccgggcgaggcacctcctccagatcatcggcaaaagtagagaccgatgaagaacaatcggttaaaaagaaaca<br>ccccaagctctctagagagtccttccatagactaagaaaagcaatcttgccaactttctttgatagaactatagtttacttttttcgccaacttgactttgttggtggtcagag<br>cattattgacacttagagttgctacccttgacggtcagcttgtgggggcattggtttcaagaagaataagggtgtttgccaagtacttgttgtactggatgcttcttggtatcc<br>ccgctgctttgacaaatgccttgttgaactggaccaaactgaacttgagcaagagcattagaatgaacttgaataataacatcatggaggaatacttgccagataact<br>tggacccaaactattattcattgatccatttgactgataacaagattagagacccaaatcagagaataaccactgatactagtcgtttgagcgatgccttggcaagctt<br>gcccggtcacatattgaagccaacgttggatatcatattgtgtgcgcaacagttaagcaagagcggtgttggtaatggggaaggtacgttggcattaggtatattggc<br>acacttctcaaccatgatcatccgtttcttctccccgccatttgccaagttggcggctgagagagctaaccttgaaggtcagttgcgttccgcgcattccaagattgttgcc<br>aacagtgaagaaattgctttcttgggtggtcatgaccgtgagttggatcacatcgaccactgctactatactttggagagattctcgaaaggcgaatattggaagcgag<br>ccatacacgaaatcacacaaacgtttattgtgaagtacttttggggtgttgcaggtttagtgttgtgttctgcaccggttttcattgccaaatacttgggtgagccggaagat<br>aagaatgttgctggtaatttcatcaccaacagaagattgttgatgagtgcctcggattccttggatcgtttaatctattctagaagatacttgttgcaagttgtcggtcatgct<br>accagagtgtctgacttcttggacactttacatgaagtggaggagaagaagagaatcacatcgaatgtgcagtttaacaacgacgagattactttcgatcatgtt<br>agattgatgactccaacggaagtgaccttgatcccagacttgaacttttccattaaaccaggtgaccatttgttgattgtggggccaaacggttcaggtaagtcgtcgtt<br>gttcagaatgttgggtgggttgtggcccgttaggtttggtactattagaattccaaacacagagaacatgttctacttgccgcaaaaggcttaccttgttgaaggatcattc<br>agagagcaaatcatttatccacacaacgtgactcaacagaagaagactgatcaacaattgaaagagatcttgaaggttttgaaattggaagattactcagggcaat<br>tggatgaggttaagaaatggagcgaagaattgtccattggtgctcaacaaagattggctatggctagattgtactaccacgaacctaagtttgctgtcttggacgaatg<br>tacttcagctgtgtcaccagacatggaacaactcatgtaccaacacgcacaaggtttgggtatcacgcttttgtccgttgcccatagacctgcattgtggcacttccaca<br>aatacttgttggaattcgacgggaagggtagttactactttggtacgttggatgaaaagcacaaaatgaagttagaagaagaagaacgactcaagaaggagaatg<br>aaaagaagagtgtcgccaagaagtag | 95 |
| *ACL1* (*Yarrowia lipolytica* CLIB122) codon optimized for *Candida* strain ATCC20336 | atgtccgccaacgaaaacatctccagattcgacgcccctgtcggtaaggaacacccagcctacgaattattccataaccacacaagatccttcgtttatggattaca<br>acctagagcctgtcaaggaatgttggacttcgacttcatctgcaagagagaaaacccatctgtcgccggagttatctatccattcggtggtcaattcgttaccaagatgt<br>actggggtaccaaggaaactcttttacctgtttaccaacaagttgaaaaggccgctgccaagcacccagaagttgatgttgtcgttaactttgcctcttccagatctgttt<br>actcttccaccatggaattgttagaatacccacaattcagaaccatcgccattattgccgaaggagttccagaaagaagggccagagaaatcttacacaaggccc<br>aaaagaagggagtcaccatcattggaccagctaccgttggcggaatcaagccaggatgtttcaaggtgggcaacaccggcggaatgatggacaacattgttgcc<br>tctaagttatacagaccaggttctgtggcctacgtttctaagtctggcggcatgtctaacgaattgaacaacattatctcccacaccaccgacggagtttacgaaggaa<br>ttgctattggaggagacagatacctggaactaccttcattgaccatatcttgagatacgaagccgacccaaagtgcaagatcatcgttttacttggagaagtgggag<br>gagtggaagaatacagagttatcgaagctgtgaagaacggtcaaatcaagaagccaatcgttgcttgggccattggaacttgcgcctctatgttcaagactgaagtg<br>caattcggtcacgccggttctatggccaactctgacttggaaactgccaaggctaagaacgccgccatgaagtccgctggtttctacgttccagataccttcgaagac<br>atgccagaagttcttgccgaattatacgaaaagatggttgccaagggtgaattgtccagaatctccgaacctgaagttccaaagatcccaattgactactcctgggcc<br>caagaacttggacttatcagaaagccagctgctttcatctctactatttctgatgacagaggtcaagaacttttgtacgctggtatgccaatttctgaagtgttcaaggaa<br>gacattggaatcggtggagttatgtccttgttgtggttcagaagaagattaccagactacgcctctaagtttcttgaaatggtgttaatgcttactgctgaccacggacca<br>gccgtatctggagccatgaacaccattatcaccaccagagctggaaaggatttaatttcctctttggtggctggattattgaccattggaaccagattcggcggagctct<br>tgacggagctgccaccgaattcaccactgcctacgacaagggattgtctccaagacaattcgtggataccatgagaaagcaaaacaagttgattcctggaattggt<br>catagagttaagtccagaaacaacccagatttcagagttgaacttgttaaggactttgtgaagaagaacttcccatctacccaattgttagactacgcccttgctgttga | 96 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| | agaagttaccacctctaagaaggacaacttgattttgaacgtggacggagctattgctgtgtcctttgttgatttaatgagatcctgtggagcctttactgtcgaagaaact gaagactacttaaagaacggagtgttaaacggattgttcgtgttaggaagatctattggattaattgcccaccatttagatcaaaagagattaaagaccggattgtaca gacatccttgggacgatatcacctacttggtgggtcaagaagctatccaaaagaagagagttgaaatcagtgccggtgacgtgtctaaggccaagactagatcata g | |
| *ACL2* (*Yarrowia lipolytica* CLIB122) codon optimized for *Candida* strain ATCC20336 | atgtcagcgaaatctattcacgaagccgacggtaaggccttgttagcacacttttgtctaaggcgccagtctgggccgaacaacaaccaatcaacacgtttgagat gggtacaccaaagttggcgtccttgacgttcgaagacggtgtcgccccagaacaaatcttcgccgccgctgagaagacctacccatggttgttggaatctggtgcca agtttgtcgccaagccagaccaattaatcaagagaagaggtaaggccggtttgttggtattaaacaagtcgtgggaagaatgtaagccatggatcgccgaacgtgc cgccaagccaatcaacgtcgaaggtattgacggcgtcttgagaacgttcttggttgaaccatttgtcccacacgaccaaaagcacgaatactacatcaacatccact ctgtcagagaaggtgactggatcttattctaccacgaaggcggcgttgacgttggtgacgtcgacgccaaggccgccaagatcttaatcccagtggacattgaaaa cgaataccatctaacgccacgttaaccaaggaattgttggcacacgtcccagaagaccaacaccaaaccttgttagacttcatcaaccgtttatacgccgtttacgt tgatttgcaatttacgtatttggaaatcaacccattggttgtcatcccaaccgcccaaggtgttgaagttcactacttggatcttgccggtaagttagaccaaaccgcaga atttgaatgtggtccaaagtgggctgctgcgcgttctccagccgctttgggtcaagttgttaccattgacgccggttctaccaaggtctctatcgacgccggtccagccat ggttttcccagctcctttcggaagagaattgtctaaggaagaagcgtacattgcggaattagattctaagaccggcgcttccttgaagttgactgtgttaaatgccaagg gtagaatctggacccttgtcgctggaggcggcgcctctgttgtttacgccgacgccattgcgtccgccggttttgctgacgaattagccaactacggtgaatactccggt gctccaaacgaaacccaaacctacgaatacgccaaaaccgtattggatttaatgacccgtggtgacgctcacccagaaggtaaggtattgttcattggtggcggca tcgccaacttcacccaagtgggctctaccttcaagggtatcatccgtgccttccgtgactaccaatcctccttgcacaaccacaaggtcaagatttacgtcagaagag gtggaccaaactggcaagaaggattgcgtctcatcaagtcggctggtgacgaattgaatttgccaatggaaatttacggtccagacatgcacgtctcgggaattgtg cctctcgctttgcttggcaagcgtccaaagaatgttaagccttttggtaccggcccttccactgaagcttctactcctttaggcgtgtaa | 97 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| *FAT1* (*Candida viswanathii* ATCC20336) | ATGTCAGGATTAGAAATAGCCGCTGCTGCCATCCTTGGTAGTCAGTTATTGGAAGCCAAATATTTAATTGCCGA<br>CGACGTGCTGTTAGCCAAGACAGTCGCTGTCAATGCCCTCCCATACTTGTGGAAAGCCAGCAGAGGTAAGGCA<br>TCATACTGGTACTTTTTCGAGCAGTCCGTGTTCAAGAACCCAAACAACAAAGCGTTGGCGTTCCCAAGACCAAG<br>AAAGAATGCCCCCACCCCCAAGACCGACGCCGAGGGATTCCAGATCTACGACGATCAGTTTGACCTAGAAGAA<br>TACACCTACAAGGAATTGTACGACATGGTTTTGAAGTACTCATACATCTTGAAGAACGAGTACGGCGTCACTGC<br>CAACGACACCATCGGTGTTTCTTGTATGAACAAGCCGCTTTTCATTGTCTTGTGGTTGGCATTGTGGAACATTGG<br>TGCCTTGCCTGCGTTCTTGAACTTCAACACCAAGGACAAGCCATTGATCCACTGTCTTAAGATTGTCAACGCTTC<br>GCAAGTTTTCGTTGACCCGGACTGTGATTCCCCAATCAGAGATACCGAGGCTCAGATCAGAGAGGAATTGCCA<br>CATGTGCAAATAAACTACATTGACGAGTTTGCCTTGTTTGACAGATTGAGACTCAAGTCGACTCCAAAACACAGA<br>GCCGAGGACAAGACCAGAAGACCAACCGATACTGACTCCTCCGCTTGTGCATTGATTTACACCTCGGGTACCA<br>CCGGTTTGCCAAAAGCCGGTATCATGTCCTGGAGAAAAGCCTTCATGGCCTCGGTTTTCTTTGGCCACATCATG<br>AAGATTGACTCGAAATCGAACGTCTTGACCGCCATGCCCTTGTACCACTCCACCGCGGCCATGTTGGGGTTGT<br>GTCCTACTTTGATTGTCGGTGGCTGTGTCTCCGTGTCCCAGAAATTCTCCGCTACTTCGTTCTGGACCCAGGCC<br>AGATTATGTGGTGCCACCCACGTGCAATACGTCGGTGAGGTCTGTCGTTACTTGTTGAACTCCAAGCCTCATCC<br>AGACCAAGACAGACACAATGTCAGAATTGCCTACGGTAACGGGTTGCGTCCAGATATATGGTCTGAGTTCAAGC<br>GCAGATTCCACATTGAAGGTATCGGTGAGTTCTACGCCGCCACCGAGTCCCCTATCGCCACCACCAACTTGCA<br>GTACGGTGAGTACGGTGTCGGCGCCTGTCGTAAGTACGGGTCCCTCATCAGCTTGTTATTGTCTACCCAGCAG<br>AAATTGGCCAAGATGGACCCAGAAGACGAGAGTGAAATCTACAAGGACCCCAAGACCGGGTTCTGTACCGAGG<br>CCGCTTACAACGAGCCAGGTGAGTTGTTGATGAGAATCTTGAACCCTAACGACGTGCAGAAATCCTTCCAGGG<br>TTATTATGGTAACAAGTCCGCCACCAACAGCAAAATCCTCACCAATGTTTTCAAAAAAGGTGACGCGTGGTACA<br>GATCCGGTGACTTGTTGAAGATGGACGAGGACAAATTGTTGTACTTTGTCGACAGATTAGGTGACACTTTCCGT<br>TGGAAGTCCGAAAACGTCTCCGCCACCGAGGTCGAGAACGAATTGATGGGCTCCAAGGCCTTGAAGCAGTCC<br>GTCGTTGTCGGTGTCAAGGTGCCAAACCACGAAGGTAGAGCCTGTTTTGCCGTCTGTGAAGCCAAGGACGAGT<br>TGAGCCATGAAGAAATCTTGAAATTGATTCACTCTCACGTGACCAAGTCTTTGCCTGTGTATGCTCAACCTGCGT<br>TCATCAAGATTGGCACCATTGAGGCTTCGCACAACCACAAGGTTCCTAAGAACCAATTCAAGAACCAAAAGTTG<br>CCAAAGGGTGAAGACGGCAAGGATTTGATCTACTGGTTGAATGGCGACAAGTACCAGGAGTTGACTGAAGACG<br>ATTGGTCTTTGATTTGTACCGGTAAAGCCAAATTGTAG | 98 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| CYP52A17 (*Candida viswanathii* ATCC20336) FROM pAA1712 | atgattgaacaactcctagaatattggtatgtcgttgtgccagtgttgtacatcatcaaacaactccttgcatacacaaagactcgcgtcttgatgaaaaagttgggtgct<br>gctccagtcacaaacaagttgtacgacaacgctttcggtatcgtcaatggatggaaggctctccagttcaagaaagagggcagggctcaagagtacaacgattac<br>aagtttgaccactccaagaacccaagcgtgggcacctacgtcagtattcttttcggcaccaggatcgtcgtgaccaaagatccagagaatatcaaagctattttggc<br>aacccagtttggtgatttttctttgggcaagaggcacactctttttaagcctttgttaggtgatgggatcttcacattggacggcgaaggctggaagcacagcagagcca<br>tgttgagaccacagtttgccagagaacaagttgctcatgtgacgtcgttggaaccacacttccagttgttgaagaagcatattcttaagcacaagggtgaatactttgat<br>atccaggaattgttctttagatttaccgttgattcggccacggagttcttatttggtgagtccgtgcactccttaaaggacgaatctattggtatcaaccaagacgatatag<br>attttgctggtagaaaggactttgctgagtcgttcaacaaagcccaggaatacttggctattagaaccttggtgcagacgttctactggttggtcaacaacaaggagttt<br>agagactgtaccaagctggtgcacaagttcaccaactactatgttcagaaagctttggatgctagcccagaagagcttgaaaagcaaagtgggtatgtgttcttgtac<br>gagcttgtcaagcagacaagagaccccaatgtgttgcgtgaccagtctttgaacatcttgttggccggaagagacaccactgctgggttgttgtcgtttgctgtctttgag<br>ttggccagacacccagagatctgggccaagttgagagaggaaattgaacaacagtttggtcttggagaagactctcgtgttgaagagattacctttgagagcttgaa<br>gagatgtgagtacttgaaagcgttccttaatgaaaccttgcgtatttacccaagtgtcccaagaaacttcagaatcgccaccaagaacacgacattgccaaggggc<br>ggtggttcagacggtacctcgccaatcttgatccaaaagggagaagctgtgtcgtatggtatcaactctactcatttggaccctgtctattacggccctgatgctgctga<br>gttcagaccagagagatggtttgagccatcaaccaaaaagctcggctgggcttacttgccattcaacggtggtccaagaatctgtttgggtcagcagtttgccttgacg<br>gaagctggctatgtgttggttagattggtgcaagagttctcccacgttaggctggacccagacgaggtgtacccgccaaagaggttgaccaacttgaccatgtgtttgc<br>aggatggtgctattgtcaagtttgactag | 99 |



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**Table 19**

| Gene (Organism) | Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted) | SEQ ID NO |
|---|---|---|
| *ADH1-2* alcohol dehydrogenase (*Candida viswanathii* ATCC20336) | Atgtctgctaatatcccaaaaactcaaaaagctgtcgtcttcgagaagaacggtggtgaattaaaatacaaagacatcccagtgccaaccccaaaggccaacga<br>attgctcatcaacgtcaagtactcgggtgtctgtcacactgatttgcacgcctggaagggtgactggccattggacaccaaattgccattggttggtggtcacgaaggt<br>gctggtgttgttgtcggcatgggtgaaaacgtcaagggctggaaaatcggtgatttcgccggtatcaaatggttgaacggttcttgtatgtcctgtgagttctgtcagcaa<br>ggtgctgaaccaaactgtggtgaagctgacttgtctggttacacccacgatggttctttcgaacaatacgccactgctgatgctgtgcaagccgccagaatcccagct<br>ggcactgatttggccgaagttgccccaatcttgtgtgctggtgtcaccgtctacaaagccttgaagactgccgacttggctgctggtcaatgggtcgctatctccggtgct<br>ggtggtggtttgggctccttggctgtccaatacgccgtcgccatgggtttgagagtcgttgccattgacggtggtgacgaaaagggtgactttgtcaagtccttgggtgct<br>gaagcctacattgatttcctcaaggaaaagggcattgttgctgctgtcaagaaggccactgatggcggtccacacggtgctatcaatgtttccgtttccgaaaaagcc<br>attgaccaatctgtcgagtacgttagaccattgggtaaggttgttttggttggtttgccagctggctccaaggtcactgctggtgttttcgaagccgttgtcaagtccattga<br>aatcaagggttcttacgtcggtaacagaaaggatactgccgaagccgttgactttttctccagaggcttgatcaagtgtccaatcaagattgtgggcttgagtgaattgc<br>cacaggtcttcaagttgatggaagaaggtaagatcttgggtagatacgtcttggatacctccaaa | 100 |
| *FAO1* fatty alcohol oxidase (*Candida viswanathii* ATCC20336) | atggctccatttttgcccgaccaggtcgactacaaacacgtcgacacccttatgttattatgtgacgggatcatccacgaaaccaccgtcgaccaaatcaaagacgtt<br>attgctcctgacttccctgctgacaagtacgaagagtacgtcaggacattcaccaaaccctccgaaaccccagggttcagggaaaccgtctacaacacagtcaac<br>gcaaacaccacggacgcaatccaccagttcattatcttgaccaatgttttggcatccagggtcttggctccagctttgaccaactcgttgacgcctatcaaggacatga<br>gcttggaagaccgtgaaaaattgttggcctcgtggcgcgactccccaatcgctgccaaaaggaagttgttcaggttggtttctacgcttaccttggtcacgttcacgag<br>attggccaatgagttgcatttgaaagccattcattatccaggaagagaagaccgtgaaaaggcttatgaaacccaggagattgacccttttaagtaccagtttttggaa<br>aaaccgaagttttacggcgctgagttgtacttgccagatattgatgtgatcattattggatctggtgccggtgctggtgttgtggcccacactttggccaacgatggcttca<br>agagtttggttttggaaaagggcaaatactttagcaactccgagttgaactttgatgacaaggacggcgttcaagaattataccaaagtggaggtactttgactacagt<br>caaccaacagttgtttgttcttgctggttccacttttggtggcggtaccactgtcaattggtcagcctgtcttaagacgccattcaaggtgcgtaaggaatggtatgatgag<br>tttggtgttgactttgctgctgatgaagcatacgataaagcgcaggattatgtttggcagcaaatgggagcttctaccgaaggcatcacccactctttggctaacgagat<br>tattattgaaggtggtaagaaattaggttacaaggccaaggtattagaccaaaacagcggtggtcatcctcagcacagatgcggtttctgttatttgggctgtaagcac<br>ggtatcaagcagggttctgttaataactggtttagagacgcagctgcccacggttcccagttcatgcaacaggttagagttttgcaaatacttaacaagaaggggatc<br>gcttacggtatcttgtgtgaggatgttgtaaccggcgccaagttcaccattactggccccaaaaagtttgttgttgctgccggtgctttgaacactccatctgtgttggtcaa<br>ctccggcttcaagaacaagaacatcggtaagaacttaactttgcacccagtttctgtcgtgtttggtgattttggcaaagacgttcaagcagaccacttccacaactcca<br>tcatgactgccctttgttcagaagccgctgatttagacggcaagggccatggatgcagaattgaaaccatcttgaacgctccattcatccaggcttcattcttaccatgg<br>agaggtagtaacgaggctagacgagacttgttgcgttacaacaacatggtggcgatgttgctccttagtcgtgacaccaccagtggttccgtttctgctcatccaacca<br>aacctgaagctttggttgtcgagtacgacgtgaacaagtttgacagaaactcgatcttgcaggcattgttggtcactgctgacttgttgtatatccaaggtgccaagaga<br>atccttagtccacaggcatgggtgccaatttttgaatccgacaagccaaaggataagagatcaatcaaggacgaggactatgtcgaatggagagccaaggttgcc<br>aagattcctttcgacacctacggctcaccttatggttcggcacatcaaatgtcttcttgccgtatgtcaggtaagggtcctaaatacggtgctgttgacaccgatggtaga<br>ttgtttgaatgttcgaatgtttatgttgccgatgcaagtcttttgccaactgcaagcggtgccaaccctatggtcaccaccatgactcttgccagacatgttgcgttaggtttg<br>gcagactccttgaagaccaaagccaagttgtag | 101 |



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**Table 19**

| Gene (Organism) | Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted) | SEQ ID NO |
|---|---|---|
| *FAS1* fatty acid synthase (*Candida viswanathii* ATCC20336) | ATGTCTACTCATAGACCTTTCCAATTGACCCACGGTTCCATCGAACACACCTTGTTGGTGCCAAACGAGTTGTTC<br>TTCAACTATTCACAGTTAAAAGACGAATTCATAAAGACCTTGCCTGAACCAACCGAAGGTTTCGCTGGCGACGA<br>TGAACCTTCCAGTCCTGCTGAATTGTACGGCAAATTCCTCGGCTACATCAGTGACAACACCGTTCAATTCCCCC<br>AGATCTTACAATTGTCCTTGCAAGACTTCCAGCAGCGATTCTTGGACAACCACGACAACATCCACTCCTTTGCC<br>GTCAGATTATTAGAAGATGAAGCTTATCCAACAACAATCACCAAAGTCAAGGAAAATATCATCAAGAACTACTAC<br>AAAGCCATCAAGTCCATCGACAAGGTCGAGTCAAACTTGTTGTACCACTGCAAACATGACGCCAAGTTGGCCG<br>CTATATTCGGTGGTCAAGGTAACACCGACGACTACTTTGAAGAATTGCGTGAATTGTACACCTTATACCAGGGC<br>TTGATTGAGGACCTCCTTATCTCCATTGCCGACAAGTTGGACGAGTTATACCCTTCTTTTGACAAGATCTACACC<br>CAGGGTTTGAACATCTTGGGCTGGTTGAAGCACCCAGAAACCACCCCTGACCAAGATTACTTGTTGTCCGTACC<br>AGTGAGTTGTCCTGTTATCTGTATCATCCAATTGTGTCACTACACCATCACCTGCAAAGTTCTTGGTTTGACCCC<br>TGGTGAATTTAGAGACTCGTTGAAGTGGTCCACCGGTCACTCCCAAGGTTTGGTTACTGCTACCGCTATTTCCA<br>GTTCCGACTCCTGGGACTCCTTCAAACAAAACTCCATTGCTGCCGTCTCCTTGATGCTTTTCATTGGTGCCAGAT<br>GTTTGATGGCTTACCCAAGAACTACCTTGCCACCAACCATGTTGCAAGACTCCTTGGAACACGGTGAAGGTAGA<br>CCATCTCCAATGTTGTCAGTTAGAGACTTGACCATCACCCAAGTTGAGAAGTTTATTGAACAGACCAACTCTCAC<br>TTGCCAAAGGAAAAGCACATTGCCGTCAGTTTGGTCAATGGTGCCAGAAATTTGGTTCTTTCTGGTCCCCCGGA<br>GTCCCTTTACGGTTTCAACTTGAACTTGAGAAACCAAAAGGCTCCAATGGGATTGGACCAATCACGTGTTCCAT<br>TCAGTGAACGTAAGTTGAAGTGTTCCAACAGATTCTTGCCAATTTTTGCACCATTCCACTCTCACTTGTTGGCTG<br>ATGCCACTGAGTACATTTTGGATGATGTCAAAGAACACCGTTTGTCTTTCCAGAAATTGAAGATTGCAGTCTACG<br>ATACCTACGACGGCTCCAACTTCCAAGAGAGCAAGGAACCAATTATTGACAGACTCGTCAAGTTGATCACCGAG<br>TTGCCAGTTCACTGGGAAACCGCCACCAACCACAGGGCCACCCACATTTTGGATTTCGGCCCAGGTGGTGTCT<br>CCGGTTTGGGTGTTTTGACCCACAGAAACAAGGAAGGTACTGGTGCTAGAATAATTGTTGCTGGTGCTCTTGAC<br>TCCAACCCAATTGACGATGAGTATGGTTTCAAGCACGAAATCTTCCAGACTTCTGCCGACAAGTCCATCAAGTG<br>GGCTAGCGATTGGTTGGAAGAATTCAAACCAACTTTGGTCAAGACTTCCCAGGGAAAGATCTACGTCAACACCA<br>AGTTCTCGCAATTGTTGGGCAGAGCTCCCTTGATGGTCCCAGGTATGACACCAACCACTGTTAACCCAGACATC<br>ATTGCCGCCTCTTTGAATGCTGGCTATCACATTGAATTAGCCGGTGGTGGTTATTTCGCCGGCAGGATCATGAC<br>CAAAGCCATTGACCAAATTGTTGCCGACATCAAGCCAGGTTACGGTTTGGGTATCAACTTGATTTACGTCAACC<br>CATTCATGTTGCAATGGGGTATTCCATTGATTAAGGAGTTGAGAGAAAAGGGTTATCCAATCCAATCTTTGACCA<br>TTGGTGCTGGTGTTCCATCTTTGGAAGTTGCCACTGAATACATTGAAGAGTTGGGTCTCACGCACTTGGGCTTG<br>AAACCAGGTTCGATTGACGCCATCAGCCAAGTCATCACCATTGCCAAGGCTCATCCAAAGTTCCCAATTGTCTT<br>GCAATGGACTGGTGGTAGAGGTGGTGGCCACCACTCTTTTGAAGATTTCCACCAACCAATCCTCCAGATGTACT<br>CCAAGATCAGAAGATGCCCAAACATTGTCTTGGTTGCTGGTTCCGGGTTTGGTTCTGACGAAGACACCTACCCA<br>TACTTGACTGGTTCTTGGTCCAAGAGATTCAACTACCCACCAATGCCATACGATGGTGTCTTGTTTGGTTCCAGA<br>GTCATGACCGCCAAGGAGGCCCACACTTCGTTGGAAGCTAAGAAATTGATTGCCTCGTGCCCAGGTGTCCCAG<br>ATGAGAAGTGGGAGCAAACCTACAAGAAGCCAACCGGTGGTATCATCACTGTTAGATCTGAAATGGGTGAGCC<br>AATCCACAAGATTGCCACCAGAGGTGTCATGTTCTGGAAGGAATTGGACGACACTATCTTCAACCTTCCAAAGC | 102 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| | CAAAAGCCTTGGAAGCCATCAAGAAGAAGAGAGACTACATCATCAAGAAGTTGAACAGCGACTTCCAAAAGCCA<br>TGGTTTGGTAAGAATGCTTCTGGTGTTTGTGACTTGCAAGAAATGACCTACGAGGAAATCGCCAACAGATTGGT<br>TGAGTTGATGTACGTCAAGAAGTCTCAAAGATGGATTGACGTTTCCTTAAGAAACTTGTACGGTGACTTTTTGAG<br>AAGAGTTGAAGAAAGATTCACTTCTGCTGCCGGCGTGGTTTCCTTGTTGCAAAACTTCATCCAGTTGAACGACC<br>CAGAAACATTCAGTGCCGAGTTCTTCAACAAGTTCCCACAAGCCAAGGAACAATTGATTTCCGAAGAAGACGCT<br>GACCACTTTTTGTTGTTGGCTGCCAGACCAGGGCAAAAGCCGGTTCCATTCGTGCCAGTCTTGGACGAAAGATT<br>TGAATTCTTCTTCAAGAAAGATTCTCTTTGGCAATCTGAAGACTTGGAAAGTGTTGTCGACGAAGACGTTCAAAG<br>AACTTGTATCTTGCACGGTCCAGTTGCCTCCCAATTCACCAAGAAGGTTGATGAACCAATTGGCGAAATCTTGG<br>ACTCTATCCACGAGGGCCATATTGCCAAGTTGATCAAGGATGAATACGCTGGTGATGCATCCAAGATCCCAGTT<br>GTTGAGTACTTCGGTGGTTTCAAGACCGACAAGGTTAATGCTAACAATGTTCAAGTCAATGCTACCAGAAAGGA<br>AACCGTCTACGAAATTGGTTCCAAGTTGCCAGCCAGGCAAGACTGGTTGGACTTGTTGGCCGGTACTGAATTG<br>AACTGGTTGCACGCTTTCATCTCCACCAACAGAATTGTCCAAGGCTCCAAGCACGTCGCCAACCCATTGCACGA<br>CATTTTGGCTCCCGTTGCCAGATCCAGTGTTTCCATTGACAAGGCTACCAAGAAATTGACTGCTTATGAAAAGG<br>TCAAGGGTGAGTTGGTTCCAGTTGTTGAAATTGAATTGGTCAAGCCAAACACCATTCAATTGTCTTTGATTGAAC<br>ACAGAACTGCTGATGGCAAACCAGTTGCTTTGCCATTCTTGTACAAGTACGACCCAACTGATGGGTTTGCACCA<br>GTCTTGGAGATCATGGAAAACAGAAACGACAGAATCAAGGAATTCTACTGGAAGTTGTGGTTCGGTGCTTCCGT<br>CCCTTACGACAATGACATCGATGTCGAAGAGCAAATCTTGGGTGACGAAATCACCATTTCTTCTCAAGACATTG<br>GTGAATTCACACACGCTATTGGTAACAAGTGTGAAGCCTTTGTCAACAGACCAGGTAAGGTCACTTTGGCTCCA<br>ATGGATTTCGCCATTGTTGTTGGTTGGAAAGCTATCATCAAGTCCATCTTCCCAAAGACCGTTGACGGTGACTT<br>GTTGAAGTTGGTCCACTTGTCCAACGGTTACAAGATGATCCCTGGTGCAGCTCCATTGCAAAAGGGCGATGTTG<br>TTTCCACTAGATCTGACATCAAGGCTGTTTTGAACCAACCAAGTGGTAAGTTGGTTGAAGTTGTTGGTACCATCT<br>TCCGTGAAGGCAAGCCAGTTATGGAAGTCACTTCACAATTCTTGTACCGTGGTGAATACGACGACTACTGCAAC<br>ACCTTCCAAAAGGTCACTGAAACTCCAGTTCAAGTCTCATTCAAGTCTCCTAAGGATTTGGCTGTTTTGAGATCC<br>AAGGAATGGTTCCATTTGGAAAAGGATGTCGAGTTTGATGCTTTGACTTTCAGATGTGAATCCACTTACAAGTTC<br>AAGTCTGCCAACGTCTACTCGTCCATCAGAACGACCGGTCAAGTTTTCTTGGAGTTGTCCACCAAAGAAGTTAT<br>CCAAGTTGGTTCTGTTGACTATGAAGCTGGTACCTCTTATGGTAACCCAGTCACTGACTACTTGAACAGAAACG<br>GTAAGACCATTGAAGAGGCTGTTACTTTTGAGAATGCCATCCCCTTGTCGTCTGGTGAAGAGTTGACCACCAAG<br>GCTCCGGGTACCAACGAGCCATATGCTATTGTTTCTGGTGACTACAACCCAATCCACGTTTCCAGAGTCTTTTCT<br>GCTTACGCCAAGTTGCCAGGTACTATCACCCACGGTATGTACTCTTCTGCCGCCATCAGAGCCTTGGTTGAAGA<br>GTGGGCTGCCAACAACGTTGCCCCAAGAGTCAGAGCCTTCAAGTGTGAATTTGTTGGTATGGTTTTGCCAAACG<br>ACACTTTGCAAACCACTATGGAACACGTTGGTATGATCAATGGCCGTAAGATCATCAAGGTCAAGACTGTCAAT<br>GCCGAGACCGAGACTCCAGTCTTGCTTGCTGAAGCCGAAATTGAACAACCAACCACCACCTATGTTTTCACTGG<br>TCAAGGTTCCCAAGAACAAGGCATGGGTATGGATTTGTACAACTCTTCTGAAGTTGCCCGTAACGTTTGGGATA<br>CCGCCGACAAGCATTTCATCAACCACTATGGCTTCTCCATCTTGGACATTGTGCAAAACAACCCTAAGGAATTG<br>ACTATCCACTTTGGAGGTGCTAAAGGTAGAGCTATCAGAGACAACTACATTGGTATGATGTTTGAAACAATTGGT | |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| | GAAGACGGTTCTTTGAAGTCCGAAAAGATCTTCAAGGACATTGACGAAAACACCACTTCCTACACCTTTGTTTCT<br>GACACTGGGTTGTTGTCTGCTACTCAATTCACCCAACCCGCTTTGACTTTGATGGAGAAGGCTGCCTACGACGA<br>TATCAAGTCTAAAGGATTGATTCCAAGTGACATCATGTTTGCTGGTCACTCTCTTGGTGAATACTCTGCTTTGAC<br>TTCCTTGGCCAACGTTATGCCTATTGAATCCTTGGTTGATGTTGTCTTCTACAGAGGTATGACCATGCAAGTTGC<br>TGTTCCAAGAGACGAGTTTGGTAGATCCAACTACGGTATGGTTGCTGTCAACCCAACCAGAGTCAGCCCAACAT<br>TTGACGATGCCGCCATGAGATTTGTTGTTGACGAGACTGCCAAGAGAACCACCTGGTTGTTGGAAATTGTCAAC<br>TACAATGTTGAAAACCAACAGTACGTTGCTGCTGGTGACTTGAGAGCCTTGGATACCTTGACCAACGTGTTGAA<br>TGTTTTGAAGATCAACAAGATTGATATTGTCAGATTGCAAGAACAATTATCCCTCGACAAGGTCAAGGAGCACTT<br>GTACGAGATTGTTGATGAAGTTGCTGCCAAGTCCATTGCTAAGCCACAACCAATTGAATTAGAAAGAGGTTTTG<br>CTGTTATCCCATTGAAGGGTATTTCTGTCCCATTCCACTCTTCCTACTTGATGTCTGGTGTCAAGCCATTCCAGA<br>GATTCTTGTGCAAGAAGATTCCAAAGGCTTCCATCAAACCACAAGATTTGATTGGCAAGTACATTCCTAACTTGA<br>CTGCTAAGCCATTTGAACTTACTAAGGAATACTTCCAGGATGTCTACGACTTGACTAAATCTGAAAAGATCAAGG<br>CTATCTTGGACAACTGGGAAAAATACGAATAG | |
| *FAS2* fatty acid synthase (*Candida viswanathii* ATCC20336) | ATGAAGCCAGAGATTGAACAAGAATTATCCCACACCTTGTTAACAGAATTGTTAGCTTATCAGTTCGCTTCTCCA<br>GTCAGATGGATCGAAACCCAAGATGTCTTCTTGAAGCAACACAACACCGAAAGAATCATCGAAATCGGCCCTTC<br>CCCAACCTTGGCCGGTATGGCCAACAGAACCATCAAGGCCAAATACCAATCCTACGACGCCGCGTTGTCCTTG<br>CAAAGAGAAGTCTTATGCTACTCTAAGGACGCCAAGGAGATCTACTACAAGCCAGATCCAGCAGATCTTGCTCC<br>AAAGGAAGAACCAAAGAAGGAAGAAGCTGCCGCCGCTCCAGCCGCTACACCAGCTGCTGCTGCTGCTGCTGC<br>TACTCCTGCTGCTGCCCCAGTCGCCGCTGCTCCAGCCCCATCTGCTGGCCCTGCTGAATCCATCCCAGATGAA<br>CCAGTCAAGGCTTCCTTGTTGATCCACGTCTTGGTTGCTCAGAAATTAAAGAAACCATTGGATGCTGTTCCAATG<br>TCCAAGGCTATCAAAGATTTAGTTAACGGTAAGTCCACTGTCCAGAACGAAATTCTTGGTGACTTGGGTAAAGA<br>ATTCGGTTCCACTCCTGAAAAACCAGAAGATACCCCATTGGAAGAATTGGCCGAACAGTTCCAAGACTCCTTCA<br>GTGGTCAATTGGGTAAGACTTCTACTTCATTGATTGGTAGATTGATGTCTTCTAAGATGCCTGGTGGTTTCTCAA<br>TCACCGCTGCCAGAAAATACTTGGAATCCAGATTCGGTTTGGGTGCCGGTAGACAAGACTCTGTCTTGTTGGTT<br>GCTTTGACCAACGAACCTGCAAGCAGATTGGGTTCTGAGGCCGAAGCTAAGACCTTCTTGGACACCATGGCTC<br>AGAAATATGCCTCATCTGCTGGTATTTCCTTGTCGTCAGCTTCTGCCGGTGCCGGTGCTGGAGGTGCCGCCGG<br>TGGCGCCGTTGTTGACAGTGCTGCTTTGGACGCCTTGACTGCTGAAAACAAGAAATTGGCTAGACAACAATTAG<br>AGGTCTTGGCTAGATACTTGCAAGTCGACTTGAACTCAGGAGCTAAGTCTTTTATCAAAGAAAAAGAAGCTTCC<br>GCTGTTTTGCAGAAAGAATTGGACTTGTGGGAAGCCGAACATGGTGAATTCTACGCCAGAGGTATCAAACCAAC<br>TTTCTCAGCTTTGAAAGCAAGAACCTATGATTCCTACTGGAACTGGGCCAGACAAGATGTTTTGTCCATGTACTT<br>TGATATTTTGTTTGGTAAGTTGACCTCCGTTGACAGAGAAACCATCGACCAATGTATCCAAATTATGAACAGATC<br>CAACCCAACTTTGATCAAGTTCATGCAATACCACATTGACCACTGCCCAGAATACAAGGGTGAGACTTACAAGT<br>TGGCCAAGAGATTGGGTCAACAGTTGATTGACAACTGTAAGCAAACCTTGAATGAAGACCCAGTGTACAAGGAC<br>GTTTCTAGAATCACTGGTCCAAAGACCACCGTCTGCGCCAAGGGTAACATTGAATACGAAGAAGCCGAAAAGG | 103 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| | ATTCTGTTAGAAAGTTTGAACAGTACGTCTACGAAATGGCCCAAGGTGGTGAAATGACCAAGATTGCCCAACCA<br>ACTATTCAAGAAGACTTGGCCAGAGTTTACAAAGCCATCTCCAAGCAAGCTTCCAGAGACAGCAAGTTGGAATT<br>GCAGAAAGTCTACGAGCAATTGTTGAAGGTTGTTGCTGGTTCAGACGAAATTGAAACTCAGCAATTAACCAAGG<br>ACATCTTGCAAGCTCCAACTGGCTCCAACACCCCAACTGATGAAGATGAAATTTCCACCGCCGACTCTGACGAT<br>GAAATTGCTTCATTGCCAGACAAGACTTCAATTGCCCAACCAGTTTCTTCAACTGTTCCACCCCAGACCATCCCA<br>TTCTTGCACATTCAAAAGAAGACCAACGAAGGCTGGGAATACGACCGCAAGTTGTCTGCCCTTTACTTGGACGG<br>TTTGGAATCCGCTGCTGTCAACGGTCTCACCTTCAAGGACAAGTACGTTTTGGTTACCGGTGCTGGTGCTGGAT<br>CCATTGGTGCCGAAATCTTGCAAGGTTTGATCAGTGGTGGTGCCAAGGTTGTTGTTACCACCTCTAGATTCTCC<br>AAGAAGGTTACTGAGTACTACCAAAACATGTACGCCAGATACGGTGCTGCCGGTTCTACTTTGATTGTTGTTCC<br>ATTCAACCAAGGTTCTAAACAAGATGTTGACGCTTTGGTTCAATACATCTACGACGATCCAAAGAAGGGTGGTTT<br>AGGCTGGGACTTGGATGCCATTATCCCATTCGCTGCTATCCCAGAAAATGGTAACGGTATCGACAACATTGATT<br>CTAAATCCGAATTTGCCCACAGAATTATGTTGACCAACCTTTTGAGATTGTTGGGTGCTGTCAAATCCAAGAAGA<br>CTACCGACACCAGACCAGCTCAATGTATCTTGCCAATGTCTCCTAACCACGGTACTTTCGGTTTCGATGGGTTG<br>TACTCTGAATCCAAGATTTCCTTGGAAACCTTGTTCAACAGATGGTACTCCGAAGATTGGGGCTCCAAGTTGAC<br>CGTCTGTGGTGCCGTTATTGGTTGGACCAGAGGTACTGGTTTGATGAGCGCCAACAACATCATTGCCGAAGGT<br>ATCGAAAAGATTGGTGTCAGAACCTTCTCCCAAAAGGAAATGGCTTTCAACATCTTGGGTTTGTTGACTCCAGA<br>GATTGTCAAGTTGTGCCAAGAAGAACCAGTTATGGCCGACTTGAACGGTGGTTTGCAATTCATTGAAAACTTGA<br>AGGATTTCACTTCCAAGTTGAGATCTGACTTGATGGAATCCGCTGAAGTTAGAAGAGCTGTCTCCATTGAATCC<br>GCCATCGAACAAAAGGTTGTCAATGGTGACAATGTTGATGCCAACTACACCAAGGTTACCGTTCAACCAAGAGC<br>CAACATGAAATTCGACTTCCCAACCTTGAAATCGTACGATGAGATCAAGAAGGTTGCTCCAGAATTGGAAGGCA<br>TGTTGGACTTGGAATCCGTCGTTGTTGTCACCGGTTTTGCCGAAGTTGGTCCATGGGGTAACGCCAGAACCAG<br>ATGGGAAATGGAATCCAAGGGTGAATTCTCCTTGGAAGGTGCCATTGAAATGGCCTGGATCATGGGTTTCATCA<br>AGTACCACAACGGTAACTTGAAGGGTAAGCCTTACTCTGGTTGGGTTGATGCCAAGACCCAAACTCCAATCGAT<br>GACAAGGACATCAAGGCCAAGTACGAAGAAGAGATCTTGGACCACTCTGGTATTAGATTGATTGAGCCAGAATT<br>GTTCAATGGCTACGATCCAAAGAAGAAGCAGATGATCCAAGAAGTTGTCATCCAACATGACTTGGAACCATTTG<br>AAGCCTCCAAGGAAACTGCTGAACAATACAAACACGAACACGGTGACAAGTGTGAGATCTTTGAAATTGAAGAA<br>TCCGGTGAATACACTGTTAGAATCTTGAAAGGTGCTACCTTGTTTGTTCCAAAGGCTTTGAGATTTGACAGATTG<br>GTTGCTGGTCAAATTCCAACTGGTTGGGATGCTCGTACCTACGGTATTCCAGAAGATACCATTAACCAAGTTGA<br>TCCTATCACTTTGTACGTCTTGGTTGCTACCGTTGAAGCTTTGTTGTCTGCTGGTATCACCGACCCATATGAATT<br>CTACAAGTACGTCCACGTTTCCGAAGTTGGTAACTGTTCTGGTTCCGGTATGGGTGGTGTCTCTGCCTTGAGAG<br>GAATGTTCAAGGACAGATACGCCGACAGACCAGTGCAAAACGATATCTTGCAAGAATCTTTCATCAACACCATG<br>TCCGCCTGGGTTAACATGTTGTTGTTGTCTTCTTCGGGTCCAATCAAGACCCCAGTTGGTGCCTGTGCTACCGC<br>TGTTGAATCCGTTGACATTGGTATTGAAACTATTTTGTCTGGTAAGGCTAAGGTTGTTATGGTTGGTGGTTACGA<br>TGACTTCCAGGAAGAAGGTTCTTATGAATTCGCCAACATGAATGCCACTTCCAACTCCCTTGACGAGTTTGCTC<br>ACGGCAGAACTCCAAAGGAGATGTCCAGACCAACTACCACCACCAGACACGGTTTCATGGAGGCCCAAGGTTC | |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| | TGGTATCCAAGTTATTATGACTGCTGACTTGGCCATCAAGATGGGTGTTCCAATTCACGCTGTGTTGGCCATGT<br>CTGCTACTGCTACCGACAAGATTGGTAGATCTGTTCCAGCTCCAGGTAAGGGTATTTTGACCACTGCCAGGGAA<br>CACCACGGTAACTTGAAGTACCCATCTCCAGCTTTGAACATCAAGTACAGAAAGAGACAATTGAAGGCTAGATT<br>AGACCAAATCAAGGCTTGGGAAGAAGCTGAAATTGCTTACTTGCAAGACGAAGCTGAGTTGGCCAAGGAAGAA<br>ATGGGCGATGAGTTCTCCATGCACGAATTCTTGAAGGAAAGAACTGAAGAAGTGTACCGTGAATCCAAGAGACA<br>AGTTTCTGACGCTAAGAAGCAATGGGGTAACCAATTCTACAAGTCTGACCCAAGAATTGCTCCATTGAGAGGTG<br>CCTTGGCTGCTTTCAACTTGACCATTGACGATCTTGGTGTTGCTTCCTTCCACGGTACTTCTACCGTCGCCAAC<br>GATAAGAACGAATCCGCCACTATTAACAGCATGATGCAACACTTGGGCAGATCTGAAGGTAACCCAGTGTTTGG<br>TGTTTTCCAGAAGTACTTGACTGGTCATCCAAAGGGTGCTGCTGGTGCTTGGATGTTGAACGGTGCCATCCAGA<br>TCTTGGAGTCTGGTATTGTTCCAGGTAACAGAAATGCCGATAACGTTGACAAGGTCTTGGAAGAATACGAGTAC<br>GTCTTGTACCCATCCAGATCCATCCAAACTGACGGTATCAAGGCCGTTTCCGTGACCTCTTTCGGTTTCGGTCA<br>AAAAGGTGCTCAAGCTGTTGTCGTCCACCCAGACTACTTGTTTGCTGTTTTGGATAGATCTACTTATGAGGACTA<br>CGCCACCAGAGTTTCTGCCAGAAACAAGAAGACTTACCGTTACATGCACAATGCTATTACTAGAAACACTATGTT<br>TGTTGCTAAGGATAAGGCTCCATATGCCGATGAATTGGAACAACCAGTTTACTTGGACCCATTAGCCCGTGTTG<br>AAAACGCTAAGGAAAAGCTTGCCTTCAGCAACAAGAGTATCCAATCCAACCAAGCTTATGCTGGTGAAAATGCC<br>AGAACCACTGCCAAGGCTTTGGCTGCCTTGAACAAGTCATCCAAGGGTGTTGGTGTCGACGTTGAATTATTGTC<br>TGAGCTCAACTTGGAGAATGAAACTTTTGTTGCAAGAAACTTCACTCCTGGTGAAATCCAATACTGCTCCAAGAG<br>TGCCAACCCACAAGCTTCATACACCGGCACTTGGTCTGCTAAAGAAGCTGTCTTCAAGGCATTAGGTGTTGAAT<br>CTAAAGGTGCTGGTGCCAGCTTGGTTGACATTGAGATCACTCGTGACGTCAACGGCGCTCCACAAGTTGTCTT<br>GCACGGTGATGCGGCAAAATCAGCCGCCAAAGCTGGTGTCAAGAACGTCAAGATTTCCATCTCCCATGACGAC<br>TTCCAAGCCACTGCTGTTGCCTTGAGTGAATTCTAG | |
| *HPD1* 3-hydroxy-propionate-dehydrogenase (*Candida viswanathii* ATCC20336) | Atgttgagatcttcagtccgtactttctccacccagtccagagtattagccaactacggtttcgtaggcttgggtctcatgggccagcacatggccagacacgtctacaa<br>ccagttgcagccagcagacaagttgtatgtccacgacgtcaacccccagcacaccacccagttcgtcaccgacgtgaccacccagaagccacagaacgccac<br>acaattgacgcccttgtcctccttgaaagagttcaccaccgagccagagtcccagttggacttcatcgtcaccatggtccccgagggcaagcacgtcaaggccgttg<br>tctccgagctagtcgaccactacaatgcgtcgggaaaatacgacccatccaagaagttgacctttgtggactcctccaccatcgacatccccacctccagggaggt<br>ccaccagctcgttgccgacaagttacaaggcgccacgttcatcgacgccccggtttcgggtggtgtcgctggtgccaggaacggaaccttgtcgttcatggtgtcgc<br>gggacaccaaggaagacgtcgaccctaacctcgtcacgcttttgaactacatgggcagcaacatcttcccatgtggtggaacccacgggaccggcttggctgcca<br>agttggcaaacaactacttgttggcgatcacgaacatcgccgtcgcagatagcttccagttggcaaactcgttcgggttgaacttgcagaactacgccaagttggtgt<br>cgacctccacaggtaagtcctgggctagtgtcgataactgcccaatccccggtgtctaccctgaaaagaacttgacttgtgataacggatacaagggtgggtttgtca<br>cgaagttgacgagaaaggatgtcgtcttggctacggagtctgctaaggctaacaaccagttccttatgcttggcgaagtcggtagatactggtacgacaaggcttgtg<br>aagatgaaaagtacgccaacagagacttgtctgttcttttcgaattcttgggtgatcttaaaaaataa | 104 |



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**Table 19**

| Gene (Organism) | Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted) | SEQ ID NO |
|---|---|---|
| *ALD6* malonate semialdehyde dehydrogenase (*Candida viswanathii* ATCC20336) | atgttatccagagttcttttcaagactaaaccaagagttcctactaaatcaatcaccgccatggccatcagaaacaaatccatcgtgactttatcctccaccacctccac atacccaaccgaccacacgaccccgtccacggagccatacatcacgccatccttcgtgaacaacgagttcatcaagtcggactccaacacctggttcgacgtgca cgacccggccacgaactacgtcgtgtccaaggtgccacagtcgacgccggaggagttggaagaggcgatcgcgtcggcccatgccgcgttccccaagtggcgc gacaccagcatcatcaagcgtcaggggatcgcgttcaagtttgtgcagttgttgcgcgagaacatggacagaatcgcaagcgtcattgtcttggaacagggtaaga cgtttgtcgatgcccagggtgacgtgactagaggattgcaggttgctgaggctgcgtgcaacatcactaatgacttgaagggtgagtcgttggaagtgtctactgatat ggagaccaagatgattagagaaccttgggtgttgtgggatccatctgtcctttaacttcccagctatggtcccattgtggtctttgcctttggttttggtcacgggtaacact gctgtgattaagccttccgagagagtcccgggcgcaagtatgattatttgtgaattggccgccaaggctggtgttccacctggtgtgttgaacattgtccacggtaagca cgacaccgtcaacaagttgattgaggacccaagaatcaaggcattgacttttgttggtggtgacaaggccggtaagtacatttacgaaaagggttccagtttgggca agagagtgcaggccaacttgggtgctaagaaccacttggttgtgttgccagacgcacacaagcagagttttgtcaatgccgtcaacggtgccgctttcggtgctgctg gacagagatgtatggctatttctgtcttggtcaccgtgggtaagaccaaggaatgggtgcaggatgtcatcaaggacgccaagttgttgaacaccggaagtggattt gacccaaagagtgacttgggtccagtcatcaacccagagtccttgactcgtgctgaagaaatcattgctgattccgtggccaacggtgccgtgttggaattggacgg aagaggatacagaccagaagacgctagattcgccaagggtaacttcttgggtccaaccatcttgaccaacgtcaagccaggcttgagagcatacgacgaagag attttcgctcctgttttgtctgtggttaacgtcgacaccattgacgaagccattgagttgatcaacaacaacaagtacggtaacggtgtttcattatttacttcctccggtggc tcagcccagtatttcaccaagagaatcgacgtcggtcaagtcggtatcaatgtcccaatccctgttccattgcctatgttctccttcactggttccagaggctccttcttggg tgacttgaacttctacggtaaggccggtatcaccttcttgaccaagccaaagaccatcactagtgcctggaagaccaacttgattgatgacgagatcttgaaaccatc tacctcgatgcctgtccaacagtaa | 105 |
| *ECI1* enoyl-CoA isomerase (*Candida viswanathii* ATCC20336) | Atgtccgacgaggaatcagatatcttatacgaggtcagagacagaaccgccatcatcaccttgaacatccccaagagattgaacgcattgaacggtgctcaatact tgaagttgggtaagttcttggagagagccaacaacgaagaggacaccgtcttgacattgatccaggccctgggcagattcttctccgccggtgccaatttcgccgac aacgatatggccaaggtcgaaatgtccaagttgttcagtcacgagtactggttggaaagattcgtcgccagaaacatctggttgaccaacttgttcaacgaccacaa gaagatcttggctgctgctgtcaatggtccagttatcggtttgagcactggtttgttgttgttggtcgatttggtctacgtccacgacttgaacaagttctacctcttggcccca tttgccaacttgggtttggttgccgaaggtgcttcctctgccactttgttcaacagattgggctggtcaaaggcttctgaggccttgttggttggccaagccaatcggcggcc aagactgttacaacgccggtttcatcaacaagcactacgacggtaagttttcctccactgaagagttcaacgaacacgtctacaaggagttgacggaagcttttgaa aacttgcacgatgactccattttgcagaacaagcaattgttgaagttgtccagagaccaggccatcaactag | 106 |
| *ECI2* enoyl-CoA isomerase (*Candida viswanathii* ATCC20336) | Atgtccgacgaccttatcacctacgaagtcaaagaccgagctgccgtgatcaccttgaacaaccccaagaagctcaacgccttatcgatcccgcagtacgacacc atctgcaagctcttagaacgagccaacgccgaagaagacaccgtcatcaccttgctccagtccacgggccgggtgttctctgccggggccaacgccgactccatc gtggggcaggatgccgagctcgagacctggttgaacatgtcggtggccaagcagacgttcttggtgcagacgttcctcgcacacaagaagatccttgccgtcgcctt gaacggccccgtgattggcttatcggcggcgttcgtggcgctctgcgacttggtctacgtgcacaacgccgcaaagacgttcttcttgaccccgttcgccaacatcgg gatccttgccgagggcggcacctcagccacgttgcccatgcgcgtggggtggtccagggccgcggaagcgttgttgttgtcaaagaggatttcgggagatgacttg cagagagcggggttcttcaataaggactacaaggggcagttcaagtccgcggaggagtttaacgaggtcgtcttgaaggagttgcttgacgccacggaaaacttg catgaggactcgatcatccagaacaaggagttgttgaaggctattttcaagccaaagatcagtgaggtcaactcgcaggaggtgtcaagaggtgtgtacaagtgg acctctggggtgccaatggatagatttaaaaaattgcttaatggtgagttgaaacataaattatag | 107 |



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**Table 19**

| Gene (Organism) | Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted) | SEQ ID NO |
|---|---|---|
| *DCR1* dienoyl-CoA reductase (*Candida viswanathii* ATCC20336) | atgccaaacactttagatcacaactaccttaagaaaagcgtctggaagtcagatatctttgccggcaaagtcatcttcatcactggtggtgccggtaccatctgtcgtgt gcaagccgaagccatggtcttgttgggtgctaatgctgccatcattggtagaaacgtcgaaaagactgaagaagccgctaaggagattgcttccttgagaccaggga gccaaggtcattggtattggtgccgttgacgtgcgtaagatccaaactatcaaggatgccgttgacagaactgttgccgagttgggcagaatcgactatgtcattgctg gtgccgccggtaacttcctttgtgacttcaaccacttgtctgccaacgccttcaagtccgtgattgacattgatttgttgggttctttcaacaccgtcaaggttacctttgacc agttgagaaagaacaaaggtgccgtgttgtttgtcagtgccactttgcactacaccggtgttcctatgcaatcgcacgttggtgctgccaagtctggtgttgatgccttga gcaacgcccttgctgttgaattgggtccattgggtatcagattcaactgtattgccccaggtgccattgctggtactgaaggtatgctgagattggccccacctaccgac acgccattggagacgaagattccattgcagagacaaggtactactgaggacattgctgatgccactgtgtttttgttctcgccagcttcctcctacattactggtgatgtttt ggttgttgatggtgctatgtggcaaactggcggaggtattcttaacgacttctacccagacatcattattcaccagaacgccgacccagaaggtaagttgtaa | 108 |
| *DCR2* dienoyl-CoA reductase (*Candida viswanathii* ATCC20336) | atgccaaacactttaactgaagcatacaaacaagaaagttcttggaagccagatttgttcaagggcaaagtcgtcttcatcaccggtggtgcaggttcgatctgccgt gtccaggccgaagccatggtcttgttgggtgccaacgctgccatcattggtagaaacgtcgaaaagaccgaatccgccgccaaagaaatcgccagcttgagatca ggagccaaggttcttggtattggtggcattgatgtgcgtaaagttgacagcttgaagagcgccgtcgataagaccgttgccgaattgggcagaatcgactttgtcattg ctggtgccgccggtaacttcctttgtgacttcaaccacttgtcttccaacgccttcaaatccatcattgacattgacttgttgggttctttcaacaccgtcaaggtcacttttga ccaattgagaaagaacaagggtgccatcttgtttgtcagtgccaccttgcactactacggtgttcctttccaaattggtgttggtgctgccaaggctggtgtcgacgcctt gagcaatgcccttgccgttgagttgggtccattgggcatcagatccaactgtattgccccaggtccaatcgacggtaccgaaggtgttgaaagattggtcagagcttc caaggccgaagccgccaagaaggttccattgcaaagattgggtactacacaagacattgctgacggtaccgtctacttgttctccccagccgcctcctttgtcactgg tgacgttttggttgttgatggtgctagctggcagatctcctctggtgttggtgccaccaactacccagtctccatcctcaatgcgatcgacgcaccgaagggtggtaagtt gtaa | 109 |
| *MCR* malonyl-CoA reductase (*Sulfolobus islandicus*) | atgaggagaactcttaaggccgccattttgggatcgactggattggttggtatcgaatatgttagaatgttagcaaaccacccttacataaaggtgggttacctagcgg gtaagggctcagtcggcaagccctacgaagaagttgtcagatggcaaaccgtcggccaagttcctaaagaaatcgctgatatggaagtcaagcctaccgatccc aagttgatggacgacgtcgacattatcttttcgccgttgccccaaggcgctgctggtcctgttgaagaggaatttgccaaacatggatttcctgtcatttccaactctcctg atcacagattcgacccagatgtccctctcctcatcccagaaatcaatccgcatacaatctcgcttattgacaaacaaagggaaaggcatgactggaggggcttcatc gttaccactcctttgtgcaccgcgcaaggtgctgccattcctttggcccctatctatatgaatttcagaatcgactccagtctcatcactaccattcaatcgctctctggcgc tgggtatccaggtatcccatcattagacgttgtcgacaacgtcttgccattaggcgataattatgacaacaaaacagttaaagagatttccaggattttgtccgagaca aagaggatggtgaacgatgacaacgatctatcgttgggcgccactacccacagaatctcgactattcacgggcattacgaggtcatttatgtaaccttcagagaaga cgtttccgtcgaaaagataagagagacgttagactcatttagaggtgaaccccagaagttgaagttgcctaccgcgcctgacaagcctatacttttgaccaaccaag acgcaagaccacaagtctactttgacaggtgggccggagaccctccaggtatgtccgtggtcgtgggtagattgagtcaaatcaacaggagaaccattaggctcgt ctcggtcgttcataataccgtcaggggagctgctggtggtggaatcttggccgctgaactactcgtagagaagggttacattgacaagaggtaa | 323 |



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| Table 19 | | |
|---|---|---|
| **Gene (Organism)** | **Nucleic acid sequence of the gene protein-encoding sequence (from START codon to STOP codon except where otherwise noted)** | **SEQ ID NO** |
| *2PS* 2-pyrone synthase (*Gerbera hybrida*) | atgggatcctactcaagtgacgacgtcgaagtcattcgtgaagccggacgagcgcaaggtctcgccaccatttagccattggcactgctactcctccgaattgtgttg<br>ctcaagctgactatgcggattattatttccgtgttactaagtctgagcatatggtggacttaaaagaaaaattcaaacggatttgcgaaaaaacggcaataaagaaac<br>ggtacctagccttgacagaggattatctccaagaaaacccaacgatgtgcgaatttatggctccaagtctcaacgctcggcaagatctagtcgttacaggcgttcca<br>atgttaggcaaagaggccgcggttaaggccattgacgagtggggactaccaaaaagtaagatcacacacttgatcttttgtacaacagctggcgtggatatgccgg<br>gtgctgattatcaattggttaaattgttaggtttgagtccttcagttaaacggtatatgctttaccaacagggatgcgccgccggcggcacggttttgcgactagccaagg<br>atttagctgagaacaacaagggctcacgggttttaatcgtttgtagtgaaatcactgctatcctctttcatggaccgaatgaaaaccacttagattcattggttgctcaag<br>ctctctttggagatggagctgcggcgttgattgtcggttcaggccctcaccttgccgtagagcgaccaatatttgaaatcgtctcaactgaccaaacgatccttcccgat<br>actgaaaaggcgatgaagctccaccttagagaaggagggcttaccttccagcttcatagagacgtaccgcttatggttgcgaagaacatagaaaacgcggcaga<br>aaaagcactttccccactagggataactgactggaactcagtgttttggatggtccacccaggtggtcgggccatacttgatcaggtcgaacggaaactaaacttaa<br>aggaggacaagctcagggcttctaggcatgtcttaagcgagtacggaaacctcatttctgcttgcgtccttttatcattgatgaagtcaggaagagatccatggcaga<br>ggggaagagcacgacaggtgagggtcttgactgtggtgtgcttttcggattcggacccggtatgactgtggaaactgtggtgttacgttctgttcgggtgactgctgcag<br>tggccaatggaaactaa | 325 |

*EXAMPLE 27: Nucleic acid sequences of plasmids and other nucleic acids referenced herein*

| Table 20 | | |
|---|---|---|
| **Plasmid** | **Nucleic acid sequence** | **SEQ ID NO** |
| pAA073 | aaacgccagcaacgcggccttttacggttcctggccttttgctggccttttgctcacatgttctttcctgcgttatcccctgattctgtggataaccgtattaccgcctttgagt<br>gagctgataccgctcgccgcagccgaacgaccgagcgcagcgagtcagtgagcgaggaagcgagttcggcatggcagatcatcatgcctgcaggagctccaa<br>ttgtaatatttcgggagaaatatcgttggggtaaaacaacagagagagagagggagagatggttctggtagaattataatctggttgttgcaaatgctactgatcgact<br>ctggcaatgtctgtagctcgctagttgtatgcaacttaggtgttatgcatacacacggttattcggttgaattgtggagtaaaaattgtctgagttgtgtcttagctactggct<br>ggcccccgcgaaagataatcaaaattacacttgtgaatttttgcacacacaccgattaacatttccctttttttgtccaccgatacacgcttgcctcttctttttttttctctgtgct<br>tcccctcctgtgacttttttccaccattgatataaaatcaactccatttccctaaaatctccccagattctaaaaacaacttcttctcttctgcttttccttttttttttgttatatttattta<br>ccatccctttttttttgaatagttattccccactaacattgttcaaatcttcacgacataagaagagcccgggtctagatgtgtgctcttccgagtgactcttttgataagagtc<br>gcaaatttgatttcataagtatatattcattatgtaaagtagtaaatggaaaattcattaaaaaaaaagcaaatttccgttgtatgcatactccgaacacaaaactagcc<br>ccggaaaaaccccttagttgatagttgcgaatttaggtcgaccatatgcgacgggtacaacgagaattgtattgaattgatcaagaacatgatcttggtgttacagaac<br>atcaagttcttggaccagactgagaatgcacagatatacaaggcgtcatgtgataaaatggatgagatttatccacaattgaagaaagagtttatggaaagtggtca<br>accagaagctaaacaggaagaagcaaacgaagaggtgaaacaagaagaagaaggtaaataagtattttgtattatataacaaacaaagtaaggaatacagat<br>ttatacaataaattgccatactagtcacgtgagatatctcatccattccccaactcccaagaaaaaaaaaaagtgaaaaaaaaaatcaaacccaaagatcaacct | 110 |



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**Table 20**

| Plasmid | Nucleic acid sequence | SEQ ID NO |
|---|---|---|
| | ccccatcatcatcgtcatcaaacccccagctcaattcgcaatggttagcacaaaaacatacacagaaagggcatcagcacacccctccaaggttgcccaacgttt attccgcttaatggagtccaaaaagaccaacctctgcgcctcgatcgacgtgaccacaaccgccgagttcctttcgctcatcgacaagctcggtccccacatctgtct cgtgaagacgcacatcgatatcatctcagacttcagctacgagggcacgattgagccgttgcttgtgcttgcagagcgccacgggttcttgatattcgaggacagga agtttgctgatatcggaaacaccgtgatgttgcagtacacctcgggggtataccggatcgcggcgtggagtgacatcacgaacgcgcacggagtgactgggaag ggcgtcgttgaagggttgaaacgcggtgcggaggggggtagaaaaggaaaggggcgtgttgatgttggcggagttgtcgagtaaaggctcgttggcgcatggtga atatacccgtgagacgatcgagattgcgaagagtgatcgggagttcgtgattgggttcatcgcgcagcgggacatgggggggtagagaagaagggtttgattggatc atcatgacgcctggtgtggggttggatgataaaggcgatgcgttgggccagcagtataggactgttgatgaggtggttctgactggtaccgatgtgattattgtcggga gagggttgtttggaaaaggaagagaccctgaggtggagggaaagagatacagggatgctggatggaaggcatacttgaagagaactggtcagttagaataaat attgtaataaataggtctatatacatacactaagcttctaggacgtcattgtagtcttcgaagttgtctgctagtttagttctcatgatttcgaaaaccaataacgcaatggat gtagcagggatggtggttagtgcgttcctgacaaacccagagtacgccgcctcaaaccacgtcacattcgcccttgcttcatccgcatcacttgcttgaaggtatcca cgtacgagttgtaatacaccttgaagaacggcttcgtctacgcgcgagacgaaagggcctcgtgatacgcctatttttataggttaatgtcatgataataatggtttctta gacgtcaggtggcacttttcggggaaatgtgcgcggaacccctatttgtttatttttctaaatacattcaaatatgtatccgctcatgagacaataaccctgataaatgcttc aataatattgaaaaaggaagagtatgagtattcaacatttccgtgtcgcccttattccctttttgcggcattttgccttcctgttttgctcacccagaaacgctggtgaaagt aaaagatgctgaagatcagttgggtgcacgagtgggttacatcgaactggatctcaacagcggtaagatccttgagagttttcgccccgaagaacgttttccaatgat gagcacttttaaagttctgctatgtggcgcggtattatcccgtattgacgccgggcaagagcaactcggtcgccgcatacactattctcagaatgacttggttgagtact caccagtcacagaaaagcatcttacggatggcatgacagtaagagaattatgcagtgctgccataaccatgagtgataacactgcggccaacttacttctgacaac gatcggaggaccgaaggagctaaccgcttttttgcacaacatgggggatcatgtaactcgccttgatcgttgggaaccggagctgaatgaagccataccaaacga cgagcgtgacaccacgatgcctgtagcaatggcaacaacgttgcgcaaactattaactggcgaactacttactctagcttcccggcaacaattaatagactggatg gaggcggataaagttgcaggaccacttctgcgctcggcccttccggctggctggtttattgctgataaatctggagccggtgagcgtgggtctcgcggtatcattgcag cactggggccagatggtaagccctcccgtatcgtagttatctacacgacggggagtcaggcaactatggatgaacgaaatagacagatcgctgagataggtgcct cactgattaagcattggtaactgtcagaccaagtttactcatatatactttagattgatttaaaacttcatttttaatttaaaaggatctaggtgaagatccttttgataatctc atgaccaaaatcccttaacgtgagttttcgttccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatcctttttttctgcgcgtaatctgctgcttgc aaacaaaaaaaccaccgctaccagcggtggtttgtttgccggatcaagagctaccaactcttttccgaaggtaactggcttcagcagagcgcagataccaaatac tgttcttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataa gtcgtgtcttaccgggttggactcaagacgatagttaccggataaggcgcagcggtcgggctgaacgggggttcgtgcacacagcccagcttggagcgaacga cctacaccgaactgagatacctacagcgtgagctatgagaaagcgccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggcagggtcgga acaggagagcgcacgagggagcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgctcgtcag gggggcggagcctatggaa | |
| pAA298 | cccgtagaaaagatcaaaggatcttcttgagatccttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgccggatca agagctaccaactctttttccgaaggtaactggcttcagcagagcgcagataccaaatactgtccttctagtgtagccgtagttaggccaccacttcaagaactctgta gcaccgcctacatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgtgtcttaccgggttggactcaagacgatagttaccggataagg cgcagcggtcgggctgaacgggggttcgtgcacacagcccagcttggagcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcg ccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggcagggtcggaacaggagagcgcacgagggagcttccagggggaaacgcctggta tctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaaacgccagcaacgcggccttttacg | 111 |



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| Table 20 | | |
|---|---|---|
| **Plasmid** | **Nucleic acid sequence** | **SEQ ID NO** |
| | gttcctgggcttttgctggccttttgctcacatgttctttcctgcgttatcccctgattctgtggataaccgtattaccgcctttgagtgagctgataccgctcgccgcagccga<br>acgaccgagcgcagcgagtcagtgagcgaggaagcggaagagcgcccaatacgcaaaccgcctctccccgcgcgttggccgattcattaatgcagctggcac<br>gacaggtttcccgactggaaagcgggcagtgagcgcaacgcaattaatgtgagttagctcactcattaggcaccccaggctttacactttatgcttccggctcgtatgt<br>tgtgtggaattgtgagcggataacaatttcacacaggaaacagctatgaccatgattacgccaagctatttaggtgacactatagaatactcaagctatgcatcaagc<br>ttggtaccgagctcggatccactagtaacggccgccagtgtgctggaattcgcccttccgttaaacaaaaatcagtctgtaaaaaaggttctaaataaatattctgtcta<br>gtgtacacattctcccaaaatagtgaaatccagctctacaatttggctttaccggtacaaatcaaagaccaatcgtcttcagtcaactcctggtacttgtcgccattcaac<br>cagtagatcaaatccttgccgtcttcacccttggcaacttttggttcttgaattggttcttaggaaccttgtggttgtgcgaagcctcaatggtgccaatcttgatgaacgca<br>ggttgagcatacacaggcaaagacttggtcacgtgagagtgaatcaatttcaagatttcttcatggctcaactcgtccttggcttcacagacggcaaaacaggctcta<br>ccttcgtggtttggcaccttgacaccgacaacgacggactgcttcaaggccttggagcccatcaattcgttctcgacctcggtggcggagacgttttcggacttccaac<br>ggaaagtgtcacctaatctgtcgacaaagtacaacaatttgtcctcgtccatcttcaacaagtcaccggatctgtaccacgcgtcacctttttgaaaacattggtgagg<br>attttgctgttggtggcggacttgttaccataataaccctggaaggatttctgcacgtcgttagggttcaagattctcatcaacaactcacctggctcgttgtaagcggcct<br>cggtacagaacccggtcttggggtccttgtagatttcactctcgtcttctgggtccatcttggccaatttctgctgggtagacaataacaagctgatgagggacccgtact<br>tacgacaggcgccgacaccgtactcaccgtactgcaagttggtggtggcgataggggactcggtggcggcgtagaactcaccgataccttcaatgtggaatctgc<br>gcttgaactcagaccatatatctggacgcaacccgttaccgtaggcaattctgacattgtgtctgtcttggtctggatgaggcttggagttcaacaagtaacgacagac<br>ctcaccgacgtattgcacgtgggtggcaccacataatctggcctgggtccagaacgaagtagcggagaatttctgggacacggagacacagccaccgacaatca<br>aagtaggacacaaccccaacatggccgcggtggagtggtacaagggcatggcggtcaagacgttcgatttcgagtcaatcttcatgatgtggccaaagaaaacc<br>gaggccatgaaggcttttctccaggacatgataccggcttttggcaaaccggtggtacccgaggtgtaaatcaatgcacaagcggaggagtcagtatcggttggtct<br>tctggtcttgtcctcggctctgtgttttggagtcgacttgagtctcaatctgtcaaacaaggcaaactcgtcaatgtagtttatttgcacatgtggcaattcctctctgatctga<br>gcctcggtatctctgattggggaatcacagtccgggtcaacgaaaacttgcgaagcgttgacaatcttaagacagtggatcaatggcttgtccttggtgttgaagttca<br>agaacgcaggcaaggcaccaatgttccacaatgccaaccacaagacaatgaaaagcggcttgttcatacaagaaacaccgatggtgtcgttggcagtgacgcc<br>gtactcgttcttcaagatgtatgagtacttcaaaaccatgtcgtacaattccttgtaggtgtattcttctaggtcaaactgatcgtcgtagatccactagtaacggccgcca<br>gtgtgctggaattcgcccttgggctaacgaaaaggaaaccgctgacgttaaaggtatctacggttgtttcggtatgacccccggggatctgacgggtacaacgaga<br>attgtattgaattgatcaagaacatgatcttggtgttacagaacatcaagttcttggaccagactgagaatgcacagatatacaaggcgtcatgtgataaaatggatga<br>gatttatccacaattgaagaaagagtttatggaaagtggtcaaccagaagctaaacaggaagaagcaaacgaagaggtgaaacaagaagaagaaggtaaat<br>aagtattttgtattatataacaaacaaagtaaggaatacagatttatacaataaattgccatactagtcacgtgagatatctcatccattccccaactcccaagaaaaa<br>aaaaaagtgaaaaaaaaaatcaaacccaaagatcaacctccccatcatcatcgtcatcaaacccccagctcaattcgcaatggttagcacaaaaacatacaca<br>gaaagggcatcagcacacccctccaaggttgcccaacgtttattccgcttaatggagtccaaaaagaccaacctctgcgcctcgatcgacgtgaccacaaccgcc<br>gagttcctttcgctcatcgacaagctcggtccccacatctgtctcgtgaagacgcacatcgatatcatctcagacttcagctacgagggcacgattgagccgttgcttgt<br>gcttgcagagcgccacgggttcttgatattcgaggacaggaagtttgctgatatcggaaacaccgtgatgttgcagtacacctcgggggtataccggatcgcggcgt<br>ggagtgacatcacgaacgcgcacggagtgactgggaagggcgtcgttgaagggttgaaacgcggtgcggaggggggtagaaaaggaaaggggcgtgttgatgt<br>tggcggagttgtcgagtaaaggctcgttggcgcatggtgaatatacccgtgagacgatcgagattgcgaagagtgatcgggagttcgtgattgggttcatcgcgcag<br>cgggacatgggggggtagagaagaagggtttgattggatcatcatgacgcctggtgtggggttggatgataaaggcgatgcgttgggccagcagtataggactgttg<br>atgaggtggttctgactggtaccgatgtgattattgtcgggagagggttgtttggaaaaggaagagaccctgaggtggaggggaaagagatacagggatgctggatg<br>gaaggcatacttgaagagaactggtcagttagaataaatattgtaataaataggtctatatacatacactaagcttctaggacgtcattgtagtcttcgaagttgtctgct<br>agtttagttctcatgatttcgaaaaccaataacgcaatggatgtagcagggatggtggttagtgcgttcctgacaaacccagagtacgccgcctcaaaccacgtcac | |



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| Table 20 | | |
|---|---|---|
| **Plasmid** | **Nucleic acid sequence** | **SEQ ID NO** |
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**Table 20**

| Plasmid | Nucleic acid sequence | SEQ ID NO |
|---|---|---|
| pAA1164 | cccgtagaaaagatcaaaggatcttcttgagatccttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgccggatca<br>agagctaccaactctttttccgaaggtaactggcttcagcagagcgcagataccaaatactgtccttctagtgtagccgtagttaggccaccacttcaagaactctgta<br>gcaccgcctacatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgtgtcttaccgggttggactcaagacgatagttaccggataagg<br>cgcagcggtcgggctgaacgggggggttcgtgcacacagcccagcttggagcgaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcg<br>ccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggcagggtcggaacaggagagcgcacgagggagcttccagggggaaacgcctggta<br>tctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaaaacgccagcaacgcggccttttacg<br>gttcctgggcttttgctggccttttgctcacatgttctttcctgcgttatcccctgattctgtggataaccgtattaccgcctttgagtgagctgataccgctcgccgcagccga<br>acgaccgagcgcagcgagtcagtgagcgaggaagcggaagagcgcccaatacgcaaaccgcctctccccgcgcgttggccgattcattaatgcagctggcac<br>gacaggtttcccgactggaaagcgggcagtgagcgcaacgcaattaatgtgagttagctcactcattaggcaccccaggctttacactttatgcttccggctcgtatgt<br>tgtgtggaattgtgagcggataacaatttcacacaggaaacagctatgaccatgattacgccaagctatttaggtgacactatagaatactcaagctatgcatcaagc<br>ttggtaccgagctcggatccactagtaacggccgccagtgtgctggaattcgcccttaaacgttgggcaaccttggaggggtgtgctgatgccctttctgtgtatgttttt<br>gtgctaaccattgcgaattgagctgggggtttgatgacgatgatgatgggggaggttgatctttgggtttgatttttttttcacttttttttttcttgggagttggggaatggatga<br>gatatctcacgtgactagtatggcaatttattgtataaatctgtattccttactttgtttgttatataatacaaaatacttatttaccttcttcttcttgtttcacctcttcgtttgcttcttc<br>ctgtttagcttctggttgaccactttccataaactctttcttcaattgtggataaatctcatccatttttatcacatgacgccttgtatatctgtgcattctcagtctggtccaagaac<br>ttgatgttctgtaacaccaagatcatgttcttgatcaattcaatacaattctcgttgtacccgtcgcatatggtcgacctaaattcgcaactatcaactaagggtttttccggg<br>gctagttttgtgttcggagtatgcatacaacggaaatttgctttttttttaatgaattttccatttactactttacataatgaatatatacttatgaaatcaaatttgcgactcttatc<br>aaaagagtcactctcttctattcttaactggacaagatttcagcagcttcttcggacttttcacctctttctctgacttcaaggtctggacggttcaacatgtcttccaaagcct<br>tggagtatggagccttggtgttttctggtgggttcaaagccttgacagtttcgaagtagtgttcgtagacgttaccatcatatctaccaatagcagcattggtcatcatgtca<br>gtcaagttgaaaccatcagtcaaaccaacaacgtttggtctaaccttggcacacaaggctgggaagtgctcggaggaaatctttctgctgacatctggggtaatgat<br>accgtactgcaagaaggtggcaccaaagttggtcaagatccagtcggcatacaagaaacccaaaggtctcaagacttcagaccattctggggtgatacgcttgaa<br>gaaaccgtcaatcaagaatctgtgagcgttcaacttggaaaccaaaaccaaacttggaccggtaatgtcgaaaccccttgtcttcagcaacagcaccaatgtctctg<br>gccaagccggaaagggcgtggtcgaaagccttaaccaacttggctgggtcgtccaagtcggcaacgctggagagaaccaatccctttttgttctggctccttcaaca<br>agtctctaaccattggcttggcaacgttcatggccaagatgttgttgtcaccttcccaggtacattggacaacccagtcggagtaggctttaccgaaaccgttgtaaga<br>cgagtaaccgtgaccaccacaagcttgacgagcttcgtcaatggcttcagcagtcaaccaggtacaggtggacttcaaacaaccggaagcgacgaacaatttctt<br>ggattcgacaatggcagcgtcaatggcttccttttcaccagcactgacagcttcgtccaacttgtcgttggttgcgttcatggtttgttctaagtacaaggcaccttgggag<br>aacaagtaagcggcagccaagaatgggaacaatctcttttgatgcaatgggtagtcaatcaacttggtttcaatggtatcggtgtccttcttcttgaattgtcttctgtggat<br>ggcgtatctcaaggcaatggtgatgaatctactggtcattctgtaggagtccatcatcatggtgactctaccaccaatcaaagccgagtaagacaattgttcagatggt<br>ggcatggtgacttcacccagacgggaaaccttacagtactttgcaacataaagaatcttgggattctgacgttggagaactggatccaaccgttatcgataccgtctc<br>tacccatcttggcaccaatgtcaccaacagtcacacctggctcgaggtcgtggttggagtctctcaatgggacaacaaaggtcttgacaccgtagtcctttccttgac<br>cttcaatctggcgtagacggagcagtgggtggcggagtgcgcagcaccaccgatccaccacttggtggcaccaatgtgtggggtgttgatgacaaactcgtcagtg<br>tcttcgtcaaaagtggcggtggtttcaataccttggacgttggaaccgtgggccaactcagtcataccaaaacaaccatagatacctctcaacttgtcgataccctattt<br>atggtccagtagaagaactgggagttggtaccgttaccacggacacaggaaacaaacaaacccaagttgacaccgattctggttccgacttgtgggtcaaagaca<br>ccgaggatcgacaatctctgggcctgttggtctgggtactcgtgctcaaagtatctggagagtctggcaatcttcttggcggtgacttctctttgttggtctttggtcaagttgt<br>agtatgaagcgtcgaccttcaagatagggtctttttgcatttgttcgaccatgttgctgatgatctcggatctttcttgggaaccttccaagaagtagttcaactcctttgggtt<br>gaacttggtgagttctctttctttttgaagttcggtaggcatgattgattgttatagtttctttctttctttttgaggatgaccagatgtttgataataatcagataggtaggaaaag | 112 |



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| Table 20 | | |
|---|---|---|
| **Plasmid** | **Nucleic acid sequence** | **SEQ ID NO** |
| | aataaaaaaaaactaatatggtaggaaagaagaaaaaaaatggtggatgtgaagaattgtgaaaaggggggccaatgcctgaagtttatataaagattcaggct<br>ccagagtttgccctgttgattctgcgggggggccacacacaagcacactgcagacaggaatagtgggggtagtgtgcatgtggataaaaatgcctgtgttttttttttctgt<br>ttcttctgatgccattgtcgcacgggcacaactttggctgtctgccccccccaggcgtaataaccgaggattatctcacaactacagttgtcacttttgccagcaaaatag<br>gaatcaacaagcaatgggttatataacactgcttatacagactactaccaatcccaatacacaccactgagcctactactcacagaaattgagcgaccaccaccc<br>aattgatcgggaatatgtctccctgattatttttccacactttaattaccccataaaacggttacatcaaggataatgaagtgggatatgcaaagtttcaatttttttcctctcc<br>gctctaaccaagggattctccatctctatccgtggtccaaacagggccaaacaccacccaagctctactctagaaccttctggtatctacgtgccagcttaattacatc<br>aactcaacgactcttcttcccttctgcaggcatgatgatctgccatgccgaactagacgaagccgttcttcaaggtgtattacaactcgtacgtggataccttcaagcaa<br>gtgatgcggatgaagcaaagggcgaatgtgacgtggtttgaggcggcgtactctgggtttgtcaggaacgcactaaccaccatccctgctacatccattgcgttattg<br>gttttcgaaatcatgagaactaaactagcagacaacttcgaagactacaatgacgtcctagaagcttagtgtatgtatatagacctatttattacaatatttattctaactg<br>accagttctcttcaagtatgccttccatccagcatcccctgtatctctttccctccacctcagggtctcttccttttccaaacaaccctctcccgacaataatcacatcggtacc<br>agtcagaaccacctcatcaacagtcctatactgctggcccaacgcatcgcctttatcatccaaccccacaccaggcgtcatgatgatccaatcaaacccttcttctcta<br>ccccccatgtcccgctgcgcgatgaacccaatcacgaactcccgatcactcttcgcaatctcgatcgtctcacgggtatattcaccatgcgccaacgagcctttactc<br>gacaactccgccaacatcaacacgccccttccttttctacccccctccgcaccgcgtttcaaccccttcaacgacgcccttcccagtcactccgtgcgcgttcgtgatgtc<br>actccacgccgcgatccggtataccccgaggtgtactgcaacatcacggtgtttccgatatcagcaaacttcctgtcctcgaatatcaagaaccccgtggcgctctgc<br>aagcacaagcaacggctcaatcgtgccctcgtagctgaagtctgagatgatatcgatgtgcgtcttcacgagacagatgtggggaccgagcttgtcgatgagcgaa<br>aggaactcggcggttgtggtcacgtcgatcgaggcgcagaggttggtcttttggactccattaagcggaaaagggcgaattctgcagatatccatcacactggcgg<br>ccgctcgagcatgcatctagagggcccaattcgccctatagtgagtcgtattacaattcactggccgtcgttttacaacgtcgtgactgggaaaaccctggcgttaccc<br>aacttaatcgccttgcagcacatccccctttcgccagctggcgtaatagcgaagaggcccgcaccgatcgcccttcccaacagttgcgcagcctatacgtacggca<br>gtttaaggtttacacctataaaagagagagccgttatcgtctgtttgtggatgtacagagtgatattattgacacgccggggcgacggatggtgatccccctggccagt<br>gcacgtctgctgtcagataaagtctcccgtgaactttacccggtggtgcatatcggggatgaaagctggcgcatgatgaccaccgatatggccagtgtgccggtctcc<br>gttatcggggaagaagtggctgatctcagccaccgcgaaaatgacatcaaaaacgccattaacctgatgttctggggaatataaatgtcaggcatgagattatcaa<br>aaaggatcttcacctagatccttttcacgtagaaagccagtccgcagaaacggtgctgaccccggatgaatgtcagctactgggctatctggacaagggaaaacg<br>caagcgcaaagagaaagcaggtagcttgcagtgggcttacatggcgatagctagactgggcggttttatggacagcaagcgaaccggaattgccagctggggc<br>gccctctggtaaggttgggaagccctgcaaagtaaactggatggctttctcgccgccaaggatctgatggcgcaggggatcaagctctgatcaagagacaggatg<br>aggatcgtttcgcatgattgaacaagatggattgcacgcaggttctccggccgcttgggtggagaggctattcggctatgactgggcacaacagacaatcggctgct<br>ctgatgccgccgtgttccggctgtcagcgcaggggcgcccggttctttttgtcaagaccgacctgtccggtgccctgaatgaactgcaagacgaggcagcgcggct<br>atcgtggctggccacgacgggcgttccttgcgcagctgtgctcgacgttgtcactgaagcgggaagggactggctgctattgggcgaagtgccggggcaggatctc<br>ctgtcatctcaccttgctcctgccgagaaagtatccatcatggctgatgcaatgcggcggctgcatacgcttgatccggctacctgcccattcgaccaccaagcgaaa<br>catcgcatcgagcgagcacgtactcggatggaagccggtcttgtcgatcaggatgatctggacgaagagcatcaggggctcgcgccagccgaactgttcgccag<br>gctcaaggcgagcatgcccgacggcgaggatctcgtcgtgacccatggcgatgcctgcttgccgaatatcatggtggaaaatggccgcttttctggattcatcgactg<br>tggccggctgggtgtggcggaccgctatcaggacatagcgttggctacccgtgatattgctgaagagcttggcggcgaatgggctgaccgcttcctcgtgctttacgg<br>tatcgccgctcccgattcgcagcgcatcgccttctatcgccttcttgacgagttcttctgaattattaacgcttacaatttcctgatgcggtattttctccttacgcatctgtgcg<br>gtatttcacaccgcatacaggtggcacttttcggggaaatgtgcgcggaacccctatttgtttatttttctaaatacattcaaatatgtatccgctcatgagacaataaccct<br>gataaatgcttcaataatagcacgtgaggagggccaccatggccaagttgaccagtgccgttccggtgctcaccgcgcgcgacgtcgccggagcggtcgagttct<br>ggaccgaccggctcgggttctcccgggacttcgtggaggacgacttcgccggtgtggtccgggacgacgtgaccctgttcatcagcgcggtccaggaccaggtgg | |



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| Table 20 | | |
|---|---|---|
| **Plasmid** | **Nucleic acid sequence** | **SEQ ID NO** |
| | tgccggacaacaccctggcctgggtgtgggtgcgcggcctggacgagctgtacgccgagtggtcggaggtcgtgtccacgaacttccgggacgcctccgggccg<br>gccatgaccgagatcggcgagcagccgtgggggcgggagttcgccctgcgcgacccggccggcaactgcgtgcacttcgtggccgaggagcaggactgaca<br>cgtgctaaaacttcatttttaatttaaaaggatctaggtgaagatccttttgataatctcatgaccaaaatcccttaacgtgagttttcgttccactgagcgtcagac | |
| *HDE1* promoter from Candida strain ATCC20336 | Aagggaagaagagtcgttgagttgatgtaattaagctggcacgtagataccagaaggttctagagtagagcttgggtggtgtttggccctgtttggaccacggatag<br>agatggagaatcccttggttagagcggagaggaaaaaattgaaactttgcatatcccacttcattatccttgatgtaaccgttttatggggtaattaaagtgtggaaaaa<br>taatcagggagacatattcccgatcaattgggtggtggtcgctcaatttctgtgagtagtaggctcagtggtgtgtattgggattggtagtagtctgtataagcagtgttata<br>taacccattgcttgttgattcctattttgctggcaaaagtgacaactgtagttgtgagataatcctcggttattacgcctgggggggcagacagccaaagttgtgcccgtg<br>cgacaatggcatcagaagaaacagaaaaaaaaaacacaggcatttttatccacatgcacactacccccactattcctgtctgcagtgtgcttgtgtggcccccg<br>cagaatcaacagggcaaactctggagcctgaatctttatataaacttcaggcattggccccctttttcacaattcttcacatccaccatttttttttcttctttcctaccatatta<br>gttttttttattcttttcctacctatctgattattatcaaacatctggtcatcctcaaaagaaagaaagaaactataacaatcaatc | 113 |
| *PGK* promoter from pAA105 | Ttgtccaatgtaataatttttccatgactaaaaagtgtgtgttggtgtaaagaagaaagtggaagggacgttggtgatggtgagttcgtctatccctttttatagttgcttgt<br>atagtaggctactcttctagggactcgatgggggaaggttcttgatatttgcttagttcgagaaggttccagatgagcgagacatttttggtacgacattgggtggatgat<br>ctgcacgacattttgtgattcttgcgacacgctgcactaccaagtgtagtctggctgaacggatcacaagataaacctctgaaaaattatctcagggcatgcaacaac<br>aattatacatagaagagggagtcacgatatacacctgtgaaggaatcatgtggtcggctctccttgaactttgaattcatgcaattattaagaagaagcacaggtgag<br>caacccaccatacgttcatttgcaccacctgatgattaaaagccaaagaaagaaaaaaaaaaaaagaaacaggcggtgggaattgttacaacccacgcgaacc<br>cgaaaatggagcaatcttccccggggcctccaaataccaactcacccgagagagagaaagagacaccacccaccacgagacggagtatatccaccaaggta<br>agtaactcagggttaatgatacaggtgtacacagctccttccctagccattgagtgggtatcacatgacactggcaggttacaaccacgtttagtagttattttgtgcaat<br>tccatggggatcaggaagtttggtttggtgggtgcgtctactgattccccctttgtctctgaaaatctttttccctagtggaacactttggctgaatgatataaattcaccttgatt<br>cccaccctcccttctttctctctctctgttacacccaattgaattttctttttttttttttacttttccctccttctttatcatcaaagataagtaagtttatcaattgcctattcaga | 114 |
| *PGK* terminator from pAA105 | Aaagtacggtgttgttgacaagttgtcccacgtttctaccggtggtggtgcttctttggaattgttagaaggtaaggaattgccaggtgtgactgctctttccaacaaggcc<br>taaatcagaaatatttattctaataaaataggatgcaaaaaaaaaaccccccttaataaaaaaaaaaagaaacgatttttatctaatgaagtctatgtatctaacaaatgt<br>atgtatcaatgtttattccgttaaacaaaaatcagtctgtaaaaaaggttctaaaataaatattctgtctagtgtacacattctcccaaaatagtgaaatccagctctacaatt<br>tagctctaccagtaca | 115 |
| *POX4* terminator from *Candida* strain ATCC20336 | Gaatagaagagagtgactcttttgataagagtcgcaaatttgatttcataagtatatattcattatgtaaagtagtaaatggaaaattcattaaaaaaaaaagcaaatttc<br>cgttgtatgcatactccgaacacaaaactagccccggaaaaacccttagttgatagttgcgaatttaggtcgac | 116 |
| *POX4* promoter from | Gagctccaattgtaatatttcgggagaaatatcgttggggtaaaacaacagagagagagagggagagatggttctggtagaattataatctggttgttgcaaatgct<br>actgatcgactctggcaatgtctgtagctcgctagttgtatgcaacttaggtgttatgcatacacacggttattcggttgaattgtggagtaaaaattgtctgagttgtgtctt | 117 |



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**Table 20**

| Plasmid | Nucleic acid sequence | SEQ ID NO |
|---|---|---|
| pAA073 | agctactggctggccccccgcgaaagataatcaaaattacacttgtgaatttttgcacacacaccgattaacatttccctttttttgtccaccgatacacgcttgcctcttctttttttctctgtgcttccccctcctgtgacttttttccaccattgatataaaatcaactccatttccctaaaatctccccagattctaaaaacaacttcttctcttctgcttttccttttttttttgttatatttatttaccatccctttttttttgaatagttattccccactaacattgttcaaatcttcacgacata | |
| G6PI promoter from pAA2218 | Aaaatcagaggctactccggagatggcacattatcacgtgggcagtcccatctctctgacaatggcactgacaatgccatgtcattctagaagtcgctcgttccattgctagtcgtgtaggtgtcttactcagaccatatgtttggctgtgtgtgtggtaaggggggtaagatatctctaggaggaggcaactctactcgaaagtacgaaatggccgacgccaatccgacctgccattgtgcaggacgcgggtcgagatccggaagtgccctccttctgaggttgttggtttgttccagttggcataagaaattaataatcgtttagtcaatggtacagtattaataattcctgacaaaaccgtgccctacaagcagtgtgagttccgggggtagcactagtggtcgtggtggtagggcccacagagagtatttccggaaccgactgatcctttgattccaatattccaagatttgggtttgtggttgtgtgtaatgcatgaggttattttgtcaacctccttccttctctacggttgttgctcctcctcctcttccttcccttttgttcatttaaatacacacacaccactccccttcccacccacaagaactttttttttttttacttttcttcttctcttacttttcttcttctttcattacctgttagattatatttatcaacctcaattggtttattatctaccacccatacaaccccccaccccctgccacatccaccaaca | 118 |
| *GPD* promoter from *Candida* strain ATCC20336 | Cggaagttgtttaccgacctgaccgtaaatttgctgctgaaagaaacgtgtcaaacaagaccaattggctcaattgaccctgtggaaatgctttgttgaccaccaatgcttccaccaaacgttacttttttttttgcaatcggatggtatgggtctggggttcacctgttttgtaaagctacagaaggtggcatatttctctgatcaggtgttttttttttcggctgctgctgctcgtggtggtgtagtggtagtggtgtgtgtgtgtgtgtgtgcgtgcgtgtggaaggacgctttttgctctctgactcctcccaatcagaagttgctatagtggtgaaacaacaatggatgataatgccccgggcggtgcgtgtccgacacaaaccactacatttttttagctgggagcctactgccactacgacccacccacccatggtcaacaaaaaaattctgacaaattataaaataacccttgaattccccccttggaaaaatttttggtatttctctctctctttttccttccctcttcttttttctctccatcaatcaattgacgttcagtaactcaattaattacatcacatccctcaattaaagaatttaatcgat | 119 |
| *TEF1* promoter from *Candida* strain ATCC20336 | Ctagcaaaggcttgatcagagaaagcaacaaaaaaaaaaaactctaatactccagaatacactcctttagaaacacacaacaaacaagcctagactaccatggactacgatgaagacgatttagattacatttctcaaggagaagaggaagagtttgacgaaaacaagttgaacaacgaagagtacgacttgttgcatgacatgcttccggagttgaagacaaaattgaaagattacaatgatgagatcccagattacgatttaaaggaagcgttatactacaactatttcgagatagaccctaccattgaagaattgaagacgaaattcaaaaagagtacgtatatacaactaacatcaacgcctttctagtttctgttctgtctccaatgcttctcctggtttcttcatggttctctctgtaccaacaaggaaaaaaaaaaaaatctggcaaaaaaaaccaaaccaaccaatgttcttactcaccaacgccctacaatc | 120 |
| *PEX11* promoter from *Candida* strain ATCC20336 | Gaagatgaagcgtatgagtattatgagtactgtcggacgttggaaggtggcagagttaagcccgagaaagcaaggaaggagtgggagatgatgagtgatgcggccaaagaggatgtgaaggctgcgtatctgtttttgatagctggtggtagccgaatagaggaaggcaagcttgttcatattggatgatgatggtagatggtggctgccaaagtggttgtaaatagaaaaaagtgggtttgggtctgttgatagttagtggtggcggctgtctgtgattacgtcagcaagtagcacctcggcagttaaaacagcagcaacagaaaaaaaaatgtgtgaaagtttgattcccccacagtctaccacacccagagttccatttatccataatatcacaagcaatagaaaaataaaaaattatcaacaaatcacaacgaaaagattctgcaaaattattttcacttcttcttttgacttcctcttcttcttgttaggttctttccatattttcccctttaaacccatacacaacgcagcc | 121 |
| pAA2214 | cgaattccagcacactggcggccgttactagtggatccgagctcggtaccaagcttgatgcatagcttgagtattctatagtgtcacctaaatagcttggcgtaatcatggtcatagctgtttcctgtgtgaaattgttatccgctcacaattccacacaacatacgagccggaagcataaagtgtaaagcctggggtgcctaatgagtgagctaactcacattaattgcgttgcgctcactgcccgctttccagtcgggaaacctgtcgtgccagctgcattaatgaatcggccaacgcgcggggagaggcggtttgcgtattgggcgctcttccgcttcctcgctcactgactcgctgcgctcggtcgttcggctgcggcgagcggtatcagctcactcaaaggcggtaatacggttatccacagaatcagggataacgcaggaaagaacatgtgagcaaaaggccagcaaaagcccaggaaccgtaaaaaggccgcgttgctggcgtttttccataggctccgccccccctgacgagcatcacaaaaatcgacgctcaagtcagaggtggcgaaacccgacaggactataaagataccaggcgtttcccccctggaagctccctcgtgcgctctcctgttcc | 122 |



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| Table 20 | | |
|---|---|---|
| **Plasmid** | **Nucleic acid sequence** | **SEQ ID NO** |
| | gaccctgccgcttaccggatacctgtccgcctttctcccttcgggaagcgtggcgctttctcatagctcacgctgtaggtatctcagttcggtgtaggtcgttcgctccaag<br>ctgggctgtgtgcacgaaccccccgttcagcccgaccgctgcgccttatccggtaactatcgtcttgagtccaacccggtaagacacgacttatcgccactggcagc<br>agccactggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttcttgaagtggtggcctaactacggctacactagaaggacagtatttggtatct<br>gcgctctgctgaagccagttaccttcggaaaaagagttggtagctcttgatccggcaaacaaaccaccgctggtagcggtggttttttgtttgcaagcagcagattac<br>gcgcagaaaaaaaggatctcaagaagatcctttgatctttctacggggtctgacgctcagtggaacgaaaactcacgttaagggattttggtcatgagattatcaaa<br>aaggatcttcacctagatccttttaaattaaaaatgaagttttagcacgtgtcagtcctgctcctcggccacgaagtgcacgcagttgccggccgggtcgcgcagggc<br>gaactcccgcccccacggctgctcgccgatctcggtcatggccggcccggaggcgtcccggaagttcgtggacacgacctccgaccactcggcgtacagctcgt<br>ccaggccgcgcacccacacccaggccagggtgttgtccggcaccacctggtcctggaccgcgctgatgaacagggtcacgtcgtcccggaccacaccggcga<br>agtcgtcctccacgaagtcccgggagaacccgagccggtcggtccagaactcgaccgctccggcgacgtcgcgcgcggtgagcaccggaacggcactggtca<br>acttggccatggtggccctcctcacgtgctattattgaagcatttatcagggttattgtctcatgagcggatacatatttgaatgtatttagaaaaataaacaaatagggt<br>tccgcgcacatttccccgaaaagtgccacctgtatgcggtgtgaaataccgcacagatgcgtaaggagaaaataccgcatcaggaaattgtaagcgttaataattc<br>agaagaactcgtcaagaaggcgatagaaggcgatgcgctgcgaatcgggagcggcgataccgtaaagcacgaggaagcggtcagcccattcgccgccaag<br>ctcttcagcaatatcacgggtagccaacgctatgtcctgatagcggtccgccacacccagccggccacagtcgatgaatccagaaaagcggccattttccaccatg<br>atattcggcaagcaggcatcgccatgggtcacgacgagatcctcgccgtcgggcatgctcgccttgagcctggcgaacagttcggctggcgcgagcccctgatgct<br>cttcgtccagatcatcctgatcgacaagaccggcttccatccgagtacgtgctcgctcgatgcgatgtttcgcttggtggtcgaatgggcaggtagccggatcaagcgt<br>atgcagccgccgcattgcatcagccatgatggatactttctcggcaggagcaaggtgagatgacaggagatcctgccccggcacttcgcccaatagcagccagtc<br>ccttcccgcttcagtgacaacgtcgagcacagctgcgcaaggaacgcccgtcgtggccagccacgatagccgcgctgcctcgtcttgcagttcattcagggcaccg<br>gacaggtcggtcttgacaaaaagaaccgggcgcccctgcgctgacagccggaacacggcggcatcagagcagccgattgtctgttgtgcccagtcatagccga<br>atagcctctccacccaagcggccggagaacctgcgtgcaatccatcttgttcaatcatgcgaaacgatcctcatcctgtctcttgatcagagcttgatccccgcgcca<br>tcagatccttggcggcgagaaagccatccagtttactttgcagggcttcccaaccttaccagagggcgccccagctggcaattccggttcgcttgctgtccataaaac<br>cgcccagtctagctatcgccatgtaagcccactgcaagctacctgctttctctttgcgcttgcgttttcccttgtccagatagcccagtagctgacattcatccggggtcag<br>caccgtttctgcggactggctttctacgtgaaaaggatctaggtgaagatccttttgataatctcatgcctgacatttatattccccagaacatcaggttaatggcgttttg<br>atgtcattttcgcggtggctgagatcagccacttcttccccgataacggagaccggcacactggccatatcggtggtcatcatgcgccagctttcatccccgatatgca<br>ccaccgggtaaagttcacgggagactttatctgacagcagacgtgcactggccaggggatcaccatccgtcgccccggcgtgtcaataatatcactctgtacatc<br>cacaaacagacgataacggctctctcttttataggtgtaaaccttaaactgccgtacgtataggctgcgcaactgttgggaagggcgatcggtgcgggcctcttcgct<br>attacgccagctggcgaaaggggatgtgctgcaaggcgattaagttgggtaacgccagggttttcccagtcacgacgttgtaaaacgacggccagtgaattgtaa<br>tacgactcactatagggcgaattgggccccatgctcgagagtgctccgaaaatacctcaagggtggttagagttacatgataaacaacaggccaaaaaaagaga<br>gggcgtttctcatagaggggttttttctttcgttcaaatctaggggggttgagaggcggaagtcctggcggaacagaagtgagctgggtaatcatacatctcaagtaatg<br>ttgcataccggagagaagaaaaaacttggccctcttctctggctgaggagtaaactagaggacggcaggtcggatagttttttttgactgtcgtagctattgcctatgg<br>ccagcaccagcaccatcacaaactccagctctcgttgacacacctgcccgcgagcatgcggcccatgcagcggaaaagagacctctttgacggaaatatgtctta<br>gggggtgttttcttactgggggggttggtaggacttcggaaacacggagtcctcttcttcggaacgccccctggttgccggccggttcgtcgcttgatccccctgcagga<br>aaatcagaggctactccggagatggcacattatcacgtgggcagtcccatctctctgacaatggcactgacaatgccatgtcattctagaagtcgctcgttccattgct<br>agtcgtgtaggtgtcttactcagaccatatgtttggctgtgtgtgtggtaaggggggtaagatatctctaggaggaggcaactctactcgaaagtacgaaatggccgac<br>gccaatccgacctgccattgtgcaggacgcgggtcgagatccggaagtgccctccttctgaggttgttggtttgttccagttggcataagaaattaataatcgtttagtca<br>atggtacagtattaataattcctgacaaaaccgtgccctacaagcagtgtgagttccgggggtagcactagtggtcgtggtggtagggcccacagagagtatttccg | |



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**Table 20**

| Plasmid | Nucleic acid sequence | SEQ ID NO |
|---|---|---|
| | gaaccgactgatcctttgattccaatattccaagatttgggtttgtggttgtgtgtaatgcatgaggttattttgtcaacctccttccttctctacggttgttgctcctcctcctcttc cttccctttgttcatttaaatacacacacaccactccccttcccacccacacaagaacttttttttttttttactttcttcttctcttacttttcttcttcttctttcattacctgttagattatattta tcaacctcaattggtttattatctaccacccatacaaccccccacccctgccacatccaccaacaatggacgacgttgattctgctttagccgataatgttaaatccttcgc tgccggtggttttggtggtatttgtgccgtgttgaccggtcatccattcgacttggtcaaggtcagattgcaaactgggttgtacaaatcgtcagtgcaatgtgtcaaagaa acaatagccaaagacgggttgtttgggttatacagaggtgtcttgcctccattgttgggtgtcaccccaatgtttgctgtttccttctggggttacgacgttggtaagaaatt ggtttcttccttcactggcaaatcagtcgacaagtttgaaatcaaagacatttcgactgccggtttcatcagtgccatcccaaccaccttggttgccgctccattcgaaag agtcaaggtcatgatgcagatccaagaaggtgccaagtccaagtccatgggtgccgttgttgctgaaatgtacagaaccggtggtatcagatccatcttcaagggta ctgtggctactttggccagagatggtccaggttctgccttgtatttcgctacttatgaatgggtcaagaaggaattgactgcccctggtgaagacttgtccttgtttgctatta ccactgctggtggtttcgctggtattgccatgtggttgggtgttttcccaattgacaccatcaagtctacccagcagtcttccaacgtcaaggtctccattgtgcaggccac taagaacatctacgctaaaggtggtatcaaggctttcttccctggtgttggtcctgccttggccagagctttcccagccaacgctgctaccttcctaggggttgaattggc cagaaagttcttggacaaagttatataagagtgactcttttgataagagtcgcaaatttgatttcataagtatatattcattatgtaaagtagtaaatggaaaattcattaaa aaaaaagcaaatttccgttgtatgcatactccgaacacaaaactagccccggaaaaacccttagttgatagttgcgaatttaggtcgaccttggacgcgtgcggccg ccagtgtgatggatatctgcagaattcgcccttcccgaaatattacaattggagctccaataaatattgtaataaataggtctatatacatacactaagcttctaggacgt cattgtagtcttcgaagttgtctgctagtttagttctcatgatttcgaaaaccaataacgcaatggatgtagcagggatggtggttagtgcgttcctgacaaacccagagt acgccgcctcaaaccacgtcacattcgccctttgcttcatccgcatcacttgcttgaaggtatccacgtacgagttgtaatacaccttgaagaacggcttcgtctcgacg ggtacaacgagaattgtattgaattgatcaagaacatgatcttggtgttacagaacatcaagttcttggaccagactgagaatgcacagatatacaaggcgtcatgtg ataaaatggatgagatttatccacaattgaagaaagagtttatggaaagtggtcaaccagaagctaaacaggaagaagcaaacgaagaggtgaaacaagaag aagaaggtaaataagtattttgtattatataacaaacaaagtaaggaatacagatttatacaataaattgccatactagtcacgtgagatatctcatccattccccaact cccaagaaaaaaaaaaagtgaaaaaaaaaatcaaacccaaagatcaacctccccatcatcatcgtcatcaaacccccagctcaattcgcaatggttagcacaa aaacatacacagaaagggcatcagcacacccctccaaggttgcccaacgtttattccgcttaatggagtccaaaaagaccaacctctgcgcctcgatcgacgtga ccacaaccgccgagttcctttcgctcatcgacaagctcggtccccacatctgtctcgtgaagacgcacatcgatatcatctcagacttcagctacgagggcacgattg agccgttgcttgtgcttgcagagcgccacgggttcttgatattcgaggacaggaagtttgctgatatcggaaacaccgtgatgttgcagtacacctcgggggtataccg gatcgcggcgtggagtgacatcacgaacgcgcacggagtgactgggaagggcgtcgttgaaggggttgaaacgcggtgcggaggggggtagaaaaggaaagg ggcgtgttgatgttggcggagttgtcgagtaaaggctcgttggcgcatggtgaatatacccgtgagacgatcgagattgcgaagagtgatcgggagttcgtgattggg ttcatcgcgcagcgggacatgggggggtagagaagaagggtttgattggatcatcatgacgcctggtgtggggttggatgataaaggcgatgcgttgggccagcagt ataggactgttgatgaggtggttctgactggtaccgatgtgattattgtcgggagagggttgtttggaaaaggaagagaccctgaggtggagggaaagagatacag ggatgctggatggaaggcatacttgaagagaactggtcagttagaataaatattgtaataaataggtctatatacatacactaagcttctaggacgtcattgtagtcttc gaagttgtctgctagtttagttctcatgatttcgaaaaccaataacgcaatggatgtagcagggatggtggttagtgcgttcctgacaaacccagagtacgccgcctca aaccacgtcacattcgccctttgcttcatccgcatcacttgcttgaaggtatccacgtacgagttgtaatacacacactagttggtagttctggggactaccggcagcaa ttccatccgtgcgttatctataatccggatcttgtgaaaaaaaaggaaaaattctatatatagaaaagaaattagatagaagcctggcgagtgtgtttctagaagacttc cggattcagatgtcgcatggctgcgatatcacaaaaaaaaaagagagactccaacattacacaaaacgtggtcttccgctcccatatctgattgcggattttagagtg acactcttccctcgttgattatcaatttcgcaactgtaggatgggctggaggtattgttgatgcatgcaccacttatcattgtcggatccactaaccgcgtataatacccac acacacacacacacatacacaaacgcttcgtcacatctctatcggtatgtatgggggagagatagaacaggaaagtcatgaggccggccaaggg | |
| pAA1116 | taaacgttgggcaaccttggaggggtgtgctgatgccctttctgtgtatgtttttgtgctaaccattgcgaattgagctgggggtttgatgacgatgatgatggggaggttg | 123 |



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| Table 20 | | |
|---|---|---|
| **Plasmid** | **Nucleic acid sequence** | **SEQ ID NO** |
| | atctttgggtttgatttttttttttcacttttttttttcttgggagttggggaatggatgagatatctcacgtgactagtatggcaatttattgtataaatctgtattccttactttgtttgtta<br>tataatacaaaatacttatttaccttcttcttcttgtttcacctcttcgtttgcttcttcctgtttagcttctggttgaccactttccataaactctttcttcaattgtggataaatctcatc<br>cattttatcacatgacgccttgtatatctgtgcattctcagtctggtccaagaacttgatgttctgtaacaccaagatcatgttcttgatcaattcaatacaattctcgttgtacc<br>cgtcgcatatggtcgacctaaattcgcaactatcaactaagggttttccggggctagttttgtgttcggagtatgcatacaacggaaatttgcttttttttttaatgaattttcca<br>tttactactttacataatgaatatatacttatgaaatcaaatttgcgactcttatcaaaagagtcactctcttctattcttaactggacaagatttcagcagcttcttcggactttt<br>cacctctttctctgacttcaaggtctggacggttcaacatgtcttccaaagccttggagtatggagccttggtgttttctggtgggttcaaagccttgacagtttcgaagtagt<br>gttcgtagacgttaccatcatatctaccaatagcagcattggtcatcatgtcagtcaagttgaaaccatcagtcaaaccaacaacgtttggtctaaccttggcacacaa<br>ggctgggaagtgctcggaggaaatctttctgctgacatctggggtaatgataccgtactgcaagaaggtggcaccaaagttggtcaagatccagtcggcatacaag<br>aaacccaaaggtctcaagacttcagaccattctggggtgatacgcttgaagaaaccgtcaatcaagaatctgtgagcgttcaacttggaaaccaaaaccaaacttg<br>gaccggtaatgtcgaaaccttgtcttcagcaacagcaccaatgtctctggccaagccggaaagggcgtggtcgaaagccttaaccaacttggctgggtcgtccaa<br>gtcggcaacgctggagagaaccaatccctttgttctggctccttcaacaagtctctaaccattggcttggcaacgttcatggccaagatgttgttgtcaccttcccaggt<br>acattggacaacccagtcggagtaggctttaccgaaaccgttgtaagacgagtaaccgtgaccaccacaagcttgacgagcttcgtcaatggcttcagcagtcaac<br>caggtacaggtggacttcaaacaaccggaagcgacgaacaatttcttggattcgacaatggcagcgtcaatggcttcctttcaccagcactgacagcttcgtccaa<br>cttgtcgttggttgcgttcatggtttgttctaagtacaaggcaccttgggagaacaagtaagcggcagccaagaatgggaacaatctctttgatgcaatgggtagtcaa<br>tcaacttggtttcaatggtatcggtgtccttcttcttgaattgtcttctgtggatggcgtatctcaaggcaatggtgatgaatctactggtcattctgtaggagtccatcatcatg<br>gtgactctaccaccaatcaaagccgagtaagacaattgttcagatggtggcatggtgacttcacccagacgggaaaccttacagtacttttgcaacataaagaatctt<br>gggattctgacgttggagaactggatccaaccgttatcgataccgtctctacccatcttggcaccaatgtcaccaacagtcacacctggctcgaggtcgtggttggagt<br>ctctcaatgggacaacaaaggtcttgacaccgtagtcctttccttgaccttcaatctggcgtagacggagcagtgggtggcggagtgcgcagcaccaccgatccac<br>cacttggtggcaccaatgtgtggggtgttgatgacaaactcgtcagtgtcttcgtcaaaagtggcggtggtttcaataccttggacgttggaaccgtgggccaactcag<br>tcataccaaaacaaccatagatacctctcaacttgtcgataccctttatttatggtccagtagaagaactgggagttggtaccgttaccacggacacaggaaacaaac<br>aaacccaagttgacaccgattctggttccgacttgtgggtcaaagacaccgaggatcgacaatctctgggcctgttggtctgggtactcgtgctcaaagtatctggag<br>agtctggcaatcttcttggcggtgacttctctttgttggtctttggtcaagttgtagtatgaagcgtcgaccttcaagatagggtctttttgcatttgttcgaccatgttgctgatg<br>atctcggatctttcttgggaaccttccaagaagtagttcaactcctttgggttgaacttggtgagttctctttcttttttgaagttcggtaggcattatgtcgtgaagatttgaaca<br>atgttagtggggaataactattcaaaaaaaagggatggtaaataaatatatacaaaaaaaaaggaaaagcagaagagaagaagttgtttttagaatctggggaga<br>ttttagggaaatggagttgattttatatcaatggtggaaaaagtcacaggaggggggaagcacagagaaaaaaaagaagaggcaagcgtgtatcggtggacaaa<br>aaagggaaatgttaatcggtgtgtgtgcaaaaattcacaagtgtaattttgattatctttcgcgggggggccagccagtagctaagacacaactcagacaatttttactcc<br>acaattcaaccgaataaccgtgtgtatgcataacacctaagttgcatacaactagcgagctacagacattgccagagtcgatcagtagcatttgcaacaaccagatt<br>ataattctaccagaaccatctctccctctctctctgttgttttaccccaacgatatttctcccgaaatattacaattggagctcctgcaggcatgatgatctgccatgccga<br>actagacgaagccgttcttcaaggtgtattacaactcgtacgtggataccttcaagcaagtgatgcggatgaagcaaagggcgaatgtgacgtggtttgaggcggc<br>gtactctgggtttgtcaggaacgcactaaccaccatccctgctacatccattgcgttattggttttcgaaatcatgagaactaaactagcagacaacttcgaagactac<br>aatgacgtcctagaagcttagtgtatgtatatagacctatttattacaatatttattctaactgaccagttctcttcaagtatgccttccatccagcatccctgtatctctttccct<br>ccacctcagggtctcttccttttccaaacaaccctctcccgacaataatcacatcggtaccagtcagaaccacctcatcaacagtcctatactgctggcccaacgcat<br>cgcctttatcatccaaccccacaccaggcgtcatgatgatccaatcaaaccccttcttctctaccccccatgtcccgctgcgcgatgaacccaatcacgaactcccgat<br>cactcttcgcaatctcgatcgtctcacgggtatattcaccatgcgccaacgagcctttactcgacaactccgccaacatcaacacgccccttccttttctaccccctccg<br>caccgcgtttcaacccttcaacgacgcccttcccagtcactccgtgcgcgttcgtgatgtcactccacgccgcgatccggtataccccccgaggtgtactgcaacatca | |



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| Table 20 | | |
|---|---|---|
| **Plasmid** | **Nucleic acid sequence** | **SEQ ID NO** |
| | cggtgtttccgatatcagcaaacttcctgtcctcgaatatcaagaacccgtggcgctctgcaagcacaagcaacggctcaatcgtgccctcgtagctgaagtctgag<br>atgatatcgatgtgcgtcttcacgagacagatgtggggaccgagcttgtcgatgagcgaaaggaactcggcggttgtggtcacgtcgatcgaggcgcagaggttgg<br>tctttttggactccattaagcggaaaagggcgaattctgcagatatccatcacactggcggccgctcgagcatgcatctagagggcccaattcgccctatagtgagtc<br>gtattacaattcactggccgtcgttttacaacgtcgtgactgggaaaaccctggcgttacccaacttaatcgccttgcagcacatccccctttcgccagctggcgtaata<br>gcgaagaggcccgcaccgatcgcccttcccaacagttgcgcagcctatacgtacggcagtttaaggtttacacctataaaagagagagccgttatcgtctgtttgtgg<br>atgtacagagtgatattattgacacgccggggcgacggatggtgatccccctggccagtgcacgtctgctgtcagataaagtctcccgtgaactttacccggtggtgc<br>atatcggggatgaaagctggcgcatgatgaccaccgatatggccagtgtgccggtctccgttatcggggaagaagtggctgatctcagccaccgcgaaaatgaca<br>tcaaaaacgccattaacctgatgttctggggaatataaatgtcaggcatgagattatcaaaaaggatcttcacctagatcctttcacgtagaaagccagtccgcaga<br>aacggtgctgaccccggatgaatgtcagctactgggctatctggacaagggaaaacgcaagcgcaaagagaaagcaggtagcttgcagtgggcttacatggcg<br>atagctagactgggcggttttatggacagcaagcgaaccggaattgccagctggggcgccctctggtaaggttgggaagccctgcaaagtaaactggatggctttc<br>tcgccgccaaggatctgatggcgcaggggatcaagctctgatcaagagacaggatgaggatcgtttcgcatgattgaacaagatggattgcacgcaggttctccg<br>gccgcttgggtggagaggctattcggctatgactgggcacaacagacaatcggctgctctgatgccgccgtgttccggctgtcagcgcaggggcgcccggttctttt<br>gtcaagaccgacctgtccggtgccctgaatgaactgcaagacgaggcagcgcggctatcgtggctggccacgacgggcgttccttgcgcagctgtgctcgacgtt<br>gtcactgaagcgggaagggactggctgctattgggcgaagtgccggggcaggatctcctgtcatctcaccttgctcctgccgagaaagtatccatcatggctgatgc<br>aatgcggcggctgcatacgcttgatccggctacctgcccattcgaccaccaagcgaaacatcgcatcgagcgagcacgtactcggatggaagccggtcttgtcga<br>tcaggatgatctggacgaagagcatcaggggctcgcgccagccgaactgttcgccaggctcaaggcgagcatgcccgacggcgaggatctcgtcgtgacccat<br>ggcgatgcctgcttgccgaatatcatggtggaaaatggccgcttttctggattcatcgactgtggccggctgggtgtggcggaccgctatcaggacatagcgttggcta<br>cccgtgatattgctgaagagcttggcggcgaatgggctgaccgcttcctcgtgctttacggtatcgccgctcccgattcgcagcgcatcgccttctatcgccttcttgacg<br>agttcttctgaattattaacgcttacaatttcctgatgcggtattttctccttacgcatctgtgcggtatttcacaccgcatacaggtggcacttttcggggaaatgtgcgcgg<br>aacccctatttgtttatttttctaaatacattcaaatatgtatccgctcatgagacaataaccctgataaatgcttcaataatagcacgtgaggagggccaccatggccaa<br>gttgaccagtgccgttccggtgctcaccgcgcgcgacgtcgccggagcggtcgagttctggaccgaccggctcgggttctcccgggacttcgtggaggacgacttc<br>gccggtgtggtccgggacgacgtgaccctgttcatcagcgcggtccaggaccaggtggtgccggacaacaccctggcctgggtgtgggtgcgcggcctggacga<br>gctgtacgccgagtggtcggaggtcgtgtccacgaacttccgggacgcctccgggccggccatgaccgagatcggcgagcagccgtgggggcgggagttcgcc<br>ctgcgcgacccggccggcaactgcgtgcacttcgtggccgaggagcaggactgacacgtgctaaaacttcatttttaatttaaaaggatctaggtgaagatccttttg<br>ataatctcatgaccaaaatcccttaacgtgagttttcgttccactgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatcctttttttctgcgcgtaatctg<br>ctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgccggatcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgcagatac<br>caaatactgtccttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatcctgttaccagtggctgctgccagtg<br>gcgataagtcgtgtcttaccgggttggactcaagacgatagttaccggataaggcgcagcggtcgggctgaacgggggttcgtgcacacagcccagcttggagc<br>gaacgacctacaccgaactgagatacctacagcgtgagctatgagaaagcgccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggcagg<br>gtcggaacaggagagcgcacgagggagcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgct<br>cgtcaggggggcggagcctatggaaaaacgccagcaacgcggcctttttacggttcctgggcttttgctggccttttgctcacatgttctttcctgcgttatcccctgattct<br>gtggataaccgtattaccgcctttgagtgagctgataccgctcgccgcagccgaacgaccgagcgcagcgagtcagtgagcgaggaagcggaagagcgcccca<br>atacgcaaaccgcctctccccgcgcgttggccgattcattaatgcagctggcacgacaggtttcccgactggaaagcgggcagtgagcgcaacgcaattaatgtg<br>agttagctcactcattaggcaccccaggctttacactttatgcttccggctcgtatgttgtgtggaattgtgagcggataacaatttcacacaggaaacagctatgaccat<br>gattacgccaagctatttaggtgacactatagaatactcaagctatgcatcaagcttggtaccgagctcggatccactagtaacggccgccagtgtgctggaattcgc | |



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**Table 20**

| Plasmid | Nucleic acid sequence | SEQ ID NO |
|---|---|---|
| | cctt | |
| pAA2311 | ttccgcttaatggagtccaaaaagaccaacctctgcgcctcgatcgacgtgaccacaaccgccgagttcctttcgctcatcgacaagctcggtccccacatctgtctc<br>gtgaagacgcacatcgatatcatctcagacttcagctacgagggcacgattgagccgttgcttgtgcttgcagagcgccacgggttcttgatattcgaggacaggaa<br>gtttgctgatatcggaaacaccgtgatgttgcagtacacctcgggggtataccggatcgcggcgtggagtgacatcacgaacgcgcacggagtgactgggaagg<br>gcgtcgttgaagggttgaaacgcggtgcggaggggggtagaaaaggaaaggggcgtgttgatgttggcggagttgtcgagtaaaggctcgttggcgcatggtgaat<br>atacccgtgagacgatcgagattgcgaagagtgatcgggagttcgtgattgggttcatcgcgcagcgggacatgggggtagagaagaagggtttgattggatcat<br>catgacgcctggtgtggggttggatgataaaggcgatgcgttgggccagcagtataggactgttgatgaggtggttctgactggtaccgatgtgattattgtcgggaga<br>gggttgtttggaaaaggaagagaccctgaggtggagggaaagagatacagggatgctggatggaaggcatacttgaagagaactggtcagttagaataaatatt<br>gtaataaataggtctatatacatacactaagcttctaggacgtcattgtagtcttcgaagttgtctgctagtttagttctcatgatttcgaaaaccaataacgcaatggatgt<br>agcagggatggtggttagtgcgttcctgacaaacccagagtacgccgcctcaaaccacgtcacattcgccctttgcttcatccgcatcacttgcttgaaggtatccacg<br>tacgagttgtaatacaccttgaagaacggcttcgtctagttcggcatggcagatcatcatgcctgcagaaaatcagaggctactccggagatggcacattatcacgtg<br>ggcagtcccatctctctgacaatggcactgacaatgccatgtcattctagaagtcgctcgttccattgctagtcgtgtaggtgtcttactcagaccatatgtttggctgtgtg<br>tgtggtaagggggtaagatatctctaggaggaggcaactctactcgaaagtacgaaatggccgacgccaatccgacctgccattgtgcaggacgcgggtcgaga<br>tccggaagtgccctccttctgaggttgttggtttgttccagttggcataagaaattaataatcgtttagtcaatggtacagtattaataattcctgacaaaaccgtgccctac<br>aagcagtgtgagttccgggggtagcactagtggtcgtggtggtagggcccacagagagtatttccggaaccgactgatcctttgattccaatattccaagatttgggttt<br>gtggttgtgtgtaatgcatgaggttattttgtcaacctccttccttctctacggttgttgctcctcctcctcttccttcccttttgttcatttaaatacacacaccactccccttccc<br>acccacaagaactttttttttttttttacttttcttcttctcttacttttcttcttcttcttttcattacctgttagattatatttatcaacctcaattggtttattatctaccacccatacaaccccca<br>cccctgccacatccaccaacaatggacgacgttgattctgctttagccgataatgttaaatccttcgctgccggtggttttggtggtatttgtgccgtgttgaccggtcatcc<br>attcgacttggtcaaggtcagattgcaaactgggttgtacaaatcgtcagtgcaatgtgtcaaagaaacaatagccaaagacgggttgtttgggttatacagaggtgt<br>cttgcctccattgttgggtgtcaccccaatgtttgctgtttccttctggggttacgacgttggtaagaaattggtttcttccttcactggcaaatcagtcgacaagtttgaaatc<br>aaagacatttcgactgccggtttcatcagtgccatcccaaccaccttggttgccgctccattcgaaagagtcaaggtcatgatgcagatccaagaaggtgccaagtc<br>caagtccatgggtgccgttgttgctgaaatgtacagaaccggtggtatcagatccatcttcaagggtactgtggctactttggccagagatggtccaggttctgccttgta<br>tttcgctacttatgaatgggtcaagaaggaattgactgcccctggtgaagacttgtccttgtttgctattaccactgctggtggtttcgctggtattgccatgtggttgggtgttt<br>tcccaattgacaccatcaagtctacccagcagtcttccaacgtcaaggtctccattgtgcaggccactaagaacatctacgctaaaggtggtatcaaggctttcttccct<br>ggtgttggtcctgccttggccagagctttcccagccaacgctgctaccttcctaggggttgaattggccagaaagttcttggacaaagttatataataagaatagaaga<br>gagtgactcttttgataagagtcgcaaatttgatttcataagtatatattcattatgtaaagtagtaaatggaaaattcattaaaaaaaaagcaaatttccgttgtatgcata<br>ctccgaacacaaaactagccccggaaaaacccttagttgatagttgcgaatttaggtcgaccatatgcgacgggtacaacgagaattgtattgaattgatcaagaa<br>catgatcttggtgttacagaacatcaagttcttggaccagactgagaatgcacagatatacaaggcgtcatgtgataaaatggatgagatttatccacaattgaagaa<br>agagtttatggaaagtggtcaaccagaagctaaacaggaagaagcaaacgaagaggtgaaacaagaagaagaaggtaaataagtattttgtattatataacaa<br>acaaagtaaggaatacagatttatacaataaattgccatactagtcacgtgagatatctcatccattccccaactcccaagaaaaaaaaaaagtgaaaaaaaaaa<br>tcaaacccaaagatcaacctccccatcatcatcgtcatcaaacccccagctcaattcgcaatggttagcacaaaaacatacacagaaagggcatcagcacaccc<br>ctccaaggttgcccaacgtttaaagggcgaattccagcacactggcggccgttactagtggatccgagctcggtaccaagcttgatgcatagcttgagtattctatagt<br>gtcacctaaatagcttggcgtaatcatggtcatagctgtttcctgtgtgaaattgttatccgctcacaattccacacaacatacgagccggaagcataaagtgtaaagc<br>ctggggtgcctaatgagtgagctaactcacattaattgcgttgcgctcactgcccgctttccagtcgggaaacctgtcgtgccagctgcattaatgaatcggccaacgc | 124 |



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| Table 20 | | |
|---|---|---|
| **Plasmid** | **Nucleic acid sequence** | **SEQ ID NO** |
| | gcggggagaggcggtttgcgtattgggcgctcttccgcttcctcgctcactgactcgctgcgctcggtcgttcggctgcggcgagcggtatcagctcactcaaaggcg gtaatacggttatccacagaatcaggggataacgcaggaaagaacatgtgagcaaaaggccagcaaaagcccaggaaccgtaaaaaggccgcgttgctggc gtttttccataggctccgccccccctgacgagcatcacaaaaatcgacgctcaagtcagaggtggcgaaacccgacaggactataaagataccaggcgtttccccct ggaagctccctcgtgcgctctcctgttccgaccctgccgcttaccggatacctgtccgcctttctcccttcgggaagcgtggcgctttctcatagctcacgctgtaggtatc tcagttcggtgtaggtcgttcgctccaagctgggctgtgtgcacgaaccccccgttcagcccgaccgctgcgccttatccggtaactatcgtcttgagtccaacccggt aagacacgacttatcgccactggcagcagccactggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttcttgaagtggtggcctaactacgg ctacactagaaggacagtatttggtatctgcgctctgctgaagccagttaccttcggaaaaagagttggtagctcttgatccggcaaacaaaccaccgctggtagcg gtggtttttttgtttgcaagcagcagattacgcgcagaaaaaaaggatctcaagaagatcctttgatcttttctacggggtctgacgctcagtggaacgaaaactcacgt taagggattttggtcatgagattatcaaaaaggatcttcacctagatccttttaaattaaaaatgaagttttagcacgtgtcagtcctgctcctcggccacgaagtgcacg cagttgccggccgggtcgcgcagggcgaactcccgccccacggctgctcgccgatctcggtcatggccggcccggaggcgtcccggaagttcgtggacacga cctccgaccactcggcgtacagctcgtccaggccgcgcacccacacccaggccagggtgttgtccggcaccacctggtcctggaccgcgctgatgaacagggtc acgtcgtcccggaccacaccggcgaagtcgtcctccacgaagtcccgggagaacccgagccggtcggtccagaactcgaccgctccggcgacgtcgcgcgcg gtgagcaccggaacggcactggtcaacttggccatggtggccctcctcacgtgctattattgaagcatttatcagggttattgtctcatgagcggatacatatttgaatgt atttagaaaaataaacaaatagggttccgcgcacatttccccgaaaagtgccacctgtatgcggtgtgaaataccgcacagatgcgtaaggagaaaataccgc atcaggaaattgtaagcgttaataattcagaagaactcgtcaagaaggcgatagaaggcgatgcgctgcgaatcgggagcggcgataccgtaaagcacgagg aagcggtcagcccattcgccgccaagctcttcagcaatatcacgggtagccaacgctatgtcctgatagcggtccgccacacccagccggccacagtcgatgaat ccagaaaagcggccattttccaccatgatattcggcaagcaggcatcgccatgggtcacgacgagatcctcgccgtcgggcatgctcgccttgagcctggcgaac agttcggctggcgcgagcccctgatgctcttcgtccagatcatcctgatcgacaagaccggcttccatccgagtacgtgctcgctcgatgcgatgtttcgcttggtggtc gaatgggcaggtagccggatcaagcgtatgcagccgccgcattgcatcagccatgatggatactttctcggcaggagcaaggtgagatgacaggagatcctgcc ccggcacttcgcccaatagcagccagtcccttcccgcttcagtgacaacgtcgagcacagctgcgcaaggaacgcccgtcgtggccagccacgatagccgcgct gcctcgtcttgcagttcattcagggcaccggacaggtcggtcttgacaaaaagaaccgggcgcccctgcgctgacagccggaacacggcggcatcagagcagcc cgattgtctgttgtgcccagtcatagccgaatagcctctccacccaagcggccggagaacctgcgtgcaatccatcttgttcaatcatgcgaaacgatcctcatcctgt ctcttgatcagagcttgatcccctgcgccatcagatccttggcggcgagaaagccatccagtttactttgcagggcttcccaaccttaccagagggcgccccagctgg caattccggttcgcttgctgtccataaaaccgcccagtctagctatcgccatgtaagcccactgcaagctacctgctttctctttgcgcttgcgttttcccttgtccagatag cccagtagctgacattcatccggggtcagcaccgtttctgcggactggctttctacgtgaaaaggatctaggtgaagatccttttgataatctcatgcctgacatttatatt ccccagaacatcaggttaatggcgtttttgatgtcattttcgcggtggctgagatcagccacttcttccccgataacggagaccggcacactggccatatcggtggtcat catgcgccagctttcatccccgatatgcaccaccgggtaaagttcacgggagactttatctgacagcagacgtgcactggccaggggatcaccatccgtcgcccc ggcgtgtcaataatatcactctgtacatccacaaacagacgataacggctctctcttttataggtgtaaaccttaaactgccgtacgtataggctgcgcaactgttggga agggcgatcggtgcgggcctcttcgctattacgccagctggcgaaaggggatgtgctgcaaggcgattaagttgggtaacgccagggttttcccagtcacgacgtt gtaaaacgacggccagtgaattgtaatacgactcactatagggcgaattgggccctctagatgcatgctcgagcggccgccagtgtgatggatatctgcagaattcg ccctt | |
| IGR5-5' genomic DNA contained in | tcatgactttcctgttctatctctccccatacataccgatagagatgtgacgaagcgtttgtgtatgtgtgtgtgtgtgtgggtattatacgcggttagtggatccgacaat gataagtggtgcatgcatcaacaatacctccagcccatcctacagttgcgaaattgataatcaacgagggaagagtgtcactctaaaatccgcaatcagatatggg agcggaagaccacgttttgtgtaatgttggagtctctctttttttttgtgatatcgcagccatgcgacatctgaatccggaagtcttctagaaacacactcgccaggcttct | 125 |



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**Table 20**

| Plasmid | Nucleic acid sequence | SEQ ID NO |
|---|---|---|
| pAA2153 | atctaatttcttttctatatatagaatttttccttttttttcacaagatccggattatagataacgcacggatggaattgctgccggtagtccccagaactaccaactagtgt | |
| IGR5-3' genomic DNA contained in pAA2153 | ggatcaagcgacgaaccggccggcaaccaggggggcgttccgaagaagaggactccgtgtttccgaagtcctaccaaccccccagtaagaaaacacccccta agacatatttccgtcaaagaggtctcttttccgctgcatgggccgcatgctcgcgggcaggtgtgtcaacgagagctggagtttgtgatggtgctggtgctggccatag gcaatagctacgacagtcaaaaaaaaaactatccgacctgccgtcctctagtttactcctcagccagagaagagggccaagtttttttcttctctccggtatgcaacatta cttgagatgtatgattacccagctcacttctgttccgccaggacttccgcctctcaaccccccctagatttgaacgaaagaaaaaccccctctatgagaaacgccctctctt tttttggcctgttgtttatcatgtaactctaaccaccctgaggtattttcggagcactctcgagcatgg | 126 |
| pAA208 | tttccataggctccgccccctgacgagcatcacaaaaatcgacgctcaagtcagaggtggcgaaacccgacaggactataaagataccaggcgtttcccccctgg aagctccctcgtgcgctctcctgttccgaccctgccgcttaccggatacctgtccgcctttctcccttcgggaagcgtggcgctttctcatagctcacgctgtaggtatctc agttcggtgtaggtcgttcgctccaagctgggctgtgtgcacgaaccccccgttcagcccgaccgctgcgccttatccggtaactatcgtcttgagtccaacccggtaa gacacgacttatcgccactggcagcagccactggtaacaggattagcagagcgaggtatgtaggcggtgctacagagttcttgaagtggtggcctaactacggcta cactagaaggacagtatttggtatctgcgctctgctgaagccagttaccttcggaaaaagagttggtagctcttgatccggcaaacaaaccaccgctggtagcggtg gtttttttgtttgcaagcagcagattacgcgcagaaaaaaaggatctcaagaagatcctttgatcttttctacggggtctgacgctcagtggaacgaaaactcacgttaa gggattttggtcatgagattatcaaaaaggatcttcacctagatccttttaaattaaaaatgaagttttaaatcaatctaaagtatatatgagtaaacttggtctgacagtta ccaatgcttaatcagtgaggcacctatctcagcgatctgtctatttcgttcatccatagttgcctgactccccgtcgtgtagataactacgatacgggagggcttaccatct ggccccagtgctgcaatgataccgcgagacccacgctcaccggctccagatttatcagcaataaaccagccagccggaagggccgagcgcagaagtggtcctg caactttatccgcctccatccagtctattaattgttgccgggaagctagagtaagtagttcgccagttaatagtttgcgcaacgttgttgccattgctacaggcatcgtggt gtcacgctcgtcgtttggtatggcttcattcagctccggttcccaacgatcaaggcgagttacatgatcccccatgttgtgcaaaaaagcggttagctccttcggtcctcc gatcgttgtcagaagtaagttggccgcagtgttatcactcatggttatggcagcactgcataattctcttactgtcatgccatccgtaagatgcttttctgtgactggtgagt actcaaccaagtcattctgagaatagtgtatgcggcgaccgagttgctcttgcccggcgtcaatacgggataataccgcgccacatagcagaactttaaaagtgctc atcattggaaaacgttcttcggggcgaaaactctcaaggatcttaccgctgttgagatccagttcgatgtaacccactcgtgcacccaactgatcttcagcatcttttactt tcaccagcgtttctgggtgagcaaaaacaggaaggcaaaatgccgcaaaaaagggaataagggcgacacggaaatgttgaatactcatactcttcctttttcaata ttattgaagcatttatcagggttattgtctcatgagcggatacatatttgaatgtatttagaaaaataaacaaatagggttccgcgcacatttccccgaaaagtgccacc tgacgtctaagaaaccattattatcatgacattaacctataaaaataggcgtatcacgaggccctttcgtctcgcgcgtttcggtgatgacggtgaaaacctctgacac atgcagctcccggagacggtcacagcttgtctgtaagcggatgccgggagcagacaagcccgtcagggcgcgtcagcgggtgttggcgggtgtcggggctggct taactatgcggcatcagagcagattgtactgagagtgcaccatatgcggtgtgaaataccgcacagatgcgtaaggagaaaataccgcatcaggcgccattcgcc attcaggctgcgcaactgttgggaagggcgatcggtgcgggcctcttcgctattacgccagctggcgaaaggggatgtgctgcaaggcgattaagttgggtaacg ccagggttttcccagtcacgacgttgtaaaacgacggccagtgaattccggttgaattgtggagtaaaaattgtctgagttgtgtcttagctactggctggcccccgcg aaagataatcaaaattacacttgtgaatttttgcacacacaccgattaacatttccctttttgtccaccgatacacgcttgcctcttctttttttctctgtgcttcccccctcctgtg actttttccaccattgatataaaatcaactccatttccctaaaatctccccagattctaaaaacaacttcttctcttctgcttttccttttttttgttatatttatttaccatccctttttttt gaatagttattccccactaacattgttcaaatcttcacgacataatgacttttacaaagaaaaacgttagtgtatcacaaggtcctgaccctagatcatccatccaaaag gaaagagacagctccaaatggaaccctcaacaaatgaactacttcttggaaggctccgtcgaaagaagtgagttgatgaaggctttggcccaacaaatggaaag agacccaatcttgttcacagacggctcctactacgacttgaccaaggaccaacaaagagaattgaccgccgtcaagatcaacagaatcgccagatacagagaa caagaatccatcgacactttcaacaagagattgtccttgattggtatctttgacccacaggtcggtaccagaattggtgtcaacctcggtttgttcctttcttgtatcagagg | 127 |



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| Table 20 | | |
|---|---|---|
| **Plasmid** | **Nucleic acid sequence** | **SEQ ID NO** |
| | taacggtaccacttcccaattgaactactgggctaacgaaaaggaaaccgctgacgttaaaggtatctacggttgtttcggtatgacccccggggatctgacgggta<br>caacgagaattgtattgaattgatcaagaacatgatcttggtgttacagaacatcaagttcttggaccagactgagaatgcacagatatacaaggcgtcatgtgataa<br>aatggatgagatttatccacaattgaagaaagagtttatggaaagtggtcaaccagaagctaaacaggaagaagcaaacgaagaggtgaaacaagaagaag<br>aaggtaaataagtattttgtattatataacaaacaaagtaaggaatacagatttatacaataaattgccatactagtcacgtgagatatctcatccattccccaactccc<br>aagaaaaaaaaaaaagtgaaaaaaaaaatcaaacccaaagatcaacctccccatcatcatcgtcatcaaacccccagctcaattcgcaatggttagcacaaaa<br>acatacacagaaagggcatcagcacacccctccnaggttgcccaacgtttattccgcttaatggagtccaaaaagaccaacctctgcgcctcgatcgacgtgacc<br>acaaccgccgagttcctttcgctcatcgacaagctcggtccccacatctgtctcgtgaagacgcacatcgatntcatctcagacttcagctacgagggcacgattgag<br>ccgttgcttgtgcttgcagagcgccacgggttcttgatattcgaggacaggaagtttgctgatatcggaaacaccgtgatgttgcagtacacctcgggggtataccgga<br>tcgcggcgtggagtgacatcacgaacgcgcacggagtgactgggaagggcgtcgttgaagggttgaaacgcggtgcggaggggggtagaaaaggaaagggg<br>cgtgttgatgtnggcggagttgtcgagtaaaggctcgttggcgcatggtgaatatacccgtgagacgatcgagattgcgaagagtgatcgggagttcgtgattgggtt<br>catcgcgcagcgggacatgggggggtagagaagaagggtttgattggatcatcatgacgcctggtgtggggttggatgataaaggcgatgcgttgggccagcagta<br>taggactgttgatgaggtggttctgactggtaccgatgtgattattgtcgggagagggttgtttggaaaaggaagagaccctgaggtggagggaaagagatacagg<br>gatgctggatggaaggcatacttgaagagaactggtcagttagaataaatattgtaataaataggtctatatacatacactaagcttctaggacgtcattgtagtcttcg<br>aagttgtctgctagtttagttctcatgatttcgaaaaccaataacgcaatggatgtagcagggatggtggttagtgcgttcctgacaaacccagagtacgccgcctcaa<br>accacgtcacattcgccctttgcttcatccgcatcacttgcttgaaggtatccacgtacgagttgtaatacaccttgaagaacggcttcgtctacggtcgacgacgggta<br>caacgagaattgtattgaattgatcaagaacatgatcttggtgttacagaacatcaagttcttggaccagactgagaatgcacagatatacaaggcgtcatgtgataa<br>aatggatgagatttatccacaattgaagaaagagtttatggaaagtggtcaaccagaagctaaacaggaagaagcaaacgaagaggtgaaacaagaagaag<br>aaggtaaataagtattttgtattatataacaaacaaagtaaggaatacagatttatacaataaattgccatactagtcacgtgagatatctcatccattccccaactccc<br>aagaaaaaaaaaaaagtgaaaaaaaaaatcaaacccaaagatcaacctccccatcatcatcgtcatcaaacccccagctcaattcgcagagctcggtacccgg<br>ggatcctctagagtcgacctgcaggcatgctaatgtttatatcgttgacggtgaaaaattttaacttacttgtaatatttacgatttggacttgaagtccaaaatgaagtata<br>aatattggttgaaaattgaaaatgatttacaagccatatacaatagttccaatccaaattggagatattgtactaaatatccaggcagaaccaacaacaacaatgac<br>gacaaagtgcaagccaaagagtagaaatcattaatctttcatattgtcatttttttattattattcagtacatatatacatatgtgtcaactatatatttcatgaaatatacata<br>cgtcaaataaaacatatcccaatcactaaaaaaaaaaaaaaatattaaccaaaactaatcttaactttaattaaatgttcatattttccttaccagacatcttcttggtatct<br>attttgaactttccaagatttaaccaattcagcagctttatcttcgtggatatctctacttttggcaacaatcttggcaattgcagcttgaacatctctagccattctactggcat<br>caccacaaacataaataattgctccactagataataactcatcaacaagagcactattttctaaaatcttatcttgaacataaactttcttagttggatcttgtcttgagaa<br>ggcattaaacatttcgaaattttctcccaatactgaggcatattcactccattcttgtttgtacaagaaatcttgttcggaatttctacatccataaaacaatacagtcttacc<br>aacattaacaccatttttaacttgttgaactctttctctaacaaaacctctcaatggtgcaacaccggtacctggaccaatcaaaataactggggtagtgctattctttggt<br>aatttgaaattagatcttctaacgtgaactggcaatctgaacttgctaaatttgcctcttggaccattcaaatcataatgaaccattggggtttcaccagttttgttttgttcaat<br>ttcaatattcttcaacaagttggtgacaacaccagtaactggtcttccatcagcttcttcttcggcttcaacaacagcagtaacattaatggtttgcttttcacttaatgagga<br>agaagaaatggagtaataacgaggagttaagtgttggacattttcaataaggaattcgaatggaacatcggaccatggtctgttgttggaagcaaataataaagcat<br>cggcaatgttgaattttctacgggtgactttactagcaaattcttgcttatcaccaccaattctagtaaatgacttttagtttcttcatcaggggcaaatccagcaatagata<br>agaaaaattgtctagaaacaggacctgaaatttccaaatggtgtctaataacagctccataagtgattggattagggaatgggatggaataagtggaatccaaagct<br>ttcaattcaataacagtatcaagtttgtcttctaaaccaaaacatttggcaaattgcttaatgttttcatcagagttagatggccagattgcaagatgatcaccggtggtata<br>tttcaagtttgattcagaaatatcaaattcaacatgaacacaatgtctgtcttcagaagtaaacaattctttagttttaacaattctagccaaaaatggatgagtatgatca<br>aatggacctttagttaagtcaacaccttcagatttaatgtatttgacatttggttcacccaaggagacatctggatcattgccagataaatcatctctttcagtcaatttaac | |



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**Table 20**

| Plasmid | Nucleic acid sequence | SEQ ID NO |
|---|---|---|
| | atttggttcgtatttcaactctttttcttcaaaattcaaatcattctttaaggaatcaaacacgttatccttccaggccaagaaatcttcatctaaagtaccagtaccatcgtca<br>ccttcaccgtattcagcaaatctgtcaccacctttttctcccaacaatctgtcaaattttctaccaatagcattgaagaattcataagttgaattacccaaaccaaaaaca<br>gtatatttcaaagtactcaaggtgtcagcttcttcagtcaaccaagtgtgaaattcgtcagcattatcggttggttcaccttcaccataagtagcaacaataaagaaaac<br>caagatatcttcagtaatatctccgaagttttcgaaatcataatcagcgaaatcagcaaccatggttttcaaaccaaatcttgaatgcaattctcttgacaatttgttggca<br>taatcttctgctgtacctgtttgggatccaaataataataacgtatttttattgttcttcttcaaagcttgtaagatatctcttgaattaccatctccactatcagtattaaggaac<br>ccggtatcttgttgttggtcaagaaactgattctttgcaaaataagctgcaattgcaaccaccaatgttataataacatataaatctaacttatctaatgccatgacttgag<br>cgattgaaacaatagtcttagttgatagagcagtaatggatcaagaaaagaaaaagaataaaaggtagaaataaaaaaaaaacaaagaaaaattggaaatttg<br>aaatgtttaaaatctttttttttattttttctctcgttcagttcgtttgaagattgtcgtatgaaatttttattcttttttttttttgttcctcttctgatgttcaattacgggacccaacgtcgttt<br>atgtattacgatactgggaccatactgactttgaaatatgtatcatttctgacagtcaggtaatgtgtaccaacttagaccaaatttcatctctataattattattgtatatata<br>actgtcctttctttttcgtctattagagttttatctcaggaggatttatcggtgcaatttcgatatcggatcatttattcactaagagctagagtgagacaaaagcagaatgcta<br>caccgaccaccgctaccaccaccattatttttggggttttactctacgcaacccccccccatcgaagcattgaatagacatgacagctcttccgcttcctcgctcactgac<br>tcgctgcgctcggtcgttcggctgcggcgagcggtatcagctcactcaaaggcggtaatacggttatccacagaatcaggggataacgcaggaaagaacatgga<br>cgctgccgttgaaaagaacgacaccaaggctatctttaagtctattgacgacatgaagtcattgtttgttgactctggttccttgaagtccactgccacttggttgggtgct<br>gaagccattgaccaatgtagacaagcctgtggtggtcacggttactcgtcctacaacggcttcggtaaagcctacaacgattgggttgtccaatgtacttgggaaggt<br>gacaacaatgtcttggccatgagtgttggtaagccaattgtcaagcaagttatcagcattgaagatgccggcaagaccgtcagaggttccaccgctttcttgaaccaa<br>ttgaaggactacactggttccaacagctccaaggttgttttgaacactgttgctgacttggacgacatcaagactgtcatcaaggctattgaagttgccatcatcagattg<br>tcccaagaagctgcttctattgtcaagaaggaatctttcgactatgtcggcgctgaattggttcaactctccaagttgaaggctcaccactacttgttgactgaatacatc<br>agaagaattgacacctttgaccaaaaggacttggttccatacttgatcaccctcggtaagttgtacgctgccactattgtcttggacagatttgccggtgtcttcttgacttt<br>caacgttgcctccaccgaagccatcactgctttggcctctgtgcaaattccaaagttgtgtgctgaagtcagaccaaacgttgttgcttacaccgactccttccaacaat<br>ccgacatgattgtcaattctgctattggtagatacgatggtgacatctatgagaactactttgacttggtcaagttgcagaacccaccatccaagaccaaggctccttac<br>tctgatgctttggaagccatgttgaacagaccaaccttggacgaaagagaaagatttgaaaagtctgatgaaaccgctgctatcttgtccaagaagcttggcgtaatc<br>atggtcatagctgtttcctgtgtgaaattgttatccgctcacaattccacacaacatacgagccggaagcataaagtgtaaagcctggggtgcctaatgagtgagcta<br>actcacattaattgcgttgcgctcactgcccgctttccagtcgggaaacctgtcgtgccagctgcattaatgaatcggccaacgcgcggggagaggcggtttgcgtatt<br>gggcgctcttccgcttcctcgctcactgactcgctgcgctcggtcgttcggctgcggcgagcggtatcagctcactcaaaggcggtaatacggttatccacagaatca<br>ggggataacgcaggaaagaacatgtgagcaaaaggccagcaaaaggccaggaaccgtaaaaaggccgcgttgctggcgtt | |
| pAA918 | ggccgcgagcactaggttttgataatttggttcttacagtttatgtattttgattcttccttttttagatacttttttttatattttattattccttattgatgtaacgacagtcccactataa<br>ttaacttaaactttgctgtaaatcagatgacaagtgtttccctgtttgcaggggagctctactagtttccttaattcatcttggttatgttgttcttgcaccgcaaggataagctg<br>ggcccgaatccaggccacgcataccaactatcgctgcccggaaaaaatacaaactagtaaactgcaaaaaaccatatataaacaataagaattttctctgtatcat<br>tcttcgaaaaacctcaccttctctctctctctctctcaccctccttccccctatcacatcttcatcttcacatcaccaactcaaaacccctaagcagccaaagtaaagaa<br>cagagaatgattccacaacatcacactatgttcttcgccgttaattaagaattcactggccgtcgttttacaacgtcgtgactgggaaaaccctggcgttacccaacttа<br>atcgccttgcagcacatccccctttcgccagctggcgtaatagcgaagaggcccgcaccgatcgcccttcccaacagttgcgcagcctgaatggcgaatggcgcct<br>gatgcggtattttctccttacgcatctgtgcggtatttcacaccgcatatggtgcactctcagtacaatctgctctgatgccgcatagttaagccagccccgacacccgcc<br>aacacccgctgacgcgccctgacgggcttgtctgctcccggcatccgcttacagacaagctgtgaccgtctccgggagctgcatgtgtcagaggttttcaccgtcatc<br>accgaaacgcgcgagacgaaagggcctcgtgatacgcctatttttataggttaatgtcatgataatgtggtttcttagacgtcaggtggcacttttcggggaaatgtgc | 128 |



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| Table 20 | | |
|---|---|---|
| **Plasmid** | **Nucleic acid sequence** | **SEQ ID NO** |
| | gcggaacccctatttgtttatttttctaaatacattcaaatatgtatccgctcatgagacaataaccctgataaatgcttcaataatattgaaaaaggaagagtatgagtat<br>tcaacatttccgtgtcgcccttattccctttttgcggcattttgccttcctgtttttgctcacccagaaacgctggtgaaagtaaaagatgctgaagatcagttgggtgcacg<br>agtgggttacatcgaactggatctcaacagcggtaagatccttgagagttttcgccccgaagaacgttttccaatgatgagcacttttaaagttctgctatgtggcgcgg<br>tattatcccgtattgacgccgggcaagagcaactcggtcgccgcatacactattctcagaatgacttggttgagtactcaccagtcacagaaaagcatcttacggatg<br>gcatgacagtaagagaattatgcagtgctgccataaccatgagtgataacactgcggccaacttacttctgacaacgatcggaggaccgaaggagctaaccgcttt<br>tttgcacaacatgggggatcatgtaactcgccttgatcgttgggaaccggagctgaatgaagccataccaaacgacgagcgtgacaccacgatgcctgtagcaat<br>ggcaacaacgttgcgcaaactattaactggcgaactacttactctagcttcccggcaacaattaatagactggatggaggcggataaagttgcaggaccacttctgc<br>gctcggcccttccggctggctggtttattgctgataaatctggagccggtgagcgtgggtctcgcggtatcattgcagcactggggccagatggtaagccctcccgtat<br>cgtagttatctacacgacggggagtcaggcaactatggatgaacgaaatagacagatcgctgagataggtgcctcactgattaagcattggtaactgtcagaccaa<br>gtttactcatatatactttagattgatttaaaacttcatttttaatttaaaaggatctaggtgaagatccttttttgataatctcatgaccaaaatcccttaacgtgagttttcgttcc<br>actgagcgtcagaccccgtagaaaagatcaaaggatcttcttgagatcctttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtgg<br>tttgtttgccggatcaagagctaccaactcttttccgaaggtaactggcttcagcagagcgcagataccaaatactgtccttctagtgtagccgtagttaggccaccact<br>tcaagaactctgtagcaccgcctacatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgtgtcttaccgggttggactcaagacgata<br>gttaccggataaggcgcagcggtcgggctgaacgggggttcgtgcacacagcccagcttggagcgaacgacctacaccgaactgagatacctacagcgtga<br>gctatgagaaagcgccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggcagggtcggaacaggagagcgcacgagggagcttccagg<br>gggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaaaacgccagc<br>aacgcggcctttttacggttcctggccttttgctggccttttgctcacatgttctttcctgcgttatcccctgattctgtggataaccgtattaccgcctttgagtgagctgatacc<br>gctcgccgcagccgaacgaccgagcgcagcgagtcagtgagcgaggaagcggaagagcgcccaatacgcaaaccgcctctccccgcgcgttggccgattca<br>ttaatgcagctggcacgacaggtttcccgactggaaagcgggcagtgagcgcaacgcaattaatgtgagttagctcactcattaggcaccccaggctttacactttat<br>gcttccggctcgtatgttgtgtggaattgtgagcggataacaatttcacacaggaaacagctatgaccatgattacgccaagcttttaattaaagataatcacaggggt<br>agagaccttggttatgggctgattggaggaagaaatggggtgatttggcacttgacagcgcgagagtggttaacacctggtttccctcatttgggttctgacatttgata<br>agttgaaagaacaatgcagaattcacatggctaatttggcctcggttccacaacgcactcagcattaaaaaaaaaatacgcaatggcagctcggtcgacgcagca<br>gaagcgccgacgtaccgtcgcgttgccccgcccatgcctcgccgacccctccaccgccatcgtttgcccattgtttgtggtagtgcgccgtgacacaaaaacttgtcc<br>tgtcacatgctgaagttacaccaacataactactatgggattacgtaatcaaaaatttcacagttttaacaaaaaaaaaaatcatacaatcaacattgggacatcttgcc<br>ctcccccacaaaacttgcttctgcatcaatcatatataaacatcatgaaataagcctaaactcacttctttttttttcatccttcctacttcttctttcatagtaactacttttttttttat<br>taccacacttattcattcataccacgctatcggccggccgcatgcggatccgagctcgcggccgccagtgtgatggatatctgcagcggccgctaaatattgtaataa<br>ataggtctatatacatacactaagcttctaggacgtcattgtagtcttcgaagttgtctgctagtttagttctcatgatttcgaaaaccaataacgcaatggatgtagcagg<br>gatggtggttagtgcgttcctgacaaacccagagtacgccgcctcaaaccacgtcacattcgccctttgcttcatccgcatcacttgcttgaaggtatccacgtacgagt<br>tgtaatacaccttgaagaacggcttcgtctcgacgggtacaacgagaattgtattgaattgatcaagaacatgatcttggtgttacagaacatcaagttcttggaccag<br>actgagaatgcacagatatacaaggcgtcatgtgataaaatggatgagatttatccacaattgaagaaagagtttatggaaagtggtcaaccagaagctaaacag<br>gaagaagcaaacgaagaggtgaaacaagaagaagaaggtaaataagtattttgtattatataacaaacaaagtaaggaatacagatttatacaataaattgccat<br>acaagtcacgtgagatatctcatccattccccaactcccaagaaaaaaaaaaagtgaaaaaaaaaatcaaacccaaagatcaacctccccatcatcatcgtcat<br>caaacccccagctcaattcgcaatggttagcacaaaaacatacacagaaagggcatcagcacacccctccaaggttgcccaacgtttattccgcttaatggagtcc<br>aaaaagaccaacctctgcgcctcgatcgacgtgaccacaaccgccgagttcctttcgctcatcgacaagctcggtccccacatctgtctcgtgaagacgcacatcg<br>atatcatctcagacttcagctacgagggcacgattgagccgttgcttgtgcttgcagagcgccacgggttcttgatattcgaggacaggaagtttgctgatatcggaaa | |



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| Table 20 | | |
|---|---|---|
| **Plasmid** | **Nucleic acid sequence** | **SEQ ID NO** |
| | caccgtgatgttgcagtacacctcgggggtataccggatcgcggcgtggagtgacatcacgaacgcgcacggagtgactgggaagggcgtcgttgaagggttga<br>aacgcggtgcggaggggtagaaaaggaaaggggcgtgttgatgttggcggagttgtcgagtaaaggctcgttggcgcatggtgaatatacccgtgagacgatc<br>gagattgcgaagagtgatcgggagttcgtgattgggttcatcgcgcagcgggacatgggggtagagaagaagggtttgattggatcatcatgacgcctggtgtgg<br>ggttggatgataaaggcgatgcgttgggccagcagtataggactgttgatgaggtggttctgactggtaccgatgtgattattgtcgggagagggttgtttggaaaagg<br>aagagaccctgaggtggagggaaagagatacagggatgctggatggaaggcatacttgaagagaactggtcagttagaataaatattgtaataaataggtctata<br>tacatacactaagcttctaggacgtcattgtagtcttcgaagttgtctgctagtttagttctcatgatttcgaaaaccaataacgcaatggatgtagcagggatggtggtta<br>gtgcgttcctgacaaacccagagtacgccgcctcaaaccacgtcacattcgccctttgcttcatccgcatcacttgcttgaaggtatccacgtacgagttgtaatacac<br>cttgaagaacggcttcgtctgc | |



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*EXAMPLE 28: Nucleic acid sequences of oligonucleotides referenced herein*

**Table 21**

| Oligo-nucleotide No. | Nucleic acid sequence | SEQ ID NO |
|---|---|---|
| oAA0124 | CACACACATATGCGACGGGTACAACGAGAATT | 129 |
| oAA0125 | CACACAACGCGTAGACGAAGCCGTTCTTCAAG | 130 |
| oAA0173 | ATGATCTGCCATGCCGAACTC | 131 |
| oAA0174 | AGCGAGTTCGGCATGGCAGATCATCATG | 132 |
| oAA0570 | CACACAGCTCTTCTAGAATGGCTTTAGACAAGTTAGA | 133 |
| oAA0571 | CACACAGCTCTTCGTTTCCAAACATCTTCTTGGTATCTATTTT | 134 |
| oAA0208 | CACACACTGCAGGAGCTCCAATTGTAATAT | 135 |
| oAA0209 | CACACATCTAGACCCGGGCTCTTCTTATGTCGTGAAGATTTGAACAAT | 136 |
| oAA0216 | GAGCCCGGGTCTAGATGTGTGCTCTTCCGAGTGACTCTTTTGATAA | 137 |
| oAA0217 | CACACACATATGGTCGACCTAAATTCGCAACTATCAA | 138 |
| oAA0519 | CACACAGCTCTTCCATAATGACTGCACAGGATATTATCGCC | 139 |
| oAA0520 | CACACAGCTCTTCCCTCTCTTCTATTCCTAATACATCTCAATGTTGGCACCG | 140 |
| oAA0572 | CACACAGCTCTTCCATAATGGCTTTAGACAAGTTAGATTTGT | 141 |
| oAA0573 | CACACAGCTCTTCCCTCTCTTCTATTCCTACCAAACATCTTCTTGGT | 142 |
| oAA2206 | TTCCGCTTAATGGAGTCCAAA | 143 |
| oAA2207 | ATGATCTGCCATGCCGAACTAGACGAAGCCGTTCTTCAAG | 144 |
| oAA2208 | CTTGAAGAACGGCTTCGTCTAGTTCGGCATGGCAGATCAT | 145 |
| oAA2209 | TAAACGTTGGGCAACCTTGG | 146 |
| oAA7409 | AAAATCAGAGGCTACTCCGGAGATGGCACATTATCACGTG | 147 |
| oAA7410 | TGTTGGTGGATGTGGCAGGGGTGGGGGTTGTATGGGTGGTA | 148 |
| oAA7403 | CCGGTTCGTCGCTTGATCCAAAATCAGAGGCTACTCCGGAGATGGCACATTATCACGTG | 149 |
| oAA7404 | CGTCGTCCATTGTTGGTGGATGTGGCAGGGGTGGGGGTTGTATGGGTGGTA | 150 |
| oAA7381 | AGAGTCACTCTTATATAACTTTGTCCAAGAACTTTCTGGCC | 151 |
| oAA7382 | CACCAACAATGGACGACGTTGATTCTG | 152 |
| oAA7379 | ACTGGCGGCCGCACGCGTCCAAGGTCGACCTAAATTCGCAAC | 153 |
| oAA7380 | AAGTTATATAAGAGTGACTCTTTTGATAAGAG | 154 |

| Table 21 | | |
|---|---|---|
| **Oligo-nucleotide No.** | **Nucleic acid sequence** | **SEQ ID NO** |
| oAA7377 | CTTGGACGCGTGCGGCCG | 155 |
| oAA7378 | GGATCAAGCGACGAACCG | 156 |
| oAA2586 | CACACAGCTCTTCCATAATGTTTAACTTTAAGTTGTCGCAACA | 157 |
| oAA2587 | CACACAGCTCTTCCCTCTCTTCTATTCTTACAACTTAGGCTTAGCATCAGTCA | 158 |
| oAA2372 | CCACCAAATCCATTATGCCA | 159 |
| oAA2373 | CCGAAACAACCGTAGATACCTTTAATGGGTTACCCTTGATGACTTCTG | 160 |
| oAA2374 | CAGAAGTCATCAAGGGTAACCCATTAAAGGTATCTACGGTTGTTTCGG | 161 |
| oAA2375 | GATTTCTTCGTCGTGGGCAGGGTACCGAGCTCTGCGAATT | 162 |
| oAA2376 | AATTCGCAGAGCTCGGTACCCTGCCCACGACGAAGAAATC | 163 |
| oAA2377 | AGTAAGCCAAACCGAAACCG | 164 |
| oAA3312 | AATTCGCAGAGCTCGGTACCCAACAAGTTGTTGTAGGCACTCAA | 165 |
| oAA3313 | TTGAGTGCCTACAACAACTTGTTGGGTACCGAGCTCTGCGAATT | 166 |
| oAA3314 | GACGAAGAATTCCAAAGGCTTGTTAAAGGTATCTACGGTTGTTTCGG | 167 |
| oAA3315 | CCGAAACAACCGTAGATACCTTTAACAAGCCTTTGGAATTCTTCGTC | 168 |
| oAA4719 | AAAGAAAGAAAGAAACTATAACAATCAATCATGCCAATTTTGAAAAAACCATTC | 169 |
| oAA4720 | TCTTATCAAAAGAGTCACTCTCTTCTATTCTTACAACTTAGGCTTAGCATCAG | 170 |
| oAA4722 | GAATAGAAGAGAGTGACTCTTTTG | 171 |
| oAA4723 | GATTGATTGTTATAGTTTCTTTCTTTC | 172 |
| oAA5319 | TTGACTGATGCTAAGTAAGAATAGAAGAGAGTGACTCTTTTG | 173 |
| oAA5320 | TCTCTTCTATTCTTACTTAGCATCAGTCAATAATCCCTTAG | 174 |
| oAA6472 | AAAAGAAAGAAAGAAACTATAACAATCAATCAATGTTTAACTTTAAGTTGTCGCAACA | 175 |
| oAA6473 | TCAAAAGAGTCACTCTCTTCTATTCTTACTTAGCATCAGTCAATAATCCCTTAGTC | 176 |
| oAA6367 | GAATAGAAGAGAGTGACTCTTTTGATAAGAGTC | 177 |
| oAA6368 | TGATTGATTGTTATAGTTTCTTTCTTTCTTTTG | 178 |
| oAA9946 | AAAGAAAGAAAGAAACTATAACAATCAATCATGTCAACTTACCAGTTCCAAG | 179 |
| oAA9947 | TCTTATCAAAAGAGTCACTCTCTTCTATTCCTAATACTCGCTTAATCTCAAC | 180 |
| oAA6669 | TGATTGATTGTTATAGTTTCTTTCTTTCTTTTG | 181 |
| oAA6771 | TCAACTTACCAGTTCCAAGAAACTTTAGAA | 182 |

| Table 21 | | |
|---|---|---|
| **Oligo-nucleotide No.** | **Nucleic acid sequence** | **SEQ ID NO** |
| oAA6772 | CAAAAGAAAGAAAGAAACTATAACAATCAATCAATGCTTTCCCGTACCACTTTAAGAGT | 183 |
| oAA6773 | TTCTAAAGTTTCTTGGAACTGGTAAGTTGATTGGTCGGTCTTGCTGTTGAA | 184 |
| oAA6774 | CAAAAGAAAGAAAGAAACTATAACAATCAATCAATGTCTGCATTAAGATCATTCCAACG | 185 |
| oAA6775 | TTCTAAAGTTTCTTGGAACTGGTAAGTTGAATAGGTTCTGACTGAGTTCTTTAAGGTGG | 186 |
| oAA6776 | CAAAAGAAAGAAAGAAACTATAACAATCAATCAATGTTTAACTTTAAGTTGTCGCAACA | 187 |
| oAA6777 | TTCTAAAGTTTCTTGGAACTGGTAAGTTGAACCCTTTGCGTGGCTGGT | 188 |
| oAA5511 | ATGGACGACGTTGATTCTGCTTTAGCCGATAATGTT | 189 |
| oAA5512 | TTATATAACTTTGTCCAAGAACTTTCTGGCC | 190 |
| oAA5553 | TTGTTTCTTTGACACATTGC | 191 |
| oAA5554 | TGCTATTACCACTGCTGG | 192 |
| oAA5555 | CAATGTGTCAAAGAAACAAGGTACCGAGCTCTGCGAA | 193 |
| oAA5556 | CAGCAGTGGTAATAGCATTAAAGGTATCTACGGTTGTTTCG | 194 |
| oAA5698 | GTAACCCCAGAAGGAAAC | 195 |
| oAA5699 | TTCAAGGGTACTGTGGCTAC | 196 |
| oAA5700 | GTTTGCTGTTTCCTTCTGGGGTTACGACGGGTACAACGAGAATTGTATTG | 197 |
| oAA5701 | CCAAAGTAGCCACAGTACCCTTGAATGCGAATTGAGCTGGGGG | 198 |
| oAA7624 | AGATCATCATGCCTGCAGAAAATCAGAGGCTACTCC | 199 |
| oAA7625 | GTCACTCTCTTCTATTCTTATATAACTTTGTCCAAGAACTTTC | 200 |
| oAA7512 | GAATAGAAGAGAGTGACTCTTTTG | 201 |
| oAA7265 | CTGCAGGCATGATGATCTG | 202 |
| oAA6369 | AAAAGAAAGAAAGAAACTATAACAATCAATCAATGGCTCCATACCTCAAAAAATTGAGA | 203 |
| oAA6370 | AGAGTCACTCTCTTCTATTCCTACAACTTGGCCCGTCTACCTTTGTTCAACTTCATGGT | 204 |
| oAA0784 | CACACAGCTCTTCCATAATGAGATGCCAAGTATCTCAACCATCAAG | 205 |
| oAA0785 | CACACAGCTCTTCCCTCTCTTCTATTCCTAGTTCAATGATTTAAGGAACTTTTCC | 206 |
| oAA0347 | CACACACTGCAGTTGTCCAATGTAATAATTTT | 207 |
| oAA0348 | CACACATCTAGACCCGGGCTCTTCTTCTGAATAGGCAATTGATAAACTTACT | 208 |

| Table 21 | | |
|---|---|---|
| **Oligo-nucleotide No.** | **Nucleic acid sequence** | **SEQ ID NO** |
| | TATC | |
| oAA0351 | GAGCCCGGGTCTAGATGTGTGCTCTTCCAAAGTACGGTGTTGTTGACA | 209 |
| oAA0352 | CACACACATATGAATTCTGTACTGGTAGAGCTAAATT | 210 |
| oAA5668 | GCTGCCAGTGTTGGTGACGACT | 211 |
| oAA5669 | TTGGACGGCGTTGTATCTGGAA | 212 |
| oAA5670 | GCTGTTTCTGACTTGTCGTTTGT | 213 |
| oAA5671 | TGCAGCATGCTTCATAGTGGTC | 214 |
| oAA5672 | GCTTTTGTTGTTGATTCCAAGAG | 215 |
| oAA5673 | CAAGTCAGAAACAGATGCAGCA | 216 |
| oAA5690 | GCTGTTTCTGACTTGGCTTTTGTTGTTGATTCCAAGAGCGAATCCACTTCCAGA | 217 |
| oAA5691 | TGCAGCATGCTTCATAGTGGTC | 218 |
| oAA6145 | TTCAAGTTGGCTCAAATCGCAGGTGCCAGATACAACGCCGTCCAATCTGC | 219 |
| oAA6146 | GCAGATTGGACGGCGTTGTATCTGGCACCTGCGATTTGAGCCAACTTGAA | 220 |
| oAA6691 | GGTGCTCGCTCATTGGCTGATGGTGGCTTGTTG | 221 |
| oAA6692 | CAACAAGCCACCATCAGCCAATGAGCGAGCACC | 222 |
| oAA7257 | CTCTACGCGTTGTACTGGTAGAGCTAAATTGTA | 223 |
| oAA7258 | CATCATGCCTGCAGGTCGACT | 224 |
| oAA7259 | TCATGACTTTCCTGTTCTATCTCTCCCC | 225 |
| oAA7260 | CCATGCTCGAGAGTGCTCCGAAAA | 226 |
| oAA6371 | AAAAGAAAGAAAGAAACTATAACAATCAATCAATGCCAGAATCAACTCAACAATCTC | 227 |
| oAA6372 | GACTCTTATCAAAAGAGTCACTCTCTTCTATTCTTATTTCTTGCAGGCATGGACA | 228 |
| oAA6470 | AAAAGAAAGAAAGAAACTATAACAATCAATCAATGACTACAGACTCAAACACCCACA | 229 |
| oAA6471 | GACTCTTATCAAAAGAGTCACTCTCTTCTATTCTTACTTTCTACCGAATTGAGCACC | 230 |
| oAA3582 | ATGCTTACACTAACTTCTGGTCCCAACC | 231 |
| oAA3583 | GACTATTGTCTCTAGCGGTCTAGATCCT | 232 |
| oAA3584 | CACATCGAGCCATGATTGAGAAC | 233 |
| oAA3585 | CGTGAGCAAACAATTGGAACG | 234 |



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| Table 21 | | |
|---|---|---|
| **Oligo-nucleotide No.** | **Nucleic acid sequence** | **SEQ ID NO** |
| oAA3586 | ATGAGTCACCCAACGCCAGA | 235 |
| oAA3587 | TCAGAGCTTCGGCGACTCAG | 236 |
| oAA3588 | ACCATGACGCTGATTGCACC | 237 |
| oAA3589 | CGAGCTACAATTTCTGCACACCT | 238 |
| oAA3590 | ATGCCCACATTCAACTACAAGGAC | 239 |
| oAA3591 | CGCTCTGTTGCAAGTATCGTCA | 240 |
| oAA3592 | ATGTCAACCACCGGAATCTACAAC | 241 |
| oAA3593 | CTACAACTTTGGAGTTGTGACCTGCTTC | 242 |
| oAA3594 | CAGACCCCGAATAATGGACAAA | 243 |
| oAA3595 | CCTCTCTATAATTTCGGGGTGATCAC | 244 |
| oAA3596 | GATCATGGAGAGATTACAGGCCG | 245 |
| oAA3597 | CGTTTTCCCCTACAACTTTGGC | 246 |
| oAA6449 | AAAGAAAGAAAGAAACTATAACAATCAATCATGAGTCACCCAACGCCAGAAG | 247 |
| oAA6450 | TCTTATCAAAAGAGTCACTCTCTTCTATTCTTACGACTCAGCTATGTACAACCTC | 248 |
| oAA2127 | CACACAGCTCTTCCATAATGGTCGCCGATTCTTTAGT | 249 |
| oAA2128 | CACACAGCTCTTCCCTCTCTTCTATTCTTAAGTGGCCTTCCACAAGT | 250 |
| oAA3156 | TTGATAGTTGCGAATTTAGGTCGACAACGATATAAACATTAGCATGCCTG | 251 |
| oAA3354 | CATTATGGTGTTCCGGTTATGAATTCGGTTGAATTGTGGAGTAAAAA | 252 |
| oAA3159 | CCCGAAATATTACAATTGGAGCTCAAGGCTATCTTTAAGTCTATTGACGACA | 253 |
| oAA3356 | GTCAGATTTCACGCCTTTACGAATTCATCAGACTTTTCAAATCTTTCTCTTTCG | 254 |
| oAA3157 | CAGGCATGCTAATGTTTATATCGTTGTCGACCTAAATTCGCAACTATCAA | 255 |
| oAA3158 | TGTCGTCAATAGACTTAAAGATAGCCTTGAGCTCCAATTGTAATATTTCGGG | 256 |
| oAA3355 | CATTATGGTGTTCCGGTTAT | 257 |
| oAA3357 | GTCAGATTTCACGCCTTTAC | 258 |
| oAA951 | CCTACTTCCACAGCTTTAATCTACTATCAT | 259 |
| oAA952 | TTTAAGAAAACAACTAAGAGAAGCCAC | 260 |
| oAA3557 | CATTGAAGAGTGTTTTGCCAGAA | 261 |
| oAA3558 | CCGAAACAACCGTAGATACCTTTAAGGCAAAAATGCAATCACTCTGT | 262 |

| Table 21 | | |
|---|---|---|
| **Oligo-nucleotide No.** | **Nucleic acid sequence** | **SEQ ID NO** |
| oAA3559 | ACAGAGTGATTGCATTTTTGCCTTAAAGGTATCTACGGTTGTTTCGG | 263 |
| oAA3560 | CGAAATATTACAATTGGAGCTCGGTACCGAGCTCTGCGAATT | 264 |
| oAA3561 | AATTCGCAGAGCTCGGTACCGAGCTCCAATTGTAATATTTCG | 265 |
| oAA3562 | CAGTGGCGGCTTTAACCTTCCGCATATGGTCGACCTAAAT | 266 |
| oAA3563 | ATTTAGGTCGACCATATGCGGAAGGTTAAAGCCGCCACTG | 267 |
| oAA3564 | GGGTCCACTCGTCATCCAAC | 268 |
| oAA5770 | AAAGAAAGAAAGAAACTATAACAATCAATCATGATTGAACAACTCCTAGAATATTG | 269 |
| oAA5771 | TCTTATCAAAAGAGTCACTCTCTTCTATTCCTAGTCAAACTTGACAATAGC | 270 |
| oAA5768 | GAATAGAAGAGAGTGACTCTTTTG | 271 |
| oAA5769 | GATTGATTGTTATAGTTTCTTTCTTTC | 272 |
| oAA2125 | CACACAGCTCTTCCATAATGGTCAACATATCGAAATT | 273 |
| oAA2126 | CACACAGCTCTTCCCTCTCTTCTATTCTTATATTTCAAATACTTTAT | 274 |
| oAA2159 | CACACAGCTCTTCCATAATGACAGTGGAGAATGCAAAA | 275 |
| oAA2162 | CACACAGCTCTTCCCTCTCTTCTATTCCTACTTCTTGGCGACACTCTTCTTTTCATTCTCCTT | 276 |
| oAA2679 | GACGGGTTATAACAAGCAGGACA | 277 |
| oAA2680 | CCGAAACAACCGTAGATACCTTTAATCAAGATCTTCCAGATAATGGTCAA | 278 |
| oAA2681 | TTGACCATTATCTGGAAGATCTTGATTAAAGGTATCTACGGTTGTTTCGG | 279 |
| oAA2682 | CAACAAATAACAAGTTCTTGCCCTTGGTACCGAGCTCTGCGAATT | 280 |
| oAA2683 | AATTCGCAGAGCTCGGTACCAAGGGCAAGAACTTGTTATTTGTTG | 281 |
| oAA2684 | CTTACTAAGAGATGGACGGTTTGAA | 282 |
| oAA2914 | AATTGACGGGTTATAACAAGCAGG | 283 |
| oAA2915 | AATTCGCAGAGCTCGGTACCGGTGATGTACTGGTCTATGTTTTGGAT | 284 |
| oAA2916 | ATCCAAAACATAGACCAGTACATCACCGGTACCGAGCTCTGCGAATT | 285 |
| oAA2917 | GGTCATCGTCATCTATATCAGCAATATTTAAAGGTATCTACGGTTGTTTCGG | 286 |
| oAA2918 | CCGAAACAACCGTAGATACCTTTAAATATTGCTGATATAGATGACGATGACC | 287 |
| oAA2919 | CACCTGGTACATCAACTCTTCCATA | 288 |
| oAA3051 | CACACAGCTCTTCCATAATGTCC | 289 |
| oAA3052 | CACACAGCTCTTCCCTCTCTTCT | 290 |
| oAA3053 | CACACAGCTCTTCCATAATGTCAG | 291 |

| Table 21 | | |
|---|---|---|
| **Oligo-nucleotide No.** | **Nucleic acid sequence** | **SEQ ID NO** |
| oAA6658 | CACACAGCTCTTCCATCATGTCCGCCAACGAAAACAT | 292 |
| oAA6659 | CACACAGCTCTTCCAGCTCATGATCTAGTCTTGGCCTTAG | 293 |
| oAA6660 | CACACAGCTCTTCCATCATGTCAGCGAAATCTATTCACGAAGCCGACGGTAAGGC | 294 |
| oAA6661 | CACACAGCTCTTCCAGCTCACACGCCTAAAGGAGTAGAAGCTTC | 295 |
| oAA540 | CACACAGCTCTTCCATAATGCCTACCGAACTTCAAAAAGAAAG | 296 |
| oAA541 | CACACAGCTCTTCCCTCTCTTCTATTCTTAACTGGACAAGATTTCAGCAGC | 297 |
| F98G fwd | GTCTTTGACCCACAAGTCGGAACCAGAATCGGTGTCAAC | 298 |
| F98G rev | GTTGACACCGATTCTGGTTCCGACTTGTGGGTCAAAGAC | 299 |
| W429F fwd | GTAAAGCCTACTCCGACTTTGTTGTCCAATGTACCTG | 300 |
| W429F rev | CAGGTACATTGGACAACAAAGTCGGAGTAGGCTTTAC | 301 |
| oAA0179 | GAATTCACATGGCTAATTTGGCCTCGGTTCCACAACGCACTCAGCATTAAAAA | 302 |
| oAA0182 | GAGCTCCCCTGCAAACAGGGAAACACTTGTCATCTGATTT | 303 |
| oAA1023 | GATATTATTCCACCTTCCCTTCATT | 304 |
| oAA1024 | CCGTTAAACAAAAATCAGTCTGTAAA | 305 |
| oAA2055 | TGCCATCCTTGGTAGTCAGTTATT | 306 |
| oAA2056 | CCGAAACAACCGTAGATACCTTTAATGGCTTGTCCTTGGTGTTGA | 307 |
| oAA2060 | TGTCGCCATTCAACCAGTAGAT | 308 |
| oAA2069 | AATTCGCAGAGCTCGGTACCGACTTGTTGAAGATGGACGAGGA | 309 |
| oAA2057 | TCAACACCAAGGACAAGCCATTAAAGGTATCTACGGTTGTTTCGG | 310 |
| oAA2068 | TCCTCGTCCATCTTCAACAAGTCGGTACCGAGCTCTGCGAATT | 311 |
| oAA2070 | TTGATCCACTGTCTTAAGATTGTCAA | 312 |
| oAA2075 | AGGATTTTGCTGTTGGTGGC | 313 |
| oAA2074 | AATTCGCAGAGCTCGGTACCATACGTCGGTGAGGTCTGTCG | 314 |
| oAA2072 | ATTCTCCGCTACTTCGTTCTGGTTAAAGGTATCTACGGTTGTTTCGG | 315 |
| oAA2073 | CGACAGACCTCACCGACGTATGGTACCGAGCTCTGCGAATT | 316 |
| oAA2071 | CCGAAACAACCGTAGATACCTTTAACCAGAACGAAGTAGCGGAGAAT | 317 |
| oAA2656 | AAGCTTTTAATTAAAGATAATCACAGGGGTAGAGACCTTG | 318 |
| oAA2657 | GGATCCGCATGCGGCCGGCCGATAGCGTGGTATGAATGAATAAGTGTG | 319 |
| oAA2658 | GGATCCGAGCTCGCGGCCGCGAGCACTAGGTTTTGATAATTTGGTTCTTAC | 320 |

| Table 21 | | |
|---|---|---|
| **Oligo-nucleotide No.** | **Nucleic acid sequence** | **SEQ ID NO** |
| oAA2659 | GAATTCTTAATTAACGGCGAAGAACATAGTGTGATG | 321 |

*EXAMPLE 29: Transformation procedure*

5 mL YPD start cultures were inoculated with a single colony of Candida strain ATCC20913 and incubated overnight at 30°C, with shaking at about 200 rpm. The following day, fresh 25 mL YPD cultures were inoculated to an initial OD600 nm of 0.4 and the culture incubated at 30°C, with shaking at about 200 rpm until an OD600 nm of 1.0-2.0 was reached. Cells were pelleted by centrifugation at 1,000 x g, 4°C for 10 minutes. Cells were washed by resuspending in 10 mL sterile water, pelleted, resuspended in 1 mL sterile water and transferred to a 1.5 mL microcentrifuge tube. The cells were then washed in 1 mL sterile TE/LiOAC solution, pH 7.5, pelleted, resuspended in 0.25 mL TE/LiOAC solution and incubated with shaking at 30°C for 30 minutes.

The cell solution was divided into 50 µL aliquots in 1.5 mL tubes to which was added 5-8 µg of linearized DNA and 5 µL of carrier DNA (boiled and cooled salmon sperm DNA, 10 mg/mL). 300 µL of sterile PEG solution (40% PEG 3500, 1X TE, 1X LiOAC) was added, mixed thoroughly and incubated at 30°C for 60 minutes with gentle mixing every 15 minutes. 40 µL of DMSO was added, mixed thoroughly and the cell solution was incubated at 42°C for 15 minutes. Cells were then pelleted by centrifugation at 1,000 x g 30 seconds, resuspended in 500 µL of YPD media and incubated at 30°C with shaking at about 200 rpm for 2 hours. Cells were then pelleted by centrifugation and resuspended in 1mL 1X TE, cells were pelleted again, resuspended in 0.2 mL 1X TE and plated on selective media. Plates were incubated at 30°C for growth of transformants.

*EXAMPLE 30: Construction of strain sAA7445 for the production of lycopene from glucose*

In order to integrate genes into the genome of *C. viswanathii*, genes were cloned into a plasmid that allows for single crossover recombination at the URA3 loci. In addition, since glucose was the primary carbon source, all genes were expressed under a GPD promoter with a POX4

terminator. The backbone for all plasmids was PCR amplified from pAA2534 with primers oAA07511 and oAA07512. The CrtE gene from *Cronobacter sakazakii* (CsCrtE) was codon optimized for *C. viswanathii*, synthesized, and then PCR amplified using primers oAA9745 and oAA9746. The CsCrtE PCR product and backbone contained overlapping sequence at their termini allowing directional ligation, generating plasmid pAA2698. The CrtB gene from *Cronobacter sakazakii* (CsCrtB) was codon optimized for *C. viswanathii*, synthesized, and then PCR amplified using primers oAA9749 and oAA9750. The CsCrtB PCR product and backbone contained overlapping sequence at their termini allowing directional ligation, generating plasmid pAA2699. The CrtI gene from *Cronobacter sakazakii* (CsCrtI) was codon optimized for *C. viswanathii*, synthesized, and then PCR amplified using primers oAA9747 and oAA9748. The CsCrtI PCR product and backbone contained overlapping sequence at their termini allowing directional ligation, generating plasmid pAA2700. Integration cassettes containing glucose inducible CsCrtE, CsCrtB, CsCrtI genes were then generated by PCR amplification of plasmids pAA2698, pAA2699, and pAA2700 with primers oAA2206 and oAA2209. All three cassettes were purified and chemically transformed into strain sAA002, which was then plated onto SCD-ura plates. Yellow/red colored colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of all three genes, and one verified isolate was saved as strain sAA7445.

*EXAMPLE 31: Shake flask characterization of sAA7445 on glucose*

Starter cultures (5 mL) of sAA7445 in YPD were incubated overnight at 30°C, with shaking at approximately 250 rpm. The overnight cultures were used to inoculate 25 mL fresh SP92-glucose media (6.7 g/L yeast nitrogen base, 3.0 g/L yeast extract, 3.0 g/L $(NH_4)_2SO_4$, 1.0 g/L $K_2HPO_4$, 1.0 g/L $KH_2PO_4$, 75 g/L glucose) to an initial OD600 nm of 0.4 and incubated approximately 24 hours at 30°C, and 300 rpm shaking. Cells were pelleted by centrifugation for 10 minutes at 3,000xg, at 4°C, and then resuspended in 12.5 mL HiP-TAB media (yeast nitrogen base without amino acids and without ammonium sulfate, 1.7 g/L; yeast extract, 3.0 g/L; potassium phosphate monobasic, 10.0 g/L; potassium phosphate dibasic, 10.0 g/L) and added to 250 mL baffled shake flasks. 500 µL of glucose was added to the shake flasks, which were shaken at approximately 300 rpm, at 30°C. Cultures were incubated for 48 hours and samples were taken at 48 hours for analysis of carotenoid production by HPLC, and results are presented in Table 22 below.

| Table 22 | | | | |
|---|---|---|---|---|
| **Strain** | **Substrate** | **Lycopene (g/L)** | **B-carotene (g/L)** | **Astaxanthin (g/L)** |
| sAA7445 | Glucose | 0.02 | | |
| sAA7882 | Glucose | 0.003 | 0.007 | |
| sAA7870 | Glucose | 0.0015 | 0.001 | 0.0015 |
| sAA7446 | Oleic Acid | 0.001 | | |
| sAA7565 | Oleic Acid | 0.011 | | |
| sAA8283 | Oleic Acid | 0.061 | | |
| sAA8283 | C10-C14 alkanes | 0.008 | | |
| sAA8283 | Methyl pentadecanoate | 0.012 | | |
| sAA8283 | Ethyl laurate | 0.022 | | |
| sAA8283 | CPO | 0.030 | | |
| sAA8283 | Ethyl PFAD | 0.051 | | |
| sAA8283 | Nonane | 0.002 | | |
| sAA8283 | Octadecane | 0.0125 | | |
| sAA8519 | Oleic Acid | 0.002 | 0.007 | |
| sAA8503 | Glucose | 0.035 | | |
| sAA8932 | Glucose | 0.064 | | |
| sAA8932 | Oleic Acid | 0.027 | | |
| sAA9432 | Oleic acid | 0.061 | | |
| sAA9670 | Oleic acid | 0.167 | | |
| sAA9811 | Oleic acid | 0.156 | | |
| sAA9812 | Oleic acid | 0.214 | | |
| sAA9814 | Oleic acid | 0.161 | | |
| sAA9817 | Oleic acid | 0.277 | | |
| sAA9819 | Oleic acid | 0.382 | | |
| sAA9821 | Oleic acid | 0.447 | | |

*EXAMPLE 32: Construction of strain sAA7446 for the production of lycopene from oleic acid*

In order to integrate genes into the genome of *C. viswanathii*, genes were cloned into a plasmid that allows for single crossover recombination at the URA3 loci. In addition, since oleic acid was the primary carbon source, all genes were expressed under a HDE1 promoter with a POX4 terminator. The backbone for all plasmids was PCR amplified from pAA1164 with primers oAA5788 and oAA5789. The CrtE gene from *Cronobacter sakazakii* (CsCrtE) was codon optimized for *C. viswanathii*, synthesized, and then PCR amplified using primers oAA9753 and oAA9754. The CsCrtE PCR product and backbone contained overlapping sequence at their termini allowing directional ligation, generating plasmid pAA2702. The CrtB gene from *Cronobacter sakazakii* (CsCrtB) was codon optimized for *C. viswanathii*, synthesized, and then PCR amplified using primers oAA9757 and oAA9758. The CsCrtB PCR product and backbone contained overlapping sequence at their termini allowing directional ligation, generating plasmid pAA2703. The CrtI gene from *Cronobacter sakazakii* (CsCrtI) was codon optimized for *C. viswanathii*, synthesized, and then PCR amplified using primers oAA9755 and oAA9756. The CsCrtI PCR product and backbone contained overlapping sequence at their termini allowing directional ligation, generating plasmid pAA2704. Integration cassettes containing oleic acid inducible CsCrtE, CsCrtB, CsCrtI genes were then generated by PCR amplification of plasmids pAA2702, pAA2703, and pAA2704 with primers oAA2206 and oAA2209. All three cassettes were purified and chemically transformed into strain sAA002; the cells were plated onto SCD-ura plates. Yellow colored colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of all three genes, and one verified isolate was saved as strain sAA7446.

*EXAMPLE 33: Shake flask characterization of sAA7446 on oleic acid*

Starter cultures (5 mL) of sAA7446 in YPD were incubated overnight at 30°C, with shaking at approximately 250 rpm. The overnight cultures were used to inoculate 25 mL fresh SP92-glycerol media (6.7g/L yeast nitrogen base, 3.0 g/L yeast extract, 3.0 g/L $(NH_4)_2SO_4$, 1.0 g/L $K_2HPO_4$, 1.0 g/L $KH_2PO_4$, 75 g/L glycerol) to an initial OD600 nm of 0.4 and incubated approximately 24 hours at 30°C, and 300 rpm shaking. Cells were pelleted by centrifugation for 10 minutes at 3,000xg, at 4°C, and then resuspended in 12.5 mL HiP-TAB media (yeast nitrogen base without amino acids and without ammonium sulfate, 1.7 g/L; yeast extract, 3.0 g/L; potassium phosphate monobasic, 10.0 g/L; potassium phosphate dibasic, 10.0 g/L) and added to 250 mL baffled shake flasks. 500 µL of oleic acid was added to the shake flasks, which were shaken at approximately 300 rpm, at 30°C. Cultures were incubated for 48 hours

and samples were taken at 48 hours for analysis of carotenoid production by HPLC, and results are presented in Table 22 above.

*EXAMPLE 34: Construction of strain sAA7449 for the production of valencene from glucose*

In order to integrate genes into the genome of *C. viswanathii*, genes were cloned into a plasmid that allows for single crossover recombination at the URA3 loci. In addition, since glucose was the primary carbon source, all genes will be expressed under a GPD promoter with a POX4 terminator. The TPS1 Gene from *Callitropsis nootkatensis* (CnTPS1) was codon optimized for *C. viswanathii*, synthesized, and then PCR amplified using primers oAA9751 and oAA9752. In addition, the plasmid backbone was PCR amplified from pAA2534 using primers oAA07511 and oAA07512. The CnTPS1 PCR product and backbone contained overlapping sequence at their termini allowing directional ligation, generating plasmid pAA2701. An integration cassette for CnTPS1 was then generated by PCR amplification of the pAA2701 plasmid with primers oAA2206 and oAA2209. The PCR product was purified and chemically transformed into strain sAA002, and plated onto SCD-ura plates. Colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of the CnTPS1 gene, and one verified isolate was saved as strain sAA7449.

*EXAMPLE 35: Shake flask characterization of sAA7449 on glucose*

Starter cultures (5 mL) of sAA7449 in YPD were incubated overnight at 30°C, with shaking at approximately 250 rpm. The overnight cultures were used to inoculate 25 mL fresh SP92-glucose media (6.7 g/L yeast nitrogen base, 3.0 g/L yeast extract, 3.0 g/L $(NH_4)_2SO_4$, 1.0 g/L $K_2HPO_4$, 1.0 g/L $KH_2PO_4$, 75 g/L glucose) to an initial OD600 nm of 0.4 and incubated approximately 24 hours at 30°C, and 300 rpm shaking. Cells were pelleted by centrifugation for 10 minutes at 3,000xg, at 4°C, and then resuspended in 12.5 mL HiP-TAB media (yeast nitrogen base without amino acids and without ammonium sulfate, 1.7 g/L; yeast extract, 3.0 g/L; potassium phosphate monobasic, 10.0 g/L; potassium phosphate dibasic, 10.0 g/L) and added to 250 mL baffled shake flasks. 500 µL of glucose was added to the shake flasks, in addition to 1.25 mL of n-Decane. The flasks were then incubated at 30°C and shaken at approximately 300 rpm. Incubation of the cultures continued for 48 hours and samples were taken from the organic layer at 48 hours for analysis of valencene production by GCMS. A Shimadzu GCMS-QP2010SE equipped with AOC-20i auto injector with a 30 m x 0.32 mm x

0.25 µm Phenomenex ZB-5HT column was used. Retention time and identity of valencene was determined by injection of a standard from Sigma-Aldrich and confirmed by NIST MS Search 2.0 library match. Positive valencene containing samples were matched by their retention times and their mass spectra (Fig 48A, Fig. 48B).

*EXAMPLE 36: Construction of strain sAA7453 for the production of valencene from oleic acid*

In order to integrate genes into the genome of *C. viswanathii*, genes were cloned into a plasmid that allows for single crossover recombination at the URA3 loci. In addition, since oleic acid was the primary carbon source, all genes will be expressed under a HDE1 promoter with a POX4 terminator. The TPS1 Gene from *Callitropsis nootkatensis* (CnTPS1) was codon optimized for *C. viswanathii*, synthesized, and then PCR amplified using primers oAA9759 and oAA9760. In addition, the plasmid backbone was PCR amplified from pAA1164 using primers oAA05788 and oAA05789. The CnTPS1 PCR product and backbone contained overlapping sequence at their termini allowing directional ligation, generating plasmid pAA2705. An integration cassette for CnTPS1 was then generated by PCR amplification of the pAA2705 plasmid with primers oAA2206 and oAA2209. The PCR product was purified and chemically transformed into strain sAA002, and plated onto SCD-ura plates. Colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of the CnTPS1 gene, and one verified isolate was saved as strain sAA7453.

*EXAMPLE 37: Shake flask characterization of sAA7453 on oleic acid*

Starter cultures (5 mL) of sAA7453 in YPD were incubated overnight at 30°C, with shaking at approximately 250 rpm. The overnight cultures were used to inoculate 25 mL fresh SP92-glucose media (6.7 g/L yeast nitrogen base, 3.0 g/L yeast extract, 3.0 g/L $(NH_4)_2SO_4$, 1.0 g/L $K_2HPO_4$, 1.0 g/L $KH_2PO_4$, 75 g/L glucose) to an initial OD600 nm of 0.4 and incubated approximately 24 hours at 30°C, and 300 rpm shaking. Cells were pelleted by centrifugation for 10 minutes at 3,000xg, at 4°C, and then resuspended in 12.5 mL HiP-TAB media (yeast nitrogen base without amino acids and without ammonium sulfate, 1.7 g/L; yeast extract, 3.0 g/L; potassium phosphate monobasic, 10.0 g/L; potassium phosphate dibasic, 10.0 g/L) and added to 250 mL baffled shake flasks. 500 µL of oleic acid was added to the shake flasks, in addition to 1.25 mL of n-Decane. The flasks were then incubated at 30°C and shaken at approximately 300 rpm. Incubation of the cultures continued for 48 hours and samples were

taken from the organic layer at 48 hours for analysis of valencene production by GCMS. A Shimadzu GCMS-QP2010SE equipped with AOC-20i auto injector with a 30 m x 0.32 mm x 0.25 µm Phenomenex ZB-5HT column was used. Retention time and identity of valencene was determined by injection of a standard from Sigma-Aldrich and confirmed by NIST MS Search 2.0 library match. Positive valencene containing samples were matched by their retention times and their mass spectra (Fig 49A, Fig. 49B).

*EXAMPLE 38: Construction of strain sAA6234*

Strain sAA5733 (described in International Patent Application Publication No. WO2016/154046) was placed on 5'FOA for the loop-out of URA3, leaving a URA3 terminator scar. One correct transformant was identified and saved as strain sAA5761. In order to knock out the final copy of CRC1 from the genome, plasmid pAA1701 with 253 bp and 375 bp homology region to the 5' and 3' respectively within the open reading frame of CRC1 in the genome was constructed with a URA3 selection cassette. Plasmid pAA1701 construction details are provided in Example 6 and Fig. 32. CRC1 knockout cassette was amplified from pAA1701 using primers oAA05511 and oAA5512 and the resulting fragment was integrated into the sAA5761 genome with URA selection. Correct crc1/crc1::URA3 transformants were identified and plated onto 5'FOA for the loop-out of URA3, leaving a URA3 promoter scar. One correct transformant was identified and saved as strain sAA6234.

*EXAMPLE 39: Construction of strain sAA7565 for the production of lycopene from oleic acid*

Plasmid pAA2311 was constructed by PCR amplification of pG6PI-CRC1 fragment from pAA2214 using primers oAA7624 and oAA7625, and PCR amplification of the plasmid backbone from pAA1116 using primers oAA7512 and oAA7265 (in certain instances, PCR amplification of the plasmid backbone was from pAA1164). The two PCR products encoded overlapping sequence at their termini allowing directional ligation, generating plasmid pAA2311. Integration cassettes for CsCrtE, CsCrtB, CsCrtI, and pG6PI-CRC1 were then generated by PCR amplification of plasmids pAA2702, pAA2703, pAA2704, and pAA2311 with primers oAA2206 and oAA2209. All four cassettes were purified and chemically transformed into strain sAA6234, which had both copies of CRC1 deleted. The transformed cells were then plated onto SCD-ura plates. Colonies were streaked onto YPD for isolation and characterization. Colony

PCR was performed to confirm the presence of all four genes, and one verified isolate was saved as strain sAA7565.

*EXAMPLE 40: Shake flask characterization of sAA7565 on oleic acid*

Starter cultures (5 mL) of sAA7565 in YPD were incubated overnight at 30°C, with shaking at approximately 250 rpm. The overnight cultures were used to inoculate 25 mL fresh SP92-glycerol media (6.7 g/L yeast nitrogen base, 3.0 g/L yeast extract, 3.0 g/L $(NH_4)_2SO_4$, 1.0 g/L $K_2HPO_4$, 1.0 g/L $KH_2PO_4$, 75 g/L glycerol) to an initial OD600 nm of 0.4 and incubated approximately 24 hours at 30°C, and 300 rpm shaking. Cells were pelleted by centrifugation for 10 minutes at 3,000xg, at 4°C, and then resuspended in 12.5 mL HiP-TAB media (yeast nitrogen base without amino acids and without ammonium sulfate, 1.7 g/L; yeast extract, 3.0 g/L; potassium phosphate monobasic, 10.0 g/L; potassium phosphate dibasic, 10.0 g/L) and added to 250 mL baffled shake flasks. 500 µL of oleic acid was added to the shake flasks, which were shaken at approximately 300 rpm, at 30°C. Cultures were incubated for 48 hours and samples were taken at 48 hours for analysis of carotenoid production by HPLC, and results are presented in Table 22 above.

*EXAMPLE 41: Construction of strain sAA7882 for the production of β-carotene from glucose*

In order to integrate genes into the genome of *C. viswanathii*, genes were cloned into a plasmid that allows for single crossover recombination at the URA3 loci. In addition, since glucose was the primary carbon source, all genes were expressed under a GPD promoter with a POX4 terminator. The CrtYB Gene from *Xanthophyllomyces dendrorho* (XdCrtYB) was codon optimized for *C. viswanathii*, and then synthesized with additional flanking regions. The XdCrtYB gene and flanking regions were PCR amplified using primers oAA9958 and oAA9959, while the plasmid backbone was PCR amplified from pAA2534 with primers oAA07511 and oAA07512. The two PCR products encoded overlapping sequence at their termini allowing directional ligation, generating plasmid pAA2866. Integration cassettes for CsCrtE, CsCrtI, and XdCrtYB were then generated by PCR amplification of plasmids pAA2698, pAA2700, and pAA2866 with primers oAA2206 and oAA2209. All three cassettes were purified and chemically transformed into strain sAA002, which was then plated onto SCD-ura plates. Yellow colored colonies were streaked onto YPD for isolation and characterization. Colony PCR was

performed to confirm the presence of all three genes, and one verified isolate was saved as strain sAA7882.

*EXAMPLE 42: Shake flask characterization of sAA7882 on glucose*

Starter cultures (5 mL) of sAA7882 in YPD were incubated overnight at 30°C, with shaking at approximately 250 rpm. The overnight cultures were used to inoculate 25 mL fresh SP92-glucose media (6.7 g/L yeast nitrogen base, 3.0 g/L yeast extract, 3.0 g/L $(NH_4)_2SO_4$, 1.0 g/L $K_2HPO_4$, 1.0 g/L $KH_2PO_4$, 75 g/L glucose) to an initial OD600 nm of 0.4 and incubated approximately 24 hours at 30°C, and 300 rpm shaking. Cells were pelleted by centrifugation for 10 minutes at 3,000xg, at 4°C, and then resuspended in 12.5 mL HiP-TAB media (yeast nitrogen base without amino acids and without ammonium sulfate, 1.7 g/L; yeast extract, 3.0 g/L; potassium phosphate monobasic, 10.0 g/L; potassium phosphate dibasic, 10.0 g/L) and added to 250 mL baffled shake flasks. 500 µL of glucose was added to the shake flasks, which were shaken at approximately 300 rpm, at 30°C. Incubation of the cultures continued for 48 hours and samples were taken at 48 hours for analysis of carotenoid production by HPLC, and results are presented in Table 22 above.

*EXAMPLE 43: Construction of strain sAA7870 for the production of astaxanthin from glucose*

In order to integrate genes into the genome of *C. viswanathii*, genes were cloned into a plasmid that allows for single crossover recombination at the URA3 loci. In addition, since glucose was the primary carbon source, all genes were expressed under a GPD promoter with a POX4 terminator. The backbone for all plasmids was amplified from pAA2534 with primers oAA07511 and oAA07512. The CrtR Gene from *Xanthophyllomyces dendrorhous* (XdCrtR) was codon optimized for *C. viswanathii*, and then synthesized with additional flanking regions. The XdCrtR gene and flanking regions were amplified using primers oAA9960 and oAA9961. The XdCrtR PCR product and the pAA2534 backbone PCR encoded overlapping sequence at their termini allowing directional ligation, generating plasmid pAA2993. The CrtS Gene from *Xanthophyllomyces dendrorho* (XdCrtS) was codon optimized for *C. viswanathii*, and then synthesized with additional flanking regions. The XdCrtS gene and flanking regions were amplified using primers oAA9962 and oAA9963. The XdCrtS PCR product and the pAA2534 backbone PCR encoded overlapping sequence at their termini allowing directional ligation, generating plasmid pAA2994. The CrtW Gene from *Agrobacterium aurantiacum* (AaCrtW) was

codon optimized for *C. viswanathii*, and then synthesized with additional flanking regions. The AaCrtW gene and flanking regions were amplified using primers oAA9964 and oAA9965. The AaCrtW PCR product and the pAA2534 backbone PCR encoded overlapping sequence at their termini allowing directional ligation, generating plasmid pAA2995. The CrtZ Gene from *Agrobacterium aurantiacum* (AaCrtZ) was codon optimized for *C. viswanathii*, and then synthesized with additional flanking regions. The AaCrtZ gene and flanking regions were amplified using primers oAA9966 and oAA9967. The AaCrtZ PCR product and the pAA2534 backbone PCR encoded overlapping sequence at their termini allowing directional ligation, generating plasmid pAA2996. Integration cassettes for CsCrtE, CsCrtI, XdCrtYB, XdCrtR, XdCrtS, AaCrtW, and AaCrtZ were then generated by PCR amplification of plasmids pAA2698, pAA2700, pAA2866, pAA2993, pAA2994, pAA2995, and pAA2996 with primers oAA2206 and oAA2209. All seven cassettes were purified and chemically transformed into strain sAA002, which was then plated onto SCD-ura plates. Yellow colored colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of all seven genes, and one verified isolate was saved as strain sAA7870.

*EXAMPLE 44: Shake flask characterization of sAA7870 on glucose*

Starter cultures (5 mL) of sAA7870 in YPD were incubated overnight at 30°C, with shaking at approximately 250 rpm. The overnight cultures were used to inoculate 25 mL fresh SP92-glucose media (6.7 g/L yeast nitrogen base, 3.0 g/L yeast extract, 3.0 g/L $(NH_4)_2SO_4$, 1.0 g/L $K_2HPO_4$, 1.0 g/L $KH_2PO_4$, 75 g/L glucose) to an initial OD600 nm of 0.4 and incubated approximately 24 hours at 30°C, and 300 rpm shaking. Cells were pelleted by centrifugation for 10 minutes at 3,000xg, at 4°C, and then resuspended in 12.5 mL HiP-TAB media (yeast nitrogen base without amino acids and without ammonium sulfate, 1.7 g/L; yeast extract, 3.0 g/L; potassium phosphate monobasic, 10.0 g/L; potassium phosphate dibasic, 10.0 g/L) and added to 250 mL baffled shake flasks. 500 µL of glucose was added to the shake flasks, which were shaken at approximately 300 rpm, at 30°C. Cultures were incubated for 48 hours and samples were taken at 48 hours for analysis of carotenoid production by HPLC, and results are presented in Table 22 above.



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*EXAMPLE 45: Construction of strain sAA8283 for the production of lycopene from oleic acid*

Integration cassettes for CsCrtE, CsCrtB, CsCrtI, and pG6PI-CRC1 were generated by PCR amplification of plasmids pAA2702, pAA2703, pAA2704, and pAA2311 with primers oAA2206 and oAA2209. All four cassettes were purified and chemically transformed into strain sAA4377, which had all copies of FAA1 (Long chain fatty acyl-CoA synthetase), FAT1 (Very long chain fatty acyl-CoA synthetase), CRC1 (Mitochondrial carnitine transporter), and POX4 (Acyl-coenzyme A oxidase) removed. The transformed cells were then plated onto SCD-ura plates. Colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of all four genes, and one verified isolate was saved as strain sAA8283.

*EXAMPLE 46: Shake flask characterization of sAA8283 on oleic acid*

Starter cultures (5 mL) of sAA8283 in YPD were incubated overnight at 30°C, with shaking at approximately 250 rpm. The overnight cultures were used to inoculate 25 mL fresh SP92-glycerol media (6.7 g/L yeast nitrogen base, 3.0 g/L yeast extract, 3.0 g/L $(NH_4)_2SO_4$, 1.0 g/L $K_2HPO_4$, 1.0 g/L $KH_2PO_4$, 75 g/L glycerol) to an initial OD600 nm of 0.4 and incubated approximately 24 hours at 30°C, and 300 rpm shaking. Cells were pelleted by centrifugation for 10 minutes at 3,000xg, at 4°C, and then resuspended in 12.5 mL HiP-TAB media (yeast nitrogen base without amino acids and without ammonium sulfate, 1.7 g/L; yeast extract, 3.0 g/L; potassium phosphate monobasic, 10.0 g/L; potassium phosphate dibasic, 10.0 g/L) and added to 250 mL baffled shake flasks. 500 µL of oleic acid was added to the shake flasks, which were shaken at approximately 300 rpm, at 30°C. Cultures were incubated for 48 hours and samples were taken at 48 hours for analysis of carotenoid production by HPLC, and results are presented in Table 22 above.

*EXAMPLE 47: Construction of strain sAA8519 for the production of β-carotene from oleic acid*

In order to integrate genes into the genome of *C. viswanathii*, genes were cloned into a plasmid that allows for single crossover recombination at the URA3 loci. In addition, since oleic acid was the primary carbon source, all genes were expressed under a HDE promoter with a POX4 terminator. The CrtYB Gene from *Xanthophyllomyces dendrorho* (XdCrtYB) was amplified from pAA2866 using primers oAA9968 and oAA2091, while the plasmid backbone was PCR



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amplified from pAA2534 with primers oAA07511 and oAA07512. The two PCR products encoded overlapping sequence at their termini allowing directional ligation, generating plasmid pAA3373. Integration cassettes for CsCrtE, XdCrtYB, CsCrtI, and pG6PI-CRC1 were then generated by PCR amplification of plasmids pAA2702, pAA3373, pAA2704, and pAA2311 with primers oAA2206 and oAA2209. All four cassettes were purified and chemically transformed into strain sAA4377. The transformed cells were then plated onto SCD-ura plates. Colonies were then streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of all four genes, and one verified isolate was saved as strain sAA8519.

*EXAMPLE 48: Shake flask characterization of sAA8519 on oleic acid*

Starter cultures (5 mL) of sAA8519 in YPD were incubated overnight at 30°C, with shaking at approximately 250 rpm. The overnight cultures were used to inoculate 25mL fresh SP92-glycerol media (6.7 g/L yeast nitrogen base, 3.0 g/L yeast extract, 3.0 g/L $(NH_4)_2SO_4$, 1.0 g/L $K_2HPO_4$, 1.0 g/L $KH_2PO_4$, 75 g/L glycerol) to an initial OD600 nm of 0.4 and incubated approximately 24 hours at 30°C, and 300 rpm shaking. Cells were pelleted by centrifugation for 10 minutes at 3,000xg, at 4°C, and then resuspended in 12.5 mL HiP-TAB media (yeast nitrogen base without amino acids and without ammonium sulfate, 1.7 g/L; yeast extract, 3.0 g/L; potassium phosphate monobasic, 10.0 g/L; potassium phosphate dibasic, 10.0 g/L) and added to 250 mL baffled shake flasks. 500 µL of oleic acid was added to the shake flasks, which were shaken at approximately 300 rpm, at 30°C. Cultures were incubated for 48 hours and samples were taken at 48 hours for analysis of carotenoid production by HPLC, and results are presented in Table 22 above.

*EXAMPLE 49: Shake flask characterization of sAA8283 on various carbon sources*

Starter cultures (5 mL) of sAA8283 in YPD were incubated overnight at 30°C, with shaking at approximately 250 rpm. The overnight cultures were used to inoculate 25 mL fresh SP92-glycerol media (6.7 g/L yeast nitrogen base, 3.0 g/L yeast extract, 3.0 g/L $(NH_4)_2SO_4$, 1.0 g/L $K_2HPO_4$, 1.0 g/L $KH_2PO_4$, 75 g/L glycerol) to an initial OD600 nm of 0.4 and incubated approximately 24 hours at 30°C, and 300 rpm shaking. Cells were pelleted by centrifugation for 10 minutes at 3,000xg, at 4°C, and then resuspended in 12.5 mL HiP-TAB media (yeast nitrogen base without amino acids and without ammonium sulfate, 1.7 g/L; yeast extract, 3.0 g/L; potassium phosphate monobasic, 10.0 g/L; potassium phosphate dibasic, 10.0 g/L) and

added to 250 mL baffled shake flasks. 500 µL of either C10-C14 mixed alkanes, methyl pentadecanoate, ethyl laurate, crude palm oil (CPO), Ethyl PFAD, nonane, or octadecane was added to the shake flasks, which were shaken at approximately 300 rpm, at 30°C. Cultures with CPO and Ethyl PFAD also contained 0.1% Triton X-100. Cultures were incubated for 48 hours and samples were taken at 48 hours for analysis of carotenoid production by HPLC, and results are presented in Table 22 above.

*EXAMPLE 50: Construction of strain sAA8503 for production of lycopene*

In order to integrate genes into the genome of *C. viswanathii*, genes were cloned into a plasmid that allows for single crossover recombination at the URA3 loci. In addition, since glucose was the primary carbon source, all genes were expressed under a GPD promoter with a POX4 terminator. Integration cassettes for XdCrtE, CsCrtB, and CsCrtI, were generated by PCR amplification of plasmids pAA3189, pAA2699, and pAA2700 with primers oAA2206 and oAA2209. All cassettes were purified and chemically transformed into strain sAA002. The transformed cells were then plated onto SCD-ura plates. Colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of all three genes, and one verified isolate was saved as strain sAA8503.

*EXAMPLE 51: Shake flask characterization of sAA8503 on glucose*

Starter cultures (5 mL) of sAA8503 in YPD were incubated overnight at 30°C, with shaking at approximately 250 rpm. The overnight cultures were used to inoculate 25 mL fresh SP92-glucose media (6.7 g/L yeast nitrogen base, 3.0 g/L yeast extract, 3.0 g/L $(NH_4)_2SO_4$, 1.0 g/L $K_2HPO_4$, 1.0 g/L $KH_2PO_4$, 75 g/L glucose) to an initial OD600 nm of 0.4 and incubated approximately 24 hours at 30°C, and 300 rpm shaking. Cells were pelleted by centrifugation for 10 minutes at 3,000xg, at 4°C, and then resuspended in 12.5 mL HiP-TAB media (yeast nitrogen base without amino acids and without ammonium sulfate, 1.7 g/L; yeast extract, 3.0 g/L; potassium phosphate monobasic, 10.0 g/L; potassium phosphate dibasic, 10.0 g/L) and added to 250 mL baffled shake flasks. 500 µL of glucose was added to the shake flasks, which were shaken at approximately 300 rpm, at 30°C. Cultures were incubated for 48 hours and samples were taken at 48 hours for analysis of carotenoid production by HPLC, and results are presented in Table 22 above.

*EXAMPLE 52: Construction of strain sAA8932 for production of lycopene*

In order to integrate genes into the genome of *C. viswanathii*, genes were cloned into a plasmid that allows for single crossover recombination at the URA3 loci. In addition, since glucose was the primary carbon source, all genes were expressed under a GPD promoter with a POX4 terminator. Integration cassettes for XdCrtE, CsCrtB, and XdCrtI, were generated by PCR amplification of plasmids pAA3189, pAA2699, and pAA3490 with primers oAA2206 and oAA2209. All cassettes were purified and chemically transformed into strain sAA002. The transformed cells were then plated onto SCD-ura plates. Colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of all three genes, and one verified isolate was saved as strain sAA8932.

*EXAMPLE 53: Shake flask characterization of sAA8932 on glucose*

Starter cultures (5 mL) of sAA8932 in YPD were incubated overnight at 30°C, with shaking at approximately 250 rpm. The overnight cultures were used to inoculate 25 mL fresh SP92-glucose media (6.7 g/L yeast nitrogen base, 3.0 g/L yeast extract, 3.0 g/L $(NH_4)_2SO_4$, 1.0 g/L $K_2HPO_4$, 1.0 g/L $KH_2PO_4$, 75 g/L glucose) to an initial OD600 nm of 0.4 and incubated approximately 24 hours at 30°C, and 300 rpm shaking. Cells were pelleted by centrifugation for 10 minutes at 3,000xg, at 4°C, and then resuspended in 12.5 mL HiP-TAB media (yeast nitrogen base without amino acids and without ammonium sulfate, 1.7 g/L; yeast extract, 3.0 g/L; potassium phosphate monobasic, 10.0 g/L; potassium phosphate dibasic, 10.0 g/L) and added to 250 mL baffled shake flasks. 500 µL of glucose was added to the shake flasks, which were shaken at approximately 300rpm, at 30°C. Cultures were incubated for 48 hours and samples were taken at 48 hours for analysis of carotenoid production by HPLC, and results are presented in Table 22 above.

*EXAMPLE 54: Shake flask characterization of sAA8932 on oleic acid*

Starter cultures (5 mL) of sAA8932 in YPD were incubated overnight at 30°C, with shaking at approximately 250 rpm. The overnight cultures were used to inoculate 25 mL fresh SP92-glycerol media (6.7g/L yeast nitrogen base, 3.0 g/L yeast extract, 3.0 g/L $(NH_4)_2SO_4$, 1.0 g/L $K_2HPO_4$, 1.0 g/L $KH_2PO_4$, 75 g/L glucose) to an initial OD600 nm of 0.4 and incubated approximately 24 hours at 30°C, and 300 rpm shaking. Cells were pelleted by centrifugation for

10 minutes at 3,000xg, at 4°C, and then resuspended in 12.5 mL HiP-TAB media (yeast nitrogen base without amino acids and without ammonium sulfate, 1.7 g/L; yeast extract, 3.0 g/L; potassium phosphate monobasic, 10.0 g/L; potassium phosphate dibasic, 10.0 g/L) and added to 250 mL baffled shake flasks. 500 µL of oleic acid was added to the shake flasks, which were shaken at approximately 300 rpm, at 30°C. Cultures were incubated for 48 hours and samples were taken at 48 hours for analysis of carotenoid production by HPLC and results are presented in Table 22 above.

*EXAMPLE 55: Carotenoid extraction*

A 0.5 ml sample of fermentation broth was sampled. Yeast cells were isolated from media by centrifugation and supernatant removal. 0.25 ml of zirconia beads and 1.0 ml of tert-Butyl methyl ether (MTBE) were added to each cell pellet. Pellets were bead-beated with a Biospec Mini-Beadbeater-16 for 1.5 minutes. Cell debris and beads were pelleted by centrifugation and the carotenoid containing MTBE extract was saved. Multiple rounds of bead-beating were conducted until remaining cell pellet was white.

*EXAMPLE 56: Carotenoid detection*

A Thermo Scientific Dionex UltiMate 3000 Liquid Chromatography System with an Acclaim C30 column was used to detect and quantify carotenoids. Column temperature was 20°C, flow rate of 1.0-1.4 mL/min, injection volume of 10 µl, and UV absorbance measured at 474 nm, 450 nm, and 285 nm. Metabolite standards were purchased from Sigma-Aldrich. Samples were diluted in MTBE.

*EXAMPLE 57: Construction of strain sAA9432 for production of lycopene*

In order to integrate genes into the genome of *C. viswanathii*, genes were cloned into a plasmid that allows for single crossover recombination at the URA3 loci. In addition, since oleic acid was the primary carbon source, all genes were expressed under a HDE promoter with a POX4 terminator. Integration cassettes for XdCrtE, CsCrtB, and XdCrtI, were generated by PCR amplification of plasmids pAA3633, pAA2703, and pAA3634 with primers oAA2206 and oAA2209. All cassettes were purified and chemically transformed into strain sAA002. The transformed cells were then plated onto SCD-ura plates. Colonies were streaked onto YPD for

isolation and characterization. Colony PCR was performed to confirm the presence of all three genes, and one verified isolate was saved as strain sAA9432.

*EXAMPLE 58: Shake flask characterization of sAA9432 on oleic acid*

Starter cultures (5 mL) of sAA9432 in YPD were incubated overnight at 30°C, with shaking at approximately 250 rpm. The overnight cultures were used to inoculate 25 mL fresh SP92-glycerol media (6.7 g/L yeast nitrogen base, 3.0 g/L yeast extract, 3.0 g/L $(NH_4)_2SO_4$, 1.0 g/L $K_2HPO_4$, 1.0 g/L $KH_2PO_4$, 75 g/L glycerol) to an initial OD600 nm of 0.4 and incubated approximately 24 hours at 30°C, and 300 rpm shaking. Cells were pelleted by centrifugation for 10 minutes at 3,000xg, at 4°C, and then resuspended in 12.5 mL HiP-TAB media (yeast nitrogen base without amino acids and without ammonium sulfate, 1.7 g/L; yeast extract, 3.0 g/L; potassium phosphate monobasic, 10.0 g/L; potassium phosphate dibasic, 10.0 g/L) and added to 250 mL baffled shake flasks. 500 µL of oleic acid was added to the shake flasks, which were shaken at approximately 300rpm, at 30°C. Cultures were incubated for 48 hours and samples were taken at 48 hours for analysis of carotenoid production by HPLC, and results are presented in Table 22 above.

*EXAMPLE 59: Construction of sAA9539, single copy of RAS2 knocked-out*

In order to knock out a single copy of RAS2 from the genome, plasmid pVZ3930 with 400 bp homology region to both the 5' and 3' region surrounding RAS2 in the genome was constructed with a URA3 selection cassette. 5' region was amplified with oAA10284 & oVZ0041, 3' region was amplified with oAA10286 & oAA10287, the URA3 cassette digested from pAA408 using restriction enzymes BamHI/PstI, and all pieces were ligated into a pUC19 vector generating pVZ3930. pVZ3930 was then digested with PacI and the resulting fragment was integrated into the sAA002 genome with URA selection. Correct ras2::URA3/RAS2 transformants were identified and plated onto 5'FOA for the loop-out of URA3, leaving a URA3 terminator scar. One correct transformant was identified and saved as strain sAA9539.

*EXAMPLE 60: Construction of strain sAA9670 for production of lycopene*

In order to integrate genes into the genome of *C. viswanathii*, genes were cloned into a plasmid that allows for single crossover recombination at the URA3 loci. In addition, since oleic acid

was the primary carbon source, all genes were expressed under a HDE promoter with a POX4 terminator. Integration cassettes for XdCrtE, CsCrtB, and XdCrtI, were generated by PCR amplification of plasmids pAA3633, pAA2703, and pAA3634 with primers oAA2206 and oAA2209. All cassettes were purified and chemically transformed into strain sAA9539. The transformed cells were then plated onto SCD-ura plates. Colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of all three genes, and one verified isolate was saved as strain sAA9670.

*EXAMPLE 61: Shake flask characterization of sAA9670 on oleic acid*

Starter cultures (5 mL) of sAA9670 in YPD were incubated overnight at 30°C, with shaking at approximately 250 rpm. The overnight cultures were used to inoculate 25 mL fresh SP92-glycerol media (6.7 g/L yeast nitrogen base, 3.0 g/L yeast extract, 3.0 g/L $(NH_4)_2SO_4$, 1.0 g/L $K_2HPO_4$, 1.0 g/L $KH_2PO_4$, 75 g/L glycerol) to an initial OD600 nm of 0.4 and incubated approximately 24 hours at 30°C, and 300 rpm shaking. Cells were pelleted by centrifugation for 10 minutes at 3,000xg, at 4°C, and then resuspended in 12.5 mL HiP-TAB media (yeast nitrogen base without amino acids and without ammonium sulfate, 1.7 g/L; yeast extract, 3.0 g/L; potassium phosphate monobasic, 10.0 g/L; potassium phosphate dibasic, 10.0 g/L) and added to 250 mL baffled shake flasks. 500 µL of oleic acid was added to the shake flasks, which were shaken at approximately 300rpm, at 30°C. Cultures were incubated for 48 hours and samples were taken at 48 hours for analysis of carotenoid production by HPLC, and results are presented in Table 22 above.

*EXAMPLE 62: Construction of sAA9684, leucine auxotrophy in sAA002 background*

In order to knock out the first copy of Leu2 from the genome, plasmid pAA3060 with a 259 bp and a 256 bp homology region to both the 5' and 3' region, respectively, of the Leu2 gene was constructed with a URA3 selection cassette. The 5' region was amplified with oAA7682 and oAA7683, the 3' region was amplified with oAA7686 and oAA7687, the URA3 cassette was amplified from pAA408 using oAA7684 and oAA7685, and all pieces were cloned by overlap PCR and topo cloned, generating plasmid pAA3060. pAA3060 was then digested with BamHI and PstI and the resulting fragment was integrated into the sAA002 genome with URA selection. Correct leu2::URA3/LEU2 transformants were identified and plated onto 5'FOA for the loop-out of URA3, leaving a URA3 terminator scar. One correct transformant was identified and saved



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as strain sAA9658. In order to knock out the second copy of Leu2 from the genome, plasmid pAA2417 with a 204 bp and a 283 bp homology region to both the 5' and 3' region nested respectively of the Leu2 gene was constructed with a URA3 selection cassette. The 5' region was amplified with oAA7941 and oAA7942, the 3' region was amplified with oAA7945 and oAA7946, the URA3 cassette was amplified from pAA408 using oAA7943 and oAA7944, and all pieces were cloned by overlap PCR and topo cloned, generating plasmid pAA2417. pAA2417 was then digested with BamHI and PstI and the resulting fragment was integrated into the sAA9658 genome with URA selection. Correct leu2/leu2::URA3 transformants were identified and plated onto 5'FOA for the loop-out of URA3, leaving a URA3 terminator scar. One correct transformant was identified and saved as strain sAA9684.

*EXAMPLE 63: Construction of sAA9703, leucine auxotrophy in a ras2/RAS2 background*

In order to knock out the first copy of Leu2 from the genome, plasmid pAA3060 was digested with BamHI and PstI and the resulting fragment was integrated into the sAA9539 genome with URA selection. Correct leu2::URA3/LEU2 transformants were identified and plated onto 5'FOA for the loop-out of URA3, leaving a URA3 terminator scar. One correct transformant was identified and saved as strain sAA9682. In order to knock out the second copy of Leu2 from the genome, plasmid pAA2417 was digested with BamHI and PstI and the resulting fragment was integrated into the sAA9682 genome with URA selection. Correct leu2/leu2::URA3 transformants were identified and plated onto 5'FOA for the loop-out of URA3, leaving a URA3 terminator scar. One correct transformant was identified and saved as strain sAA9703.

*EXAMPLE 64: Construction of strain sAA9748 for production of lycopene with leucine auxotrophy*

In order to integrate genes into the genome of *C. viswanathii*, genes were cloned into a plasmid that allows for single crossover recombination at the URA3 loci. In addition, since oleic acid was the primary carbon source, all genes were expressed under a HDE promoter with a POX4 terminator. Integration cassettes for XdCrtE, CsCrtB, and XdCrtI, were generated by PCR amplification of plasmids pAA3633, pAA2703, and pAA3634 with primers oAA2206 and oAA2209. All cassettes were purified and chemically transformed into strain sAA9684. The transformed cells were then plated onto SCD-ura plates with 1.60 g/L leucine supplementation. Colonies were streaked onto YPD for isolation and characterization. Colony PCR was

performed to confirm the presence of all three genes, and one verified isolate was saved as strain sAA9748.

*EXAMPLE 65: Construction of strain sAA9750 for production of lycopene with leucine auxotrophy*

In order to integrate genes into the genome of *C. viswanathii*, genes were cloned into a plasmid that allows for single crossover recombination at the URA3 loci. In addition, since oleic acid was the primary carbon source, all genes were expressed under a HDE promoter with a POX4 terminator. Integration cassettes for XdCrtE, CsCrtB, and XdCrtI, were generated by PCR amplification of plasmids pAA3633, pAA2703, and pAA3634 with primers oAA2206 and oAA2209. All cassettes were purified and chemically transformed into strain sAA9703. The transformed cells were then plated onto SCD-ura plates with leucine supplementation. Colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of all three genes, and one verified isolate was saved as strain sAA9750.

*EXAMPLE 66: Construction of single crossover cassettes with leucine selection*

In order to integrate genes at the leu2 loci within the genome of C. viswanathii, single crossover plasmid pVZ4045 was generated by amplifying the native LEU2 promoter, open reading frame, and terminator in two pieces split in the open reading frame. The front portion was amplified using primers oVZ339 and oVZ340 using genomic DNA from sAA001, and back portion was amplified using primers oVZ341 and oVZ342 using genomic DNA from sAA001, and the base vector was pUC19 amplified using primers oVZ337 and oVZ338. Fragments were assembled such that the back fragment was 5' of the front fragment using overlap PCR and the resulting correct plasmid was designated pVZ4045. DNA constructs for integration with the single crossover cassettes were cloned in between the back and front portions. Fragments for integration were amplified using primers oVZ373 and oVZ374.

In order to integrate carotenoid genes at the leu2 loci within the genome of *C. viswanathii*, XdCrtE, CsCrtB, and XdCrtI were cloned using overlapping PCR along with the HDE promoter and POX4 terminator from plasmid pAA3633, pAA2703, and pAA3634 respectively using primers oVZ371 and oVZ372 into pVZ4045 amplified with primers oVZ369 and oVZ370, generating plasmids pVZ4056, pVZ4057, and pVZ4058 respectively.

In order to integrate mevalonate genes at the leu2 loci with the genome of *C. viswanathii*, each gene was cloned under the POX18 promoter and terminator. First, plasmid pVZ4083 was generated by cloning the POX18 promoter and terminator from genomic DNA isolated from sAA001 using primers oVZ376 and oVZ377, and cloned using overlap PCR into plasmid pVZ4045 amplified with primers oVZ378 and oVZ379. Each gene from the mevalonate pathway was then cloned into pVZ4083. Erg12 was amplified from genomic DNA isolated from sAA001 using primers oVZ394 and oVZ395, and pVZ4083 was amplified using primers oVZ392 and oVZ393, and overlap PCR was used to generate plasmid pVZ4101. Erg13 was amplified from genomic DNA isolated from sAA001 using primers oVZ386 and oVZ387, and pVZ4083 was amplified using primers oVZ384 and oVZ385, and overlap PCR was used to generate plasmid pVZ4099. Erg20 was amplified from genomic DNA isolated from sAA001 using primers oVZ410 and oVZ411, and pVZ4083 was amplified using primers oVZ408 and oVZ409, and overlap PCR was used to generate plasmid pVZ4105. HMG1 was amplified from genomic DNA isolated from sAA001 using primers oVZ390 and oVZ391, and pVZ4083 was amplified using primers oVZ388 and oVZ389, and overlap PCR was used to generate plasmid pVZ4100. IDI1 was amplified from genomic DNA isolated from sAA001 using primers oVZ406 and oVZ407, and pVZ4083 was amplified using primers oVZ404 and oVZ405, and overlap PCR was used to generate plasmid pVZ4104. Erg8 was amplified from genomic DNA isolated from sAA001 using primers oVZ481 and oVZ399, and pVZ4083 was amplified using primers oVZ396 and oVZ480, and overlap PCR was used to generate plasmid pVZ4122. MVD1 was amplified from genomic DNA isolated from sAA001 using primers oVZ402 and oVZ479, and pVZ4083 was amplified using primers oVZ478 and oVZ401, and overlap PCR was used to generate plasmid pVZ4123.

*EXAMPLE 67: Construction of strains sAA9811, sAA9812, and sAA9814 for production of lycopene*

In order to integrate genes into the genome of *C. viswanathii*, genes were cloned into a plasmid that allows for single crossover recombination at the leu2 loci. Three libraries of genes were integrated into sAA9748 using leucine selection. Integration cassettes for XdCrtE, CsCrtB, and XdCrtI were generated by PCR amplification of plasmids pVZ4056, pVZ4057, and pVZ4058 respectively using primers oVZ373 and oVZ374. All cassettes were purified and chemically transformed into strain sAA9748. The transformed cells were then plated onto SCD-leu plates. Colonies were streaked onto YPD for isolation and characterization. Colony PCR was

performed to confirm the presence of all three genes, and one verified isolate was saved as strain sAA9811.

Integration cassettes for Erg10, Erg13, HMG1, Erg12, Erg8, MVD1, IDI1, and Erg20 were generated by PCR amplification of plasmids pVZ4098, pVZ4099, pVZ4100, pVZ4101, pVZ4122, pVZ4123, pVZ4104, and pVZ4105 using primers oVZ373 and oVZ374. All cassettes were purified and chemically transformed into strain sAA9748. The transformed cells were then plated onto SCD-leu plates. Colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of all eight genes, and one verified isolate was saved as strain sAA9812.

Integration cassettes for XdCrtE, CsCrtB, XdCrtI, Erg10, Erg13, HMG1, Erg12, Erg8, MVD1, IDI1, and Erg20 were generated by PCR amplification of plasmids pVZ4056, pVZ4057, pVZ4058, pVZ4098, pVZ4099, pVZ4100, pVZ4101, pVZ4122, pVZ4123, pVZ4104, and pVZ4105 respectively using primers oVZ373 and oVZ374. All cassettes were purified and chemically transformed into strain sAA9748. The transformed cells were then plated onto SCD-leu plates. Colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of all 11 genes. One verified isolate contained all genes except overexpression of Erg8, and was saved as strain sAA9814.

*EXAMPLE 68: Construction of strains sAA9817, sAA9819, and sAA9821 for production of lycopene*

In order to integrate genes into the genome of *C. viswanathii*, genes were cloned into a plasmid that allows for single crossover recombination at the leu2 loci. Three libraries of genes were integrated into sAA9750 using leucine selection. Integration cassettes for XdCrtE, CsCrtB, and XdCrtI were generated by PCR amplification of plasmids pVZ4056, pVZ4057, and pVZ4058 respectively using primers oVZ373 and oVZ374. All cassettes were purified and chemically transformed into strain sAA9750. The transformed cells were then plated onto SCD-leu plates. Colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of all three genes, and one verified isolate was saved as strain sAA9819.



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Integration cassettes for Erg10, Erg13, HMG1, Erg12, Erg8, MVD1, IDI1, and Erg20 were generated by PCR amplification of plasmids pVZ4098, pVZ4099, pVZ4100, pVZ4101, pVZ4122, pVZ4123, pVZ4104, and pVZ4105 using primers oVZ373 and oVZ374. All cassettes were purified and chemically transformed into strain sAA9750. The transformed cells were then plated onto SCD-leu plates. Colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of all eight genes. One verified isolate contained all genes except overexpression of Erg8, and was saved as strain sAA9817.

Integration cassettes for XdCrtE, CsCrtB, XdCrtI, Erg10, Erg13, HMG1, Erg12, Erg8, MVD1, IDI1, and Erg20 were generated by PCR amplification of plasmids pVZ4056, pVZ4057, pVZ4058, pVZ4098, pVZ4099, pVZ4100, pVZ4101, pVZ4122, pVZ4123, pVZ4104, and pVZ4105 respectively using primers oVZ373 and oVZ374. All cassettes were purified and chemically transformed into strain sAA9750. The transformed cells were then plated onto SCD-leu plates. Colonies were streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of all eleven genes. One verified isolate contained all genes except overexpression of Erg8 and IDI1, and was saved as strain sAA9821.

*EXAMPLE 69: Shake flask characterization of sAA9811, sAA9812, sAA9814, sAA9817, sAA9819, and sAA9821 on oleic acid*

Starter cultures (5 mL) of sAA9811, sAA9812, sAA9814, sAA9817, sAA9819, and sAA9821 in YPD were incubated overnight at 30°C, with shaking at approximately 250 rpm. The overnight cultures were used to inoculate 25 mL fresh SP92-glycerol media (6.7 g/L yeast nitrogen base, 3.0 g/L yeast extract, 3.0 g/L $(NH_4)_2SO_4$, 1.0 g/L $K_2HPO_4$, 1.0 g/L $KH_2PO_4$, 75 g/L glycerol) to an initial OD600 nm of 0.4 and incubated approximately 24 hours at 30°C, and 300 rpm shaking. Cells were pelleted by centrifugation for 10 minutes at 3,000xg, at 4°C, and then resuspended in 12.5 mL HiP-TAB media (yeast nitrogen base without amino acids and without ammonium sulfate, 1.7 g/L; yeast extract, 3.0 g/L; potassium phosphate monobasic, 10.0 g/L; potassium phosphate dibasic, 10.0 g/L) and added to 250 mL baffled shake flasks. 500 µL of oleic acid was added to the shake flasks, which were shaken at approximately 300rpm, at 30°C. Cultures were incubated for 48 hours and samples were taken at 48 hours for analysis of carotenoid production by HPLC, and results are presented in Table 22 above.

*EXAMPLE 70: Genes*

Details for certain genes described herein are provided in Tables 23, 24 and 25 below.

**Table 23**

| Gene | Organism | Protein sequence | SEQ ID NO |
|---|---|---|---|
| CsCrtE | Chronobacter sakazakii | MNANAVKSSGQEIELQALRDALQTRLDELLPPGQERDLVCAAMREGALTPGKRVRPLLLILAARD LGCDASQPALMDLACAVEMVHAASLMLDDIPCMDNALLRRGKPTIHRQYGESVAILAAVALLSRA FGVVAQANPLSDSCKTQAVSELSSAVGLQGLVQGQFRDLSEGNQARSAEAILATNDLKTSVLFD ATLQIAAIAAGASASVRHKLREFSRHLGQAFQLLDDLADGLNHTGKDINKDAGKSTLVAMLGPEA VHQRLRDHLLRADEHLTGACSRGASTRRFMYAWFDKQLAMFG | 326 |
| CsCrtI | Chronobacter sakazakii | MTKTVVIGSGFGGLALAIRLQAAGVPTLLLEQRDKPGGRAYVYEDKGFTFDAGPTVITDPSAIEEL FTLAGKNIADYVDLLPVTPFYRLCWENGQVFNYDNDQASLEAQIARFNPRDVEGYRQFLAYSQA VFKEGYLKLGAVPFLSFRDMLRAGPQLARLQAWRSVYGMVSKFIENDHLRQAFSFHSLLVGGNP FATSSIYTLIHALERQWGVWFARGGTGALVQGLVKLFTDLGGEIELNAKVTRLDTQGDKISGVTLA DGRRIPARAVASNADVVHTYNNLLGHHPRGVSQAASLRRKRMSNSLFVLYFGLNHHHSQLAHHT VCFGPRYKGLIEDIFKRDSLADDFSLYLHAPCVTDPSLAPPGCGSYYVLAPVPHLGTANLNWDVE GPRLRDRIFEYLEQHYMPGLRDQLVTHRMFTPFDFRDQLGAYHGSAFSVEPILTQSAWFRPHNR DSRIDNLYLVGAGTHPGAGIPGVIGSAKATAGLMLEGHA | 327 |
| CsCrtB | Chronobacter sakazakii | MSDKPLLTHATETIEAGSKSFATASKLFDAKTRRSALMLYAWCRHCDDVTDGQALGFRAADAPT DTPQARIALLRALTLEAYAGKPMREPNFAAFQEVALAHQIPPALALDHLEGFAMDVREERYHTFD DTLRYCYHVAGVVGLMMARVMGVRDEAVLDRACDLGLAFQLTNIARDIVEDAAIGRCYLPEAWL QEEGLCADTLTDRAHRPALARLAARLVDEAEPYYASARAGLAGLPLRSAWAIATAHGVYREIGVK VKRAGVNAWETRQGTSKAEKLALLAKGAVMAVSSRGASSSPRPSALWQRPRAQDDRYAHAAP PAA | 328 |
| XdCrtE | Xanthophyllomyces dendrorhous | MDYANILTAIPLEFTPQDDIVLLEPYHYLGKNPGKEIRSQLIEAFNYWLDVKKEDLEVIQNVVGMLH TASLLMDDVEDSSVLRRGSPVAHLIYGIPQTINTANYVYFLAYQEIFKLRPTPIPMPVIPPSSASLQS SVSSASSSSSASSENGGTSTPNSQIPFSKDTYLDKVITDEMLSLHRGQGLELFWRDSLTCPSEEE YVKMVLGKTGGLFRIAVRLMMAKSECDIDFVQLVNLISIYFQIRDDYMNLQSSEYAHNKNFAEDLT EGKFSFPTIHSIHANPSSRLVINTLQKKSTSPEILHHCVNYMRTETHSFEYTQEVLNTLSGALEREL GRLQGEFAEANSKIDLGDVESEGRTGKNVKLEAILKKLADIPL | 329 |



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| Table 23 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **Protein sequence** | **SEQ ID NO** |
| XdCrtI | Xanthophyllomyces dendrorhous | MGKEQDQDKPTAIIVGCGIGGIATAARLAKEGFQVTVFEKNDYSGGRCSLIERDGYRFDQGPSLL LLPDLFKQTFEDLGEKMEDWVDLIKCEPNYVCHFHDEETFTFSTDMALLKREVERFEGKDGFDR FLSFIQEAHRHYELAVVHVLQKNFPGFAAFLRLQFIGQILALHPFESIWTRVCRYFKTDRLRRVFSF AVMYMGQSPYSAPGTYSLLQYTELTEGIWYPRGGFWQVPNTLLQIVKRNNPSAKFNFNAPVSQV LLSPAKDRATGVRLESGEEHHADVVIVNADLVYASEHLIPDDARNKIGQLGEVKRSWWADLVGG KKLKGSCSSLSFYWSMDRIVDGLGGHNIFLAEDFKGSFDTIFEELGLPADPSFYVNVPSRIDPSAA PEGKDAIVILVPCGHIDASNPQDYNKLVARARKFVIQTLSAKLGLPDFEKMIVAEKVHDAPSWEKE FNLKDGSILGLAHNFMQVLGFRPSTRHPKYDKLFFVGASTHPGTGVPIVLAGAKLTANQVLESFD RSPAPDPNMSLSVPYGKPLKSNGTGIDSQVQLKFMDLERWVYLLVLLIGAVIARSVGVLAF | 330 |
| XdCrtY B | Xanthophyllomyces dendrorhous | MTALAYYQIHLIYTLPILGLLGLLTSPILTKFDIYKISILVFIAFSATTPWDSWIIRNGAWTYPSAESGQ GVFGTFLDVPYEEYAFFVIQTVITGLVYVLATRHLLPSLALPKTRSSALSLALKALIPLPIIYLFTAHP SPSPDPLVTDHYFYMRALSLLITPPTMLLAALSGEYAFDWKSGRAKSTIAAIMIPTVYLIWVDYVAV GQDSWSINDEKIVGWRLGGVLPIEEAMFFLLTNLMIVLGLSACDHTQALYLLHGRTIYGNKKMPSS FPLITPPVLSLFFSSRPYSSQPKRDLELAVKLLEEKSRSFFVASAGFPSEVRERLVGLYAFCRVTD DLIDSPEVSSNPHATIDMVSDFLTLLFGPPLHPSQPDKILSSPLLPPSHPSRPTGMYPLPPPPSLSP AELVQFLTERVPVQYHFAFRLLAKLQGLIPRYPLDELLRGYTTDLIFPLSTEAVQARKTPIETTADLL DYGLCVAGSVAELLVYVSWASAPSQVPATIEEREAVLVASREMGTALQLVNIARDIKGDATEGRF YLPLSFFGLRDESKLAIPTDWTEPRPQDFDKLLSLSPSSTLPSSNASESFRFEWKTYSLPLVAYAE DLAKHSYKGIDRLPTEVQAGMRAACASYLLIGREIKVVWKGDVGERRTVAGWRRVRKVLSVVMS GWEGQ | 331 |
| PaCrtE | Pantoea ananatis | MTVCAKKHVHLTRDAAEQLLADIDRRLDQLLPVEGERDVVGAAMREGALAPGKRIRPMLLLLTAR DLGCAVSHDGLLDLACAVEMVHAASLILDDMPCMDDAKLRRGRPTIHSHYGEHVAILAAVALLSK AFGVIADADGLTPLAKNRAVSELSNAIGMQGLVQGQFKDLSEGDKPRSAEAILMTNHFKTSTLFC ASMQMASIVANASSEARDCLHRFSLDLGQAFQLLDDLTDGMTDTGKDSNQDAGKSTLVNLLGPR AVEERLRQHLQLASEHLSAACQHGHATQHFIQAWFDKKLAAVS | 332 |
| PaCrtI | Pantoea ananatis | MKPTTVIGAGFGGLALAIRLQAAGIPVLLLEQRDKPGGRAYVYEDQGFTFDAGPTVITDPSAIEELF ALAGKQLKEYVELLPVTPFYRLCWESGKVFNYDNDQTRLEAQIQQFNPRDVEGYRQFLDYSRAV FKEGYLKLGTVPFLSFRDMLRAAPQLAKLQAWRSVYSKVASYIEDEHLRQAFSFHSLLVGGNPFA TSSIYTLIHALEREWGVWFPRGGTGALVQGMIKLFQDLGGEVVLNARVSHMETTGNKIEAVHLED GRRFLTQAVASNADVVHTYRDLLSQHPAAVKQSNKLQTKRMSNSLFVLYFGLNHHHDQLAHHTV CFGPRYRELIDEIFNHDGLAEDFSLYLHAPCVTDSSLAPEGCGSYYVLAPVPHLGTANLDWTVEG PKLRDRIFAYLEQHYMPGLRSQLVTHRMFTPFDFRDQLNAYHGSAFSVEPVLTQSAWFRPHNRD KTITNLYLVGAGTHPGAGIPGVIGSAKATAGLMLEDLI | 333 |



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| Table 23 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **Protein sequence** | **SEQ ID NO** |
| PaCrtB | Pantoea ananatis | MNNPSLLNHAVETMAVGSKSFATASKLFDAKTRRSVLMLYAWCRHCDDVIDDQTLGFQARQPAL QTPEQRLMQLEMKTRQAYAGSQMHEPAFAAFQEVAMAHDIAPAYAFDHLEGFAMDVREAQYSQ LDDTLRYCYHVAGVVGLMMAQIMGVRDNATLDRACDLGLAFQLTNIARDIVDDAHAGRCYLPAS WLEHEGLNKENYAAPENRQALSRIARRLVQEAEPYYLSATAGLAGLPLRSAWAIATAKQVYRKIG VKVEQAGQQAWDQRQSTTTPEKLTLLLAASGQALTSRMRAHPPRPAHLWQRPL | 334 |
| XdCrtR | Xanthophyllomyces dendrorhous | MATLSDLVILLLGALLALGFYNKDRLLGSSSSSASTTSGSSAATANGSKPTYSNGNGNAFKGDPR DFVARMKDQKKRLAVFYGSQTGTAEEYATRIAKEAKSRFGVSSLVCDIEEYDFEKLDQVPEDCAI VFCMATYGEGEPTDNAVQFIEMISQDDPEFSEGSTLDGLKYVVFGLGNKTYEQYNVVGRQLDAR LTALGATRVGERGEGDDDKSMEEDYLAWKDDMFAALATTLSFEEGASGETPDFVVTEVPNHPIE KVFQGELSSRALLGSKGVHDAKNPYASPVLACRELFTGGDRNCIHLEFDITGSGITYQTGDHVAV WPSNPDVEVERLLAVLGLTSPEKRRMIIQVVSLDPTLAKVPFPTPTTYDAVFRHYLDISAVASRQT LAVLAKYAPSEQAAEFLTRLGTDKQAYHTEVVGGHLRLAEVLQLAAGNDITVMPTAENTTVWNIP FDHVVSDVSRLQPRFYSISSSPKLHPNSIHVTAVILKYESQATDRHPARWVFGLGTNYLLNVKQA ANNETTPMISDGQDDVPEHVSAPKYTLEGPRGSYKHDDQLFKVPIHVRRSTFRLPTSPKIPVIMIG PGTGVAPFRGFIQERIALARRSIAKNGPDALADWAPIYLFYGSRDEQDFLYAEEWPAYEAELQGK FKIHVAFSRSGPRKPDGSKIYVQDLLWDQKEVIKSAIVEKRASVYICGDGRNMSKDVEQKLAAML AESKNGSAAVEGAAEVKSLKERSRLLMDVWS | 335 |
| XdCrtS | Xanthophyllomyces dendrorhous | MFILVLLTGALGLAAFSWASIAFFSLYLAPRRSSLYNLQGPNHTNYFTGNFLDILSARTGEEHAKY REKYGSTLRFAGIAGAPVLNSTDPKVFNHVMKEAYDYPKPGMAARVLRIATGDGVVTAEGEAHK RHRRIMIPSLSAQAVKSMVPIFLEKGMELVDKMMEDAAEKDMAVGESAGEKKATRLETEGVDVK DWVGRATLDVMALAGFDYKSDSLQNKTNELYVAFVGLTDGFAPTLDSFKAIMWDFVPYFRTMKR RHEIPLTQGLAVSRRVGIELMEQKKQAVLGSASDQAVDKKDVQGRDILSLLVRANIAANLPESQKL SDEEVLAQISNLLFAGYETSSTVLTWMFHRLSEDKAVQDKLREEICQIDTDMPTLDELNALPYLEA FVKESLRLDPPSPYANRECLKDEDFIPLAEPVIGRDGSVINEVRITKGTMVMLPLFNINRSKFIYGE DAEEFRPERWLEDVTDSLNSIEAPYGHQASFISGPRACFGWRFAVAEMKAFLFVTLRRVQFEPII SHPEYEHITLIISRPRIVGREKEGYQMRLQVKPVE | 336 |
| ERG10 | Candida viswanathii | MTLPPVYIVSTARTPIGSFQGSLSSLTYSDLGAHAVKAALAKVPQIKPQDVDEIVFGGVLQANVGQ APARQVALKAGLPDSIIASTINKVCASGMKAVIIGAQNIICGTSDIVVVGGAESMSNTPYYLPSARS GARYGDAVMVDGVQKDGLLDVYEEKLMGVAAEKCAKDHGFSREDQDNFAINSYKKAGKALSEG KFKSEIAPVTIKGFRGKPDTVIENDEEIGKFNEDRLKSARTVFQKENGTVTAPNASKLNDGGAALV LVSEAKLKQLGLKPLAKISGWGEAARTPFDFTIAPALAVPKAVKHAGLTVDRVDFFELNEAFSVVG LANAELVKIPLEKLNVYGGAVAMGHPLGCSGARIIVTLLSVLTQEGGRFGAAGVCNGGGGASAIVI EKIDSDAKL | 337 |



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| Table 23 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **Protein sequence** | **SEQ ID NO** |
| ERG13 | Candida viswanathii | MTNAPQNIGIKGIEVYIPGQAVNQSDLEKFDGIPAGKYTIGLGQTNMAFVNDREDIYSIALTVVSRLI KHYNVDTNNVGRLEVGTETLLDKSKSVKSVLMQLFPDNNDIEGIDTVNACYGGTSAVINAINWIES SSWDGRDAIVVAGDIAIYDKGAARPTGGAGSIAMLIGPDAPIVFDSVRGSFMEHAYDFYKPDFTSE YPVVDGHFSLSCYVKAVDNCYKNYSKKVTGNVDKTVGVYDHFDYNAFHVPTCKLVTKSYARLLY NDYKSDPSKFADLIDESTRQHIDSLSYEASLTDKVLEKTFVTLAKEETKKRVQPALQVPTNTGNMY TASSWVSLASLLYYVGAENLKEKRIGLFSYGSGLASTLLSVTVVGDVSPITKVLDFDYKLGEGRKI QSPEEYLAAIELREKAHLQKSFKPQGSLDNLSQGTYYLTEVDDKFRRSYAIKE | 338 |
| HMG1 | Candida viswanathii | MLSFITEASGRIAQTAAHRPIHFMVIPALLASIAYLSIIDDYVPEHIRAQYMSGVSYFHPQGSASDLD KWIEIHDTTQYANANQISVIPLRFRRFHDSIPQIANAIKISNNEQILIVPSDKAESTVEGLSEITENGIT WRARNNDKLSKYYDYARYGLLRIQDAIHNADNFDILLVFVAYLGMWYSLIKVFIDMRRIGSKFWLA FGTLTSSTFSFLFALVISNKFLDAKVSLRSLSECIPFLVAIIGFKHKVAITTSVAQSSTSSPEDVPHVV GKAVSDQCLFILRDHLVVIIGFLACAAYGNELKGLRNFCILGALILSFDIVSVYTFFSAILALKVEINRA RRTQDLQHVLEEDGISSLVAARVAERNATIEHPNETNFFSSNNSSIVYFKVIMSLGFFAFHAFWLG SSWLYNTSDGGSHGSFSFLSNIPLLTQDISNSIPIGRRGTVVTILPTTFYMPSGIIIQFEDMVYLALS KISSAIRDSLISKCIVFALTISIVTNIYFLNAARFQVSATRKLIDQEMSRPKQAAAAAAAAPSAAKSVA PEEDEDETSSEELEIKAPVKPLSLEECTRILKEGKVKTLSNAEVSSLVVGGKLPLYALEKQLGDNK RAVAVRRKAIAKLANAPVLETNRLPYSHYDYDRVFGACCENVIGYMPIPVGVAGPLVIDGKPYHIP MATTEGCLVASTMRGCKAINAGGGVETVLTKDGMTRGPCVRFPTLKRAGAAKLWIDSEEGQNTIK KAFNSTSRFARLQHIQTALAGTSLFIRFRTTTGDAMGMNMISKGVEYSLKHMVEECGWDDMEVIS VSGNYCTDKKPAAINWIEGRGKSVVAAATIPADVVTKVLKSDVDALVELNVSKNLVGSAMAGSVG GFNAHAANLVTAVYLACGQDPAQNVESSNCITLMEKDKQTGDLVISVSMPSIEVGTIGGGTILEPQ AAMLDLLGVRGPHPTNPGDNARQLAKIVASAVLAAELSLCSALAAGHLVQSHMQHNRKGATPAA APAISNGSAKGTKTNGSINGKDLKRLKDGSVTCIKS | 339 |
| ERG12 | Candida viswanathii | MSVSPFVVSAPGKVIIFGEHSAVYGKPAIAAALSLRCYLLVSPSVDDANTIRLQFPDIQLDHSWDIN DIPWDEIKPFVKYDANNKPLTSSELVPEIVDKLSPLLTDFDNKMHYYACFCFLYLYVNLCTAETPG TTFIVRSTLPIGAGLGSSASTSVCLSSALALLGGWISEPSISATDKILNEDIPDLEFVDKWSLIGEKC FHGNPSGIDNAVATFGGAVMFQRTSAPEQPSIRTNMRNFPAIKLLLTNTKVPKSTADLVAGVGKL NAEFNPITTSILTAMEHLSQEAYKVMITPGFGKDETNTLRKLVNINHGLLVALGVSHPSLETVKIIGD RHKIGATKLTGAGGGGCAITLVNDNVEESAIQEAIKEFAGEGYESFETSLGGKGVGVLFSGDVSV DGKFSQSVFCNYPDRASIEDALGMINVKEWKFW | 340 |
| ERG8 | Candida viswanathii | MSKAFSAPGKALLAGGYLVLEPTYDAYVTALSSRMHAIITPQKPASISKIKISSPQFANGEWEYHV TSNEKPKDIKSRSNPFLEATIFIVLSYIQPTEPFDLDLVIYSDPGYHSQEHTTQKVSSNGKKKFLYH SRAINDVEKTGLGSSAGLVSVVTTSLLSYFIPGIEESNKDMLHNVAQIAHCFAQKKIGSGFDVATAI | 341 |



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| Table 23 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **Protein sequence** | **SEQ ID NO** |
| | | YGSIVYRRFQPSLINDVFEILEETPGRFPGALKSLVESNWEFKHERCVLPPKIKLLMGDIKGGSET PKLVSKILQWKKDKPEESGLVYDQLNSANVAFMKKISTLNESSQVQEIDELSDYISAVRKGLQELT EKSKVPVEPPVQTELLDRIAKLPGCLGGVVPGAGGYDAIAVLVLEKEVENFRNKTLENPEYYHNV YWVDLEEETEGVVVENWEDYIGL | |
| MVD1 | Candida viswanathii | MYSASVTAPVNIATLKYWGKRDKTLNLPTNSSISVTLSQDDLRTLTTAAASTTFDKDQLWLNGKLE SLDTPRTQACLVDLRKLRADVEQANADLPKLSTMKLHIVSENNFPTAAGLASSAAGFAALITAIAKL YELPQDMSELSKIARKGSGSACRSLFGGFVAWEMGDAADGQDSKAVEVAPLDHWPSMRAVILV VSDDKKDTPSTTGMQATVQTSDLFAHRVTKVVPQCFEEMKKAIVAKDFPKFTELTMKDSNSFHA VCLDSYPPIFYLNDTSKKIIKLVEAINKHATIAAYTFDAGPNAVIYYDAANEDEVLSQLYKSFGHVQG WKKAYTAETAVAGVSRIIQTSIGQGPQVTNESLINESGLPN | 342 |
| IDI1 | Candida viswanathii | MSSEYAKLVASFSPNDILAKWPEVTPLKKISGIPRSAESDSSNGSHNNTELFNGHDEEQIRLMEEL CIVLDYDDKPVGAGTKKLCHIMDNINEGLLHRAFSVFLFNEDGKLLLQQRADEKITFPAMWTNTCC SHPLCVPSELGVDADAKDVNNLDNAVHGAKVAAQRKLDHELGIPFSDTPLDQFTYLTRIHYKSAS GAEDSKWGEHEIDYILILKTKNDITINANYNEVKDYKYVDAKELQEMFEDKDLVFTPWFKLICQSFL FKWWNNLSDLEKYQDTEIHRLL | 343 |
| ERG20 | Candida viswanathii | MSDKAAARERFLSVFECAVEELKEVLVSHKMPQEAIDWFVKNLNYNTPGGKLNRGLSVVDTYAIL NNTTADKLNDEQYKKVALLGWSIELLQAYFLVADDMMDQSKTRRGQKCWYLVEGVGNIAINDSF MLEGAIYVLLKKHFRQDPYYVDLLDLFHEVTFQTELGQLLDLVTADEEVVDLDKFSLDKHSFIVIFK TAYYSFYLPVALAMYMSGISSEEDLKQVRDILIPLGEYFQIQDDFLDCFGTPEQIGKIGTDIKDNKC SWVVNQALLHATPEQRKLLDDNYGKKDDESEQRCKDLFKSMGIEKIYHDYEESIVAKLREQIDKV DESRGLKKDVLTAFLGKVYKRSK | 344 |
| BTS1 | Candida viswanathii | MSFNIDSLIQPGAAYDPSMTDAIMKPYRYISEVPSNNHNVRTRFLLAFNELFYGMKNEDLLHRISHI ISVFHNSSLLIDDIEDDSQLRRGMPVAHVKYGVPLTINCGNMMYFVAVQKAIDLAGEAGSAELKFE TSQILVDEMMNVHHGQGLDIYWRDYLKDLEHLPEIEDYLGMVKDKTGSLFRLAIKLLSLHSDVGE DNGLVAIANLLGIIYQIRDDYLNLVDIKYSAMKGVTCEDLIEGKLSLPILHCLRTTTNSPVHEILYNYN TSAERAKQNALIEECLTYMKNKSRSLQYTLDLIKTLERKIKAMMTKYPNSENSGLIKIIDRLCDL | 345 |
| CRC1 | Candida viswanathii | MDDVDSALADNVKSFAAGGFGGICAVLTGHPFDLVKVRLQTGLYKSSVQCVKETIAKDGLFGLYR GVLPPLLGVTPMFAVSFWGYDVGKKLVSSFTGKSVDKFEIKDISTAGFISAIPTTLVAAPFERVKV MMQIQEGAKSKSMGAVVAEMYRTGGIRSIFKGTVATLARDGPGSALYFATYEWVKKELTAPGED LSLFAITTAGGFAGIAMWLGVFPIDTIKSTQQSSNVKVSIVQATKNIYAKGGIKAFFPGVGPALARA FPANAATFLGVELARKFLDKVI | 346 |



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| Table 23 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **Protein sequence** | **SEQ ID NO** |
| FAA1 | Candida viswanathii | MGAPLTVAVGEAKPGETAPRRKAAQKMASVERPTDSKATTLPDFIEECFARNGTRDAMAWRDL<br>VEIHVETKQVTKIIDGEQKKVDKDWIYYEMGPYNYISYPKLLTLVKNYSKGLLELGLAPDQESKLMI<br>FASTSHKWMQTFLASSFQGIPVVTAYDTLGESGLTHSLVQTESDAVFTDNQLLSSLIRPLEKATSV<br>KYVIHGEKIDPNDKRQGGKIYQDAEKAKEKILQIRPDIKFISFDEVVALGEQSSKELHFPKPEDPICI<br>MYTSGSTGAPKGVVITNANIVAAVGGISTNATRDLVRTVDRVIAFLPLAHIFELAFELVTFWWGAPL<br>GYANVKTLTEASCRNCQPDLIEFKPTIMVGVAAVWESVRKGVLSKLKQASPIQQKIFWAAFNAKS<br>TLNRYGLPGGGLFDAVFKKVKAATGGQLRYVLNGGSPISVDAQVFISTLLAPMLLGYGLTETCAN<br>TTIVEHTRFQIGTLGTLVGSVTAKLVDVADAGYYAKNNQGEIWLKGGPVVKEYYKNEEETKAAFT<br>EDGWFKTGDIGEWTADGGLNIIDRKKNLVKTLNGEYIALEKLESIYRSNHLILNLCVYADQTKVKPI<br>AIVLPIEANLKSMLKDEKIIPDADSQELSSLVHNKKVAQAVLRHLLQTGKQQGLKGIELLQNVVLLD<br>DEWTPQNGFVTSAQKLQRKKILESCKKEVEEAYKSS | 347 |
| FAT1 | Candida viswanathii | MSGLEIAAAAILGSQLLEAKYLIADDVSLAKTVAVNALPYLWKASRGKASYWYFFEQSVFKNPNN<br>KALAFPRPRKNAPTPKTDAEGFQIYDDQFDLEEYTYKELYDMVLKYSYILKNEYGVTANDTIGVSC<br>MNKPLFIVLWLALWNIGALPAFLNFNTKDKPLIHCLKIVNASQVFVDPDCDSPIRDTEAQIREELPH<br>VQINYIDEFALFDRLRLKSTPKHRAEDKTRRPTDTDSSACALIYTSGTTGLPKAGIMSWRKAFMAS<br>VFFGHIMKIDSKSNVLTAMPLYHSTAAMLGLCPTLIVGGCVSVSQKFSATSFWTQARLCGATHVQ<br>YVGEVCRYLLNSKPHPDQDRHNVRIAYGNGLRPDIWSEFKRRFHIEGIGEFYAATESPIATTNLQY<br>GEYGVGACRKYGSLISLLLSTQQKLAKMDPEDESEIYKDPKTGFCTEAAYNEPGELLMRILNPND<br>VQKSFQGYYGNKSATNSKILTNVFKKGDAWYRSGDLLKMDEDKLLYFVDRLGDTFRWKSENVS<br>ATEVENELMGSKALKQSVVVGVKVPNHEGRACFAVCEAKDELSHEEILKLIHSHVTKSLPVYAQP<br>AFIKIGTIEASHNHKVPKNQFKNQKLPKGEDGKDLIYWLNGDKYQELTEDDWSLICTGKAKL | 348 |
| LEU2 | Candida viswanathii | MSVKTKTITILPGDHVGTEIVAEAIKSLHAIESSTPYQKVHFEFKHHLIGGAAIDATGVPLPDDALAA<br>AKSSDAVLLGAVGGPKWGTGAVRPEQGLLKIRKELNLYANIRPCNFASDSLLELSPLRPEVVKGT<br>NLIIVRELVGGIYFGERQEQEESEDGKSAWDTEKYTVDEVARITRMAAFMALQHTPPLPIWSLDKA<br>NVLASSRLWRKTVDKIISEEFPTLAVQHQLIDSAAMILIQNPTKLNGIIITSNMFGDIISDEASVIPGSL<br>GLLPSASLASLPDTNTAFGLYEPCHGSAPDLPENKVNPIATILSVASMLRLSLDCVKEAEALEQAV<br>KEVLDKGIRTADLRGSSTTTEVGDAVAETVSRILKEAKA | 349 |
| AaCrtZ | Agrobacterium aurantiacum | MTNFLIVVATVLVMELTAYSVHRWIMHGPLGWGWHKSHHEEHDHALEKNDLYGLVFAVIATVLFT<br>VGWIWAPVLWWIALGMTVYGLIYFVLHDGLVHQRWPFRYIPRKGYARRLYQAHRLHHAVEGRD<br>HCVSFGFIYAPPVDKLKQDLKMSGVLRAEAQERT | 350 |
| AaCrtW | Agrobacterium aurantiacum | MSAHALPKADLTATSLIVSGGIIAAWLALHVHALWFLDAAAHPILAIANFLGLTWLSVGLFIIAHDAM<br>HGSVVPGRPRANAAMGQLVLWLYAGFSWRKMIVKHMAHHRHAGTDDDPDFDHGGPVRWYAR | 351 |



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| Table 23 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **Protein sequence** | **SEQ ID NO** |
| | | FIGTYFGWREGLLLPVIVTVYALILGDRWMYVVFWPLPSILASIQLFVFGTWLPHRPGHDAFPDRH NARSSRISDPVSLLTCFHFGGYHHEHHLHPTVPWWRLPSTRTKGDTA | |
| CnTPS 1 | Callitropsis nootkatensis | MAEMFNGNSSNDGSSCMPVKDALRRTGNHHPNLWTDDFIQSLNSPYSDSSYHKHREILIDEIRD MFSNGEGDEFGVLENIWFVDVVQRLGIDRHFQEEIKTALDYIYKFWNHDSIFGDLNMVALGFRILR LNRYVASSDVFKKFKGEEGQFSGFESSDQDAKLEMMLNLYKASELDFPDEDILKEARAFASMYL KHVIKEYGDIQESKNPLLMEIEYTFKYPWRCRLPRLEAWNFIHIMRQQDCNISLANNLYKIPKIYMK KILELAILDFNILQSQHQHEMKLISTWWKNSSAIQLDFFRHRHIESYFWWASPLFEPEFSTCRINCT KLSTKMFLLDDIYDTYGTVEELKPFTTTLTRWDVSTVDNHPDYMKIAFNFSYEIYKEIASEAERKHG PFVYKYLQSCWKSYIEAYMQEAEWIASNHIPGFDEYLMNGVKSSGMRILMIHALILMDTPLSDEIL EQLDIPSSKSQALLSLITRLVDDVKDFEDEQAHGEMASSIECYMKDNHGSTREDALNYLKIRIESC VQELNKELLEPSNMHGSFRNLYLNVGMRVIFFMLNDGDLFTHSNRKEIQDAITKFFVEPIIP | 352 |
| tHMG1 | Candida viswanathii | MDQEMSRPKQAAAAAVAPSAAKSVAPEEDEDETSSEELEIKAPVKPLPLEECTRILKEGKVKTLS NAEVSSLVVGGKLPLYALEKQLGDHKRAVAVRRKAIAKLANAPVLETNRLPYSHYDYDRVFGACC ENVIGYMPIPVGVAGPLVIDGKPYHIPMATTEGCLVASTMRGCKAINAGGGVETVLTKDGMTRGP CVRFPTLKRAGAAKLWIDSEEGQITIKKAFNSTSRFARLQHIQTALAGTSLFIRFRTTTGDAMGMN MISKGVEYSLKYMVEECGWDDMEVISVSGNYCTDKKPAAINWIEGRGKSIVAAATIPADVVTKVLK SDVDALVELNVSKNLVGSAMAGSVGGFNAHAANLVTAVYLACGQDPAQNVESSNCITLMEKDK QTGDLVISVSMPSIEVGTIGGGTILEPQAAMLDLLGVRGPHPTNPGDNARQLAKIVASAVLAAELS LCSALAAGHLVQSHMQHNRKGATAAAAAAAAPAIANGRANGTKTNGSINGKDLKRLKDGSVTCIK S | 353 |
| POX4 | Candida viswanathii | MTFTKKNVSVSQGPDPRSSIQKERDSSKWNPQQMNYFLEGSVERSELMKALAQQMERDPILFT DGSYYDLTKDQQRELTAVKINRIARYREQESIDTFNKRLSLIGIFDPQVGTRIGVNLGLFLSCIRGN GTTSQLNYWANEKETADVKGIYGCFGMTELAHGSNVAGLETTATFDKESDEFVINTPHIGATKW WIGGAAHSATHCSVYARLIVDGQDYGVKTFVVPLRDSNHDLMPGVTVGDIGAKMGRDGIDNGWI QFSNVRIPRFFMLQKFCKVSAEGEVTLPPLEQLSYSALLGGRVMMVLDSYRMLARMSTIALRYAI GRRQFKGDNVDPKDPNALETQLIDYPLHQKRLFPYLAAAYVISAGALKVEDTIHNTLAELDAAVEK NDTKAIFKSIDDMKSLFVDSGSLKSTATWLGAEAIDQCRQACGGHGYSSYNGFGKAYNDWVVQ CTWEGDNNVLAMSVGKPIVKQVISIEDAGKTVRGSTAFLNQLKDYTGSNSSKVVLNTVADLDDIK TVIKAIEVAIIRLSQEAASIVKKESFDYVGAELVQLSKLKAHHYLLTEYIRRIDTFDQKDLVPYLITLGK LYAATIVLDRFAGVFLTFNVASTEAITALASVQIPKLCAEVRPNVVAYTDSFQQSDMIVNSAIGRYD GDIYENYFDLVKLQNPPSKTKAPYSDALEAMLNRPTLDERERFEKSDETAAILSK | 354 |
| POX5 | Candida viswanathii | MPTELQKERELTKFNPKELNYFLEGSQERSEIISNMVEQMQKDPILKVDASYYNLTKDQQREVTA | 355 |



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| Table 23 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **Protein sequence** | **SEQ ID NO** |
| | | KKIARLSRYFEHEYPDQQAQRLSILGVFDPQVFTRIGVNLGLFVSCVRGNGTNSQFFYWTINKGID<br>KLRGIYGCFGMTELAHGSNVQGIETTATFDEDTDEFVINTPHIGATKWWIGGAAHSATHCSVYAR<br>LKVKGKDYGVKTFVVPLRDSNHDLEPGVTVGDIGAKMGRDGIDNGWIQFSNVRIPRFFMLQKYC<br>KVSRLGEVTMPPSEQLSYSALIGGRVTMMMDSYRMTSRFITIALRYAIHRRQFKKKDTDTIETKLID<br>YPLHQKRLFPFLAAAYLFSQGALYLEQTMNATNDKLDEAVSAGEKEAIDAAIVESKKLFVASGCLK<br>STCTWLTAEAIDEARQACGGHGYSSYNGFGKAYSDWVVQCTWEGDNNILAMNVAKPMVRDLLK<br>EPEQKGLVLSSVADLDDPAKLVKAFDHALSGLARDIGAVAEDKGFDITGPSLVLVSKLNAHRFLID<br>GFFKRITPEWSEVLRPLGFLYADWILTNFGATFLQYGIITPDVSRKISSEHFPALCAKVRPNVVGLT<br>DGFNLTDMMTNAAIGRYDGNVYEHYFETVKALNPPENTKAPYSKALEDMLNRPDLEVRERGEKS<br>EEAAEILSS | |
| URA3 | Candida viswanathii | MVSTKTYTERASAHPSKVAQRLFRLMESKKTNLCASIDVTTTAEFLSLIDKLGPHICLVKTHIDIISD<br>FSYEGTIEPLLVLAERHGFLIFEDRKFADIGNTVMLQYTSGVYRIAAWSDITNAHGVTGKGVVEGL<br>KRGAEGVEKERGVLMLAELSSKGSLAHGEYTRETIEIAKSDREFVIGFIAQRDMGGREEGFDWIIM<br>TPGVGLDDKGDALGQQYRTVDEVVLTGTDVIIVGRGLFGKGRDPEVEGKRYRDAGWKAYLKRT<br>GQLE | 356 |

| Table 24 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **C.V. Codon Optimized Sequence** | **SEQ ID NO** |
| CsCrtE | Chronobacter sakazakii | ATGAATGCTAATGCCGTTAAGTCCTCCGGTCAGGAAATCGAATTGCAAGCTTTGAGGGATGC<br>CTTGCAAACACGCTTGGACGAATTGCTCCCACCCGGGCAGGAGAGAGACTTGGTTTGCGCA<br>GCCATGCGCGAAGGGGCATTGACGCCAGGGAAGAGAGTTCGTCCATTACTTTTGATCTTGG<br>CAGCGAGGGACTTGGGTTGCGACGCATCCCAGCCAGCCTTGATGGATTTAGCATGTGCAGT<br>AGAGATGGTTCATGCGGCCTCACTCATGTTGGATGATATTCCCTGTATGGACAACGCCCTTTT<br>GAGACGAGGTAAACCTACAATCCATCGACAGTACGGTGAGTCGGTGGCAATATTGGCGGCT<br>GTTGCGTTGCTTAGCAGAGCATTCGGCGTTGTGGCACAGGCGAACCCTCTATCTGACTCATG<br>TAAGACCCAAGCAGTGTCGGAGCTTTCCTCGGCCGTTGGTTTGCAGGGGTTGGTGCAGGGT<br>CAATTTCGTGATTTGTCCGAAGGGAACCAGGCAAGGAGTGCAGAAGCAATTTTGGCCACCAA<br>CGATTTGAAAACCTCAGTGTTGTTCGACGCTACCTTACAGATTGCGGCCATTGCGGCAGGCG<br>CAAGTGCGTCAGTTAGACACAAGTTGAGAGAGTTCTCTCGACACTTGGGGCAAGCCTTCCAG<br>TTGTTGGACGATTTAGCCGATGGATTAAACCACACGGGAAAGGATATCAACAAAGACGCTGG<br>CAAGAGCACCCTTGTCGCAATGTTGGGTCCGGAAGCTGTGCACCAACGCTTGCGAGACCAT | 357 |



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| Table 24 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **C.V. Codon Optimized Sequence** | **SEQ ID NO** |
| | | CTTCTTCGCGCAGATGAACACTTGACTGGTGCATGTTCAAGAGGTGCCTCCACCCGTCGCTT<br>CATGTATGCATGGTTTGACAAACAGCTTGCAATGTTTGGGTAA | |
| CsCrtI | Chronobacter sakazakii | ATGACCAAAACGGTTGTAATAGGATCAGGATTTGGGGGTCTCGCACTAGCCATTAGACTTCA<br>AGCCGCTGGTGTACCAACCTTGTTGCTTGAGCAGCGAGACAAGCCGGGAGGCCGTGCATAT<br>GTGTACGAGGACAAAGGATTTACCTTTGACGCCGGGCCCACTGTCATTACGGACCCATCCG<br>CTATCGAAGAGTTATTTACCCTTGCCGGTAAAAATATAGCTGATTACGTGGATTTGTTGCCAG<br>TAACCCCCTTCTACCGTTTGTGTTGGGAAAATGGTCAAGTGTTTAATTACGATAACGACCAAG<br>CCAGTTTGGAAGCACAAATCGCACGTTTTAACCCAAGGGATGTGGAGGGTTACCGACAATTC<br>CTCGCGTATTCACAAGCAGTATTTAAAGAGGGGTACTTGAAACTAGGCGCGGTTCCATTCTT<br>GAGCTTCAGAGACATGTTGCGTGCGGGACCCCAATTGGCCAGACTCCAAGCCTGGAGGAGT<br>GTCTATGGTATGGTTAGCAAGTTTATCGAGAACGATCATTTGCGACAAGCTTTCAGCTTCCAC<br>AGTTTGTTGGTGGGTGGCAACCCATTCGCAACAAGCTCCATCTATACACTCATCCATGCCTT<br>GGAAAGGCAGTGGGGCGTCTGGTTTGCCAGAGGAGGCACCGGAGCTTTGGTTCAGGGCTT<br>AGTAAAGTTGTTTACTGATTTGGGGGGAGAAATTGAACTCAACGCAAAGGTCACCAGGTTGG<br>ATACCCAAGGAGACAAAATAAGTGGCGTGACTTTGGCAGATGGTAGGCGGATTCCAGCTAG<br>GGCCGTCGCATCCAACGCAGATGTGGTCCATACCTACAACAATTTGTTGGGTCATCATCCGA<br>GAGGCGTTTCGCAAGCTGCCTCTCTTAGAAGAAAGCGCATGAGCAATTCCCTCTTTGTGTTG<br>TATTTCGGGCTCAACCATCACCACTCCCAATTAGCCCACCATACGGTCTGTTTTGGACCACGT<br>TATAAGGGGTTGATAGAAGACATTTTCAAGAGAGACAGCTTGGCAGACGATTTTTCCTTGTAT<br>CTTCACGCTCCGTGTGTGACAGACCCTTCATTGGCTCCACCAGGATGTGGATCATACTACGT<br>ATTGGCTCCTGTGCCACACTTGGGTACTGCAAACTTGAACTGGGATGTAGAGGGGCCACGAT<br>TAAGAGACAGGATCTTTGAATACTTAGAACAACACTATATGCCGGGTCTTAGAGATCAACTTG<br>TTACACACCGTATGTTCACTCCGTTTGACTTTAGAGACCAATTAGGTGCCTACCACGGTTCCG<br>CTTTCTCCGTTGAGCCAATCCTAACTCAATCTGCTTGGTTTCGCCCACACAACAGAGACTCTC<br>GAATTGATAACCTTTATTTGGTTGGAGCTGGAACGCATCCCGGTGCAGGAATTCCGGGTGTC<br>ATCGGGAGCGCGAAAGCAACGGCCGGCTTAATGTTGGAGGGCCACGCGTAA | 358 |
| CsCrtB | Chronobacter sakazakii | ATGAGTGACAAGCCTTTGTTAACACATGCTACAGAGACCATTGAGGCCGGATCAAAGAGTTT<br>TGCGACCGCCAGTAAGCTCTTTGACGCCAAGACACGTAGAAGTGCTCTTATGTTGTACGCTT<br>GGTGTCGTCATTGCGATGACGTCACTGACGGTCAAGCCTTGGGATTTAGGGCTGCAGACGC<br>TCCTACAGATACACCACAGGCTCGTATCGCATTGTTGCGTGCTCTAACCTTGGAAGCGTATG<br>CTGGTAAACCAATGCGTGAACCAAACTTTGCAGCTTTCCAGGAGGTTGCTCTAGCACATCAG<br>ATCCCACCCGCATTGGCACTCGATCACTTGGAAGGGTTTGCGATGGATGTACGTGAAGAACG<br>CTACCATACCTTTGACGATACCCTAAGATACTGTTATCATGTTGCAGGCGTTGTTGGGTTGAT<br>GATGGCTAGAGTTATGGGTGTCCGAGACGAGGCTGTCTTAGATAGAGCCTGCGATTTAGGTT | 359 |



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| Table 24 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **C.V. Codon Optimized Sequence** | **SEQ ID NO** |
| | | TGGCTTTTCAGTTAACAAATATTGCCCGAGACATTGTCGAAGATGCGGCAATCGGACGATGC<br>TATCTCCCAGAGGCCTGGTTGCAAGAAGAAGGACTTTGTGCGGATACATTGACTGACAGAGC<br>ACACAGACCAGCTTTGGCTAGGCTAGCCGCACGATTGGTGGATGAGGCAGAGCCATACTAT<br>GCCTCAGCGAGAGCTGGATTGGCCGGACTCCCATTAAGGTCAGCCTGGGCCATCGCTACTG<br>CCCACGGGGTTTACAGAGAAATCGGCGTAAAGGTGAAGAGGGCTGGTGTGAACGCGTGGG<br>AAACGCGACAGGGAACGTCGAAGGCCGAGAAACTCGCCTTGTTGGCCAAGGGGGCGGTGA<br>TGGCAGTCTCCTCCAGAGGAGCCAGTTCAAGTCCACGACCTTCCGCGTTGTGGCAAAGACC<br>ACGCGCTCAGGATGATCGGTACGCGCATGCCGCCCCTCCTGCCGCTTAA | |
| XdCrtE | Xanthophyllomyces dendrorhous | ATGGACTACGCCAACATCCTCACTGCCATTCCATTGGAATTTACGCCACAAGATGATATAGTT<br>TTGTTGGAACCCTACCACTACCTCGGTAAAAACCCCGGGAAGGAAATCCGATCACAATTGAT<br>TGAGGCATTCAACTACTGGTTAGATGTTAAAAAGGAGGATCTCGAGGTTATTCAGAATGTAGT<br>CGGAATGTTGCACACAGCATCCCTTTTGATGGATGATGTCGAAGACTCTTCTGTACTACGAC<br>GTGGATCACCGGTCGCGCATTTGATTTACGGAATCCCACAAACCATTAACACCGCCAACTAC<br>GTTTATTTCCTAGCTTATCAGGAAATATTCAAATTGAGGCCTACCCCAATCCCTATGCCAGTC<br>ATCCCCCCATCGTCGGCCTCATTGCAGTCCAGCGTAAGCTCAGCTAGCTCCTCGTCATCTGC<br>GTCATCGGAAAACGGGGGAACATCAACACCCAACAGTCAAATTCCATTCAGCAAGGACACCT<br>ACCTTGACAAAGTAATCACCGATGAAATGCTTTCCTTGCACAGGGGCCAGGGCTTAGAACTT<br>TTCTGGAGAGACTCGCTAACATGTCCCAGTGAGGAAGAGTACGTTAAGATGGTCCTTGGCAA<br>AACAGGGGGGCTATTCCGAATTGCTGTCAGGTTGATGATGGCTAAAAGTGAATGCGATATTG<br>ACTTCGTTCAATTGGTCAATTTAATTTCGATATATTTCCAGATTAGAGATGACTACATGAACTT<br>GCAATCGTCCGAGTATGCTCACAACAAGAACTTCGCGGAGGATCTAACGGAGGGCAAGTTTT<br>CATTCCCTACGATTCACTCGATTCACGCAAATCCATCAAGTCGTTTGGTCATCAACACTTTGC<br>AAAAGAAGTCAACAAGCCCTGAAATCCTTCATCACTGTGTGAATTACATGAGAACTGAAACTC<br>ACTCCTTTGAGTACACCCAAGAGGTATTGAACACACTCAGTGGAGCCTTAGAGAGAGAACTT<br>GGTCGGTTGCAAGGAGAGTTTGCCGAAGCCAACAGCAAAATCGACTTGGGGGATGTGGAAA<br>GTGAAGGTCGCACAGGTAAAAACGTAAAACTTGAGGCAATCTTGAAAAAGCTTGCCGACATC<br>CCATTATAA | 360 |
| XdCrtI | Xanthophyllomyces dendrorhous | ATGGGCAAGGAACAAGATCAAGACAAACCTACTGCCATTATCGTCGGTTGTGGTATAGGCGG<br>CATCGCTACCGCCGCTCGTCTTGCTAAGGAGGGCTTTCAAGTCACGGTGTTCGAGAAGAAC<br>GATTATAGCGGAGGCAGGTGTTCGCTAATCGAACGCGATGGTTACCGATTTGACCAGGGTC<br>CGAGTTTGTTATTGCTCCCGGACTTGTTTAAACAAACCTTTGAAGACCTTGGAGAAAAGATGG<br>AGGATTGGGTAGACTTAATAAAATGCGAACCGAACTACGTTTGTCATTTTCATGACGAAGAGA<br>CTTTCACCTTTAGTACGGACATGGCATTGTTGAAGAGAGAAGTCGAGAGATTTGAAGGAAAG<br>GATGGTTTCGACAGATTTCTATCCTTTATTCAAGAAGCCCACCGTCATTATGAGTTGGCTGTC | 361 |



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| Table 24 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **C.V. Codon Optimized Sequence** | **SEQ ID NO** |
| | | GTGCACGTCTTACAAAAAAATTTCCCAGGTTTCGCTGCATTCCTTCGCCTTCAATTTATAGGC<br>CAGATCTTAGCATTGCACCCATTTGAATCAATATGGACCCGCGTGTGCAGATACTTCAAGAC<br>TGACCGCTTGAGAAGAGTTTTTTCGTTCGCAGTCATGTACATGGGTCAAAGCCCTTACTCAG<br>CGCCGGGAACATATAGTTTGTTGCAGTACACCGAGTTAACGGAGGGAATATGGTACCCAAGG<br>GGCGGTTTCTGGCAGGTTCCAAATACCTTGTTGCAAATTGTTAAACGAAACAACCCTTCAGCT<br>AAATTCAACTTCAATGCACCAGTGAGTCAGGTCCTCTTGAGTCCTGCCAAAGACAGAGCAAC<br>CGGAGTGAGGTTGGAGAGTGGCGAAGAGCATCACGCGGACGTTGTCATCGTCAACGCAGAC<br>CTTGTTTATGCTAGCGAGCATCTTATTCCTGATGACGCTAGGAACAAGATTGGGCAATTAGGA<br>GAGGTGAAAAGGTCTTGGTGGGCGGATTTGGTGGGAGGTAAGAAACTCAAAGGTTCGTGTT<br>CTTCATTGTCATTCTACTGGTCCATGGACCGAATAGTGGACGGTTTGGGGGGTCACAATATC<br>TTTTTGGCCGAGGACTTCAAGGGTAGTTTTGACACCATTTTCGAGGAACTAGGATTGCCGGC<br>TGATCCTTCTTTTTACGTAAATGTTCCATCTCGCATCGATCCGTCAGCTGCCCCGGAAGGTAA<br>GGATGCCATTGTGATACTAGTGCCATGTGGTCATATTGATGCCAGCAACCCACAAGATTACA<br>ATAAGTTGGTTGCTAGGGCTAGGAAGTTCGTGATTCAGACATTGTCGGCAAAGTTGGGACTT<br>CCAGACTTCGAAAAAATGATTGTTGCGGAAAAGGTGCACGATGCTCCGTCGTGGGAAAAGGA<br>GTTCAACCTCAAGGATGGTTCAATCTTGGGTTTGGCGCACAACTTCATGCAAGTGCTCGGGT<br>TCAGGCCGTCGACTAGACACCCTAAGTATGACAAACTCTTTTTTGTTGGAGCTAGCACACATC<br>CTGGTACAGGGGTTCCAATCGTCCTTGCTGGTGCCAAATTGACAGCTAATCAAGTGTTGGAG<br>TCCTTCGACAGATCTCCAGCCCCCGATCCAAATATGAGCCTATCTGTTCCATACGGCAAGCC<br>TTTAAAGTCGAACGGGACGGGCATCGATAGTCAAGTCCAGTTGAAGTTCATGGATTTGGAAA<br>GATGGGTTTACTTGTTGGTTTTGTTAATCGGAGCCGTTATTGCCAGAAGCGTTGGAGTTCTCG<br>CATTTTAA | |
| XdCrtYB | Xanthophyllomyces dendrorhous | ATGACGGCATTGGCGTACTACCAAATCCACTTGATTTATACCTTGCCCATACTAGGGTTGTTG<br>GGTTTGTTAACTTCTCCAATCCTTACCAAATTTGATATATATAAAATCTCTATCTTGGTTTTTAT<br>TGCATTTTCAGCAACAACTCCATGGGACAGTTGGATAATTAGAAATGGGGCATGGACTTACC<br>CTTCGGCCGAGTCGGGTCAAGGCGTCTTTGGGACTTTTCTAGATGTTCCATATGAAGAATAC<br>GCTTTCTTCGTTATCCAAACGGTTATAACAGGTTTGGTCTACGTCCTCGCCACAAGGCATTTG<br>TTACCATCGCTCGCCTTGCCAAAGACACGATCCTCGGCATTAAGCCTCGCGTTGAAGGCATT<br>GATTCCGCTCCCAATTATCTATTTGTTCACAGCCCACCCAAGTCCTTCACCAGATCCCTTGGT<br>TACCGATCATTACTTCTATATGAGGGCGTTGTCACTCTTGATCACGCCTCCCACCATGTTACT<br>TGCTGCCTTGAGCGGGGAATACGCGTTCGACTGGAAGTCCGGCCGAGCAAAAAGCACGATT<br>GCAGCTATCATGATTCCAACGGTATACCTAATTTGGGTCGATTACGTCGCAGTAGGACAGGA<br>CAGCTGGAGTATCAACGACGAAAAAATCGTGGGCTGGCGCCTTGGGGGGGTCCTTCCTATA<br>GAAGAGGCAATGTTCTTTTTGTTGACGAATCTCATGATCGTGTTAGGGCTTTCAGCATGCGAC | 362 |



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| Table 24 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **C.V. Codon Optimized Sequence** | **SEQ ID NO** |
| | | CATACTCAAGCCTTGTATCTTCTCCATGGTAGAACTATTTATGGTAATAAAAAAATGCCGTCGT<br>CGTTCCCATTGATTACCCCGCCAGTGCTTTCTTTGTTCTTCTCAAGTAGACCGTATTCATCGC<br>AACCCAAGCGTGATTTGGAATTGGCGGTTAAGTTGTTGGAAGAGAAATCCCGTAGCTTCTTC<br>GTGGCAAGTGCCGGTTTTCCAAGTGAAGTGAGAGAGAGGTTGGTGGGCTTATACGCGTTTT<br>GCAGAGTCACGGACGACCTTATAGACAGTCCAGAAGTGTCCTCTAACCCACACGCGACGAT<br>CGATATGGTTAGCGATTTTTTGACCTTGCTTTTTGGACCACCCTTGCACCCATCCCAACCAGA<br>TAAAATTTTGAGCTCACCTCTACTCCCGCCCAGTCACCCAAGCAGGCCCACGGGGATGTACC<br>CACTCCCCCCGCCACCGAGTTTGTCTCCAGCCGAATTGGTTCAGTTCTTGACGGAACGCGTT<br>CCAGTGCAGTATCACTTTGCGTTCAGGTTGTTGGCGAAATTACAAGGGTTGATCCCAAGATAT<br>CCGTTAGACGAGCTATTGAGAGGTTACACCACCGATTTGATATTCCCTTTGTCCACCGAGGC<br>TGTGCAAGCCAGAAAAACCCCAATCGAGACTACGGCAGATCTTTTGGATTACGGTTTGTGCG<br>TAGCGGGGAGCGTCGCCGAGTTATTAGTCTACGTTTCGTGGGCATCCGCTCCATCACAGGTT<br>CCAGCCACTATTGAGGAAAGAGAGGCCGTGTTGGTCGCTAGCAGAGAGATGGGTACAGCGT<br>TGCAATTAGTGAATATTGCTCGAGATATTAAAGGTGATGCCACTGAGGGCCGTTTCTACTTGC<br>CATTGAGTTTTTTCGGTTTGCGTGACGAATCAAAACTCGCAATACCAACAGATTGGACTGAAC<br>CTAGGCCACAAGACTTCGATAAATTGTTGTCGCTCTCCCCAAGCTCCACTCTTCCATCATCAA<br>ATGCGTCCGAATCCTTCAGATTTGAGTGGAAGACGTACAGTCTTCCCCTAGTGGCCTACGCC<br>GAAGACTTGGCGAAACACAGTTACAAGGGAATTGACCGTCTACCCACCGAGGTGCAGGCAG<br>GTATGAGGGCGGCGTGCGCCAGTTACCTTTTGATAGGGCGCGAAATCAAGGTTGTGTGGAA<br>GGGTGACGTAGGTGAGCGACGGACGGTGGCCGGTTGGCGAAGAGTCAGAAAAGTTCTTTCT<br>GTGGTTATGTCGGGTTGGGAGGGACAATAA | |
| PaCrtE | Pantoea ananatis | ATGACGGTGTGTGCTAAGAAGCACGTCCACTTGACTAGAGACGCCGCCGAACAATTATTGGC<br>CGACATTGACAGGAGACTTGACCAACTTTTGCCGGTTGAAGGTGAGAGGGATGTTGTGGGA<br>GCTGCAATGCGAGAGGGGGCTTTAGCCCCAGGTAAGAGGATAAGACCTATGTTACTTCTATT<br>GACAGCGAGGGATCTAGGTTGTGCTGTCTCACATGATGGTTTGCTAGATTTGGCCTGTGCGG<br>TCGAGATGGTCCATGCCGCCAGCCTCATCCTCGACGATATGCCCTGTATGGATGACGCTAAA<br>TTGAGACGTGGTCGGCCAACGATTCACTCGCACTACGGGGAACATGTTGCAATCCTAGCCG<br>CAGTCGCCTTGTTGTCAAAGGCCTTCGGCGTTATCGCCGACGCCGACGGCTTGACGCCATT<br>GGCAAAAAACAGAGCGGTGAGTGAGTTGAGTAACGCTATAGGTATGCAAGGGTTGGTTCAA<br>GGACAATTCAAAGATTTGTCCGAGGGGGACAAGCCAAGAAGTGCTGAAGCAATTTTGATGAC<br>CAATCACTTCAAAACATCCACATTGTTCTGCGCATCTATGCAAATGGCATCCATTGTTGCAAA<br>CGCCTCATCGGAGGCCAGAGATTGTCTACATCGATTTTCTCTTGATTTGGGCCAAGCGTTCC<br>AGTTGTTGGACGACCTTACAGACGGTATGACCGATACTGGCAAAGATTCGAACCAAGACGCA<br>GGTAAAAGTACGTTGGTAAATCTCTTGGGTCCTAGAGCTGTCGAGGAGAGGCTTAGACAACA | 363 |



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| Table 24 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **C.V. Codon Optimized Sequence** | **SEQ ID NO** |
| | | TCTCCAGTTAGCATCCGAACATCTATCAGCTGCCTGCCAACACGGTCACGCCACTCAACACT TTATTCAAGCGTGGTTTGACAAGAAGTTGGCGGCTGTAAGCTAA | |
| PaCrtI | Pantoea ananatis | ATGAAACCCACAACCGTTATTGGTGCTGGCTTTGGCGGTTTGGCATTGGCCATAAGATTGCA AGCTGCCGGCATACCTGTGTTATTGCTTGAACAGCGGGACAAGCCAGGAGGACGGGCGTAC GTCTATGAAGATCAGGGTTTCACTTTTGATGCTGGGCCCACGGTTATCACAGACCCGTCGGC GATCGAGGAACTTTTTGCCTTGGCTGGAAAGCAATTGAAAGAATATGTCGAGTTACTTCCAGT TACACCATTTTATCGATTGTGCTGGGAATCCGGTAAGGTGTTTAATTACGACAACGACCAAAC TAGGCTTGAGGCCCAAATCCAACAATTCAACCCCCGGGATGTTGAGGGTTATCGCCAATTCC TCGACTATAGCAGAGCTGTCTTCAAGGAAGGGTACTTGAAATTGGGCACTGTGCCATTCTTG TCTTTCCGAGATATGTTACGAGCAGCCCCACAGTTGGCGAAATTGCAAGCATGGAGAAGCGT TTACAGTAAAGTTGCTTCTTATATTGAGGACGAACATTTGAGACAGGCCTTTTCATTTCACAG CTTGTTGGTGGGAGGTAACCCATTCGCTACTAGCTCTATTTACACCTTGATTCACGCCCTAGA AAGGGAATGGGGTGTTTGGTTCCCAAGAGGTGGGACAGGCGCTTTAGTGCAAGGAATGATC AAATTGTTCCAAGACTTAGGAGGGGAAGTGGTGTTGAACGCGCGTGTGTCTCACATGGAAAC TACAGGGAACAAGATAGAAGCAGTGCATCTCGAAGACGGCCGTCGATTCTTGACACAAGCA GTTGCATCGAATGCCGACGTCGTTCACACCTACCGCGACTTGCTATCTCAACATCCAGCCGC TGTGAAGCAATCCAATAAATTACAAACAAAAAGAATGTCAAACTCCTTATTTGTTTTGTACTTC GGTCTAAATCACCACCATGATCAACTCGCTCATCACACAGTCTGCTTCGGTCCAAGATATAGA GAATTGATCGACGAGATATTCAATCATGACGGGTTGGCCGAAGATTTTTCTTTGTATCTACAC GCTCCATGCGTCACTGATAGCTCTCTCGCTCCTGAGGGGTGCGGTTCTTACTACGTTTTGGC CCCTGTACCTCACTTGGGTACGGCGAACTTGGATTGGACCGTAGAGGGTCCCAAGCTACGT GACAGAATCTTTGCATACTTAGAACAACACTACATGCCGGGGTTACGCAGCCAATTGGTTAC ACATAGAATGTTCACGCCGTTCGACTTCCGAGACCAACTAAACGCGTATCACGGCTCTGCTT TTTCAGTTGAGCCAGTGCTCACGCAAAGCGCATGGTTTAGGCCTCACAACAGGGACAAAACG ATCACTAACTTATACCTCGTGGGGGCCGGGACCCACCCAGGAGCAGGAATACCAGGGGTCA TTGGTTCGGCTAAGGCGACTGCCGGCTTGATGCTTGAGGACTTGATTTAA | 364 |
| PaCrtB | Pantoea ananatis | ATGAACAACCCCTCATTGTTGAACCATGCCGTGGAGACAATGGCAGTCGGTTCTAAGAGCTT TGCTACCGCATCGAAATTGTTTGATGCCAAAACTCGGAGGTCAGTTTTGATGCTCTACGCGT GGTGTCGTCATTGTGACGATGTTATCGATGACCAAACCCTCGGCTTCCAAGCTCGGCAGCCG GCATTGCAAACCCCAGAACAAAGGTTGATGCAATTGGAGATGAAGACCAGGCAAGCTTATGC CGGCAGCCAGATGCACGAACCCGCCTTTGCGGCTTTTCAAGAGGTTGCAATGGCTCATGATA TAGCACCTGCTTATGCCTTTGACCACTTGGAGGGTTTCGCTATGGACGTCAGGGAAGCCCAG TATAGCCAATTGGATGATACCTTAAGGTACTGTTATCATGTCGCAGGAGTTGTGGGATTGATG ATGGCGCAGATCATGGGGGTTCGAGACAACGCTACCTTGGATAGAGCATGTGATTTGGGGT | 365 |



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| Table 24 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **C.V. Codon Optimized Sequence** | **SEQ ID NO** |
| | | TGGCGTTTCAATTGACTAATATCGCGCGAGATATTGTCGACGATGCACACGCAGGTAGATGC<br>TACTTGCCAGCCTCTTGGCTAGAACACGAAGGCCTCAACAAGGAGAATTACGCCGCCCCTGA<br>AAATCGACAAGCTTTGTCAAGAATAGCGAGAAGATTGGTGCAAGAGGCCGAGCCATATTATT<br>TGAGTGCGACAGCTGGTTTAGCCGGATTGCCATTGAGGAGTGCATGGGCCATAGCCACGGC<br>GAAACAAGTATACCGTAAAATAGGGGTCAAAGTCGAGCAAGCTGGTCAGCAAGCATGGGATC<br>AGCGGCAATCAACCACAACGCCAGAGAAGTTGACTTTGCTCTTGGCCGCCAGTGGACAGGC<br>TCTAACCTCCCGTATGAGAGCCCATCCCCCAAGGCCAGCTCACTTGTGGCAGAGGCCATTGT<br>AA | |
| XdCrtR | Xanthophyllomyces dendrorhous | ATGGCAACCCTATCTGACTTGGTTATCTTGTTGTTGGGTGCCTTGTTAGCATTGGGGTTCTAT<br>AACAAGGACAGATTGCTCGGTTCAAGTTCCAGCAGTGCCTCTACCACATCAGGTTCAAGCGC<br>TGCAACTGCTAACGGTAGTAAGCCCACATATTCTAATGGCAACGGGAACGCCTTTAAAGGCG<br>ACCCACGAGATTTCGTCGCGAGAATGAAGGATCAAAAGAAAAGGTTGGCAGTTTTTTATGGG<br>TCCCAAACTGGAACTGCTGAGGAGTACGCCACCCGAATCGCCAAGGAAGCGAAATCTCGTTT<br>CGGCGTTTCGAGTTTGGTGTGTGACATTGAGGAATATGATTTTGAAAAATTGGATCAAGTCCC<br>CGAGGATTGCGCCATTGTTTTTTGCATGGCTACCTATGGTGAAGGTGAACCAACAGACAATG<br>CTGTCCAATTCATCGAAATGATCAGCCAGGATGACCCAGAGTTTAGTGAAGGTAGTACTTTG<br>GATGGTTTGAAGTACGTTGTCTTTGGATTGGGGAATAAGACATACGAGCAATACAATGTTGTC<br>GGCCGGCAGTTGGACGCTAGATTAACCGCACTTGGTGCGACCAGAGTAGGTGAGCGAGGT<br>GAAGGAGATGATGACAAAAGTATGGAGGAAGATTATCTCGCATGGAAGGATGATATGTTCGC<br>CGCTTTGGCCACCACATTAAGTTTTGAAGAGGGAGCTTCGGGGGAGACTCCAGATTTCGTGG<br>TCACTGAAGTCCCGAACCACCCCATCGAAAAGGTCTTCCAGGGCGAATTGTCCAGCAGAGC<br>CTTGTTGGGCTCTAAAGGCGTTCACGACGCCAAGAATCCATACGCTTCGCCAGTTTTGGCCT<br>GCCGGGAATTATTCACAGGCGGAGATCGAAACTGTATCCACCTTGAGTTTGACATCACCGGG<br>TCCGGAATCACCTACCAAACAGGTGATCACGTCGCAGTTTGGCCATCCAATCCTGACGTTGA<br>AGTGGAAAGGCTTTTGGCAGTGTTGGGTCTCACATCTCCAGAGAAGAGACGGATGATTATCC<br>AAGTGGTGAGCCTTGATCCAACTTTGGCCAAGGTTCCATTCCCTACACCAACCACCTATGAT<br>GCCGTTTTCAGACATTACCTTGACATTAGTGCAGTGGCTTCACGCCAGACCTTGGCTGTCCT<br>TGCCAAATACGCCCCTTCCGAACAGGCTGCCGAATTCTTAACCAGGTTGGGAACTGACAAGC<br>AAGCGTACCATACTGAAGTGGTTGGAGGTCACCTAAGATTGGCCGAGGTGCTTCAATTGGCT<br>GCAGGGAACGATATTACTGTTATGCCTACAGCTGAAAATACAACCGTCTGGAACATACCATTC<br>GACCACGTTGTGTCTGACGTTTCCCGCTTGCAACCCCGATTTTACTCGATAAGCAGTTCTCCT<br>AAGCTTCACCCGAACAGCATTCACGTAACGGCTGTGATACTTAAATACGAAAGTCAAGCCAC<br>TGACCGTCACCCAGCGAGATGGGTGTTTGGTTTGGGGACAAACTATTTGCTTAATGTTAAGC<br>AAGCAGCAAACAATGAAACCACTCCAATGATCTCTGACGGACAAGACGATGTGCCAGAACAC | 366 |



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| Table 24 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **C.V. Codon Optimized Sequence** | **SEQ ID NO** |
| | | GTGTCAGCGCCAAAATACACGTTGGAGGGACCCCGTGGCTCCTATAAGCACGACGATCAATT<br>GTTTAAAGTGCCAATCCATGTGCGTAGGTCCACTTTCAGACTTCCAACCAGCCCTAAGATCC<br>CTGTGATCATGATTGGGCCAGGTACCGGTGTCGCTCCTTTCAGAGGATTCATCCAAGAAAGA<br>ATTGCCCTTGCCAGAAGGTCCATTGCTAAGAATGGGCCTGACGCCTTGGCGGATTGGGCCC<br>CAATCTATCTTTTCTACGGTAGTCGCGATGAGCAAGACTTCTTGTACGCGGAAGAGTGGCCT<br>GCTTATGAAGCGGAGTTGCAAGGGAAATTCAAGATTCATGTTGCTTTCAGTAGATCCGGGCC<br>AAGAAAGCCAGATGGGAGTAAGATTTACGTGCAAGATCTTTTGTGGGATCAAAAGGAAGTCA<br>TCAAATCTGCCATAGTGGAAAAACGTGCATCTGTTTATATTTGTGGGGACGGTCGAAATATGT<br>CGAAAGATGTCGAGCAGAAGTTGGCCGCGATGTTGGCCGAAAGTAAAAACGGTAGTGCGGC<br>CGTGGAAGGAGCCGCGGAAGTCAAGTCATTGAAAGAGAGATCTCGATTATTGATGGATGTGT<br>GGAGCTAA | |
| XdCrtS | Xanthophyllomyces dendrorhous | ATGTTCATTTTGGTGTTGTTGACCGGTGCTTTGGGTTTGGCAGCTTTTAGCTGGGCTTCGATT<br>GCCTTCTTTTCTCTTTATTTGGCGCCCCGTCGCAGTAGCTTGTATAATTTGCAAGGTCCAAAC<br>CACACTAACTATTTCACCGGCAATTTTTTAGATATACTCTCGGCTAGGACGGGTGAGGAGCAT<br>GCTAAGTATAGGGAAAAATACGGATCCACGTTGAGATTTGCCGGCATCGCCGGTGCCCCAG<br>TTTTGAACTCAACTGATCCAAAAGTCTTCAACCATGTGATGAAGGAGGCATATGATTACCCAA<br>AACCTGGTATGGCTGCGAGAGTGTTGAGGATTGCAACGGGTGACGGAGTGGTTACGGCTGA<br>GGGTGAGGCACATAAACGACATCGAAGAATCATGATCCCTTCCTTGTCAGCACAAGCCGTTA<br>AGTCCATGGTTCCCATCTTCCTTGAGAAGGGCATGGAGCTCGTTGACAAGATGATGGAGGAC<br>GCAGCCGAGAAAGACATGGCAGTGGGCGAATCCGCAGGTGAGAAGAAGGCTACTCGACTTG<br>AAACGGAAGGCGTTGACGTAAAGGACTGGGTTGGGAGAGCAACTTTGGATGTCATGGCTTT<br>GGCCGGATTTGATTACAAATCAGATTCGCTACAAAATAAGACGAACGAATTGTATGTCGCTTT<br>CGTGGGGCTTACAGACGGATTTGCACCCACCTTGGACAGTTTTAAGGCCATCATGTGGGATT<br>TCGTGCCGTACTTTCGCACAATGAAGAGAAGACATGAGATTCCCTTGACCCAGGGTTTGGCT<br>GTTAGTCGCCGTGTTGGCATTGAACTAATGGAGCAAAAGAAGCAAGCAGTGTTGGGTTCGGC<br>ATCGGATCAAGCCGTGGACAAAAAGGACGTGCAGGGACGCGACATTCTTAGTCTCTTGGTCA<br>GAGCGAACATTGCCGCAAATCTTCCCGAATCTCAAAAGTTGTCGGACGAAGAGGTTCTTGCT<br>CAAATTTCGAATTTGCTATTCGCGGGTTATGAGACTTCAAGTACTGTTCTCACATGGATGTTC<br>CATCGATTGTCGGAGGACAAAGCAGTGCAAGATAAATTAAGGGAGGAAATCTGTCAAATAGA<br>TACAGACATGCCAACACTAGACGAGTTGAATGCATTGCCGTACTTGGAGGCGTTTGTGAAAG<br>AGTCACTAAGACTCGATCCCCCGTCTCCATATGCTAATAGGGAGTGTTTGAAGGATGAAGAT<br>TTCATTCCATTGGCCGAACCTGTCATTGGACGCGATGGGTCCGTGATCAATGAAGTCAGAAT<br>CACAAAGGGGACAATGGTGATGCTTCCATTGTTCAACATCAATAGATCGAAATTCATATACGG<br>TGAGGACGCCGAAGAATTTAGACCAGAAAGGTGGTTGGAGGATGTTACCGACTCCCTTAACA | 367 |



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| Table 24 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **C.V. Codon Optimized Sequence** | **SEQ ID NO** |
| | | GCATAGAAGCTCCATATGGCCATCAAGCGTCCTTCATATCAGGACCAAGAGCCTGCTTTGGA<br>TGGCGGTTCGCGGTCGCAGAAATGAAAGCATTCTTGTTCGTAACACTAAGGAGAGTGCAATT<br>TGAGCCGATCATAAGCCATCCAGAGTACGAACATATTACTCTCATTATCAGCAGACCCCGGA<br>TCGTTGGCAGAGAGAAGGAAGGATATCAGATGCGTTTGCAGGTAAAGCCAGTGGAGTAA | |
| AaCrtZ | Agrobacterium aurantiacum | ATGACAAACTTCTTGATCGTTGTGGCCACCGTGCTTGTTATGGAATTGACCGCCTACTCGGT<br>GCATAGATGGATAATGCACGGACCATTGGGTTGGGGATGGCACAAATCACACCATGAGGAA<br>CACGATCACGCCTTAGAAAAAAATGACCTCTATGGCTTGGTGTTCGCTGTCATTGCGACCGT<br>TTTGTTCACCGTCGGTTGGATCTGGGCACCAGTGTTGTGGTGGATTGCACTTGGTATGACGG<br>TTTATGGCCTCATATATTTTGTGCTCCACGATGGTCTAGTTCACCAAAGGTGGCCTTTCAGAT<br>ACATTCCCAGGAAGGGTTATGCACGGCGATTATATCAAGCCCATCGTCTTCATCACGCTGTG<br>GAGGGAAGGGATCACTGTGTCAGCTTTGGGTTCATCTACGCACCACCCGTTGACAAGTTGAA<br>ACAAGATTTGAAAATGTCTGGTGTCTTGCGGGCTGAGGCCCAAGAGAGGACGTAA | 368 |
| AaCrtW | Agrobacterium aurantiacum | ATGAGTGCCCATGCCTTGCCAAAGGCAGATCTAACTGCCACTAGTTTAATTGTGTCGGGCGG<br>TATTATCGCGGCTTGGTTGGCATTGCATGTACATGCATTGTGGTTCTTGGACGCGGCAGCGC<br>ACCCTATCCTCGCAATCGCCAATTTTCTTGGACTAACGTGGTTGTCAGTAGGTCTATTTATCA<br>TCGCCCACGATGCGATGCACGGATCCGTAGTCCCTGGTAGACCTAGAGCAAATGCTGCAAT<br>GGGCCAGTTGGTGCTTTGGTTGTACGCTGGCTTCAGTTGGAGAAAGATGATTGTTAAGCATA<br>TGGCACATCACCGTCACGCAGGTACCGATGACGACCCAGATTTTGATCATGGAGGTCCAGT<br>GCGATGGTACGCACGCTTCATAGGAACCTACTTTGGCTGGAGAGAAGGTTTGCTTTTGCCAG<br>TCATAGTAACCGTCTACGCTTTAATACTTGGAGATCGATGGATGTACGTCGTGTTCTGGCCAT<br>TGCCATCAATCTTGGCCTCTATCCAATTGTTCGTTTTTGGGACTTGGTTACCACATAGACCCG<br>GTCACGATGCATTCCCAGATAGACACAACGCCCGATCATCCAGAATATCCGATCCAGTTTCTT<br>TGCTAACTTGCTTCCACTTCGGTGGTTATCACCACGAACACCATTTGCATCCGACGGTCCCAT<br>GGTGGAGGTTGCCTAGCACTAGAACTAAAGGTGATACCGCTTAA | 369 |
| CnTPS 1 | Callitropsis nootkatensis | ATGGCAGAAATGTTCAATGGAAACAGCAGTAATGACGGGAGCTCATGTATGCCGGTAAAGGA<br>TGCATTGAGACGTACGGGCAACCACCACCCAAATTTGTGGACTGACGATTTCATTCAATCGC<br>TTAATAGTCCATACTCGGACTCCTCCTACCATAAACATCGCGAAATATTGATCGATGAGATTC<br>GCGATATGTTTTCAAATGGGGAAGGGGACGAATTTGGCGTGTTGGAAAATATTTGGTTTGTC<br>GATGTTGTTCAACGGTTAGGAATCGACCGTCACTTTCAGGAAGAGATTAAAACAGCGTTGGA<br>CTACATCTACAAGTTTTGGAACCACGACTCGATATTTGGTGACCTTAACATGGTCGCTTTGGG<br>TTTCAGAATCCTAAGATTGAACCGTTACGTGGCAAGCAGCGACGTTTTCAAGAAGTTTAAGG<br>GTGAAGAGGGCCAATTTTCAGGCTTCGAAAGTTCAGACCAAGATGCGAAGTTGGAAATGATG<br>CTAAACTTGTATAAAGCAAGTGAGCTAGATTTTCCAGACGAGGATATCCTTAAGGAAGCTAGG | 370 |



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| Table 24 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **C.V. Codon Optimized Sequence** | **SEQ ID NO** |
| | | GCCTTTGCGAGTATGTACTTAAAGCACGTAATCAAGGAATACGGTGATATCCAAGAGAGCAA<br>GAACCCATTGCTTATGGAGATAGAATACACATTCAAGTACCCATGGCGTTGTCGTTTGCCTCG<br>ACTTGAGGCTTGGAATTTCATTCACATTATGAGACAACAAGACTGTAATATTTCATTGGCTAAC<br>AACCTTTACAAGATTCCGAAGATATACATGAAAAAGATCTTGGAGTTGGCCATCTTGGACTTT<br>AATATTCTCCAGTCACAACACCAACATGAAATGAAACTCATCTCCACGTGGTGGAAGAACTCT<br>AGCGCTATCCAATTGGACTTTTTCAGACACCGCCACATCGAAAGCTACTTCTGGTGGGCTTC<br>ACCGTTGTTCGAGCCCGAGTTTTCTACATGTCGGATCAACTGTACGAAGTTGTCAACTAAAAT<br>GTTTCTACTTGACGATATATACGACACATACGGTACTGTGGAAGAACTAAAGCCGTTTACGAC<br>CACATTAACGAGGTGGGACGTGTCTACAGTCGACAACCATCCAGACTACATGAAGATCGCTT<br>TTAACTTCAGCTATGAAATCTACAAGGAGATCGCCTCCGAGGCGGAACGTAAACACGGTCCA<br>TTCGTGTATAAATATTTGCAATCATGCTGGAAGTCCTATATAGAAGCTTACATGCAAGAAGCA<br>GAGTGGATTGCTTCAAATCATATTCCAGGCTTCGATGAGTACCTCATGAATGGTGTAAAGTCA<br>TCCGGCATGAGAATATTGATGATCCACGCATTAATCCTAATGGATACACCATTGAGCGACGA<br>GATTTTGGAACAATTAGATATACCGAGTTCGAAGTCACAGGCCTTGTTGTCGTTGATTACCAG<br>ACTCGTGGATGATGTCAAGGACTTTGAGGACGAACAAGCCCATGGGGAAATGGCATCCTCC<br>ATTGAATGTTACATGAAAGATAACCACGGTTCAACAAGAGAAGATGCATTAAACTACTTGAAG<br>ATCCGAATCGAGTCGTGTGTCCAGGAGTTGAATAAGGAATTGTTAGAACCTTCGAATATGCA<br>CGGTAGTTTTAGGAATCTTTATTTGAATGTTGGCATGCGTGTCATATTTTTCATGCTCAACGAT<br>GGGGACTTGTTCACTCATTCGAACAGGAAGGAAATTCAGGATGCCATCACCAAGTTCTTCGT<br>CGAACCGATTATACCTTAA | |

| Table 25 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **Native Cv sequence** | **SEQ ID NO** |
| ERG10 | Candida viswanathii | ATGACCCTCCCACCAGTCTACATCGTCTCCACAGCAAGAACTCCAATTGGTTCCTTCCAAGG<br>TTCGTTATCTTCCTTGACCTACTCCGATTTGGGTGCCCACGCCGTTAAGGCAGCTTTAGCCAA<br>GGTCCCACAGATCAAGCCACAGGACGTCGACGAGATCGTCTTTGGTGGTGTCTTGCAAGCC<br>AACGTTGGTCAGGCACCAGCCAGACAAGTCGCCTTGAAGGCAGGCTTGCCAGACTCTATCA<br>TTGCCTCCACCATTAACAAAGTCTGTGCCTCCGGTATGAAGGCCGTCATTATCGGTGCCCAA<br>AACATCATCTGTGGAACCAGTGACATCGTCGTTGTTGGTGGTGCCGAATCCATGTCTAACAC<br>TCCATACTACTTGCCAAGCGCCAGAAGCGGGGCCAGATACGGTGACGCCGTCATGGTTGAT<br>GGTGTCCAGAAGGATGGTTTGTTGGATGTCTACGAAGAAAAATTGATGGGTGTTGCTGCTGA | 371 |



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| Table 25 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **Native Cv sequence** | **SEQ ID NO** |
| | | AAAGTGTGCCAAGGACCACGGATTCAGCAGAGAAGACCAGGACAACTTTGCCATCAACTCTT<br>ACAAGAAGGCTGGCAAGGCTTTGAGTGAAGGTAAGTTCAAATCAGAAATCGCCCCAGTCACC<br>ATTAAAGGATTCAGAGGCAAGCCAGATACTGTTATTGAAAATGATGAAGAGATTGGCAAATTC<br>AACGAAGACAGACTCAAGTCTGCCAGAACTGTCTTCCAAAAGGAAAACGGTACTGTTACTGC<br>TCCAAACGCTTCTAAATTGAACGATGGTGGTGCTGCCTTGGTCTTGGTTTCTGAAGCCAAGTT<br>GAAGCAATTGGGCTTGAAGCCATTGGCCAAGATCTCTGGTTGGGGTGAAGCTGCCAGAACT<br>CCATTCGATTTCACCATTGCCCCAGCTTTGGCTGTTCCAAAGGCTGTCAAGCACGCTGGTTT<br>GACCGTTGACAGAGTCGACTTCTTTGAATTGAACGAAGCCTTCTCCGTTGTTGGTTTGGCCA<br>ACGCTGAATTGGTCAAGATCCCATTGGAAAAATTGAACGTCTACGGTGGTGCTGTCGCCATG<br>GGTCACCCATTGGGTTGCAGTGGTGCTAGAATTATTGTCACCTTGTTGTCCGTCTTGACCCA<br>AGAAGGCGGTAGATTTGGTGCTGCTGGTGTCTGTAACGGTGGTGGTGGTGCCTCTGCCATC<br>GTCATTGAAAAGATTGACTCCGATGCCAAGTTGTAA | |
| ERG13 | Candida viswanathii | ATGACTAACGCACCACAAAACATTGGTATCAAAGGAATCGAAGTCTACATTCCAGGCCAAGC<br>TGTCAATCAATCAGACTTGGAAAAATTCGACGGCATCCCAGCCGGCAAGTACACCATCGGCT<br>TGGGCCAGACAAACATGGCTTTTGTCAACGACAGAGAGGACATCTACTCCATCGCCTTGACT<br>GTTGTCTCCAGATTGATCAAGCATTACAATGTTGACACCAACAACGTCGGTCGTTTAGAGGTC<br>GGTACCGAGACCTTGTTGGACAAGTCCAAGTCTGTCAAGTCCGTGTTGATGCAATTGTTCCC<br>CGACAACAACGACATTGAAGGTATCGACACTGTCAACGCCTGCTACGGTGGTACCTCCGCTG<br>TCATCAATGCCATCAACTGGATCGAGTCGTCTTCCTGGGACGGCAGAGACGCCATTGTTGTT<br>GCTGGTGACATTGCTATCTACGACAAGGGTGCTGCTAGACCAACTGGTGGTGCTGGTTCCAT<br>TGCTATGTTGATTGGTCCTGACGCTCCTATTGTCTTTGACTCTGTGCGTGGCTCATTCATGGA<br>ACACGCCTATGATTTCTACAAGCCAGACTTCACCAGTGAATACCCAGTTGTCGATGGTCACTT<br>CTCCTTGTCCTGTTACGTTAAGGCTGTTGACAACTGTTACAAGAACTACTCCAAGAAGGTCAC<br>CGGCAACGTCGACAAGACCGTCGGCGTTTACGACCACTTTGACTACAACGCTTTCCACGTGC<br>CAACCTGTAAGTTGGTCACCAAGAGTTACGCCAGATTGTTGTACAACGACTACAAGTCGGAT<br>CCATCCAAGTTTGCTGACTTGATTGACGAAAGCACCAGACAACACATTGACAGTTTGTCCTAC<br>GAAGCATCCTTGACCGACAAGGTCTTGGAAAAAACTTTCGTCACATTGGCCAAAGAAGAGAC<br>CAAAAAGAGAGTGCAACCAGCCTTGCAAGTGCCAACCAACACCGGTAACATGTACACTGCTT<br>CGTCATGGGTCTCCTTGGCCTCGTTGTTGTACTATGTTGGCGCTGAAAACTTGAAAGAAAAG<br>AGAATCGGTCTCTTCTCCTACGGTTCAGGTTTGGCCTCCACCTTGTTGTCCGTCACTGTTGTC<br>GGCGACGTTTCTCCAATCACCAAGGTCTTGGACTTCGACTACAAATTAGGCGAAGGAAGAAA<br>GATCCAGAGCCCAGAAGAATACTTGGCTGCCATTGAATTGAGAGAAAAGGCACACTTGCAAA<br>AGAGCTTCAAGCCACAAGGTTCCCTCGATAACTTGAGCCAAGGCACCTACTACTTGACTGAG<br>GTTGACGACAAGTTTAGAAGAAGTTATGCTATAAAGGAATAA | 372 |



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| Table 25 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **Native Cv sequence** | **SEQ ID NO** |
| HMG1 | Candida viswanathii | ATGCTAAGTTTCATCACCGAAGCAAGTGGCAGAATAGCCCAGACTGCAGCCCACAGACCAAT<br>TCACTTCATGGTGATACCTGCGTTACTAGCATCCATAGCATACTTGTCCATCATCGATGATTA<br>CGTCCCCGAACACATCAGAGCCCAGTACATGTCAGGCGTCAGCTATTTCCACCCTCAAGGGT<br>CCGCCTCGGATTTGGACAAGTGGATCGAAATCCACGACACTACCCAATACGCCAACGCCAAC<br>CAAATCAGTGTCATCCCTTTGAGATTCAGACGATTCCATGACTCAATCCCACAGATCGCTAAC<br>GCCATCAAGATTTCAAACAATGAACAGATCTTGATTGTTCCTTCGGATAAAGCAGAGTCGACA<br>GTAGAAGGTTTGAGCGAGATCACTGAGAATGGCATCACTTGGAGGGCTAGAAATAACGACAA<br>GTTGTCCAAGTATTATGATTACGCCAGGTACGGGTTGCTTAGAATCCAGGACGCTATACACA<br>ATGCTGATAATTTCGACATCCTCTTGGTGTTTGTTGCCTACCTTGGAATGTGGTACTCACTTAT<br>CAAAGTGTTTATTGACATGAGACGCATTGGCTCCAAGTTTTGGTTAGCCTTTGGCACGTTGAC<br>TTCCTCGACTTTTTCATTCTTGTTTGCCTTGGTCATTTCAAACAAATTTTTGGACGCTAAAGTTT<br>CCTTGAGAAGTCTTTCTGAATGCATCCCATTTTTGGTTGCCATTATTGGGTTCAAACATAAAGT<br>GGCCATCACGACTAGTGTTGCCCAGTCATCCACTTCTTCACCCGAGGATGTTCCTCATGTCG<br>TGGGCAAAGCTGTCTCAGACCAGTGTTTGTTTATCTTGAGAGACCATTTGGTTGTCATTATTG<br>GATTTCTCGCCTGTGCGGCTTATGGCAATGAATTGAAAGGTTTAAGAAACTTCTGTATCTTGG<br>GGGCATTGATTTTGTCCTTTGACATTGTTTCCGTTTATACTTTCTTTTCTGCGATTTTGGCCTT<br>GAAAGTCGAGATTAACCGTGCCCGCCGTACTCAAGATTTACAACACGTATTGGAAGAGGACG<br>GCATTTCATCCTTAGTTGCCGCTAGAGTTGCCGAACGAAATGCAACTATTGAACACCCCAAC<br>GAAACCAACTTCTTCTCATCTAATAATTCTTCCATTGTTTATTTCAAAGTTATTATGAGTCTTGG<br>GTTTTTCGCCTTCCATGCATTTTGGTTGGGCAGCTCGTGGTTATACAACACTTCTGATGGCGG<br>TAGCCATGGAAGCTTTTCGTTCCTCAGCAACATCCCATTATTGACACAAGACATCTCCAACTC<br>CATCCCAATTGGCAGAAGAGGAACCGTGGTTACCATTTTGCCAACCACCTTCTACATGCCAT<br>CCGGTATCATCATCCAATTTGAAGATATGGTCTACTTAGCATTGAGCAAGATCAGCAGTGCTA<br>TCAGAGACAGTTTGATTTCCAAGTGTATTGTGTTTGCATTGACCATTTCCATTGTCACCAACAT<br>CTACTTCTTGAATGCTGCCCGTTTCCAAGTTTCAGCTACCCGTAAGTTGATTGACCAAGAAAT<br>GTCTCGTCCAAAACAAGCCGCCGCCGCCGCTGCTGCTGCTCCATCTGCTGCAAAATCAGTG<br>GCGCCAGAAGAAGACGAAGACGAAACCTCCAGTGAAGAACTTGAAATCAAAGCTCCTGTAAA<br>GCCGTTATCACTTGAAGAATGTACCAGAATCCTCAAAGAGGGCAAAGTCAAGACCTTGTCCA<br>ACGCTGAAGTGTCTAGCTTGGTTGTTGGCGGTAAATTGCCATTGTACGCTTTGGAGAAACAA<br>TTGGGCGACAACAAGAGAGCCGTGGCTGTTCGTCGTAAGGCTATTGCAAAATTAGCTAACGC<br>GCCAGTATTAGAAACCAACCGTTTGCCTTACTCCCACTATGATTATGATAGAGTCTTTGGTGC<br>TTGTTGTGAGAATGTTATTGGGTACATGCCAATCCCTGTTGGTGTTGCTGGACCATTAGTGAT<br>TGATGGCAAGCCATACCACATCCCCATGGCCACTACCGAAGGTTGTCTTGTTGCCTCTACTA<br>TGCGTGGCTGTAAAGCTATTAACGCTGGAGGTGGTGTTGAAACCGTCTTAACCAAAGATGGT<br>ATGACTAGAGGTCCTTGTGTTCGTTTCCCAACTTTGAAAAGAGCCGGTGCTGCAAAGTTGTG | 373 |



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| Table 25 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **Native Cv sequence** | **SEQ ID NO** |
| | | GATTGACTCCGAAGAAGGTCAAATCACCATCAAAAAGGCATTCAACTCGACTTCTAGATTTGC<br>TCGTTTGCAACATATTCAAACTGCCCTCGCTGGTACCTCGTTGTTTATTAGATTTAGAACTACT<br>ACTGGTGATGCTATGGGCATGAACATGATTTCCAAAGGTGTTGAATATTCCTTGAAGCACATG<br>GTTGAAGAATGTGGCTGGGACGATATGGAAGTTATCTCTGTTTCTGGTAACTACTGTACCGAT<br>AAGAAACCAGCTGCTATTAACTGGATTGAAGGTAGAGGTAAGTCTATTGTTGCTGCTGCTACC<br>ATCCCTGCTGATGTTGTCACTAAAGTCTTGAAATCCGATGTCGATGCCTTAGTTGAATTGAAT<br>GTTTCCAAAAACTTGGTTGGCTCAGCCATGGCCGGTTCAGTTGGTGGTTTCAATGCTCATGC<br>AGCCAACTTGGTCACTGCTGTTTACTTGGCCTGTGGTCAAGACCCCGCACAGAATGTCGAAT<br>CCTCAAACTGTATTACCTTGATGGAAAAGGACAAACAGACTGGCGACTTGGTCATTTCTGTTT<br>CTATGCCATCTATTGAAGTTGGTACGATTGGTGGTGGTACCATTTTGGAACCACAAGCAGCC<br>ATGTTGGACTTGTTGGGCGTGCGTGGCCCTCACCCAACCAATCCAGGTGACAATGCAAGAC<br>AATTGGCCAAGATTGTTGCTTCGGCAGTGTTAGCAGCTGAATTGTCACTTTGTTCTGCTTTGG<br>CCGCTGGTCACTTGGTGCAATCCCACATGCAACACAACCGTAAAGGAGCCACCCCAGCTGC<br>GGCCCCTGCAATCAGCAACGGAAGTGCCAAGGGAACCAAAACCAATGGGTCTATAAATGGG<br>AAAGACTTGAAGCGTCTCAAAGACGGATCCGTTACATGTATAAAGTCATAG | |
| ERG12 | Candida viswanathii | ATGTCAGTCTCGCCTTTCGTTGTCAGTGCTCCAGGGAAAGTCATCATCTTTGGAGAACATTCA<br>GCAGTTTATGGGAAACCCGCCATTGCAGCCGCCTTGAGTTTGCGATGTTACCTACTCGTTTC<br>CCCGTCAGTTGATGATGCAAACACAATCAGATTGCAGTTCCCAGACATCCAATTGGACCATTC<br>CTGGAACATCAACGATATCCCCTGGGATGAAATCAAACCGTTCGTTAAATATGACGCCAATAA<br>CAAACCACTCACCCCATCGGAGTTGGTACCAGAGATCCTCGATAAGTTGTCTCCCTTGTTGA<br>CTGATTTCGATAACAAAATGCATTACTACGCTTGTTTTTGTTTCCTTTACTTGTACGTCAATTTA<br>TGTTCCACTGAAACTCCAGGAACCACCTTCATCGTCAGATCCACCTTGCCAATCGGTGCCGG<br>GTTAGGGTCGTCTGCATCCACGTCTGTGTGCTTGTCGTCTGCATTAGCATTGTTAGGCGGCT<br>GTATCAGTGAGCCTTCGATATCAGCCACCGACAAGATCCTCAACGAAGACATACCCGATTTG<br>GAGTTCGTCGATAAGTGGTCCTTGATAGGTGAGAAGTGCTTCCACGGGAACCCATCCGGTAT<br>CGACAACGCAGTAGCCACGTTTGGCGGTGCCGTCATGTTTCAAAGAACTTCGGCTCCTGAG<br>CAACCATCCATCAGAACCAACATGAGAAACTTCCCAGCTATCAAACTCTTACTTACAAACACC<br>AAAGTCCCAAAAAGTACAGCTGATTTAGTCGCCGGTGTTGGCAAACTCAACGCGGAGTTCAA<br>CCCGATAACCACGTCGATTTTGACCGCTATGGAGCATTTGTCACAGGAAGCTTACAAAGTCA<br>TGATCACTCCGGGGTTCGGCAAGGACGAAACAAACACGTTGCGTAAGTTGGTCAATATCAAC<br>CATGGTCTATTGGTAGCGTTGGGCGTGTCACATCCGTCGCTAGAAACCGTCAAGATCATTGG<br>TGACAGACACAAGATTGGCGCTACTAAGTTGACTGGCGCTGGTGGTGGCGGGTGTGCGATC<br>ACTTTGGTTAATGATAATGTCGAGGAGTCTGTTATCCAAGAAGCTATCAAAGAGTTTGCAACT<br>GAGGGATATGAGAGTTTTGAAACGTCGTTGGGTGGTAAAGGTGTCGGTGTCCTATTTAGTGG | 374 |



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| Table 25 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **Native Cv sequence** | **SEQ ID NO** |
| | | TGATGTTTCTGATAATGGCAAGTTTTCTCAATCTGTGTTCTGCAACTATCCCGACCGTGCTAG<br>CATTGAAGATGCTTTAGGAATGATAAACGTTAAAGAATGGAAGTTCTGGTGA | |
| ERG8 | Candida viswanathii | ATGTCAAAAGCTTTTAGTGCCCCTGGTAAAGCATTGTTAGCTGGCGGATACTTGGTTCTTGAG<br>CCAACATATGATGCATATGTGACGGCATTGTCCTCGAGAATGCACGCTATAATAACACCCCA<br>GAAGCCAGCATCCATTTCAAAAATCAAAATCAGTTCGCCTCAGTTTGCCAATGGTGAGTGGG<br>AGTATCACGTCACGTCTAATGAGAAACCCAAGGACATCAAGTCCAGAAGCAATCCGTTTTTG<br>GAGGCTACCATTTTCATTGTTTTGTCTTATATCCAACCAACAGAACCATTCGATCTCGATTTGG<br>TCATATATTCAGACCCAGGATACCATTCACAAGAACACACCACGCAGAAAGTGTCCAGCAAC<br>GGCAAGAAAAAGTTCCTTTATCATTCGCGTGCAATTAACGATGTTGAGAAGACAGGATTAGGT<br>TCTTCGGCCGGTCTAGTTTCTGTCGTCACTACCAGTTTGTTATCCTACTTTATTCCGGGAATT<br>GAAGAGTCAAACAAGGACATGTTGCACAATGTAGCACAGATTGCCCATTGTTTTGCCCAGAA<br>GAAGATTGGGTCCGGGTTCGATGTGGCCACTGCCATCTATGGTCTGATTGTCTACCGAAGAT<br>TCCAGCCAAGCTTGATCAACGACGTTTTCGAGATCTTAGAGGAAACCCCGGGTAGATTCCCC<br>GGTGCTTTGAAGTCGTTAGTTGAGTCTAATTGGGAATTCAAACATGAAAGGTGCGTACTACCA<br>CCAAAGATCAAGCTCTTGATGGGAGATATCAAAGGAGGATCAGAGACTCCGAAATTGGTTTC<br>CAAAATACTCCAATGGAAGAAGGACAAACCAGAAGAAAGTGGGTTGGTGTATGATCAACTTA<br>ATAGCGCAAATGTGGCGTTCATGAAGAAGATCAGTACATTGAATGAATCGAGTCAGGTCCAA<br>GAGATTGACGAATTGTCTGATTACATAAGCGCGGTACGCAAAGGGTTGCAAGAATTGACAGA<br>AAAATCCAAAGTCCCAGTTGAACCACCTGTGCAAACTGAATTGTTGGACCGCATTGCAAAACT<br>CCCAGGTTGTCTAGGTGGGGTTGTGCCCGGTGCTGGTGGGTACGATGCTATAGCTGTGCTT<br>GTGTTGGAAAAGGAGGTTGAGAACTTTAGAAACAAGACACTTGAAAACCCAGAGTACTACCA<br>CAACGTGTACTGGGTTGACTTGGAGGAAGAAACCGAGGGTGTAGTGGTGGAAAACTGGGAG<br>GACTATATCGGTTTATAG | 375 |
| MVD1 | Candida viswanathii | ATGTATTCAGCTTCAGTCACAGCACCTGTCAACATAGCGGTATGTATGTTCCCACCTAGTTGC<br>TCCTCCCCGACTCATCACTAACACCCTTATAGACCCTCAAGTACTGGGGGAAACGCGACAAG<br>ACCCTAAACTTGCCAACCAACTCCTCCATATCAGTCACCCTCTCCCAGGATGATTTGCGAACC<br>TTAACCACAGCAGCAGCGTCCACCACTTTCGACAAGGACCAATTGTGGCTCAATGGCAAGTT<br>GGAGTCGTTGGACACTCCGAGAACGCAAGCGTGCTTGGTCGATTTAAGGAAATTGAGAGCC<br>GATGTTGAGCAAGCCAACGCTGACCTCCCAAAGCTTTCCACGATGAAGCTCCACATTGTTTC<br>GGAAAACAACTTCCCCACCGCCGCTGGTTTGGCTTCCTCGGCTGCTGGGTTTGCTGCGTTG<br>ATCACAGCCATCGCCAAGTTGTACGAATTGCCCCAAGACATGTCGGAGTTGTCCAAGATTGC<br>TCGTAAAGGTTCTGGTTCTGCGTGCAGATCCCTCTTTGGCGGGTTTGTTGCGTGGGAGATGG<br>GCGACGCCGCCGACGGACAAGACTCCAAGGCAGTTGAAGTTGCGCCTTTGGACCACTGGCC<br>TAGCATGAGGGCGGTTATTTTGGTGGTCAGCGACGACAAAAAGGACACCCCAAGCACCACC | 376 |



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| Table 25 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **Native Cv sequence** | **SEQ ID NO** |
| | | GGTATGCAAGCCACCGTGCAAACCTCCGACTTGTTTGCCCACCGCGTCACCAAAGTCGTCC<br>CCCAGTGTTTCGAAGAGATGAAGAAGGCCATCGTCGCCAAGGACTTTCCTAAATTTACCGAG<br>TTAACCATGAAGGATTCCAACTCGTTCCACGCTGTTTGTTTGGACTCGTACCCTCCAATCTTC<br>TACCTCAACGACACCTCCAAGAAGATCATCAAGTTGGTGGAGGCCATCAACAAGCACGCCAC<br>CATCGCTGCCTACACGTTCGACGCGGGCCCCAACGCCGTCATCTACTACGACGCCGCTAAC<br>GAAGACGAGGTCTTGTCCCAGCTCTACAAGTCGTTTGGCCACGTTCAAGGATGGAAGAAAGC<br>CTACACCGCGGAAACAGCCGTTGCCGGTGTTTCACGTATTATTCAAACATCTATTGGTCAAG<br>GACCGCAAGTTACAAACGAATCATTAATCAACGAAAGTGGGTTACCAAACTAA | |
| IDI1 | Candida viswanathii | ATGTCATCAGAATACGCTAAACTAGTGGCAAGTTTCTCCCCAAACGACATATTGGCTAAATGG<br>CCAGAGGTCACTCCATTGAAGAAGATATCAGGCATCCCAAGATCAGCCGAGTCAGACAGCTC<br>CAACGGCTCCCACAACAACACAGAACTTTTCAATGGCCACGACGAGGAGCAAATCAGATTGA<br>TGGAGGAGCTTTGCATTGTTTTGGACTACGACGACAAGCCTGTTGGGGCAGGAACAAAGAA<br>GTTGTGTCATATCATGGACAATATCAACGAAGGATTGTTGCACCGTGCCTTCTCGGTGTTTTT<br>ATTCAATGAGGACGGGAAATTATTATTACAACAACGTGCCGATGAAAAAATCACCTTCCCTGC<br>CATGTGGACAAATACTTGCTGTTCCCATCCTTTGTGTGTTCCTAGTGAGTTGGGGGTTGATGC<br>CGATGCCAAAGATGTCAACAACTTGGATAACGCCGTGCATGGAGCCAAGGTGGCCGCACAA<br>AGAAAATTGGACCATGAGTTGGGCATCCCATTCAGTGACACTCCCTTGGACCAGTTCACGTA<br>CTTGACTAGAATTCACTATAAATCAGCCAGTGGTGCAGAAGACTCCAAATGGGGTGAGCATG<br>AAATAGACTATATTTTGATATTGAAGACCAAGAATGACATCACCATCAATGCCAACTATAATGA<br>AGTCAAGGATTATAAGTATGTGGACGCCAAGGAGTTGCAGGAGATGTTTGAAGATAAGGACT<br>TGGTGTTCACCCCGTGGTTTAAATTGATCTGTCAATCTTTCTTATTCAAATGGTGGAATAATTT<br>AAGTGATTTAGAAAAGTACCAAGATACTGAGATACATAGATTACTTTAA | 377 |
| ERG20 | Candida viswanathii | ATGTCTGATAAAGCAGCCGCTAGAGAGAGATTCCTCTCTGTTTTTGAGTGTGCCGTCGAGGA<br>ATTGAAAGAAGTCTTGGTTTCTCACAAGATGCCGCAAGAAGCAATTGACTGGTTTGTCAAGAA<br>CTTGAACTACAACACCCCCGGCGGTAAGTTGAACAGAGGTTTGTCTGTTGTCGACACCTACG<br>CTATCTTGAACAACACCACCGCTGACAAGTTGAACGATGAACAATACAAGAAGGTCGCCTTG<br>TTGGGCTGGTCAATTGAATTGTTGCAAGCTTACTTTTTGGTTGCCGATGATATGATGGACCAA<br>TCCAAGACCAGAAGAGGACAGAAATGTTGGTACTTGGTCGAAGGTGTTGGAAACATTGCAAT<br>TAATGACTCCTTCATGTTGGAAGGTGCCATTTACGTCTTGTTGAAGAAGCACTTCCGTCAAGA<br>TCCATACTATGTCGACTTGTTGGACTTGTTCCACGAAGTCACCTTCCAGACCGAATTGGGTCA<br>ATTATTGGACTTGGTGACTGCTGATGAAGAAGTCGTCGACTTGGACAAGTTCTCCTTGGACA<br>AGCACTCGTTCATTGTCATTTTCAAAACCGCATACTACTCCTTCTACTTGCCTGTTGCTTTGGC<br>CATGTACATGAGCGGTATCAGCAGCGAAGAAGACTTGAAGCAAGTCAGAGATATCTTGATCC<br>CATTGGGTGAGTACTTCCAAATCCAGGACGATTTCTTGGACTGTTTCGGAACCCCAGAACAA | 378 |



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| Table 25 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **Native Cv sequence** | **SEQ ID NO** |
| | | ATTGGCAAGATCGGTACTGATATCAAAGACAACAAGTGTTCCTGGGTGGTCAACCAAGCTTT<br>GTTGCATGCTACTCCAGAACAACGTAAGTTGTTGGACGACAACTACGGTAAGAAAGACGACG<br>AGTCTGAACAGAGATGCAAGGACTTGTTCAAGTCCATGGGCATTGAAAAGATCTACCACGAC<br>TACGAAGAGTCAATTGTTGCTAAATTAAGAGAACAAATCGATAAAGTTGATGAATCAAGAGGT<br>TTGAAAAAAGATGTCTTGACCGCTTTCTTGGGCAAGGTTTACAAGAGATCCAAATAG | |
| BTS1 | Candida viswanathii | ATGTCATTCAACATCGATAGCTTAATCCAACCAGGCGCCGCCTACGACCCATCTATGACCGA<br>CGCCATCATGAAGCCATACAGGTACATCTCCGAGGTCCCCAGCAACAACCACAACGTGCGTA<br>CGCGCTTCTTGCTTGCATTCAACGAGTTGTTCTACGGCATGAAGAACGAGGACTTGCTCCAC<br>CGCATCAGCCACATCATCTCCGTGTTCCACAACTCGTCCTTGTTGATCGACGACATCGAGGA<br>CGACTCGCAGTTGCGCCGCGGCATGCCGGTGGCACACGTCAAGTACGGCGTCCCGTTGAC<br>CATCAACTGCGGCAACATGATGTACTTCGTCGCGGTCCAGAAAGCGATTGACTTGGCGGGC<br>GAGGCTGGGTCCGCGGAGTTGAAGTTCGAGACGTCGCAGATCTTGGTTGACGAAATGATGA<br>ATGTTCACCATGGACAAGGCTTGGATATTTACTGGAGAGACTACTTGAAGGACCTCGAGCAC<br>TTGCCTGAGATCGAGGATTACTTGGGCATGGTCAAGGACAAGACTGGGAGCTTGTTCAGATT<br>GGCCATCAAATTGTTGCTGTTGCATTCGGACGTGGGCGAGGACAATGGTTTGGTCGCGATTG<br>CCAACTTGTTGGGCATAATCTACCAAATCAGAGACGACTACTTGAACTTGGTCGATATCAAGT<br>ACTCCGCCATGAAGGGCGTTACTTGTGAAGACTTGATTGAAGGTAAGTTATCCTTGCCTATTT<br>TGCATTGCTTGAGAACCACGACTAATTCCCCGGTTCACGAGATCCTATACAACTACAACACCA<br>GCGCTGAGCGCGCCAAACAGAACGCCTTGATTGAAGAGTGCTTGACTTACATGAAGAACAAG<br>TCGAGATCCTTGCAATATACTTTGGACTTGATCAAGACCTTGGAGCGCAAGATCAAGGCTAT<br>GATGACCAAGTATCCAAACCTGGAGAACTCCGGCTTGATAAAAATCATTGACAGGTTGTGTG<br>ATCTT | 379 |
| CRC1 | Candida viswanathii | ATGGACGACGTTGATTCTGCTTTAGCCGATAATGTTAAATCCTTCGCTGCCGGTGGTTTTGGT<br>GGTATTTGTGCCGTGTTGACCGGTCATCCATTCGACTTGGTCAAGGTCAGATTGCAAACTGG<br>GTTGTACAAATCGTCAGTGCAATGTGTCAAAGAAACAATAGCCAAAGACGGGTTGTTTGGGT<br>TATACAGAGGTGTCTTGCCTCCATTGTTGGGTGTCACCCCAATGTTTGCTGTTTCCTTCTGGG<br>GTTACGACGTTGGTAAGAAATTGGTTTCTTCCTTCACTGGCAAATCAGTCGACAAGTTTGAAA<br>TCAAAGACATTTCGACTGCCGGTTTCATCAGTGCCATCCCAACCACCTTGGTTGCCGCTCCA<br>TTCGAAAGAGTCAAGGTCATGATGCAGATCCAAGAAGGTGCCAAGTCCAAGTCCATGGGTGC<br>CGTTGTTGCTGAAATGTACAGAACCGGTGGTATCAGATCCATCTTCAAGGGTACTGTGGCTA<br>CTTTGGCCAGAGATGGTCCAGGTTCTGCCTTGTATTTCGCTACTTATGAATGGGTCAAGAAG<br>GAATTGACTGCCCCTGGTGAAGACTTGTCCTTGTTTGCTATTACCACTGCTGGTGGTTTCGCT<br>GGTATTGCCATGTGGTTGGGTGTTTTCCCAATTGACACCATCAAGTCTACCCAGCAGTCTTCC<br>AACGTCAAGGTCTCCATTGTGCAGGCCACTAAGAACATCTACGCTAAAGGTGGTATCAAGGC | 380 |



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| Table 25 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **Native Cv sequence** | **SEQ ID NO** |
| | | TTTCTTCCCTGGTGTTGGTCCTGCCTTGGCCAGAGCTTTCCCAGCCAACGCTGCTACCTTCC<br>TAGGGGTTGAATTGGCCAGAAAGTTCTTGGACAAAGTTATATAA | |
| FAA1 | Candida viswanathii | ATGGGTGCCCCTTTAACAGTCGCCGTTGGCGAAGCAAAACCAGGCGAAACCGCTCCAAGAA<br>GAAAAGCCGCTCAAAAAATGGCCTCTGTCGAACGCCCAACAGACTCAAAGGCAACCACTTTG<br>CCAGACTTCATTGAAGAGTGTTTTGCCAGAAACGGCACCAGAGATGCCATGGCCTGGAGAG<br>ACTTGGTCGAAATCCACGTCGAAACCAAACAGGTTACCAAAATCATTGACGGCGAACAGAAA<br>AAGGTCGATAAGGACTGGATCTACTACGAAATGGGTCCTTACAACTACATATCCTACCCCAA<br>GTTGTTGACGTTGGTCAAGAACTACTCCAAGGGTTTGTTGGAGTTGGGCTTGGCCCCAGATC<br>AAGAATCCAAGTTGATGATCTTTGCCAGTACCTCCCACAAGTGGATGCAGACCTTCTTAGCCT<br>CCAGTTTCCAAGGTATCCCCGTTGTCACCGCCTACGACACCTTGGGTGAGTCGGGCTTGAC<br>CCACTCCTTGGTGCAAACCGAATCCGATGCCGTGTTCACCGACAACCAATTGTTGTCCTCCT<br>TGATTCGTCCTTTGGAGAAGGCCACCTCCGTCAAGTATGTCATCCACGGGGAAAAGATTGAC<br>CCTAACGACAAGAGACAGGGCGGCAAAATCTACCAGGATGCGGAAAAGGCCAAGGAGAAGA<br>TTTTACAAATTAGACCAGATATTAAATTTATTTCTTTCGACGAGGTTGTTGCATTGGGTGAACA<br>ATCGTCCAAAGAATTGCATTTCCCAAAACCAGAAGACCCAATCTGTATCATGTACACCTCGGG<br>TTCCACCGGTGCTCCAAAGGGTGTGGTTATCACCAATGCCAACATTGTTGCCGCCGTGGGTG<br>GTATCTCCACCAATGCTACTAGAGACTTGGTTAGAACTGTCGACAGAGTGATTGCATTTTTGC<br>CATTGGCCCACATTTTCGAGTTGGCCTTTGAGTTGGTTACCTTCTGGTGGGGGGCTCCATTG<br>GGTTACGCCAATGTCAAGACTTTGACCGAAGCCTCCTGCAGAAACTGTCAGCCAGACTTGAT<br>TGAATTCAAACCAACCATCATGGTTGGTGTTGCTGCCGTTTGGGAATCGGTCAGAAAGGGTG<br>TCTTGTCTAAATTGAAACAGGCTTCTCCAATCCAACAAAAGATCTTCTGGGCTGCATTCAATG<br>CCAAGTCTACTTTGAACCGTTATGGCTTGCCAGGCGGTGGGTTGTTTGACGCTGTCTTCAAG<br>AAGGTTAAAGCCGCCACTGGTGGCCAATTGCGTTATGTGTTGAATGGTGGGTCCCCAATCTC<br>TGTTGATGCCCAAGTGTTTATCTCCACCTTGCTTGCGCCAATGTTGTTGGGTTACGGTTTGAC<br>TGAAACCTGTGCCAATACCACCATTGTCGAACACACGCGCTTCCAGATTGGTACTTTGGGTA<br>CCTTGGTTGGATCTGTCACTGCCAAGTTGGTTGATGTTGCTGATGCTGGATACTACGCCAAG<br>AACAACCAGGGTGAAATCTGGTTGAAAGGCGGTCCAGTTGTCAAGGAATACTACAAGAACGA<br>AGAAGAAACCAAGGCTGCATTCACCGAAGATGGCTGGTTCAAGACTGGTGATATTGGTGAAT<br>GGACCGCCGACGGTGGTTTGAACATCATTGACCGTAAGAAGAACTTGGTCAAGACTTTGAAT<br>GGTGAATACATTGCTTTGGAGAAATTGGAAAGTATTTACAGATCCAACCACTTGATTTTGAAC<br>TTGTGTGTTTACGCTGACCAAACCAAGGTCAAGCCAATTGCTATTGTCTTGCCAATTGAAGCC<br>AACTTGAAGTCTATGTTGAAGGACGAAAAGATTATCCCAGATGCTGATTCACAAGAATTGAGC<br>AGCTTGGTTCACAACAAGAAGGTTGCCCAAGCTGTCTTGAGACACTTGCTCCAAACCGGTAA<br>ACAACAAGGTTTGAAAGGTATTGAATTGTTGCAGAATGTTGTCTTGTTGGATGACGAGTGGAC | 381 |



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| Table 25 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **Native Cv sequence** | **SEQ ID NO** |
| | | CCCACAGAATGGTTTTGTTACTTCTGCCCAAAAGTTGCAGAGAAAGAAGATTTTAGAAAGTTG TAAAAAAGAAGTTGAAGAGGCATACAAGTCGTCTTAG | |
| FAT1 | Candida viswanathii | ATGTCAGGATTAGAAATAGCCGCTGCTGCCATCCTTGGTAGTCAGTTATTGGAAGCCAAATAT TTAATTGCCGACGACGTGCTGTTAGCCAAGACAGTCGCTGTCAATGCCCTCCCATACTTGTG GAAAGCCAGCAGAGGTAAGGCATCATACTGGTACTTTTTCGAGCAGTCCGTGTTCAAGAACC CAAACAACAAAGCGTTGGCGTTCCCAAGACCAAGAAAGAATGCCCCCACCCCCAAGACCGA CGCCGAGGGATTCCAGATCTACGACGATCAGTTTGACCTAGAAGAATACACCTACAAGGAAT TGTACGACATGGTTTTGAAGTACTCATACATCTTGAAGAACGAGTACGGCGTCACTGCCAAC GACACCATCGGTGTTTCTTGTATGAACAAGCCGCTTTTCATTGTCTTGTGGTTGGCATTGTGG AACATTGGTGCCTTGCCTGCGTTCTTGAACTTCAACACCAAGGACAAGCCATTGATCCACTGT CTTAAGATTGTCAACGCTTCGCAAGTTTTCGTTGACCCGGACTGTGATTCCCCAATCAGAGAT ACCGAGGCTCAGATCAGAGAGGAATTGCCACATGTGCAAATAAACTACATTGACGAGTTTGC CTTGTTTGACAGATTGAGACTCAAGTCGACTCCAAAACACAGAGCCGAGGACAAGACCAGAA GACCAACCGATACTGACTCCTCCGCTTGTGCATTGATTTACACCTCGGGTACCACCGGTTTG CCAAAAGCCGGTATCATGTCCTGGAGAAAAGCCTTCATGGCCTCGGTTTTCTTTGGCCACAT CATGAAGATTGACTCGAAATCGAACGTCTTGACCGCCATGCCCTTGTACCACTCCACCGCGG CCATGTTGGGGTTGTGTCCTACTTTGATTGTCGGTGGCTGTGTCTCCGTGTCCCAGAAATTC TCCGCTACTTCGTTCTGGACCCAGGCCAGATTATGTGGTGCCACCCACGTGCAATACGTCGG TGAGGTCTGTCGTTACTTGTTGAACTCCAAGCCTCATCCAGACCAAGACAGACACAATGTCA GAATTGCCTACGGTAACGGGTTGCGTCCAGATATATGGTCTGAGTTCAAGCGCAGATTCCAC ATTGAAGGTATCGGTGAGTTCTACGCCGCCACCGAGTCCCCTATCGCCACCACCAACTTGCA GTACGGTGAGTACGGTGTCGGCGCCTGTCGTAAGTACGGGTCCCTCATCAGCTTGTTATTGT CTACCCAGCAGAAATTGGCCAAGATGGACCCAGAAGACGAGAGTGAAATCTACAAGGACCC CAAGACCGGGTTCTGTACCGAGGCCGCTTACAACGAGCCAGGTGAGTTGTTGATGAGAATC TTGAACCCTAACGACGTGCAGAAATCCTTCCAGGGTTATTATGGTAACAAGTCCGCCACCAA CAGCAAAATCCTCACCAATGTTTTCAAAAAAGGTGACGCGTGGTACAGATCCGGTGACTTGT TGAAGATGGACGAGGACAAATTGTTGTACTTTGTCGACAGATTAGGTGACACTTTCCGTTGG AAGTCCGAAAACGTCTCCGCCACCGAGGTCGAGAACGAATTGATGGGCTCCAAGGCCTTGA AGCAGTCCGTCGTTGTCGGTGTCAAGGTGCCAAACCACGAAGGTAGAGCCTGTTTTGCCGT CTGTGAAGCCAAGGACGAGTTGAGCCATGAAGAAATCTTGAAATTGATTCACTCTCACGTGA CCAAGTCTTTGCCTGTGTATGCTCAACCTGCGTTCATCAAGATTGGCACCATTGAGGCTTCG CACAACCACAAGGTTCCTAAGAACCAATTCAAGAACCAAAAGTTGCCAAAGGGTGAAGACGG CAAGGATTTGATCTACTGGTTGAATGGCGACAAGTACCAGGAGTTGACTGAAGACGATTGGT CTTTGATTTGTACCGGTAAAGCCAAATTGTAG | 382 |



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| Table 25 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **Native Cv sequence** | **SEQ ID NO** |
| tHMG1 | Candida viswanathii | ATGGACCAAGAAATGTCTCGTCCAAAACAAGCCGCTGCTGCTGCTGTTGCTCCATCTGCCGC<br>AAAATCAGTGGCGCCAGAAGAAGACGAAGACGAAACCTCCAGCGAGGAACTTGAAATCAAA<br>GCTCCTGTCAAGCCATTACCACTTGAAGAATGTACCAGAATCCTCAAAGAGGGCAAAGTCAA<br>GACCTTGTCCAATGCTGAAGTGTCTAGTTTGGTTGTTGGTGGTAAATTGCCATTGTACGCTTT<br>GGAGAAGCAATTGGGCGACCACAAGAGAGCCGTGGCTGTTCGTCGTAAGGCTATTGCAAAA<br>TTAGCTAATGCGCCAGTATTAGAAACCAACCGTTTACCTTACTCCCACTATGATTATGATAGA<br>GTTTTTGGTGCTTGTTGTGAGAATGTTATTGGATACATGCCAATCCCTGTTGGTGTTGCTGGA<br>CCATTAGTTATTGATGGCAAGCCATACCACATCCCCATGGCCACTACTGAAGGTTGTCTTGTT<br>GCCTCTACTATGCGTGGTTGTAAAGCTATTAACGCTGGAGGTGGTGTTGAAACTGTCTTGAC<br>CAAAGATGGTATGACTAGAGGTCCTTGTGTTCGTTTCCCAACTTTGAAAAGAGCCGGCGCTG<br>CAAAGTTGTGGATTGACTCCGAAGAAGGTCAAATCACCATCAAAAAGGCATTCAACTCGACTT<br>CTAGATTTGCTCGTTTGCAACATATTCAAACTGCCCTCGCTGGTACCTCGTTGTTTATTAGATT<br>TAGAACTACTACTGGTGATGCCATGGGTATGAACATGATTTCCAAAGGTGTTGAGTATTCCTT<br>GAAGTACATGGTTGAAGAATGTGGCTGGGACGATATGGAAGTTATCTCTGTTTCTGGTAACTA<br>CTGTACCGATAAGAAACCAGCTGCTATTAACTGGATCGAAGGTAGAGGTAAGTCTATTGTTG<br>CCGCTGCTACCATTCCTGCTGATGTTGTCACTAAAGTCTTGAAATCCGATGTCGATGCATTAG<br>TTGAATTGAATGTTTCTAAGAACTTGGTTGGTTCAGCCATGGCCGGTTCAGTTGGTGGTTTCA<br>ACGCCCATGCAGCCAACTTGGTCACAGCTGTTTATTTGGCCTGTGGTCAGGACCCTGCACAA<br>AACGTCGAATCTTCAAACTGTATTACTTTGATGGAAAAGGACAAACAGACTGGTGACTTGGTG<br>ATTTCTGTTTCTATGCCATCTATCGAAGTTGGTACGATTGGTGGTGGTACCATTTTGGAACCA<br>CAAGCAGCCATGTTGGACTTGTTGGGCGTGCGTGGTCCTCACCCAACCAACCCTGGTGACA<br>ATGCAAGACAATTGGCCAAGATTGTTGCTTCGGCAGTGTTAGCAGCTGAATTGTCACTTTGTT<br>CCGCTTTGGCTGCTGGTCACTTGGTGCAATCCCACATGCAACACAACCGTAAAGGAGCTACC<br>GCCGCCGCCGCCGCCGCAGCAGCCCCTGCAATCGCCAACGGCCGTGCCAATGGAACCAAA<br>ACCAATGGGTCTATCAATGGGAAAGACTTGAAGCGTCTCAAAGACGGATCAGTTACATGTAT<br>AAAGTCA | 383 |
| POX4 | Candida viswanathii | ATGACTTTTACAAAGAAAAACGTTAGTGTATCACAAGGTCCTGACCCTAGATCATCCATCCAA<br>AAGGAAAGAGACAGCTCCAAATGGAACCCTCAACAAATGAACTACTTCTTGGAAGGCTCCGT<br>CGAAAGAAGTGAGTTGATGAAGGCTTTGGCCCAACAAATGGAAAGAGACCCAATCTTGTTCA<br>CAGACGGCTCCTACTACGACTTGACCAAGGACCAACAAAGAGAATTGACCGCCGTCAAGATC<br>AACAGAATCGCCAGATACAGAGAACAAGAATCCATCGACACTTTCAACAAGAGATTGTCCTTG<br>ATTGGTATCTTTGACCCACAGGTCGGTACCAGAATTGGTGTCAACCTCGGTTTGTTCCTTTCT<br>TGTATCAGAGGTAACGGTACCACTTCCCAATTGAACTACTGGGCTAACGAAAAGGAAACCGC<br>TGACGTTAAAGGTATCTACGGTTGTTTCGGTATGACCGAATTGGCCCACGGTTCCAACGTTG | 384 |



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| Table 25 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **Native Cv sequence** | **SEQ ID NO** |
| | | CTGGTTTGGAAACCACCGCCACATTTGACAAGGAATCTGACGAGTTTGTCATCAACACCCCA<br>CACATTGGTGCCACCAAGTGGTGGATTGGTGGTGCTGCTCACTCCGCCACCCACTGTTCTGT<br>CTACGCCAGATTGATTGTTGACGGTCAAGATTACGGTGTCAAGACTTTTGTTGTCCCATTGAG<br>AGACTCCAACCACGACCTCATGCCAGGTGTCACTGTTGGTGACATTGGTGCCAAGATGGGTA<br>GAGATGGTATCGATAACGGTTGGATCCAATTCTCCAACGTCAGAATCCCAAGATTCTTTATGT<br>TGCAAAAGTTCTGTAAGGTTTCTGCTGAAGGTGAAGTCACCTTGCCACCTTTGGAACAATTGT<br>CTTACTCCGCCTTGTTGGGTGGTAGAGTCATGATGGTTTTGGACTCCTACAGAATGTTGGCT<br>AGAATGTCCACCATTGCCTTGAGATACGCCATTGGTAGAAGACAATTCAAGGGTGACAATGT<br>CGATCCAAAAGATCCAAACGCTTTGGAAACCCAATTGATAGATTACCCATTGCACCAAAAGAG<br>ATTGTTCCCATACTTGGCTGCTGCCTACGTCATCTCCGCTGGTGCCCTCAAGGTTGAAGACA<br>CCATCCATAACACCTTGGCTGAATTGGACGCTGCCGTTGAAAAGAACGACACCAAGGCTATC<br>TTTAAGTCTATTGACGACATGAAGTCATTGTTTGTTGACTCTGGTTCCTTGAAGTCCACTGCC<br>ACTTGGTTGGGTGCTGAAGCCATTGACCAATGTAGACAAGCCTGTGGTGGTCACGGTTACTC<br>GTCCTACAACGGCTTCGGTAAAGCCTACAACGATTGGGTTGTCCAATGTACTTGGGAAGGTG<br>ACAACAATGTCTTGGCCATGAGTGTTGGTAAGCCAATTGTCAAGCAAGTTATCAGCATTGAAG<br>ATGCCGGCAAGACCGTCAGAGGTTCCACCGCTTTCTTGAACCAATTGAAGGACTACACTGGT<br>TCCAACAGCTCCAAGGTTGTTTTGAACACTGTTGCTGACTTGGACGACATCAAGACTGTCATC<br>AAGGCTATTGAAGTTGCCATCATCAGATTGTCCCAAGAAGCTGCTTCTATTGTCAAGAAGGAA<br>TCTTTCGACTATGTCGGCGCTGAATTGGTTCAACTCTCCAAGTTGAAGGCTCACCACTACTTG<br>TTGACTGAATACATCAGAAGAATTGACACCTTTGACCAAAAGGACTTGGTTCCATACTTGATC<br>ACCCTCGGTAAGTTGTACGCTGCCACTATTGTCTTGGACAGATTTGCCGGTGTCTTCTTGACT<br>TTCAACGTTGCCTCCACCGAAGCCATCACTGCTTTGGCCTCTGTGCAAATTCCAAAGTTGTGT<br>GCTGAAGTCAGACCAAACGTTGTTGCTTACACCGACTCCTTCCAACAATCCGACATGATTGTC<br>AATTCTGCTATTGGTAGATACGATGGTGACATCTATGAGAACTACTTTGACTTGGTCAAGTTG<br>CAGAACCCACCATCCAAGACCAAGGCTCCTTACTCTGATGCTTTGGAAGCCATGTTGAACAG<br>ACCAACCTTGGACGAAAGAGAAAGATTTGAAAAGTCTGATGAAACCGCTGCTATCTTGTCCAA<br>GTAA | |
| POX5 | Candida viswanathii | ATGCCTACCGAACTTCAAAAAGAAAGAGAACTCACCAAGTTCAACCCAAAGGAGTTGAACTA<br>CTTCTTGGAAGGTTCCCAAGAAAGATCCGAGATCATCAGCAACATGGTCGAACAAATGCAAA<br>AAGACCCTATCTTGAAGGTCGACGCTTCATACTACAACTTGACCAAAGACCAACAAAGAGAA<br>GTCACCGCCAAGAAGATTGCCAGACTCTCCAGATACTTTGAGCACGAGTACCCAGACCAACA<br>GGCCCAGAGATTGTCGATCCTCGGTGTCTTTGACCCACAAGTCTTCACCAGAATCGGTGTCA<br>ACTTGGGTTTGTTTGTTTCCTGTGTCCGTGGTAACGGTACCAACTCCCAGTTCTTCTACTGGA<br>CCATAAATAAGGGTATCGACAAGTTGAGAGGTATCTATGGTTGTTTTGGTATGACTGAGTTGG | 385 |



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| Table 25 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **Native Cv sequence** | **SEQ ID NO** |
| | | CCCACGGTTCCAACGTCCAAGGTATTGAAACCACCGCCACTTTTGACGAAGACACTGACGAG<br>TTTGTCATCAACACCCCACACATTGGTGCCACCAAGTGGTGGATCGGTGGTGCTGCGCACTC<br>CGCCACCCACTGCTCCGTCTACGCCAGATTGAAGGTCAAAGGAAAGGACTACGGTGTCAAG<br>ACCTTTGTTGTCCCATTGAGAGACTCCAACCACGACCTCGAGCCAGGTGTGACTGTTGGTGA<br>CATTGGTGCCAAGATGGGTAGAGACGGTATCGATAACGGTTGGATCCAGTTCTCCAACGTCA<br>GAATCCCAAGATTCTTTATGTTGCAAAAGTACTGTAAGGTTTCCCGTCTGGGTGAAGTCACCA<br>TGCCACCATCTGAACAATTGTCTTACTCGGCTTTGATTGGTGGTAGAGTCACCATGATGATGG<br>ACTCCTACAGAATGACCAGTAGATTCATCACCATTGCCTTGAGATACGCCATCCACAGAAGA<br>CAATTCAAGAAGAAGGACACCGATACCATTGAAACCAAGTTGATTGACTACCCATTGCATCAA<br>AAGAGATTGTTCCCATTCTTGGCTGCCGCTTACTTGTTCTCCCAAGGTGCCTTGTACTTAGAA<br>CAAACCATGAACGCAACCAACGACAAGTTGGACGAAGCTGTCAGTGCTGGTGAAAAGGAAG<br>CCATTGACGCTGCCATTGTCGAATCCAAGAAATTGTTCGTCGCTTCCGGTTGTTTGAAGTCCA<br>CCTGTACCTGGTTGACTGCTGAAGCCATTGACGAAGCTCGTCAAGCTTGTGGTGGTCACGGT<br>TACTCGTCTTACAACGGTTTCGGTAAAGCCTACTCCGACTGGGTTGTCCAATGTACCTGGGA<br>AGGTGACAACAACATCTTGGCCATGAACGTTGCCAAGCCAATGGTTAGAGACTTGTTGAAGG<br>AGCCAGAACAAAAGGGATTGGTTCTCTCCAGCGTTGCCGACTTGGACGACCCAGCCAAGTT<br>GGTTAAGGCTTTCGACCACGCCCTTTCCGGCTTGGCCAGAGACATTGGTGCTGTTGCTGAAG<br>ACAAGGGTTTCGACATTACCGGTCCAAGTTTGGTTTTGGTTTCCAAGTTGAACGCTCACAGAT<br>TCTTGATTGACGGTTTCTTCAAGCGTATCACCCCAGAATGGTCTGAAGTCTTGAGACCTTTGG<br>GTTTCTTGTATGCCGACTGGATCTTGACCAACTTTGGTGCCACCTTCTTGCAGTACGGTATCA<br>TTACCCCAGATGTCAGCAGAAAGATTTCCTCCGAGCACTTCCCAGCCTTGTGTGCCAAGGTT<br>AGACCAAACGTTGTTGGTTTGACTGATGGTTTCAACTTGACTGACATGATGACCAATGCTGCT<br>ATTGGTAGATATGATGGTAACGTCTACGAACACTACTTCGAAACTGTCAAGGCTTTGAACCCA<br>CCAGAAAACACCAAGGCTCCATACTCCAAGGCTTTGGAAGACATGTTGAACCGTCCAGACCT<br>TGAAGTCAGAGAAAGAGGTGAAAAGTCCGAAGAAGCTGCTGAAATCTTGTCCAGTTAA | |
| URA3 | Candida viswanathii | ATGGTTAGCACAAAAACATACACAGAAAGGGCATCAGCACACCCCTCCAAGGTTGCCCAACG<br>TTTATTCCGCTTAATGGAGTCCAAAAAGACCAACCTCTGCGCCTCGATCGACGTGACCACAA<br>CCGCCGAGTTCCTTTCGCTCATCGACAAGCTCGGTCCCCACATCTGTCTCGTGAAGACGCAC<br>ATCGATATCATCTCAGACTTCAGCTACGAGGGCACGATTGAGCCGTTGCTTGTGCTTGCAGA<br>GCGCCACGGGTTCTTGATATTCGAGGACAGGAAGTTTGCTGATATCGGAAACACCGTGATGT<br>TGCAGTACACCTCGGGGGTATACCGGATCGCGGCGTGGAGTGACATCACGAACGCGCACG<br>GAGTGACTGGGAAGGGCGTCGTTGAAGGGTTGAAACGCGGTGCGGAGGGGGTAGAAAAGG<br>AAAGGGGCGTGTTGATGTTGGCGGAGTTGTCGAGTAAAGGCTCGTTGGCGCATGGTGAATA<br>TACCCGTGAGACGATCGAGATTGCGAAGAGTGATCGGGAGTTCGTGATTGGGTTCATCGCG | 386 |



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| Table 25 | | | |
|---|---|---|---|
| **Gene** | **Organism** | **Native Cv sequence** | **SEQ ID NO** |
| | | CAGCGGGACATGGGGGGTAGAGAAGAAGGGTTTGATTGGATCATCATGACGCCTGGTGTGG<br>GGTTGGATGATAAAGGCGATGCGTTGGGCCAGCAGTATAGGACTGTTGATGAGGTGGTTCT<br>GACTGGTACCGATGTGATTATTGTCGGGAGAGGGTTGTTTGGAAAAGGAAGAGACCCTGAG<br>GTGGAGGGAAAGAGATACAGGGATGCTGGATGGAAGGCATACTTGAAGAGAACTGGTCAGT<br>TAGAATAA | |
| LEU2 | Candida viswanathii | ATGTCCGTTAAAACCAAAACCATCACTATTCTCCCAGGTGACCACGTCGGTACCGAGATCGT<br>TGCCGAGGCAATCAAGTCCTTACATGCCATTGAGTCCCTGACCCCATACCAAAAAGTTCATTT<br>TGAGTTCAAGCACCACTTGATAGGCGGTGCCGCCATTGACGCCACTGGTGTGCCGCTCCCA<br>GACGACGCCTTAGCCGCCGCCAAGTCCTCAGACGCCGTGTTGCTCGGGGCCGTCGGTGGA<br>CCCAAATGGGGAACCGGCGCTGTGCGTCCAGAACAGGGATTGTTGAAGATCCGTAAGGAGT<br>TGAACCTTTATGCCAACATCAGACCATGCAATTTCGCTAGCGACTCCTTGCTTGAGCTCTCTC<br>CGTTACGTCCTGAGGTCGTCAAGGGCACCAACTTGATCATTGTGCGTGAGCTAGTTGGTGG<br>GATTTACTTTGGTGAGAGACAGGAGCAGGAAGAAAGCGAGGATGGGAAATCGGCTTGGGAT<br>ACTGAAAAGTACACCGTTGATGAAGTCGCGAGAATTACACGCATGGCTGCGTTCATGGCATT<br>GCAACACACCCCACCATTGCCAATCTGGTCCTTGGACAAAGCCAACGTGCTTGCCTCGTCAA<br>GATTGTGGAGAAAGACAGTGGATAAAATCATCAGTGAAGAGTTCCCTACCTTGGCTGTCCAG<br>CACCAGTTGATTGACTCCGCGGCTATGATCTTGATCCAGAACCCAACGAAGTTGAACGGGAT<br>CATCATCACGTCGAACATGTTTGGTGATATCATATCCGATGAAGCGTCCGTGATCCCAGGGT<br>CCTTGGGATTGTTGCCATCTGCGTCATTGGCTTCGTTACCGGACACAAACACTGCGTTTGGT<br>CTCTATGAGCCATGCCATGGTTCGGCACCTGATTTGCCAGAGAACAAAGTCAACCCTATTGC<br>GACAATCTTGTCCGTTGCTAGTATGTTGAGATTGAGTTTGGATTGTGTTAAGGAAGCCGAGG<br>CTTTGGAACAGGCCGTGAAGGAAGTCTTGGATAAGGGGATCAGAACGGCGGATTTGAGAGG<br>TAGTAGCACAACCACTGAAGTCGGTGATGCCGTTGCTGAAACCGTCTCCAGAATCTTGAAAG<br>AAGCTAAGGCTTGA | 387 |



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*EXAMPLE 71: Plasmids*

Certain plasmids described herein are further characterized in Table 26 below.

**Table 26**

| Plasmid No. | Description |
|---|---|
| pAA1116 | POX4 promoter + POX5F98G + POX4 terminator in AFC |
| pAA1164 | HDE1 promoter + POX5F98G + POX4 terminator in AFC |
| pAA1701 | CRC1 knockout cassette 1 |
| pAA2214 | G6PI promoter + CRC1 + terminator POX4 @ int5 Integration site |
| pAA2311 | G6PI promoter + CRC1 + terminator POX4 in AFC |
| pAA2534 | GPD promoter + Caenorhabditis elegans FAT5 CvCO + POX4 terminator in AFC |
| pAA2698 | GPD promoter + CsCrtE CvCO + POX4 terminator in AFC |
| pAA2699 | GPD promoter + CsCrtB CvCO + POX4 terminator in AFC |
| pAA2700 | GPD promoter + CsCrtI CvCO + POX4 terminator in AFC |
| pAA2701 | GPD promoter + CnTPS1 CvCO + POX4 terminator in AFC |
| pAA2702 | HDE1 promoter + CsCrtE CvCO + POX4 terminator in AFC |
| pAA2703 | HDE1 promoter + CsCrtB CvCO + POX4 terminator in AFC |
| pAA2704 | HDE1 promoter + CsCrtI CvCO + POX4 terminator in AFC |
| pAA2705 | HDE1 promoter + CnTPS1 CvCO + POX4 terminator in AFC |
| pAA2866 | GPD promoter + XdCrtYB CvCO + POX4 terminator in AFC |
| pAA2993 | GPD promoter + XdCrtR CvCO + POX4 terminator in AFC |
| pAA2994 | GPD promoter + XdCrtS CvCO + |

| Table 26 | |
|---|---|
| **Plasmid No.** | **Description** |
| | POX4 terminator in AFC |
| pAA2995 | GPD promoter + AaCrtW CvCO + POX4 terminator in AFC |
| pAA2996 | GPD promoter + AaCrtZ CvCO + POX4 terminator in AFC |
| pAA2998 | Plasmid from Invitrogen containing XdCrtR CvCO |
| pAA2999 | Plasmid from Invitrogen containing XdCrtS CvCO |
| pAA3000 | Plasmid from Invitrogen containing AaCrtW CvCO |
| pAA3001 | Plasmid from Invitrogen containing AaCrtZ CvCO |
| pAA3373 | HDE1 promoter + XdCrtYB CvCO + POX4 terminator in AFC |
| pAA3189 | GPD promoter + XdCrtE + POX4 terminator in AFC |
| pAA3190 | GPD promoter + PaCrtE + POX4 terminator in AFC |
| pAA3378 | GPD promoter + PaCrtB + POX4 terminator in AFC |
| pAA3490 | GPD promoter + XdCrtI + POX4 terminator in AFC |
| pAA3633 | HDE promoter + XdCrtE CvCO + POX4 terminator in AFC |
| pAA3634 | HDE promoter + XdCrtI CvCO + POX4 terminator in AFC |
| pAA408 | Contains URA3 terminator + URA3 promoter + URA3 + URA3 terminator |
| pAA3060 | LEU2 knock-out cassette 1 |
| pAA2417 | LEU2 knock-out cassette 2 |
| pVZ3930 | RAS2 knock-out cassette |
| pVZ4045 | LEU2 single crossover AFC |
| pVZ4056 | HDE promoter + XdCrtE CvCO + POX4 terminator in AFC, leucine |
| pVZ4057 | HDE promoter + CsCrtB CvCO + POX4 terminator in AFC, leucine |

| Table 26 | |
|---|---|
| **Plasmid No.** | **Description** |
| pVZ4058 | HDE promoter + XdCrtI CvCO + POX4 terminator in AFC, leucine |
| pVZ4083 | Ppox18 -Tpox18 in AFC, leucine |
| pVZ4098 | POX18 promoter + Erg10 + POX18 terminator in AFC, leucine |
| pVZ4099 | POX18 promoter + Erg13 + POX18 terminator in AFC, leucine |
| pVZ4100 | POX18 promoter + HMG1 + POX18 terminator in AFC, leucine |
| pVZ4101 | POX18 promoter + Erg12 + POX18 terminator in AFC, leucine |
| pVZ4122 | POX18 promoter + Erg8 + POX18 terminator in AFC, leucine |
| pVZ4123 | POX18 promoter + Mvd1 + POX18 terminator in AFC, leucine |
| pVZ4104 | POX18 promoter + Idi1 + POX18 terminator in AFC, leucine |
| pVZ4105 | POX18 promoter + Erg20 + POX18 terminator in AFC, leucine |

AFC = antibiotic free cassette, uracil marker unless indicated otherwise
CvCO = Codon optimized for Candida viswanathii

*EXAMPLE 72: Oligonucleotides*

Certain oligonucleotides described herein are provided in Table 27 below.

| Table 27 | | |
|---|---|---|
| **Oligo No.** | **Sequence** | **SEQ ID NO** |
| oAA02206 | TTCCGCTTAATGGAGTCCAAA | 388 |
| oAA2091 | GCATACAACGGAAATTTGCTTT | 389 |
| oAA02209 | TAAACGTTGGGCAACCTTGG | 390 |



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| Table 27 | | |
|---|---|---|
| **Oligo No.** | **Sequence** | **SEQ ID NO** |
| oAA5511 | ATGGACGACGTTGATTCTGCTTTAGCCGATAATGTT | 391 |
| oAA5512 | TTATATAACTTTGTCCAAGAACTTTCTGGCC | 392 |
| oAA05788 | GAATAGAAGAGAGTGACTCTTTTG | 393 |
| oAA05789 | GATTGATTGTTATAGTTTCTTTCTTTC | 394 |
| oAA07265 | CTGCAGGCATGATGATCTG | 395 |
| oAA07511 | ATCGATTAAATTCTTTAATTGAGGG | 396 |
| oAA07512 | GAATAGAAGAGAGTGACTCTTTTG | 397 |
| oAA07624 | AGATCATCATGCCTGCAGAAAATCAGAGGCTACTCC | 398 |
| oAA07625 | GTCACTCTCTTCTATTCTTATATAACTTTGTCCAAGAACTTTC | 399 |
| oAA09745 | CCCTCAATTAAAGAATTTAATCGATATGAATGCTAATGCCGTTAAGTC | 400 |
| oAA09746 | TCAAAAGAGTCACTCTCTTCTATTCTTACCCAAACATTGCAAGCTGT | 401 |
| oAA09747 | CCCTCAATTAAAGAATTTAATCGATATGACCAAAACGGTTGTAATAGGATCAGG | 402 |
| oAA09748 | TCAAAAGAGTCACTCTCTTCTATTCTTACGCGTGGCCCTCCAACATT | 403 |
| oAA09749 | CCCTCAATTAAAGAATTTAATCGATATGAGTGACAAGCCTTTGTTAACACATGCTACAGA | 404 |
| oAA09750 | TCAAAAGAGTCACTCTCTTCTATTCTTAAGCGGCAGGAGGGGCGGCA | 405 |
| oAA09751 | CCCTCAATTAAAGAATTTAATCGATATGGCAGAAATGTTCAATGGAAAC | 406 |
| oAA09752 | TCAAAAGAGTCACTCTCTTCTATTCTTAAGGTATAATCGGTTCGACG | 407 |
| oAA09753 | AAGAAAGAAACTATAACAATCAATCATGAATGCTAATGCCGTTAAGTC | 408 |
| oAA09754 | TCAAAAGAGTCACTCTCTTCTATTCTTACCCAAACATTGCAAGCTGT | 409 |
| oAA09755 | AAGAAAGAAACTATAACAATCAATCATGACCAAAACGGTTGTAATAGGATCAGG | 410 |
| oAA09756 | TCAAAAGAGTCACTCTCTTCTATTCTTACGCGTGGCCCTCCAACATT | 411 |
| oAA09757 | AAGAAAGAAACTATAACAATCAATCATGAGTGACAAGCCTTTGTTAACACATGCTACAGA | 412 |
| oAA09758 | TCAAAAGAGTCACTCTCTTCTATTCTTAAGCGGCAGGAGGGGCGGCA | 413 |
| oAA09759 | AAGAAAGAAACTATAACAATCAATCATGGCAGAAATGTTCAATGGAAAC | 414 |
| oAA09760 | TCAAAAGAGTCACTCTCTTCTATTCTTAAGGTATAATCGGTTCGACG | 415 |
| oAA9960 | CCCTCAATTAAAGAATTTAATCGATATGGCAACCCTATCTGACTTGG | 416 |

| Table 27 | | |
|---|---|---|
| **Oligo No.** | **Sequence** | **SEQ ID NO** |
| oAA9961 | TCAAAAGAGTCACTCTCTTCTATTCTTAGCTCCACACATCCATCAATAATC | 417 |
| oAA9962 | CCCTCAATTAAAGAATTTAATCGATATGTTCATTTTGGTGTTGTTGACC | 418 |
| oAA9963 | TCAAAAGAGTCACTCTCTTCTATTCTTACTCCACTGGCTTTACCTGC | 419 |
| oAA9964 | CCCTCAATTAAAGAATTTAATCGATATGAGTGCCCATGCCTTGCCAA | 420 |
| oAA9965 | TCAAAAGAGTCACTCTCTTCTATTCTTAAGCGGTATCACCTTTAGTTCTAGTGC | 421 |
| oAA9966 | CCCTCAATTAAAGAATTTAATCGATATGACAAACTTCTTGATCGTTGTGG | 422 |
| oAA9967 | TCAAAAGAGTCACTCTCTTCTATTCTTACGTCCTCTCTTGGGCCTCA | 423 |
| oAA9968 | GATCCACAAGTTTTCACTAGAATTGG | 424 |
| oAA10284 | CACTGCTTAATTAAGCGCTGTCACTCTTATATCAGTATGACC | 425 |
| oAA10286 | CACTGCGGATCCGGTACCCCACCACGACAACAGGATTCCCC | 426 |
| oAA10287 | CGAGATTTAATTAATTAGCCAACAGGACAATAGAGTTAAAAAGAATGAATTG | 427 |
| oVZ0041 | CGAGATCTGCAGGCGGCCGCTTCTGTGAGTATTTGTTATTGAAAATGAAGGGATGG | 428 |
| oAA7682 | CCACGTCGGTACCGAGATCGTTGCCGAGGCAATCAAGTCCTT | 429 |
| oAA7683 | AACGGCTTCGTCTAAACAACCACGGATCTTCAACAATCCCTGTTCTGGAC | 430 |
| oAA7684 | GTCCAGAACAGGGATTGTTGAAGATCCGTGGTTGTTTAGACGAAGCCGTT | 431 |
| oAA7685 | AGTGTTTGTGTCCGGTAACGACCGAAATATTACAATTGGAGCTCC | 432 |
| oAA7686 | GGAGCTCCAATTGTAATATTTCGGTCGTTACCGGACACAAACACT | 433 |
| oAA7687 | TTTCAGCAACGGCATCACC | 434 |
| oAA7941 | ACCTTTATGCCAACATCAGACC | 435 |
| oAA7942 | AACGGCTTCGTCTAAACAACCCATCAACGGTGTACTTTTCAGTATCC | 436 |
| oAA7943 | GGATACTGAAAAGTACACCGTTGATGGGTTGTTTAGACGAAGCCGTT | 437 |
| oAA7944 | TTGCAATGCCATGAACGCCCGAAATATTACAATTGGAGCTCC | 438 |
| oAA7945 | GGAGCTCCAATTGTAATATTTCGGGCGTTCATGGCATTGCAA | 439 |
| oAA7946 | CAGATGGCAACAATCCCAAG | 440 |
| oVZ337 | AATTAACCTATGGTGCAC | 441 |
| oVZ338 | TTAATTAAAAGCTTGGCGTAATC | 442 |

| Table 27 | | |
|---|---|---|
| **Oligo No.** | **Sequence** | **SEQ ID NO** |
| oVZ339 | CTCGTGCTAGTCAGTCTTGCACGCTTTGGGTG | 443 |
| oVZ340 | ATGATTACGCCAAGCTTTTAATTAACAACACGGCGTCTGAGGAC | 444 |
| oVZ341 | ACTGAGAGTGCACCATAGGTTAATTAACTCGGGGCCGTCGGTGGA | 445 |
| oVZ342 | AAGCGTGCAAGACTGACTAGCACGAGCGAAGATGGGG | 446 |
| oVZ373 | CTC GGG GCC GTC GGT GGA | 447 |
| oVZ374 | CAACACGGCGTCTGAGGACTTGG | 448 |
| oVZ369 | GCTCAACAATTGTCTGACAAGATCTC | 449 |
| oVZ370 | GAGATTGTTAACTTTATGGGGCAATAAC | 450 |
| oVZ371 | TCCCCATCTTCGCTCGTGCTAGTCAAAGGGAAGAAGAGTCGTTG | 451 |
| oVZ372 | CGTCGGCACCCAAAGCGTGCAAGACGTCGACCTAAATTCGCAAC | 452 |
| oVZ376 | GTGCTAGTCAAGATAAACCGAGGCATGGAAG | 453 |
| oVZ377 | CGTGCAAGACGCATAGACTGCAAGTAAGCAGC | 454 |
| oVZ378 | CAGTCTATGCGTCTTGCACGCTTTGGGTGC | 455 |
| oVZ379 | CGGTTTATCTTGACTAGCACGAGCGAAGATGG | 456 |
| oVZ384 | AAAGGAATAAGCTGGGTGTTTGCTCATTTTG | 457 |
| oVZ385 | CGTTAGTCATTTTTGTTAATTGGTGTTTTGTTTAATAATGGGG | 458 |
| oVZ386 | AATTAACAAAAATGACTAACGCACCACAAAACATTG | 459 |
| oVZ387 | AACACCCAGCTTATTCCTTTATAGCATAACTTCTTCTAAACTTGTCG | 460 |
| oVZ388 | AAAGTCATAGGCTGGGTGTTTGCTCATTTTG | 461 |
| oVZ389 | AACTTAGCATTTTTGTTAATTGGTGTTTTGTTTAATAATGGGG | 462 |
| oVZ390 | AATTAACAAAAATGCTAAGTTTCATCACCGAAGC | 463 |
| oVZ391 | AACACCCAGCCTATGACTTTATACATGTAACGGATCCGTC | 464 |
| oVZ392 | GTTCTGGTGAGCTGGGTGTTTGCTCATTTTG | 465 |
| oVZ393 | AGACTGACATTTTTGTTAATTGGTGTTTTGTTTAATAATGGGG | 466 |
| oVZ394 | AATTAACAAAAATGTCAGTCTCGCCTTTCGTTG | 467 |
| oVZ395 | AACACCCAGCTCACCAGAACTTCCATTCTTTAACGTTTATC | 468 |
| oVZ396 | CGGTTTATAGGCTGGGTGTTTGCTCATTTTG | 469 |
| oVZ399 | AACACCCAGCCTATAAACCGATATAGTCCTCCCAGTTTTC | 470 |
| oVZ401 | CTGAATACATTTTTGTTAATTGGTGTTTTGTTTAATAATGGGG | 471 |
| oVZ402 | AATTAACAAAAATGTATTCAGCTTCAGTCACAGC | 472 |
| oVZ404 | GATTACTTTAAGCTGGGTGTTTGCTCATTTTG | 473 |
| oVZ405 | CTGATGACATTTTTGTTAATTGGTGTTTTGTTTAATAATGGGG | 474 |



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| Table 27 | | |
|---|---|---|
| **Oligo No.** | **Sequence** | **SEQ ID NO** |
| oVZ406 | AATTAACAAAAATGTCATCAGAATACGCTAAACTAGTGG | 475 |
| oVZ407 | AACACCCAGCTTAAAGTAATCTATGTATCTCAGTATCTTGGTACTTTTC | 476 |
| oVZ408 | ATCCAAATAGGCTGGGTGTTTGCTCATTTTG | 477 |
| oVZ409 | TATCAGACATTTTTGTTAATTGGTGTTTTGTTTAATAATGGGG | 478 |
| oVZ410 | AATTAACAAAAATGTCTGATAAAGCAGCCGCTAG | 479 |
| oVZ411 | AACACCCAGCCTATTTGGATCTCTTGTAAACCTTGCC | 480 |
| oVZ478 | TACCAAACTAAGCTGGGTGTTTGCTCATTTTG | 481 |
| oVZ479 | AACACCCAGCTTAGTTTGGTAACCCACTTTCGTTG | 482 |
| oVZ480 | CTTTTGCACATTTTTGTTAATTGGTGTTTTGTTTAATAATGGGG | 483 |
| oVZ481 | CCAATTAACAAAAATGTCAAAAGCTTTTAGTGCCCC | 484 |

*EXAMPLE 73: Promoters and terminators*

Certain promoters and terminators described herein are provided in Tables 28 and 29 below.

| Table 28 | | | |
|---|---|---|---|
| **Promoter** | **Sequence** | **SEQ ID NO** | **Length** |
| HDE1 | AAGGGAAGAAGAGTCGTTGAGTTGATGTAATTAAGCTGGCACGTAGATACCAGAAGGTTCTAGAGTAGAGCTTGGGTGGTGTTTGGCCCTGTTTGGACCACGGATAGAGATGGAGAATCCCTTGGTTAGAGCGGAGAGGAAAAAATTGAAACTTTGCATATCCCACTTCATTATCCTTGATGTAACCGTTTTATGGGGTAATTAAAGTGTGGAAAAATAATCAGGGAGACATATTCCCGATCAATTGGGTGGTGGTCGCTCAATTTCTGTGAGTAGTAGGCTCAGTGGTGTGTATTGGGATTGGTAGTAGTCTGTATAAGCAGTGTTATATAACCCATTGCTTGTTGATTCCTATTTTGCTGGCAAAAGTGACAACTGTAGTTGTGAGATAATCCTCGGTTATTACGCCTGGGGGGGCAGACAGCCAAAGTTGTGCCCGTGCGACAATGGCATCAGAAGAAACAGAAAAAAAAAACACAGGCATTTTTATCCACATGCACACTACCCCCACTATTCCTGTCTGCAGTGTGCTTGTGTGTGGCCCCCCGCAGAATCAACAGGGCAAACTCTGGAGCCTGAATCTTTATATAAACTTCAGGCATTGGCCCCCCTTTTCACAATTCTTCACATCCACCATTTTTTTTCTTCTTTCCTACCATATTAGTTTTTT | 485 | 751 |

| Table 28 | | | |
|---|---|---|---|
| **Promoter** | **Sequence** | **SEQ ID NO** | **Length** |
| | TTTATTCTTTTCCTACCTATCTGATTATTATCAA<br>ACATCTGGTCATCCTCAAAAGAAAGAAAGAAA<br>CTATAACAATCAATC | | |
| POX4 | GAGCTCCAATTGTAATATTTCGGGAGAAATAT<br>CGTTGGGGTAAAACAACAGAGAGAGAGAGGG<br>AGAGATGGTTCTGGTAGAATTATAATCTGGTT<br>GTTGCAAATGCTACTGATCGACTCTGGCAATG<br>TCTGTAGCTCGCTAGTTGTATGCAACTTAGGT<br>GTTATGCATACACACGGTTATTCGGTTGAATT<br>GTGGAGTAAAAATTGTCTGAGTTGTGTCTTAG<br>CTACTGGCTGGCCCCCCGCGAAAGATAATCA<br>AAATTACACTTGTGAATTTTTGCACACACACC<br>GATTAACATTTCCCTTTTTTGTCCACCGATACA<br>CGCTTGCCTCTTCTTTTTTTTCTCTGTGCTTCC<br>CCCTCCTGTGACTTTTTCCACCATTGATATAAA<br>ATCAACTCCATTTCCCTAAAATCTCCCCAGATT<br>CTAAAAACAACTTCTTCTCTTCTGCTTTTCCTT<br>TTTTTTTGTTATATTTATTTACCATCCCTTTTTT<br>TTGAATAGTTATTCCCCACTAACATTGTTCAAA<br>TCTTCACGACATA | 486 | 531 |
| URA3 | CGACGGGTACAACGAGAATTGTATTGAATTGA<br>TCAAGAACATGATCTTGGTGTTACAGAACATC<br>AAGTTCTTGGACCAGACTGAGAATGCACAGAT<br>ATACAAGGCGTCATGTGATAAAATGGATGAGA<br>TTTATCCACAATTGAAGAAAGAGTTTATGGAA<br>AGTGGTCAACCAGAAGCTAAACAGGAAGAAG<br>CAAACGAAGAGGTGAAACAAGAAGAAGAAGG<br>TAAATAAGTATTTTGTATTATATAACAAACAAA<br>GTAAGGAATACAGATTTATACAATAAATTGCC<br>ATACTAGTCACGTGAGATATCTCATCCATTCC<br>CCAACTCCCAAGAAAAAAAAAAAGTGAAAAAA<br>AAAATCAAACCCAAAGATCAACCTCCCCATCA<br>TCATCGTCATCAAACCCCCAGCTCAATTCGCA | 487 | 415 |
| G6PI | AAAATCAGAGGCTACTCCGGAGATGGCACAT<br>TATCACGTGGGCAGTCCCATCTCTCTGACAAT<br>GGCACTGACAATGCCATGTCATTCTAGAAGTC<br>GCTCGTTCCATTGCTAGTCGTGTAGGTGTCTT<br>ACTCAGACCATATGTTTGGCTGTGTGTGTGGT<br>AAGGGGGTAAGATATCTCTAGGAGGAGGCAA<br>CTCTACTCGAAAGTACGAAATGGCCGACGCC<br>AATCCGACCTGCCATTGTGCAGGACGCGGGT<br>CGAGATCCGGAAGTGCCCTCCTTCTGAGGTT<br>GTTGGTTTGTTCCAGTTGGCATAAGAAATTAA<br>TAATCGTTTAGTCAATGGTACAGTATTAATAAT<br>TCCTGACAAAACCGTGCCCTACAAGCAGTGT<br>GAGTTCCGGGGGGTAGCACTAGTGGTCGTGGT<br>GGTAGGGCCCACAGAGAGTATTTCCGGAACC<br>GACTGATCCTTTGATTCCAATATTCCAAGATTT<br>GGGTTTGTGGTTGTGTGTAATGCATGAGGTTA<br>TTTTGTCAACCTCCTTCCTTCTCTACGGTTGTT | 488 | 745 |

| Table 28 | | | |
|---|---|---|---|
| **Promoter** | **Sequence** | **SEQ ID NO** | **Length** |
| | GCTCCTCCTCCTCTTCCTTCCCTTTGTTCATTT AAATACACACACACCACTCCCCTTCCCACCCA CAAGAACTTTTTTTTTTTTTTACTTTCTTCTTCTC TTACTTTCTTCTTCTTCTTTCATTACCTGTTAG ATTATATTTATCAACCTCAATTGGTTTATTATCT ACCACCCATACAACCCCCACCCCTGCCACAT CCACCAACA | | |
| GPD | CGGAAGTTGTTTACCGACCTGACCGTAAATTT GCTGCTGAAAGAAACGTGTCAAACAAGACCA ATTGGCTCAATTGACCCTGTGGAAATGCTTTG TTGACCACCAATGCTTCCACCAAACGTTACTT TTTTTTTGCAATCGGATGGTATGGGTCTGGGG TTCACCTGTTTTGTAAAGCTACAGAAGGTGGC ATATTTCTCTGATCAGGTGTTTTTTTTTTCGGC TGCTGCTGCTCGTGGTGGTGTAGTGGTAGTG GTGTGTGTGTGTGTGTGTGCGTGCGTGTGGA AGGACGCTTTTTGCTCTCTGACTCCTCCCAAT CAGAAGTTGCTATAGTGGTGAAACAACAATGG ATGATAATGCCCCGGGCGGTGCGTGTCCGAC ACAAACCACTACATTTTTTAGCTGGGAGCCTA CTGCCACTACGACCCACCCACCCATGGTCAA CAAAAAAATTCTGACAAATTATAAAATAACCCT TGAATTCCCCCTTGGAAAAATTTTTGGTATTTC TCTCTCTCTTTTCCTTTCCCTCTTCTTTTTCTCT CCATCAATCAATTGACGTTCAGTAACTCAATTA ATTACATCACATCCCTCAATTAAAGAATTTAAT CGAT | 489 | 614 |
| POX18 | AGATAAACCGAGGCATGGAAGAAGTCAGAGA CTTCCTAATTGGGAAATATCACATGAAAATGT ATGGATGTGTGCAGGACACGTCTACTGAGTG CCATACTTTGTGCCCACCGCGAAATTAAGGG CAGCCATCAGATTACGTAAGCAGTGGTATGCA GTTGTTACAGCGTCAAGTTTTTCTGTATTACC CCAAAGTTTTCTTCTCTCCCCTGCAAAAGTTTT CATATGCAAAACTGATCCCACAGCAGTATAAA TACCCAATAACTTTCCCCAACCAACAGCAACA ACCTCTCATTCTTTTTTTTCCTATTCTTTTCTTT AATCAACCCCCATTATTAAACAAAACACCAATT AACAAAA | 490 | 360 |
| LEU2 | GTCTTGCACGCTTTGGGTGCCGACGACGCCT TGGCCCACTCAAGTATTAGATTCGGTATTGGT AGATTCACCACTGAGGCAGAGGTCGACTATG TCATCAAGGCTATTAACGAAAGAGTTGATTTC TTGAGAAAGATGTCTCCATTGTGGGAGATGGT GCAAGAAGGCATTGACTTGGACTCAATTGAAT GGAGTGGTCATTAGTGTGACCCCCTATCGCT ACGGTCTTCCTCTTCGTTTCATTTAAGTATGAC TTGTTTTGTTTATTTATCTCGTGTATAGAATAG TAATTATTTTTGGTATAAGCCATGTTGGTATTC GTGAGTCAGATGTGAGCTGTATGTTCTCCTGA | 491 | 500 |

| Table 28 | | | |
|---|---|---|---|
| **Promoter** | **Sequence** | **SEQ ID NO** | **Length** |
| | ACCGGCGTAACTTCCCTTCCGTTGCCGCGGG AATGCGACAACAACGACGGGAAAAAAATCCC AGTATAAAACCACCCCAAATGACGTGTGTGAA ATTTTTCACTCCAGGTTTCTCTCCCTCTTTTTT GTCCCCCCAAACCAATCACCA | | |

| Table 29 | | | |
|---|---|---|---|
| **Terminator** | **Sequence** | **SEQ ID NO** | **Length** |
| POX4 | GAATAGAAGAGAGTGACTCTTTTGATAAGAGT CGCAAATTTGATTTCATAAGTATATATTCATTA TGTAAAGTAGTAAATGGAAAATTCATTAAAAAA AAAGCAAATTTCCGTTGTATGCATACTCCGAA CACAAAACTAGCCCCGGAAAAACCCTTAGTTG ATAGTTGCGAATTTAGGTCGAC | 492 | 184 |
| URA3 | TAAATATTGTAATAAATAGGTCTATATACATAC ACTAAGCTTCTAGGACGTCATTGTAGTCTTCG AAGTTGTCTGCTAGTTTAGTTCTCATGATTTCG AAAACCAATAACGCAATGGATGTAGCAGGGAT GGTGGTTAGTGCGTTCCTGACAAACCCAGAG TACGCCGCCTCAAACCACGTCACATTCGCCC TTTGCTTCATCCGCATCACTTGCTTGAAGGTA TCCACGTACGAGTTGTAATACACCTTGAAGAA CGGCTTCGTCT | 493 | 267 |
| POX18 | GCTGGGTGTTTGCTCATTTTGTTTCAATGCAT GGATGCACACTGGTCTTTTTATGATTTGAAAT GAATAGATTTGATCATCATCATTTTTTTTTTGC AAATATTTATCATATAATGTGAGTTTTCTTTGG TGTATATTTGTTTTCTACAGTATTTAGATAATAT TATTTGAAAAGAATATATGCTTAAAAGAAACTA CAAGTTAGAGTTCGACGATGAAGTGGTTGTCA TGTCGGCGACTTTACCCCAGAGTAAGAAGGA ATTGTCGTGGTCGATATTGGTTGAAACATATC GGGACTAGTCTTGCGACCGAGCGCAGGGCAA TTGTGGAGTCACACATACAATGAAAATTGGCT GCTTACTTGCAGTCTATGC | 494 | 374 |
| LEU2 | GGATCCTCTACATATGAACATTCTATATTGAAA AATGCATGTATATTATTGTCGTTAACGTTTTTT AAAAATTCATGGTCTTTTCTAAAAATGCAGATA GTTGAGCAACAGAATCCTCTTTATCAATCACC ACAACCACCGACTGTTTGCTACTCTCATTCGT AACAATGAATCTATTCGACGTGACCGTAGCCT CACTAGACTCAGATTGGCTGGTGTTCCTCGCA AGATTGGGTTCTTCATTAGGCGAGTAAAACTC CTCTTCCTCGTCCTCCTCCCCATCTTCGCTCG TGCTAGTCA | 495 | 300 |

*EXAMPLE 74: Strains*

Certain strains described herein are further characterized in Table 30 below.

**Table 30**

| Strain No. | Description | Construction |
|---|---|---|
| sAA001 | Candida viswanathii ATCC 20336 | Acquired from ATCC |
| sAA002 | Candida viswanathii ATCC 20913, Δura3 | Acquired from ATCC |
| sAA4377 | Δpox4, Δfaa1, Δfat1, Δcrc1, Δura3 | Described in Example 21 and Table 11 |
| sAA5733 | ald6/ald6::URA3, crc1:: ura3 promoter/CRC1 | Described in International Patent Application Publication No. WO2016/154046 |
| sAA5761 | ald6/ald6, crc1:: ura3 promoter /CRC1 | 5'FOA of sAA5733 |
| sAA6234 | crc1::ura3 promoter/crc1::ura3 promoter, ald6::ura3 promoter/ald6::ura3 promoter | 5'FOA of sAA5761 + pAA1701 |
| sAA7443 | GPD promoter - CsCrtE CvCO, GPD promoter - CsCrtB CvCO, GPD promoter - CsCrtI CvCO | sAA002 + PCR cassettes amplified from pAA2698, pAA2699, and pAA2700 using primers oAA02206 and oAA02209 |
| sAA7444 | GPD promoter - CsCrtE CvCO, GPD promoter - CsCrtB CvCO, GPD promoter - CsCrtI CvCO | sAA002 + PCR cassettes amplified from pAA2698, pAA2699, and pAA2700 using primers oAA02206 and oAA02209 |
| sAA7445 | GPD promoter - CsCrtE CvCO, GPD promoter - CsCrtB CvCO, GPD promoter - CsCrtI CvCO | sAA002 + PCR cassettes amplified from pAA2698, pAA2699, and pAA2700 using primers oAA02206 and oAA02209 |
| sAA7446 | HDE1 promoter - CsCrtE CvCO, HDE1 promoter - CsCrtB CvCO, HDE1 promoter - CsCrtI CvCO | sAA002 + PCR cassettes amplified from pAA2702, pAA2703 , and pAA2704 using primers oAA02206 and oAA02209 |
| sAA7449 | GPD promoter - CnTPS1 CvCO | sAA002 + PCR cassettes amplified from pAA2701 using primers oAA02206 and oAA02209 |

| Table 30 | | |
|---|---|---|
| **Strain No.** | **Description** | **Construction** |
| sAA7453 | HDE1 promoter - CnTPS1 CvCO | sAA0002 + PCR cassettes amplified from pAA2705 using primers oAA02206 and oAA02209 |
| sAA7565 | crc1::ura3 promoter/crc1::ura3 promoter, ald6::ura3 promoter/ald6::ura3 promoter, HDE1 promoter - CsCrtE CvCO, HDE1 promoter - CsCrtB CvCO, HDE1 promoter - CsCrtI CvCO | sAA06234 +PCR cassettes amplified from pAA2702, pAA2703, pAA2704, and pAA2311 using primers oAA02206 and oAA02209 |
| sAA7870 | GPD promoter - CsCrtE CvCO, GPD promoter - CsCrtI CvCO, GPD promoter - XdCrtYB CvCO, GPD promoter - XdCrtR CvCO, GPD promoter - AaCrtZ CvCO, GPD promoter - XdCrtS CvCO, GPD promoter - AaCrtW CvCO | sAA002 + PCR cassettes amplified from pAA2698, pAA2866, pAA2700, pAA2993, pAA2994, pAA2995, and pAA2996 using primers oAA02206 and oAA02209 |
| sAA7882 | GPD promoter - CsCrtE CvCO, GPD promoter - CsCrtI CvCO, GPD promoter - XdCrtYB CvCO | sAA002 + PCR cassettes amplified from pAA2698, pAA2866, and pAA2700 using primers oAA02206 and oAA02209 |
| sAA8283 | Δpox4, Δfaa1, Δfat1, Δcrc1, Δura3, G6PIpromoter - CRC1, HDE1 promoter - CsCrtE CvCO, HDE1 promoter - CsCrtB CvCO, HDE1 promoter - CsCrtI CvCO | sAA4377 + PCR cassettes amplified from pAA2311, pAA2702, pAA2703, sAA2704 |
| sAA8519 | Δpox4, Δfaa1, Δfat1, Δcrc1, Δura3, G6PIpromoter -CRC1, HDE1 promoter - CsCrtE CvCO, HDE1 promoter - XdCrtYB CvCO, HDE1 promoter - CsCrtI CvCO | sAA4377 + PCR cassettes amplified from pAA2311, pAA2702, pAA3373, sAA2704 |
| sAA8503 | GPD promoter - XdCrtE CvCO, GPD promoter - CsCrtB CvCO, GPD promoter - CsCrtI CvCO | sAA002 + PCR cassettes amplifieid from pAA3189, pAA2699, and pAA2700 |
| sAA8932 | GPD promoter - XdCrtE CvCO, GPD promoter - CsCrtB CvCO, GPD promoter - XdCrtI CvCO | sAA002 + PCR cassettes amplifieid from pAA3189, pAA2699, and pAA3490 |
| sAA9432 | HDE1 promoter - XdCrtE CvCO, HDE1 promoter - CsCrtB CvCO, HDE1 promoter - XdCrtI CvCO | sAA002 + PCR cassettes amplified from pAA3633, pAA2703, and pAA3634 |
| sAA9539 | Δras2/RAS2, Δura3 | sAA002 + pVZ3930, after 5'FOA |
| sAA9670 | Δras2/RAS2, HDE1 promoter - XdCrtE CvCO, HDE1 promoter - | sAA9539 + PCR cassettes amplified from pAA3633, |

| Table 30 | | |
|---|---|---|
| **Strain No.** | **Description** | **Construction** |
| | CsCrtB CvCO, HDE1 promoter - XdCrtI CvCO | pAA2703, and pAA3634 |
| sAA9658 | Δura3, Δleu2::tURA3/LEU2 | sAA002 + pAA3060 (BamHI/PstI) after 5'FOA |
| sAA9682 | Δras2/RAS2, Δura3, Δleu2::tURA3/LEU2 | sAA9539 + pAA3060 (BamHI/PstI) after 5'FOA |
| sAA9684 | Δura3, Δleu2 | sAA9658 + pAA2417 (BamHI/PstI) after 5'FOA |
| sAA9703 | Δras2/RAS2, Δura3, Δleu2 | sAA9682 + pAA3060 (BamHI/PstI) after 5'FOA |
| sAA9748 | Δleu2, HDE1 promoter - XdCrtE CvCO (URA3), HDE1 promoter - CsCrtB CvCO (URA3), HDE1 promoter - XdCrtI CvCO (URA3) | sAA9684 + PCR cassettes amplified from pAA3633, pAA2703, and pAA3634 |
| sAA9750 | Δleu2, Δras2/RAS2, HDE1 promoter - XdCrtE CvCO (URA3), HDE1 promoter - CsCrtB CvCO (URA3), HDE1 promoter - XdCrtI CvCO (URA3) | sAA9703 + PCR cassettes amplified from pAA3633, pAA2703, and pAA3634 |
| sAA9812 | HDE1 promoter - XdCrtE CvCO (URA3), HDE1 promoter - CsCrtB CvCO (URA3), HDE1 promoter - XdCrtI CvCO (URA3) +leu2 library of (Ppox18Erg10, Ppox18Erg13, Ppox18HMG1, Ppox18Erg12, Ppox18Erg8, Ppox18Mvd1, Ppox18IDI1, Ppox18Erg20 | sAA9748 + PCR cassettes amplified from pVZ4098, pVZ4099, pVZ4100, pVZ4101, pVZ4122, pVZ4123, pVZ4104, pVZ4105 |
| sAA9811 | HDE1 promoter - XdCrtE CvCO (URA3), HDE1 promoter - CsCrtB CvCO (URA3), HDE1 promoter - XdCrtI CvCO (URA3) +leu2 library of (Phde-XdCrtE, Phde-CsCrtB, Phde-XdCrtI) | sAA9748 + PCR cassettes amplified from pVZ4056, pVZ4057, pVZ4058 |
| sAA9814 | HDE1 promoter - XdCrtE CvCO (URA3), HDE1 promoter - CsCrtB CvCO (URA3), HDE1 promoter - XdCrtI CvCO (URA3) +leu2 library of (Phde-XdCrtE, Phde-CsCrtB, Phde-XdCrtI, Ppox18Erg10, Ppox18Erg13, Ppox18HMG1, Ppox18Erg12, Ppox18Mvd1, Ppox18IDI1, Ppox18Erg20) | sAA9748 + PCR cassettes amplified from pVZ4098, pVZ4099, pVZ4100, pVZ4101, pVZ4122, pVZ4123, pVZ4104, pVZ4105, pVZ4056, pVZ4057, pVZ4058 |
| sAA9819 | Δras2/RAS2, HDE1 promoter - XdCrtE CvCO (URA3), HDE1 | sAA9750 + PCR cassettes amplified from pVZ4056, |

| Table 30 | | |
|---|---|---|
| **Strain No.** | **Description** | **Construction** |
| | promoter - CsCrtB CvCO (URA3), HDE1 promoter - XdCrtI CvCO (URA3) +leu2 library of (Phde-XdCrtE, Phde-CsCrtB, Phde-XdCrtI) | pVZ4057, pVZ4058 |
| sAA9817 | Δras2/RAS2, HDE1 promoter - XdCrtE CvCO (URA3), HDE1 promoter - CsCrtB CvCO (URA3), HDE1 promoter - XdCrtI CvCO (URA3) + leu2 library of (Ppox18Erg10, Ppox18Erg13, Ppox18HMG1, Ppox18Erg12, Ppox18Mvd1, Ppox18IDI1, Ppox18Erg20) | sAA9750 + PCR cassettes amplified from pVZ4098, pVZ4099, pVZ4100, pVZ4101, pVZ4122, pVZ4123, pVZ4104, pVZ4105 |
| sAA9821 | Δras2/RAS2, HDE1 promoter - XdCrtE CvCO (URA3), HDE1 promoter - CsCrtB CvCO (URA3), HDE1 promoter - XdCrtI CvCO (URA3) + leu2 library of (Ppox18Erg10, Ppox18Erg13, Ppox18HMG1, Ppox18Erg12, Ppox18Mvd1, Ppox18Erg20, Phde-XdCrtE, Phde-CsCrtB, Phde-XdCrtI) | sAA9750 + PCR cassettes amplified from pVZ4098, pVZ4099, pVZ4100, pVZ4101, pVZ4122, pVZ4123, pVZ4104, pVZ4105, pVZ4056, pVZ4057, pVZ4058 |

*EXAMPLE 75: Examples of embodiments*

The examples set forth below illustrate certain embodiments and do not limit the technology.

A1. A genetically modified microorganism, comprising:

one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides, wherein expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide.

A2. The microorganism of embodiment A1, wherein the microorganism is a fungus.

A3. The microorganism of embodiment A2, wherein the fungus is a yeast.

A4. The microorganism of embodiment A3, wherein the yeast is chosen from *Candida* spp, *Yarrowia* spp, *Rhodotorula* spp, *Rhodosporidium* spp, *Cryptococcus* spp, *Trichosporon* spp, *Lipomyces* spp, and *Blastobotrys* spp.

A5. The microorganism of any one of embodiments A1 to A4, wherein the one or more heterologous nucleic acids encode one or more terpene biosynthesis polypeptides chosen from terpene synthase, phytoene synthase, geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, zeaxanthin glucosyltransferase, valencene synthase, and cytochrome p450 reductase.

A6. The microorganism of any one of embodiments A1 to A5, wherein the fatty acid is a saturated fatty acid or an unsaturated fatty acid.

A7. The microorganism of any one of embodiments A1 to A6, wherein the fatty acid is chosen from one or more of oleic acid, palmitoleic acid, erucic acid, linoleic acid, palmitic acid, caproic acid, enanthic acid, caprylic acid pelargonic acid, capric acid, undecylic acid, lauric acid, myristic acid, pentadecanoic acid, margaric acid, stearic acid arachidic acid, behenic acid, tridecylic acid, and linolenic acid.

A7.1 The microorganism of any one of embodiments A1 to A5, wherein the alkane is chosen from one or more of hexane, heptane, nonane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, hexadecane, heptadecane, and octadecane

A8. The microorganism of any one of embodiments A1 to A7.1, wherein the nucleic acid that provides for fatty acid or alkane induction of expression of a terpene biosynthesis polypeptide comprises a fatty acid response element or an alkane response element.

A9. The microorganism of embodiment A8, wherein the fatty acid response element comprises an oleic acid response element.

A9.1 The microorganism of embodiment A8, wherein the alkane response element comprises an alkane response element 1 (ARE1) sequence.



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A10. The microorganism of any one of embodiments A1 to A9, wherein the nucleic acid that provides for fatty acid induction of expression of a terpene biosynthesis polypeptide comprises a promoter region chosen from promoter regions of genes encoding hydratase-dehydrogenase-epimerase (HDE), acyl co-A oxidase (POX), acyl co-A thiolase (POT), peroxin (PEX) and peroxisomal adenine nucleotide transporter protein (ANT1).

A11. The microorganism of any one of embodiments A1 to A10, wherein the microorganism is *Candida viswanathii.*

A12. The microorganism of any one of embodiments A1 to A10, wherein the microorganism is *Blastobotrys adeninivorans*.

A13. The microorganism of any one of embodiments A1 to A12, wherein the one or more heterologous nucleic acids encode phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase.

A14. The microorganism of embodiment A13, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase is regulated by a promoter region of the gene encoding a hydratase-dehydrogenase-epimerase (HDE).

A14.1 The microorganism of embodiment A13, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase is regulated by a promoter region of the gene encoding a *Candida* hydratase-dehydrogenase-epimerase (HDE).

A14.2 The microorganism of embodiment A13, A14 or A14.1, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase is regulated by a terminator region of the gene encoding an acyl co-A oxidase 4 (POX4).

A14.3 The microorganism of embodiment A13, A14 or A14.1, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase is regulated by a terminator region of the gene encoding a *Candida* acyl co-A oxidase 4 (POX4).



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A15. The microorganism of any one of embodiments A1 to A12, wherein the one or more heterologous nucleic acids encode valencene synthase.

A16. The microorganism of embodiment A15, wherein expression of the heterologous nucleic acids encoding valencene synthase is regulated by a promoter region of the gene encoding a hydratase-dehydrogenase-epimerase (HDE).

A16.1 The microorganism of embodiment A15, wherein expression of the heterologous nucleic acids encoding valencene synthase is regulated by a promoter region of the gene encoding a *Candida* hydratase-dehydrogenase-epimerase (HDE).

A16.2 The microorganism of embodiment A15, A16 or A16.1, wherein expression of the heterologous nucleic acids encoding valencene synthase is regulated by a terminator region of the gene encoding an acyl co-A oxidase 4 (POX4).

A16.3 The microorganism of embodiment A15, A16 or A16.1, wherein expression of the heterologous nucleic acids encoding valencene synthase is regulated by a terminator region of the gene encoding a *Candida* acyl co-A oxidase 4 (POX4).

A17. The microorganism of any one of embodiments A1 to A12, wherein the one or more heterologous nucleic acids encode geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase.

A18. The microorganism of embodiment A17, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a promoter region of the gene encoding a hydratase-dehydrogenase-epimerase (HDE).

A18.1 The microorganism of embodiment A17, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a promoter region of the gene encoding a *Candida* hydratase-dehydrogenase-epimerase (HDE).



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A18.2 The microorganism of embodiment A17, A18 or A18.1, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a terminator region of the gene encoding an acyl co-A oxidase 4 (POX4).

A18.3 The microorganism of embodiment A17, A18 or A18.1, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a terminator region of the gene encoding a *Candida* acyl co-A oxidase 4 (POX4).

A19. The microorganism of any one of embodiments A1 to A18.3, wherein the one or more heterologous nucleic acids encoding the one or more terpene biosynthesis polypeptides are endogenously expressed in a microorganism chosen from *Cronobacter* spp, *Callitropsis* spp, *Xanthophyllomyces* spp, *Agrobacterium* spp, and *Pantoea* spp.

A20. The microorganism of any one of embodiments A1 to A19, wherein the amount and/or activity of a Ras2 protein has been decreased.

A21. The microorganism of any one of embodiments A1 to A20, wherein the microorganism has been genetically modified to reduce or eliminate expression of an endogenous *RAS2* gene.

A22. The microorganism of any one of embodiments A1 to A21, wherein the amount and/or activity of an Faa1 protein has been decreased.

A23. The microorganism of any one of embodiments A1 to A22, wherein the microorganism has been genetically modified to reduce or eliminate expression of an endogenous *FAA1* gene.

A24. The microorganism of any one of embodiments A1 to A23, wherein the amounts and/or activities of one or more proteins chosen from acetyl-CoA C-acetyltransferase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, and isopentyl diphosphate delta isomerase, have been increased.



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A25. The microorganism of any one of embodiments A1 to A24, wherein the microorganism has been genetically modified to increase expression of one or more endogenous genes chosen from *ERG10*, *ERG13*, *HMG1*, *ERG12*, *ERG8*, *MVD1*, and *IDI1*.

A26. The microorganism of any one of embodiments A1 to A25, wherein the amount and/or activity of dimethylallyltranstransferase or farnesyl diphosphate synthetase has been increased.

A27. The microorganism of any one of embodiments A1 to A26, wherein the microorganism has been genetically modified to increase expression of an endogenous *ERG20* gene.

A28. The microorganism of embodiment A25, wherein expression of *ERG10*, *ERG13*, *HMG1*, *ERG12*, *ERG8*, *MVD1*, and/or *IDI1* is regulated by a promoter region of the gene encoding oleate-induced peroxisomal protein (POX18).

A29. The microorganism of embodiment A25 or A28, wherein expression of *ERG10*, *ERG13*, *HMG1*, *ERG12*, *ERG8*, *MVD1*, and/or *IDI1* is regulated by a terminator region of the gene encoding oleate-induced peroxisomal protein (POX18).

A30. The microorganism of embodiment A27, wherein expression of *ERG20* is regulated by a promoter region of the gene encoding oleate-induced peroxisomal protein (POX18).

A31. The microorganism of embodiment A27 or A30, wherein expression of *ERG20* is regulated by a terminator region of the gene encoding oleate-induced peroxisomal protein (POX18).

B1. A genetically modified microorganism, comprising:

one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides, and

a genetic modification that alters the expression of a polypeptide providing for transport of acetyl-carnitine in the microorganism.

B1.1 The microorganism of embodiment B1, wherein expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide.



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B1.2 The microorganism of embodiment B1, wherein expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for glucose induction of expression of the terpene biosynthesis polypeptide.

B2. The microorganism of embodiment B1, B1.1 or B1.2, wherein the microorganism is a fungus.

B3. The microorganism of embodiment B2, wherein the fungus is a yeast.

B4. The microorganism of embodiment B3, wherein the yeast is chosen from *Candida* spp, *Yarrowia* spp, *Rhodotorula* spp, *Rhodosporidium* spp, *Cryptococcus* spp, *Trichosporon* spp, *Lipomyces* spp, and *Blastobotrys* spp.

B5. The microorganism of any one of embodiments B1 to B4, wherein the one or more heterologous nucleic acids encode one or more terpene biosynthesis polypeptides chosen from terpene synthase, phytoene synthase, geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, zeaxanthin glucosyltransferase, valencene synthase, and cytochrome p450 reductase.

B6. The microorganism of any one of embodiments B1 to B5, wherein the genetic modification reduces expression of the polypeptide providing for transport of acetyl-carnitine in the microorganism relative to a microorganism that does not have the genetic modification.

B6.1 The microorganism of embodiment B6, wherein the genetic modification is a disruption, deletion or knockout of (i) a polynucleotide that encodes a polypeptide providing for transport of acetyl-carnitine, or (ii) a promoter operably linked to a polynucleotide that encodes a polypeptide providing for transport of acetyl-carnitine, whereby endogenous activity of a polypeptide providing for transport of acetyl-carnitine is reduced or abolished.

B7. The microorganism of embodiment B6, further comprising a nucleic acid encoding a polypeptide providing for transport of acetyl-carnitine wherein expression of the nucleic acid encoding a polypeptide providing for transport of acetyl-carnitine is regulated by a promoter that provides for reduced expression relative to endogenous expression.



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B7.1 The microorganism of embodiment B7, wherein the genetic modification comprises replacing the promoter of an endogenous gene encoding the polypeptide providing for transport of acetyl-carnitine in the microorganism with a promoter that provides for reduced expression of the polypeptide in the microorganism relative to a microorganism that does not have the genetic modification.

B8. The microorganism of embodiment B7 or B7.1, wherein the promoter that provides for reduced expression relative to endogenous expression is a promoter for a glucose-6-phosphate isomerase (G6PI) gene.

B9. The microorganism of embodiment B7 or B7.1, wherein the promoter that provides for reduced expression relative to endogenous expression is a promoter for a *Candida* glucose-6-phosphate isomerase (G6PI) gene.

B10. The microorganism of any one of embodiments B1 to B9, wherein the polypeptide providing for transport of acetyl-carnitine in the microorganism is an acetyl-carnitine translocase (CRC).

B11. The microorganism of embodiment B10, wherein the acetyl-carnitine translocase (CRC) is acetyl-carnitine translocase 1 (CRC1).

B12. The microorganism of any one of embodiments B1 to B11, wherein the microorganism is *Candida viswanathii*.

B13. The microorganism of any one of embodiments B1 to B11, wherein the microorganism is *Blastobotrys adeninivorans*.

B14. The microorganism of any one of embodiments B1 to B13, wherein the one or more heterologous nucleic acids encode phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase.

B15. The microorganism of embodiment B14, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase is regulated by a promoter region of the gene encoding a hydratase-dehydrogenase-epimerase (HDE).



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B15.1 The microorganism of embodiment B14, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase is regulated by a promoter region of the gene encoding a *Candida* hydratase-dehydrogenase-epimerase (HDE).

B15.2 The microorganism of embodiment B14, B15 or B15.1, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase is regulated by a terminator region of the gene encoding an acyl co-A oxidase 4 (POX4).

B15.3 The microorganism of embodiment B14, B15 or B15.1, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase is regulated by a terminator region of the gene encoding a *Candida* acyl co-A oxidase 4 (POX4).

B16. The microorganism of any one of embodiments B1 to B13, wherein the one or more heterologous nucleic acids encode geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase.

B17. The microorganism of embodiment B16, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a promoter region of the gene encoding a hydratase-dehydrogenase-epimerase (HDE).

B17.1 The microorganism of embodiment B16, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a promoter region of the gene encoding a *Candida* hydratase-dehydrogenase-epimerase (HDE).

B17.2 The microorganism of embodiment B16, B17 or B17.1, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a terminator region of the gene encoding an acyl co-A oxidase 4 (POX4).



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B17.3 The microorganism of embodiment B16, B17 or B17.1, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a terminator region of the gene encoding a *Candida* acyl co-A oxidase 4 (POX4).

B18. The microorganism of any one of embodiments B1 to B17.3, wherein the one or more heterologous nucleic acids encoding the one or more terpene biosynthesis polypeptides are endogenously expressed in a microorganism chosen from *Cronobacter* spp, *Callitropsis* spp, *Xanthophyllomyces* spp, *Agrobacterium* spp, and *Pantoea* spp.

B19. The microorganism of any one of embodiments B1 to B18, wherein the amount and/or activity of a Ras2 protein has been decreased.

B20. The microorganism of any one of embodiments B1 to B19, wherein the microorganism has been genetically modified to reduce or eliminate expression of an endogenous *RAS2* gene.

B21. The microorganism of any one of embodiments B1 to B20, wherein the amount and/or activity of an Faa1 protein has been decreased.

B22. The microorganism of any one of embodiments B1 to B21, wherein the microorganism has been genetically modified to reduce or eliminate expression of an endogenous *FAA1* gene.

B23. The microorganism of any one of embodiments B1 to B22, wherein the amounts and/or activities of one or more proteins chosen from acetyl-CoA C-acetyltransferase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, and isopentyl diphosphate delta isomerase, have been increased.

B24. The microorganism of any one of embodiments B1 to B23, wherein the microorganism has been genetically modified to increase expression of one or more endogenous genes chosen from *ERG10*, *ERG13*, *HMG1*, *ERG12*, *ERG8*, *MVD1*, and *IDI1*.



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B25. The microorganism of any one of embodiments B1 to B24, wherein the amount and/or activity of dimethylallyltranstransferase or farnesyl diphosphate synthetase has been increased.

B26. The microorganism of any one of embodiments B1 to B25, wherein the microorganism has been genetically modified to increase expression of an endogenous *ERG20* gene.

B27. The microorganism of embodiment B24, wherein expression of *ERG10*, *ERG13*, *HMG1*, *ERG12*, *ERG8*, *MVD1*, and/or *IDI1* is regulated by a promoter region of the gene encoding oleate-induced peroxisomal protein (POX18).

B28. The microorganism of embodiment B24 or B27, wherein expression of *ERG10*, *ERG13*, *HMG1*, *ERG12*, *ERG8*, *MVD1*, and/or *IDI1* is regulated by a terminator region of the gene encoding oleate-induced peroxisomal protein (POX18).

B29. The microorganism of embodiment B26, wherein expression of *ERG20* is regulated by a promoter region of the gene encoding oleate-induced peroxisomal protein (POX18).

B30. The microorganism of embodiment B26 or B29, wherein expression of *ERG20* is regulated by a terminator region of the gene encoding oleate-induced peroxisomal protein (POX18).

C1. A genetically modified *Candida viswanathii* yeast, comprising one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides.

C1.1 The genetically modified *Candida viswanathii* yeast of embodiment C1, wherein expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide.

C1.2 The genetically modified *Candida viswanathii* yeast of embodiment C1, wherein expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for glucose induction of expression of the terpene biosynthesis polypeptide.

C2. The genetically modified *Candida viswanathii* yeast of embodiment C1, C1.1 or C1.2, wherein the one or more heterologous nucleic acids encode one or more terpene biosynthesis polypeptides chosen from terpene synthase, phytoene synthase, geranylgeranyl diphosphate synthase,

phytoene desaturase, lycopene cyclase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, zeaxanthin glucosyltransferase, valencene synthase, and cytochrome p450 reductase.

C3. The genetically modified *Candida viswanathii* yeast of any one of embodiments C1 to C2, wherein the one or more heterologous nucleic acids encode phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase

C4. The genetically modified *Candida viswanathii* yeast of embodiment C3, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase is regulated by a promoter region of the gene encoding a *Candida* hydratase-dehydrogenase-epimerase (HDE).

C4.1 The genetically modified *Candida viswanathii* yeast of embodiment C4, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase is regulated by a terminator region of the gene encoding a *Candida* acyl co-A oxidase 4 (POX4).

C5. The genetically modified *Candida viswanathii* yeast of embodiment C3, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase is regulated by a promoter region of the gene encoding a *Candida* glyceraldehyde-3-phosphate dehydrogenase (GPD).

C6. The genetically modified *Candida viswanathii* yeast of any one of embodiments C1 to C2, wherein the one or more heterologous nucleic acids encode valencene synthase.

C7. The genetically modified *Candida viswanathii* yeast of embodiment C6, wherein expression of the heterologous nucleic acids encoding valencene synthase is regulated by a promoter region of the gene encoding a *Candida* hydratase-dehydrogenase-epimerase (HDE).

C7.1 The genetically modified *Candida viswanathii* yeast of embodiment C7, wherein expression of the heterologous nucleic acids valencene synthase is regulated by a terminator region of the gene encoding a *Candida* acyl co-A oxidase 4 (POX4).



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C8. The genetically modified *Candida viswanathii* yeast of embodiment C6, wherein expression of the heterologous nucleic acids encoding valencene synthase is regulated by a promoter region of the gene encoding a *Candida* glyceraldehyde-3-phosphate dehydrogenase (GPD).

C9. The genetically modified *Candida viswanathii* yeast of any one of embodiments C1 to C2, wherein the one or more heterologous nucleic acids encode geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase.

C10. The genetically modified *Candida viswanathii* yeast of embodiment C9, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a promoter region of the gene encoding a *Candida* hydratase-dehydrogenase-epimerase (HDE).

C10.1 The genetically modified *Candida viswanathii* yeast of embodiment C10, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a terminator region of the gene encoding a *Candida* acyl co-A oxidase 4 (POX4).

C11. The genetically modified *Candida viswanathii* yeast of embodiment C9, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a promoter region of the gene encoding a *Candida* glyceraldehyde-3-phosphate dehydrogenase (GPD).

C12. The genetically modified *Candida viswanathii* yeast of any one of embodiments C1 to C2, wherein the one or more heterologous nucleic acids encode geranylgeranyl diphosphate synthase, phytoene desaturase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, and cytochrome p450 reductase.

C13. The genetically modified *Candida viswanathii* yeast of embodiment C12, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, and cytochrome p450 reductase is regulated by a promoter region of the gene encoding a *Candida* hydratase-dehydrogenase-epimerase (HDE).



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C13.1 The genetically modified *Candida viswanathii* yeast of embodiment C13, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, and cytochrome p450 reductase is regulated by a terminator region of the gene encoding a *Candida* acyl co-A oxidase 4 (POX4).

C14. The genetically modified *Candida viswanathii* yeast of embodiment C12, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, and cytochrome p450 reductase is regulated by a promoter region of the gene encoding a *Candida* glyceraldehyde-3-phosphate dehydrogenase (GPD).

C15. The genetically modified *Candida viswanathii* yeast of any one of embodiments C1 to C14, wherein the one or more heterologous nucleic acids encoding the one or more terpene biosynthesis polypeptides are endogenously expressed in a microorganism chosen from *Cronobacter* spp, *Callitropsis* spp, *Xanthophyllomyces* spp, *Agrobacterium* spp, and *Pantoea* spp.

C16. The genetically modified *Candida viswanathii* yeast of any one of embodiments C1 to C15, further comprising a genetic modification that alters the expression of one or more nucleic acids encoding one or more endogenous polypeptides.

C17. The genetically modified *Candida viswanathii* yeast of embodiment C16, wherein the one or more endogenous polypeptides are chosen from polypeptides having one or more of the following activities: acyl-CoA synthetase activity, acyl-CoA oxidase activity, ATP-binding cassette transporter activity, carnitine acetyltransferase activity, transport of acetyl-carnitine, acyl-CoA thioesterase activity, acyl-CoA hydrolase activity, aldehyde dehydrogenase activity, monooxygenase activity, or monooxgenase reductase activity.

C18. The genetically modified *Candida viswanathii* yeast of any one of embodiments C1 to C17, wherein the amount and/or activity of a Ras2 protein has been decreased.

C19. The genetically modified *Candida viswanathii* yeast of any one of embodiments C1 to C18, wherein the yeast has been genetically modified to reduce or eliminate expression of an endogenous *RAS2* gene.



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C20. The genetically modified *Candida viswanathii* yeast of any one of embodiments C1 to C19, wherein the amount and/or activity of an Faa1 protein has been decreased.

C21. The genetically modified *Candida viswanathii* yeast of any one of embodiments C1 to C20, wherein the yeast has been genetically modified to reduce or eliminate expression of an endogenous *FAA1* gene.

C22. The genetically modified *Candida viswanathii* yeast of any one of embodiments C1 to C21, wherein the amounts and/or activities of one or more proteins chosen from acetyl-CoA C-acetyltransferase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, and isopentyl diphosphate delta isomerase, have been increased.

C23. The genetically modified *Candida viswanathii* yeast of any one of embodiments C1 to C22, wherein the yeast has been genetically modified to increase expression of one or more endogenous genes chosen from *ERG10*, *ERG13*, *HMG1*, *ERG12*, *ERG8*, *MVD1*, and *IDI1*.

C24. The genetically modified *Candida viswanathii* yeast of any one of embodiments C1 to C23, wherein the amount and/or activity of dimethylallyltranstransferase or farnesyl diphosphate synthetase has been increased.

C25. The genetically modified *Candida viswanathii* yeast of any one of embodiments C1 to C24, wherein the yeast has been genetically modified to increase expression of an endogenous *ERG20* gene.

C26. The genetically modified *Candida viswanathii* yeast of embodiment C23, wherein expression of *ERG10*, *ERG13*, *HMG1*, *ERG12*, *ERG8*, *MVD1*, and/or *IDI1* is regulated by a promoter region of the gene encoding oleate-induced peroxisomal protein (POX18).

C27. The genetically modified *Candida viswanathii* yeast of embodiment C23 or C26, wherein expression of *ERG10*, *ERG13*, *HMG1*, *ERG12*, *ERG8*, *MVD1*, and/or *IDI1* is regulated by a terminator region of the gene encoding oleate-induced peroxisomal protein (POX18).

C28. The genetically modified *Candida viswanathii* yeast of embodiment C25, wherein expression of *ERG20* is regulated by a promoter region of the gene encoding oleate-induced peroxisomal protein (POX18).

C29. The genetically modified *Candida viswanathii* yeast of embodiment C25 or C28, wherein expression of *ERG20* is regulated by a terminator region of the gene encoding oleate-induced peroxisomal protein (POX18).

D1. A method for producing a terpene comprising:

contacting the genetically modified microorganism of any one of embodiments A1 to A31 with a feedstock comprising a carbon source, and

culturing the microorganism under conditions in which the terpenes are produced from the feedstock.

D2. A method for producing a terpene comprising:

contacting the genetically modified microorganism of any one of embodiments B1 to B30 with a feedstock comprising a carbon source, and

culturing the microorganism under conditions in which the terpenes are produced from the feedstock.

D3. A method for producing a terpene comprising:

contacting the genetically modified *Candida viswanathii* yeast of any one of embodiments C1 to C29 with a feedstock comprising a carbon source, and

culturing the microorganism under conditions in which the terpenes are produced from the feedstock.

D4. The method of any one of embodiments D1 to D3, wherein the feedstock comprises one or more fatty acids.

D5. The method of embodiment D4, wherein the feedstock comprises one or more saturated fatty acids or one or more unsaturated fatty acids; or one or more saturated fatty acids and one or more unsaturated fatty acids.



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D6. The method of embodiment D4 or D5, wherein the feedstock comprises one or more fatty acids chosen from oleic acid, palmitoleic acid, erucic acid, linoleic acid, palmitic acid, caproic acid, enanthic acid, caprylic acid pelargonic acid, capric acid, undecylic acid, lauric acid, myristic acid, pentadecanoic acid, margaric acid, stearic acid arachidic acid, behenic acid, tridecylic acid, and linolenic acid.

D7. The method of embodiment D6 wherein the feedstock comprises oleic acid.

D8. The method of any one of embodiments D1 to D3, wherein the feedstock comprises one or more sugars.

D9. The method of D8, wherein the feedstock comprises glucose.

D10. The method of any one of embodiments D1 to D3, wherein the feedstock comprises one or more alkane hydrocarbons.

D10.1 The method of embodiment D10, wherein the feedstock comprises one or more alkane hydrocarbons chosen from one or more of hexane, heptane, nonane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, hexadecane, heptadecane, and octadecane.

D11. The method of embodiment D10, wherein the feedstock comprises one or more alkane hydrocarbons chosen from C10 to C14 alkane hydrocarbons, nonane and octadecane.

D12. The method of any one of embodiments D1 to D3, wherein the feedstock comprises one or more vegetable oils, derivatives thereof, or byproducts thereof.

D13. The method of embodiment D12, wherein the feedstock comprises crude palm oil (CPO).

D14. The method of embodiment D12, wherein the feedstock comprises palm oil fatty acid distillate (PFAD).

D15. The method of any one of embodiments D1 to D14, wherein the terpene is chosen from lycopene, beta carotene and astaxanthin.



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D16. The method of any one of embodiments D1 to D15, wherein the yield or titer of the terpene is between about 0.001 g/L to about 2.0 g/L.

D16.1 The method of any one of embodiments D1 to D15, wherein the yield or titer of the terpene is between about 0.05 g/L to about 0.5 g/L.

D17. The method of any one of embodiments D1 to D15, wherein the yield or titer of the terpene is between about 0.001 g/L to about 0.2 g/L.

D18. The method of embodiment D16, wherein the yield or titer of lycopene is between about 0.03 g/L to about 2.0 g/L.

D18.1 The method of embodiment D16.1, wherein the yield or titer of lycopene is between about 0.05 g/L to about 0.5 g/L.

D19. The method of embodiment D17, wherein the yield or titer of lycopene is between about 0.001 g/L to about 0.2 g/L.

D20. The method of embodiment D16, wherein the yield or titer of beta carotene is between about 0.1 g/L to about 0.2 g/L.

D21. The method of embodiment D17, wherein the yield or titer of beta carotene is between about 0.01 g/L to about 0.1 g/L.

D22. The method of embodiment D17, wherein the yield or titer of beta carotene is between about 0.001 g/L to about 0.01 g/L.

D23. The method of embodiment D16, wherein the yield or titer of astaxanthin is between about 0.005 g/L to about 0.01 g/L.

D24. The method of embodiment D17, wherein the yield or titer of astaxanthin is between about 0.001 g/L to about 0.005 g/L.

D25. The method of any one of embodiments D1 to D24, further comprising isolating the terpene.



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E1. A genetically modified yeast comprising one or more heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase.

E2. A genetically modified yeast comprising one or more heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase.

E3. A genetically modified yeast comprising one or more heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, and cytochrome p450 reductase.

E4. The genetically modified yeast of embodiment E1, E2 or E3, wherein the amount and/or activity of a Ras2 protein has been decreased.

E5. The genetically modified yeast of any one of embodiments E1 to E4, wherein the yeast has been genetically modified to reduce or eliminate expression of an endogenous *RAS2* gene.

E6. The genetically modified yeast of any one of embodiments E1 to E5, wherein the amount and/or activity of an Faa1 protein has been decreased.

E7. The genetically modified yeast of any one of embodiments E1 to E6, wherein the yeast has been genetically modified to reduce or eliminate expression of an endogenous *FAA1* gene.

E8. The genetically modified yeast of any one of embodiments E1 to E7, wherein the amounts and/or activities of one or more proteins chosen from acetyl-CoA C-acetyltransferase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, and isopentyl diphosphate delta isomerase, have been increased.

E9. The genetically modified yeast of any one of embodiments E1 to E8, wherein the yeast has been genetically modified to increase expression of one or more endogenous genes chosen from *ERG10*, *ERG13*, *HMG1*, *ERG12*, *ERG8*, *MVD1*, and *IDI1*.

E10. The genetically modified yeast of any one of embodiments E1 to E9, wherein the amount and/or activity of dimethylallyltranstransferase or farnesyl diphosphate synthetase has been increased.

E11. The genetically modified yeast of any one of embodiments E1 to E10, wherein the yeast has been genetically modified to increase expression of an endogenous *ERG20* gene.

E12. The genetically modified yeast of any one of embodiments E1 to E11, wherein the yeast is *Candida viswanathii.*

E13. The genetically modified yeast of any one of embodiments E1 to E11, wherein the yeast is *Blastobotrys adeninivorans*.

E14. Use of the genetically modified yeast of any one of embodiments E1 to E13 for the production of a terpene.

E15. Use of the genetically modified yeast of any one of embodiments E1 and E4 to E14 for the production of lycopene.

E16. Use of the genetically modified yeast of any one of embodiments E2 and E4 to E14 for the production of beta carotene.

E17. Use of the genetically modified yeast of any one of embodiments E3 to E14 for the production of astaxanthin.

* * *

The entirety of each patent, patent application, publication and document referenced herein hereby is incorporated by reference. Citation of the above patents, patent applications, publications and documents is not an admission that any of the foregoing is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents. Their citation is not an indication of a search for relevant disclosures. All statements regarding the date(s) or contents



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of the documents is based on available information and is not an admission as to their accuracy or correctness.

Modifications may be made to the foregoing without departing from the basic aspects of the technology. Although the technology has been described in substantial detail with reference to one or more specific embodiments, those of ordinary skill in the art will recognize that changes may be made to the embodiments specifically disclosed in this application, yet these modifications and improvements are within the scope and spirit of the technology.

The technology illustratively described herein suitably may be practiced in the absence of any element(s) not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising," "consisting essentially of," and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and use of such terms and expressions do not exclude any equivalents of the features shown and described or portions thereof, and various modifications are possible within the scope of the technology claimed. The term "a" or "an" can refer to one of or a plurality of the elements it modifies (e.g., "a reagent" can mean one or more reagents) unless it is contextually clear either one of the elements or more than one of the elements is described. The term "about" as used herein refers to a value within 10% of the underlying parameter (i.e., plus or minus 10%), and use of the term "about" at the beginning of a string of values modifies each of the values (i.e., "about 1, 2 and 3" refers to about 1, about 2 and about 3). For example, a weight of "about 100 grams" can include weights between 90 grams and 110 grams. Further, when a listing of values is described herein (e.g., about 50%, 60%, 70%, 80%, 85% or 86%) the listing includes all intermediate and fractional values thereof (e.g., 54%, 85.4%). Thus, it should be understood that although the present technology has been specifically disclosed by representative embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and such modifications and variations are considered within the scope of this technology.

Certain embodiments of the technology are set forth in the claim(s) that follow(s).



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What is claimed is:

1. A genetically modified *Candida viswanathii* yeast, comprising:

heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and phytoene synthase,

a genetic modification to reduce or eliminate expression of an endogenous *RAS2* gene,

wherein:

a) expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and phytoene synthase is regulated by a promoter region of the gene encoding a *Candida* hydratase-dehydrogenase-epimerase (HDE); and

b) expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and phytoene synthase are regulated by a terminator region of the gene encoding a *Candida* acyl co-A oxidase 4 (POX4).

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PATENT
VRD-3002-PV

# BIOLOGICAL METHODS FOR PREPARING TERPENES


## Abstract

The technology relates in part to biological methods for producing terpenes and to engineered cells and microorganisms capable of such production.




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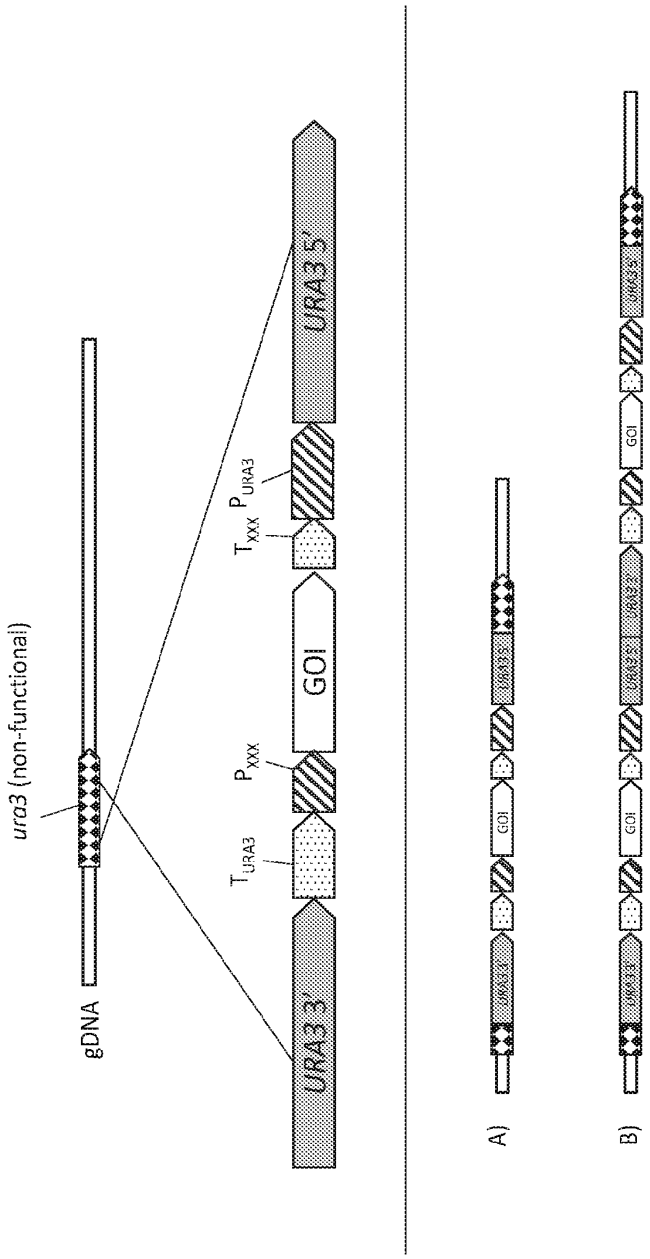

ura3 (non-functional)
gDNA
URA3 3'
T URA3
P XXX
GOI
T XXX
P URA3
URA3 5'
A)
B)
GOI

FIG. 1

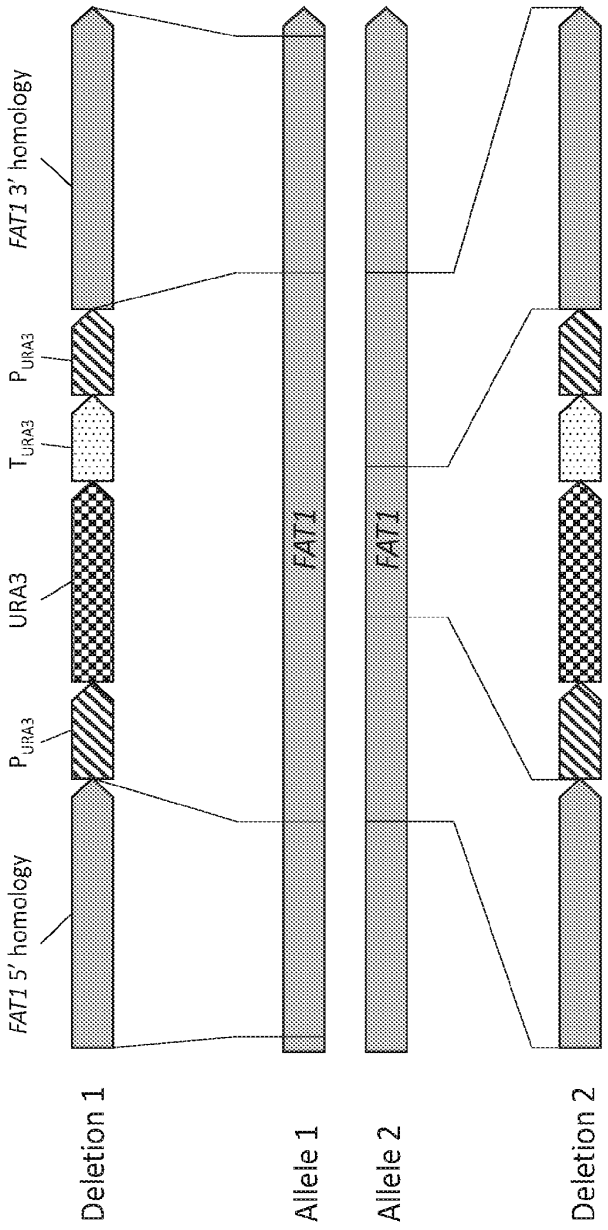

FAT1 5' homology
P URA3
URA3
T URA3
P URA3
FAT1 3' homology
FAT1
FAT1
Deletion 1
Allele 1
Allele 2
Deletion 2

FIG. 2

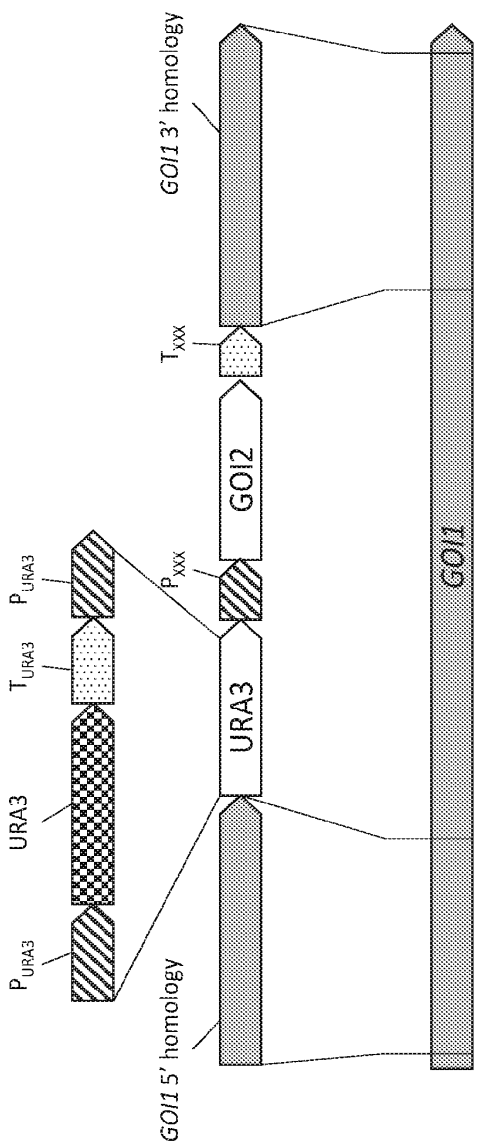



FIG. 3A

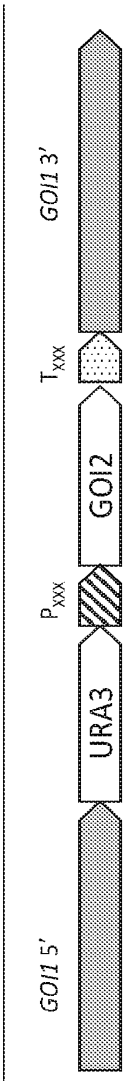



FIG. 3B




FIG. 3C

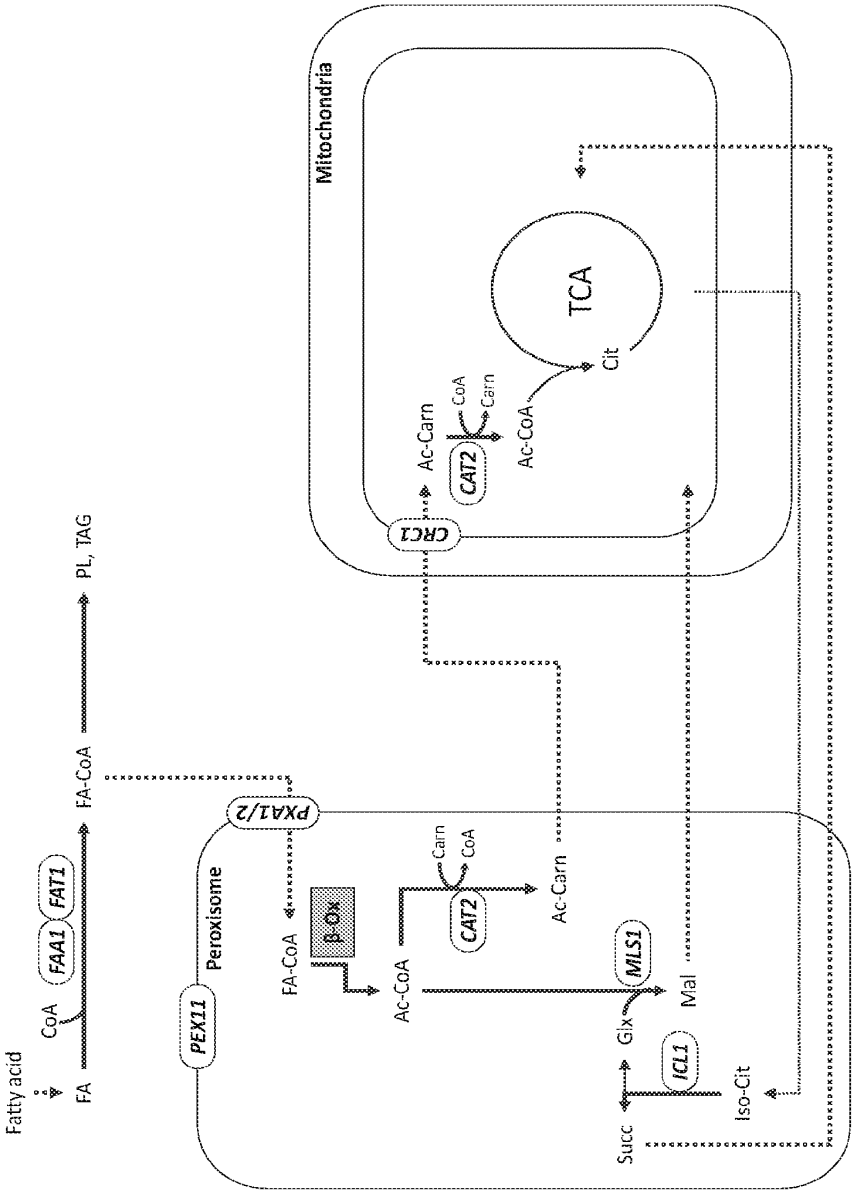

Fatty acid
FA
CoA
FAA1
FAT1
FA-CoA
PL, TAG
PEX11
Peroxisome
PXA1/2
FA-CoA
β-Ox
Ac-CoA
Carn
CoA
CAT2
Ac-Carn
MLS1
Glx
Mal
ICL1
Succ
Iso-Cit
Mitochondria
CRC1
Ac-Carn
CoA
Carn
CAT2
Ac-CoA
TCA
Cit

FIG. 4

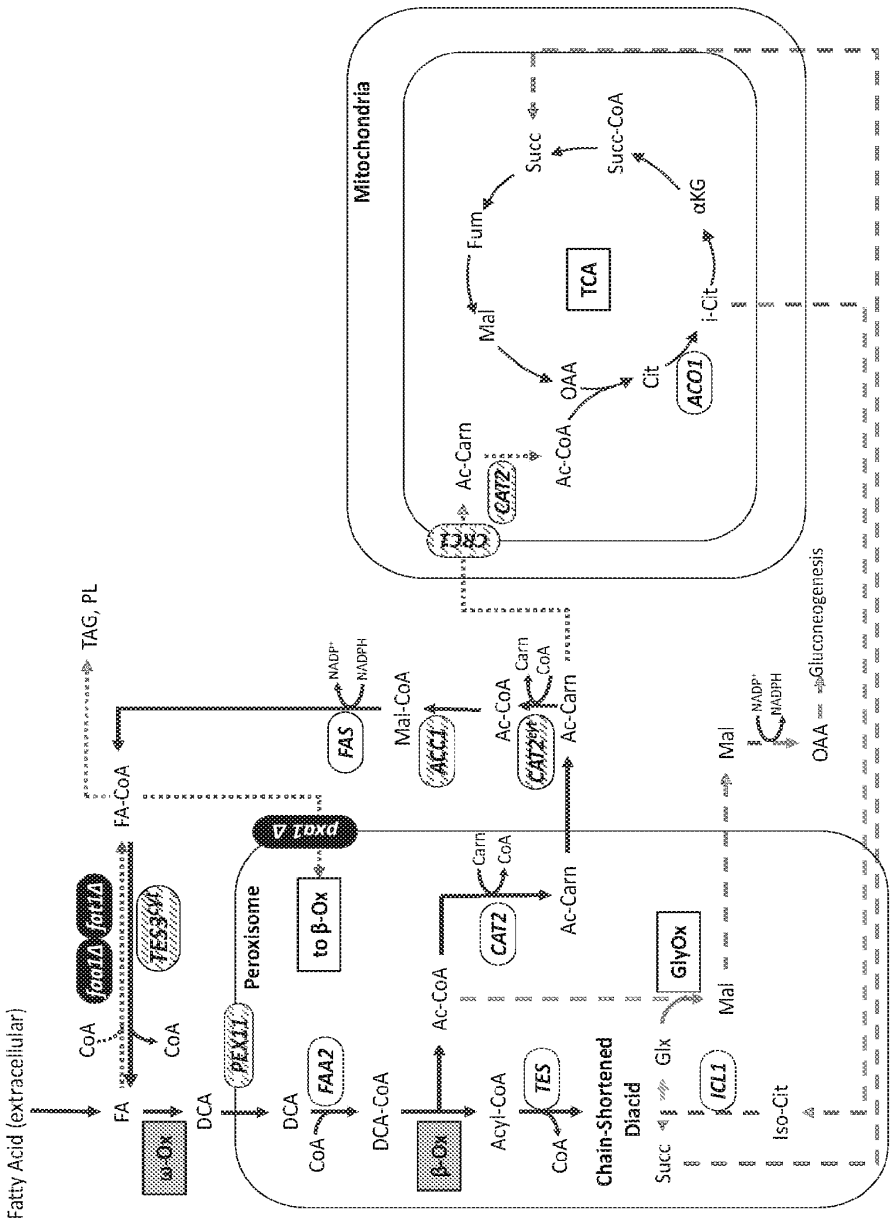

Fatty Acid (extracellular)
FA
CoA
ω-Ox
DCA
PEX11
Peroxisome
DCA
CoA
FAA2
DCA-CoA
β-Ox
Acyl-CoA
TES
CoA
Chain-Shortened Diacid
Succ
Glx
ICL1
Iso-Cit
Mal
GlyOx
Ac-CoA
CAT2
Carn
CoA
Ac-Carn
Pxa1 Δ
to β-Ox
FA-CoA
TAG, PL
FAS
NADP+
NADPH
Mal-CoA
ACC1
Ac-CoA
CAT2(P)
Carn
CoA
Ac-Carn
Mal
NADP+
NADPH
OAA
Gluconeogenesis
Mitochondria
CRC1
Ac-Carn
CAT2
Ac-CoA
OAA
Cit
ACO1
i-Cit
αKG
Succ-CoA
Succ
Fum
Mal
TCA

FIG. 5

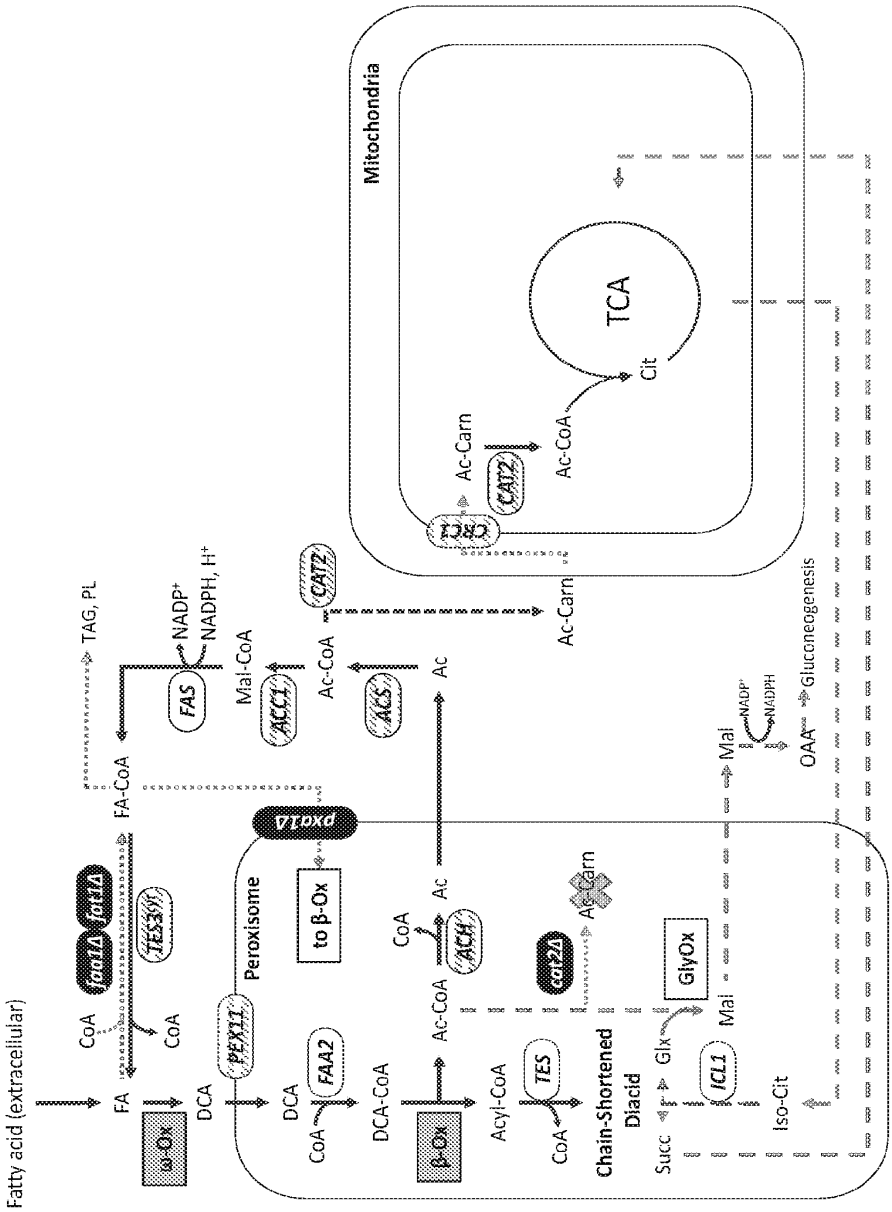

Fatty acid (extracellular)
FA
CoA
FAT1
FAT4
TES3
CoA
FA-CoA
TAG, PL
ω-Ox
DCA
PEX11
Peroxisome
FAS
NADP+
NADPH, H+
Mal-CoA
ACC1
Ac-CoA
CAT2
ACS
Ac
Ac-Carn
Mitochondria
CRC1
Ac-Carn
CAT2
Ac-CoA
TCA
Cit
POX1A
DCA
CoA
FAA2
DCA-CoA
to β-Ox
β-Ox
CoA
Ac
Ac-CoA
ACH
Acyl-CoA
TES
CoA
FAT2A
Ac-Carn
Chain-Shortened
Diacid
Succ
Glx
GlyOx
ICL1
Mal
Iso-Cit
Mal
NADP+
NADPH
OAA
Glucneogenesis

FIG. 6

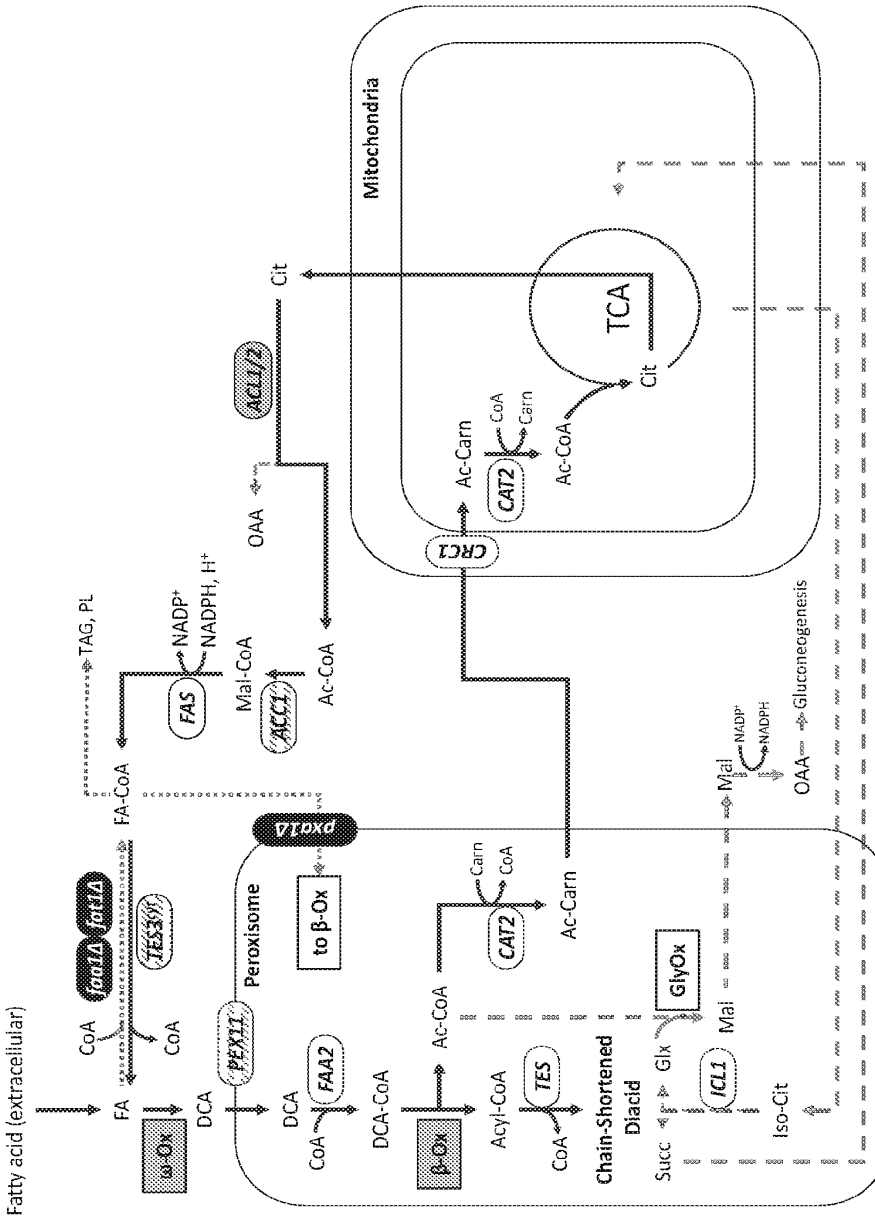

Fatty acid (extracellular)
FA
CoA
CoA
ω-Ox
DCA
PEX11
Peroxisome
DCA
CoA
FAA2
DCA-CoA
β-Ox
Ac-CoA
Acyl-CoA
CoA
TES
Chain-Shortened Diacid
Succ
Glx
ICL1
Iso-Cit
Mal
GlyOx
Carn
CoA
CAT2
Ac-Carn
to β-Ox
pxo1A
FA-CoA
TAG, PL
FAS
NADP+
NADPH, H+
Mal-CoA
ACC1
Ac-CoA
OAA
ACL1/2
Cit
CRC1
Mitochondria
Ac-Carn
CoA
Carn
CAT2
Ac-CoA
TCA
Cit
Mal
NADP+
NADPH
OAA
Gluconeogenesis

FIG. 7

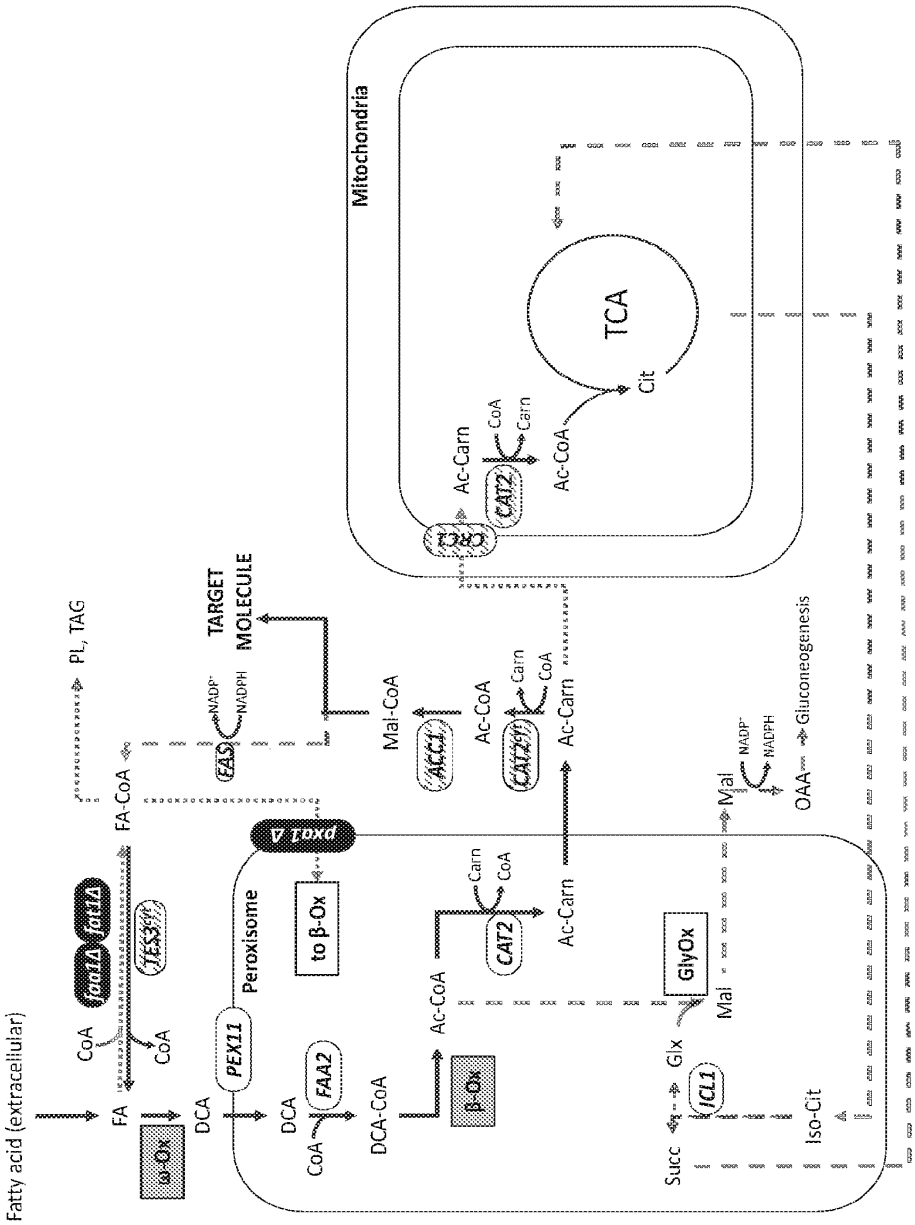

Fatty acid (extracellular)
FA
CoA
ω-Ox
DCA
PEX11
Peroxisome
DCA
CoA
FAA2
DCA-CoA
β-Ox
Ac-CoA
Carn
CoA
CAT2
Ac-Carn
to β-Ox
Δ POX
FA-CoA
CoA
TES3(P)
PL, TAG
FAD
NADP+
NADPH
TARGET MOLECULE
Mal-CoA
ACC1
Ac-CoA
CAT2(P)
Carn
CoA
Ac-Carn
Succ
Glx
ICL1
Iso-Cit
GlyOx
Mal
Mal
NADP+
NADPH
OAA
Gluconeogenesis
Mitochondria
CRC
Ac-Carn
CAT2
CoA
Carn
Ac-CoA
TCA
Cit

FIG. 8

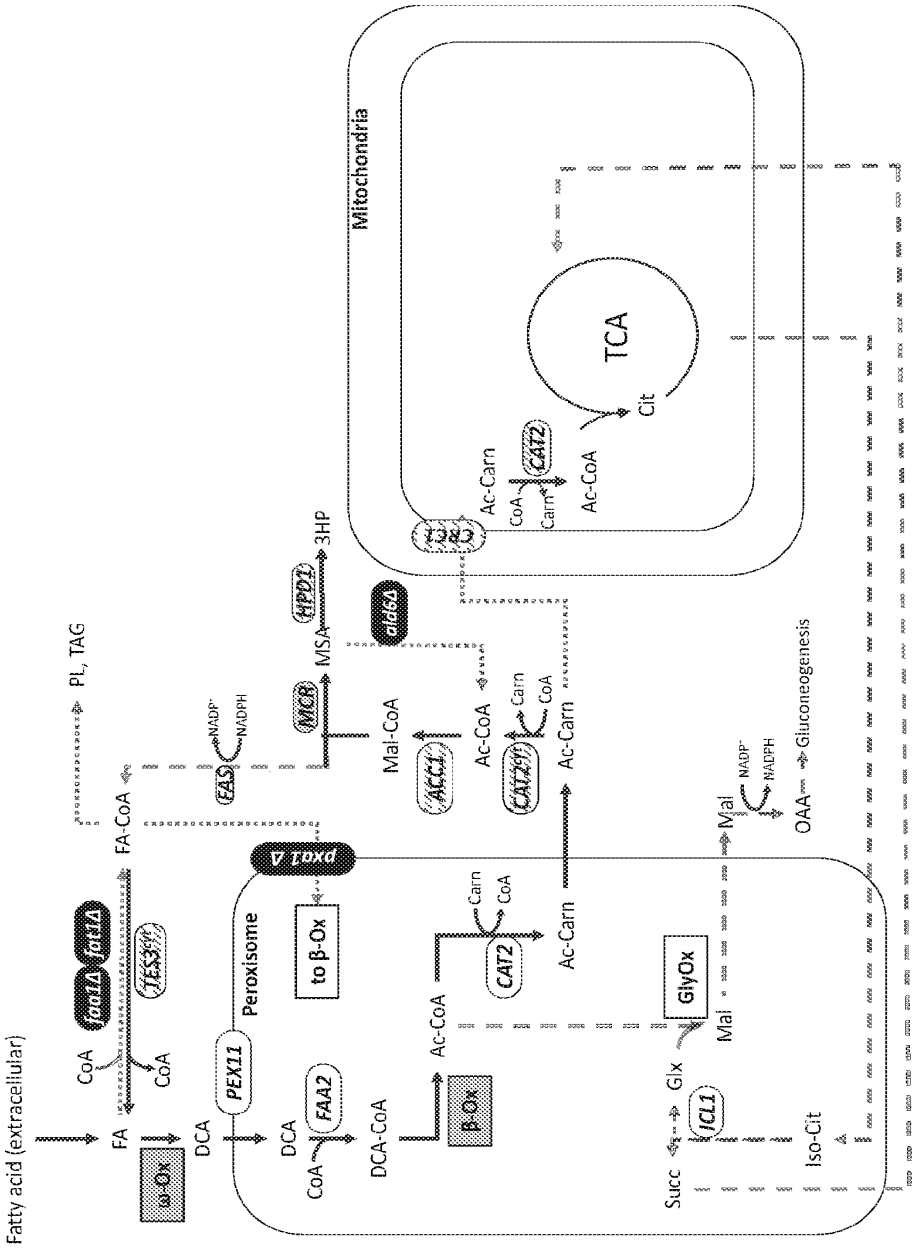

Fatty acid (extracellular)
FA
ω-Ox
DCA
CoA
FA-CoA
PL, TAG
Peroxisome
PEX11
FAA2
DCA
CoA
DCA-CoA
β-Ox
Ac-CoA
to β-Ox
Pxa1 Δ
CAT2
Carn
CoA
Ac-Carn
Succ
Glx
ICL1
Iso-Cit
GlyOx
Mal
NADP+
NADPH
OAA
Gluconeogenesis
FAD
MCR
MSA
HPD1
3HP
Mal-CoA
ACC1
CRC1
Mitochondria
TCA
Cit
Ac-CoA

FIG. 9

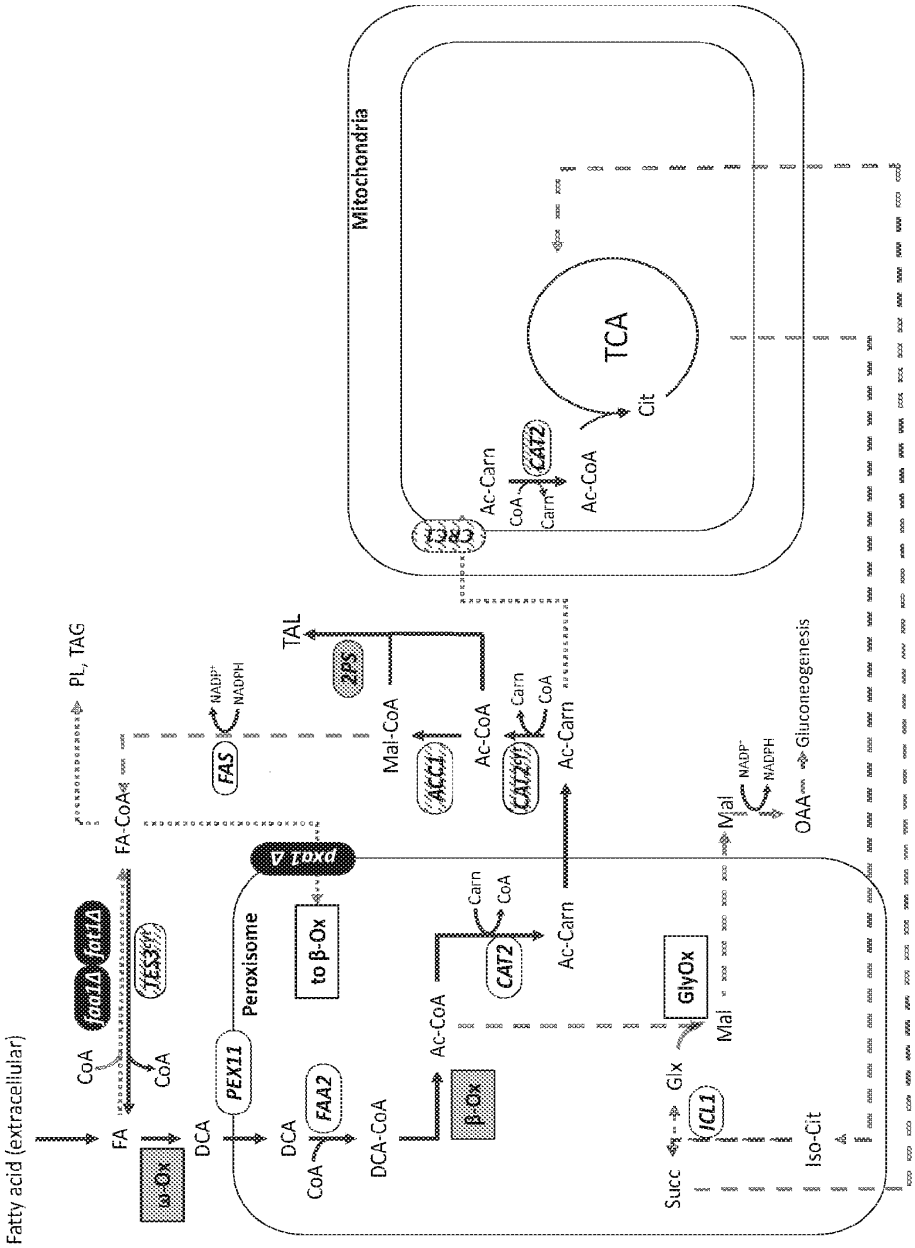

Fatty acid (extracellular)
FA
CoA
CoA
ω-Ox
DCA
PEX11
Peroxisome
DCA
CoA
FAA2
DCA-CoA
β-Ox
Ac-CoA
CAT2
Carn
CoA
Ac-Carn
Δ POX1
to β-Ox
FA-CoA
PL, TAG
FAS
NADP+
NADPH
TAL
2PS
Mal-CoA
ACC1
Ac-CoA
CAT2(p)
Carn
CoA
Ac-Carn
Succ
Glx
ICL1
Iso-Cit
Mal
GlyOx
Mal
NADP+
NADPH
OAA
Gluconeogenesis
Mitochondria
CRC1
Ac-Carn
CoA
Carn
CAT2
Ac-CoA
TCA
Cit

FIG. 10

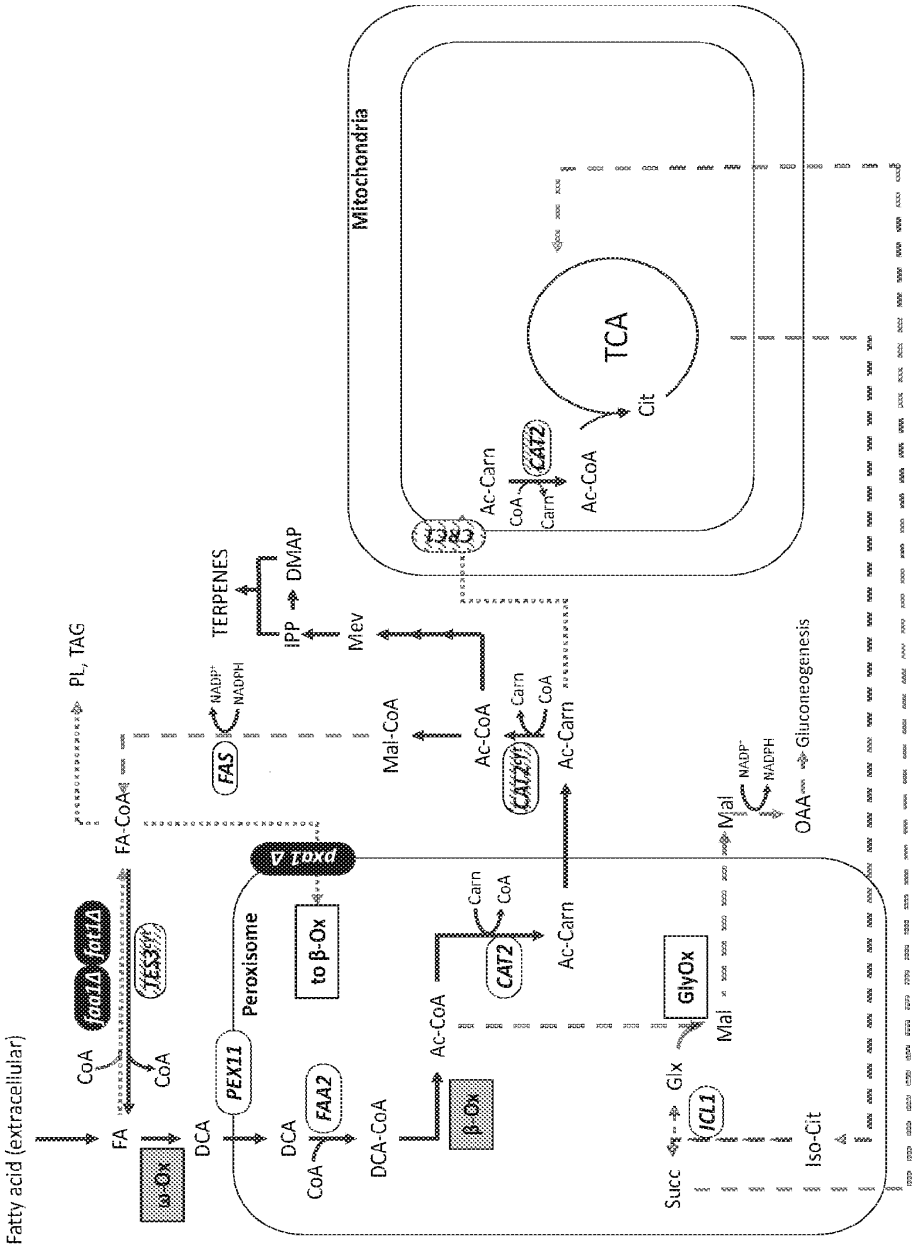

Fatty acid (extracellular)
FA
ω-Ox
DCA
CoA
FA-CoA
POX4 POX5
TES3(P)
PEX11
Peroxisome
DCA
CoA
FAA2
DCA-CoA
β-Ox
Ac-CoA
Carn
CoA
CAT2
Ac-Carn
POX1-6
to β-Ox
Ac-CoA
Carn
CoA
CAT2(P)
Ac-Carn
FAS
NADP+
NADPH
PL, TAG
TERPENES
iPP
DMAP
Mev
Mal-CoA
Ac-CoA
Carn
CoA
Ac-Carn
Succ
Glx
ICL1
Iso-Cit
GlyOx
Mal
Mal
NADP+
NADPH
OAA
Gluconeogenesis
Mitochondria
CRC1
Ac-Carn
CoA
Carn
CAT2
Ac-CoA
TCA
Cit

FIG. 11

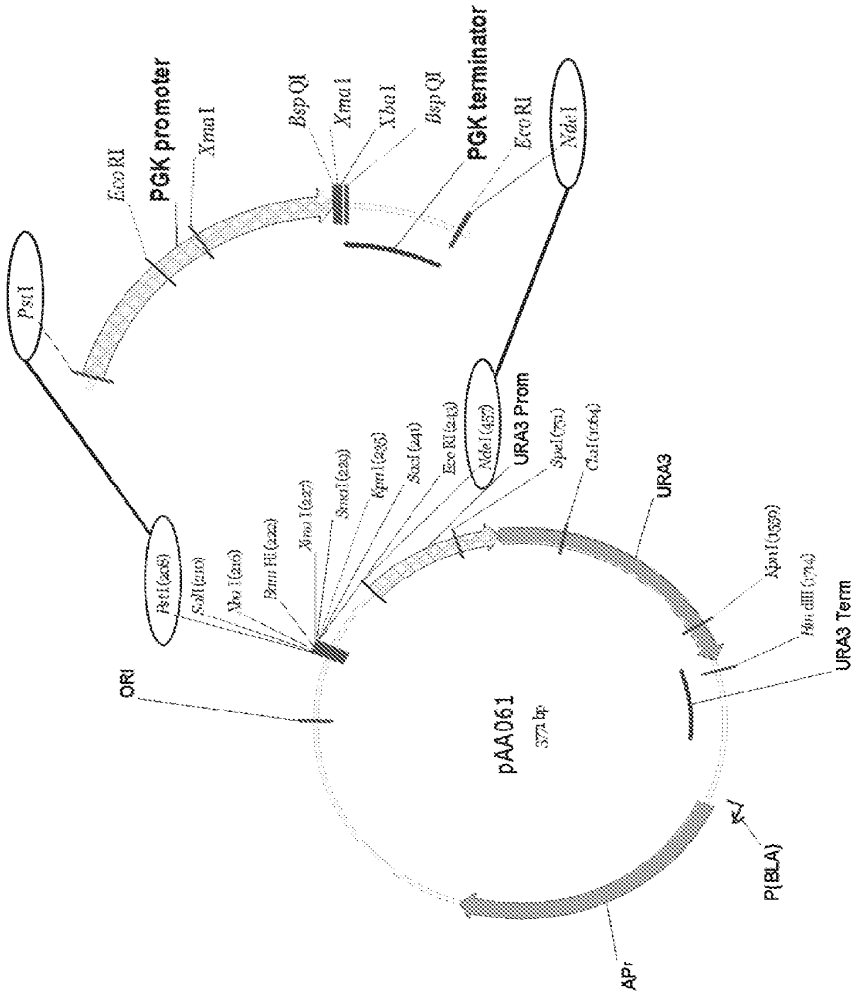

PGK promoter
PGK terminator
URA3 Prom
URA3
URA3 Term
ORI
pAA061
3774 bp
P(BLA)
APr
Pst I
Eco RI
Xma I
Bsp QI
Xba I
Nde I
Sal I
Xho I
Sma I
Kpn I
Sac I
Spe I
Cla I
Apa I
Hin dIII

FIG. 12

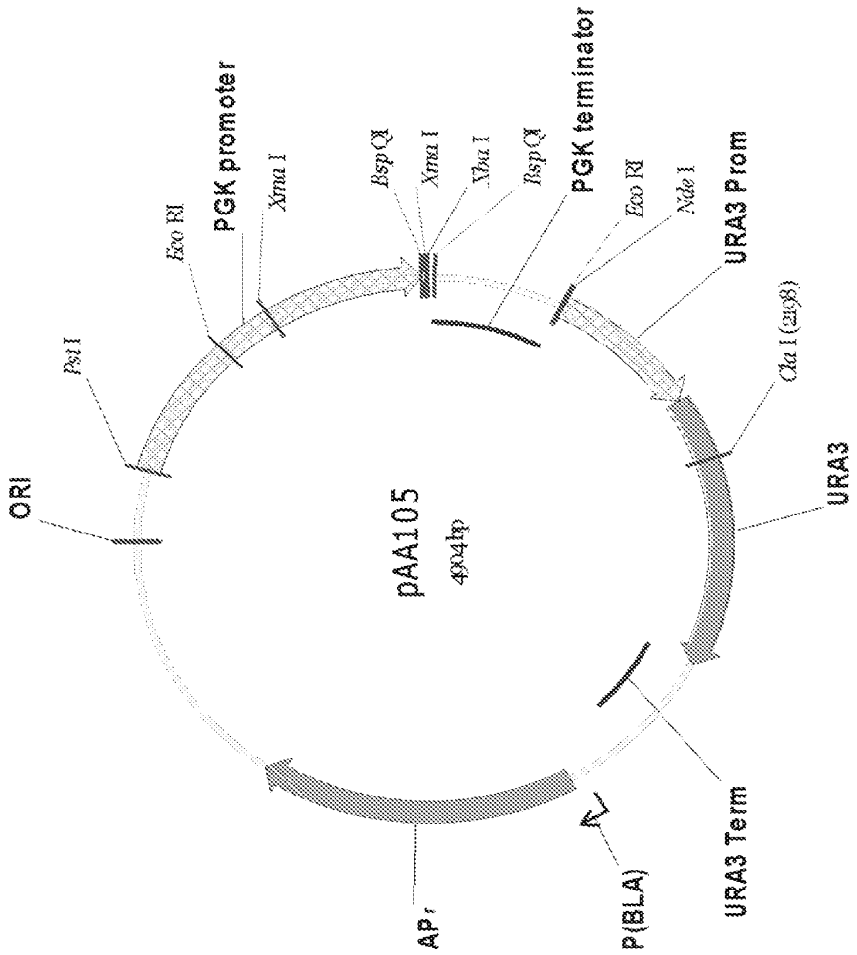

pAA105
4904p
ORI
Pst I
Eco RI
PGK promoter
Xma I
Bsp QI
Xma I
Xba I
Bsp QI
PGK terminator
Eco RI
Nde I
URA3 Prom
Cla I (2µB)
URA3
URA3 Term
P(BLA)
AP r

FIG. 13

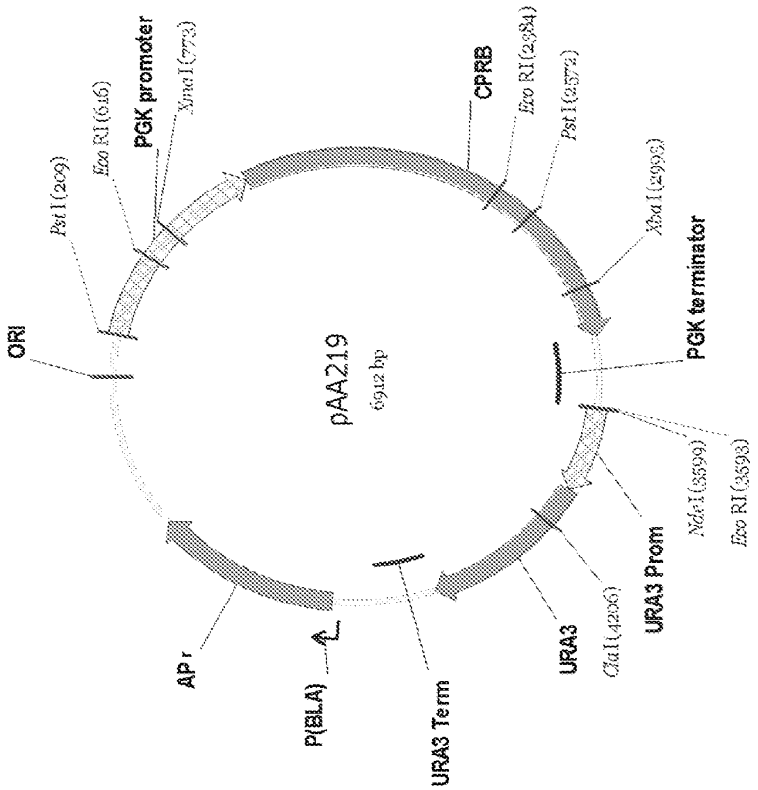

pAA219
6912 bp
ORI
Pst I (209)
Eco RI (646)
PGK promoter
Xma I (773)
CPRB
Eco RI (2384)
Pst I (2572)
Xba I (2993)
PGK terminator
Nde I (3599)
Eco RI (3593)
URA3 Prom
URA3
Cla I (4296)
URA3 Term
P(BLA)
AP r

FIG. 14

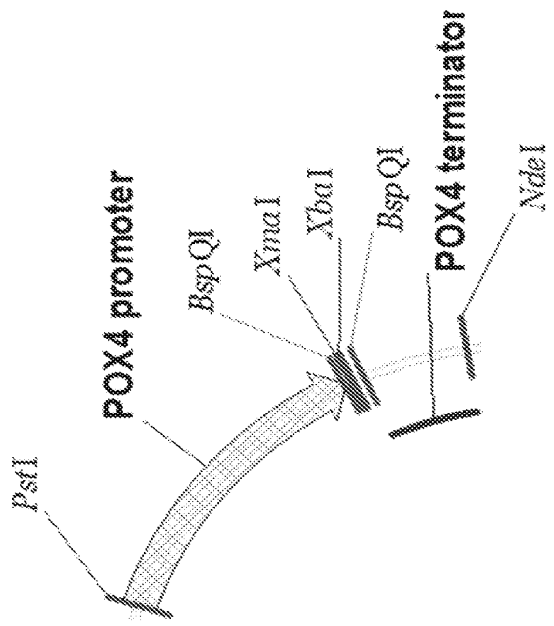

POX4 promoter
POX4 terminator
PstI
BspQI
XmaI
XbaI
BspQI
NdeI

FIG. 15

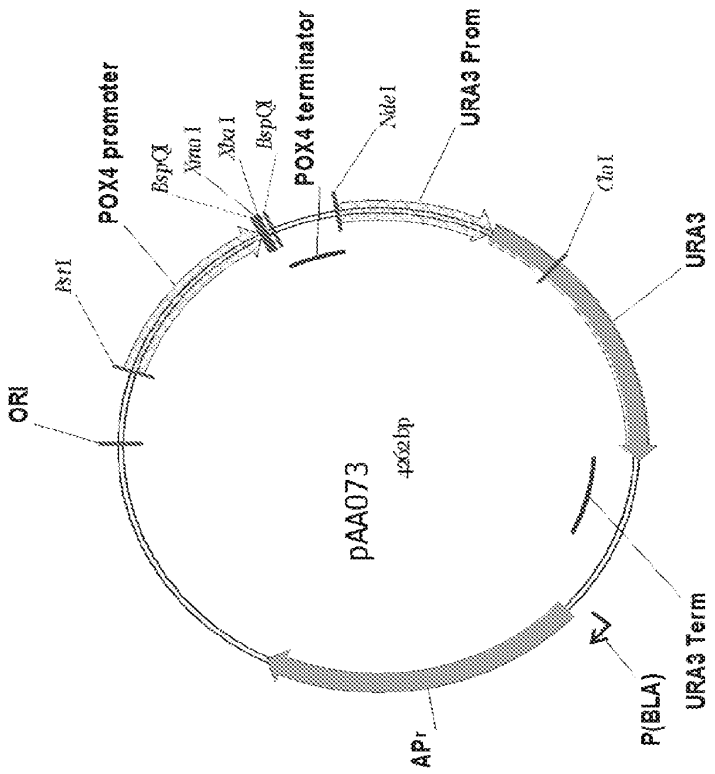

POX4 promoter
BspQI
XmaI
XbaI
BspQI
POX4 terminator
NdeI
URA3 Prom
ClaI
URA3
BstI
ORI
pAA073
4962bp
APr
P(BLA)
URA3 Term

FIG. 16

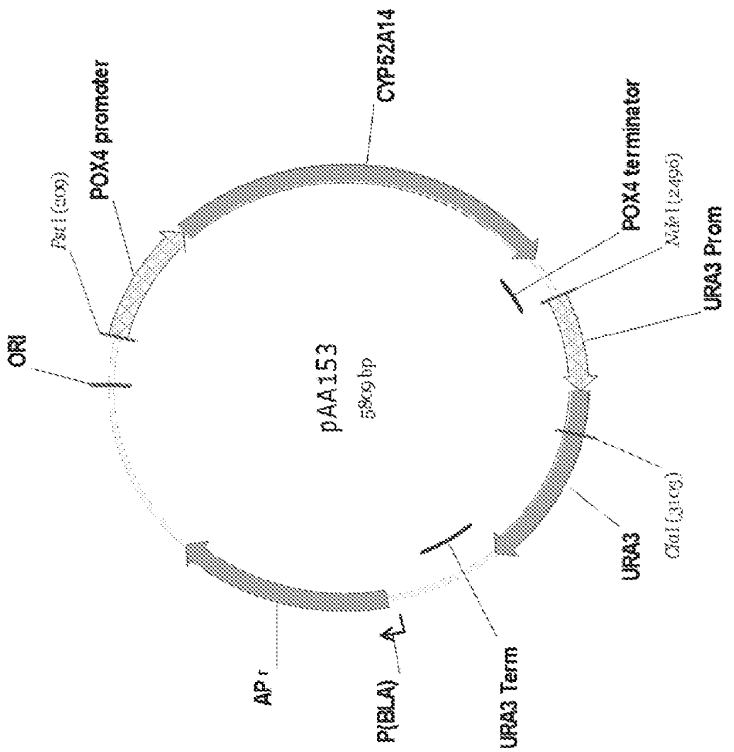

CYP52A14
POX4 promoter
POX4 terminator
URA3 Prom
ORI
pAA153
5805 bp
URA3
URA3 Term
P(BLA)
AP r

FIG. 17

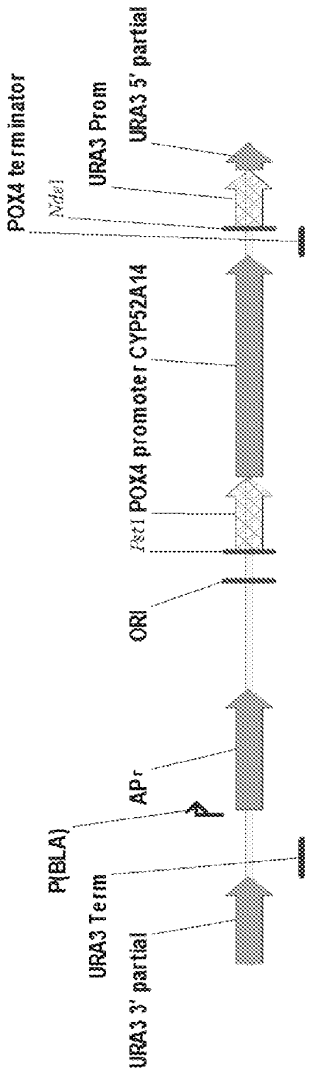

POX4 terminator
NdeI
URA3 Prom
URA3 5' partial
PstI POX4 promoter CYP52A14
ORI
APr
P(BLA)
URA3 Term
URA3 3' partial
pAA153 linearized at ClaI
5809bp

FIG. 18

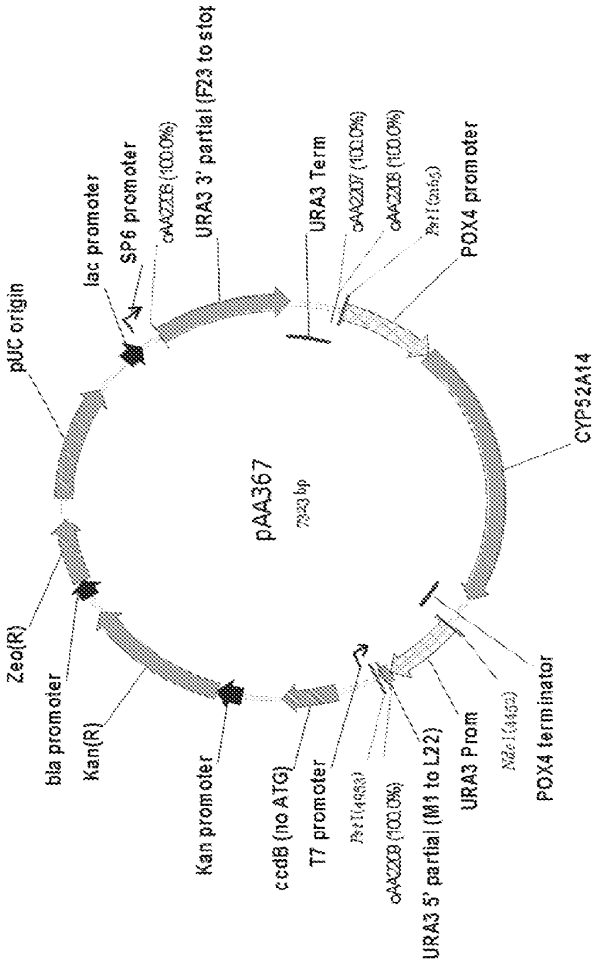

pAA367
7923 bp
pUC origin
lac promoter
SP6 promoter
URA3 3' partial (F23 to stop
URA3 Term
POX4 promoter
CYP52A14
POX4 terminator
URA3 Prom
URA3 5' partial (M1 to L22)
T7 promoter
ccdB (no ATG)
Kan promoter
Kan(R)
bla promoter
Zeo(R)

FIG. 19

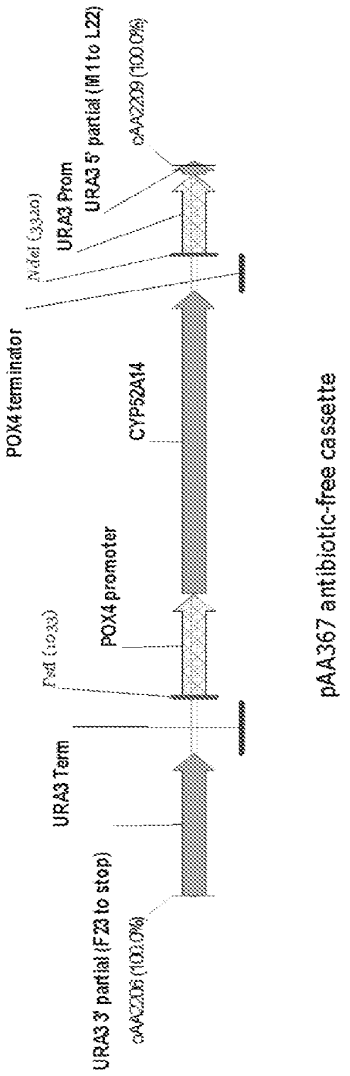

URA3 3' partial (F23 to stop)
cAA2206 (100.0%)
URA3 Term
FseI (6933)
POX4 promoter
CYP52A14
POX4 terminator
AvrII (9320)
URA3 Prom
URA3 5' partial (M1 to L22)
cAA2209 (100.0%)
pAA367 antibiotic-free cassette
3804 bp

FIG. 20

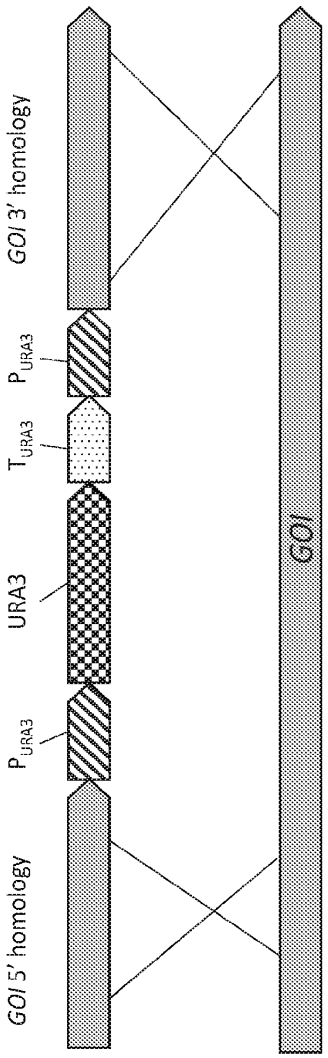



FIG. 21A

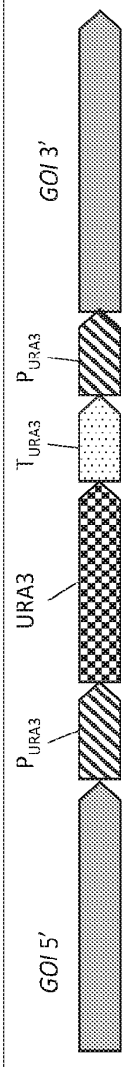



FIG. 21B

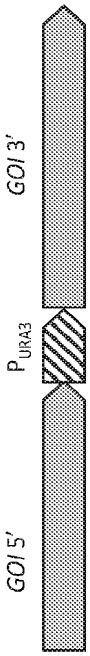



FIG. 21C

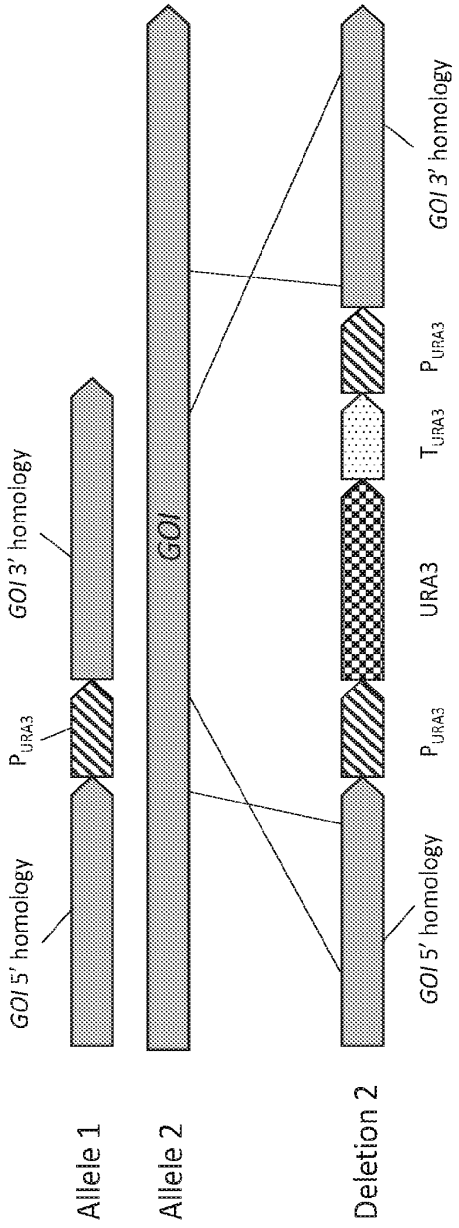

GOI 5' homology
P URA3
GOI 3' homology
GOI
URA3
T URA3
P URA3
GOI 3' homology
GOI 5' homology
P URA3
Allele 1
Allele 2
Deletion 2

FIG. 22

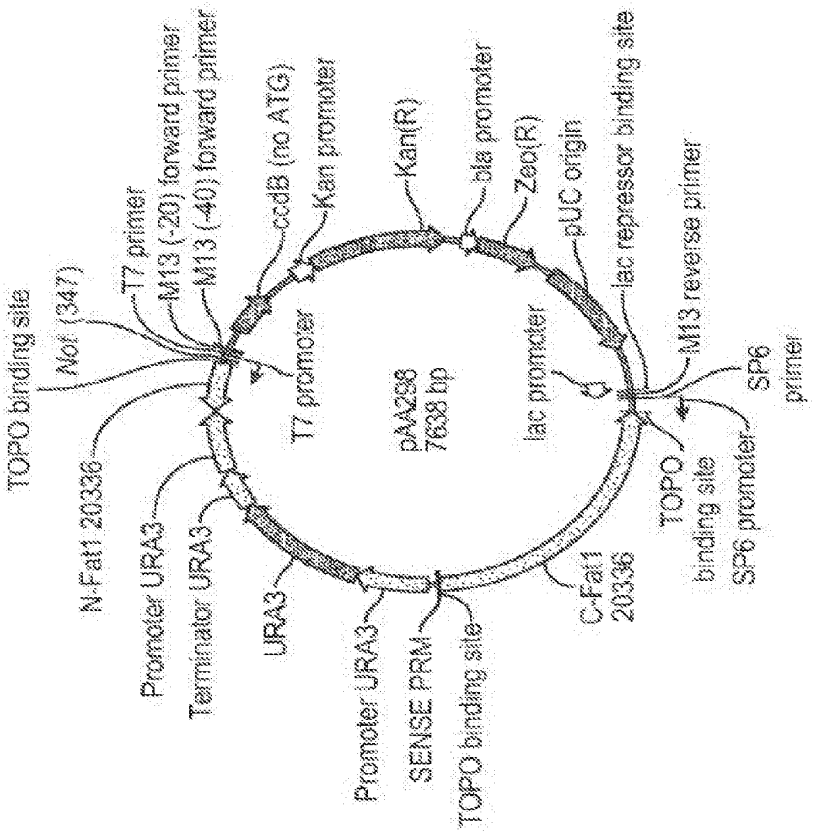

TOPO binding site
Not (347)
T7 primer
M13 (-20) forward primer
M13 (-40) forward primer
ccdB (no ATG)
Kan promoter
Kan(R)
bla promoter
Zeo(R)
pUC origin
lac repressor binding site
M13 reverse primer
SP6 primer
N-Fat1 20336
Promoter URA3
Terminator URA3
URA3
Promoter URA3
SENSE PRM
TOPO binding site
T7 promoter
pAA238
7638 bp
lac promoter
C-Fat1 20336
TOPO binding site
SP6 promoter

FIG. 23


pAA1519
6486 bp
pUC origin
lac promoter
SP6 promoter
oAA2377 (100.0%)
Cv CAT2 3' homology
Promoter URA3
Terminator URA3
Cv URA3
Promoter URA3
Cv CAT2 5' homology
oAA2372 (100.0%)
T7 promoter
ccdB (no ATG)
Kan promoter
Kan(R)
bla promoter
Zeo(R)
pAA1520
7028 bp
pUC origin
lac promoter
SP6 promoter
oAA2372 (100.0%)
Cv CAT2 5' homology
Promoter URA3
Terminator URA3
Cv URA3
Promoter URA3
Cv CAT2 3' homology
oAA2377 (100.0%)
T7 promoter
ccdB (no ATG)
Kan promoter
Kan(R)
bla promoter
Zeo(R)

FIG. 24

FIG. 25A

```
                                                1                                                50
CvCAT2 from pAA426  (1) MFNFKLSQQVLKNSTKSLMPILKKPFSTSHAKGDLFKYQSQLPKLPVFTL
          CvCAT2(-mts)  (1) ----------------------------MPILKKPFSTSHAKGDLFKYQSQLPKLPVFTL
     CvCAT2(-mts-pts)   (1) ----------------------------MPILKKPFSTSHAKGDLFKYQSQLPKLPVFTL
          CvCAT2(-pts)  (1) MFNFKLSQQVLKNSTKSLMPILKKPFSTSHAKGDLFKYQSQLPKLPVFTL
```

FIG. 25B

```
                                                601                                              628
CvCAT2 from pAA426  (601) KWYLVDSANEMKDVLTKGLLTDAKPKL-
          CvCAT2(-mts)  (583) KWYLVDSANEMKDVLTKGLLTDAKPKL-
     CvCAT2(-mts-pts)   (583) KWYLVDSANEMKDVLTKGLLTDAK----
          CvCAT2(-pts)  (601) KWYLVDSANEMKDVLTKGLLTDAK-----
```

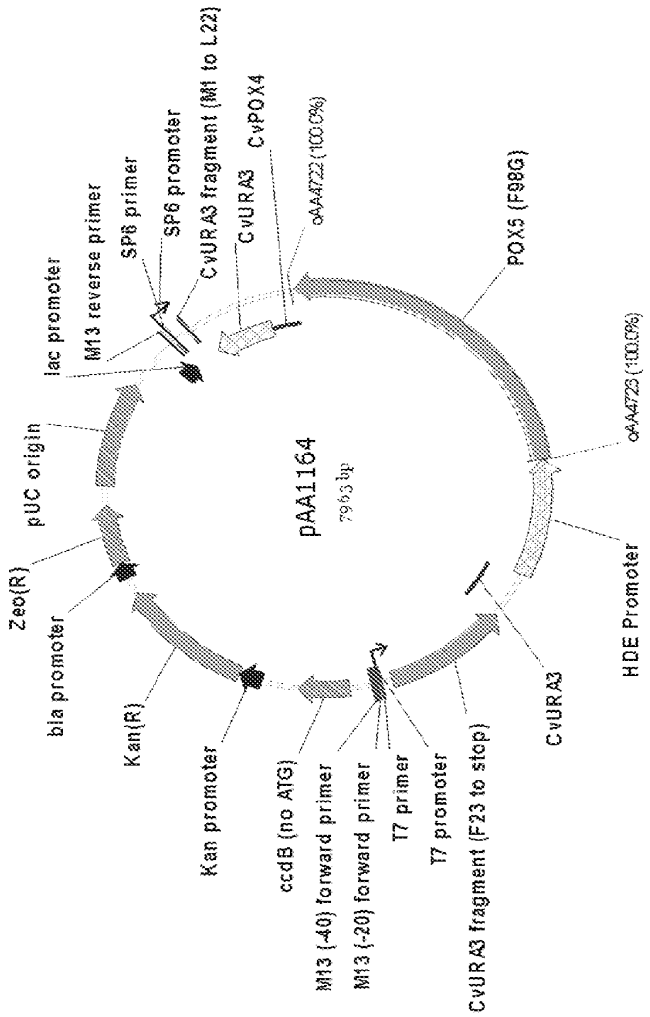

pAA1164
7963 bp
lac promoter
M13 reverse primer
SP6 primer
SP6 promoter
CvURA3 fragment (M1 to L22)
CvURA3
CvPOX4
oAA4722 (100.0%)
POX5 (F98G)
oAA4723 (100.0%)
HDE Promoter
CvURA3
CvURA3 fragment (F23 to stop)
T7 promoter
T7 primer
M13 (-20) forward primer
M13 (-40) forward primer
ccdB (no ATG)
Kan promoter
Kan(R)
bla promoter
Zeo(R)
pUC origin

FIG. 26

```
                         1                                                50
CvYat1p            (1) ---------------------------------MSTYQFQETLEKLPIPD
CvYat1p+CAT2mts    (1) MFNFKLSQQVLKNSTKSIMPILKKP-FSTSHAKGSTYQFQETLEKLPIPD
CvYAT1p+CIT1mts    (1) -------MSALRSFQRSSNVAKST--LKNSVRTYSTYQFQETLEKLPIPD
CvYat1p+COX4mts    (1) ----MLSRTTLRVARQQTRLLSTSRILFNSKTDQSTYQFQETLEKLPIPD
```

FIG. 27

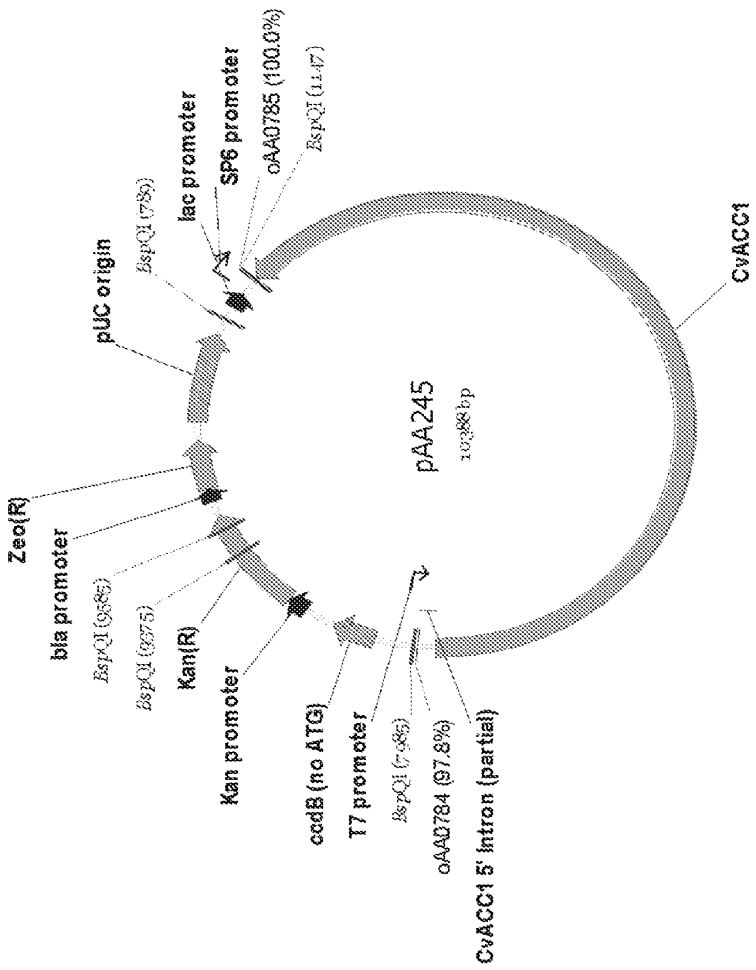

pAA245
10388 bp
Zeo(R)
bla promoter
EspQI (9585)
EspQI (9575)
Kan(R)
Kan promoter
ccdB (no ATG)
T7 promoter
EspQI (7585)
oAA0784 (97.8%)
CvACC1 5' intron (partial)
CvACC1
EspQI (1147)
oAA0785 (100.0%)
SP6 promoter
lac promoter
EspQI (1785)
pUC origin

FIG. 28

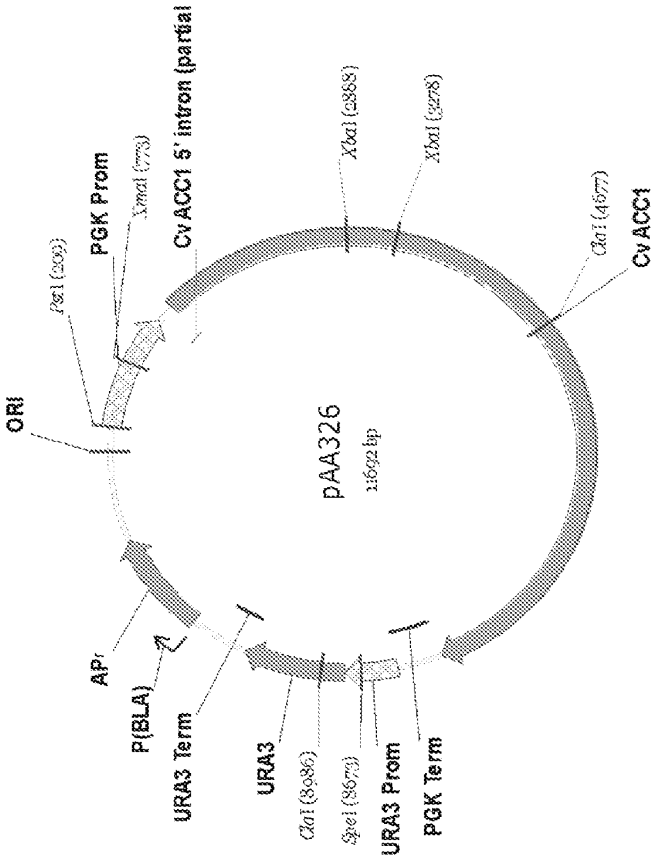

pAA326
16692 bp
ORI
PGK Prom
Cv ACC1 5' intron (partial
Cv ACC1
AP^r
P(BLA)
URA3 Term
URA3
URA3 Prom
PGK Term
PstI (209)
XmaI (773)
XbaI (2858)
XbaI (3278)
ClaI (4677)
ClaI (8686)
SpeI (8673)

FIG. 29

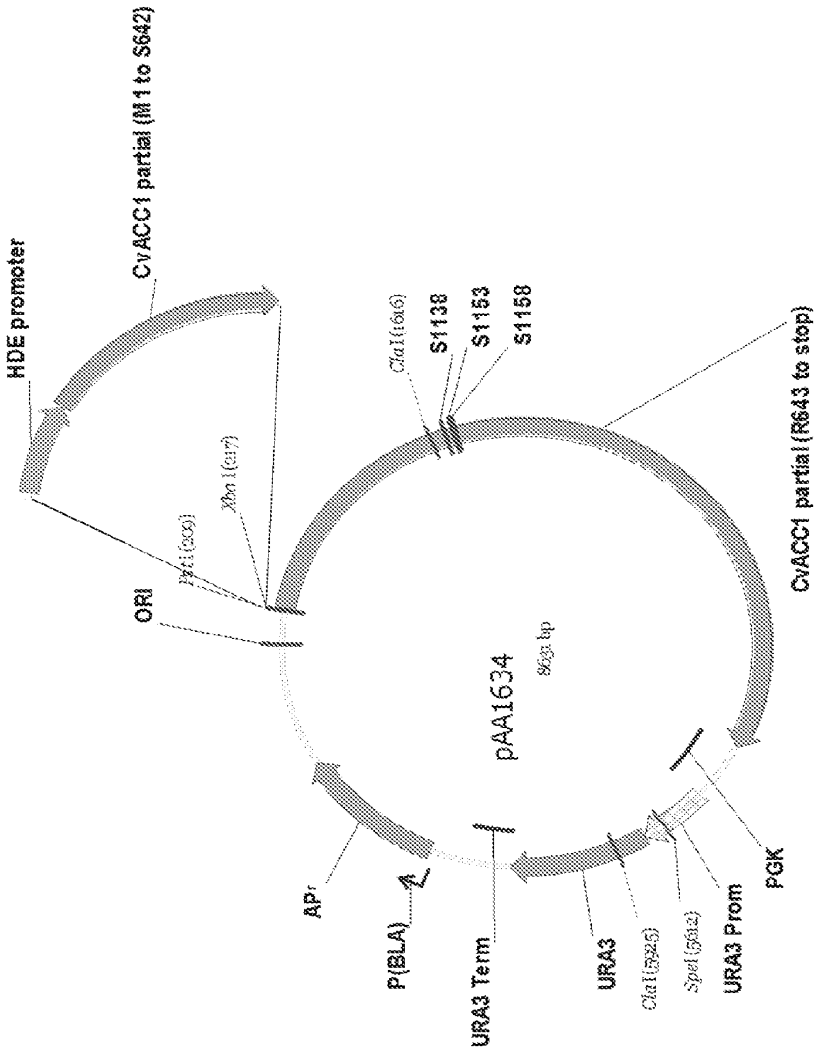

HDE promoter
CvACC1 partial (M1 to S642)
ClaI (1616)
S1138
S1153
S1158
CvACC1 partial (R643 to stop)
XbaI (2317)
PstI (2500)
ORI
pAA1634
8633 bp
AP^r
P(BLA)
URA3 Term
URA3
ClaI (5926)
SpeI (5932)
URA3 Prom
PGK

FIG. 30

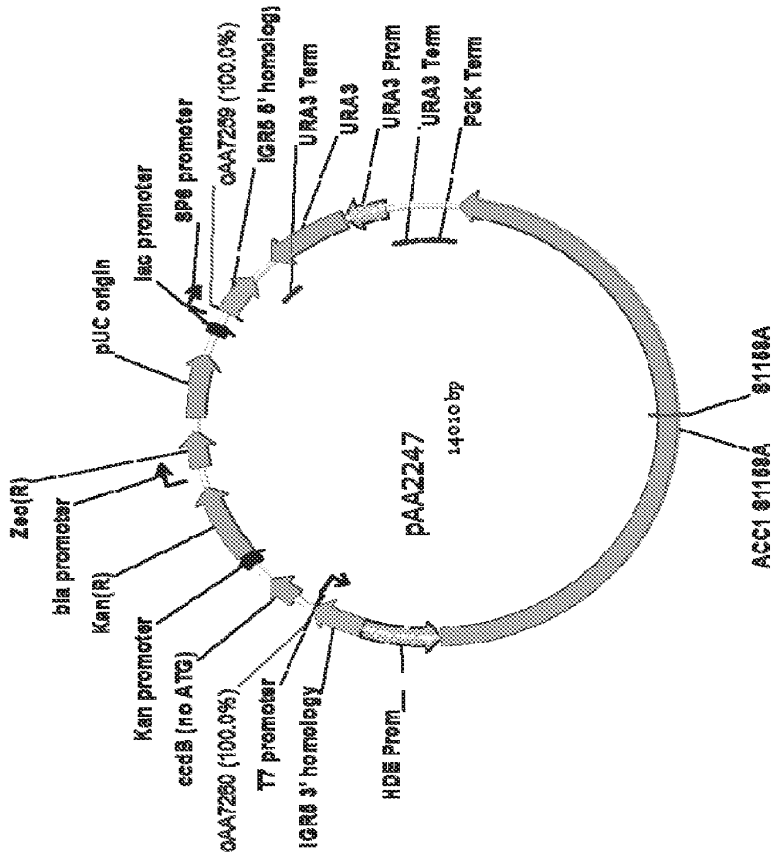

pAA2247
14010 bp
Zeo(R)
bla promoter
Kan(R)
Kan promoter
ccdB (no ATG)
pAA7280 (100.0%)
T7 promoter
IGR8 3' homology
HDE Prom
ACC1 81188A
81188A
pUC origin
lac promoter
8P6 promoter
pAA7259 (100.0%)
IGR8 5' homolog
URA3 Term
URA3
URA3 Prom
URA3 Term
PGK Term

FIG. 31


pAA1613
5901 bp
pUC origin
lac promoter
SP6 promoter
pAA5512 (100.0%)
CRC1 5' homology
URA3 Prom
URA3
URA3 Term
URA3 Prom
CRC1 5' homology
pAA5511 (100.0%)
T7 promoter
ccdB (no ATG)
Kan promoter
Kan(R)
bla promoter
Zeo(R)
pAA1701
6054 bp
pUC origin
lac promoter
SP6 promoter
pAA5512 (100.0%)
CRC1 3' homology
URA3 Prom
URA3 Term
URA3
URA3 Prom
CRC1 5' homology
pAA5511 (100.0%)
T7 promoter
ccdB (no ATG)
Kan promoter
Kan(R)
bla promoter
Zeo(R)

FIG. 32

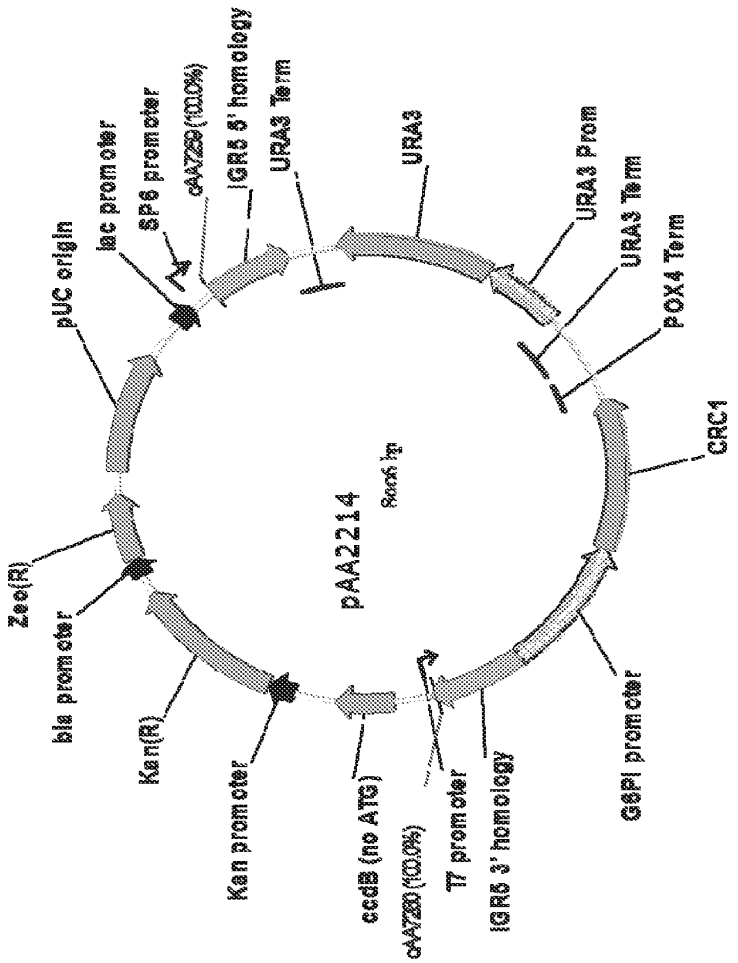

pAA2214
8xxx bp
pUC origin
lac promoter
SP6 promoter
cAA7250 (100.0%)
IGR5 5' homology
URA3 Term
URA3
URA3 Prom
URA3 Term
POX4 Term
CRC1
G6PI promoter
IGR5 3' homology
T7 promoter
cAA7250 (100.0%)
ccdB (no ATG)
Kan promoter
Kan(R)
bla promoter
Zeo(R)

FIG. 33

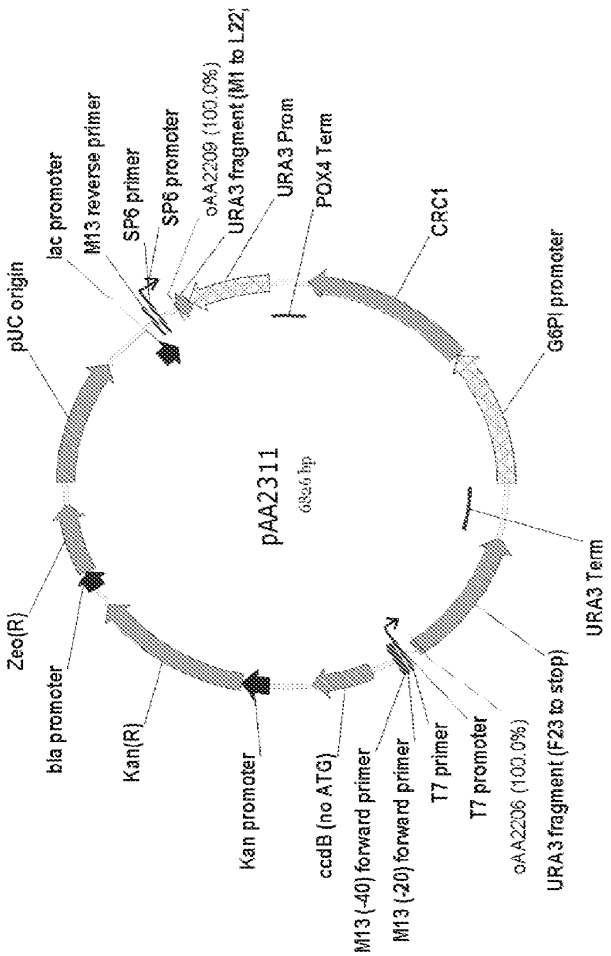

pAA2311
6826 bp
pUC origin
lac promoter
M13 reverse primer
SP6 primer
SP6 promoter
oAA2209 (100.0%)
URA3 fragment (M1 to L22)
URA3 Prom
POX4 Term
CRC1
G6PI promoter
URA3 Term
URA3 fragment (F23 to stop)
oAA2206 (100.0%)
T7 promoter
T7 primer
M13 (-20) forward primer
M13 (-40) forward primer
ccdB (no ATG)
Kan promoter
Kan(R)
bla promoter
Zeo(R)

FIG. 34

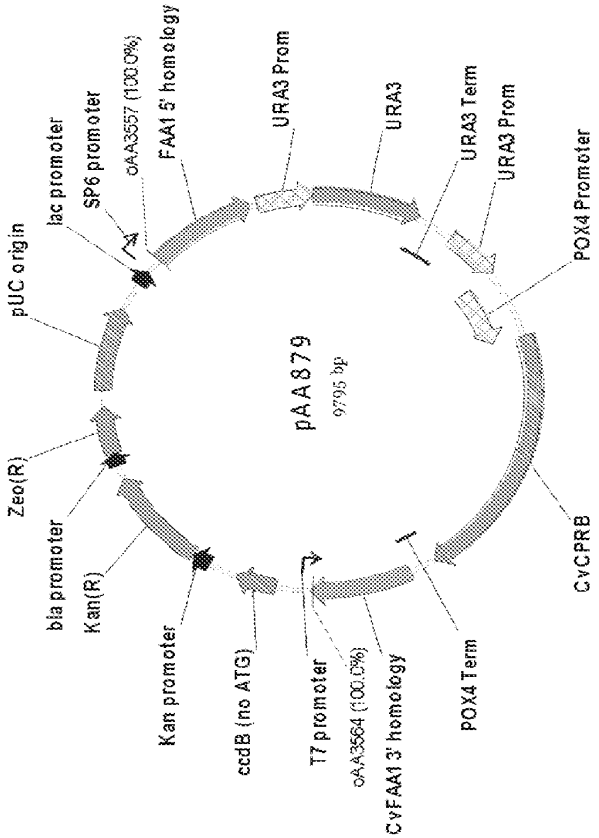

pAA879
9795 bp
pUC origin
lac promoter
SP6 promoter
oAA3557 (100.0%)
FAA1 5' homology
URA3 Prom
URA3
URA3 Term
URA3 Prom
POX4 Promoter
CvCPRB
POX4 Term
CvFAA1 3' homology
oAA3564 (100.0%)
T7 promoter
ccdB (no ATG)
Kan promoter
Kan(R)
bla promoter
Zeo(R)

FIG. 35

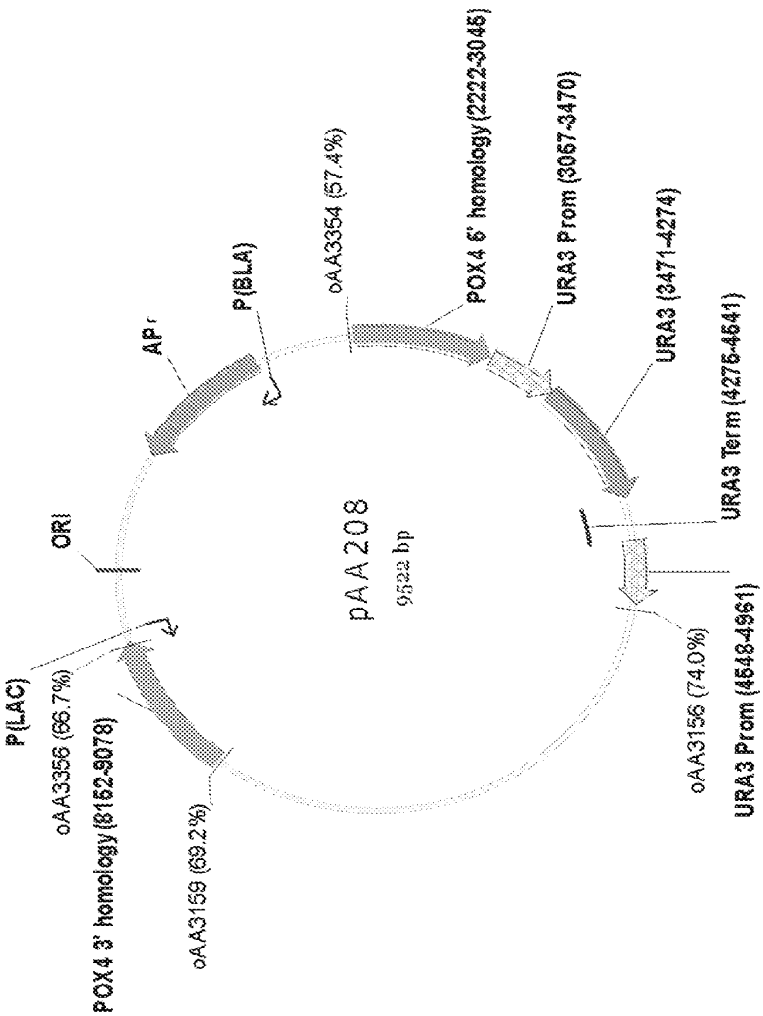

pAA208
9522 bp
P(LAC)
oAA3356 (66.7%)
POX4 3' homology (8162-9078)
oAA3159 (69.2%)
ORI
AP r
P(BLA)
oAA3354 (57.4%)
POX4 5' homology (2222-3045)
URA3 Prom (3057-3470)
URA3 (3471-4274)
URA3 Term (4276-4641)
oAA3156 (74.0%)
URA3 Prom (4648-4961)

FIG. 36

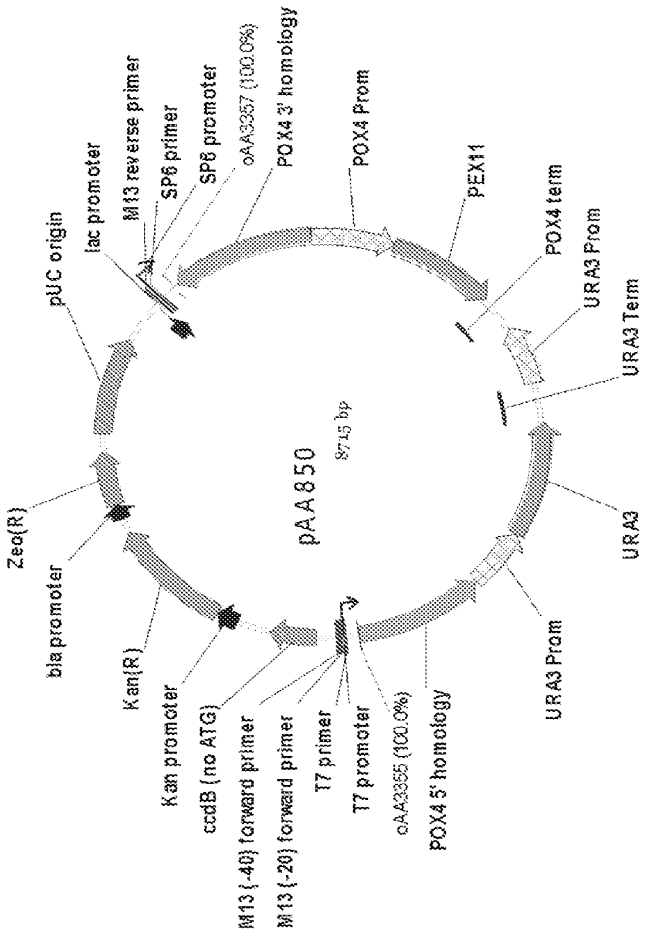
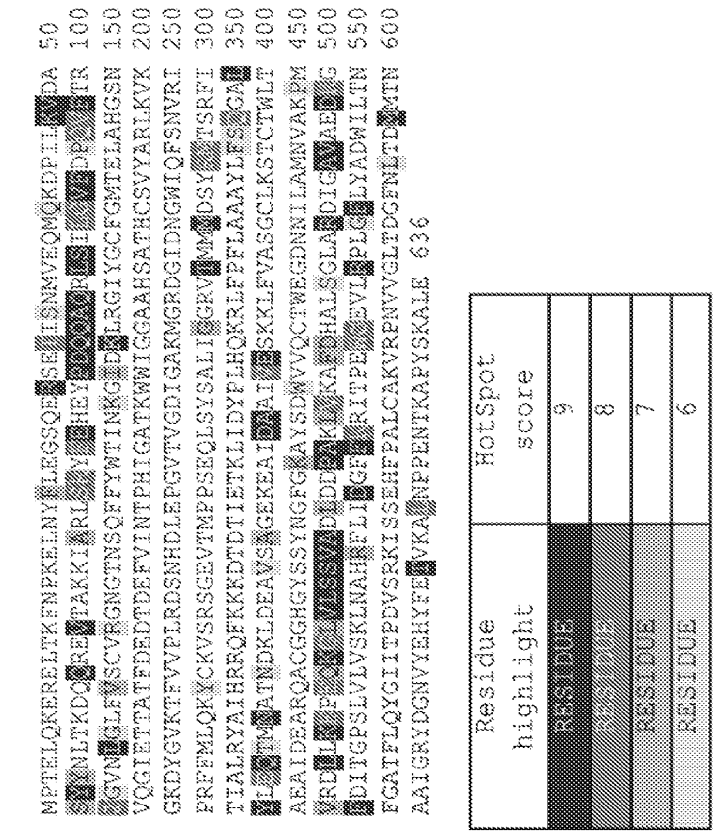

pAA850
8745 bp
pUC origin
lac promoter
M13 reverse primer
SP6 primer
SP6 promoter
oAA3357 (100.0%)
POX4 3' homology
POX4 Prom
PEX11
POX4 term
URA3 Prom
URA3 Term
URA3
URA3 Prom
POX4 5' homology
oAA3355 (100.0%)
T7 promoter
T7 primer
M13 (-20) forward primer
M13 (-40) forward primer
ccdB (no ATG)
Kan promoter
Kan(R)
bla promoter
Zeo(R)

FIG. 37




| Residue highlight | HotSpot score |
|---|---|
| RESIDUE | 9 |
| RESIDUE | 8 |
| RESIDUE | 7 |
| RESIDUE | 6 |

FIG. 38

| | |
|---|---|
| Oligo A | oAA540 |
| Oligo D | oAA541 |
| Oligo C | F98G fwd |
| Oligo B | F98G rev |
| Oligo C | W429F fwd |
| Oligo B | W429F rev |

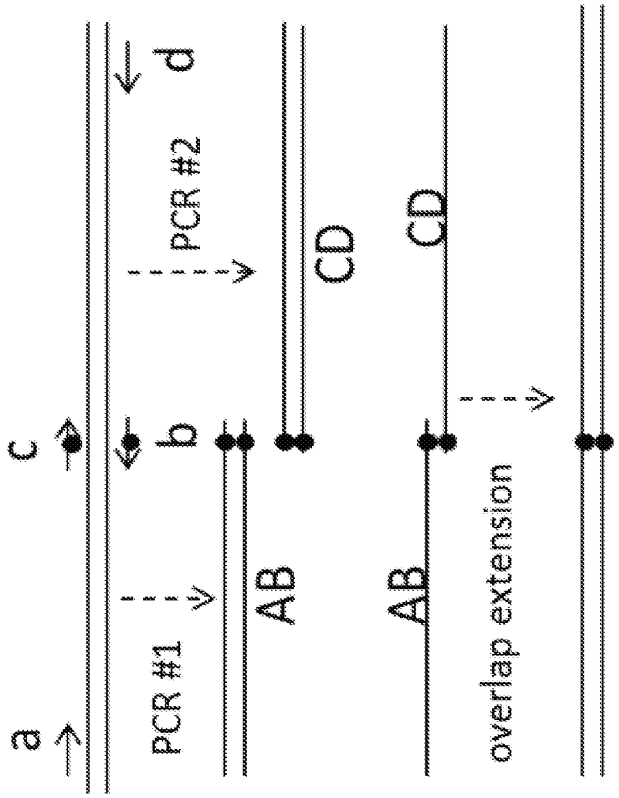



FIG. 39

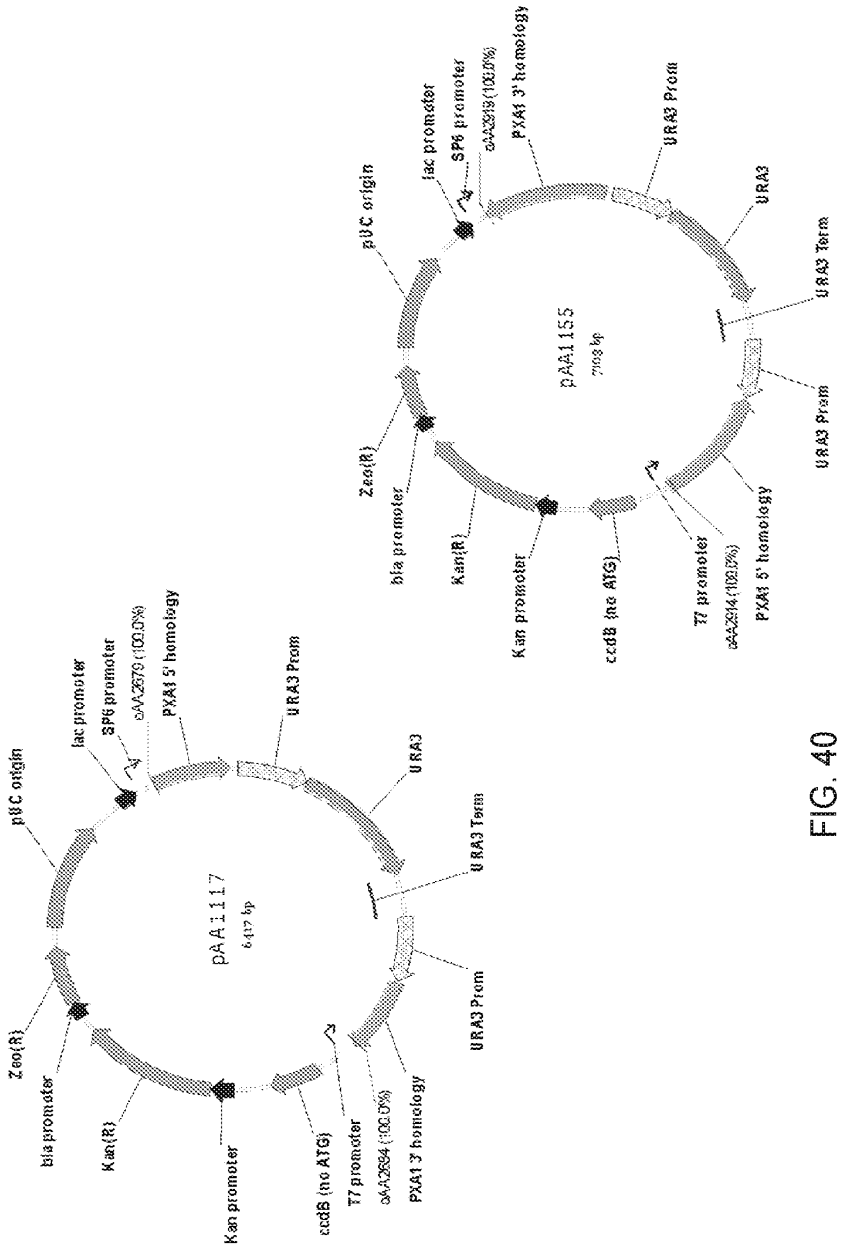

pAA1117
6417 bp
pUC origin
lac promoter
SP6 promoter
PXA1 5' homology
URA3 Prom
URA3
URA3 Term
URA3 Prom
PXA1 3' homology
T7 promoter
ccdB (no ATG)
Kan promoter
Kan(R)
bla promoter
Zeo(R)
pAA1155
pUC origin
lac promoter
SP6 promoter
PXA1 3' homology
URA3 Prom
URA3
URA3 Term
URA3 Prom
PXA1 5' homology
T7 promoter
ccdB (no ATG)
Kan promoter
Kan(R)
bla promoter
Zeo(R)

FIG. 40

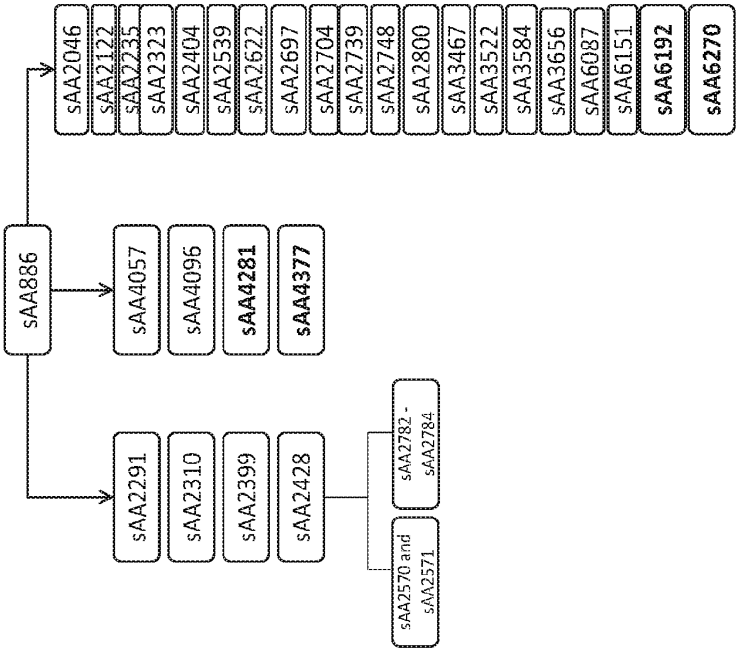

sAA886
sAA2046
sAA2122
sAA2235
sAA2323
sAA2404
sAA2539
sAA2622
sAA2697
sAA2704
sAA2739
sAA2748
sAA2800
sAA3467
sAA3522
sAA3584
sAA3656
sAA6087
sAA6151
sAA6192
sAA6270
sAA4057
sAA4096
sAA4281
sAA4377
sAA2291
sAA2310
sAA2399
sAA2428
sAA2782 - sAA2784
sAA2570 and sAA2571

FIG. 41

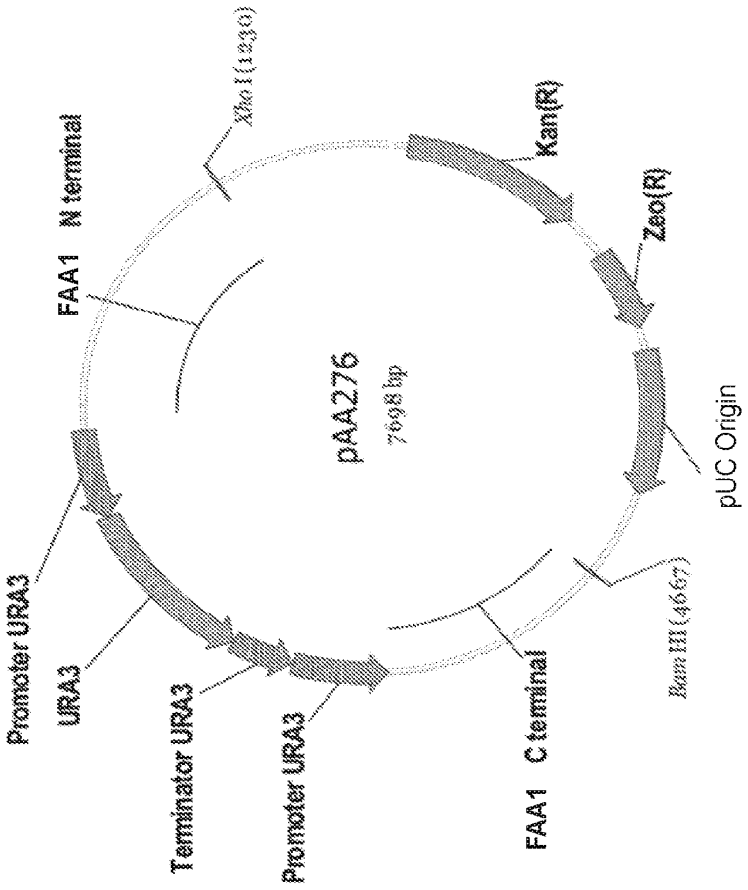

pAA276
7698 bp
FAA1 N terminal
Xho I (1230)
Kan(R)
Zeo(R)
pUC Origin
BamH I (4667)
FAA1 C terminal
Promoter URA3
Terminator URA3
URA3
Promoter URA3

FIG. 42

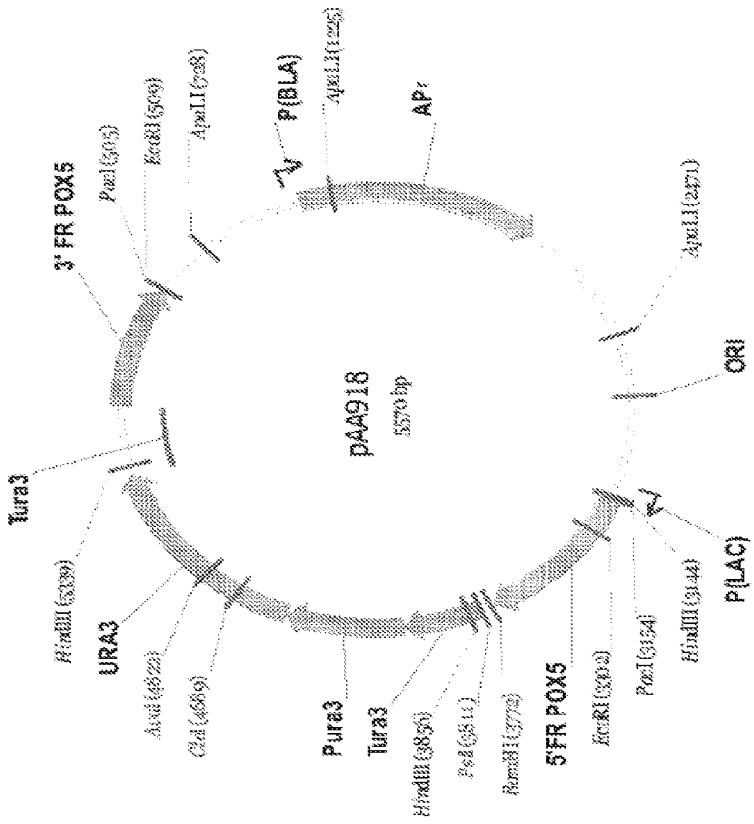

pAA918
5570 bp
Tura3
3' FR POX5
URA3
Pura3
Tura3
5'FR POX5
P(BLA)
APr
ORI
P(LAC)
HindIII (5339)
AvaI (4622)
ClaI (4569)
HindIII (3856)
PstI (3814)
BamHI (3772)
EcoRI (3073)
PmeI (3124)
HindIII (3144)
AgeI (2971)
AgeI (2225)
EcoRI (5069)
AgeI (5268)
PmeI (5015)

FIG. 43

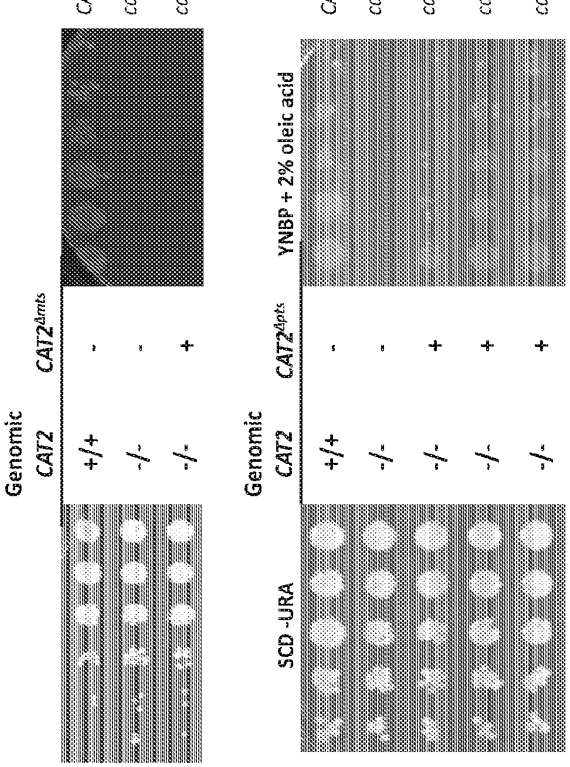

Genomic
CAT2
CAT2Δmts
+/+
-/-
-/-
-
-
+
CAT2/CAT2
cat2-Δ1::PURA3/cat2-Δ2::URA3
cat2-Δ1::PURA3/cat2-Δ2::PURA3 + pAA1610
SCD -URA
YNBP + 2% oleic acid
Genomic
CAT2
CAT2Δmts
+/+
-/-
-/-
-/-
-/-
-
-
+
+
+
CAT2/CAT2
cat2-Δ1::PURA3/cat2-Δ2::URA3
cat2-Δ1::PURA3/cat2-Δ2::PURA3 + pAA1876
cat2-Δ1::PURA3/cat2-Δ2::PURA3 + pAA1876
cat2-Δ1::PURA3/cat2-Δ2::PURA3 + pAA1876

FIG. 44

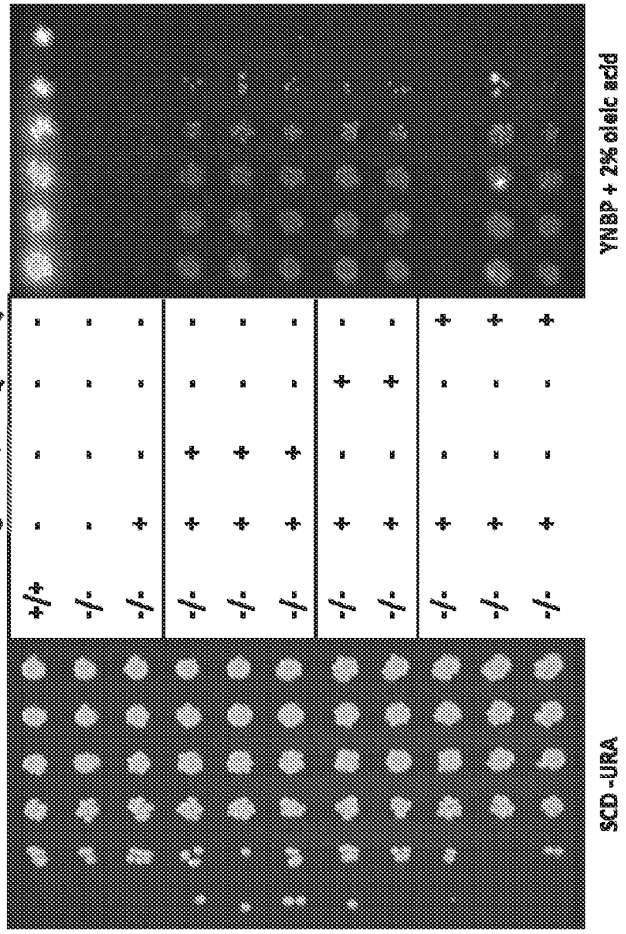

Genomic CAT2
CAT2pex
VAT1OPEX
VAT1ORTH
VAT1CYTOP
CAT2/CAT2
cat2-Δ1::PUAS1/cat2-Δ2::URA3
cat2-Δ1::PUAS1/cat2-Δ2::PUAS1 + pAA1610
cat2-Δ1::PUAS1/cat2-Δ2::PUAS1 + pAA1610/pAA1967
cat2-Δ1::PUAS1/cat2-Δ2::PUAS1 + pAA1610/pAA1967
cat2-Δ1::PUAS1/cat2-Δ2::PUAS1 + pAA1610/pAA1967
cat2-Δ1::PUAS1/cat2-Δ2::PUAS1 + pAA1610/pAA1968
cat2-Δ1::PUAS1/cat2-Δ2::PUAS1 + pAA1610/pAA1968
cat2-Δ1::PUAS1/cat2-Δ2::PUAS1 + pAA1610/pAA1969
cat2-Δ1::PUAS1/cat2-Δ2::PUAS1 + pAA1610/pAA1969
cat2-Δ1::PUAS1/cat2-Δ2::PUAS1 + pAA1610/pAA1969
+/+
-/-
-/-
-/-
-/-
-/-
-/-
-/-
-/-
-/-
SCD -URA
YNBP + 2% oleic acid

FIG. 45

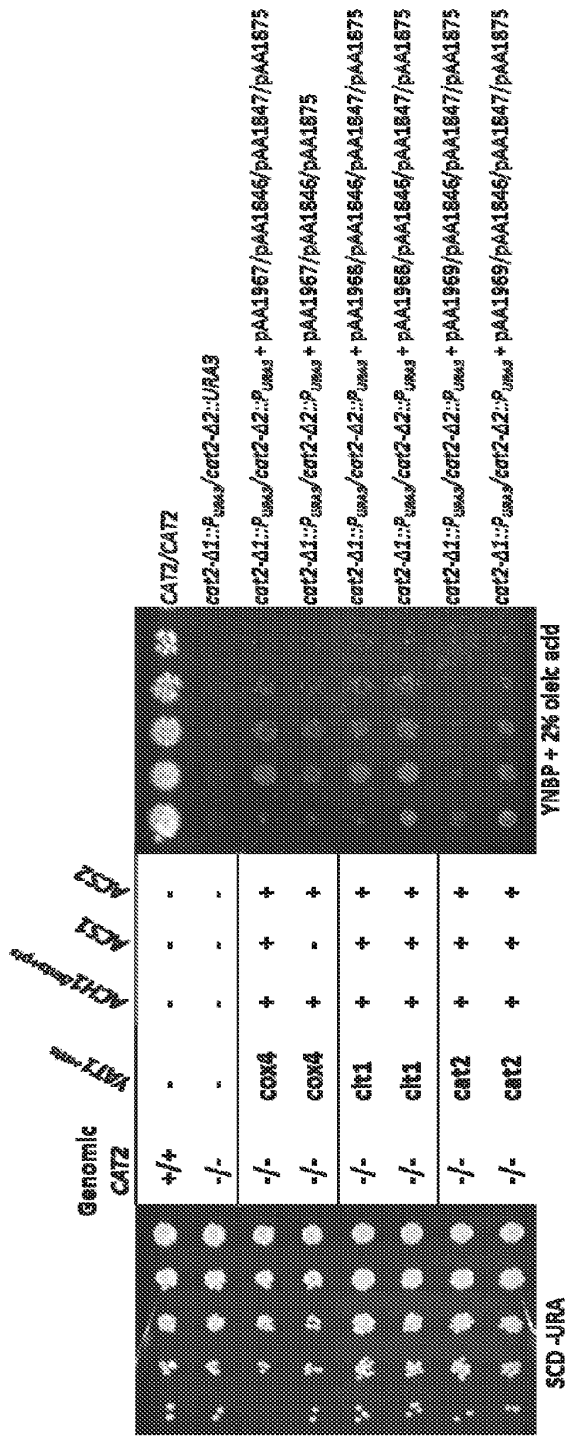

Genomic CAT2
YBT1
ACH1
ACS1
ACS2
CAT2/CAT2
cat2-Δ1::P_URA3/cat2-Δ2::URA3
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1967/pAA1846/pAA1875
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1967/pAA1846/pAA1875
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1968/pAA1846/pAA1875
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1968/pAA1845/pAA1847/pAA1875
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1969/pAA1846/pAA1847/pAA1875
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1969/pAA1846/pAA1847/pAA1875
+/+ - - - -
-/- - - - -
-/- cox4 + + +
-/- cox4 + - +
-/- cit1 + + +
-/- cit1 + + +
-/- cat2 + + +
-/- cat2 + + +
SCD -URA
YNBP + 2% oleic acid

FIG. 46

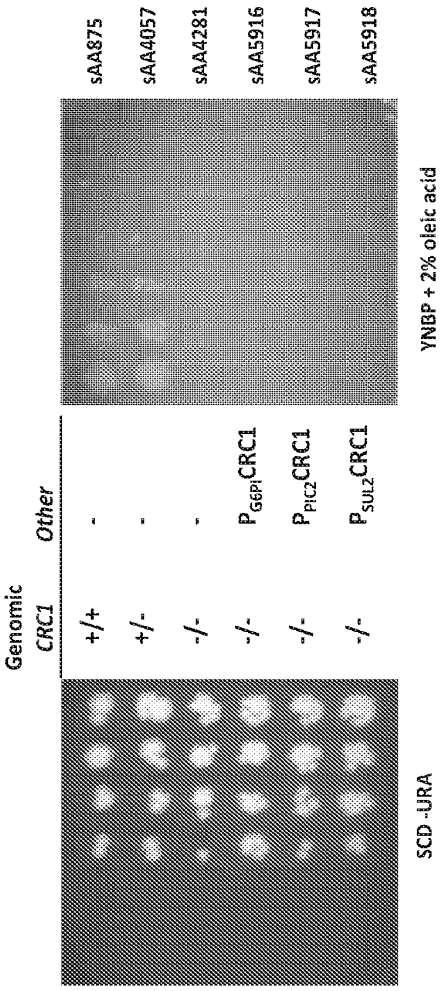

Genomic
CRC1
Other
+/+
+/-
-/-
-/-
-/-
-/-
-
-
-
$P_{G6PI}$CRC1
$P_{PIC2}$CRC1
$P_{SUL2}$CRC1
sAA875
sAA4057
sAA4281
sAA5916
sAA5917
sAA5918
SCD -URA
YNBP + 2% oleic acid
Genomic
CRC1
+/+
+/-
-/-
+/+
ATCC20336
sAA4368
sAA9398
sAA875
SCD -URA
YNBP + 2% oleic acid

FIG. 47

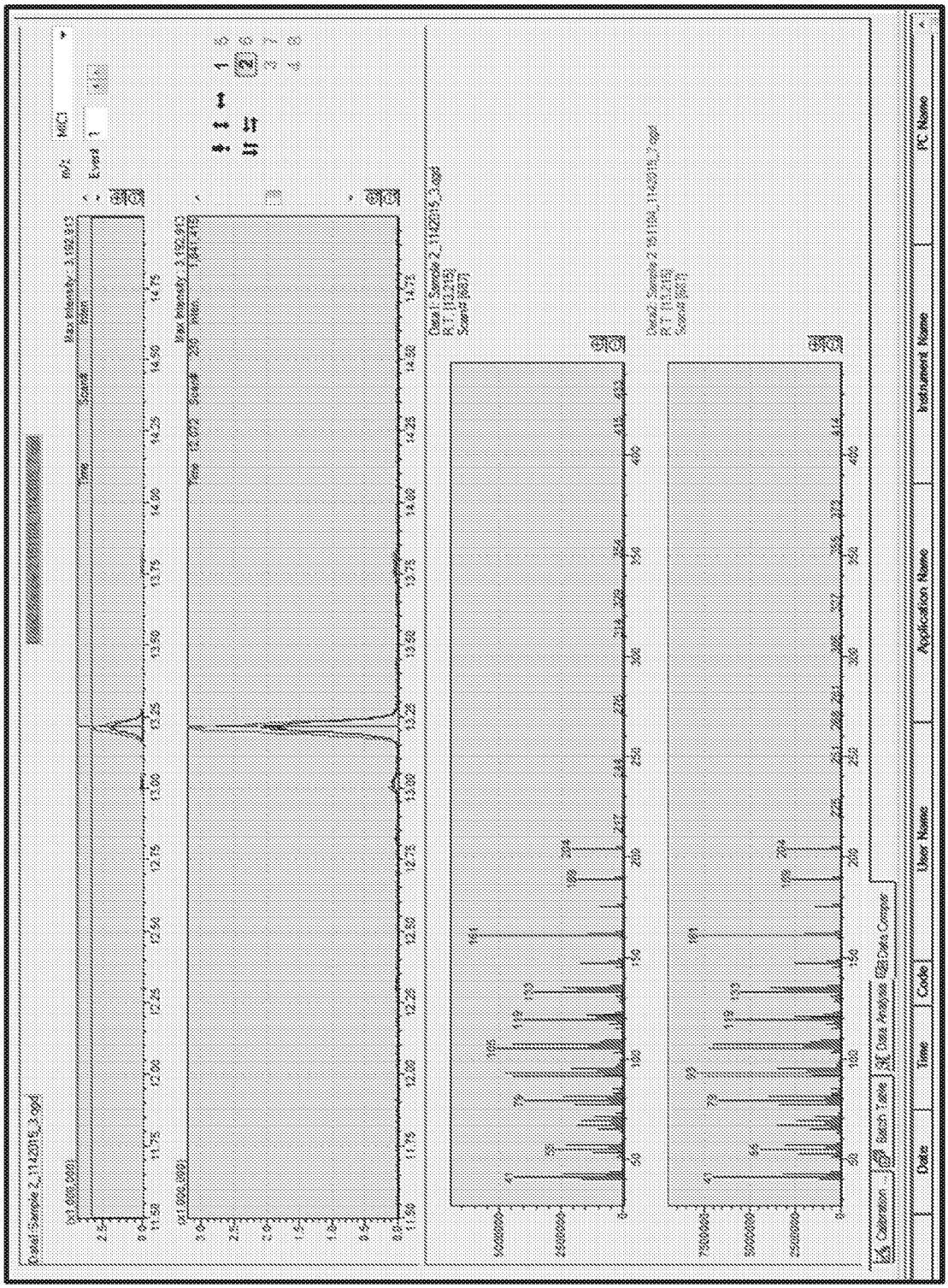


Fig. 48A

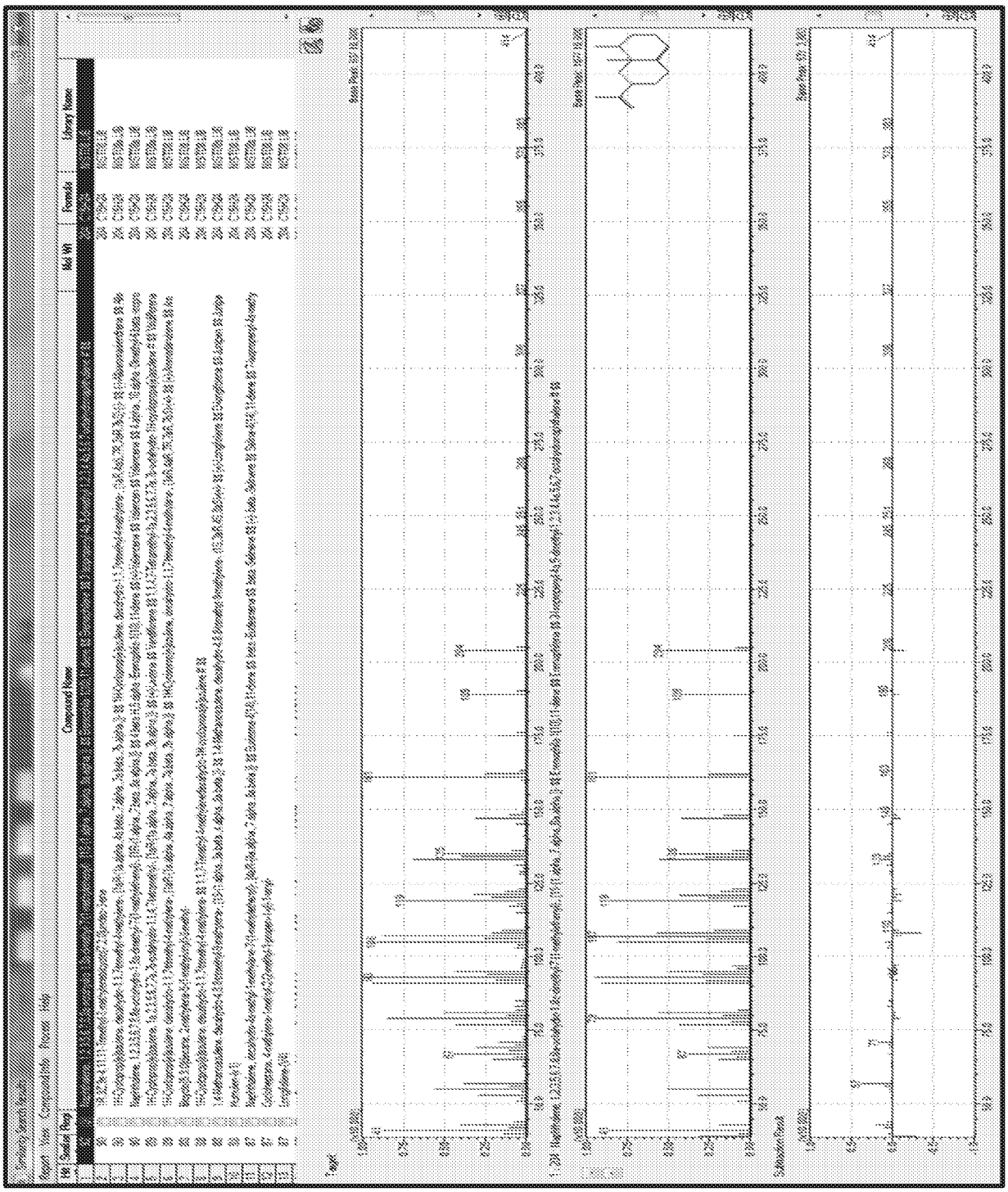


Fig. 48B

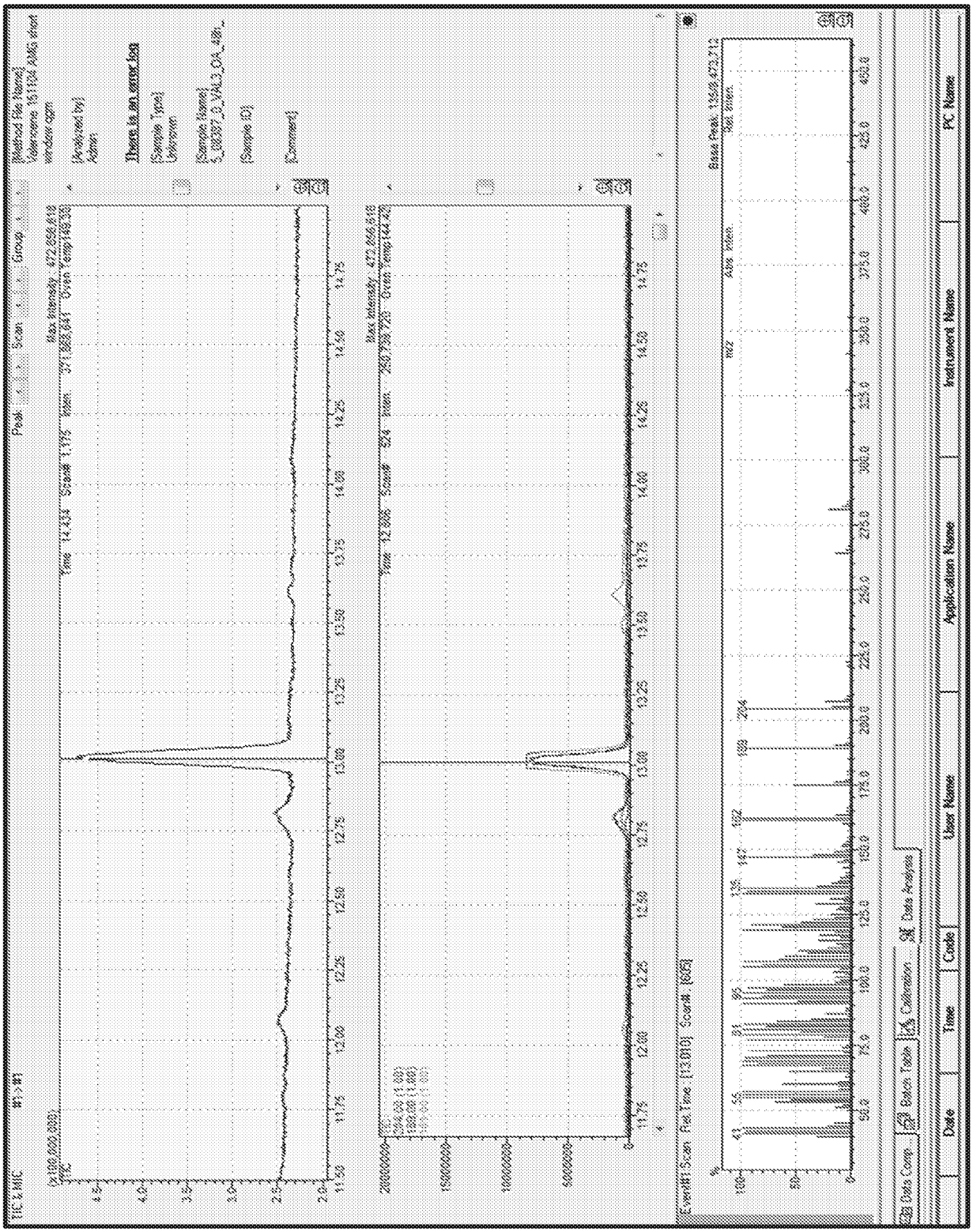


Fig. 49A

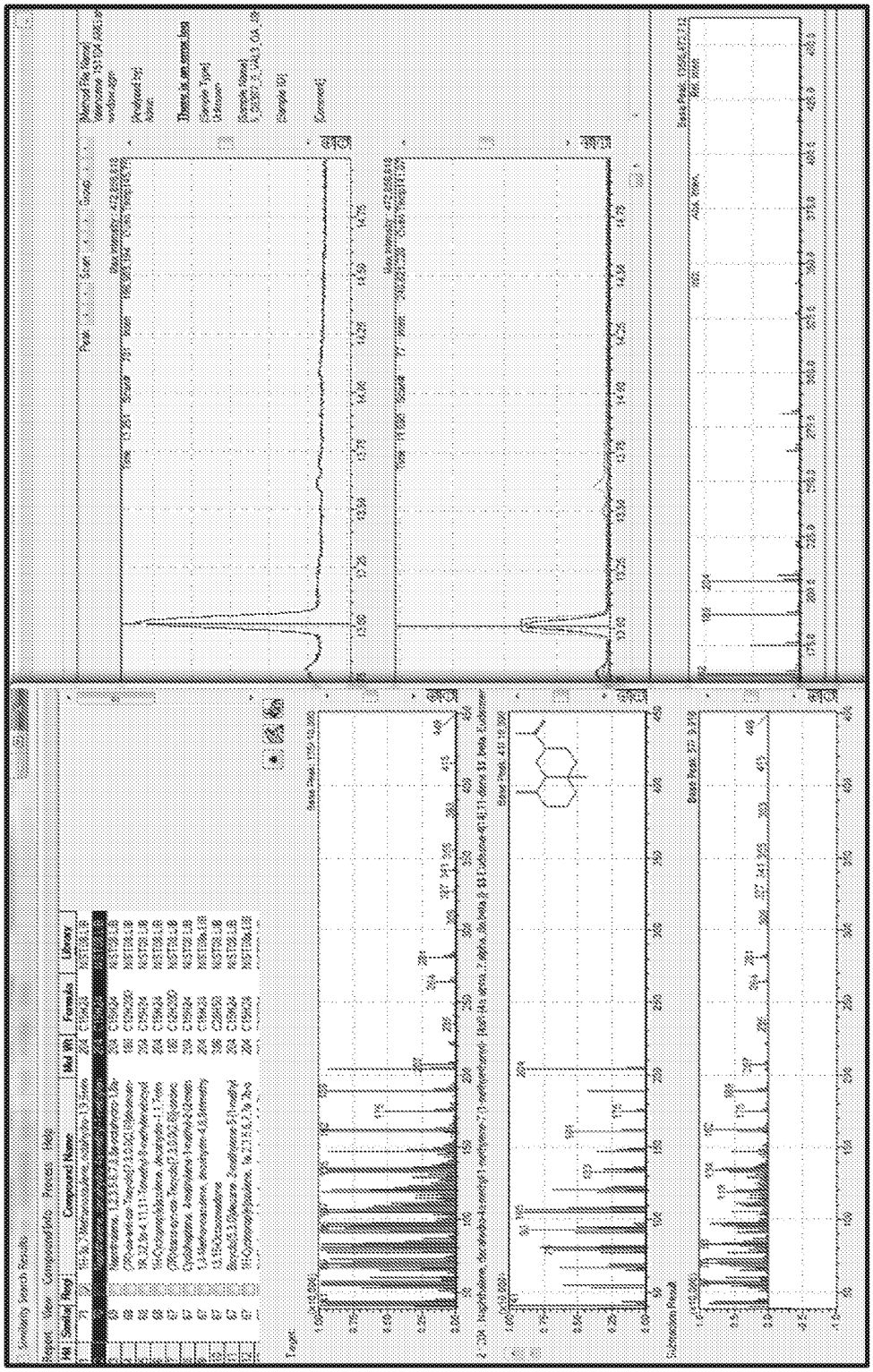


Fig. 49B

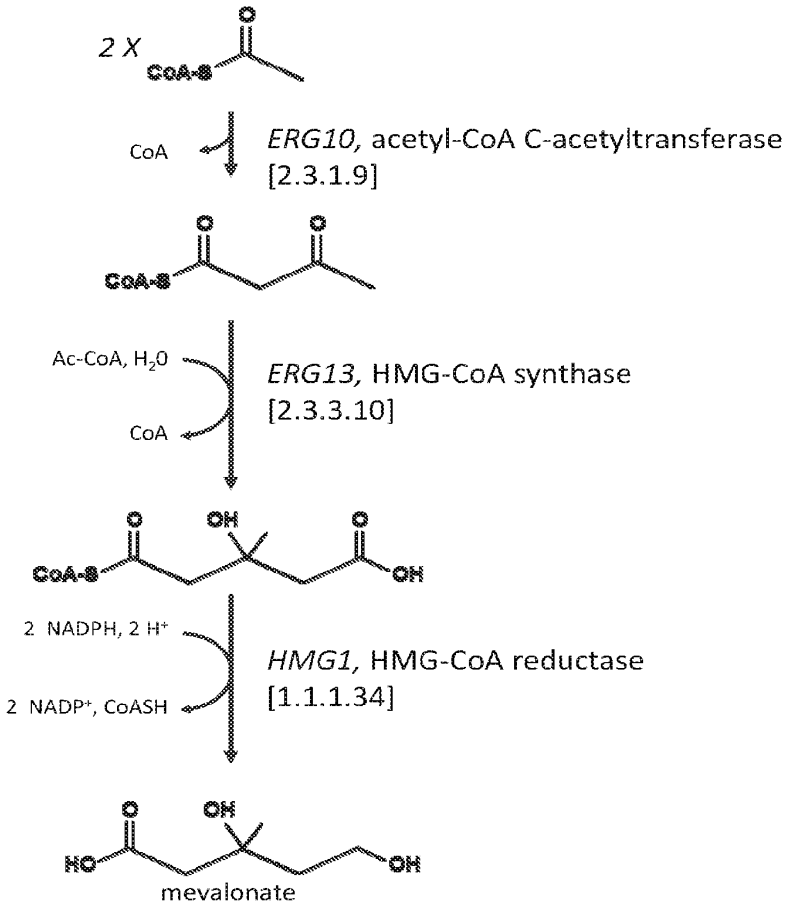

2 X
CoA-S
CoA
ERG10, acetyl-CoA C-acetyltransferase
[2.3.1.9]
CoA-S
Ac-CoA, H2O
CoA
ERG13, HMG-CoA synthase
[2.3.3.10]
CoA-S
OH
OH
2 NADPH, 2 H+
2 NADP+, CoASH
HMG1, HMG-CoA reductase
[1.1.1.34]
HO
OH
OH
mevalonate

Fig. 50

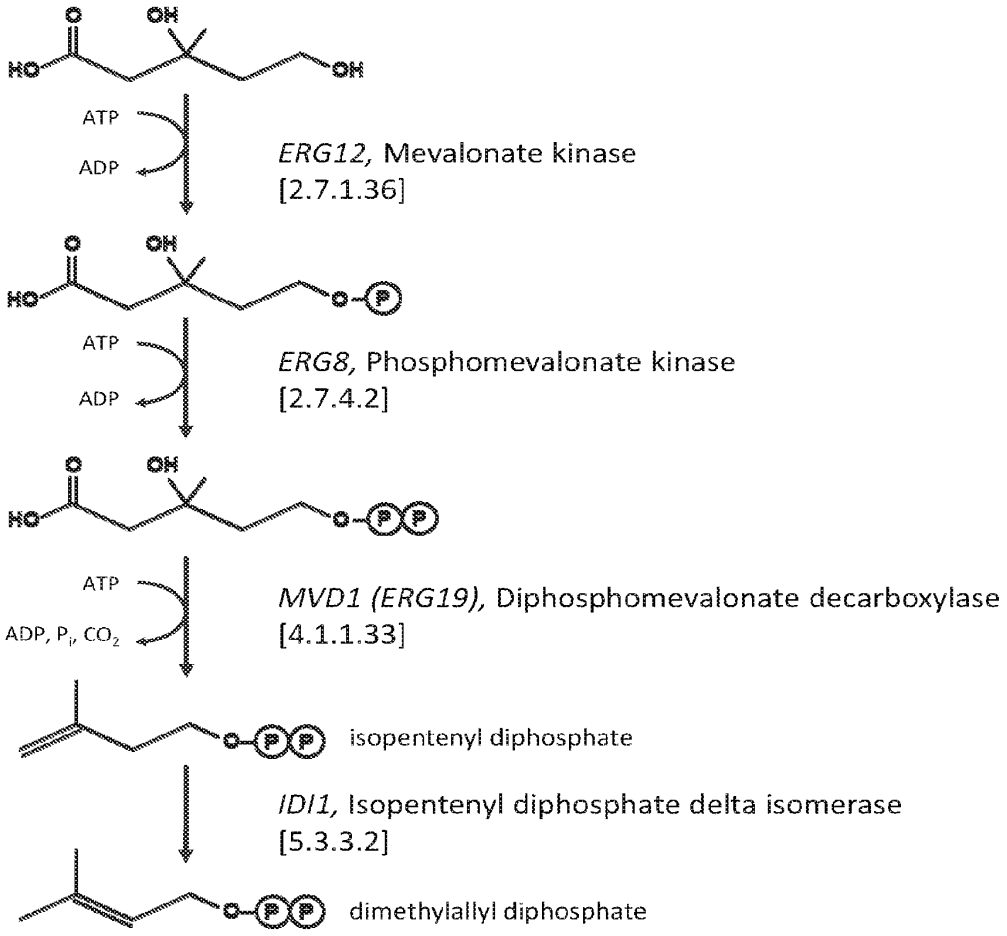

ATP
ADP
ERG12, Mevalonate kinase
[2.7.1.36]
ATP
ADP
ERG8, Phosphomevalonate kinase
[2.7.4.2]
ATP
ADP, Pi, CO2
MVD1 (ERG19), Diphosphomevalonate decarboxylase
[4.1.1.33]
isopentenyl diphosphate
IDI1, Isopentenyl diphosphate delta isomerase
[5.3.3.2]
dimethylallyl diphosphate

Fig. 51

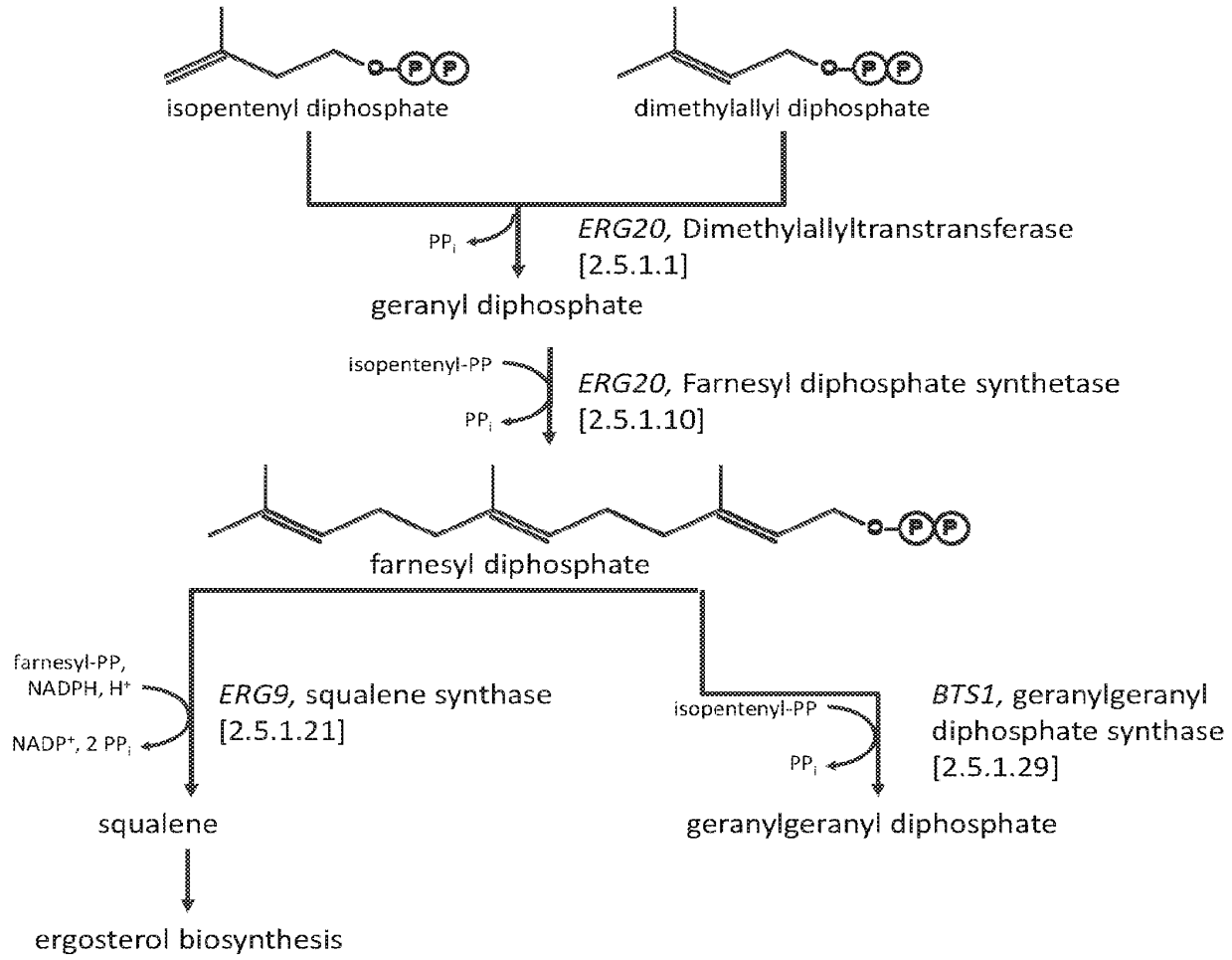

isopentenyl diphosphate
dimethylallyl diphosphate
PPi
ERG20, Dimethylallyltranstransferase
[2.5.1.1]
geranyl diphosphate
isopentenyl-PP
PPi
ERG20, Farnesyl diphosphate synthetase
[2.5.1.10]
farnesyl diphosphate
farnesyl-PP,
NADPH, H+
NADP+, 2 PPi
ERG9, squalene synthase
[2.5.1.21]
squalene
ergosterol biosynthesis
isopentenyl-PP
PPi
BTS1, geranylgeranyl
diphosphate synthase
[2.5.1.29]
geranylgeranyl diphosphate

Fig. 52

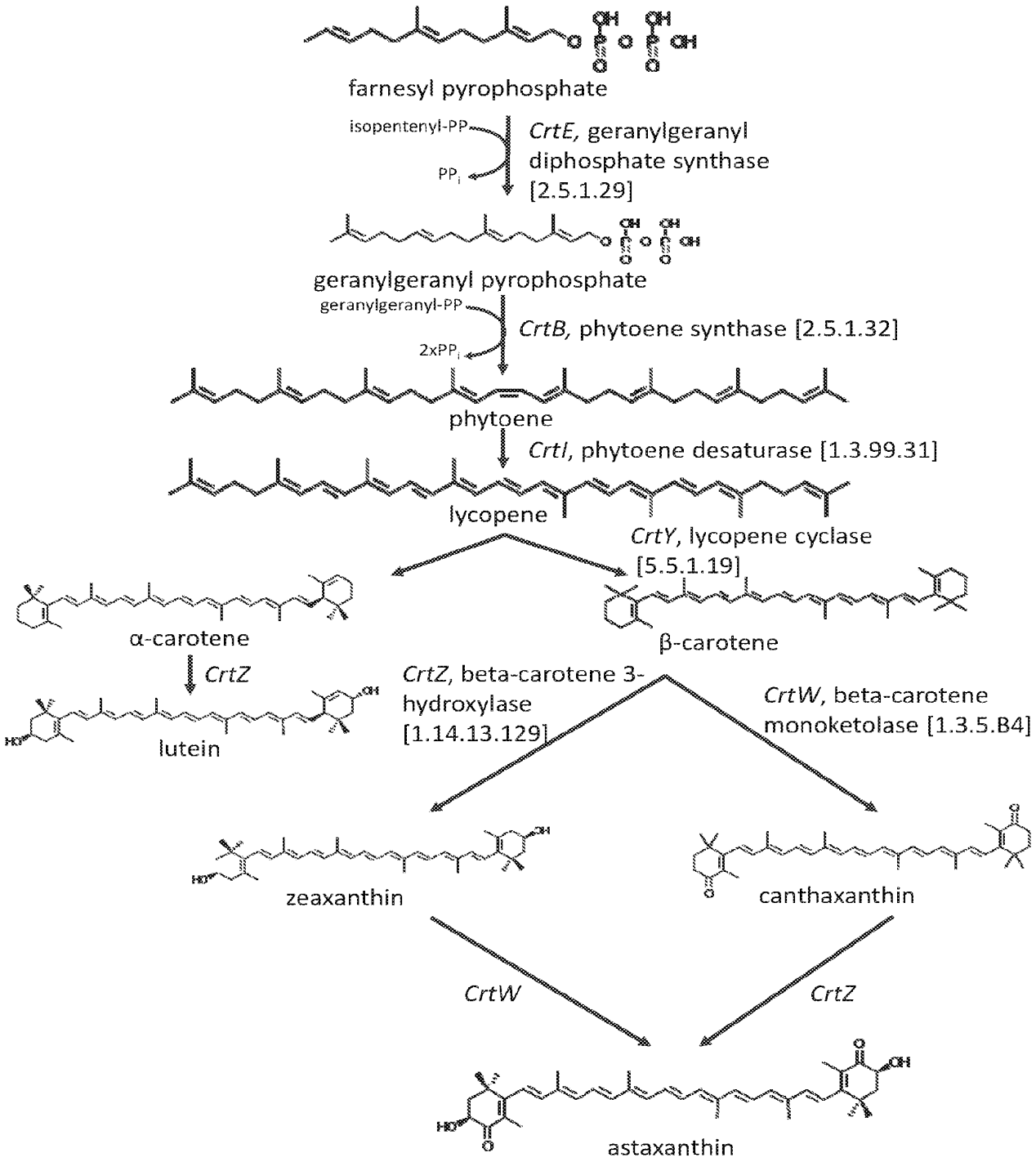

farnesyl pyrophosphate
isopentenyl-PP
PPi
CrtE, geranylgeranyl diphosphate synthase [2.5.1.29]
geranylgeranyl pyrophosphate
geranylgeranyl-PP
2xPPi
CrtB, phytoene synthase [2.5.1.32]
phytoene
CrtI, phytoene desaturase [1.3.99.31]
lycopene
CrtY, lycopene cyclase [5.5.1.19]
α-carotene
β-carotene
CrtZ
lutein
CrtZ, beta-carotene 3-hydroxylase [1.14.13.129]
CrtW, beta-carotene monoketolase [1.3.5.B4]
zeaxanthin
canthaxanthin
CrtW
CrtZ
astaxanthin

Fig. 53

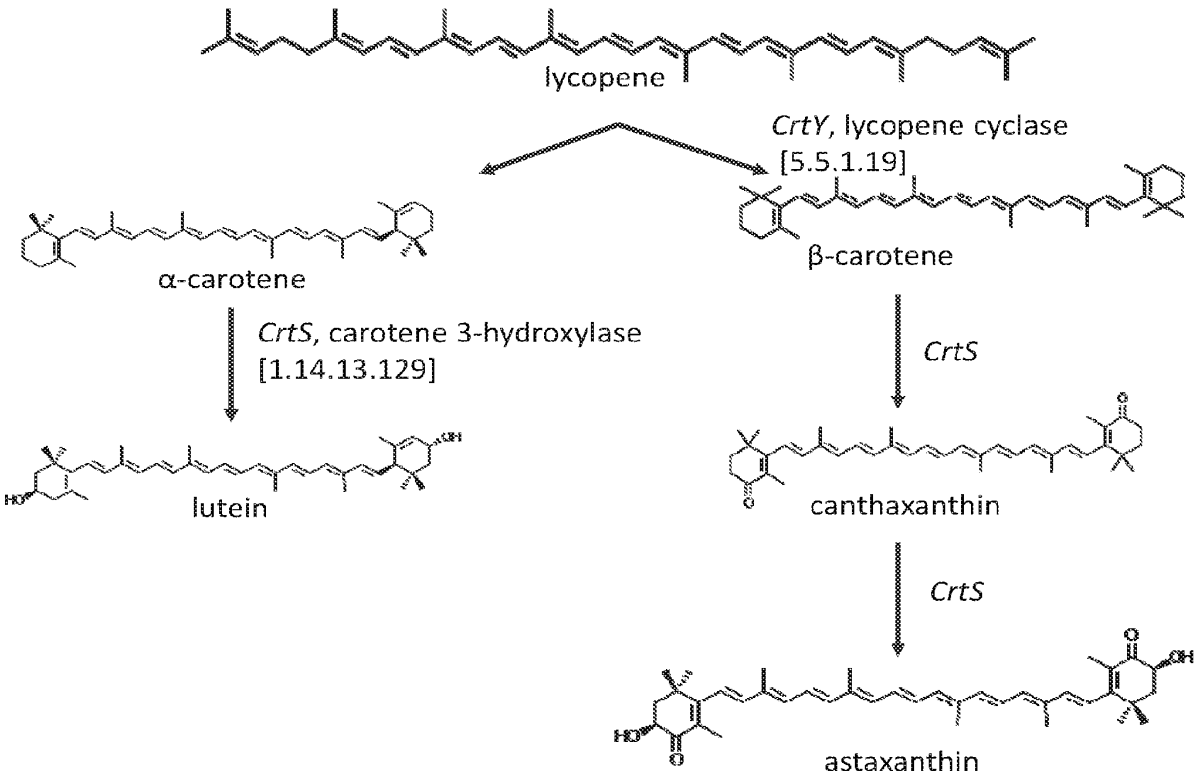

lycopene
CrtY, lycopene cyclase
[5.5.1.19]
α-carotene
β-carotene
CrtS, carotene 3-hydroxylase
[1.14.13.129]
CrtS
lutein
canthaxanthin
CrtS
astaxanthin

Fig. 54

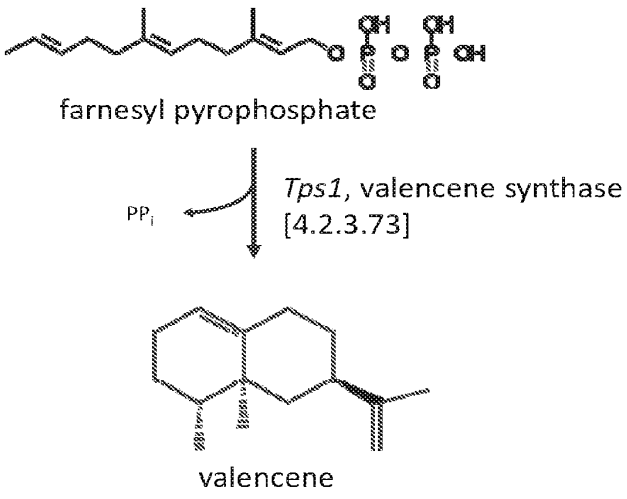

farnesyl pyrophosphate
PPi
Tps1, valencene synthase
[4.2.3.73]
valencene

Fig. 55

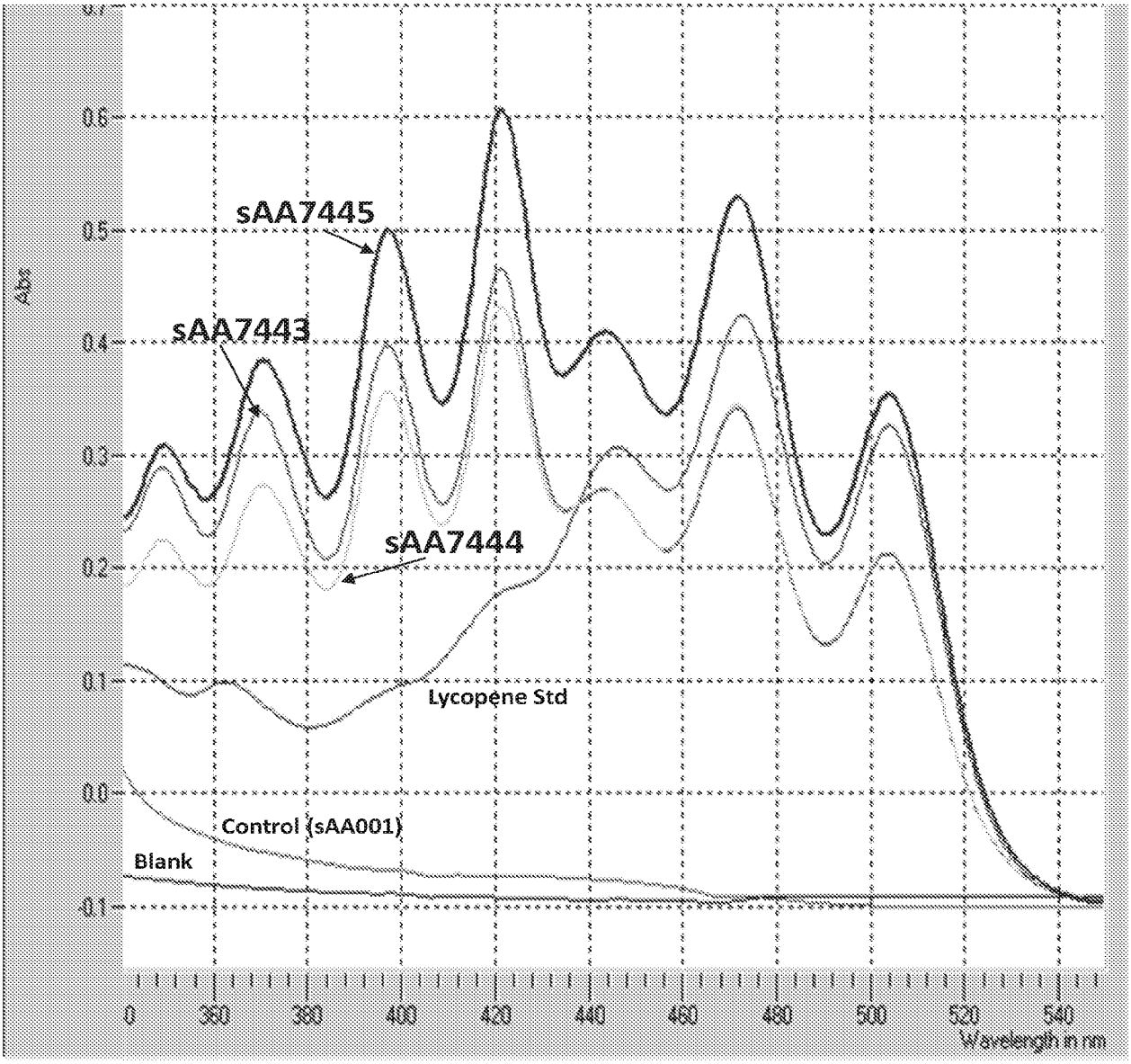

sAA7445
sAA7443
sAA7444
Lycopene Std
Control (sAA001)
Blank
Abs
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
0
360
380
400
420
440
460
480
500
520
540
Wavelength in nm

Fig. 56

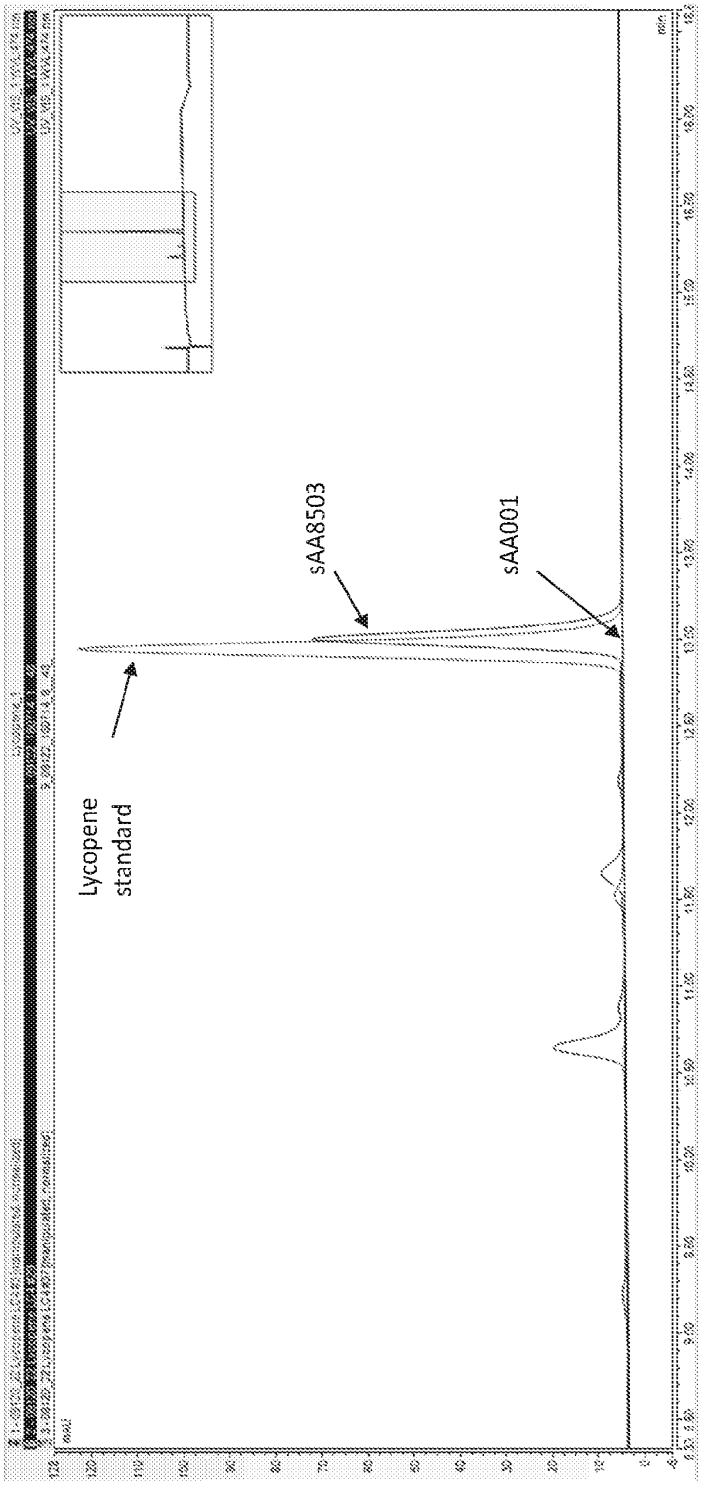



Fig. 57

| Electronic Acknowledgement Receipt | |
|---|---|
| **EFS ID:** | 29779065 |
| **Application Number:** | 62532297 |
| **International Application Number:** | |
| **Confirmation Number:** | 2178 |
| **Title of Invention:** | BIOLOGICAL METHODS FOR PREPARING TERPENES |
| **First Named Inventor/Applicant Name:** | Kimberly Ann Aeling |
| **Customer Number:** | 69403 |
| **Filer:** | Kari Ann Dickinson/Alexandra Williamson |
| **Filer Authorized By:** | Kari Ann Dickinson |
| **Attorney Docket Number:** | VRD-3002-PV |
| **Receipt Date:** | 13-JUL-2017 |
| **Filing Date:** | |
| **Time Stamp:** | 19:47:36 |
| **Application Type:** | Provisional |

## Payment information:

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|---|---|
| Submitted with Payment | yes |
| Payment Type | CARD |
| Payment was successfully received in RAM | $1730 |
| RAM confirmation Number | 071417INTEFSW19482300 |
| Deposit Account | 503473 |
| Authorized User | Alexandra Williamson |
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## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Application Data Sheet | VRD-3002-PV_ADS_signed_uspto.pdf | 119348<br>031ad1057910b9235e79b7d0a5fa059b466693b88 | no | 8 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| This is not an USPTO supplied ADS fillable form | | | | | |
| 2 | | VRD-3002-PV_SPEC_FINAL_2017-07-13_uspto.pdf | 2109063<br>a283d1d80ef65eacd59fd6bf3d929af6f6a08e031 | yes | 551 |
| | **Multipart Description/PDF files in .zip description** | | | | |
| | **Document Description** | | **Start** | **End** | |
| | Specification | | 1 | 549 | |
| | Claims | | 550 | 550 | |
| | Abstract | | 551 | 551 | |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 3 | Drawings-only black and white line drawings | VRD-3002-PV_Drawings_2017-07-13_uspto.pdf | 5019296<br>fca1cb1effefe311fac8ecde5fcb51a6497d523a | no | 59 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 4 | Fee Worksheet (SB06) | fee-info.pdf | 31795<br>7f6fa1019e203f6483a6797f7fb7d6ae76815766 | no | 2 |
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| | | |
|---|---|---|
| International application number: | | **PCT/US2018/041579** |
| International filing date: | | **11 July 2018 (11.07.2018)** |
| Document type: | | **Certified copy of priority document** |
| Document details: | Country/Office: | **US** |
| | Number: | **62/532,297** |
| | Filing date: | **13 July 2017 (13.07.2017)** |
| Date of receipt at the International Bureau: | | **22 July 2018 (22.07.2018)** |

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a),(b) or (b-*bis*)

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Silvia Salvadori, P.C.
270 Madison Avenue, 8th Floor
New York, NY 10016
ÉTATS-UNIS D'AMÉRIQUE

Date of mailing *(day/month/year)*
07 January 2020 (07.01.2020)

Applicant's or agent's file reference
SS118-1026

**IMPORTANT NOTIFICATION**

International application No.
PCT/US2018/041579

International filing date *(day/month/year)*
11 July 2018 (11.07.2018)

1. The following indications appeared on record concerning:

☐ the applicant ☒ the inventor ☐ the agent ☐ the common representative

Name and Address

State of Nationality | State of Residence

Telephone No.

Facsimile No.

E-mail address

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☒ the person ☐ the name ☐ the address ☐ the nationality ☐ the residence

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Carlsbad, CA 92010
United States of America

State of Nationality | State of Residence

Telephone No.

Facsimile No.

E-mail address
☐ Notifications by e-mail authorized

3. Further observations, if necessary:
The person identified in Box 2 has been added as inventor only.

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New York, NY 10016
ÉTATS-UNIS D'AMÉRIQUE

Date of mailing *(day/month/year)*
15 May 2019 (15.05.2019)

Applicant's or agent's file reference
SS118-1026

**IMPORTANT NOTIFICATION**

International application No.
PCT/US2018/041579

International filing date *(day/month/year)*
11 July 2018 (11.07.2018)

1. The following indications appeared on record concerning:

☒ the applicant ☐ the inventor ☐ the agent ☐ the common representative

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Menlo Park, CA 94025
United States of America

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State of Residence: US
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Facsimile No.: 925-365-3818
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State of Residence: IT
Telephone No.:
Facsimile No.:
E-mail address:
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3. Further observations, if necessary:

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34, chemin des Colombettes
1211 Geneva 20, Switzerland

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New York, NY 10016
ÉTATS-UNIS D'AMÉRIQUE

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07 January 2020 (07.01.2020)

Applicant's or agent's file reference
SS118-1026

**IMPORTANT NOTIFICATION**

International application No.
PCT/US2018/041579

International filing date *(day/month/year)*
11 July 2018 (11.07.2018)

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☐ the applicant ☒ the inventor ☐ the agent ☐ the common representative

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2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

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Encinitas, CA 92024
United States of America

State of Nationality | State of Residence

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Facsimile No.

E-mail address
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3. Further observations, if necessary:
The person identified in Box 2 has been added as inventor only.

4. A copy of this notification has been sent to:

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International Application No.

11 July 2018 (11.07.2018)
International Filing Date

US PCT INTERNATIONAL APPLICATION
Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* VAL-3002-PC

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**Box No. II APPLICANT** ☐ This person is also inventor

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

Verdezyne (ABC), LLC
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Menlo Park, CA 94025
UNITED STATES OF AMERICA

Telephone No.
925 302 4298

Facsimile No.
925 365 3818

Applicant's registration No. with the Office

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This person is applicant for the purposes of: ☒ all designated States ☐ the States indicated in the Supplemental Box

**Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)**

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

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The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

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c/o CPA Global
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Telephone No.
858-412-1084

Facsimile No.
858-623-3224

Agent's registration No. with the Office
60,632

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☐ as advance copies followed by paper notifications; or ☒ exclusively in electronic form (no paper notifications will be sent).

E-mail address: docketing@cpaglobal.com

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

**Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)**

*If none of the following sub-boxes is used, this sheet should not be included in the request.*

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

AELING, Kimberly Ann
324 Plaza Estival
San Clemente, CA 92672
UNITED STATES OF AMERICA

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☒ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:
- ☐ applicant only
- ☐ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

This person is applicant for the purposes of: ☐ all designated States ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.



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**Supplemental Box** *If the Supplemental Box is not used, this sheet should not be included in the request.*

1. *If, in any of the Boxes, except Boxes Nos. VIII(i) to (v) for which a special continuation box is provided,* ***the space is insufficient*** *to furnish all the information: in such case, write "Continuation of Box No...." (indicate the number of the Box) and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:*

(i) ***if more than one person is to be indicated as applicant and/or inventor*** *and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;*

(ii) *if, in Box No. II or in any of the sub-boxes of Box No. III, the indication* ***"the States indicated in the Supplemental Box"*** *is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;*

(iii) *if, in Box No. II or in any of the sub-boxes of Box No. III,* ***the inventor or the inventor/applicant is not inventor for the purposes of all designated States****: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;*

(iv) *if, in addition to the agent(s) indicated in Box No. IV, there are* ***further agents****: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;*

(v) *if, in Box No. VI, there are* ***more than three earlier applications whose priority is claimed****: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI.*

2. *If the applicant intends to make an indication of the wish that the international application be treated, in certain designated States, as an application for a patent of addition, certificate of addition, inventor's certificate of addition or utility certificate of addition: in such case, write the name or two-letter code of each designated State concerned and the indication "****patent of addition,****" "****certificate of addition,****" "****inventor's certificate of addition****" or "****utility certificate of addition,****" the number of the parent application or parent patent or other parent grant and the date of grant of the parent patent or other parent grant or the date of filing of the parent application (Rules 4.11(a)(i) and 49bis.1(a) or (b)).*

3. *If the applicant intends to make an indication of the wish that the international application be treated, in the United States of America, as a continuation or continuation-in-part of an earlier application: in such case, write "United States of America" or "US" and the indication "****continuation****" or "****continuation-in-part****" and the number and the filing date of the parent application (Rules 4.11(a)(ii) and 49bis.1(d)).*

Continuation of Box IV:

Bruce D. Grant, Registration No.: 47,608
Kari A. Dickinson, Registration No.: 60,632
Sheryl R. Silverstein, Registration No.: 40,812
Gary H. Silverstein, Registration No.: 39,372



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**Box No. V DESIGNATIONS**

The filing of this request **constitutes under Rule 4.9(a) the designation** of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.

However,

☐ DE Germany **is not designated** for any kind of national protection

☐ JP Japan **is not designated** for any kind of national protection

☐ KR Republic of Korea **is not designated** for any kind of national protection

*(The check-boxes above may only be used to exclude (irrevocably) the designations concerned if, at the time of filing or subsequently under Rule 26bis.1, the international application contains in Box No. VI a priority claim to an earlier national application filed in the particular State concerned, in order to avoid the ceasing of the effect, under the national law, of this earlier national application.)*

**Box No. VI PRIORITY CLAIM AND DOCUMENT**

**The priority of the following earlier application(s) is hereby claimed:**

| Filing date of earlier application *(day/month/year)* | Number of earlier application | Where earlier application is: | | |
|---|---|---|---|---|
| | | national application: country or Member of WTO | regional application: regional Office | international application: receiving Office |
| item (1) 13 July, 2017 (13.07.2017) | 62/532,297 | US | | |
| item (2) | | | | |
| item (3) | | | | |

☐ Further priority claims are indicated in the Supplemental Box.

**Furnishing the priority document(s):**

☒ The **receiving Office** is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(only if the earlier application(s) was filed with the receiving Office which, for the purposes of this international application, is the receiving Office)* identified above as:

☐ all items ☒ item (1) ☐ item (2) ☐ item (3) ☐ other, see Supplemental Box

☐ The **International Bureau** is requested to obtain from a digital library a certified copy of the earlier application(s) identified above, using, where applicable, the access code(s) indicated below *(if the earlier application(s) is available to it from a digital library)*:

☐ item (1) access code _____ ☐ item (2) access code _____ ☐ item (3) access code _____ ☐ other, see Supplemental Box

**Restore the right of priority:** the receiving Office is requested to restore the right of priority for the earlier application(s) identified above or in the Supplemental Box as item(s) (_____). *(See also the Notes to Box No. VI; further information **must** be provided to support a request to restore the right of priority.)*

**Incorporation by reference:** where an element of the international application referred to in Article 11(1)(iii)(d) or (e) or a part of the description, claims or drawings referred to in Rule 20.5(a) is not otherwise contained in this international application but is completely contained in an earlier application whose priority is claimed on the date on which one or more elements referred to in Article 11(1)(iii) were first received by the receiving Office, that element or part is, subject to confirmation under Rule 20.6, incorporated by reference in this international application for the purposes of Rule 20.6.

**Box No. VII INTERNATIONAL SEARCHING AUTHORITY**

**Choice of International Searching Authority (ISA)** *(if more than one International Searching Authority is competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)*:

ISA/ US



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Sheet No. 5

**Box No. IX CHECK LIST for EFS-Web filings** - this sheet is only to be used when filing an international application with RO/US via EFS-Web

| This international application **contains** the following: | Number of sheets |
|---|---|
| (a) request form PCT/RO/101 (including any declarations and supplemental sheets) | 5 |
| (b) description (excluding any sequence listing part of the description, see (f), below) | 550 |
| (c) claims | 21 |
| (d) abstract | 1 |
| (e) drawings (if any) | 59 |
| (f) sequence listing part of the description in the form of an **image file** (e.g. PDF) | 0 |
| **Total number of sheets** (including the sequence listing part of the description if **filed as an image file**) | 636 |

(g) sequence listing part of the description

- ☐ filed in the form of an **Annex C/ST.25 text file**
- ☐ WILL BE filed separately on physical data carrier(s), on the same day and in the form of an **Annex C/ST.25 text file**

Indicate type and number of physical data carrier(s)

| This international application is **accompanied by** the following item(s) *(mark the applicable check-boxes below and indicate in right column the number of each item)*: | Number of items |
|---|---|
| 1. ☒ fee calculation sheet | 1 |
| 2. ☐ original separate power of attorney | |
| 3. ☐ original general power of attorney | |
| 4. ☐ copy of general power of attorney; reference number: | |
| 5. ☐ priority document(s) identified in Box No. VI as item(s) | |
| 6. ☐ Translation of international application into *(language)*: | |
| 7. ☐ separate indications concerning deposited microorganism or other biological material | |
| 8. ☐ *(only where item (f) is marked in the left column)* copy of the sequence listing in electronic form (Annex C/ST.25 text file) not forming part of the international application but **furnished only for the purposes of international search** under Rule 13*ter* | |
| 9. ☐ *(only where item (f) (in the left column) and item 8 (above) are marked)* a statement confirming that "the information recorded in electronic form submitted under Rule 13*ter* is identical to the sequence listing as contained in the international application" as filed via EFS-Web: | |
| 10. ☐ copy of results of earlier search(es) (Rule 12*bis*.1(a)) | |
| 11. ☐ other *(specify)*: | |

| **Figure of the drawings** which should accompany the abstract: | 1 | **Language of filing** of the international application: | English |
|---|---|---|---|

**Box No. X SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE**

*Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).*

/Kari A. Dickinson/

Kari A. Dickinson, Reg No. 60,632

For receiving Office use only

| | | |
|---|---|---|
| 1. Date of actual receipt of the purported international application: | 11 July 2018 (11.07.2018) | 2. Drawings: ☐ received: |
| 3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application: | | |
| 4. Date of timely receipt of the required corrections under PCT Article 11(2): | | ☐ not received: |
| 5. International Searching Authority (if two or more are competent): ISA / US | 6. ☐ Transmittal of search copy delayed until search fee is paid | |

For International Bureau use only

Date of receipt of the record copy by the International Bureau:



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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

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WIPO | PCT

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*C12N 1/19* (2006.01) *C12N 15/09* (2006.01)
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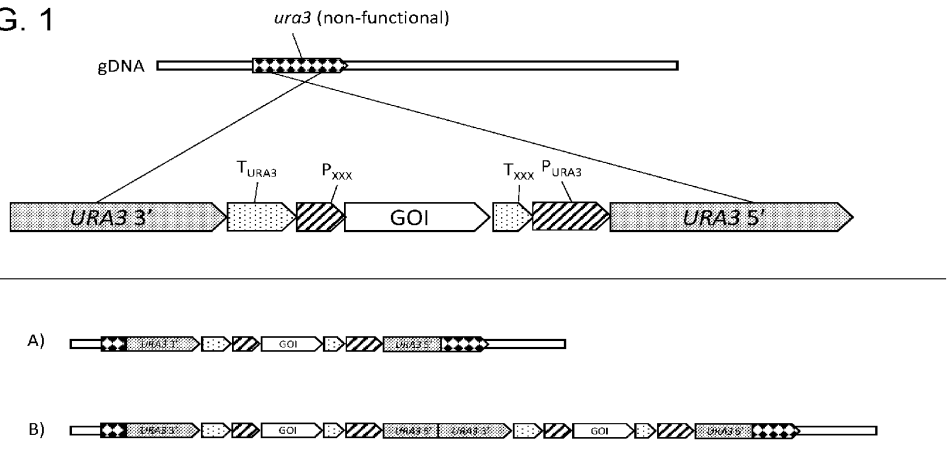
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

**Published:**
— *with international search report (Art. 21(3))*

(54) Title: BIOLOGICAL METHODS FOR PREPARING TERPENES

FIG. 1




(57) Abstract: The technology relates in part to biological methods for producing terpenes and to engineered cells and microorganisms capable of such production.

WO 2019/014310 A1

What is claimed is:

1. A genetically modified microorganism, comprising:

one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides, wherein expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide.

2. The microorganism of claim 1, wherein the microorganism is a fungus.

3. The microorganism of claim 2, wherein the fungus is a yeast.

4. The microorganism of claim 3, wherein the yeast is chosen from *Candida* spp, *Yarrowia* spp, *Rhodotorula* spp, *Rhodosporidium* spp, *Cryptococcus* spp, *Trichosporon* spp, *Lipomyces* spp, and *Blastobotrys* spp.

5. The microorganism of any one of claims 1 to 4, wherein the one or more heterologous nucleic acids encode one or more terpene biosynthesis polypeptides chosen from terpene synthase, phytoene synthase, geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, zeaxanthin glucosyltransferase, valencene synthase, and cytochrome p450 reductase.

6. The microorganism of any one of claims 1 to 5, wherein the fatty acid is a saturated fatty acid or an unsaturated fatty acid.

7. The microorganism of any one of claims 1 to 6, wherein the fatty acid is chosen from one or more of oleic acid, palmitoleic acid, erucic acid, linoleic acid, palmitic acid, caproic acid, enanthic acid, caprylic acid pelargonic acid, capric acid, undecylic acid, lauric acid, myristic acid, pentadecanoic acid, margaric acid, stearic acid arachidic acid, behenic acid, tridecylic acid, and linolenic acid.



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8. The microorganism of any one of claims 1 to 5, wherein the alkane is chosen from one or more of hexane, heptane, nonane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, hexadecane, heptadecane, and octadecane

9. The microorganism of any one of claims 1 to 8, wherein the nucleic acid that provides for fatty acid or alkane induction of expression of a terpene biosynthesis polypeptide comprises a fatty acid response element or an alkane response element.

10. The microorganism of claim 9, wherein the fatty acid response element comprises an oleic acid response element.

11. The microorganism of claim 9, wherein the alkane response element comprises an alkane response element 1 (ARE1) sequence.

12. The microorganism of any one of claims 1 to 11, wherein the nucleic acid that provides for fatty acid induction of expression of a terpene biosynthesis polypeptide comprises a promoter region chosen from promoter regions of genes encoding hydratase-dehydrogenase-epimerase (HDE), acyl co-A oxidase (POX), acyl co-A thiolase (POT), peroxin (PEX) and peroxisomal adenine nucleotide transporter protein (ANT1).

13. The microorganism of any one of claims 1 to 12, wherein the microorganism is *Candida viswanathii.*

14. The microorganism of any one of claims 1 to 12, wherein the microorganism is *Blastobotrys adeninivorans*.

15. The microorganism of any one of claims 1 to 14, wherein the one or more heterologous nucleic acids encode phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase.

16. The microorganism of claim 15, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene



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desaturase is regulated by a promoter region of the gene encoding a hydratase-dehydrogenase-epimerase (HDE).

17. The microorganism of claim 15, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase is regulated by a promoter region of the gene encoding a *Candida* hydratase-dehydrogenase-epimerase (HDE).

18. The microorganism of claim 15, 16 or 17, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase is regulated by a terminator region of the gene encoding an acyl co-A oxidase 4 (POX4).

19. The microorganism of claim 15, 16 or 17, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase is regulated by a terminator region of the gene encoding a *Candida* acyl co-A oxidase 4 (POX4).

20. The microorganism of any one of claims 1 to 14, wherein the one or more heterologous nucleic acids encode valencene synthase.

21. The microorganism of claim 20, wherein expression of the heterologous nucleic acids encoding valencene synthase is regulated by a promoter region of the gene encoding a hydratase-dehydrogenase-epimerase (HDE).

22. The microorganism of claim 20, wherein expression of the heterologous nucleic acids encoding valencene synthase is regulated by a promoter region of the gene encoding a *Candida* hydratase-dehydrogenase-epimerase (HDE).

23. The microorganism of claim 20, 21 or 22, wherein expression of the heterologous nucleic acids encoding valencene synthase is regulated by a terminator region of the gene encoding an acyl co-A oxidase 4 (POX4).



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24. The microorganism of claim 20, 21 or 22, wherein expression of the heterologous nucleic acids encoding valencene synthase is regulated by a terminator region of the gene encoding a *Candida* acyl co-A oxidase 4 (POX4).

25. The microorganism of any one of claims 1 to 14, wherein the one or more heterologous nucleic acids encode geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase.

26. The microorganism of claim 25, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a promoter region of the gene encoding a hydratase-dehydrogenase-epimerase (HDE).

27. The microorganism of claim 25, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a promoter region of the gene encoding a *Candida* hydratase-dehydrogenase-epimerase (HDE).

28. The microorganism of claim 25, 26 or 27, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a terminator region of the gene encoding an acyl co-A oxidase 4 (POX4).

29. The microorganism of claim 25, 26 or 27, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a terminator region of the gene encoding a *Candida* acyl co-A oxidase 4 (POX4).

30. The microorganism of any one of claims 1 to 29, wherein the one or more heterologous nucleic acids encoding the one or more terpene biosynthesis polypeptides are endogenously expressed in a microorganism chosen from *Cronobacter* spp, *Callitropsis* spp, *Xanthophyllomyces* spp, *Agrobacterium* spp, and *Pantoea* spp.



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31. The microorganism of any one of claims 1 to 30, wherein the amount and/or activity of a Ras2 protein has been decreased.

32. The microorganism of any one of claims 1 to 31, wherein the microorganism has been genetically modified to reduce or eliminate expression of an endogenous *RAS2* gene.

33. The microorganism of any one of claims 1 to 32, wherein the amount and/or activity of an Faa1 protein has been decreased.

34. The microorganism of any one of claims 1 to 33, wherein the microorganism has been genetically modified to reduce or eliminate expression of an endogenous *FAA1* gene.

35. The microorganism of any one of claims 1 to 34, wherein the amounts and/or activities of one or more proteins chosen from acetyl-CoA C-acetyltransferase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, and isopentyl diphosphate delta isomerase, have been increased.

36. The microorganism of any one of claims 1 to 35, wherein the microorganism has been genetically modified to increase expression of one or more endogenous genes chosen from *ERG10*, *ERG13*, *HMG1*, *ERG12*, *ERG8*, *MVD1*, and *IDI1*.

37. The microorganism of any one of claims 1 to 36, wherein the amount and/or activity of dimethylallyltranstransferase or farnesyl diphosphate synthetase has been increased.

38. The microorganism of any one of claims 1 to 37, wherein the microorganism has been genetically modified to increase expression of an endogenous *ERG20* gene.

39. The microorganism of claim 36, wherein expression of *ERG10*, *ERG13*, *HMG1*, *ERG12*, *ERG8*, *MVD1*, and/or *IDI1* is regulated by a promoter region of the gene encoding oleate-induced peroxisomal protein (POX18).



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40. The microorganism of claim 36 or 39, wherein expression of *ERG10*, *ERG13*, *HMG1*, *ERG12*, *ERG8*, *MVD1*, and/or *IDI1* is regulated by a terminator region of the gene encoding oleate-induced peroxisomal protein (POX18).

41. The microorganism of claim 38, wherein expression of *ERG20* is regulated by a promoter region of the gene encoding oleate-induced peroxisomal protein (POX18).

42. The microorganism of claim 38 or 41, wherein expression of *ERG20* is regulated by a terminator region of the gene encoding oleate-induced peroxisomal protein (POX18).

43. A genetically modified microorganism, comprising:

one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides, and

a genetic modification that alters the expression of a polypeptide providing for transport of acetyl-carnitine in the microorganism.

44. The microorganism of claim 43, wherein expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide.

45. The microorganism of claim 43, wherein expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for glucose induction of expression of the terpene biosynthesis polypeptide.

46. The microorganism of claim 43, 44 or 45, wherein the microorganism is a fungus.

47. The microorganism of claim 46, wherein the fungus is a yeast.

48. The microorganism of claim 47, wherein the yeast is chosen from *Candida* spp, *Yarrowia* spp, *Rhodotorula* spp, *Rhodosporidium* spp, *Cryptococcus* spp, *Trichosporon* spp, *Lipomyces* spp, and *Blastobotrys* spp.

49. The microorganism of any one of claims 43 to 48, wherein the one or more heterologous nucleic acids encode one or more terpene biosynthesis polypeptides



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chosen from terpene synthase, phytoene synthase, geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, zeaxanthin glucosyltransferase, valencene synthase, and cytochrome p450 reductase.

50. The microorganism of any one of claims 43 to 49, wherein the genetic modification reduces expression of the polypeptide providing for transport of acetyl-carnitine in the microorganism relative to a microorganism that does not have the genetic modification.

51. The microorganism of claim 50, wherein the genetic modification is a disruption, deletion or knockout of (i) a polynucleotide that encodes a polypeptide providing for transport of acetyl-carnitine, or (ii) a promoter operably linked to a polynucleotide that encodes a polypeptide providing for transport of acetyl-carnitine, whereby endogenous activity of a polypeptide providing for transport of acetyl-carnitine is reduced or abolished.

52. The microorganism of claim 50, further comprising a nucleic acid encoding a polypeptide providing for transport of acetyl-carnitine wherein expression of the nucleic acid encoding a polypeptide providing for transport of acetyl-carnitine is regulated by a promoter that provides for reduced expression relative to endogenous expression.

53. The microorganism of claim 52, wherein the genetic modification comprises replacing the promoter of an endogenous gene encoding the polypeptide providing for transport of acetyl-carnitine in the microorganism with a promoter that provides for reduced expression of the polypeptide in the microorganism relative to a microorganism that does not have the genetic modification.

54. The microorganism of claim 52 or 53, wherein the promoter that provides for reduced expression relative to endogenous expression is a promoter for a glucose-6-phosphate isomerase (G6PI) gene.



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55. The microorganism of claim 52 or 53, wherein the promoter that provides for reduced expression relative to endogenous expression is a promoter for a *Candida* glucose-6-phosphate isomerase (G6PI) gene.

56. The microorganism of any one of claims 43 to 55, wherein the polypeptide providing for transport of acetyl-carnitine in the microorganism is an acetyl-carnitine translocase (CRC).

57. The microorganism of claim 56, wherein the acetyl-carnitine translocase (CRC) is acetyl-carnitine translocase 1 (CRC1).

58. The microorganism of any one of claims 43 to 57, wherein the microorganism is *Candida viswanathii*.

59. The microorganism of any one of claims 43 to 57, wherein the microorganism is *Blastobotrys adeninivorans*.

60. The microorganism of any one of claims 43 to 59, wherein the one or more heterologous nucleic acids encode phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase.

61. The microorganism of claim 60, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase is regulated by a promoter region of the gene encoding a hydratase-dehydrogenase-epimerase (HDE).

62. The microorganism of claim 60, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase is regulated by a promoter region of the gene encoding a *Candida* hydratase-dehydrogenase-epimerase (HDE).

63. The microorganism of claim 60, 61 or 62, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and



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phytoene desaturase is regulated by a terminator region of the gene encoding an acyl co-A oxidase 4 (POX4).

64. The microorganism of claim 60, 61 or 62, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase is regulated by a terminator region of the gene encoding a *Candida* acyl co-A oxidase 4 (POX4).

65. The microorganism of any one of claims 43 to 59, wherein the one or more heterologous nucleic acids encode geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase.

66. The microorganism of claim 65, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a promoter region of the gene encoding a hydratase-dehydrogenase-epimerase (HDE).

67. The microorganism of claim 65, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a promoter region of the gene encoding a *Candida* hydratase-dehydrogenase-epimerase (HDE).

68. The microorganism of claim 65, 66 or 67, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a terminator region of the gene encoding an acyl co-A oxidase 4 (POX4).

69. The microorganism of claim 65, 66 or 67, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a terminator region of the gene encoding a *Candida* acyl co-A oxidase 4 (POX4).

70. The microorganism of any one of claims 43 to 69, wherein the one or more heterologous nucleic acids encoding the one or more terpene biosynthesis polypeptides



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are endogenously expressed in a microorganism chosen from *Cronobacter* spp, *Callitropsis* spp, *Xanthophyllomyces* spp, *Agrobacterium* spp, and *Pantoea* spp.

71. The microorganism of any one of claims 43 to 70, wherein the amount and/or activity of a Ras2 protein has been decreased.

72. The microorganism of any one of claims 43 to 71, wherein the microorganism has been genetically modified to reduce or eliminate expression of an endogenous *RAS2* gene.

73. The microorganism of any one of claims 43 to 72, wherein the amount and/or activity of an Faa1 protein has been decreased.

74. The microorganism of any one of claims 43 to 73, wherein the microorganism has been genetically modified to reduce or eliminate expression of an endogenous *FAA1* gene.

75. The microorganism of any one of claims 43 to 74, wherein the amounts and/or activities of one or more proteins chosen from acetyl-CoA C-acetyltransferase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, and isopentyl diphosphate delta isomerase, have been increased.

76. The microorganism of any one of claims 43 to 75, wherein the microorganism has been genetically modified to increase expression of one or more endogenous genes chosen from *ERG10*, *ERG13*, *HMG1*, *ERG12*, *ERG8*, *MVD1*, and *IDI1*.

77. The microorganism of any one of claims 43 to 76, wherein the amount and/or activity of dimethylallyltranstransferase or farnesyl diphosphate synthetase has been increased.

78. The microorganism of any one of claims 43 to 77, wherein the microorganism has been genetically modified to increase expression of an endogenous *ERG20* gene.



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79. The microorganism of claim 76, wherein expression of *ERG10*, *ERG13*, *HMG1*, *ERG12*, *ERG8*, *MVD1*, and/or *IDI1* is regulated by a promoter region of the gene encoding oleate-induced peroxisomal protein (POX18).

80. The microorganism of claim 76 or 79, wherein expression of *ERG10*, *ERG13*, *HMG1*, *ERG12*, *ERG8*, *MVD1*, and/or *IDI1* is regulated by a terminator region of the gene encoding oleate-induced peroxisomal protein (POX18).

81. The microorganism of claim 78, wherein expression of *ERG20* is regulated by a promoter region of the gene encoding oleate-induced peroxisomal protein (POX18).

82. The microorganism of claim 78 or 81, wherein expression of *ERG20* is regulated by a terminator region of the gene encoding oleate-induced peroxisomal protein (POX18).

83. A genetically modified *Candida viswanathii* yeast, comprising one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides.

84. The genetically modified *Candida viswanathii* yeast of claim 83, wherein expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide.

85. The genetically modified *Candida viswanathii* yeast of claim 83, wherein expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for glucose induction of expression of the terpene biosynthesis polypeptide.

86. The genetically modified *Candida viswanathii* yeast of claim 83, 84 or 85, wherein the one or more heterologous nucleic acids encode one or more terpene biosynthesis polypeptides chosen from terpene synthase, phytoene synthase, geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, zeaxanthin glucosyltransferase, valencene synthase, and cytochrome p450 reductase.



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87. The genetically modified *Candida viswanathii* yeast of any one of claims 83 to 86, wherein the one or more heterologous nucleic acids encode phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase

88. The genetically modified *Candida viswanathii* yeast of claim 87, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase is regulated by a promoter region of the gene encoding a *Candida* hydratase-dehydrogenase-epimerase (HDE).

89. The genetically modified *Candida viswanathii* yeast of claim 88, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase is regulated by a terminator region of the gene encoding a *Candida* acyl co-A oxidase 4 (POX4).

90. The genetically modified *Candida viswanathii* yeast of claim 87, wherein expression of the heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase is regulated by a promoter region of the gene encoding a *Candida* glyceraldehyde-3-phosphate dehydrogenase (GPD).

91. The genetically modified *Candida viswanathii* yeast of any one of claims 83 to 86, wherein the one or more heterologous nucleic acids encode valencene synthase.

92. The genetically modified *Candida viswanathii* yeast of claim 91, wherein expression of the heterologous nucleic acids encoding valencene synthase is regulated by a promoter region of the gene encoding a *Candida* hydratase-dehydrogenase-epimerase (HDE).

93. The genetically modified *Candida viswanathii* yeast of claim 92, wherein expression of the heterologous nucleic acids valencene synthase is regulated by a terminator region of the gene encoding a *Candida* acyl co-A oxidase 4 (POX4).

94. The genetically modified *Candida viswanathii* yeast of claim 91, wherein expression of the heterologous nucleic acids encoding valencene synthase is regulated by a



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promoter region of the gene encoding a *Candida* glyceraldehyde-3-phosphate dehydrogenase (GPD).

95. The genetically modified *Candida viswanathii* yeast of any one of claims 83 to 86, wherein the one or more heterologous nucleic acids encode geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase.

96. The genetically modified *Candida viswanathii* yeast of claim 95, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a promoter region of the gene encoding a *Candida* hydratase-dehydrogenase-epimerase (HDE).

97. The genetically modified *Candida viswanathii* yeast of claim 96, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a terminator region of the gene encoding a *Candida* acyl co-A oxidase 4 (POX4).

98. The genetically modified *Candida viswanathii* yeast of claim 95, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase is regulated by a promoter region of the gene encoding a *Candida* glyceraldehyde-3-phosphate dehydrogenase (GPD).

99. The genetically modified *Candida viswanathii* yeast of any one of claims 83 to 86, wherein the one or more heterologous nucleic acids encode geranylgeranyl diphosphate synthase, phytoene desaturase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, and cytochrome p450 reductase.

100. The genetically modified *Candida viswanathii* yeast of claim 99, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, and cytochrome p450



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reductase is regulated by a promoter region of the gene encoding a *Candida* hydratase-dehydrogenase-epimerase (HDE).

101. The genetically modified *Candida viswanathii* yeast of claim 100, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, and cytochrome p450 reductase is regulated by a terminator region of the gene encoding a *Candida* acyl co-A oxidase 4 (POX4).

102. The genetically modified *Candida viswanathii* yeast of claim 99, wherein expression of the heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, and cytochrome p450 reductase is regulated by a promoter region of the gene encoding a *Candida* glyceraldehyde-3-phosphate dehydrogenase (GPD).

103. The genetically modified *Candida viswanathii* yeast of any one of claims 83 to 102, wherein the one or more heterologous nucleic acids encoding the one or more terpene biosynthesis polypeptides are endogenously expressed in a microorganism chosen from *Cronobacter* spp, *Callitropsis* spp, *Xanthophyllomyces* spp, *Agrobacterium* spp, and *Pantoea* spp.

104. The genetically modified *Candida viswanathii* yeast of any one of claims 83 to 103, further comprising a genetic modification that alters the expression of one or more nucleic acids encoding one or more endogenous polypeptides.

105. The genetically modified *Candida viswanathii* yeast of claim 104, wherein the one or more endogenous polypeptides are chosen from polypeptides having one or more of the following activities: acyl-CoA synthetase activity, acyl-CoA oxidase activity, ATP-binding cassette transporter activity, carnitine acetyltransferase activity, transport of acetyl-carnitine, acyl-CoA thioesterase activity, acyl-CoA hydrolase activity, aldehyde dehydrogenase activity, monooxygenase activity, or monooxgenase reductase activity.



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106. The genetically modified *Candida viswanathii* yeast of any one of claims 83 to 105, wherein the amount and/or activity of a Ras2 protein has been decreased.

107. The genetically modified *Candida viswanathii* yeast of any one of claims 83 to 106, wherein the yeast has been genetically modified to reduce or eliminate expression of an endogenous *RAS2* gene.

108. The genetically modified *Candida viswanathii* yeast of any one of claims 83 to 107, wherein the amount and/or activity of an Faa1 protein has been decreased.

109. The genetically modified *Candida viswanathii* yeast of any one of claims 83 to 108, wherein the yeast has been genetically modified to reduce or eliminate expression of an endogenous *FAA1* gene.

110. The genetically modified *Candida viswanathii* yeast of any one of claims 83 to 109, wherein the amounts and/or activities of one or more proteins chosen from acetyl-CoA C-acetyltransferase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, and isopentyl diphosphate delta isomerase, have been increased.

111. The genetically modified *Candida viswanathii* yeast of any one of claims 83 to 110, wherein the yeast has been genetically modified to increase expression of one or more endogenous genes chosen from *ERG10*, *ERG13*, *HMG1*, *ERG12*, *ERG8*, *MVD1*, and *IDI1*.

112. The genetically modified *Candida viswanathii* yeast of any one of claims 83 to 111, wherein the amount and/or activity of dimethylallyltranstransferase or farnesyl diphosphate synthetase has been increased.

113. The genetically modified *Candida viswanathii* yeast of any one of claims 83 to 112, wherein the yeast has been genetically modified to increase expression of an endogenous *ERG20* gene.



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114. The genetically modified *Candida viswanathii* yeast of claim 111, wherein expression of *ERG10*, *ERG13*, *HMG1*, *ERG12*, *ERG8*, *MVD1*, and/or *IDI1* is regulated by a promoter region of the gene encoding oleate-induced peroxisomal protein (POX18).

115. The genetically modified *Candida viswanathii* yeast of claim 111 or 114, wherein expression of *ERG10*, *ERG13*, *HMG1*, *ERG12*, *ERG8*, *MVD1*, and/or *IDI1* is regulated by a terminator region of the gene encoding oleate-induced peroxisomal protein (POX18).

116. The genetically modified *Candida viswanathii* yeast of claim 113, wherein expression of *ERG20* is regulated by a promoter region of the gene encoding oleate-induced peroxisomal protein (POX18).

117. The genetically modified *Candida viswanathii* yeast of claim 113 or 116, wherein expression of *ERG20* is regulated by a terminator region of the gene encoding oleate-induced peroxisomal protein (POX18).

118. A method for producing a terpene comprising:

contacting the genetically modified microorganism of any one of claims 1 to 42 with a feedstock comprising a carbon source, and

culturing the microorganism under conditions in which the terpenes are produced from the feedstock.

119. A method for producing a terpene comprising:

contacting the genetically modified microorganism of any one of claims 43 to 82 with a feedstock comprising a carbon source, and

culturing the microorganism under conditions in which the terpenes are produced from the feedstock.

120. A method for producing a terpene comprising:

contacting the genetically modified *Candida viswanathii* yeast of any one of claims 83 to 117 with a feedstock comprising a carbon source, and

culturing the microorganism under conditions in which the terpenes are produced from the feedstock.



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121. The method of any one of claims 118 to 120, wherein the feedstock comprises one or more fatty acids.

122. The method of claim 121, wherein the feedstock comprises one or more saturated fatty acids or one or more unsaturated fatty acids; or one or more saturated fatty acids and one or more unsaturated fatty acids.

123. The method of claim 121 or 122, wherein the feedstock comprises one or more fatty acids chosen from oleic acid, palmitoleic acid, erucic acid, linoleic acid, palmitic acid, caproic acid, enanthic acid, caprylic acid pelargonic acid, capric acid, undecylic acid, lauric acid, myristic acid, pentadecanoic acid, margaric acid, stearic acid arachidic acid, behenic acid, tridecylic acid, and linolenic acid.

124. The method of claim 123 wherein the feedstock comprises oleic acid.

125. The method of any one of claims 118 to 120, wherein the feedstock comprises one or more sugars.

126. The method of 125, wherein the feedstock comprises glucose.

127. The method of any one of claims 118 to 120, wherein the feedstock comprises one or more alkane hydrocarbons.

128. The method of claim 127, wherein the feedstock comprises one or more alkane hydrocarbons chosen from one or more of hexane, heptane, nonane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, hexadecane, heptadecane, and octadecane.

129. The method of claim 127, wherein the feedstock comprises one or more alkane hydrocarbons chosen from C10 to C14 alkane hydrocarbons, nonane and octadecane.

130. The method of any one of claims 118 to 120, wherein the feedstock comprises one or more vegetable oils, derivatives thereof, or byproducts thereof.



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131. The method of claim 130, wherein the feedstock comprises crude palm oil (CPO).

132. The method of claim 130, wherein the feedstock comprises palm oil fatty acid distillate (PFAD).

133. The method of any one of claims 118 to 132, wherein the terpene is chosen from lycopene, beta carotene and astaxanthin.

134. The method of any one of claims 118 to 133, wherein the yield or titer of the terpene is between about 0.001 g/L to about 2.0 g/L.

135. The method of any one of claims 118 to 133, wherein the yield or titer of the terpene is between about 0.05 g/L to about 0.5 g/L.

136. The method of any one of claims 118 to 133, wherein the yield or titer of the terpene is between about 0.001 g/L to about 0.2 g/L.

137. The method of claim 134, wherein the yield or titer of lycopene is between about 0.03 g/L to about 2.0 g/L.

138. The method of claim 135, wherein the yield or titer of lycopene is between about 0.05 g/L to about 0.5 g/L.

139. The method of claim 136, wherein the yield or titer of lycopene is between about 0.001 g/L to about 0.2 g/L.

140. The method of claim 134, wherein the yield or titer of beta carotene is between about 0.1 g/L to about 0.2 g/L.

141. The method of claim 136, wherein the yield or titer of beta carotene is between about 0.01 g/L to about 0.1 g/L.



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142. The method of claim 136, wherein the yield or titer of beta carotene is between about 0.001 g/L to about 0.01 g/L.

143. The method of claim 134, wherein the yield or titer of astaxanthin is between about 0.005 g/L to about 0.01 g/L.

144. The method of claim 136, wherein the yield or titer of astaxanthin is between about 0.001 g/L to about 0.005 g/L.

145. The method of any one of claims 118 to 144, further comprising isolating the terpene.

146. A genetically modified yeast comprising one or more heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase.

147. A genetically modified yeast comprising one or more heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase and bifunctional lycopene cyclase/phytoene synthase.

148. A genetically modified yeast comprising one or more heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, and cytochrome p450 reductase.

149. The genetically modified yeast of claim 146, 147 or 148, wherein the amount and/or activity of a Ras2 protein has been decreased.

150. The genetically modified yeast of any one of claims 146 to 149, wherein the yeast has been genetically modified to reduce or eliminate expression of an endogenous *RAS2* gene.

151. The genetically modified yeast of any one of claims 146 to 150, wherein the amount and/or activity of an Faa1 protein has been decreased.



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152. The genetically modified yeast of any one of claims 146 to 151, wherein the yeast has been genetically modified to reduce or eliminate expression of an endogenous *FAA1* gene.

153. The genetically modified yeast of any one of claims 146 to 152, wherein the amounts and/or activities of one or more proteins chosen from acetyl-CoA C-acetyltransferase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, diphosphomevalonate decarboxylase, and isopentyl diphosphate delta isomerase, have been increased.

154. The genetically modified yeast of any one of claims 146 to 153, wherein the yeast has been genetically modified to increase expression of one or more endogenous genes chosen from *ERG10*, *ERG13*, *HMG1*, *ERG12*, *ERG8*, *MVD1*, and *IDI1*.

155. The genetically modified yeast of any one of claims 146 to 154, wherein the amount and/or activity of dimethylallyltranstransferase or farnesyl diphosphate synthetase has been increased.

156. The genetically modified yeast of any one of claims 146 to 155, wherein the yeast has been genetically modified to increase expression of an endogenous *ERG20* gene.

157. The genetically modified yeast of any one of claims 146 to 156, wherein the yeast is *Candida viswanathii.*

158. The genetically modified yeast of any one of claims 146 to 156, wherein the yeast is *Blastobotrys adeninivorans*.

159. Use of the genetically modified yeast of any one of claims 146 to 158 for the production of a terpene.

160. Use of the genetically modified yeast of any one of claims 146 and 149 to 159 for the production of lycopene.



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161. Use of the genetically modified yeast of any one of claims 147 and 149 to 159 for the production of beta carotene.

162. Use of the genetically modified yeast of any one of claims 148 to 159 for the production of astaxanthin.



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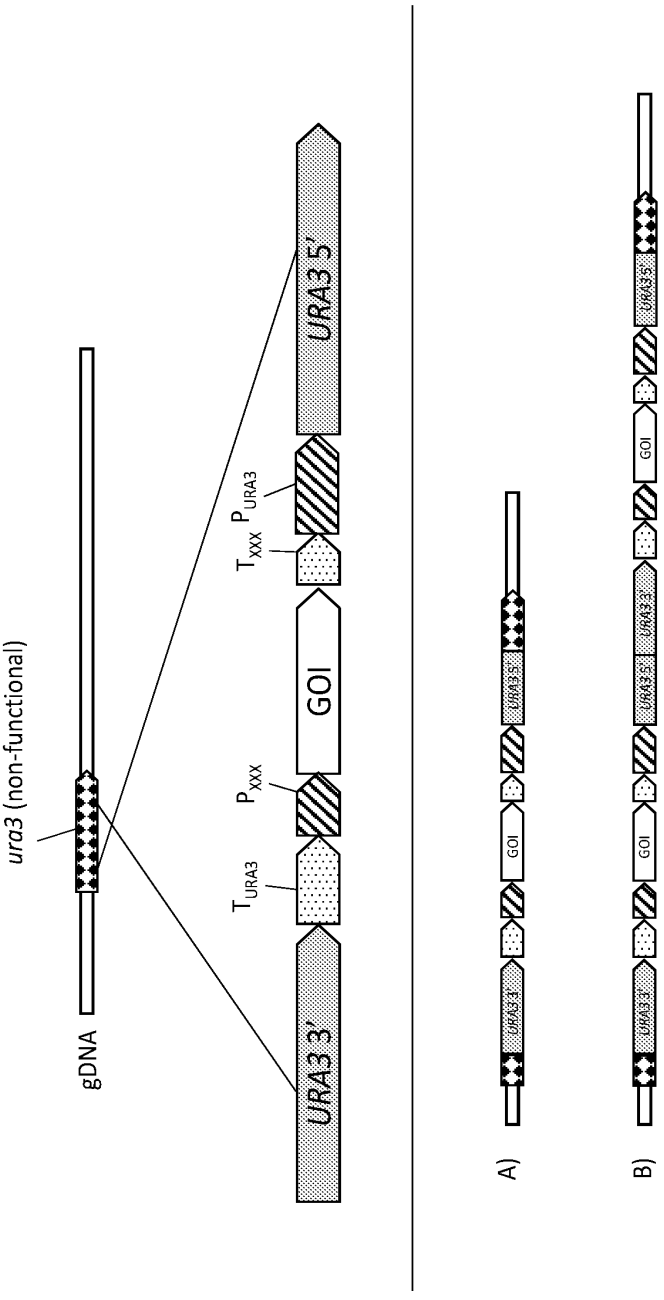

ura3 (non-functional)
gDNA
URA3 3'
T URA3
P XXX
GOI
T XXX
P URA3
URA3 5'
A)
B)
GOI

FIG. 1

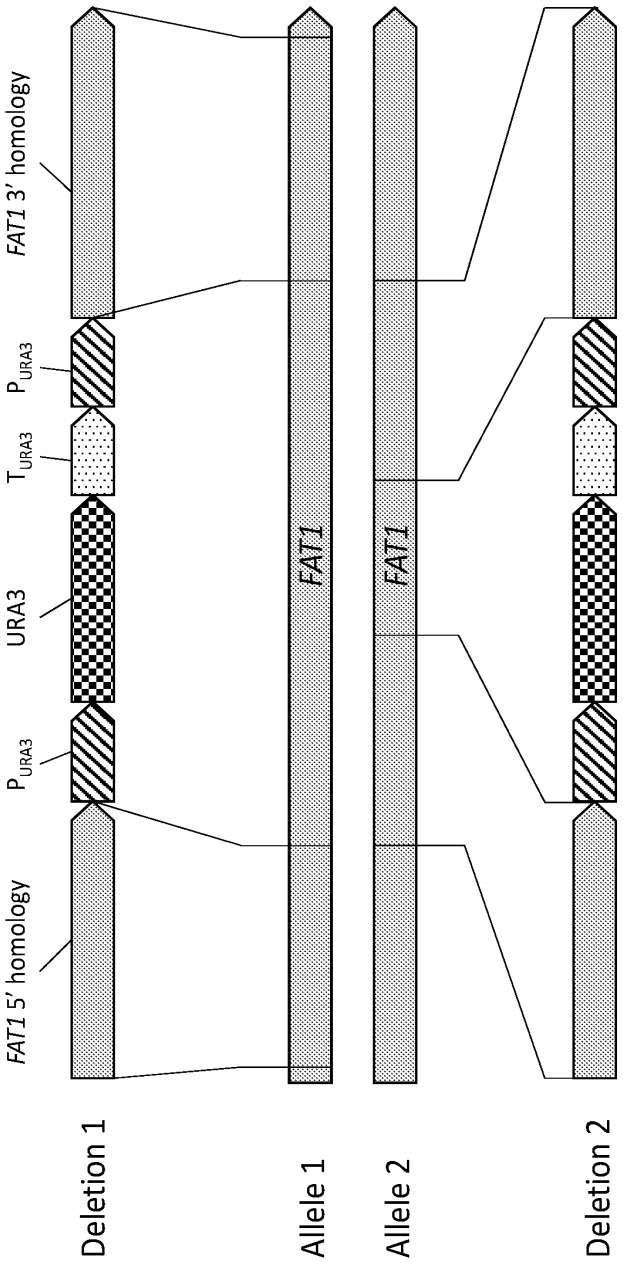

FAT1 3' homology
P URA3
T URA3
URA3
P URA3
FAT1 5' homology
FAT1
FAT1
Deletion 1
Allele 1
Allele 2
Deletion 2

FIG. 2

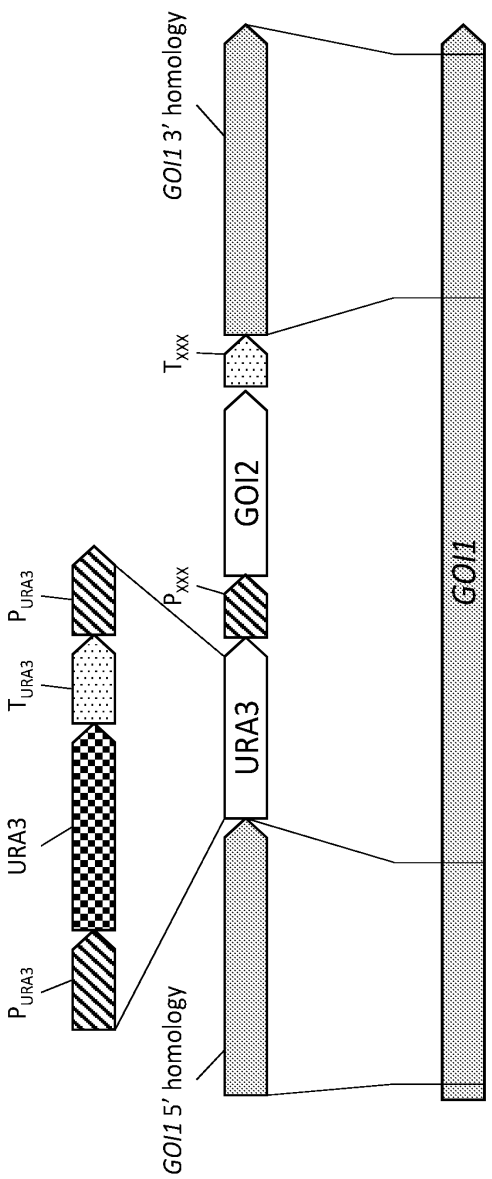



FIG. 3A

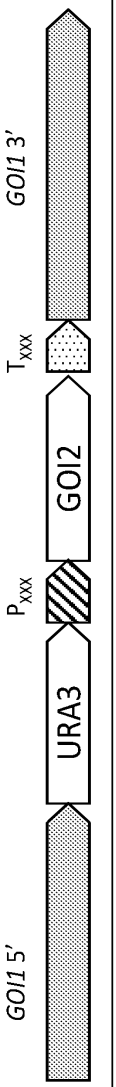



FIG. 3B




FIG. 3C

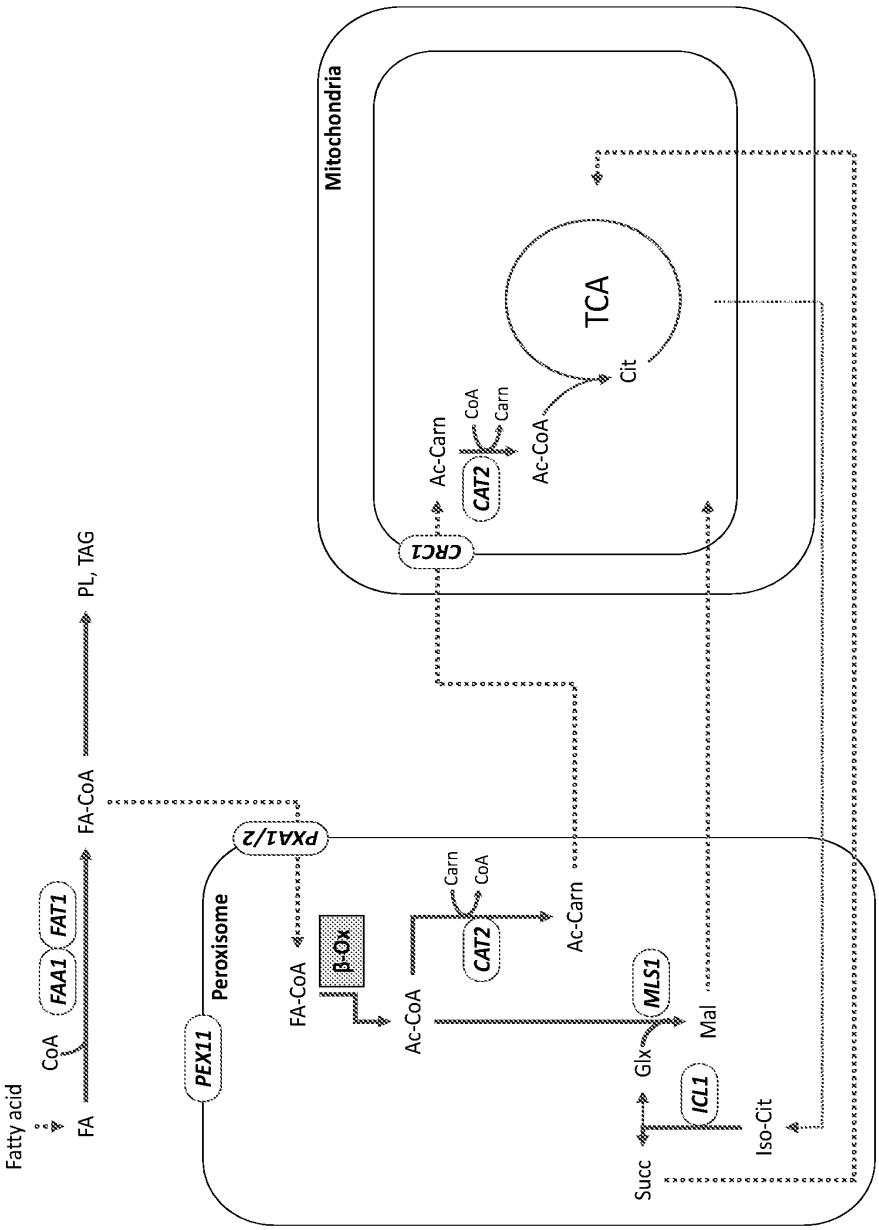

Fatty acid
FA
CoA
FAA1
FAT1
FA-CoA
PL, TAG
Peroxisome
PEX11
PXA1/2
FA-CoA
β-Ox
Ac-CoA
Carn
CoA
CAT2
Ac-Carn
MLS1
Glx
Mal
ICL1
Iso-Cit
Succ
Mitochondria
CRC1
Ac-Carn
CoA
Carn
CAT2
Ac-CoA
TCA
Cit

FIG. 4

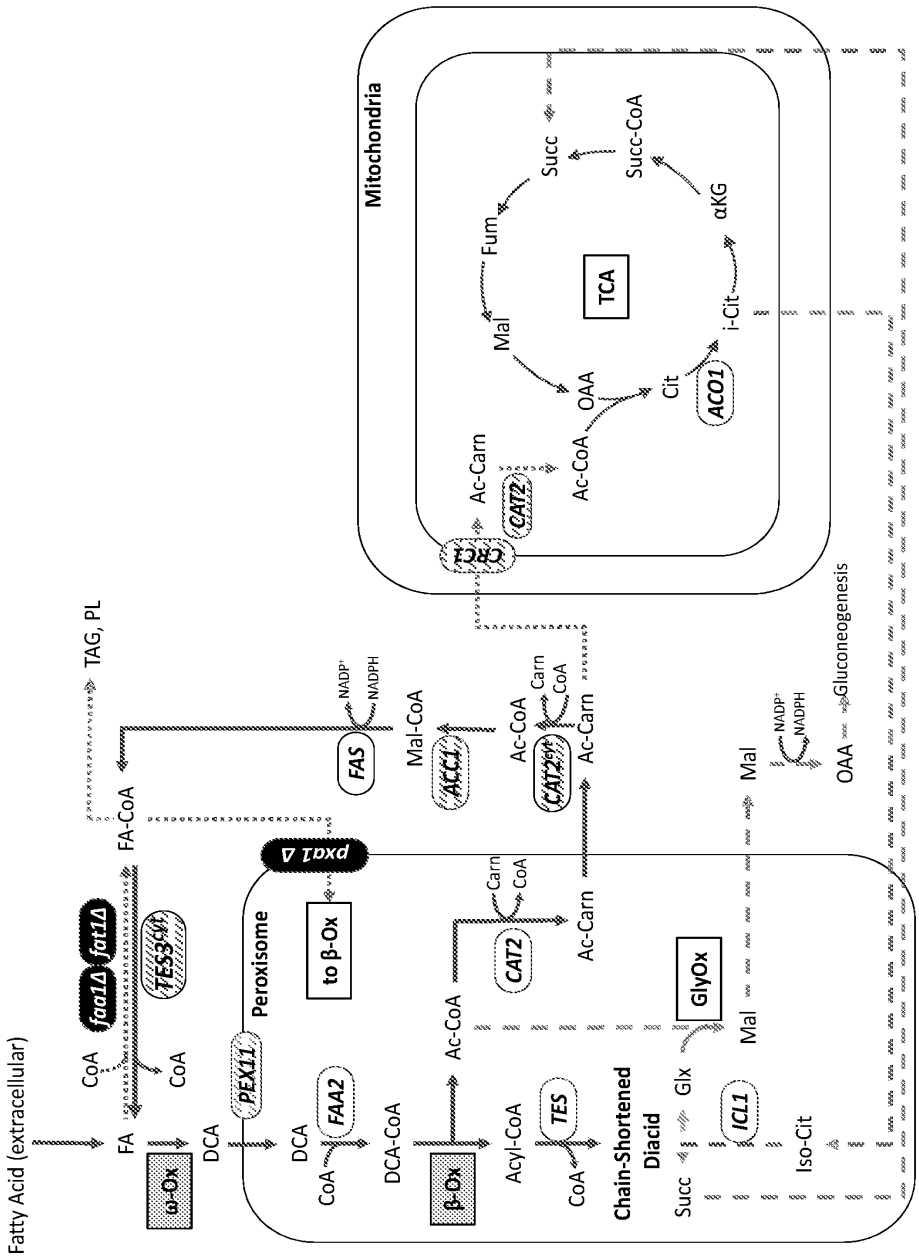

Fatty Acid (extracellular)
FA
CoA
faa1Δ fat1Δ
TES3Δ
TAG, PL
FA-CoA
α-Ox
DCA
PEX11
Peroxisome
pxo1 Δ
DCA
CoA
FAA2
DCA-CoA
to β-Ox
β-Ox
Acyl-CoA
CoA
TES
Ac-CoA
Carn
CoA
CAT2
Ac-Carn
Chain-Shortened Diacid
Succ
Glx
ICL1
Iso-Cit
GlyOx
Mal
FAS
NADP+
NADPH
Mal-CoA
ACC1
Ac-CoA
Carn
CoA
CAT2
Ac-Carn
Mal
NADP+
NADPH
OAA
Gluconeogenesis
Mitochondria
CRC1
Ac-Carn
CAT2
Ac-CoA
OAA
Cit
ACO1
i-Cit
αKG
Succ-CoA
Succ
Fum
Mal
TCA

FIG. 5

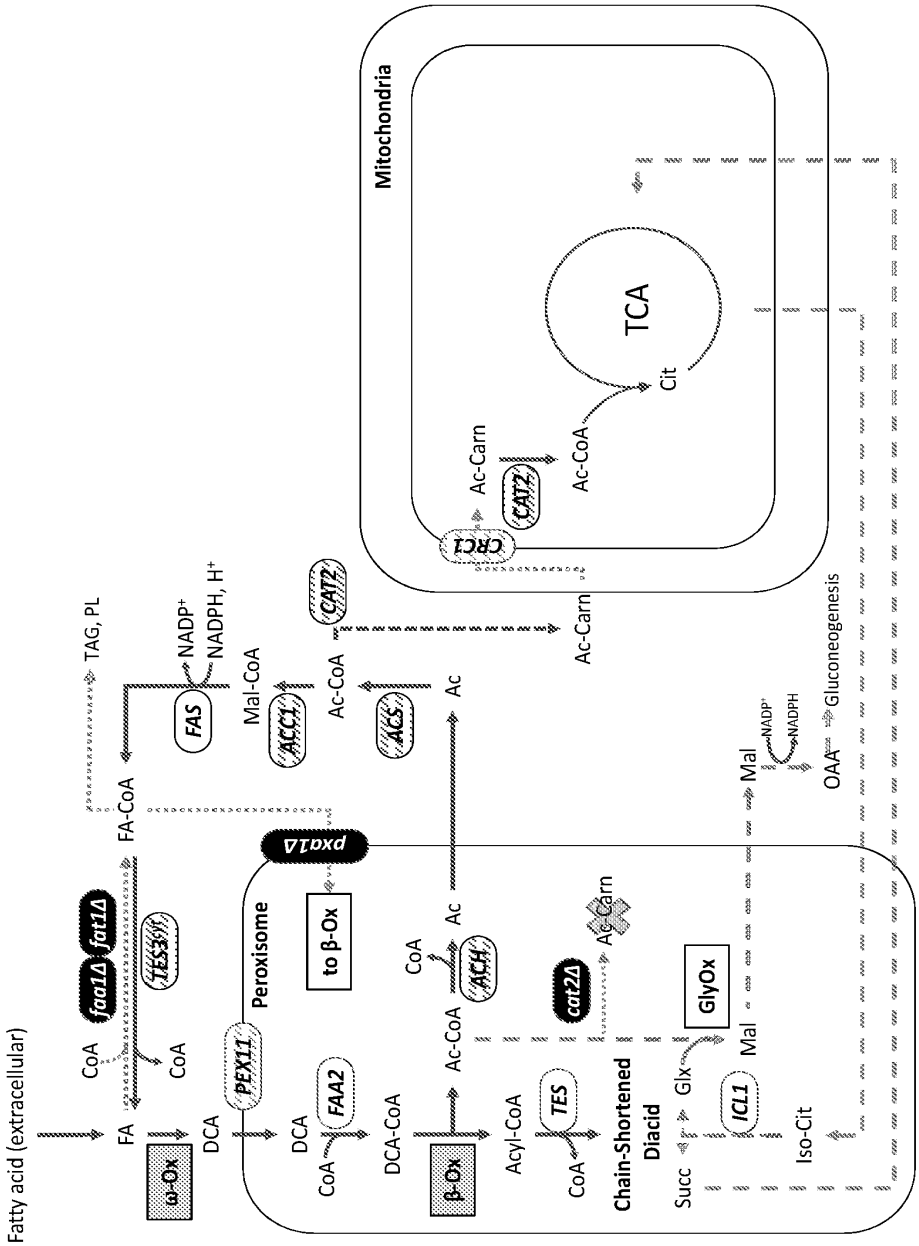

Fatty acid (extracellular)
FA
CoA
faa1Δ fat1Δ
α-Ox
DCA
PEX11
Peroxisome
pxa1Δ
TES1/2
FA-CoA
TAG, PL
FAS
NADP+
NADPH, H+
Mal-CoA
ACC1
Ac-CoA
ACS
Ac
CAT2
Ac-Carn
Mitochondria
CRC1
Ac-Carn
CAT2
Ac-CoA
TCA
Cit
DCA
CoA
FAA2
DCA-CoA
to β-Ox
β-Ox
Acyl-CoA
TES
CoA
Ac-CoA
CoA
Ac
ACH
cat2Δ
Ac-Carn
Chain-Shortened
Diacid
Succ
Glx
GlyOx
ICL1
Mal
Iso-Cit
Mal
NADP+
NADPH
OAA
Gluconeogenesis

FIG. 6

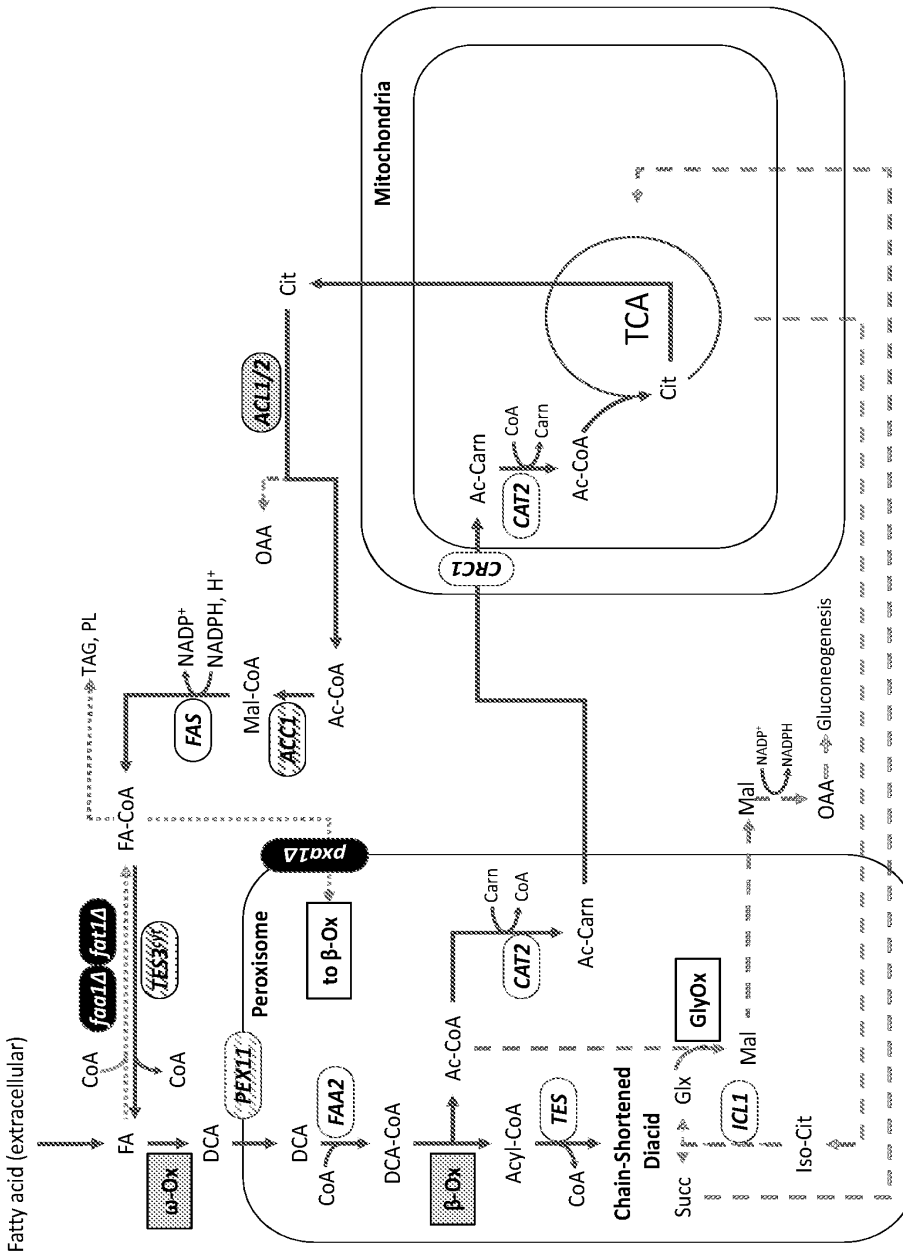

Fatty acid (extracellular)
FA
CoA
faa1Δ fat1Δ
FA-CoA
TAG, PL
α-Ox
DCA
PEX11
Peroxisome
pxa1Δ
DCA
CoA
FAA2
DCA-CoA
to β-Ox
β-Ox
Acyl-CoA
Ac-CoA
CAT2
Carn
CoA
Ac-Carn
TES
CoA
Chain-Shortened Diacid
Succ
Glx
GlyOx
ICL1
Mal
Iso-Cit
NADP+
NADPH, H+
FAS
Mal-CoA
ACC1
Ac-CoA
OAA
ACL1/2
Cit
Mitochondria
CRC1
Ac-Carn
CAT2
CoA
Carn
Ac-CoA
TCA
Cit
Mal
NADP+
NADPH
OAA
Gluconeogenesis

FIG. 7

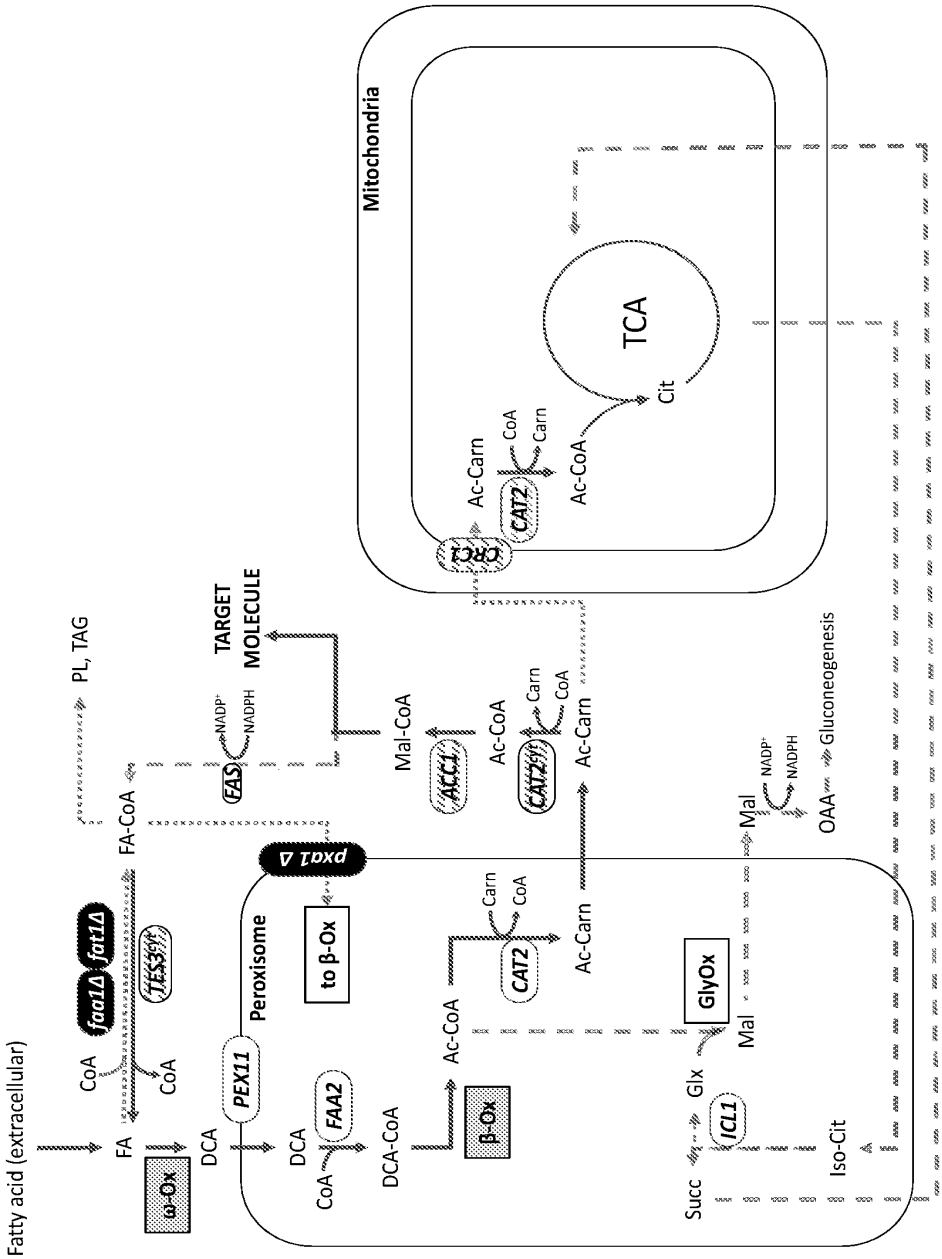

Fatty acid (extracellular)
FA
CoA
faa1Δ fat1Δ
FA-CoA
PL, TAG
TES3on
α-Ox
DCA
PEX11
Peroxisome
pxo1 Δ
DCA
CoA
FAA2
DCA-CoA
to β-Ox
β-Ox
Ac-CoA
Carn
CoA
CAT2
Ac-Carn
EAS
NADP+
NADPH
TARGET MOLECULE
Mal-CoA
ACC1
Ac-CoA
CAT2on
Carn
CoA
Ac-Carn
Succ
Glx
ICL1
Iso-Cit
GlyOx
Mal
Mal
NADP+
NADPH
OAA
Gluconeogenesis
Mitochondria
CRC1
Ac-Carn
CAT2
CoA
Carn
Ac-CoA
TCA
Cit

FIG. 8

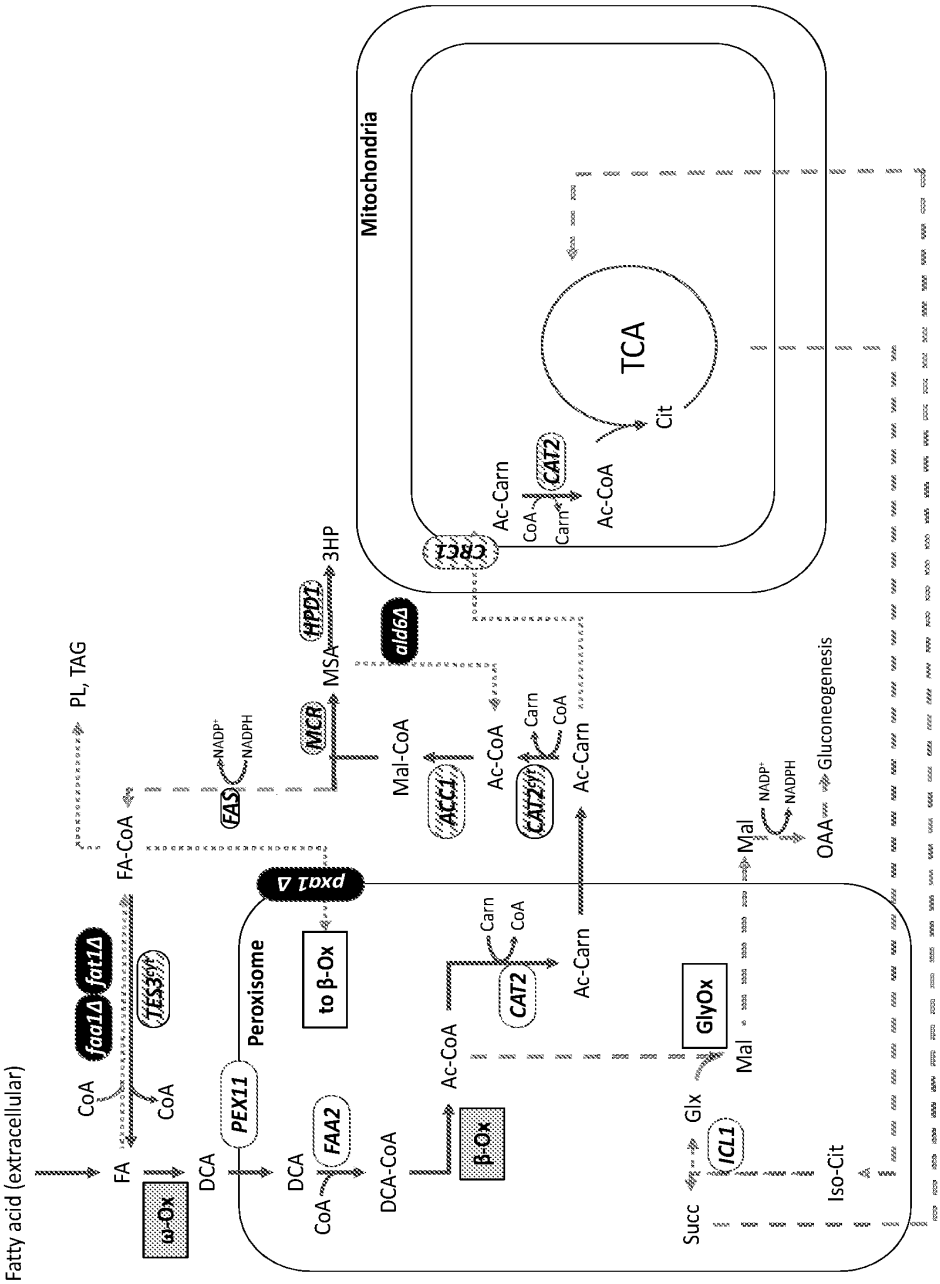

Fatty acid (extracellular)
FA
CoA
faa1Δ fat1Δ
FA-CoA
PL, TAG
TES3(P)
CoA
α-Ox
DCA
PEX11
Peroxisome
pxo1 Δ
DCA
CoA
FAA2
DCA-CoA
to β-Ox
Ac-CoA
β-Ox
Carn
CoA
CAT2
Ac-Carn
NADP+
NADPH
EAS
MCR
MSA
HPD1
3HP
Mal-CoA
ACC1
Ac-CoA
CAT2(P)
Carn
CoA
Ac-Carn
ald6Δ
Succ
Glx
ICL1
Iso-Cit
GlyOx
Mal
Mal
NADP+
NADPH
OAA
Gluconeogenesis
Mitochondria
CRC1
Ac-Carn
CoA
Carn
CAT2
Ac-CoA
TCA
Cit

FIG. 9

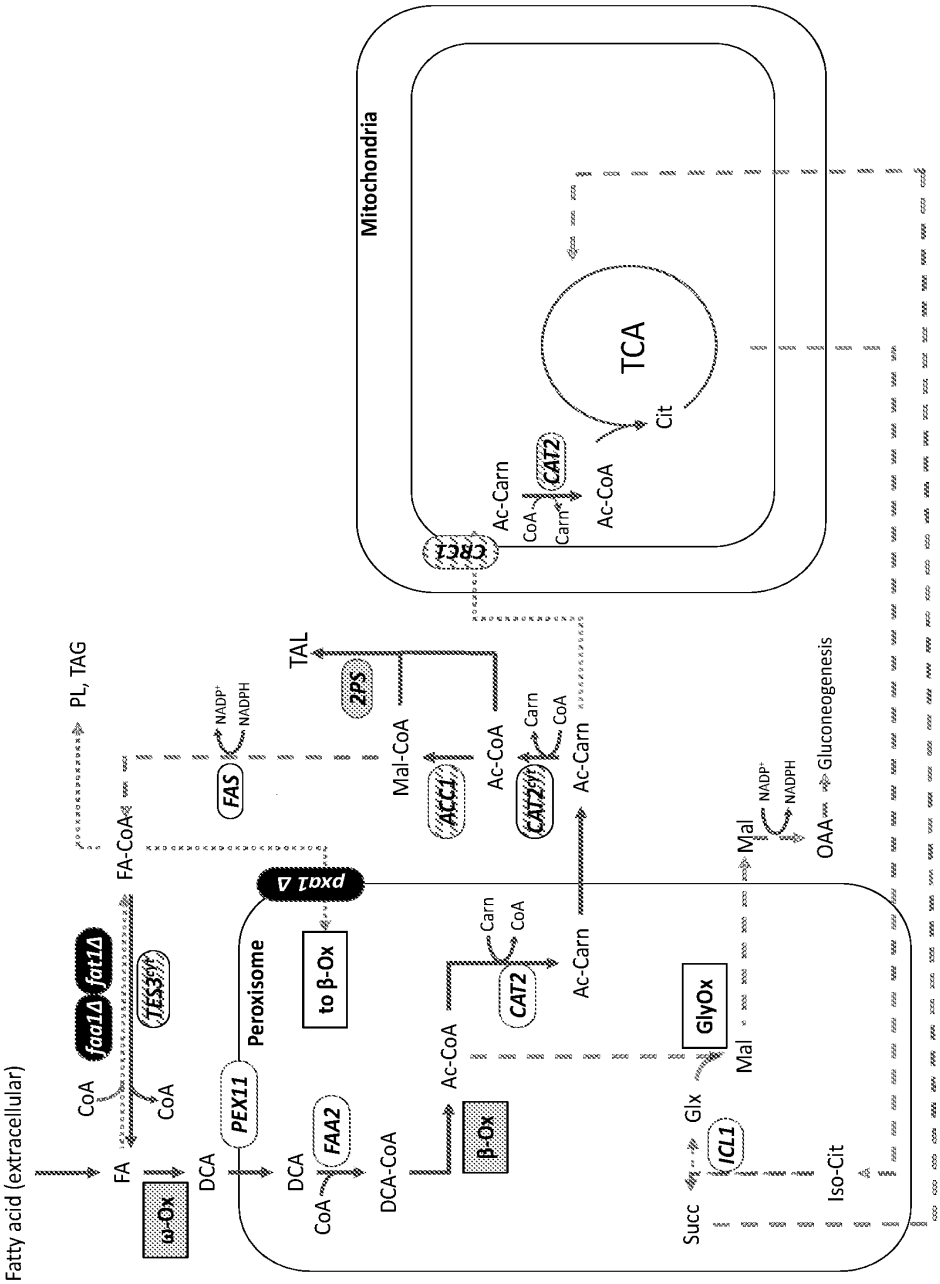

Fatty acid (extracellular)
FA
CoA
faa1Δ fat1Δ
FA-CoA
PL, TAG
TES3(P)
CoA
α-Ox
DCA
PEX11
Peroxisome
pxo1 Δ
DCA
CoA
FAA2
DCA-CoA
to β-Ox
β-Ox
Ac-CoA
Carn
CoA
CAT2
Ac-Carn
FAS
NADP+
NADPH
Mal-CoA
ACC1
Ac-CoA
CAT2(P)
Carn
CoA
Ac-Carn
TAL
2PS
Succ
Glx
ICL1
Iso-Cit
GlyOx
Mal
Mal
NADP+
NADPH
OAA
Gluconeogenesis
Mitochondria
CRC1
Ac-Carn
CoA
Carn
CAT2
Ac-CoA
TCA
Cit

FIG. 10

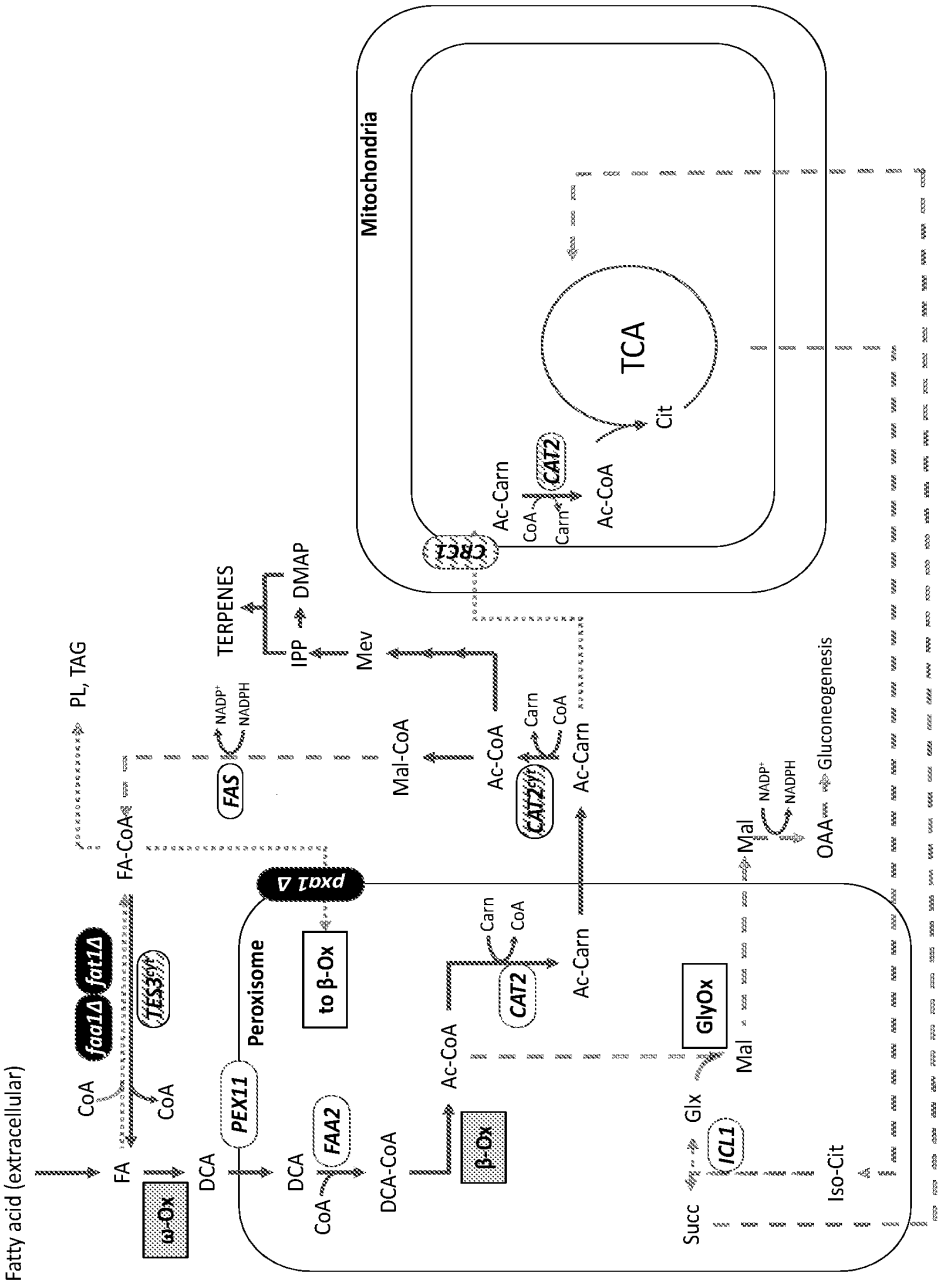

Fatty acid (extracellular)
FA
CoA
faa1Δ fat1Δ
TES3(p)
FA-CoA
PL, TAG
α-Ox
DCA
PEX11
Peroxisome
pxo1 Δ
DCA
CoA
FAA2
DCA-CoA
to β-Ox
β-Ox
Ac-CoA
Carn
CoA
CAT2
Ac-Carn
FAS
NADP+
NADPH
TERPENES
IPP
DMAP
Mev
Mal-CoA
Ac-CoA
Carn
CoA
CAT2(p)
Ac-Carn
Mitochondria
CRC1
Ac-Carn
CoA
Carn
CAT2
Ac-CoA
TCA
Cit
Succ
Glx
ICL1
Iso-Cit
GlyOx
Mal
Mal
NADP+
NADPH
OAA
Gluconeogenesis

FIG. 11

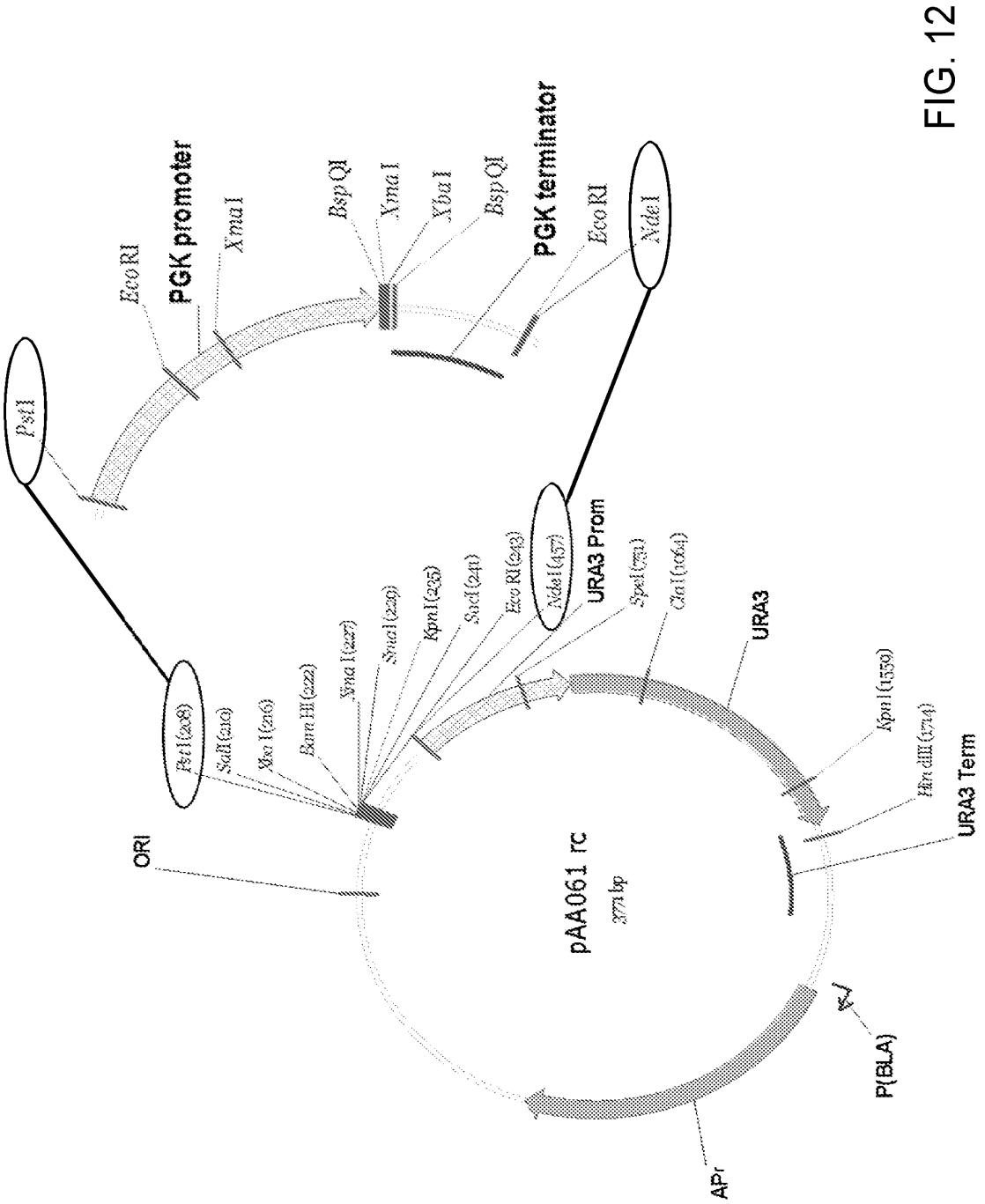



FIG. 12



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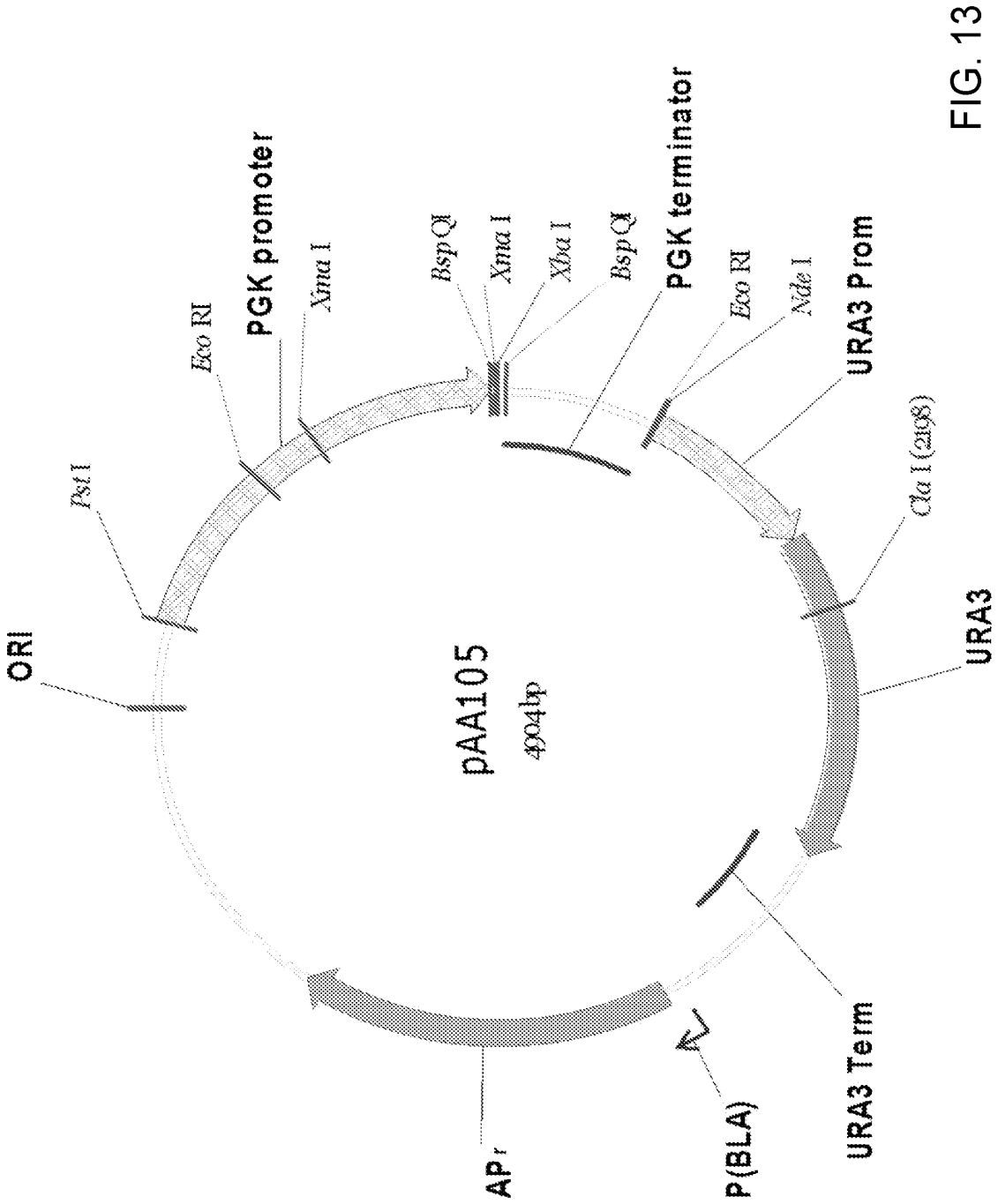



FIG. 13



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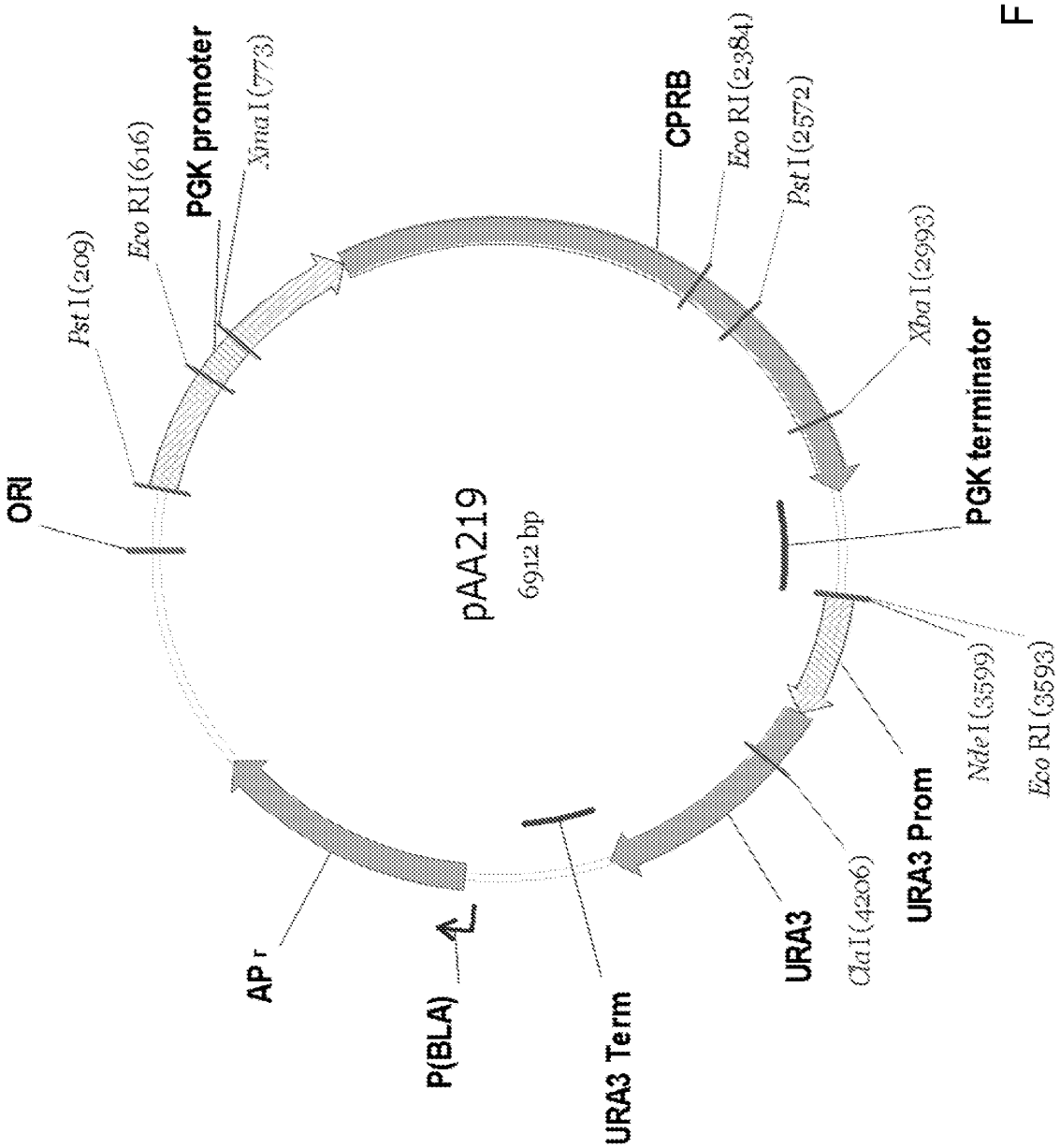



FIG. 14



LCY Biotechnology Holding, Inc.
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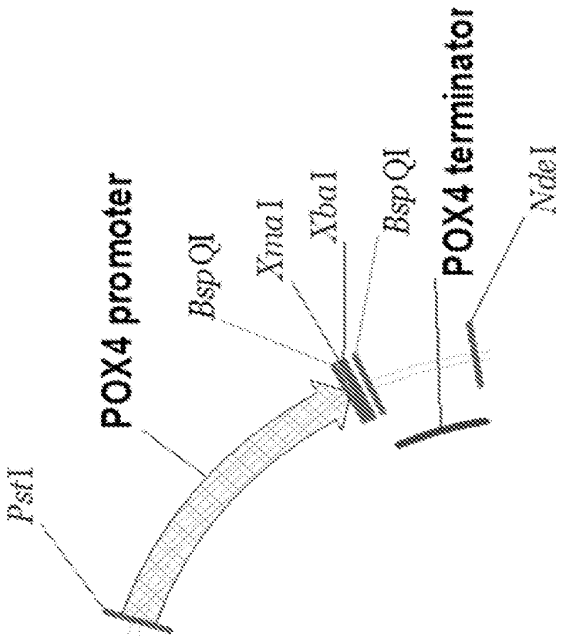

POX4 promoter
POX4 terminator
PstI
BspQI
XmaI
XbaI
BspQI
NdeI

FIG. 15

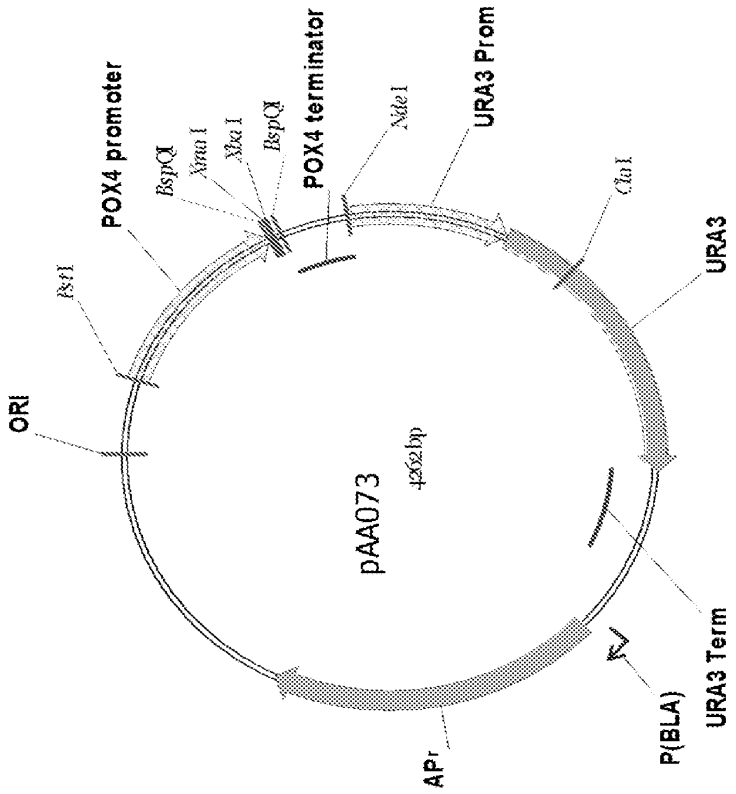

POX4 promoter
BspQI
Xma I
Xba I
BspQI
POX4 terminator
Nde I
URA3 Prom
Cla I
URA3
Bst1
ORI
pAA073
4262bp
APr
P(BLA)
URA3 Term

FIG. 16

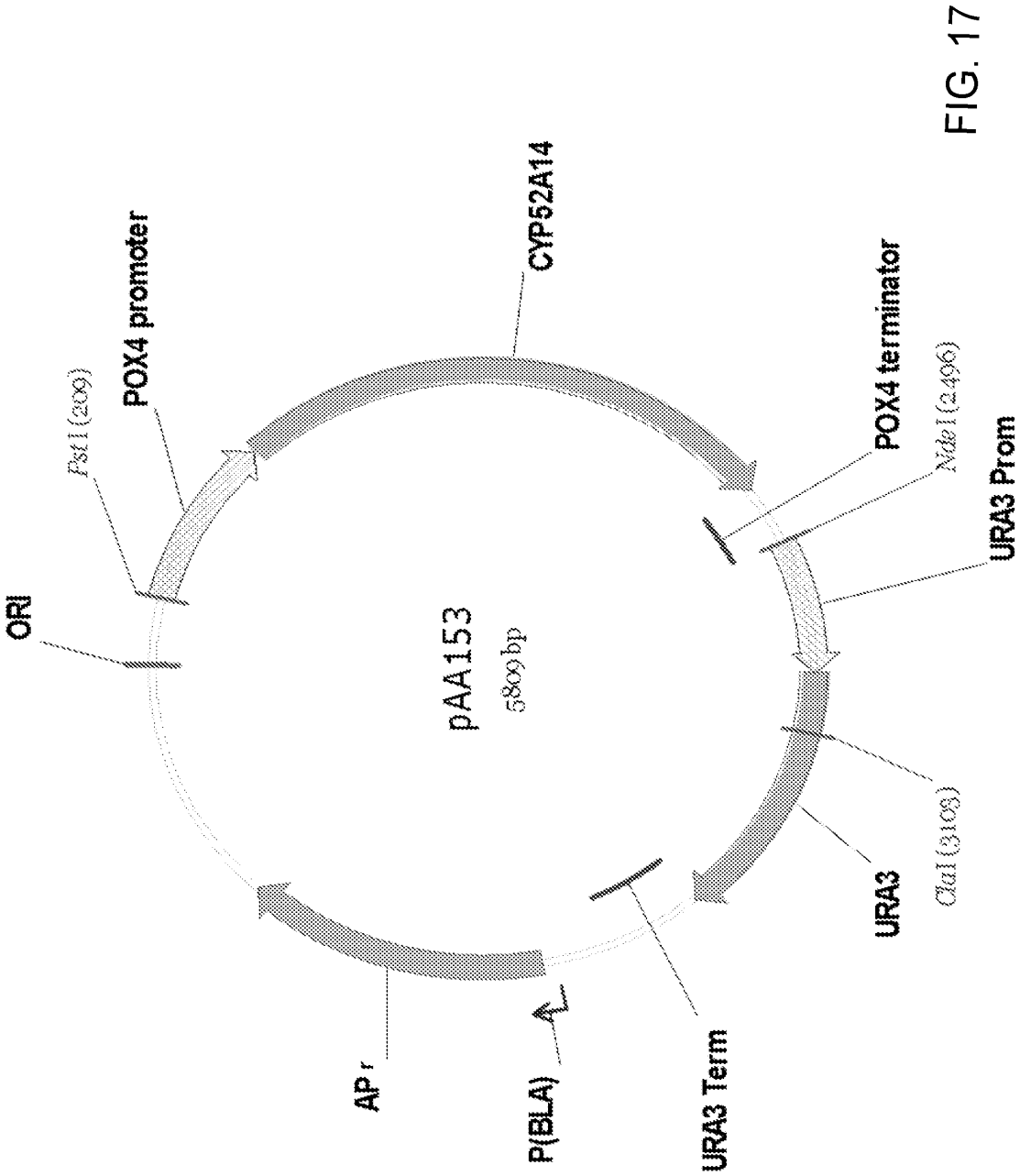



FIG. 17



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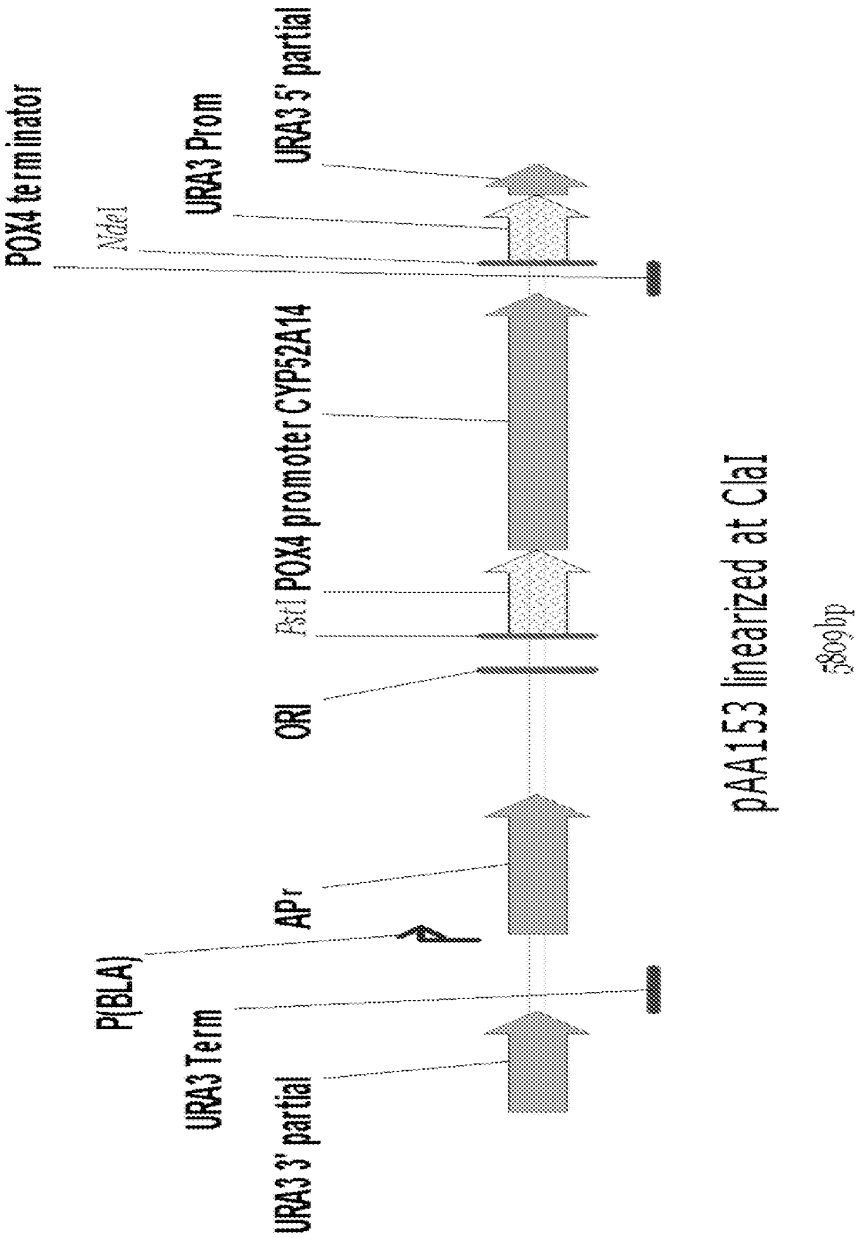



FIG. 18



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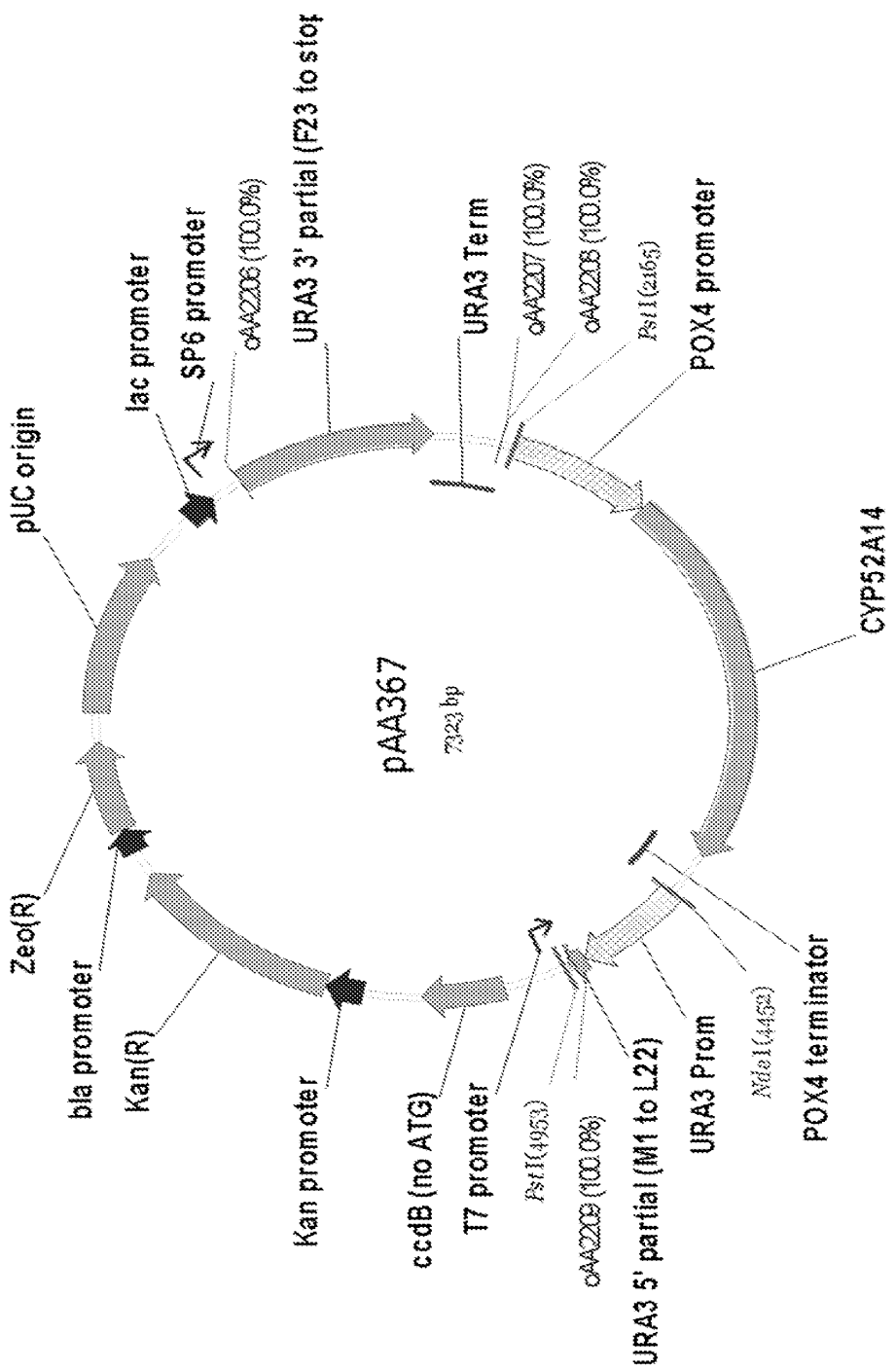



FIG. 19



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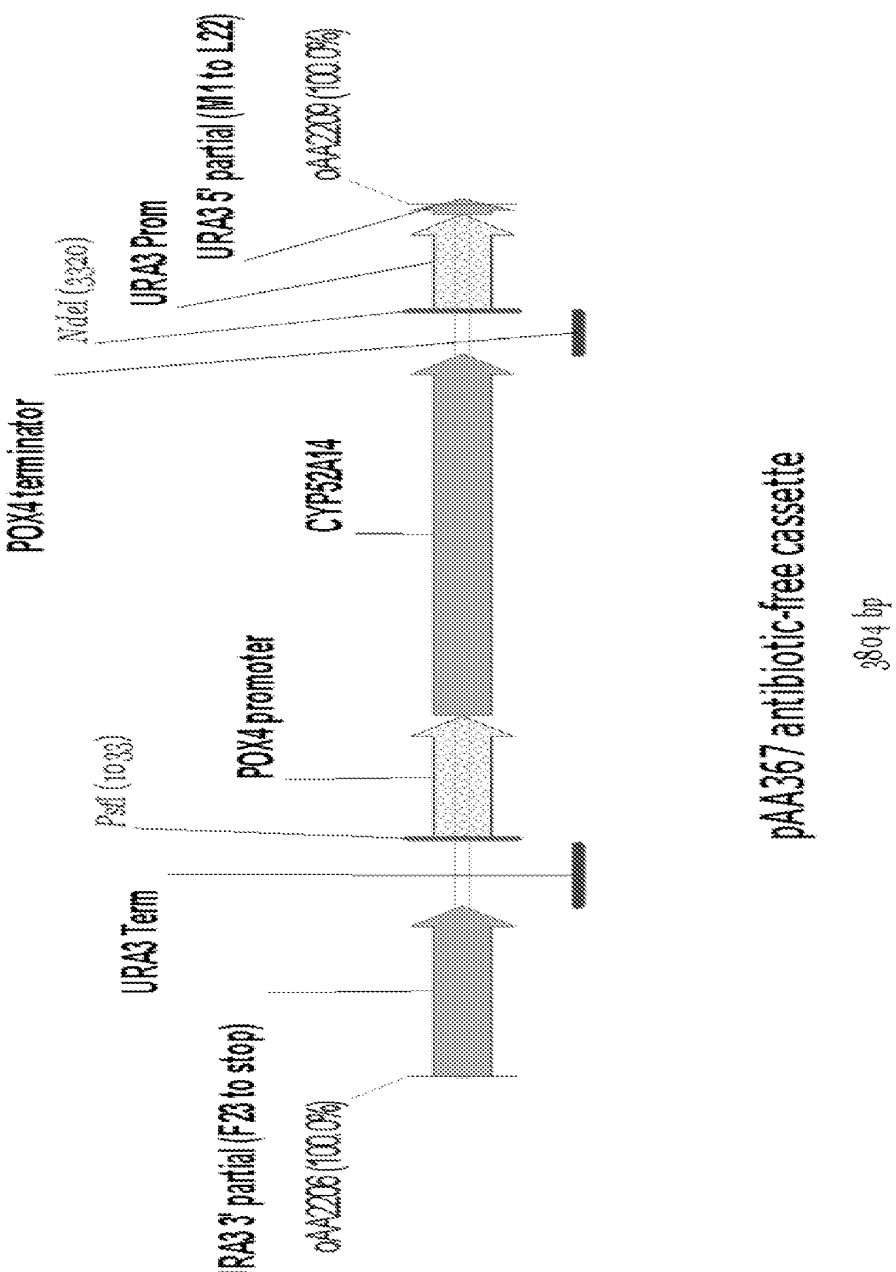



FIG. 20



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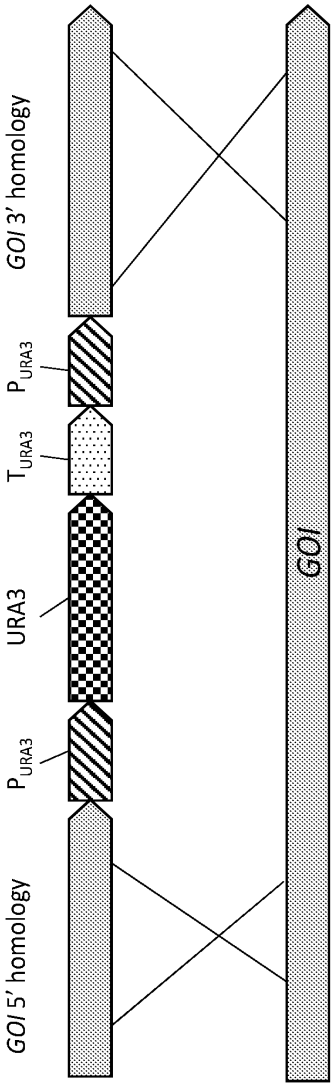



FIG. 21A

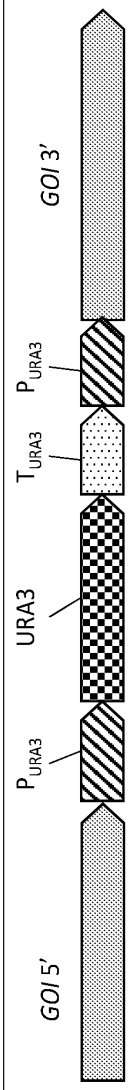



FIG. 21B

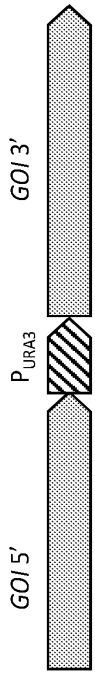



FIG. 21C

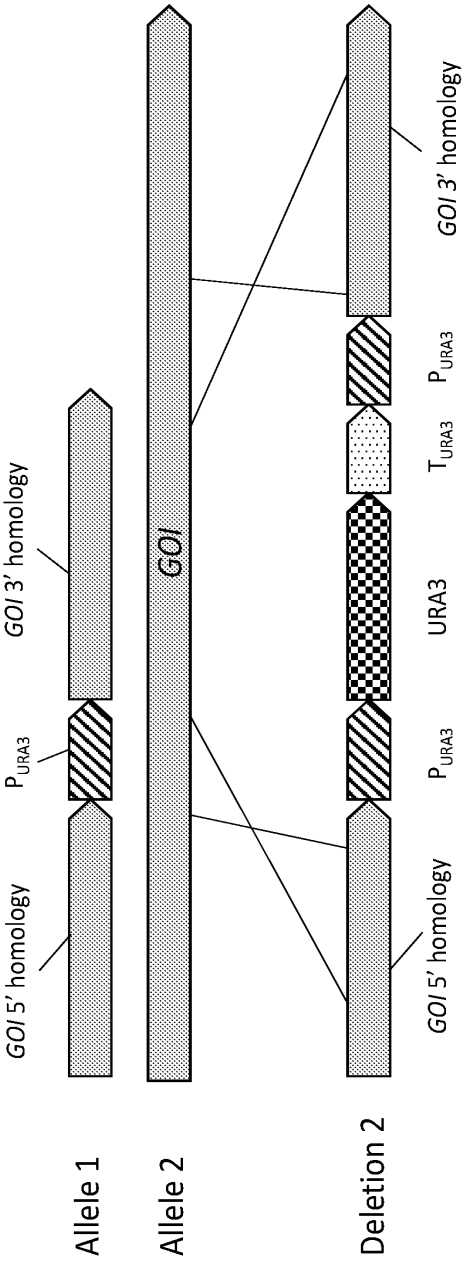

GOI 5' homology
P URA3
GOI 3' homology
GOI
GOI 5' homology
P URA3
URA3
T URA3
P URA3
GOI 3' homology
Allele 1
Allele 2
Deletion 2

FIG. 22

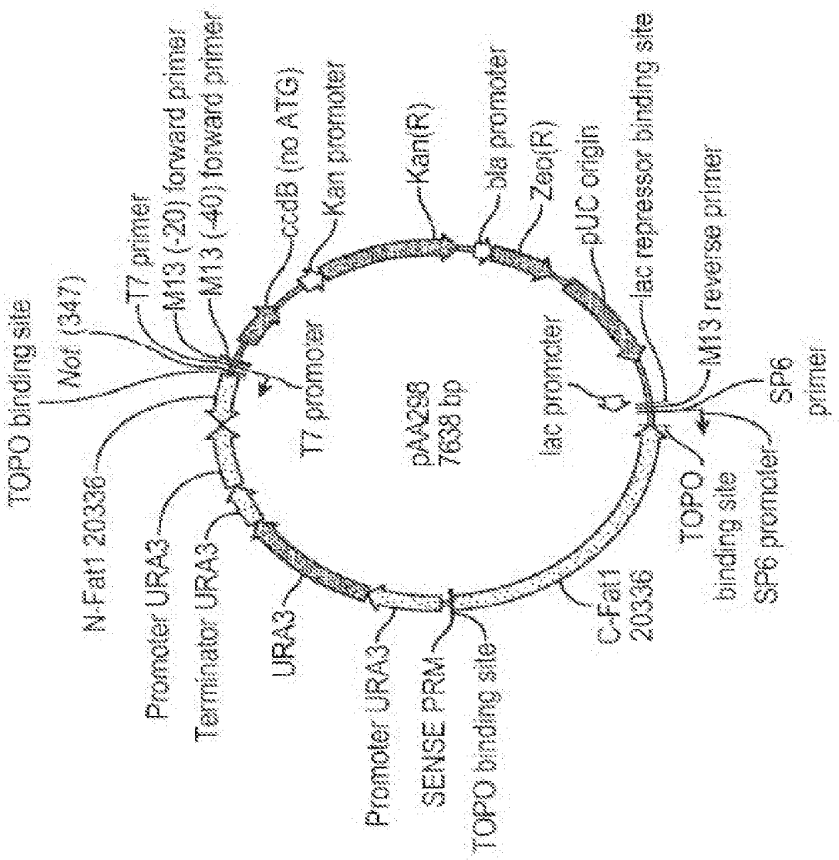

TOPO binding site
Not (347)
T7 primer
M13 (-20) forward primer
M13 (-40) forward primer
ccdB (no ATG)
Kan promoter
Kan(R)
bla promoter
Zeo(R)
pUC origin
lac repressor binding site
M13 reverse primer
SP6 primer
SP6 promoter
TOPO binding site
C-Fat1 20336
lac promoter
T7 promoter
pAA293 7638 bp
N-Fat1 20336
Promoter URA3
Terminator URA3
URA3
Promoter URA3
SENSE PRM
TOPO binding site

FIG. 23




FIG. 24



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FIG. 25A

```
                                                                  1                                                  50
CvCAT2 from pAA426      (1) MFNFKLSQQVLKNSTKSIMPILKKPFSTSHAKGDLFKYQSQLPKLPVPTL
     CvCAT2(-mts)       (1) ----------------MPILKKPFSTSHAKGDLFKYQSQLPKLPVPTL
CvCAT2(-mts-pts)        (1) ----------------MPILKKPFSTSHAKGDLFKYQSQLPKLPVPTL
     CvCAT2(-pts)       (1) MFNFKLSQQVLKNSTKSIMPILKKPFSTSHAKGDLFKYQSQLPKLPVPTL
```

FIG. 25B

```
                                                                601                                                628
CvCAT2 from pAA426      (601) KWYLVDSANEMKDVLTKGLLTDAKPKL-
     CvCAT2(-mts)       (583) KWYLVDSANEMKDVLTKGLLTDAKPKL-
CvCAT2(-mts-pts)        (583) KWYLVDSANEMKDVLTKGLLTDAK----
     CvCAT2(-pts)       (601) KWYLVDSANEMKDVLTKGLLTDAK----
```

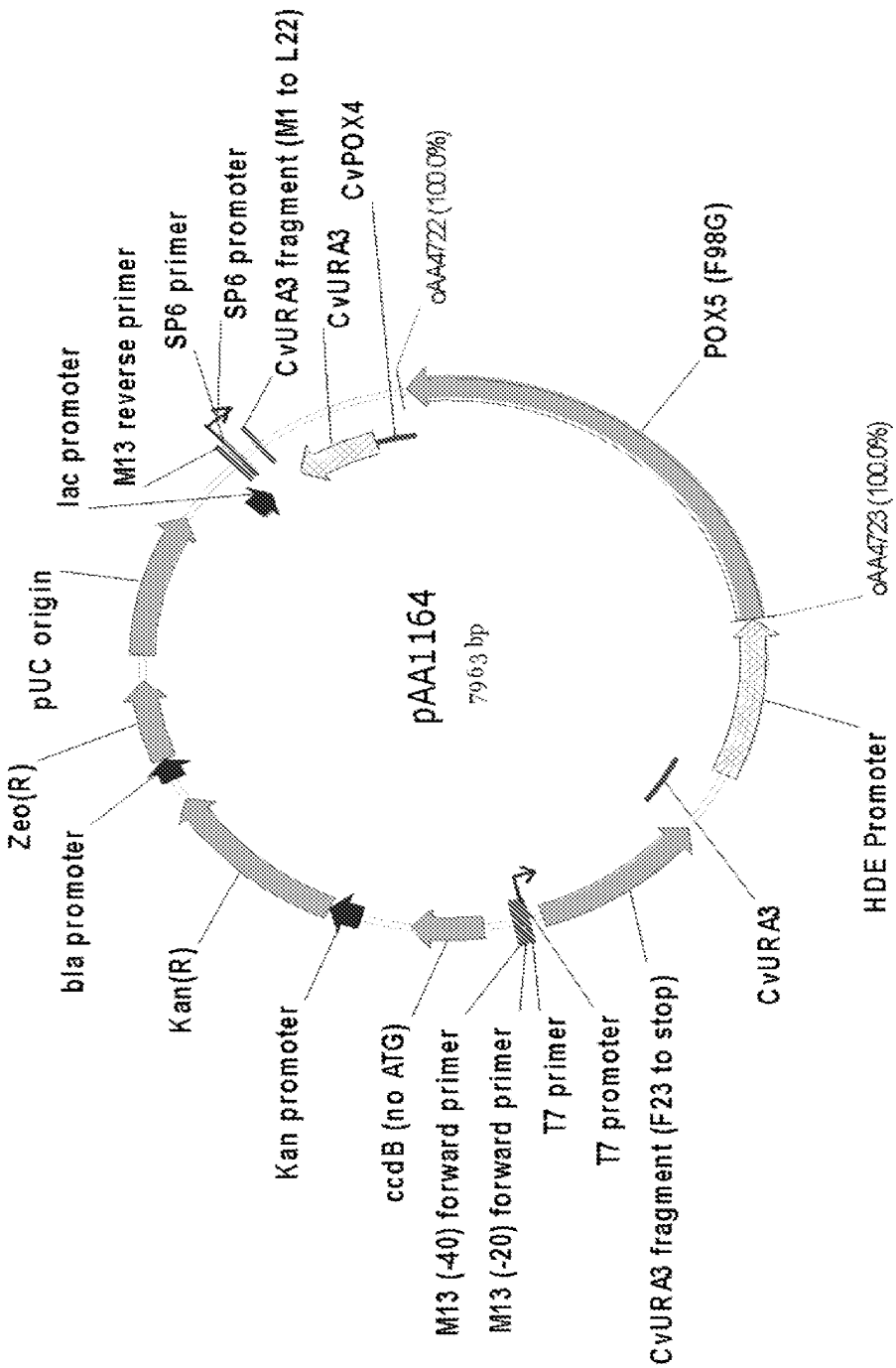



FIG. 26



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```
                1                                                  50
CvYat1p     (1) ----------------------------------MSTYQFQETLEKLPIPD
CvYat1p+CAT2mts (1) MFNFKLSQQVLKNSTKSIMPILKKP-FSTSHAKGSTYQFQETLEKLPIPD
CvYAT1p+CIT1mts (1) ------MSALRSFQRSSNVAKST--LKNSVRTYSTYQFQETLEKLPIPD
CvYat1p+COX4mts (1) ---MLSRTTLRVARQQTRLLSTSRILFNSKTDQSTYQFQETLEKLPIPD
```

FIG. 27

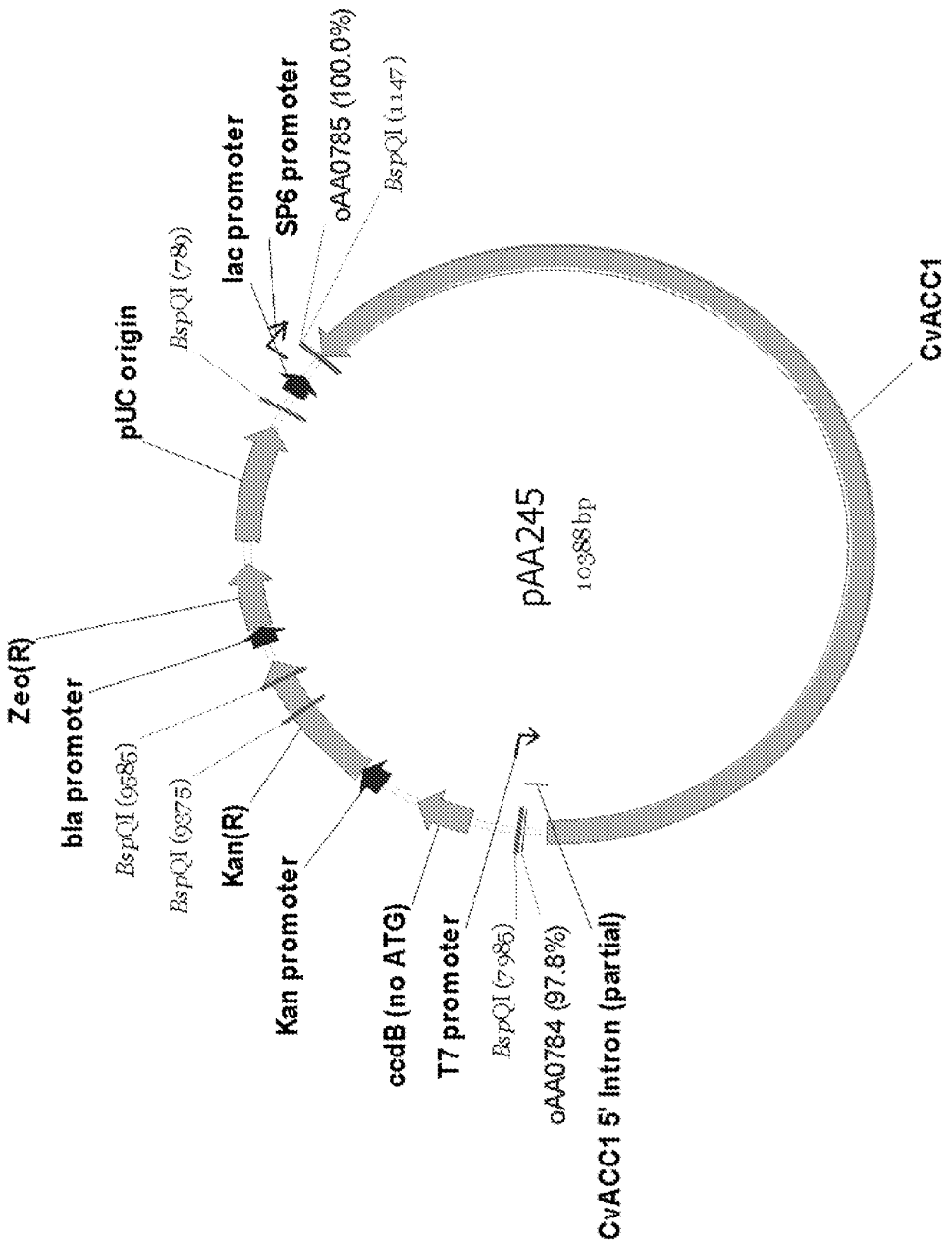



FIG. 28



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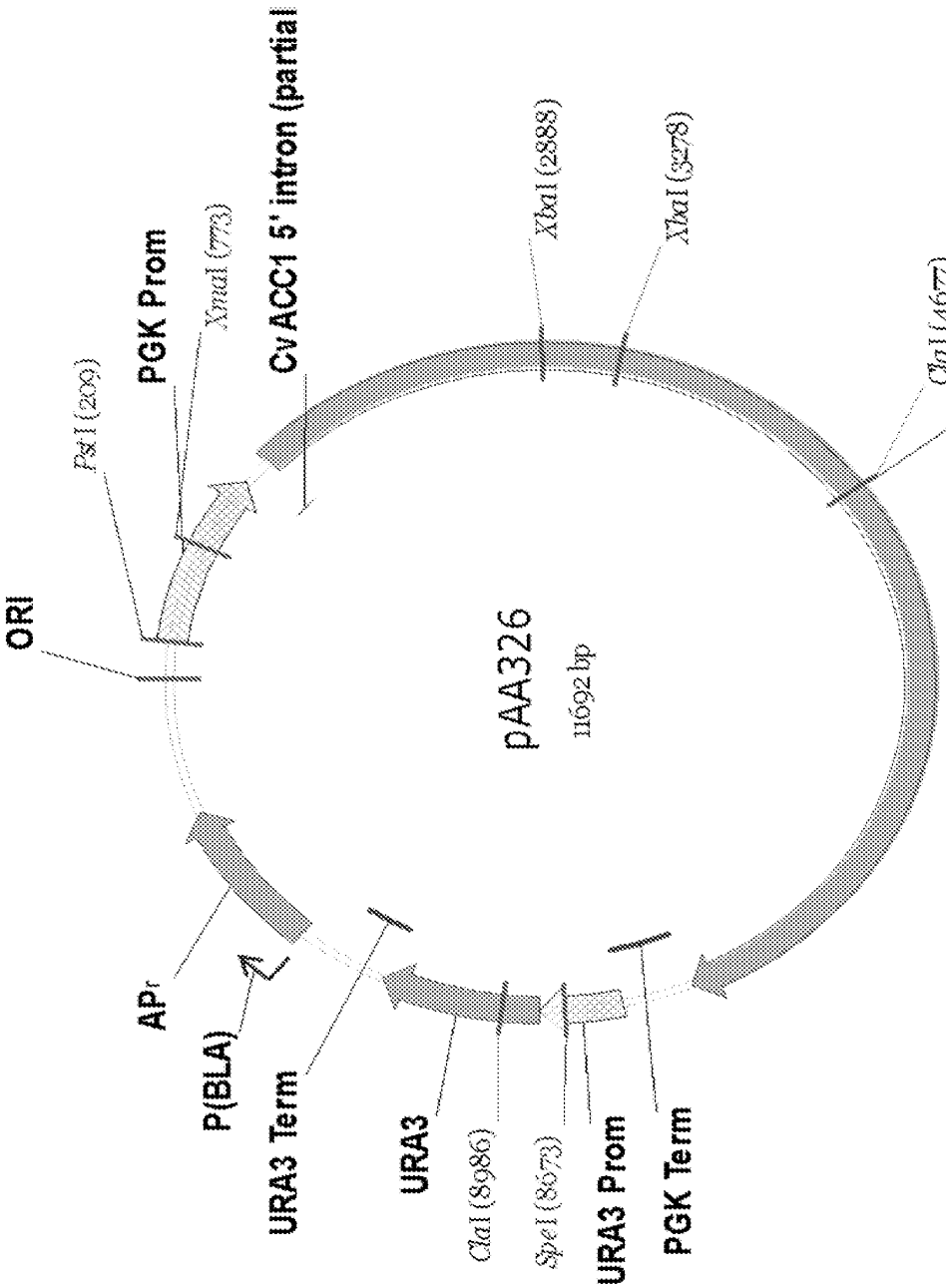



FIG. 29



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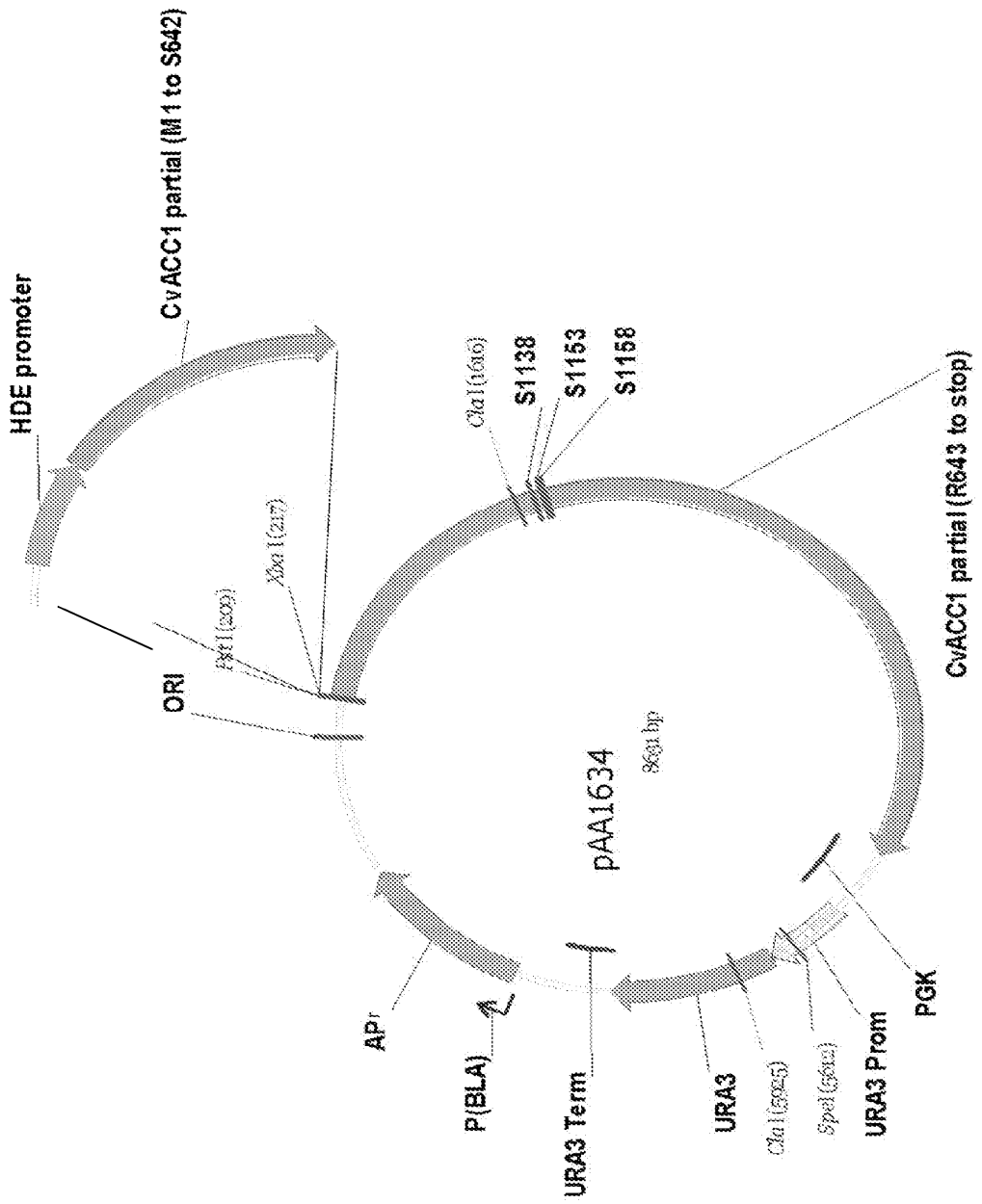



FIG. 30



LCY Biotechnology Holding, Inc.
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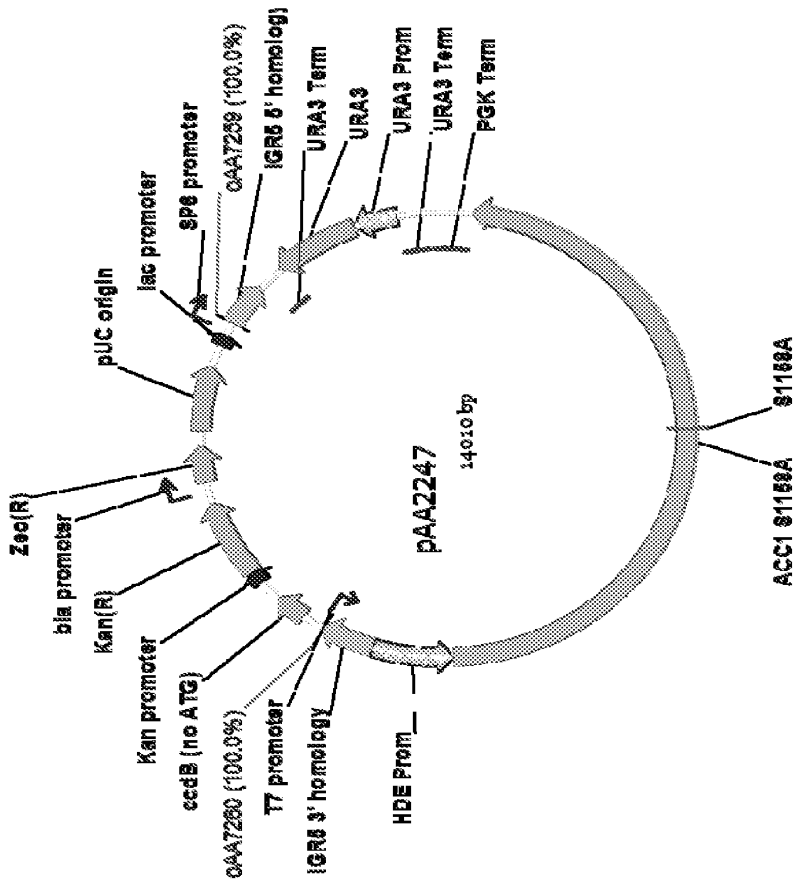

pAA2247
14010 bp
Zeo(R)
bla promoter
Kan(R)
Kan promoter
ccdB (no ATG)
cAA7260 (100.0%)
T7 promoter
IGR8 3' homology
HDE Prom_
ACC1 S1158A
S1158A
pUC origin
lac promoter
SP6 promoter
cAA7259 (100.0%)
IGR8 5' homolog
URA3 Term
URA3
URA3 Prom
URA3 Term
PGK Term

FIG. 31

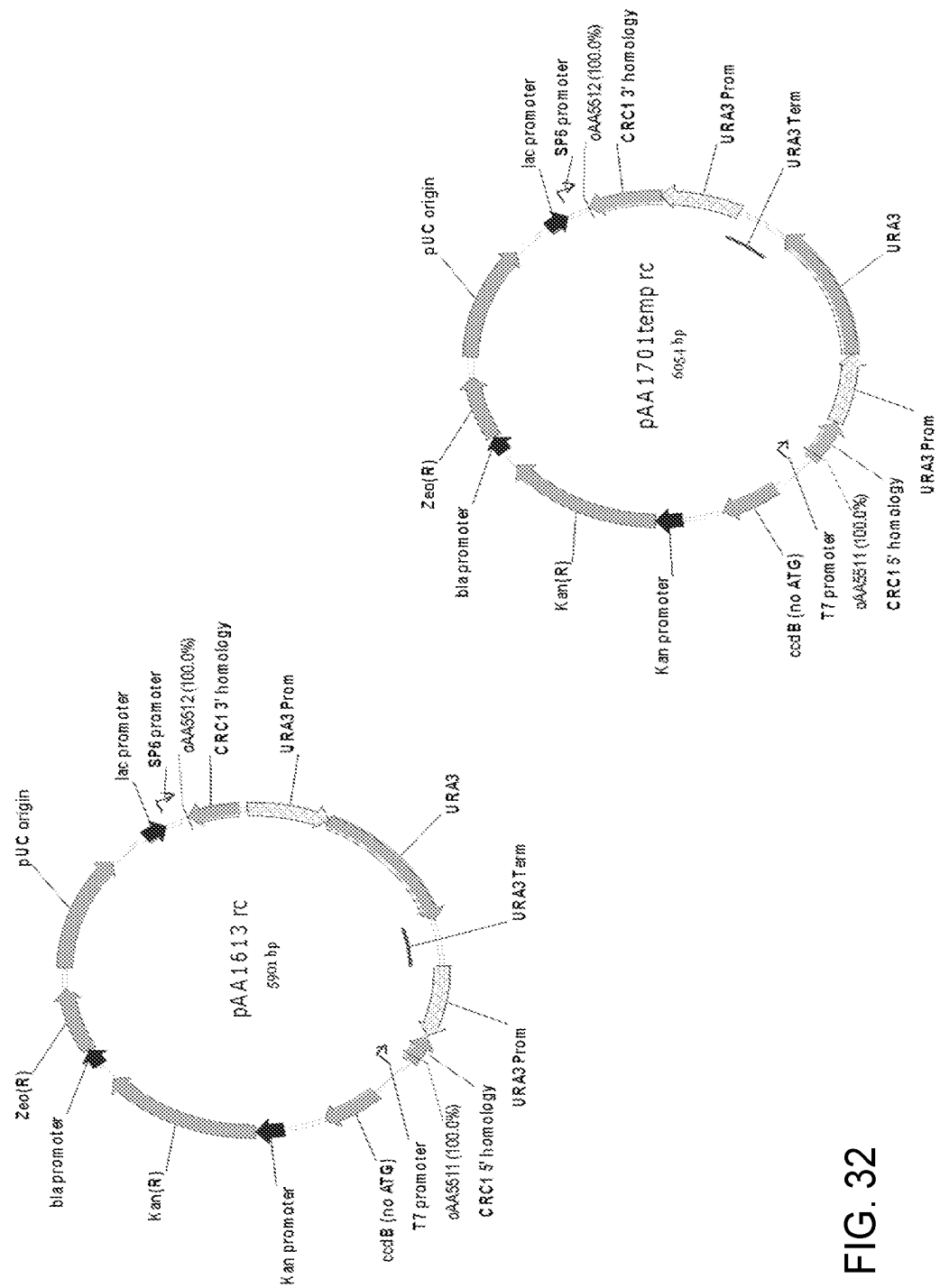



FIG. 32



LCY Biotechnology Holding, Inc.
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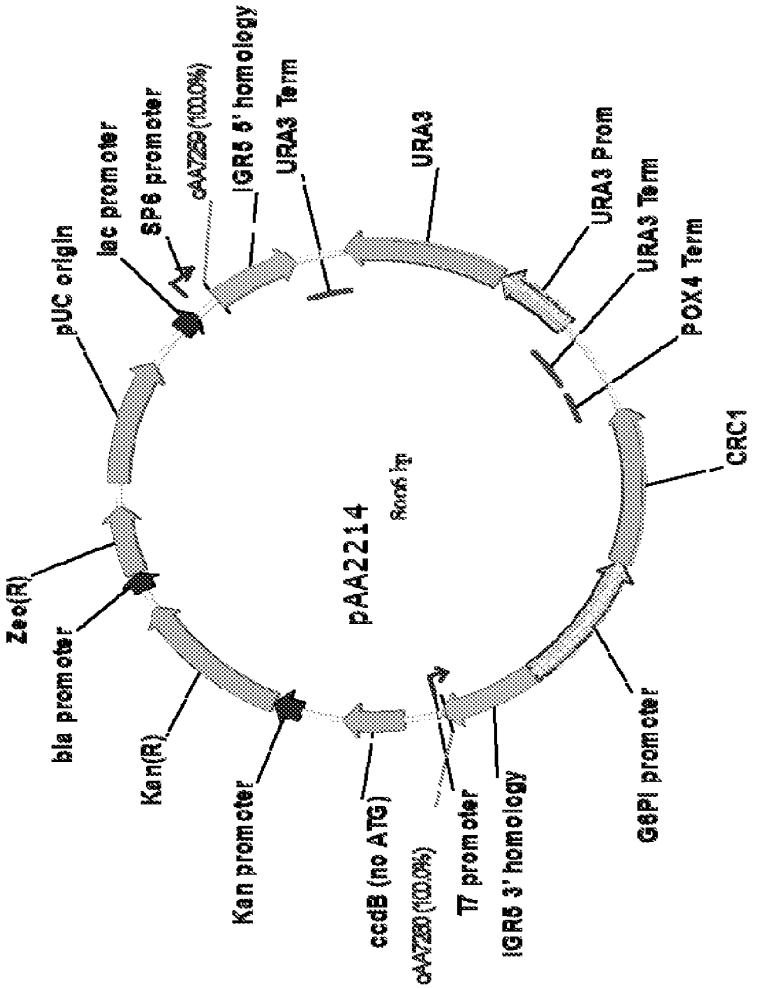

pAA2214
8005 bp
pUC origin
lac promoter
SP6 promoter
cAA729 (100.0%)
IGR5 5' homology
URA3 Term
URA3
URA3 Prom
URA3 Term
POX4 Term
CRC1
G6PI promoter
IGR5 3' homology
T7 promoter
cAA720 (100.0%)
ccdB (no ATG)
Kan promoter
Kan(R)
bla promoter
Zeo(R)

FIG. 33

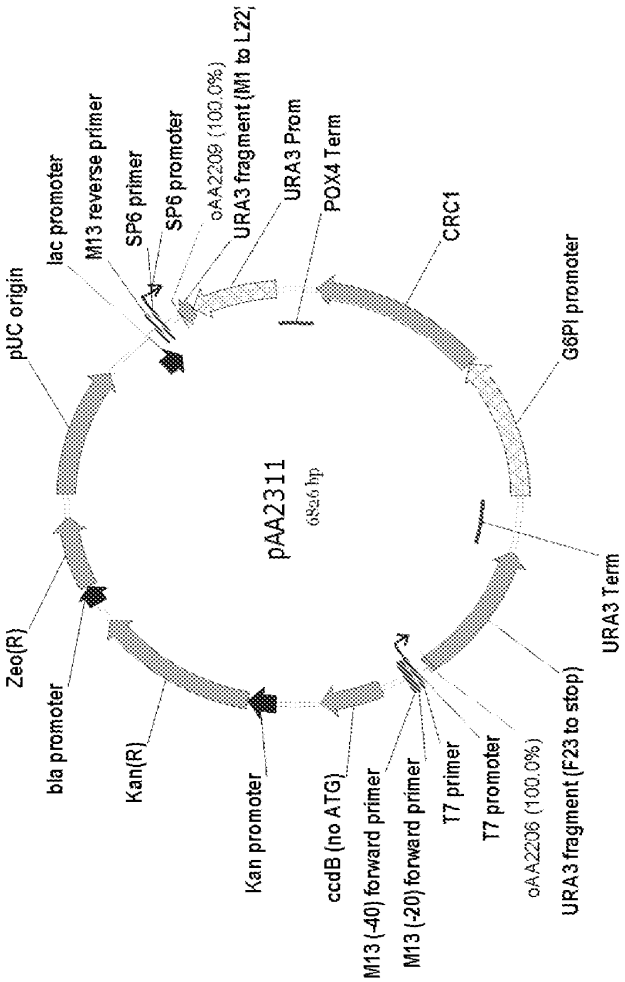

pAA2311
6826 bp
pUC origin
lac promoter
M13 reverse primer
SP6 primer
SP6 promoter
oAA2209 (100.0%)
URA3 fragment (M1 to L22)
URA3 Prom
POX4 Term
CRC1
G6PI promoter
URA3 Term
URA3 fragment (F23 to stop)
oAA2206 (100.0%)
T7 promoter
T7 primer
M13 (-20) forward primer
M13 (-40) forward primer
ccdB (no ATG)
Kan promoter
Kan(R)
bla promoter
Zeo(R)

FIG. 34

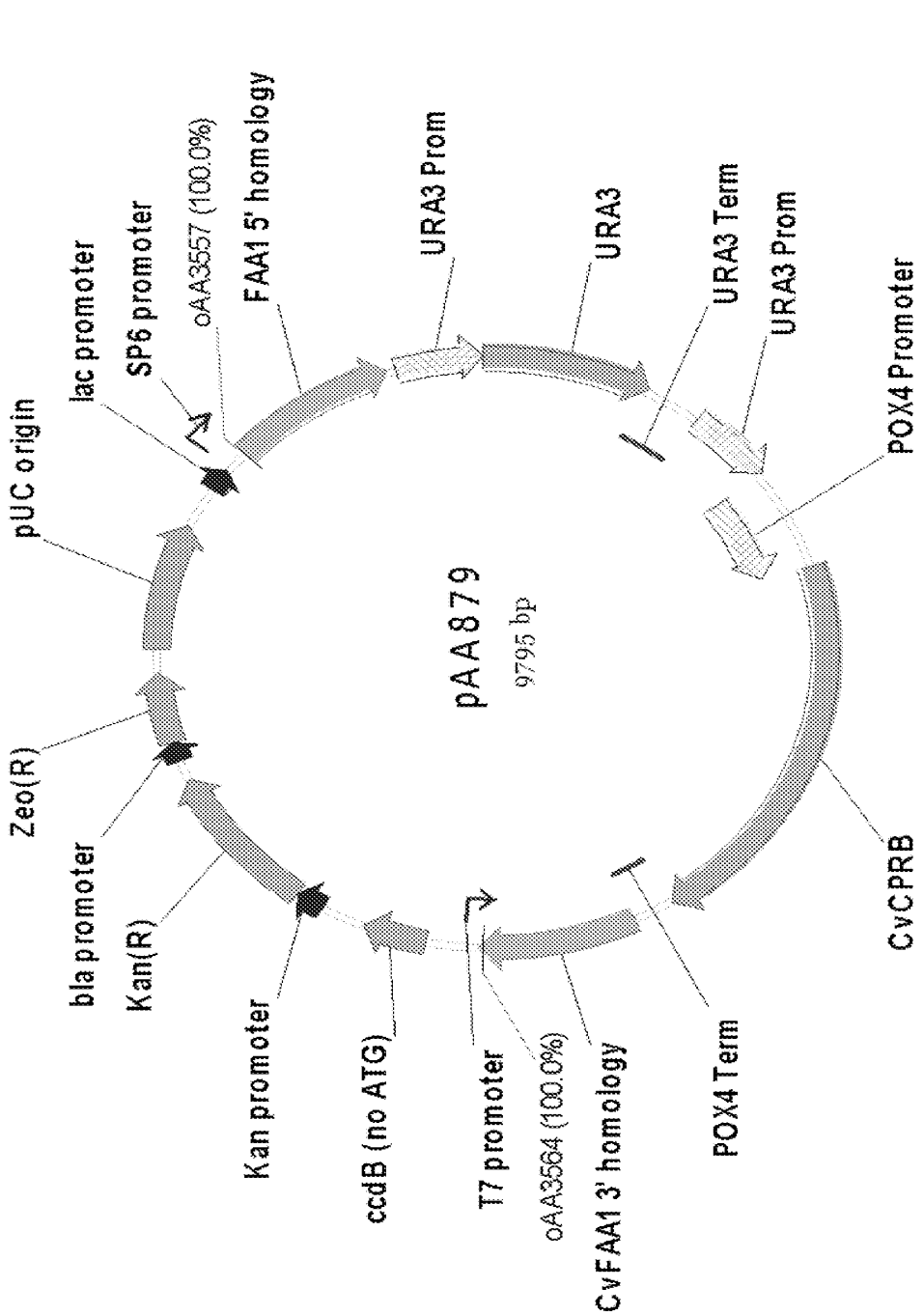



FIG. 35



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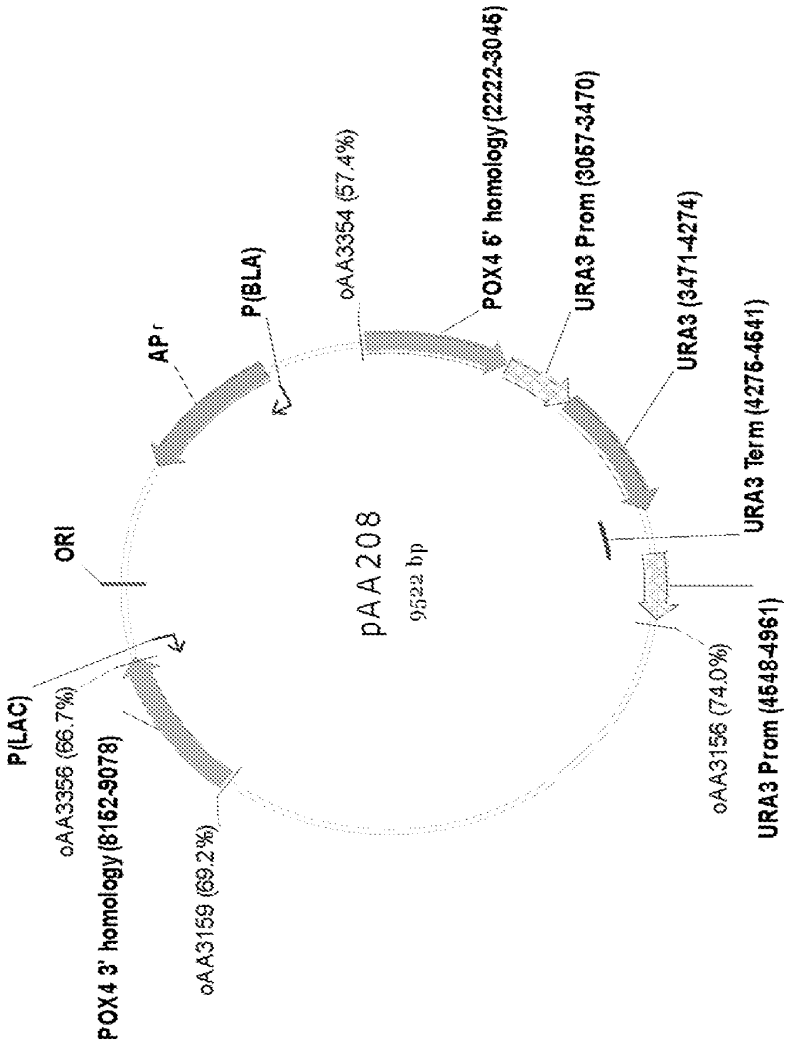

pAA208
9522 bp
oAA3354 (57.4%)
POX4 5' homology (2222-3045)
URA3 Prom (3067-3470)
URA3 (3471-4274)
URA3 Term (4276-4641)
oAA3156 (74.0%)
URA3 Prom (4548-4961)
oAA3159 (69.2%)
POX4 3' homology (8162-9078)
oAA3356 (66.7%)
P(LAC)
ORI
AP r
P(BLA)

FIG. 36

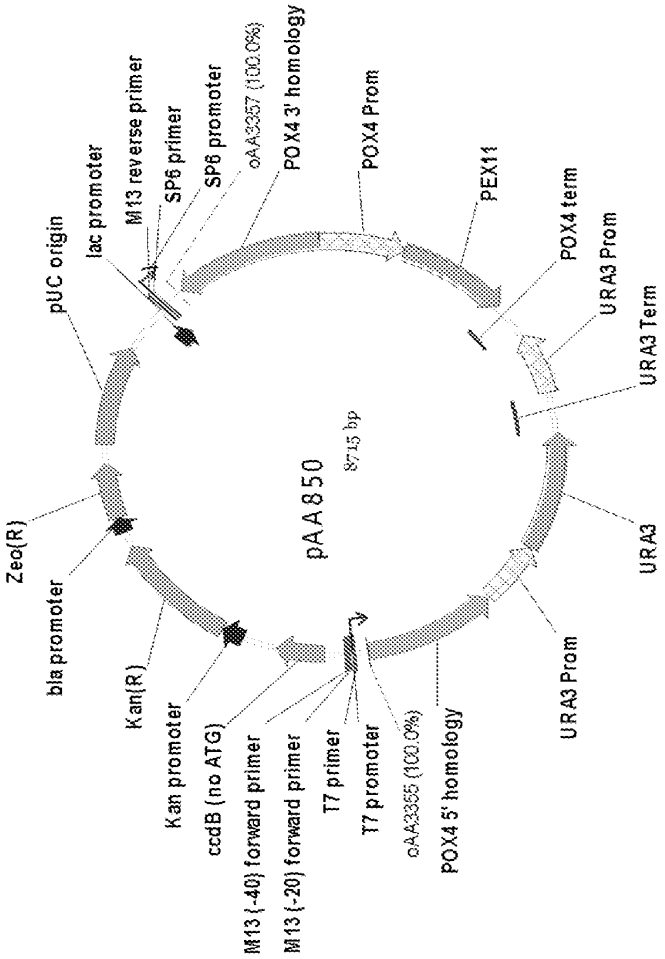
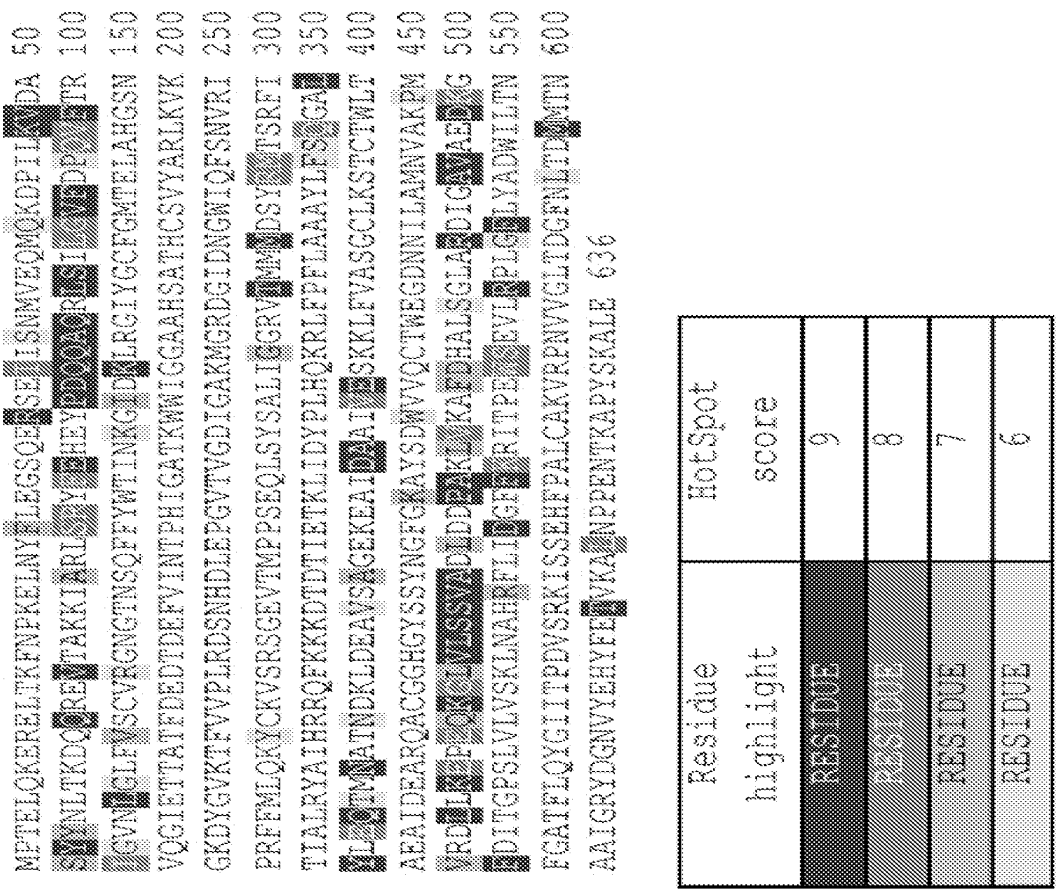

pAA850
8745 bp
pUC origin
lac promoter
M13 reverse primer
SP6 primer
SP6 promoter
oAA3357 (100.0%)
POX4 3' homology
POX4 Prom
PEX11
POX4 term
URA3 Prom
URA3 Term
URA3
URA3 Prom
POX4 5' homology
oAA3355 (100.0%)
T7 promoter
T7 primer
M13 (-20) forward primer
M13 (-40) forward primer
ccdB (no ATG)
Kan promoter
Kan(R)
bla promoter
Zeo(R)

FIG. 37

FIG. 38






LCY Biotechnology Holding, Inc.
Ex. 1002
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| | |
|---|---|
| Oligo A | oAA540 |
| Oligo D | oAA541 |
| Oligo C | F98G fwd |
| Oligo B | F98G rev |
| Oligo C | W429F fwd |
| Oligo B | W429F rev |

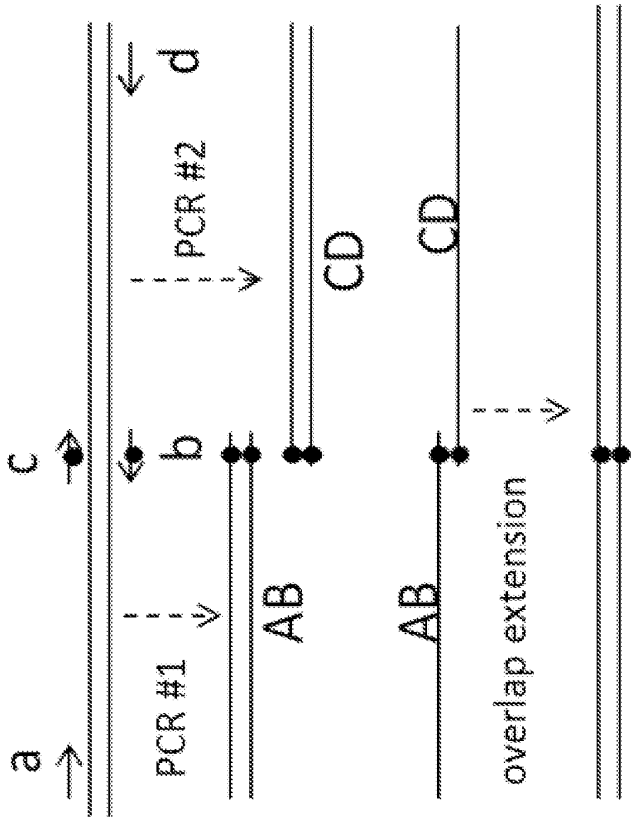



FIG. 39

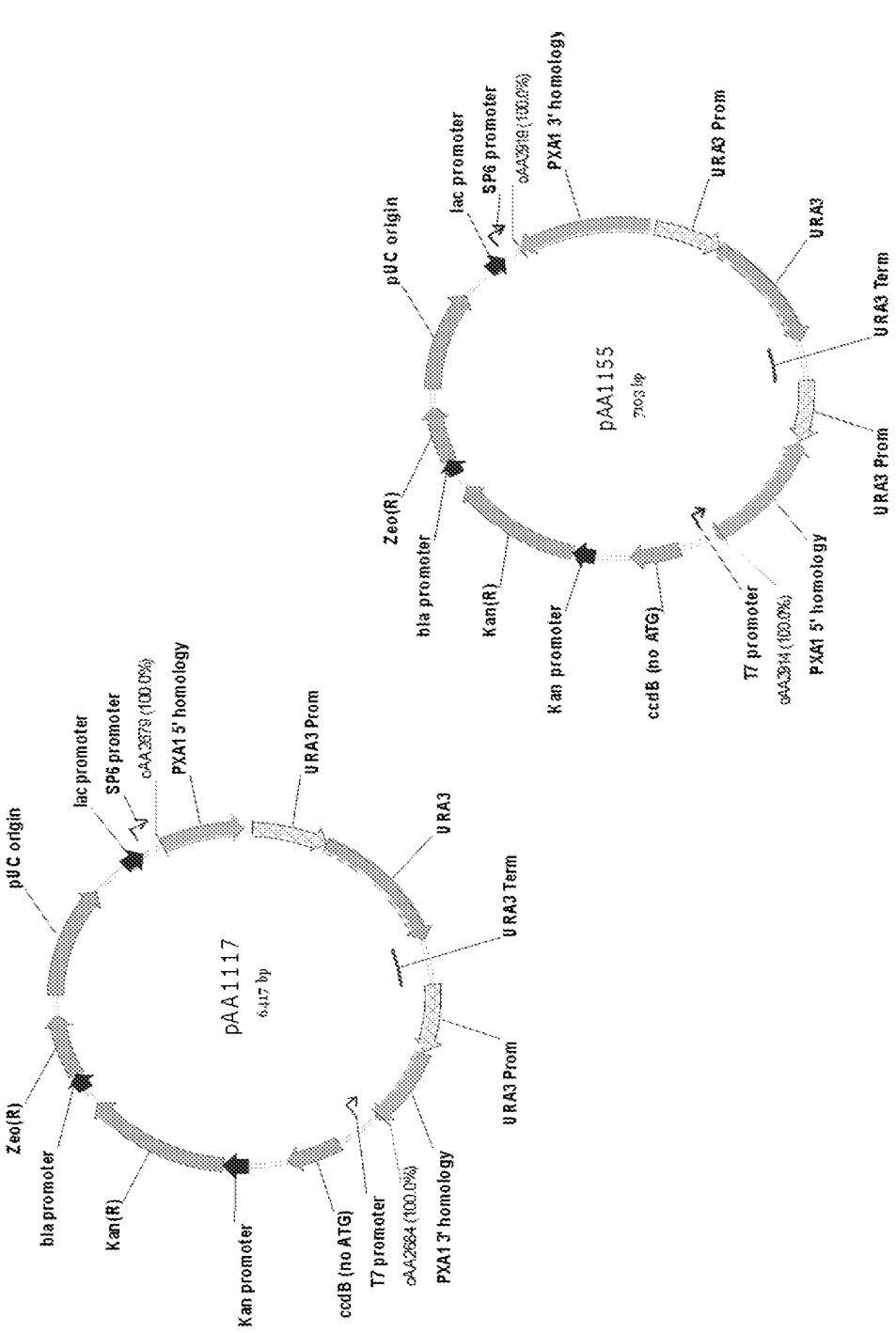



FIG. 40



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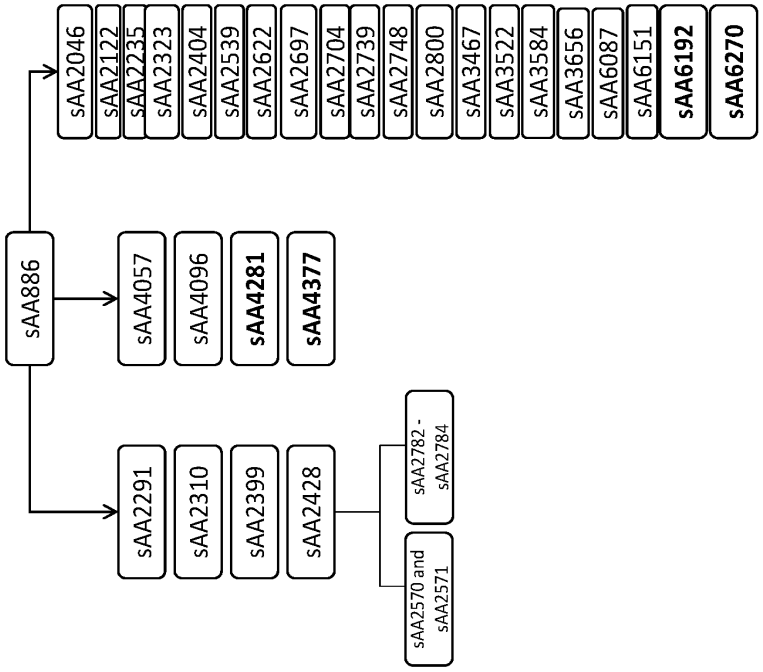

sAA886
sAA2046
sAA2122
sAA2235
sAA2323
sAA2404
sAA2539
sAA2622
sAA2697
sAA2704
sAA2739
sAA2748
sAA2800
sAA3467
sAA3522
sAA3584
sAA3656
sAA6087
sAA6151
sAA6192
sAA6270
sAA4057
sAA4096
sAA4281
sAA4377
sAA2291
sAA2310
sAA2399
sAA2428
sAA2782 - sAA2784
sAA2570 and sAA2571

FIG. 41

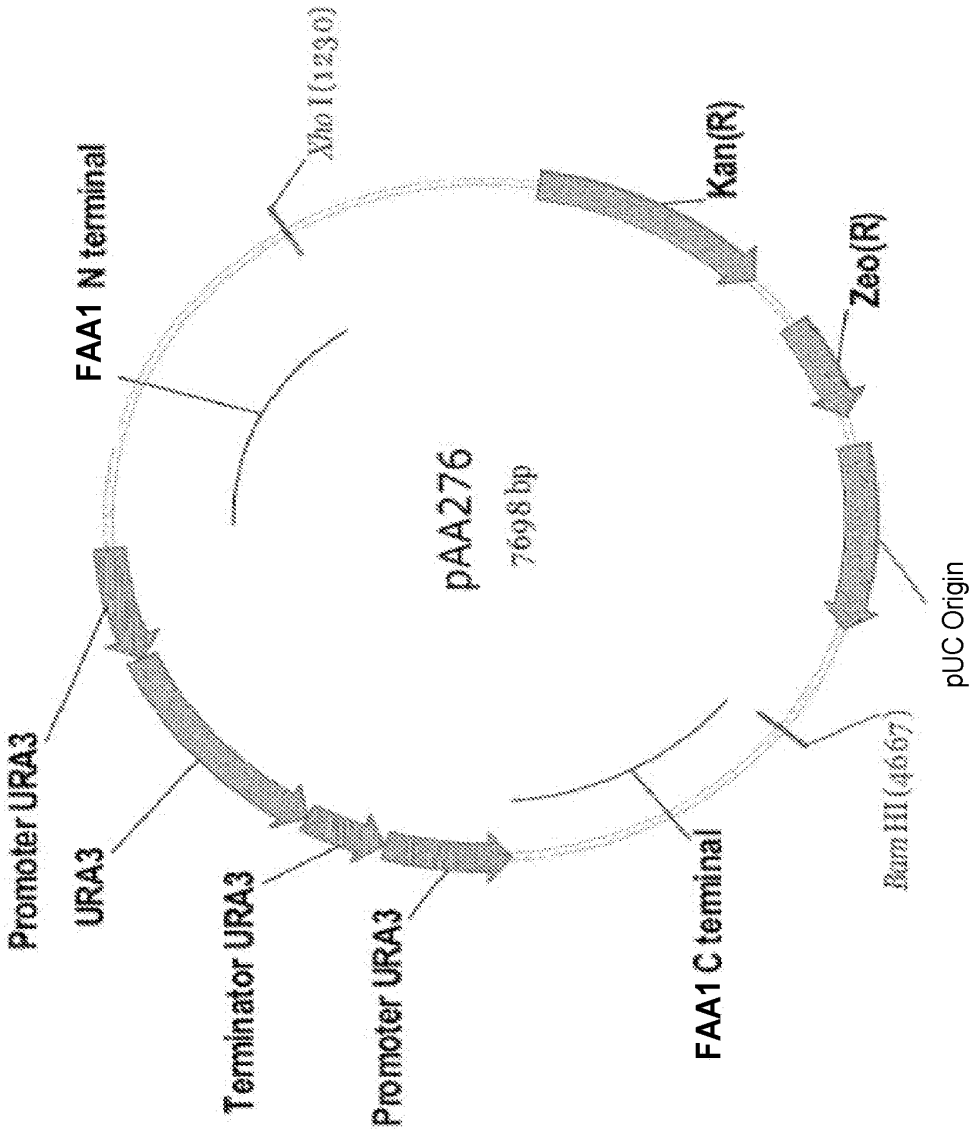



FIG. 42



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Ex. 1002
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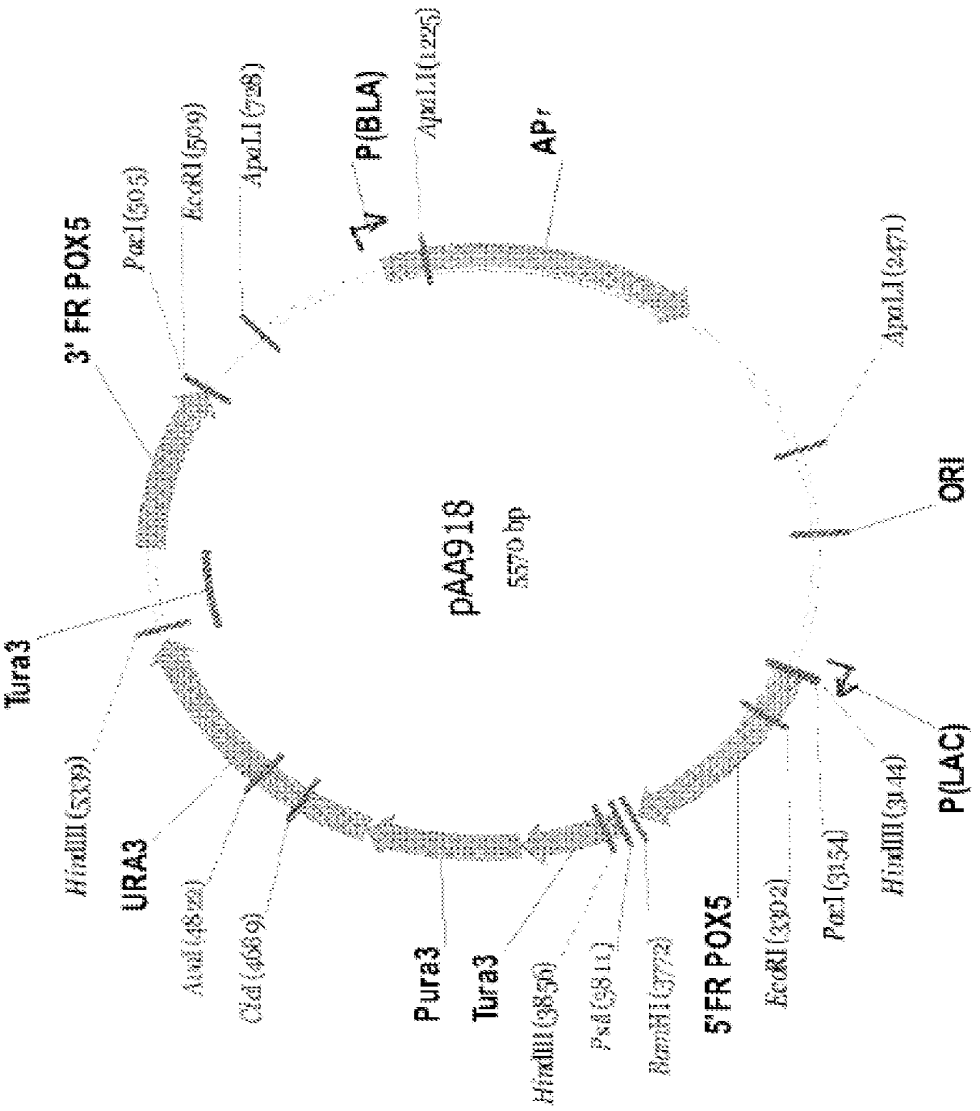



FIG. 43



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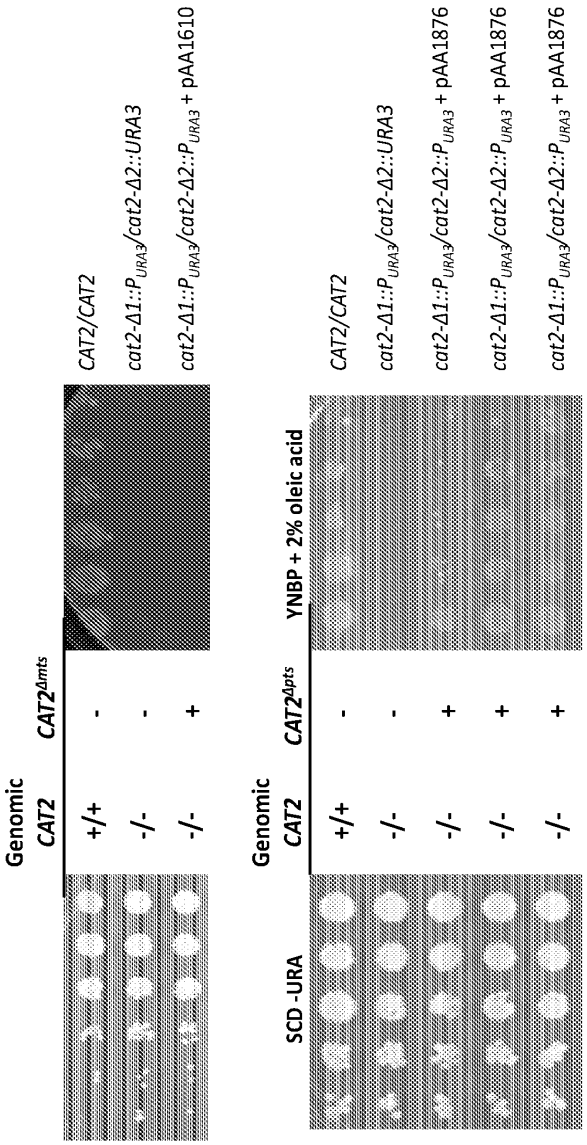

Genomic
CAT2
CAT2Δmts
+/+
-
-/-
-
-/-
+
CAT2/CAT2
cat2-Δ1::PURA3/cat2-Δ2::URA3
cat2-Δ1::PURA3/cat2-Δ2::PURA3 + pAA1610
SCD -URA
YNBP + 2% oleic acid
Genomic
CAT2
CAT2Δpts
+/+
-
-/-
-
-/-
+
-/-
+
-/-
+
CAT2/CAT2
cat2-Δ1::PURA3/cat2-Δ2::URA3
cat2-Δ1::PURA3/cat2-Δ2::PURA3 + pAA1876
cat2-Δ1::PURA3/cat2-Δ2::PURA3 + pAA1876
cat2-Δ1::PURA3/cat2-Δ2::PURA3 + pAA1876

FIG. 44

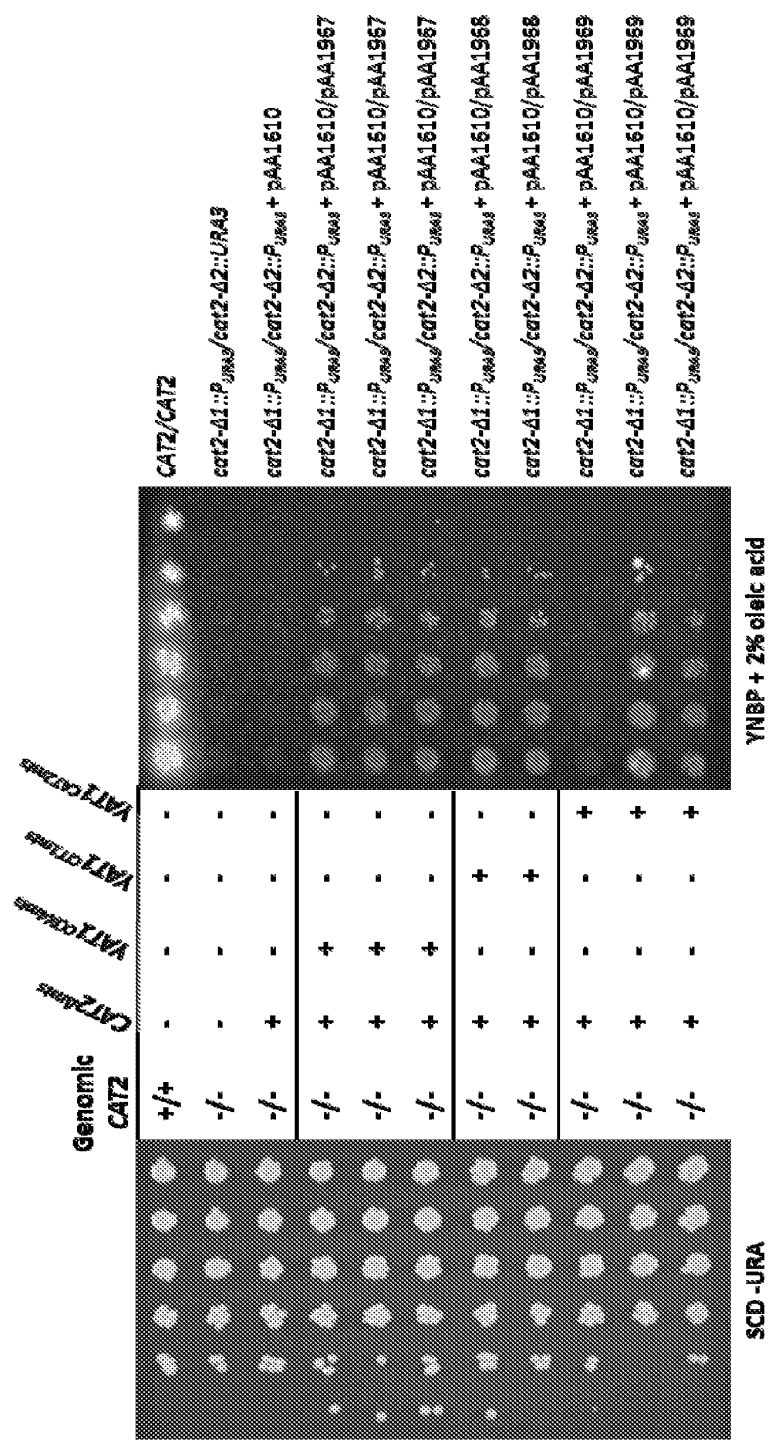

SCD -URA
YNBP + 2% oleic acid
Genomic CAT2
CAT2 Atrans
VAT1 Ctrans
VAT1 Atrans
VAT1 Ctrans
CAT2/CAT2
cat2-Δ1::P_URA3/cat2-Δ2::URA3
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1610
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1610/pAA1967
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1610/pAA1957
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1610/pAA1967
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1610/pAA1968
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1610/pAA1968
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1610/pAA1969
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1610/pAA1969
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1610/pAA1969

FIG. 45

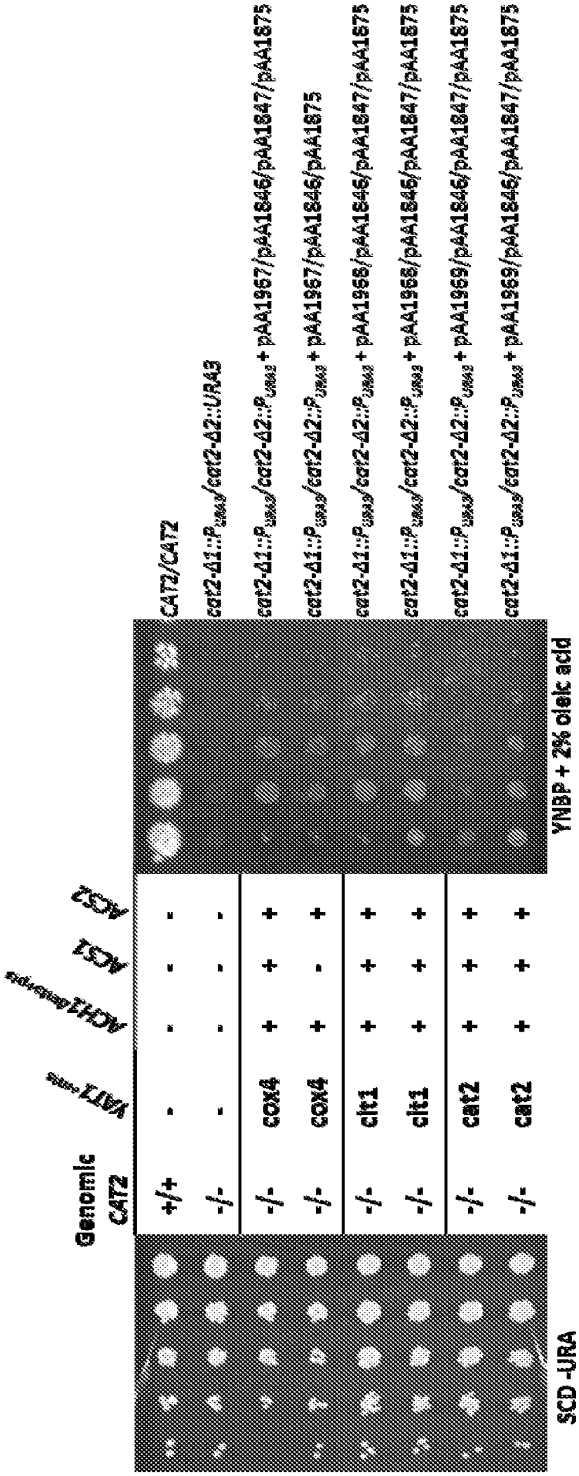

Genomic CAT2
VAT1 orth.
ACH1 Saccharo.
ACS1
ACS2
+/+ - - - -
-/- - - - -
-/- cox4 + + +
-/- cox4 + - +
-/- clt1 + + +
-/- clt1 + + +
-/- cat2 + + +
-/- cat2 + + +
SCD -URA
YNBP + 2% oleic acid
CAT2/CAT2
cat2-Δ1::P_URA3/cat2-Δ2::URA3
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1967/pAA1846/pAA1847/pAA1875
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1967/pAA1846/pAA1875
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1968/pAA1846/pAA1847/pAA1875
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1968/pAA1846/pAA1847/pAA1875
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1969/pAA1846/pAA1847/pAA1875
cat2-Δ1::P_URA3/cat2-Δ2::P_URA3 + pAA1969/pAA1846/pAA1847/pAA1875

FIG. 46

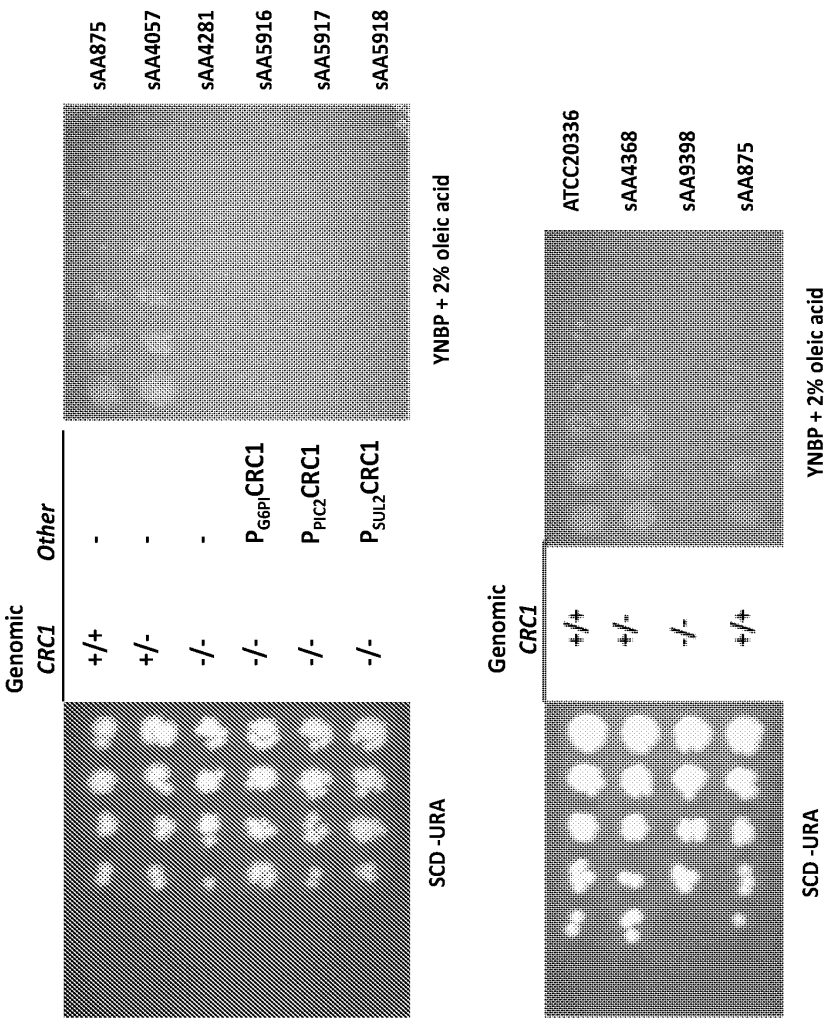

Genomic CRC1
Other
+/+ -
+/- -
-/- -
-/- P_G6PI CRC1
-/- P_PIC2 CRC1
-/- P_SUL2 CRC1
SCD -URA
sAA875
sAA4057
sAA4281
sAA5916
sAA5917
sAA5918
YNBP + 2% oleic acid
Genomic CRC1
+/+
+/-
-/-
+/+
SCD -URA
ATCC20336
sAA4368
sAA9398
sAA875
YNBP + 2% oleic acid

FIG. 47

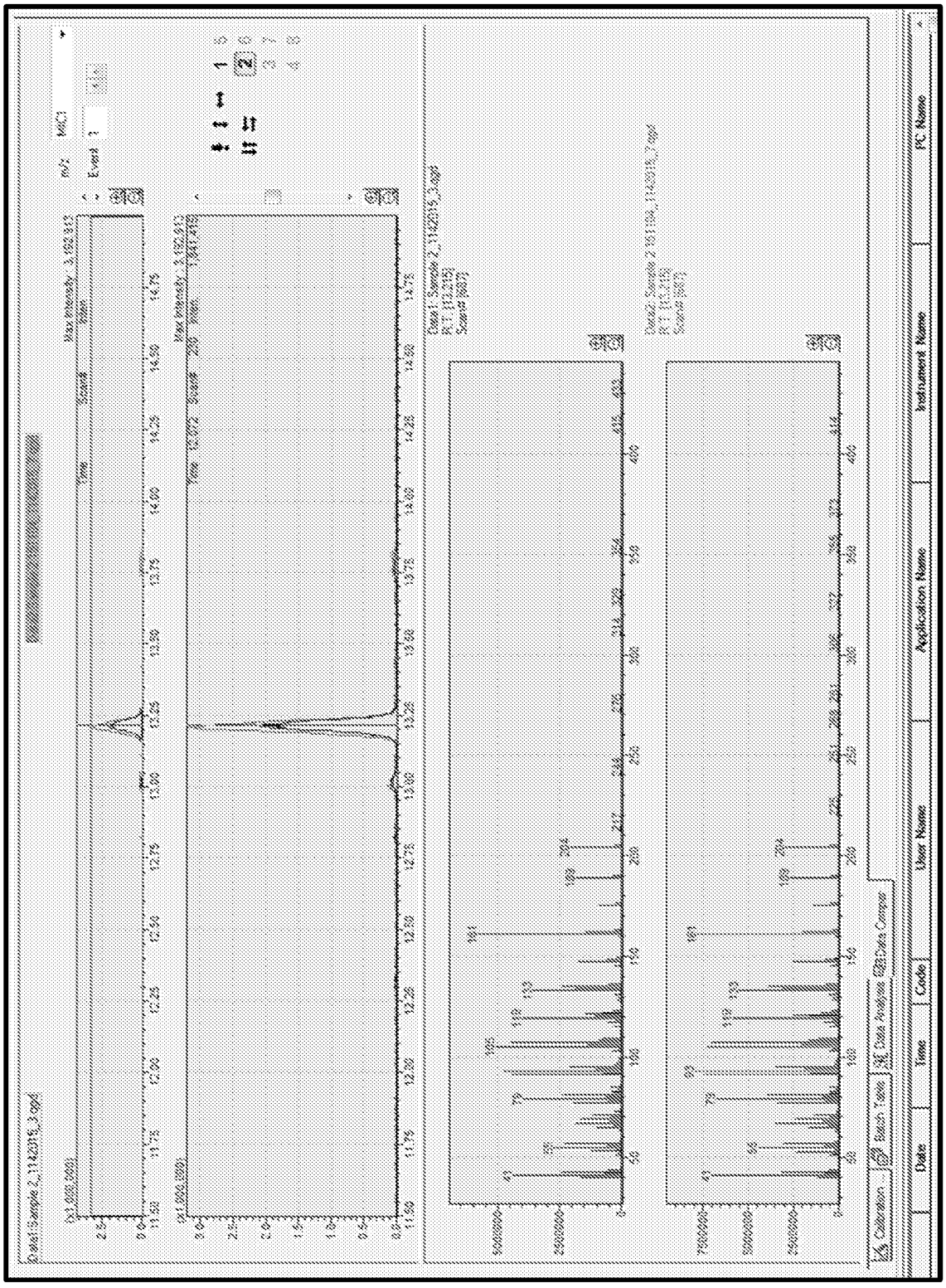


Fig. 48A

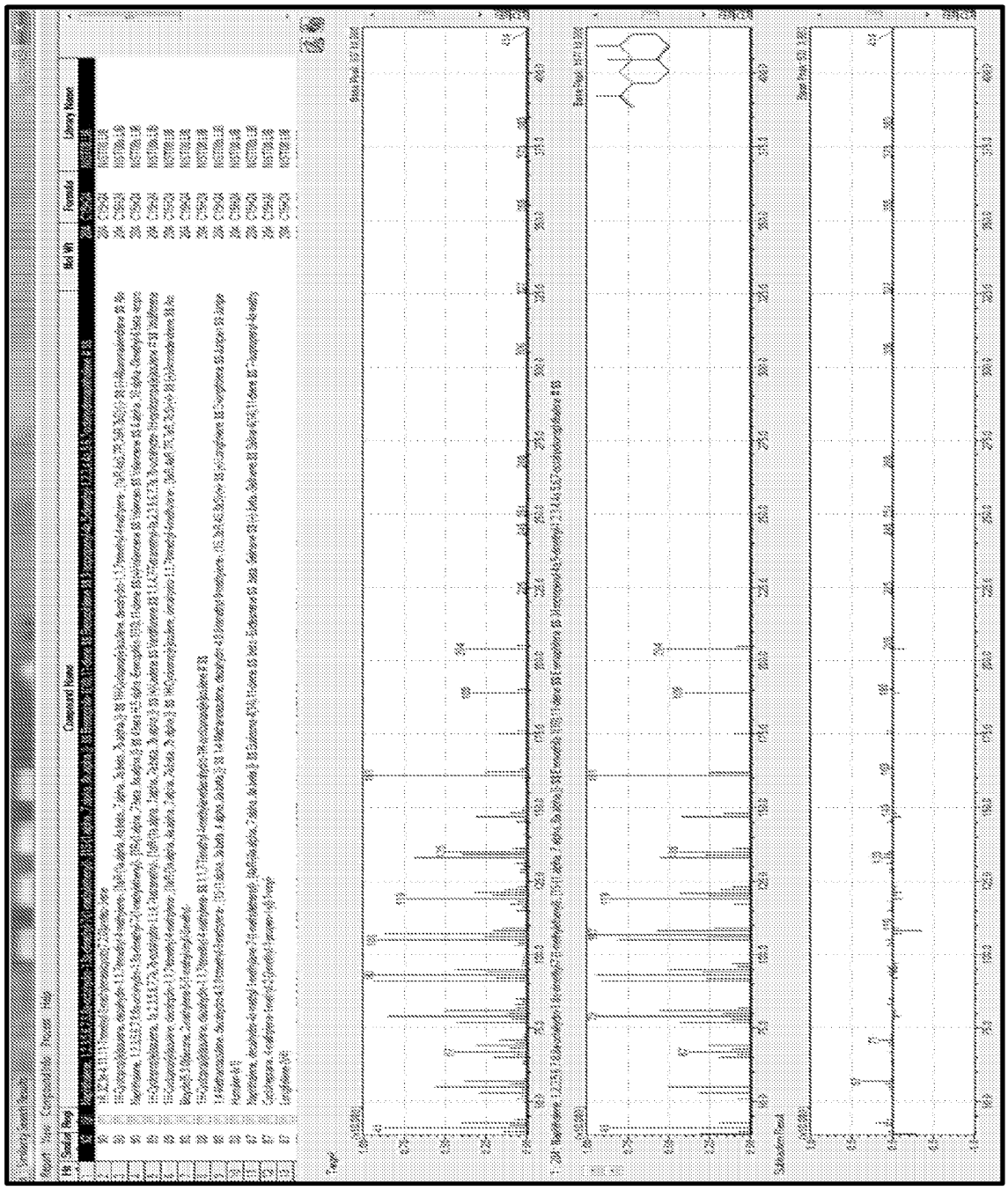


Fig. 48B

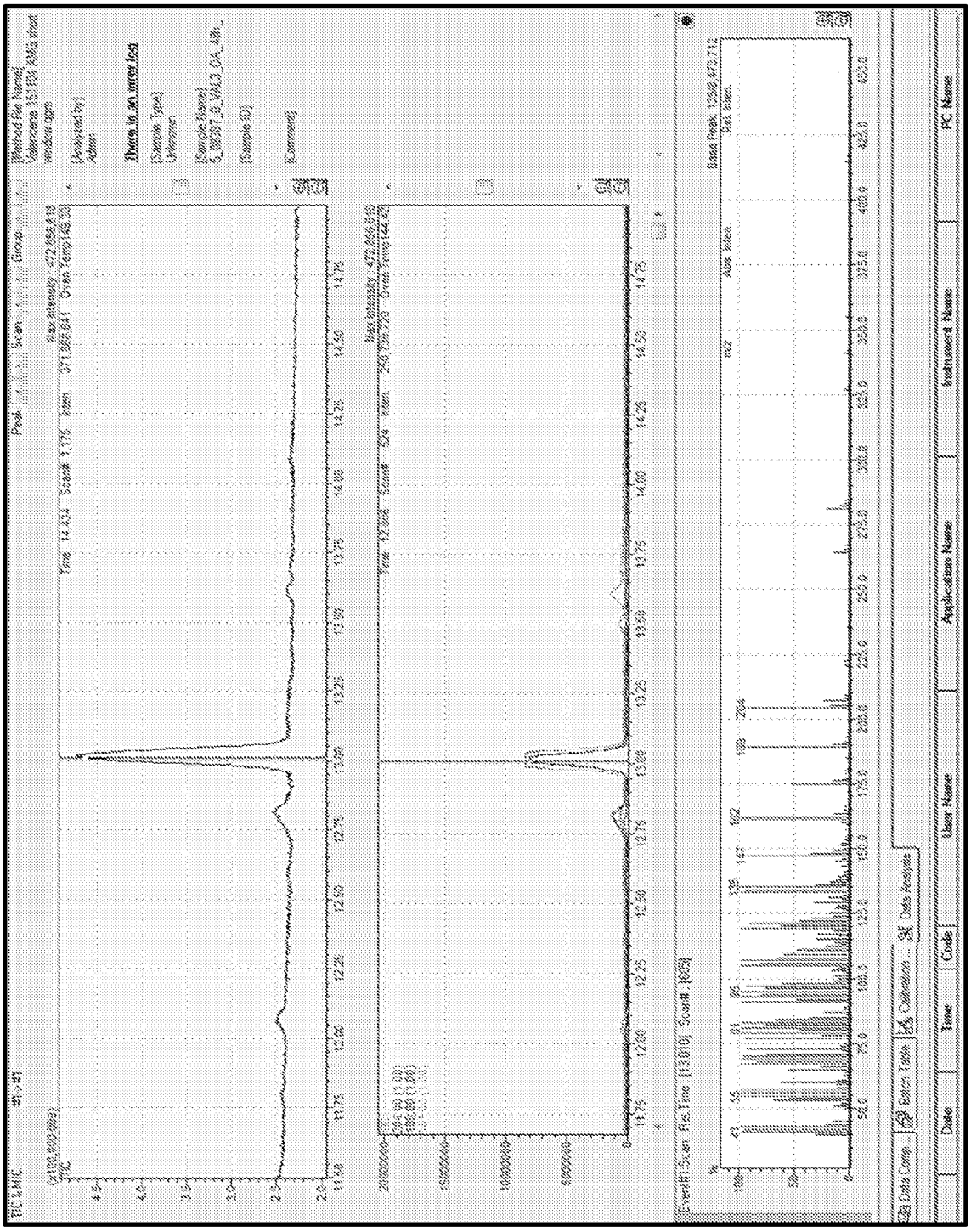


Fig. 49A

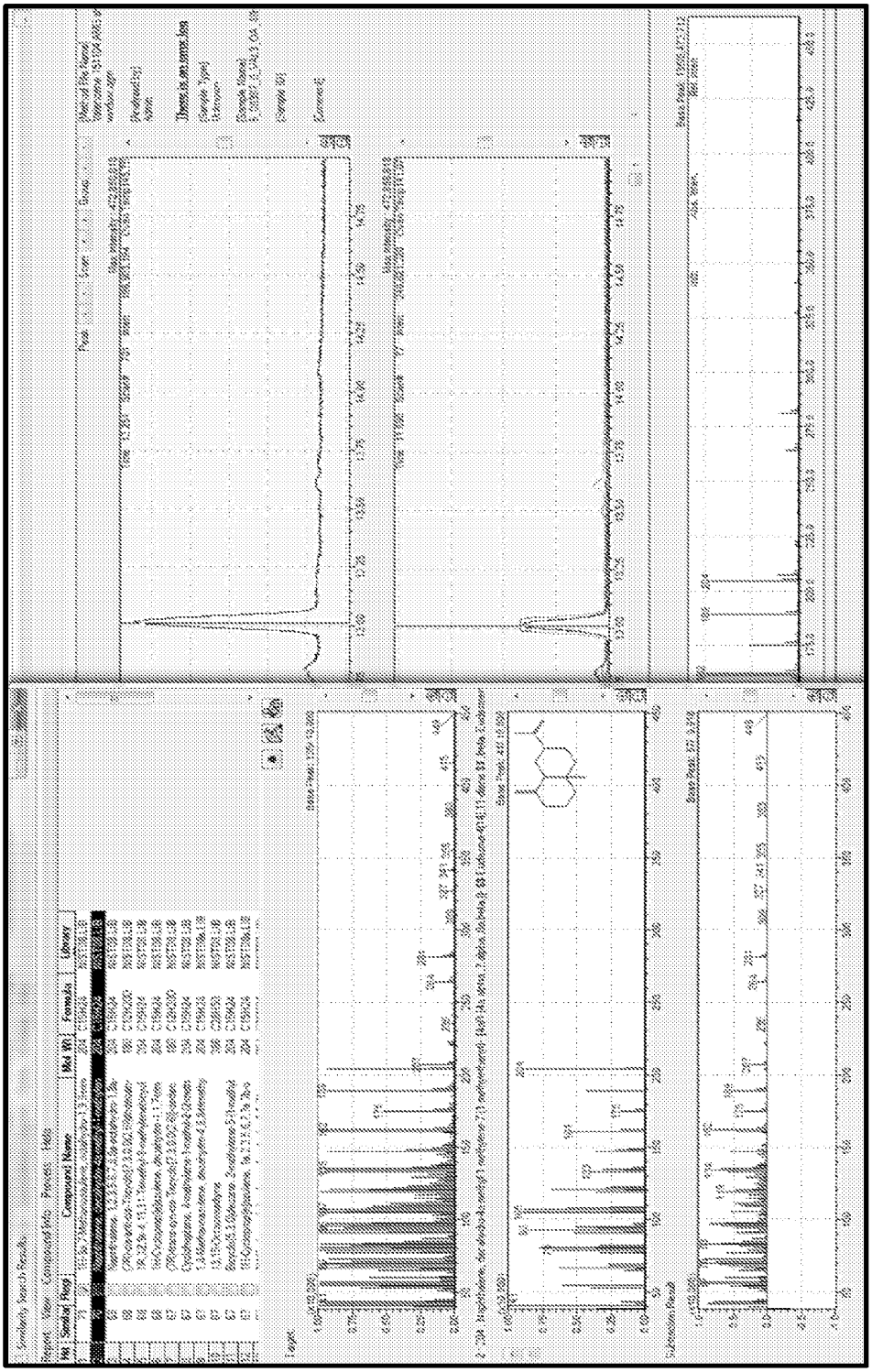


Fig. 49B

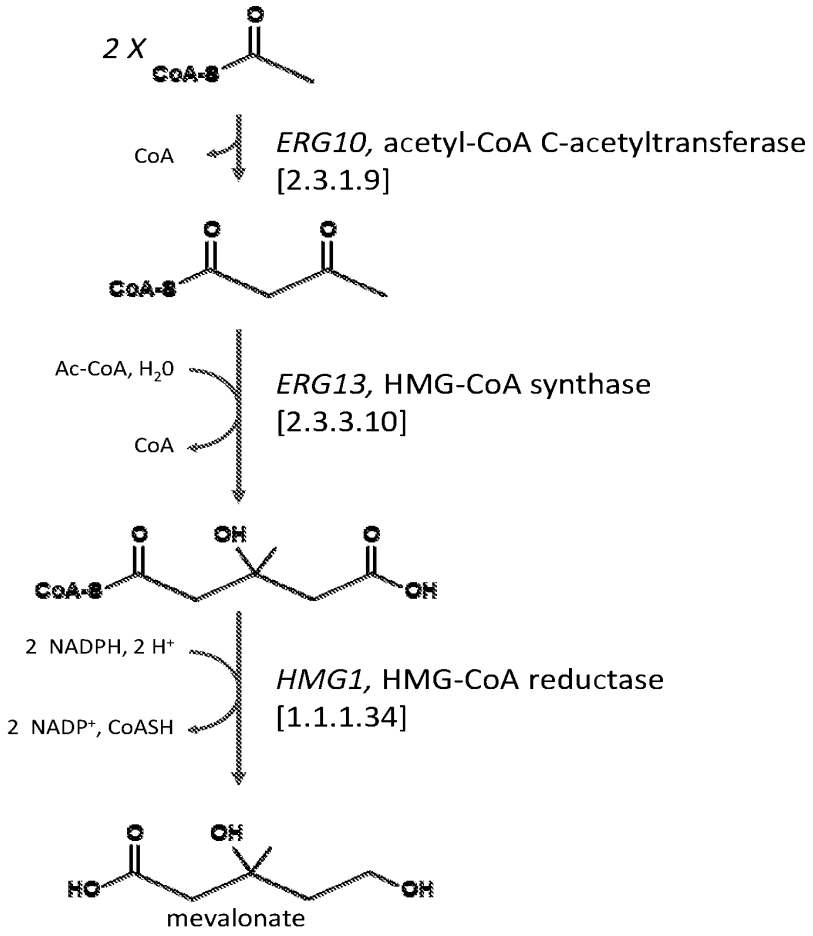

2 X
CoA-S
CoA
ERG10, acetyl-CoA C-acetyltransferase
[2.3.1.9]
CoA-S
Ac-CoA, H2O
CoA
ERG13, HMG-CoA synthase
[2.3.3.10]
CoA-S
OH
OH
2 NADPH, 2 H+
2 NADP+, CoASH
HMG1, HMG-CoA reductase
[1.1.1.34]
HO
OH
OH
mevalonate

Fig. 50

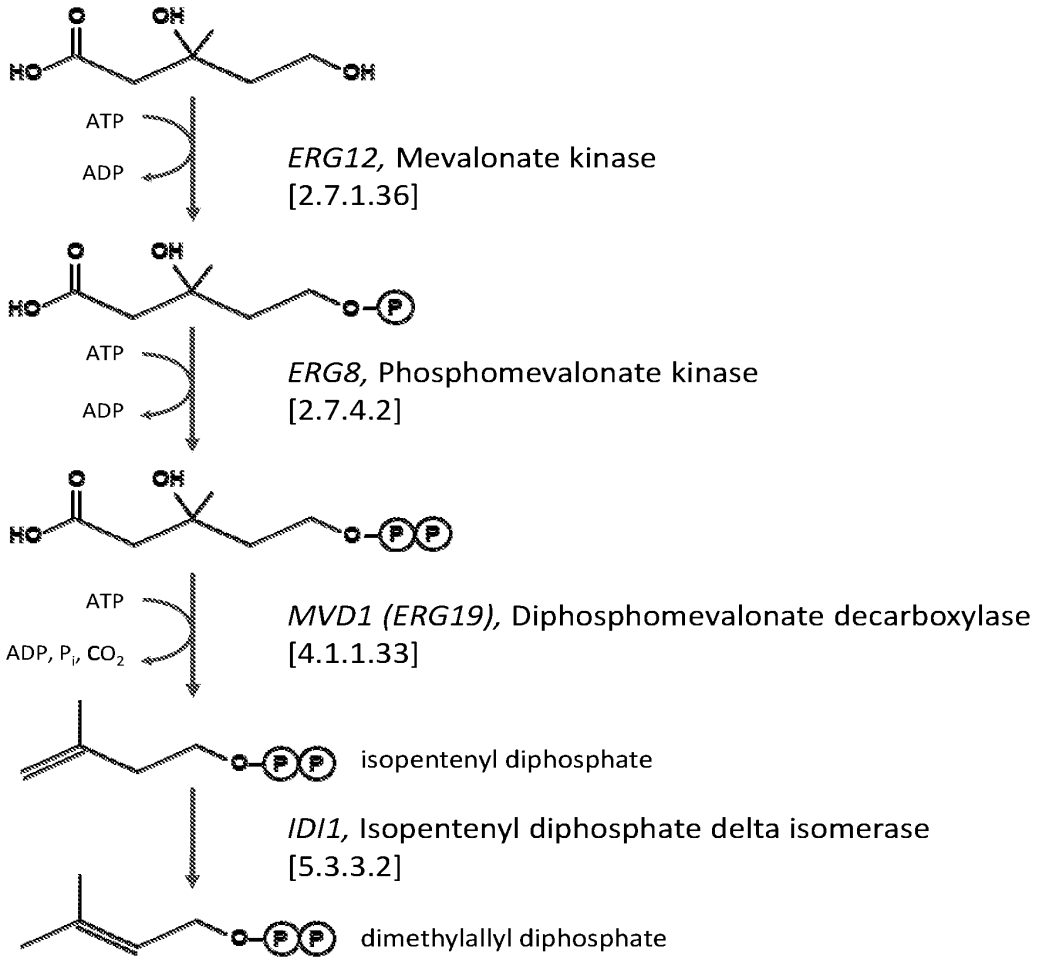

ATP
ADP
ERG12, Mevalonate kinase
[2.7.1.36]
ATP
ADP
ERG8, Phosphomevalonate kinase
[2.7.4.2]
ATP
ADP, Pi, CO2
MVD1 (ERG19), Diphosphomevalonate decarboxylase
[4.1.1.33]
isopentenyl diphosphate
IDI1, Isopentenyl diphosphate delta isomerase
[5.3.3.2]
dimethylallyl diphosphate

Fig. 51

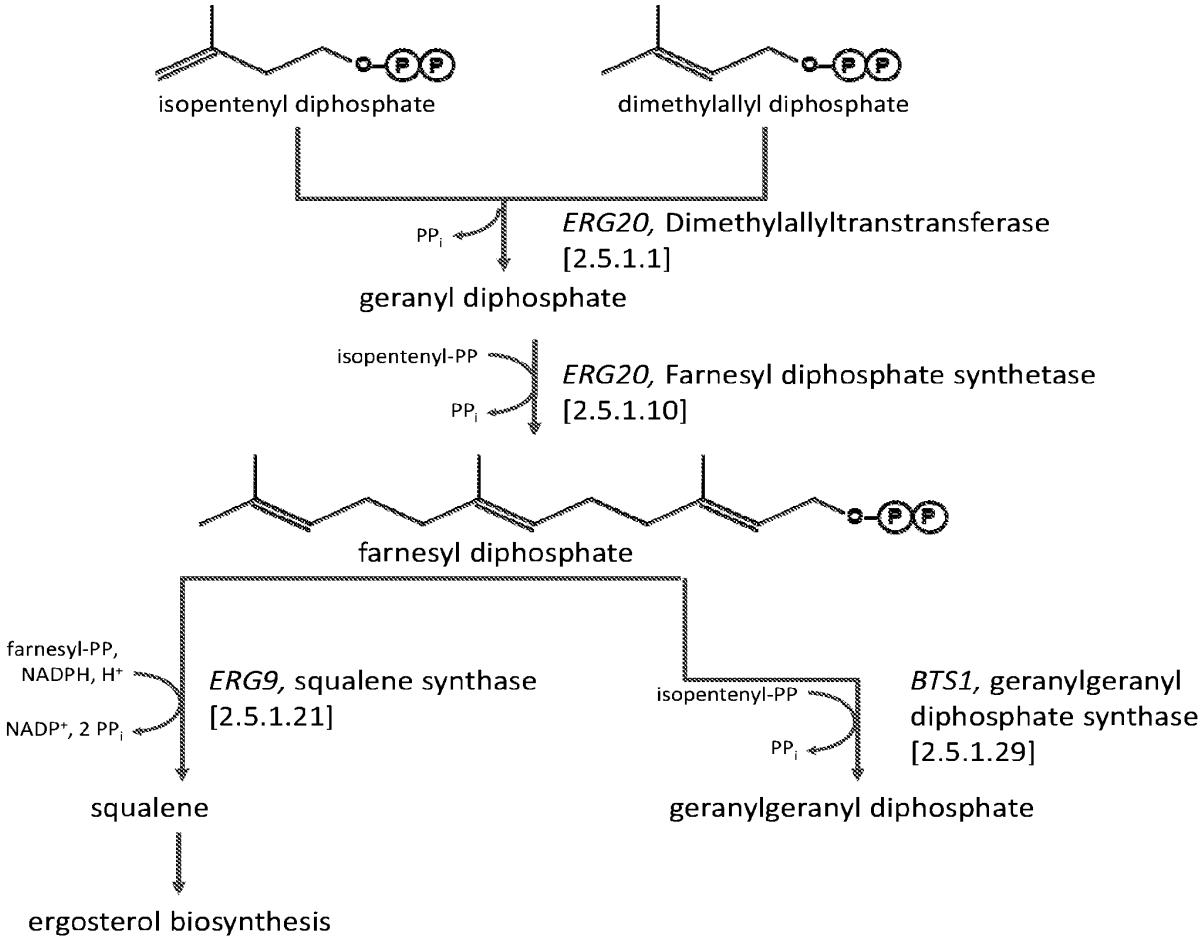

isopentenyl diphosphate
dimethylallyl diphosphate
PPi
ERG20, Dimethylallyltranstransferase
[2.5.1.1]
geranyl diphosphate
isopentenyl-PP
PPi
ERG20, Farnesyl diphosphate synthetase
[2.5.1.10]
farnesyl diphosphate
farnesyl-PP,
NADPH, H+
NADP+, 2 PPi
ERG9, squalene synthase
[2.5.1.21]
squalene
ergosterol biosynthesis
isopentenyl-PP
PPi
BTS1, geranylgeranyl
diphosphate synthase
[2.5.1.29]
geranylgeranyl diphosphate

Fig. 52

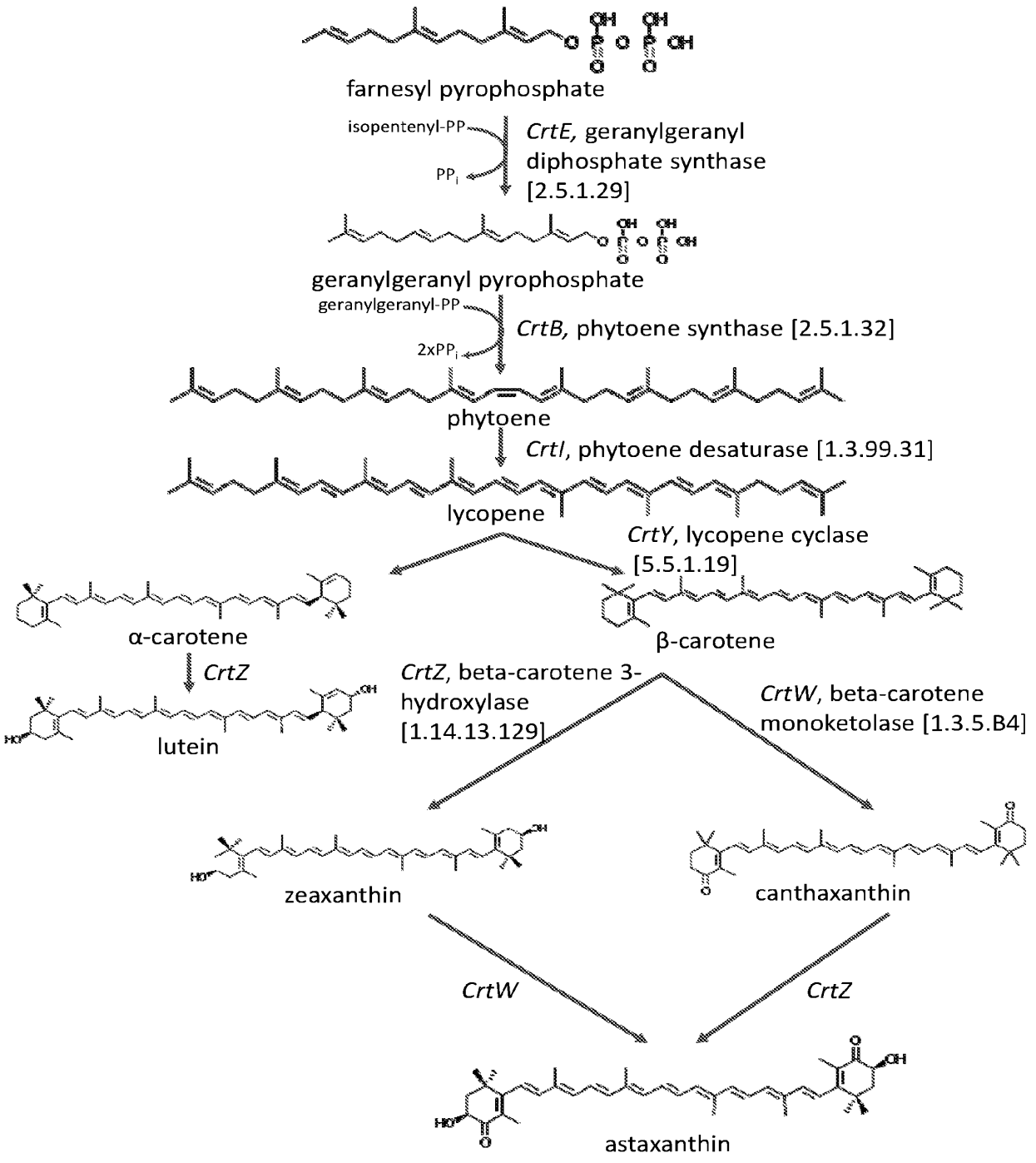

farnesyl pyrophosphate
isopentenyl-PP
PPi
CrtE, geranylgeranyl diphosphate synthase [2.5.1.29]
geranylgeranyl pyrophosphate
geranylgeranyl-PP
2xPPi
CrtB, phytoene synthase [2.5.1.32]
phytoene
CrtI, phytoene desaturase [1.3.99.31]
lycopene
CrtY, lycopene cyclase [5.5.1.19]
α-carotene
β-carotene
CrtZ
lutein
CrtZ, beta-carotene 3-hydroxylase [1.14.13.129]
CrtW, beta-carotene monoketolase [1.3.5.B4]
zeaxanthin
canthaxanthin
CrtW
CrtZ
astaxanthin

Fig. 53

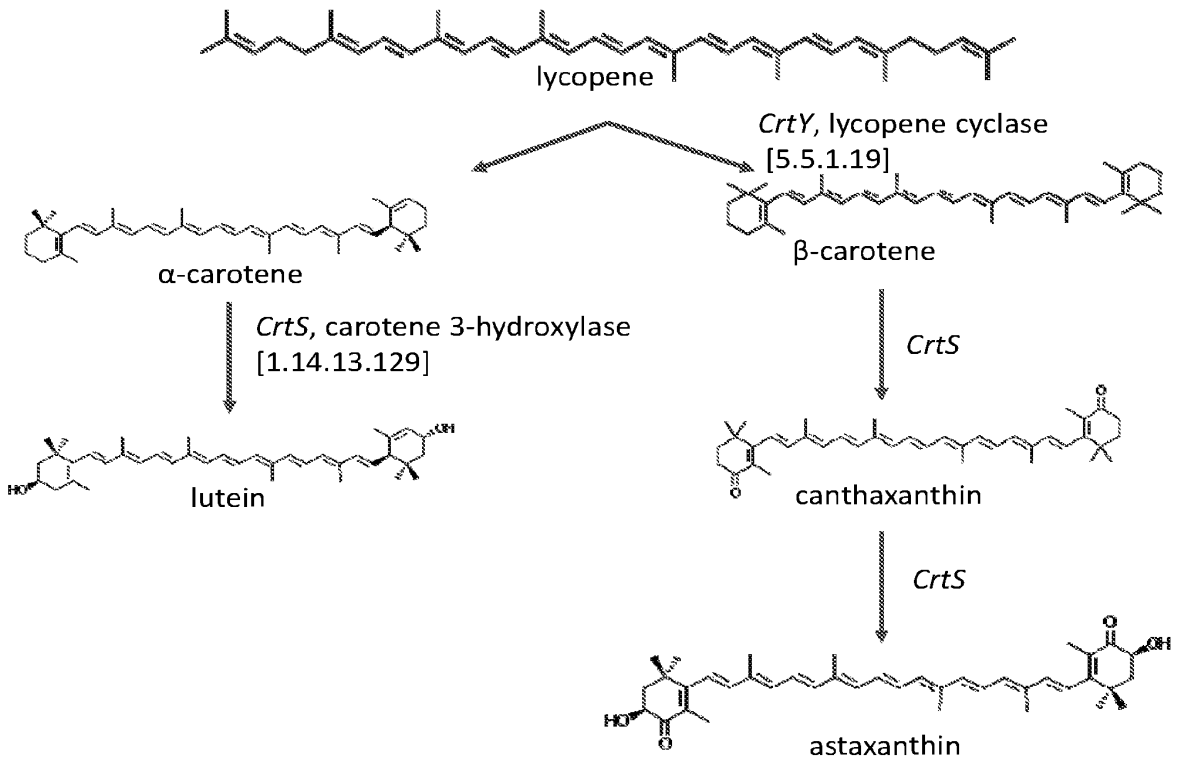

lycopene
CrtY, lycopene cyclase
[5.5.1.19]
α-carotene
β-carotene
CrtS, carotene 3-hydroxylase
[1.14.13.129]
CrtS
lutein
canthaxanthin
CrtS
astaxanthin

Fig. 54

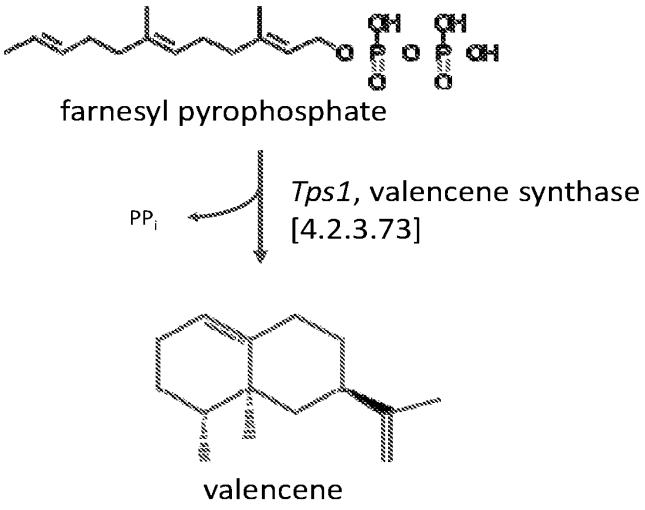

farnesyl pyrophosphate
PPi
Tps1, valencene synthase
[4.2.3.73]
valencene

Fig. 55

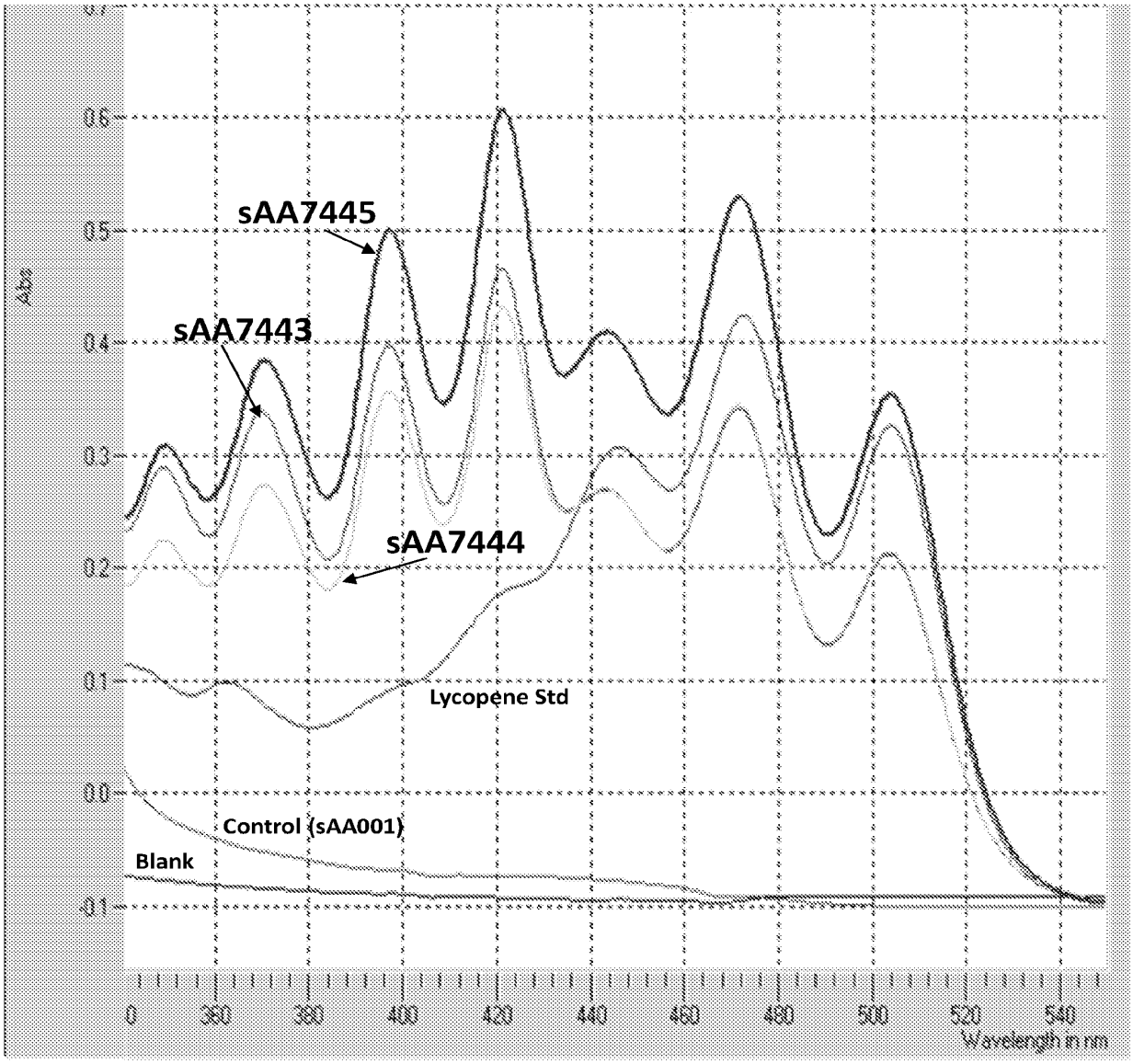

sAA7445
sAA7443
sAA7444
Lycopene Std
Control (sAA001)
Blank
Abs
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.1
0
360
380
400
420
440
460
480
500
520
540
Wavelength in nm

Fig. 56

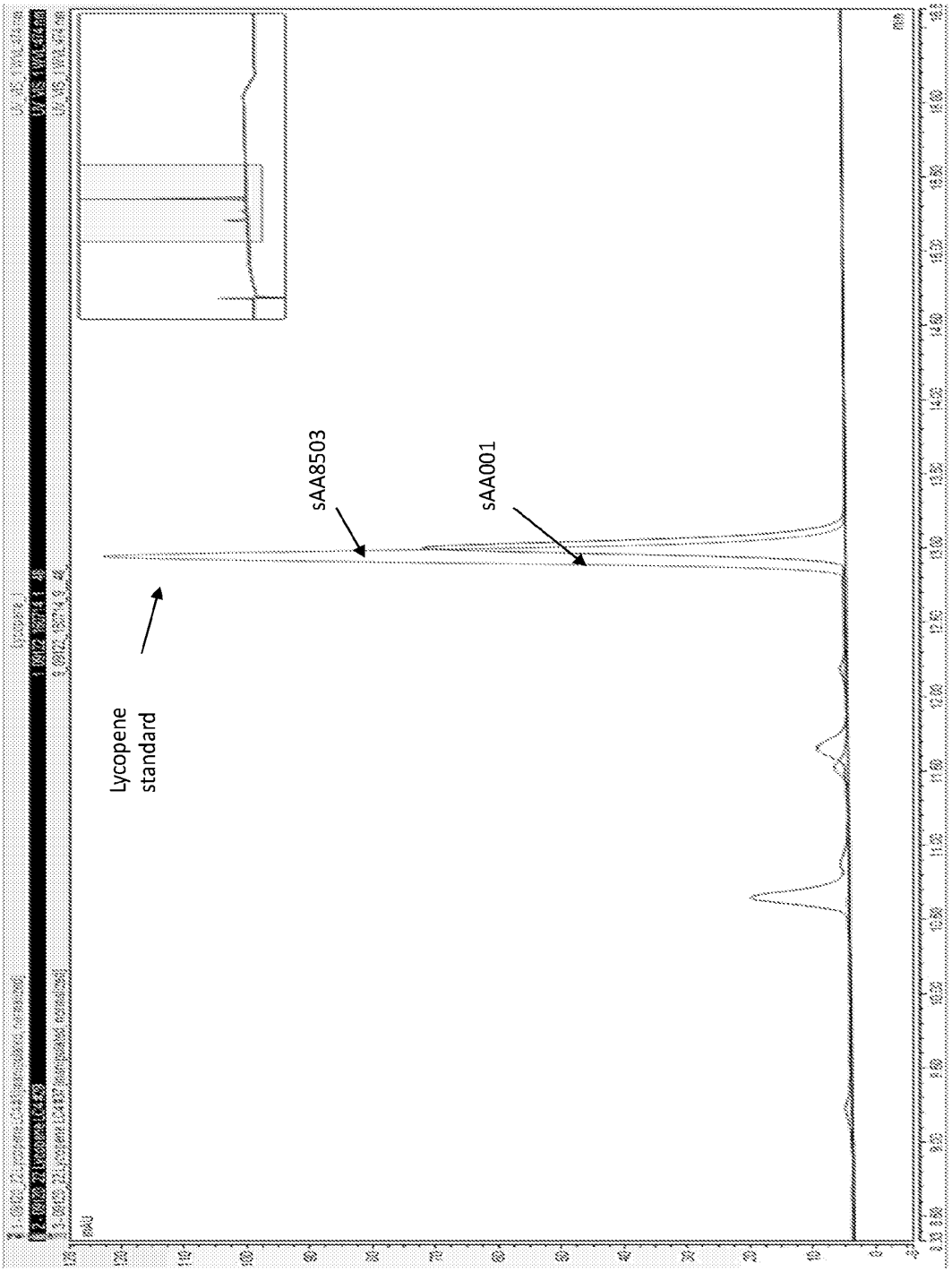



FIG. 57



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## PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 16/630,567

### APPLICATION AS FILED - PART I

| FOR | (Column 1) NUMBER FILED | (Column 2) NUMBER EXTRA | SMALL ENTITY RATE($) | SMALL ENTITY FEE($) | OR | OTHER THAN SMALL ENTITY RATE($) | OTHER THAN SMALL ENTITY FEE($) |
|---|---|---|---|---|---|---|---|
| BASIC FEE (37 CFR 1.16(a), (b), or (c)) | N/A | N/A | N/A | | | N/A | 320 |
| SEARCH FEE (37 CFR 1.16(k), (i), or (m)) | N/A | N/A | N/A | | | N/A | 140 |
| EXAMINATION FEE (37 CFR 1.16(o), (p), or (q)) | N/A | N/A | N/A | | | N/A | 800 |
| TOTAL CLAIMS (37 CFR 1.16(i)) | 21 minus 20 = | * 1 | | | OR | x 100 = | 100 |
| INDEPENDENT CLAIMS (37 CFR 1.16(h)) | 6 minus 3 = | * 3 | | | | x 480 = | 1440 |
| APPLICATION SIZE FEE (37 CFR 1.16(s)) | If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s). | | | | | | 4620 |
| MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j)) | | | | | | | 0.00 |
| * If the difference in column 1 is less than zero, enter "0" in column 2. | | | TOTAL | | | TOTAL | 7420 |

### APPLICATION AS AMENDED - PART II

| | | (Column 1) CLAIMS REMAINING AFTER AMENDMENT | | (Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR | (Column 3) PRESENT EXTRA | SMALL ENTITY RATE($) | SMALL ENTITY ADDITIONAL FEE($) | OR | OTHER THAN SMALL ENTITY RATE($) | OTHER THAN SMALL ENTITY ADDITIONAL FEE($) |
|---|---|---|---|---|---|---|---|---|---|---|
| AMENDMENT A | Total (37 CFR 1.16(i)) | * | Minus | ** | = | x = | | OR | x = | |
| | Independent (37 CFR 1.16(h)) | * | Minus | *** | = | x = | | OR | x = | |
| | Application Size Fee (37 CFR 1.16(s)) | | | | | | | | | |
| | FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j)) | | | | | | | OR | | |
| | | | | | | TOTAL ADD'L FEE | | OR | TOTAL ADD'L FEE | |

| | | (Column 1) CLAIMS REMAINING AFTER AMENDMENT | | (Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR | (Column 3) PRESENT EXTRA | RATE($) | ADDITIONAL FEE($) | OR | RATE($) | ADDITIONAL FEE($) |
|---|---|---|---|---|---|---|---|---|---|---|
| AMENDMENT B | Total (37 CFR 1.16(i)) | * | Minus | ** | = | x = | | OR | x = | |
| | Independent (37 CFR 1.16(h)) | * | Minus | *** | = | x = | | OR | x = | |
| | Application Size Fee (37 CFR 1.16(s)) | | | | | | | | | |
| | FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j)) | | | | | | | OR | | |
| | | | | | | TOTAL ADD'L FEE | | OR | TOTAL ADD'L FEE | |

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

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| U.S. APPLICATION NO. | FIRST NAMED INVENTOR | ATTY. DOCKET NO. |
|---|---|---|
| 16/630,567 | Kimberly Ann Aeling | SS118-1026US |

122515
Silvia Salvadori, P.C.
Silvia Salvadori
485 Central Park West Unit 5C
New York, NY 10025

| INTERNATIONAL APPLICATION NO. | |
|---|---|
| PCT/US2018/041579 | |
| I.A. FILING DATE | PRIORITY DATE |
| 07/11/2018 | 07/13/2017 |

**CONFIRMATION NO. 5356**
**371 ACCEPTANCE LETTER**

*OC000000121914016*

Date Mailed: 12/11/2020

## NOTICE OF ACCEPTANCE OF APPLICATION UNDER 35 U.S.C 371 AND 37 CFR 1.495

The applicant is hereby advised that the United States Patent and Trademark Office, in its capacity as a Designated / Elected Office (37 CFR 1.495), has ACCEPTED the above identified international application for national patentability examination in the United States Patent and Trademark Office.

The United States Application Number assigned to the application is shown above. A Filing Receipt will be issued for the present application in due course. **THE DATE APPEARING ON THE FILING RECEIPT AS THE "FILING DATE or 371(c) DATE" IS THE DATE ON WHICH THE LAST OF THE 35 U.S.C. 371 (c)(1) and (c)(2) REQUIREMENTS HAS BEEN RECEIVED IN THE OFFICE. THIS DATE IS SHOWN BELOW.** The filing date of the above identified application is the international filing date of the international application (Article 11(3) and 35 U.S.C. 363)

01/13/2020
DATE OF RECEIPT OF 35 U.S.C.
371(c)(1) and (c)(2) REQUIREMENTS

The following items have been received:

- Copy of the International Application filed on 01/13/2020
- Copy of the International Search Report filed on 01/13/2020
- Copy of IPE Report filed on 01/13/2020
- Preliminary Amendments filed on 01/13/2020
- Information Disclosure Statements filed on 01/13/2020
- Sequence Listing txt file (EFS or physical medium) filed on 01/13/2020
- Request for Immediate Examination filed on 01/13/2020
- U.S. Basic National Fees filed on 01/13/2020
- Assignee Statement for PGPUB filed on 01/13/2020
- Authorize Access to Search Results filed on 01/13/2020
- Priority Documents filed on 01/13/2020
- Specification filed on 01/13/2020
- Claims filed on 01/13/2020
- Abstracts filed on 01/13/2020
- Drawings filed on 01/13/2020
- Authorization to Permit Access filed on 01/13/2020
- Application Data Sheet (37 CFR 1.76) filed on 01/13/2020



FORM PCT/DO/EO/903 (371 Acceptance Notice)

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Ex. 1002
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Applicant is notified that the above-identified application contains the deficiencies noted below. No period for reply is set forth in this notice for correction of these deficiencies. However, if a deficiency relates to the inventor's oath or declaration, the applicant must file an oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each actual inventor no later than the expiration of the time period set in the "Notice of Allowability" to avoid abandonment. See 37 CFR 1.495(c).

- Properly executed inventor's oath or declaration for the following inventor(s) has not been submitted: **Kimberly Ann Aeling**, **Pierre-Yves De Wals**, **Eric Scott**, **Eric Knight**, **Jian Yi**, **Michael Waldbridge**, and **Thomas Beardslee**

Applicant is reminded that any communications to the United States Patent and Trademark Office must be mailed to the address given in the heading and include the U.S. application no. shown above (37 CFR 1.5)

LESLIE R CHASE

Telephone: (703) 756-1026



FORM PCT/DO/EO/903 (371 Acceptance Notice)

LCY Biotechnology Holding, Inc.
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UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NUMBER | FILING or 371(c) DATE | GRP ART UNIT | FIL FEE REC'D | ATTY.DOCKET.NO | TOT CLAIMS | IND CLAIMS |
|---|---|---|---|---|---|---|
| 16/630,567 | 01/13/2020 | | 8600 | SS118-1026US | 21 | 6 |

**CONFIRMATION NO. 5356**

**FILING RECEIPT**

122515
Silvia Salvadori, P.C.
Silvia Salvadori
485 Central Park West Unit 5C
New York, NY 10025

OC000000121914015*

Date Mailed: 12/11/2020

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

**Please verify the accuracy of the data presented on this receipt.** If an error is noted on this Filing Receipt, please submit a written request for a corrected Filing Receipt, including a properly marked-up ADS showing the changes with strike-through for deletions and underlining for additions. If you received a "Notice to File Missing Parts" or other Notice requiring a response for this application, please submit any request for correction to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections provided that the request is grantable.

**Inventor(s)**

Kimberly Ann Aeling, San Clemente, CA;
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Eric Scott, Encinitas, CA;
Eric Knight, Carlsbad, CA;
Jian Yi, San Diego, CA;
Michael Waldbridge, Saugus, MA;
Thomas Beardslee, Carlsbad, CA;

**Applicant(s)**

Radici Chimica S.p.A., Bergamo, ITALY;

**Assignment For Published Patent Application**

Radici Chimica S.p.A., Bergamo, ITALY

**Power of Attorney:** None

**Domestic Priority data as claimed by applicant**

This application is a 371 of PCT/US2018/041579 07/11/2018
which claims benefit of 62/532,297 07/13/2017

**Foreign Applications** for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
*Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.*

**Permission to Access Application via Priority Document Exchange:** Yes

**Permission to Access Search Results:** Yes

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**If Required, Foreign Filing License Granted:** 12/09/2020
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 16/630,567**
**Projected Publication Date:** 03/18/2021
**Non-Publication Request:** No
**Early Publication Request:** No
**Title**

BIOLOGICAL METHODS FOR PREPARING TERPENES

**Preliminary Class**

**Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications:** No

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Ex. 1002
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## Title 37, Code of Federal Regulations, 5.11 & 5.15

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---

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| MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET<br><br>Substitute for Form PTO-1360<br>(For use with Form PTO/SB/06) | Application Number<br>16630567 | Filing Date |
|---|---|---|
| | Applicant(s) Kimberly Aeling | |

* May be used for additional claims or amendments

| CLAIMS | AS FILED | | AFTER FIRST AMENDMENT | | AFTER SECOND AMENDMENT | |
|---|---|---|---|---|---|---|
| | Indep | Depend | Indep | Depend | Indep | Depend |
| 1 | 1 | | --- | --- | | |
| 2 | | 1 | --- | --- | | |
| 3 | | 1 | --- | --- | | |
| 4 | | 1 | --- | --- | | |
| 5 | | 4 | --- | --- | | |
| 6 | | ( 1 ) | --- | --- | | |
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| 43 | 1 | | --- | --- | | |
| 44 | | 1 | --- | --- | | |
| 45 | | 1 | --- | --- | | |
| 46 | | 3 | --- | --- | | |
| 47 | | 3 | --- | --- | | |
| 48 | | 3 | --- | --- | | |
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| 50 | | ( 1 ) | --- | --- | | |

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| | Indep | Depend | Indep | Depend | Indep | Depend |
| 51 | | ( 1 ) | --- | --- | | |
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| 86 | | 3 | --- | --- | | |
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| 100 | | ( 1 ) | --- | --- | | |

| MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET<br><br>Substitute for Form PTO-1360<br>(For use with Form PTO/SB/06) | Application Number<br>16630567 | Filing Date |
|---|---|---|
| | Applicant(s) Kimberly Aeling | |

* May be used for additional claims or amendments

| CLAIMS | AS FILED | | AFTER FIRST AMENDMENT | | AFTER SECOND AMENDMENT | |
|---|---|---|---|---|---|---|
| | Indep | Depend | Indep | Depend | Indep | Depend |
| 101 | | ( 1 ) | --- | --- | | |
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| 147 | 1 | | --- | --- | | |
| 148 | 1 | | --- | --- | | |
| 149 | | 3 | --- | --- | | |
| 150 | | ( 1 ) | --- | --- | | |
| Total Indep | 6 | | 6 | | 0 | |
| Total Depend | 169 | ↵ | 15 | ↵ | 0 | ↵ |
| Total Claims | 175 | | 21 | | 0 | |

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| 177 | | | | 1 | | |
| 178 | | | | 1 | | |
| 179 | | | | 1 | | |
| 180 | | | | 1 | | |
| 181 | | | 1 | | | |
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| Application Data Sheet 37 CFR 1.76 | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
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## Inventor Information:

**Inventor 1** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | | | | |

**Residence Information (Select One)** ◉ US Residency ○ Non US Residency Active US Military Service

| City | | State/Province | | Country of Residence[i] | |
|---|---|---|---|---|---|

**Mailing Address of Inventor:**

| Address 1 | |
|---|---|
| Address 2 | |

| City | | State/Province | |
|---|---|---|---|
| Postal Code | | Country[i] | |

**Inventor 2** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | | | | |

**Residence Information (Select One)** ◉ US Residency ○ Non US Residency Active US Military Service

| City | | State/Province | CA | Country of Residence[i] | US |
|---|---|---|---|---|---|

**Mailing Address of Inventor:**

| Address 1 | |
|---|---|
| Address 2 | |

| City | | State/Province | |
|---|---|---|---|
| Postal Code | | Country[i] | |

**Inventor 3** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | | | | |

**Residence Information (Select One)** ◉ US Residency ○ Non US Residency Active US Military Service

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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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| Application Data Sheet 37 CFR 1.76 | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

| City | | State/Province | | Country of Residence[i] | |
|---|---|---|---|---|---|

**Mailing Address of Inventor:**

| Address 1 | | | |
|---|---|---|---|
| Address 2 | | | |
| City | | State/Province | |
| Postal Code | | Country[i] | |

**Inventor 4** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | ~~Eric~~ | | ~~Knight~~ | |

**Residence Information (Select One)** (•) US Residency ( ) Non US Residency Active US Military Service

| City | ~~Carlsbad~~ | State/Province | ~~CA~~ | Country of Residence[i] | ~~US~~ |
|---|---|---|---|---|---|

**Mailing Address of Inventor:**

| Address 1 | ~~2744 Loker Ave West, unit 100~~ | | |
|---|---|---|---|
| Address 2 | | | |
| City | | State/Province | |
| Postal Code | ~~92010~~ | Country[i] | ~~US~~ |

**Inventor 5** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | ~~Jian~~ | | ~~Yi~~ | |

**Residence Information (Select One)** (•) US Residency ( ) Non US Residency Active US Military Service

| City | ~~San Diego~~ | State/Province | ~~CA~~ | Country of Residence[i] | ~~US~~ |
|---|---|---|---|---|---|

**Mailing Address of Inventor:**

| Address 1 | ~~13313 Thunderhead Street~~ | | |
|---|---|---|---|
| Address 2 | | | |
| City | ~~San Diego~~ | State/Province | ~~CA~~ |
| Postal Code | ~~92129~~ | Country[i] | ~~US~~ |

**Inventor 6** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | | | | |

**Residence Information (Select One)** (•) US Residency ( ) Non US Residency Active US Military Service

| City | | State/Province | | Country of Residence[i] | |
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| Application Data Sheet 37 CFR 1.76 | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

| Mailing Address of Inventor: | | | |
|---|---|---|---|
| Address 1 | | | |
| Address 2 | | | |
| City | | State/Province | |
| Postal Code | | Country | |

Inventor 7 — Remove

Legal Name

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | | | | |

Residence Information (Select One) (•) US Residency ( ) Non US Residency ( ) Active US Military Service

| City | | State/Province | | Country of Residence | |
|---|---|---|---|---|---|

| Mailing Address of Inventor: | | | |
|---|---|---|---|
| Address 1 | | | |
| Address 2 | | | |
| City | | State/Province | |
| Postal Code | | Country | |

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. — Add

## Correspondence Information:

**Enter either Customer Number or complete the Correspondence Information section below.**
**For further information see 37 CFR 1.33(a).**

☐ **An Address is being provided for the correspondence Information of this application.**

| Customer Number | | | |
|---|---|---|---|
| Email Address | | Add Email | Remove Email |

## Application Information:

| Title of the Invention | | | |
|---|---|---|---|
| Attorney Docket Number | | Small Entity Status Claimed | ☐ |
| Application Type | | | |
| Subject Matter | | | |
| Total Number of Drawing Sheets (if any) | | Suggested Figure for Publication (if any) | |

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| **Application Data Sheet 37 CFR 1.76** | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

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For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

| Application number of the previously filed application | Filing date (YYYY-MM-DD) | Intellectual Property Authority or Country |
|---|---|---|
| | | |

## Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

## Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

| Please Select One: | ◉ Customer Number | ○ US Patent Practitioner | ○ Limited Recognition (37 CFR 11.9) |
|---|---|---|---|
| Customer Number | | | |

## Domestic Benefit/National Stage Information:

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When referring to the current application, please leave the "Application Number" field blank.

| Prior Application Status | Pending | | Remove |
|---|---|---|---|
| Application Number | Continuity Type | Prior Application Number | Filing or 371(c) Date (YYYY-MM-DD) |
| | | | |

PTO/AIA/14 (02-18)
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| **Application Data Sheet 37 CFR 1.76** | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

| Prior Application Status | Expired | | Remove |
|---|---|---|---|
| Application Number | Continuity Type | Prior Application Number | Filing or 371(c) Date (YYYY-MM-DD) |
| | | | |
| Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button. | | | Add |

## Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)[i] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

| | | | Remove |
|---|---|---|---|
| Application Number | Country[i] | Filing Date (YYYY-MM-DD) | Access Code[i] (if applicable) |
| | | | |
| Additional Foreign Priority Data may be generated within this form by selecting the **Add** button. | | | Add |

## Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

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| Application Data Sheet 37 CFR 1.76 | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

## Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

**NOTE**: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

**1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)**

**A. Priority Document Exchange (PDX)** - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

**B. Search Results from U.S. Application to EPO** - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

**2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)**

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

**NOTE:** Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

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| **Application Data Sheet 37 CFR 1.76** | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

## Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

**Applicant 1** Remove

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

| ◉ Assignee | ○ Legal Representative under 35 U.S.C. 117 | ○ Joint Inventor |
|---|---|---|
| ○ Person to whom the inventor is obligated to assign. | ○ Person who shows sufficient proprietary interest | |

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

| Organization Name | | | |
|---|---|---|---|
| **Mailing Address Information For Applicant:** | | | |
| **Address 1** | | | |
| Address 2 | | | |
| **City** | | **State/Province** | |
| **Country**[i] IT | | Postal Code | |
| Phone Number | | Fax Number | |
| Email Address | | | |

Additional Applicant Data may be generated within this form by selecting the Add button. Add

## Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

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| **Application Data Sheet 37 CFR 1.76** | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

**Assignee 1**

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

Remove

If the Assignee or Non-Applicant Assignee is an Organization check here. ☒

| Organization Name | |
|---|---|

**Mailing Address Information For Assignee including Non-Applicant Assignee:**

| **Address 1** | | | |
|---|---|---|---|
| Address 2 | | | |
| **City** | | **State/Province** | |
| **Country** i | | Postal Code | |
| Phone Number | | Fax Number | |
| Email Address | | | |

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button. Add

## Signature:

Remove

**NOTE:** This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

| **Signature** | /Silvia Salvadori/ | | | Date (YYYY-MM-DD) | 2021-02-22 |
|---|---|---|---|---|---|
| First Name | Silvia | Last Name | Salvadori | Registration Number | 48265 |

Additional Signature may be generated within this form by selecting the Add button. Add

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| **Application Data Sheet 37 CFR 1.76** | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

# Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1 The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3 A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent CooperationTreaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.



LCY Biotechnology Holding, Inc.
Ex. 1002
Page 2577 of 3077

# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 41981337 |
| **Application Number:** | 16630567 |
| **International Application Number:** | |
| **Confirmation Number:** | 5356 |
| **Title of Invention:** | BIOLOGICAL METHODS FOR PREPARING TERPENES |
| **First Named Inventor/Applicant Name:** | Kimberly Ann Aeling |
| **Customer Number:** | 122515 |
| **Filer:** | Silvia Salvadori |
| **Filer Authorized By:** | |
| **Attorney Docket Number:** | SS118-1026US |
| **Receipt Date:** | 22-FEB-2021 |
| **Filing Date:** | 13-JAN-2020 |
| **Time Stamp:** | 12:43:33 |
| **Application Type:** | U.S. National Stage under 35 USC 371 |

## Payment information:

| | |
|---|---|
| Submitted with Payment | no |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Application Data Sheet | Updated_ADS.pdf | 139799<br>c74eae0870342f578ae9ac13ec67587fc18f55ea | no | 10 |

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| Information: | |
|---|---|
| This is not an USPTO supplied ADS fillable form | |
| **Total Files Size (in bytes):** | 139799 |

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**New Applications Under 35 U.S.C. 111**
**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**
**National Stage of an International Application under 35 U.S.C. 371**
**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**
**New International Application Filed with the USPTO as a Receiving Office**
**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NUMBER | FILING OR 371(C) DATE | FIRST NAMED APPLICANT | ATTY. DOCKET NO./TITLE |
|---|---|---|---|
| 16/630,567 | 01/13/2020 | Kimberly Ann Aeling | SS118-1026US |

**CONFIRMATION NO. 5356**

122515
Silvia Salvadori, P.C.
Silvia Salvadori
485 Central Park West Unit 5C
New York, NY 10025

**PUBLICATION NOTICE**

*OC000000124194559*

**Title:**BIOLOGICAL METHODS FOR PREPARING TERPENES

**Publication No.**US-2021-0079408-A1
**Publication Date:**03/18/2021

## NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Public Records Division. The Public Records Division can be reached by telephone at (571) 272-3150 or (800) 972-6382, by facsimile at (571) 273-3250, by mail addressed to the United States Patent and Trademark Office, Public Records Division, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently https://portal.uspto.gov/pair/PublicPair. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

PTO/AIA/80 (07-17)
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# POWER OF ATTORNEY TO PROSECUTE APPLICATIONS BEFORE THE USPTO

I hereby revoke all previous powers of attorney given in the application identified in the attached statement under 37 CFR 3.73(c).

I hereby appoint:

[X] Practitioners associated with Customer Number: 122515

**OR**

[ ] Practitioner(s) named below (if more than ten patent practitioners are to be named, then a customer number must be used):

| Name | Registration Number | Name | Registration Number |
|---|---|---|---|
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |

As attorney(s) or agent(s) to represent the undersigned before the United States Patent and Trademark Office (USPTO) in connection with any and all patent applications assigned only to the undersigned according to the USPTO assignment records or assignment documents attached to this form in accordance with 37 CFR 3.73(c).

Please change the correspondence address for the application identified in the attached statement under 37 CFR 3.73(c) to:

[X] The address associated with Customer Number: 122515

**OR**

[ ]

| | | |
|---|---|---|
| Firm or individual name | | |
| Address | | |
| City | State | Zip |
| Country | | |
| Telephone | Email | |

Assignee name and address: Radici Chimica S.p.A.
Via G. Verdi, 11 Bergamo 24121
Italy

**A copy of this form, together with a statement under 37 CFR 3.73(c) (Form PTO/AIA/96 or equivalent) is required to be filed in each application in which this form is used. The statement under 37 CFR 3.73(c) may be completed by one of the practitioners appointed in this form, and must identify the application in which this Power of Attorney is to be filed.**

**SIGNATURE of Assignee of Record**

The individual whose signature and title is supplied below is authorized to act on behalf of the assignee.

| | |
|---|---|
| Signature RADICI CHIMICA S.P.A. | Date 7 May 2020 |
| Name Angelo RADICI | Telephone |
| Title - CEO - | |

This collection of information is required by 37 CFR 1.31, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public, which is to update (and by the USPTO to process) the file of a patent or reexamination proceeding. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 18 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
*If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.*

## Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant *(i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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# STATEMENT UNDER 37 CFR 3.73(c)

Applicant/Patent Owner: Radici Chimica S.p.A.

Application No./Patent No.: 16/630,567 Filed/Issue Date: 2020-01-13

Titled: BIOLOGICAL METHODS FOR PREPARING TERPENES

Radici Chimica S.p.A., a Corporation

(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that, for the patent application/patent identified above, it is (choose **one** of options 1, 2, 3 or 4 below):

1. [x] The assignee of the entire right, title, and interest.

2. [ ] An assignee of less than the entire right, title, and interest (check applicable box):

   [ ] The extent (by percentage) of its ownership interest is _____%. Additional Statement(s) by the owners holding the balance of the interest must be submitted to account for 100% of the ownership interest.

   [ ] There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. [ ] The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. [ ] The recipient, via a court proceeding or the like (*e.g.*, bankruptcy, probate), of an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

The interest identified in option 1, 2 or 3 above (not option 4) is evidenced by either (choose **one** of options A or B below):

A. [✓] An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.

B. [ ] A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: _____ To: _____

   The document was recorded in the United States Patent and Trademark Office at

   Reel _____, Frame _____, or for which a copy thereof is attached.

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This collection of information is required by 37 CFR 3.73(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

*If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.*

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## STATEMENT UNDER 37 CFR 3.73(c)

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☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

☑ As required by 37 CFR 3.73(c)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

| | |
|---|---|
| /Silvia Salvadori/ | October 4, 2021 |
| Signature | Date |
| Silvia Salvadori | 48265 |
| Printed or Typed Name | Title or Registration Number |

# Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

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- ☐ *Foreign rights, title and interest in the invention*
- ☐ *United States Patent Application Serial No.*
  *Date of Execution: _____ Date of Filing:*
- ☐ *United States Provisional Patent Application Serial No. _____*
- ☐ *United States Patent No(s). _____*
- ☒ *International (PCT) Patent Application No. PCT/US2018/041579*
  *Date of Filing: 11 July 2018*
- ☐ *Other (specify)_*

*Title of the Invention:*

**BIOLOGICAL METHOD FOR PREPARING TERPENES**

*Inventors (assignors):*

| *Name* | *Address* |
|---|---|
| Kimberly Ann Aeling | 324 Plaza Estival, San Clemente, CA 92672 (US) |
| Pierre-Yves De Wals | 925 North Vulcan Ave. Apt. #205, Encinitas, CA 92024 (US) |
| Erin Scott | 1579 Caudor Street, Encinitas, CA 92024 (US) |
| [redacted] | |
| Michael Walbridge | 4 Diane Dr, Saugus MA 01906 (US) |
| Thomas Beardslee | 6827 Carnation Drive, Carlsbad, CA 92011 (US) |

*Assignee:*

| *Name* | *Address* |
|---|---|
| RADICI CHIMICA S.P.A. | Via G. Verdi, 11 24121 Bergamo (Italy) |

Whereas, we, the above-identified Inventor(s), have invented certain new and useful improvements in the Invention identified above and described in the above-identified patent application(s) and /or patent(s) (hereinafter referred to as "Invention");

And, whereas we desire to assign our above-identified rights, title and interest in the Invention to the above-identified Assignee;

Now, this indenture witnesseth, that for good and valuable consideration, the receipt whereof is hereby acknowledged;

We hereby assign, sell and transfer our above-identified rights, title and interest in said Invention, said application(s) as identified above, including any divisions, continuations, and continuations-in-part thereof, and in and to any and all Letters Patent of the United States, and countries foreign thereto, which may be granted for said Invention, and in and to any and all reissues and reexaminations thereof, which may be granted or have granted for said Invention, and in and to any and all priority rights, Convention rights, and other benefits accruing or to accrue to us with respect to the filing of applications for patents or securing of patents in the United States and countries foreign thereto, unto said Assignee;

And we hereby authorize and request the Commissioner of Patents and Trademarks to issue any United States Letters Patent which may issue for said Invention to said Assignee, as assignee of the whole right, title and interest thereto;

And we further agree to sign and execute all necessary and lawful future documents, including applications for foreign patents, for filing divisions, continuations and continuation-in-part of said application for patent and/or for obtaining any reissue or reissues of any Letters Patent which may be granted for my aforesaid Invention, as the Assignee or its Designee(s) may from time to time require and prepare at its own expense.

The undersigned hereby grant(s) the firm SILVIA SALVADORI, P.C., 270 Madison Avenue, 8th Floor, New York, NY 10016 the power to insert on this assignment any further identification which may be necessary or desirable in order to comply with the rules of the United States Patent Office for recordation of this document.

***Inventors' Signatures:***

| Name | Signature/ Date |
|---|---|
| Kimberly Ann Aeling | 11/7/2020 |
| Pierre-Yves De Wals | |
| Eron Scott | |
| [redacted] | |



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| | |
|---|---|
| Michael Walbridge | |
| Thomas Beardslee | |

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- ☐ *United States Patent No(s).*_____
- ☒ *International (PCT) Patent Application No. PCT/US2018/041579*
  *Date of Filing: 11 July 2018*
- ☐ *Other (specify)*_

*Title of the Invention:*

**BIOLOGICAL METHOD FOR PREPARING TERPENES**

*Inventors (assignors):*

| *Name* | *Address* |
|---|---|
| Kimberly Ann Aeling | 324 Plaza Estival, San Clemente, CA 92672 (US) |
| Pierre-Yves De Wals | 925 North Vulcan Ave. Apt. #205, Encinitas, CA 92024 (US) |
| Erin Scott | 1579 Caudor Street, Encinitas, CA 92024 (US) |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| Michael Walbridge | 4 Diane Dr, Saugus MA 01906 (US) |
| Thomas Beardslee | 6827 Carnation Drive, Carlsbad, CA 92011 (US) |

*Assignee:*

| *Name* | *Address* |
|---|---|
| RADICI CHIMICA S.P.A. | Via G. Verdi, 11 24121 Bergamo (Italy) |

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And we hereby authorize and request the Commissioner of Patents and Trademarks to issue any United States Letters Patent which may issue for said Invention to said Assignee, as assignee of the whole right, title and interest thereto;

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***Inventors' Signatures:***

| Name | Signature/ Date |
|---|---|
| Kimberly Ann Aeling | |
| Pierre-Yves De Wals | 01/20/2020 |
| Erin Scott | |
| [redacted] | |
| [redacted] | |

| | |
|---|---|
| Michael Walbridge | |
| Thomas Beardslee | |



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Ex. 1002
Page 2591 of 3077

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  *Date of Filing: 11 July 2018*
- ☐ *Other (specify)_*

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| [redacted] | |
| Michael Walbridge | 4 Diane Dr, Saugus MA 01906 (US) |
| Thomas Beardslee | 6827 Carnation Drive, Carlsbad, CA 92011 (US) |

*Assignee:*

| *Name* | *Address* |
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***Inventors' Signatures:***

| Name | Signature/ Date |
|---|---|
| Kimberly Ann Aching | |
| Pierre-Yves De Wals | |
| Erin Scott | 1/20/20 |
| [redacted] | |

| | |
|---|---|
| Michael Walbridge | |
| Thomas Beardslee | |

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- ☐ *United States Patent No(s).*_____
- ☒ *International (PCT) Patent Application No. PCT/US2018/041579*
  *Date of Filing: 11 July 2018*
- ☐ *Other (specify)*_

*Title of the Invention:*

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*Inventors (assignors):*

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| [redacted] | |
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| Thomas Beardslee | 6827 Carnation Drive, Carlsbad, CA 92011 (US) |

*Assignee:*

| *Name* | *Address* |
|---|---|
| RADICI CHIMICA S.P.A. | Via G. Verdi, 11 24121 Bergamo (Italy) |



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|---|---|
| Michael Walbridge |  |
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- ☒ *International (PCT) Patent Application No. PCT/US2018/041576*
  *Date of Filing: 11 July 2018*
- ☐ *Other (specify)*_

*Title of the Invention:*

**BIOLOGICAL METHOD FOR MODIFYING CELLULAR CARBON FLUX**

*Inventors (assignors):*

| *Name* | *Address* |
|---|---|
| Thomas Beardslee | 6827 Carnation Drive, Carlsbad, CA 92011 (US) |

*Assignee:*

| *Name* | *Address* |
|---|---|
| RADICI CHIMICA S.P.A. | Via G. Verdi, 11 24121 Bergamo (Italy) |

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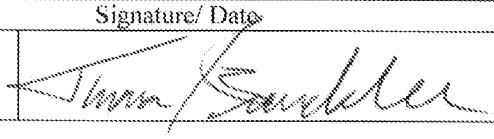
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***Inventors' Signatures:***

| Name | Signature/ Date |
|---|---|
| Thomas Beardslee |  |

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# DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

| Title of Invention | BIOLOGICAL METHODS FOR PREPARING TERPENES |
|---|---|

As the below named inventor, I hereby declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number PCT/US2018/041579

filed on 11 July 2018

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

## WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Kimberly Ann Aeling Date (Optional): 1/17/2020

Signature: K-Ael

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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# DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

| Title of Invention | BIOLOGICAL METHODS FOR PREPARING TERPENES |
|---|---|

As the below named inventor, I hereby declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number PCT/US2018/041579 filed on 11 July 2018.

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

## WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Pierre-Yves De Wals Date (Optional): 01/20/2020

Signature: 

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

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*If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.*

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LEGAL NAME OF INVENTOR

Inventor: Erin Scott Date (Optional): 1/20/20

Signature: Erin Scott

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

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LEGAL NAME OF INVENTOR

Inventor: Michael Waldbridge Date (Optional) :

Signature: 

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| Title of Invention | BIOLOGICAL METHODS FOR MODIFYING CELLULAR CARBON FLUX |
|---|---|

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- ☒ United States application or PCT international application number PCT/US2018/041576 filed on 11 July 2018.

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LEGAL NAME OF INVENTOR

Inventor: Tom Beardslee Date (Optional): 16-JAN-2020

Signature: Tom Beardslee

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# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 43935052 |
| **Application Number:** | 16630567 |
| **International Application Number:** | |
| **Confirmation Number:** | 5356 |
| **Title of Invention:** | BIOLOGICAL METHODS FOR PREPARING TERPENES |
| **First Named Inventor/Applicant Name:** | Kimberly Ann Aeling |
| **Customer Number:** | 122515 |
| **Filer:** | Silvia Salvadori |
| **Filer Authorized By:** | |
| **Attorney Docket Number:** | SS118-1026US |
| **Receipt Date:** | 04-OCT-2021 |
| **Filing Date:** | 13-JAN-2020 |
| **Time Stamp:** | 10:18:19 |
| **Application Type:** | U.S. National Stage under 35 USC 371 |

## Payment information:

| | |
|---|---|
| Submitted with Payment | no |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Application Data Sheet | ADS.pdf | 442169<br>6cabe2370c14be7ce6cfafe05e5a9dfeb02fd340 | no | 10 |
| **Warnings:** | | | | | |

| | | | | | |
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| **Information:** | | | | | |
| This is not an USPTO supplied ADS fillable form | | | | | |
| 2 | Power of Attorney | power.pdf | 652945<br>9a2164a48f2d247b5bd9e148946ea94ef230b8ac | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 3 | Assignee showing of ownership per 37 CFR 3.73 | statement.pdf | 7937842<br>c6f8e1360f6fd6c4b15382bedc42d7fa874b81cb | no | 16 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 4 | Oath or Declaration filed | Declaration.pdf | 4580007<br>fecbbdb210706842e7ffce6f2377f4e746a56aea | no | 10 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| | | | **Total Files Size (in bytes):** | 13612963 | |

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**New Applications Under 35 U.S.C. 111**

**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**

**National Stage of an International Application under 35 U.S.C. 371**

**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**

**New International Application Filed with the USPTO as a Receiving Office**

**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

PTO/AIA/14 (02-18)
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| Application Data Sheet 37 CFR 1.76 | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

## Secrecy Order 37 CFR 5.2:

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

## Inventor Information:

**Inventor 1** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | Kimberly | Ann | Aeling | |

**Residence Information (Select One)** ◉ US Residency ○ Non US Residency ○ Active US Military Service

| City | San Clemente | State/Province | CA | Country of Residence | US |
|---|---|---|---|---|---|

**Mailing Address of Inventor:**

| Address 1 | 324 Plaza Estival, |
|---|---|
| Address 2 | |

| City | San Clemente | State/Province | CA |
|---|---|---|---|
| Postal Code | 92672 | Country | US |

**Inventor 2** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | Pierre-Yves | | De Wals | |

**Residence Information (Select One)** ◉ US Residency ○ Non US Residency ○ Active US Military Service

| City | Encinitas | State/Province | CA | Country of Residence | US |
|---|---|---|---|---|---|

**Mailing Address of Inventor:**

| Address 1 | 925 North Vulcan Ave. Apt. #205 |
|---|---|
| Address 2 | |

| City | Encinitas | State/Province | CA |
|---|---|---|---|
| Postal Code | 92024 | Country | US |

**Inventor 3** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | Eric | | Scott | |

**Residence Information (Select One)** ◉ US Residency ○ Non US Residency ○ Active US Military Service

| Application Data Sheet 37 CFR 1.76 | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

| City | Encinitas | State/Province | CA | Country of Residence | US |
|---|---|---|---|---|---|

**Mailing Address of Inventor:**

| Address 1 | 1579 Caudor Street | | |
|---|---|---|---|
| Address 2 | | | |
| City | Encinitas | State/Province | CA |
| Postal Code | 92024 | Country | US |

**Inventor 4** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | ~~Eric~~ | | ~~Knight~~ | |

**Residence Information (Select One)** (•) US Residency ( ) Non US Residency ( ) Active US Military Service

| City | ~~Carlsbad~~ | State/Province | ~~CA~~ | Country of Residence | ~~US~~ |
|---|---|---|---|---|---|

**Mailing Address of Inventor:**

| Address 1 | ~~2744 Loker Ave West, unit 100~~ | | |
|---|---|---|---|
| Address 2 | | | |
| City | ~~Carlsbad~~ | State/Province | ~~CA~~ |
| Postal Code | ~~92010~~ | Country | ~~US~~ |

**Inventor 5** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | ~~Jian~~ | | ~~Yi~~ | |

**Residence Information (Select One)** (•) US Residency ( ) Non US Residency ( ) Active US Military Service

| City | ~~San Diego~~ | State/Province | ~~CA~~ | Country of Residence | ~~US~~ |
|---|---|---|---|---|---|

**Mailing Address of Inventor:**

| Address 1 | ~~13313 Thunderhead Street~~ | | |
|---|---|---|---|
| Address 2 | | | |
| City | ~~San Diego~~ | State/Province | ~~CA~~ |
| Postal Code | ~~92129~~ | Country | ~~US~~ |

**Inventor 6** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | Michael | | Waldbridge | |

**Residence Information (Select One)** (•) US Residency ( ) Non US Residency ( ) Active US Military Service

| City | Saugus | State/Province | MA | Country of Residence | US |
|---|---|---|---|---|---|

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|---|---|---|
| | Application Number | |
| Title of Invention | | |

| Mailing Address of Inventor: | | | |
|---|---|---|---|
| **Address 1** | 4 Diane Dr | | |
| **Address 2** | | | |
| **City** | Saugus | **State/Province** | MA |
| **Postal Code** | 01906 | **Country** | US |

**Inventor 7** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | Thomas | | Beardslee | |

**Residence Information (Select One)** (•) US Residency ( ) Non US Residency ( ) Active US Military Service

| City | Carlsbad | State/Province | CA | Country of Residence | US |
|---|---|---|---|---|---|

| Mailing Address of Inventor: | | | |
|---|---|---|---|
| **Address 1** | 6827 Carnation Drive | | |
| **Address 2** | | | |
| **City** | Carlsbad | **State/Province** | CA |
| **Postal Code** | 92011 | **Country** | US |

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

## Correspondence Information:

**Enter either Customer Number or complete the Correspondence Information section below.**
**For further information see 37 CFR 1.33(a).**

☐ **An Address is being provided for the correspondence Information of this application.**

| | | | |
|---|---|---|---|
| **Customer Number** | | | |
| **Email Address** | | Add Email | Remove Email |

## Application Information:

| | | | |
|---|---|---|---|
| **Title of the Invention** | | | |
| **Attorney Docket Number** | | **Small Entity Status Claimed** | ☐ |
| **Application Type** | | | |
| **Subject Matter** | | | |
| **Total Number of Drawing Sheets (if any)** | | **Suggested Figure for Publication (if any)** | |

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| **Application Data Sheet 37 CFR 1.76** | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

## Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

| Application number of the previously filed application | Filing date (YYYY-MM-DD) | Intellectual Property Authority or Country |
|---|---|---|
| | | |

## Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

## Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

| Please Select One: | ◉ Customer Number | ○ US Patent Practitioner | ○ Limited Recognition (37 CFR 11.9) |
|---|---|---|---|
| Customer Number | | | |

## Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the "Application Number" field blank.

| Prior Application Status | Pending | | Remove |
|---|---|---|---|
| Application Number | Continuity Type | Prior Application Number | Filing or 371(c) Date (YYYY-MM-DD) |
| | | | |

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|---|---|---|
| | Application Number | |
| Title of Invention | | |

| Prior Application Status | Expired | | Remove |
|---|---|---|---|
| Application Number | Continuity Type | Prior Application Number | Filing or 371(c) Date (YYYY-MM-DD) |
| | | | |
| Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button. | | | Add |

## Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)[i] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

| | | | Remove |
|---|---|---|---|
| Application Number | Country[i] | Filing Date (YYYY-MM-DD) | Access Code[i] (if applicable) |
| | | | |
| Additional Foreign Priority Data may be generated within this form by selecting the **Add** button. | | | Add |

## Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

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|---|---|---|
| | Application Number | |
| Title of Invention | | |

## Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

**NOTE**: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

**1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)**

**A. Priority Document Exchange (PDX)** - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

**B. Search Results from U.S. Application to EPO** - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

**2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)**

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

**NOTE:** Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

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| Application Data Sheet 37 CFR 1.76 | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

## Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

**Applicant 1** Remove

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

| | | |
|---|---|---|
| ◉ Assignee | ○ Legal Representative under 35 U.S.C. 117 | ○ Joint Inventor |
| ○ Person to whom the inventor is obligated to assign. | ○ Person who shows sufficient proprietary interest | |

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

| | | | |
|---|---|---|---|
| Organization Name | | | |
| **Mailing Address Information For Applicant:** | | | |
| **Address 1** | | | |
| Address 2 | | | |
| **City** | | **State/Province** | |
| **Country** | | Postal Code | |
| Phone Number | | Fax Number | |
| Email Address | | | |

Additional Applicant Data may be generated within this form by selecting the Add button. Add

## Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

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|---|---|---|
| | Application Number | |
| Title of Invention | | |

**Assignee 1**

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

Remove

If the Assignee or Non-Applicant Assignee is an Organization check here. ☒

| Organization Name | |
|---|---|

**Mailing Address Information For Assignee including Non-Applicant Assignee:**

| **Address 1** | | | |
|---|---|---|---|
| Address 2 | | | |
| **City** | | **State/Province** | |
| **Country** i | | Postal Code | |
| Phone Number | | Fax Number | |
| Email Address | | | |

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button. Add

## Signature:

Remove

**NOTE:** This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

| **Signature** | /Silvia Salvadori/ | | | Date (YYYY-MM-DD) | 2021-10-04 |
|---|---|---|---|---|---|
| First Name | Silvia | Last Name | Salvadori | Registration Number | 48265 |

Additional Signature may be generated within this form by selecting the Add button. Add

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|---|---|---|
| | Application Number | |
| Title of Invention | | |

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

# Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1 The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3 A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent CooperationTreaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.



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Ex. 1002
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# POWER OF ATTORNEY TO PROSECUTE APPLICATIONS BEFORE THE USPTO

I hereby revoke all previous powers of attorney given in the application identified in the attached statement under 37 CFR 3.73(c).

I hereby appoint:

[X] Practitioners associated with Customer Number: 122515

**OR**

[ ] Practitioner(s) named below (if more than ten patent practitioners are to be named, then a customer number must be used):

| Name | Registration Number | Name | Registration Number |
|---|---|---|---|
| | | | |
| | | | |
| | | | |
| | | | |
| | | | |

As attorney(s) or agent(s) to represent the undersigned before the United States Patent and Trademark Office (USPTO) in connection with any and all patent applications assigned only to the undersigned according to the USPTO assignment records or assignment documents attached to this form in accordance with 37 CFR 3.73(c).

Please change the correspondence address for the application identified in the attached statement under 37 CFR 3.73(c) to:

[X] The address associated with Customer Number: 122515

**OR**

[ ]

| | | |
|---|---|---|
| Firm or individual name | | |
| Address | | |
| City | State | Zip |
| Country | | |
| Telephone | Email | |

Assignee name and address: Radici Chimica S.p.A.
Via G. Verdi, 11 Bergamo 24121
Italy

**A copy of this form, together with a statement under 37 CFR 3.73(c) (Form PTO/AIA/96 or equivalent) is required to be filed in each application in which this form is used. The statement under 37 CFR 3.73(c) may be completed by one of the practitioners appointed in this form, and must identify the application in which this Power of Attorney is to be filed.**

**SIGNATURE of Assignee of Record**

The individual whose signature and title is supplied below is authorized to act on behalf of the assignee.

| | |
|---|---|
| Signature RADICI CHIMICA S.P.A. | Date 7 May 2020 |
| Name Angelo RADICI | Telephone |
| Title - CEO - | |

This collection of information is required by 37 CFR 1.31, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public, which is to update (and by the USPTO to process) the file of a patent or reexamination proceeding. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 18 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**
*If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.*

## Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant *(i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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# STATEMENT UNDER 37 CFR 3.73(c)

Applicant/Patent Owner: Radici Chimica S.p.A.

Application No./Patent No.: 16/630,567 Filed/Issue Date: 2020-01-13

Titled: BIOLOGICAL METHODS FOR PREPARING TERPENES

Radici Chimica S.p.A., a Corporation

(Name of Assignee) (Type of Assignee, e.g., corporation, partnership, university, government agency, etc.)

states that, for the patent application/patent identified above, it is (choose **one** of options 1, 2, 3 or 4 below):

1. [x] The assignee of the entire right, title, and interest.

2. [ ] An assignee of less than the entire right, title, and interest (check applicable box):

   [ ] The extent (by percentage) of its ownership interest is _____%. Additional Statement(s) by the owners holding the balance of the interest must be submitted to account for 100% of the ownership interest.

   [ ] There are unspecified percentages of ownership. The other parties, including inventors, who together own the entire right, title and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

3. [ ] The assignee of an undivided interest in the entirety (a complete assignment from one of the joint inventors was made). The other parties, including inventors, who together own the entire right, title, and interest are:

   Additional Statement(s) by the owner(s) holding the balance of the interest must be submitted to account for the entire right, title, and interest.

4. [ ] The recipient, via a court proceeding or the like (*e.g.*, bankruptcy, probate), of an undivided interest in the entirety (a complete transfer of ownership interest was made). The certified document(s) showing the transfer is attached.

The interest identified in option 1, 2 or 3 above (not option 4) is evidenced by either (choose **one** of options A or B below):

A. [✓] An assignment from the inventor(s) of the patent application/patent identified above. The assignment was recorded in the United States Patent and Trademark Office at Reel _____, Frame _____, or for which a copy thereof is attached.

B. [ ] A chain of title from the inventor(s), of the patent application/patent identified above, to the current assignee as follows:

1. From: _____ To: _____

   The document was recorded in the United States Patent and Trademark Office at

   Reel _____, Frame _____, or for which a copy thereof is attached.

2. From: _____ To: _____

   The document was recorded in the United States Patent and Trademark Office at

   Reel _____, Frame _____, or for which a copy thereof is attached.



This collection of information is required by 37 CFR 3.73(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450**.

*If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.*

PTO/AIA/96 (08-12)
Approved for use through 01/31/2013. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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## STATEMENT UNDER 37 CFR 3.73(c)

3. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

4. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

5. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

6. From: _____ To: _____

The document was recorded in the United States Patent and Trademark Office at

Reel _____, Frame _____, or for which a copy thereof is attached.

☐ Additional documents in the chain of title are listed on a supplemental sheet(s).

☑ As required by 37 CFR 3.73(c)(1)(i), the documentary evidence of the chain of title from the original owner to the assignee was, or concurrently is being, submitted for recordation pursuant to 37 CFR 3.11.

[NOTE: A separate copy (i.e., a true copy of the original assignment document(s)) must be submitted to Assignment Division in accordance with 37 CFR Part 3, to record the assignment in the records of the USPTO. See MPEP 302.08]

The undersigned (whose title is supplied below) is authorized to act on behalf of the assignee.

| /Silvia Salvadori/ | October 5, 2021 |
|---|---|
| Signature | Date |
| Silvia Salvadori | 48265 |
| Printed or Typed Name | Title or Registration Number |

# Privacy Act Statement

The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

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# ASSIGNMENT OF RIGHTS, TITLE AND INTEREST IN INVENTION

*This is an assignment of the following rights, title and interest (check all that apply):*

- ☒ *United States of America rights, title and interest in the invention*
- ☐ *Foreign rights, title and interest in the invention*
- ☐ *United States Patent Application Serial No.*
  *Date of Execution: _____ Date of Filing:*
- ☐ *United States Provisional Patent Application Serial No. _____*
- ☐ *United States Patent No(s). _____*
- ☒ *International (PCT) Patent Application No. PCT/US2018/041579*
  *Date of Filing: 11 July 2018*
- ☐ *Other (specify)_*

*Title of the Invention:*

**BIOLOGICAL METHOD FOR PREPARING TERPENES**

*Inventors (assignors):*

| *Name* | *Address* |
|---|---|
| Kimberly Ann Aeling | 324 Plaza Estival, San Clemente, CA 92672 (US) |
| Pierre-Yves De Wals | 925 North Vulcan Ave. Apt. #205, Encinitas, CA 92024 (US) |
| Erin Scott | 1579 Caudor Street, Encinitas, CA 92024 (US) |
| [redacted] | |
| Michael Walbridge | 4 Diane Dr, Saugus MA 01906 (US) |
| Thomas Beardslee | 6827 Carnation Drive, Carlsbad, CA 92011 (US) |

*Assignee:*

| *Name* | *Address* |
|---|---|
| RADICI CHIMICA S.P.A. | Via G. Verdi, 11 24121 Bergamo (Italy) |



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Ex. 1002
Page 2626 of 3077

Whereas, we, the above-identified Inventor(s), have invented certain new and useful improvements in the Invention identified above and described in the above-identified patent application(s) and /or patent(s) (hereinafter referred to as "Invention");

And, whereas we desire to assign our above-identified rights, title and interest in the Invention to the above-identified Assignee;

Now, this indenture witnesseth, that for good and valuable consideration, the receipt whereof is hereby acknowledged;

We hereby assign, sell and transfer our above-identified rights, title and interest in said Invention, said application(s) as identified above, including any divisions, continuations, and continuations-in-part thereof, and in and to any and all Letters Patent of the United States, and countries foreign thereto, which may be granted for said Invention, and in and to any and all reissues and reexaminations thereof, which may be granted or have granted for said Invention, and in and to any and all priority rights, Convention rights, and other benefits accruing or to accrue to us with respect to the filing of applications for patents or securing of patents in the United States and countries foreign thereto, unto said Assignee;

And we hereby authorize and request the Commissioner of Patents and Trademarks to issue any United States Letters Patent which may issue for said Invention to said Assignee, as assignee of the whole right, title and interest thereto;

And we further agree to sign and execute all necessary and lawful future documents, including applications for foreign patents, for filing divisions, continuations and continuation-in-part of said application for patent and/or for obtaining any reissue or reissues of any Letters Patent which may be granted for my aforesaid Invention, as the Assignee or its Designee(s) may from time to time require and prepare at its own expense.

The undersigned hereby grant(s) the firm SILVIA SALVADORI, P.C., 270 Madison Avenue, 8th Floor, New York, NY 10016 the power to insert on this assignment any further identification which may be necessary or desirable in order to comply with the rules of the United States Patent Office for recordation of this document.

***Inventors' Signatures:***

| Name | Signature/ Date |
|---|---|
| Kimberly Ann Aeling | 11/7/2020 |
| Pierre-Yves De Wals | |
| Eron Scott | |
| [redacted] | |

| | |
|---|---|
| Michael Walbridge | |
| Thomas Beardslee | |



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Ex. 1002
Page 2628 of 3077

# ASSIGNMENT OF RIGHTS, TITLE AND INTEREST IN INVENTION

*This is an assignment of the following rights, title and interest (check all that apply):*

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- ☐ *United States Patent No(s).*_____
- ☒ *International (PCT) Patent Application No. PCT/US2018/041579*
  *Date of Filing: 11 July 2018*
- ☐ *Other (specify)*_

*Title of the Invention:*

**BIOLOGICAL METHOD FOR PREPARING TERPENES**

*Inventors (assignors):*

| *Name* | *Address* |
|---|---|
| Kimberly Ann Aeling | 324 Plaza Estival, San Clemente, CA 92672 (US) |
| Pierre-Yves De Wals | 925 North Vulcan Ave. Apt. #205, Encinitas, CA 92024 (US) |
| Erin Scott | 1579 Caudor Street, Encinitas, CA 92024 (US) |
| [redacted] | [redacted] |
| [redacted] | [redacted] |
| Michael Walbridge | 4 Diane Dr, Saugus MA 01906 (US) |
| Thomas Beardslee | 6827 Carnation Drive, Carlsbad, CA 92011 (US) |

*Assignee:*

| *Name* | *Address* |
|---|---|
| RADICI CHIMICA S.P.A. | Via G. Verdi, 11 24121 Bergamo (Italy) |



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Ex. 1002
Page 2629 of 3077

Whereas, we, the above-identified Inventor(s), have invented certain new and useful improvements in the Invention identified above and described in the above-identified patent application(s) and /or patent(s) (hereinafter referred to as "Invention");

And, whereas we desire to assign our above-identified rights, title and interest in the Invention to the above-identified Assignee;

Now, this indenture witnesseth, that for good and valuable consideration, the receipt whereof is hereby acknowledged;

We hereby assign, sell and transfer our above-identified rights, title and interest in said Invention, said application(s) as identified above, including any divisions, continuations, and continuations-in-part thereof, and in and to any and all Letters Patent of the United States, and countries foreign thereto, which may be granted for said Invention, and in and to any and all reissues and reexaminations thereof, which may be granted or have granted for said Invention, and in and to any and all priority rights, Convention rights, and other benefits accruing or to accrue to us with respect to the filing of applications for patents or securing of patents in the United States and countries foreign thereto, unto said Assignee;

And we hereby authorize and request the Commissioner of Patents and Trademarks to issue any United States Letters Patent which may issue for said Invention to said Assignee, as assignee of the whole right, title and interest thereto;

And we further agree to sign and execute all necessary and lawful future documents, including applications for foreign patents, for filing divisions, continuations and continuation-in-part of said application for patent and/or for obtaining any reissue or reissues of any Letters Patent which may be granted for my aforesaid Invention, as the Assignee or its Designee(s) may from time to time require and prepare at its own expense.

The undersigned hereby grant(s) the firm SILVIA SALVADORI, P.C., 270 Madison Avenue, 8th Floor, New York, NY 10016 the power to insert on this assignment any further identification which may be necessary or desirable in order to comply with the rules of the United States Patent Office for recordation of this document.

***Inventors' Signatures:***

| Name | Signature/ Date |
|---|---|
| Kimberly Ann Aeling | |
| Pierre-Yves De Wals | 01/20/2020 |
| Erin Scott | |
| [redacted] | |
| [redacted] | |

| | |
|---|---|
| Michael Walbridge | |
| Thomas Beardslee | |



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Page 2631 of 3077

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- ☐ *Other (specify)_*

*Title of the Invention:*

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| [redacted] | |
| Michael Walbridge | 4 Diane Dr, Saugus MA 01906 (US) |
| Thomas Beardslee | 6827 Carnation Drive, Carlsbad, CA 92011 (US) |

*Assignee:*

| *Name* | *Address* |
|---|---|
| RADICI CHIMICA S.P.A. | Via G. Verdi, 11 24121 Bergamo (Italy) |

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And we hereby authorize and request the Commissioner of Patents and Trademarks to issue any United States Letters Patent which may issue for said invention to said Assignee, as assignee of the whole right, title and interest thereto;

And we further agree to sign and execute all necessary and lawful future documents, including applications for foreign patents, for filing divisions, continuations and continuation-in-part of said application for patent and/or for obtaining any reissue or reissues of any Letters Patent which may be granted for my aforesaid Invention, as the Assignee or its Designee(s) may from time to time require and prepare at its own expense.

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***Inventors' Signatures:***

| Name | Signature/ Date |
|---|---|
| Kimberly Ann Asling | |
| Pierre-Yves De Wals | |
| Erin Scott | 1/20/20 |
| [redacted] | |

| | |
|---|---|
| Michael Walbridge | |
| Thomas Beardslee | |



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Ex. 1002
Page 2634 of 3077

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  *Date of Filing: 11 July 2018*
- ☐ *Other (specify)*_

*Title of the Invention:*

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|---|---|
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| [redacted] | |
| Michael Walbridge | 4 Diane Dr, Saugus MA 01906 (US) |
| Thomas Beardslee | 6827 Carnation Drive, Carlsbad, CA 92011 (US) |

*Assignee:*

| *Name* | *Address* |
|---|---|
| RADICI CHIMICA S.P.A. | Via G. Verdi, 11 24121 Bergamo (Italy) |

| | |
|---|---|
| Michael Walbridge | 01/20/2020 |
| Thomas Beardslee | |

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LCY Biotechnology Holding, Inc.
Ex. 1002
Page 2637 of 3077

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| Erin Scott | |
| [redacted] | |

| | |
|---|---|
| Michael Walbridge | |
| Thomas Beardslee | 1/20/2020 |

# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 43945712 |
| **Application Number:** | 16630567 |
| **International Application Number:** | |
| **Confirmation Number:** | 5356 |
| **Title of Invention:** | BIOLOGICAL METHODS FOR PREPARING TERPENES |
| **First Named Inventor/Applicant Name:** | Kimberly Ann Aeling |
| **Customer Number:** | 122515 |
| **Filer:** | Silvia Salvadori |
| **Filer Authorized By:** | |
| **Attorney Docket Number:** | SS118-1026US |
| **Receipt Date:** | 05-OCT-2021 |
| **Filing Date:** | 13-JAN-2020 |
| **Time Stamp:** | 03:15:08 |
| **Application Type:** | U.S. National Stage under 35 USC 371 |

## Payment information:

| | |
|---|---|
| Submitted with Payment | no |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Power of Attorney | power.pdf | 652945<br>9a2164a48f2d247b5bd9e148946ea94ef230b8ac | no | 2 |
| **Warnings:** | | | | | |

| Information: | | | | | |
|---|---|---|---|---|---|
| 2 | Assignee showing of ownership per 37 CFR 3.73 | statement.pdf | 9397518<br>8f26c641e5f37534fcb103f57e322508adc8a728 | no | 17 |
| Warnings: | | | | | |
| Information: | | | | | |
| | | | Total Files Size (in bytes): | 10050463 | |

**This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.**

**New Applications Under 35 U.S.C. 111**

**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**

**National Stage of an International Application under 35 U.S.C. 371**

**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**

**New International Application Filed with the USPTO as a Receiving Office**

**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

# Electronic Patent Application Fee Transmittal

| | |
|---|---|
| **Application Number:** | 16630567 |
| **Filing Date:** | 13-Jan-2020 |
| **Title of Invention:** | BIOLOGICAL METHODS FOR PREPARING TERPENES |
| **First Named Inventor/Applicant Name:** | Kimberly Ann Aeling |
| **Filer:** | Silvia Salvadori |
| **Attorney Docket Number:** | SS118-1026US |

Filed as Large Entity

**Filing Fees for U.S. National Stage under 35 USC 371**

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Basic Filing:** | | | | |
| **Pages:** | | | | |
| **Claims:** | | | | |
| **Miscellaneous-Filing:** | | | | |
| PROCESSING FEE, EXCEPT PROV. APPLS. | 1830 | 1 | 140 | 140 |
| **Petition:** | | | | |
| **Patent-Appeals-and-Interference:** | | | | |
| **Post-Allowance-and-Post-Issuance:** | | | | |

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Extension-of-Time:** | | | | |
| **Miscellaneous:** | | | | |
| | | **Total in USD ($)** | | **140** |

| Electronic Acknowledgement Receipt | |
|---|---|
| **EFS ID:** | 43969391 |
| **Application Number:** | 16630567 |
| **International Application Number:** | |
| **Confirmation Number:** | 5356 |
| **Title of Invention:** | BIOLOGICAL METHODS FOR PREPARING TERPENES |
| **First Named Inventor/Applicant Name:** | Kimberly Ann Aeling |
| **Customer Number:** | 122515 |
| **Filer:** | Silvia Salvadori |
| **Filer Authorized By:** | |
| **Attorney Docket Number:** | SS118-1026US |
| **Receipt Date:** | 07-OCT-2021 |
| **Filing Date:** | 13-JAN-2020 |
| **Time Stamp:** | 08:41:58 |
| **Application Type:** | U.S. National Stage under 35 USC 371 |

## Payment information:

| | |
|---|---|
| Submitted with Payment | yes |
| Payment Type | CARD |
| Payment was successfully received in RAM | $140 |
| RAM confirmation Number | E202107943311367 |
| Deposit Account | 506423 |
| Authorized User | Silvia Salvadori |
| The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:<br>37 CFR 1.21 (Miscellaneous fees and charges) | |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Fee Worksheet (SB06) | fee-info.pdf | 38093<br>7e54ccfe246227f4f1885d9199104db01f9a36eb | no | 2 |

**Warnings:**

**Information:**

| Total Files Size (in bytes): | 38093 |
|---|---|

**This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.**

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**New International Application Filed with the USPTO as a Receiving Office**

**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NUMBER | FILING OR 371(C) DATE | FIRST NAMED APPLICANT | ATTY. DOCKET NO./TITLE |
|---|---|---|---|
| 16/630,567 | 01/13/2020 | Kimberly Ann Aeling | SS118-1026US |

**CONFIRMATION NO. 5356**

122515
Silvia Salvadori, P.C.
Silvia Salvadori
485 Central Park West Unit 5C
New York, NY 10025

**POA ACCEPTANCE LETTER**

*OC000000128913731*

Date Mailed: 10/07/2021

## NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 10/04/2021.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/qtran/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NUMBER | FILING OR 371(C) DATE | FIRST NAMED APPLICANT | ATTY. DOCKET NO./TITLE |
|---|---|---|---|
| 16/630,567 | 01/13/2020 | Kimberly Ann Aeling | SS118-1026US |

**CONFIRMATION NO. 5356**

122515
Silvia Salvadori, P.C.
Silvia Salvadori
485 Central Park West Unit 5C
New York, NY 10025

**37 CFR 1.48 ACKNOWLEDGEMENT LETTER**

*OC000000128913687*

Date Mailed: 10/07/2021

## IMPROPER SUBMISSION OF REQUEST UNDER 37 CFR 1.48(a)

The request under 37 CFR 1.48(a) (request to change inventorship) submitted on 10/04/2021 in the above-identified application is not accepted because:

- The request to correct inventorship under 37 CFR 1.48(a) is deficient because the fee set forth in 37 CFR 1.17(i) has not been submitted.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/qtran/

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 16/630,567 | 01/13/2020 | Kimberly Ann Aeling | SS118-1026US | 5356 |

122515 7590 10/27/2021

Silvia Salvadori, P.C.
Silvia Salvadori
485 Central Park West Unit 5C
New York, NY 10025

| EXAMINER | |
|---|---|
| NOAKES, SUZANNE MARIE | |

| ART UNIT | PAPER NUMBER |
|---|---|
| 1656 | |

| NOTIFICATION DATE | DELIVERY MODE |
|---|---|
| 10/27/2021 | ELECTRONIC |

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

eofficeaction@appcoll.com
silvia30121@me.com
silvia@salvadorilaw.com

| *Office Action Summary* | **Application No.** 16/630,567 | **Applicant(s)** Aeling et al. | |
|---|---|---|---|
| | **Examiner** SUZANNE M NOAKES | **Art Unit** 1656 | **AIA (FITF) Status** Yes |

*-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --*

**Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 2 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

1)☑ Responsive to communication(s) filed on 01/13/2020.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims***

5) ☑ Claim(s) 163-183 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☐ Claim(s) _____ is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☑ Claim(s) 163-183 are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

**Application Papers**

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

**Priority under 35 U.S.C. § 119**

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

**Certified copies:**

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

1) ☑ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.



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Ex. 1002
Page 2649 of 3077

## DETAILED ACTION

### *Election/Restriction*

1. REQUIREMENT FOR UNITY OF INVENTION

As provided in 37 CFR 1.475(a), a national stage application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept ("requirement of unity of invention"). Where a group of inventions is claimed in a national stage application, the requirement of unity of invention shall be fulfilled only when there is a technical relationship among those inventions involving one or more of the same or corresponding special technical features. The expression "special technical features" shall mean those technical features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art.

The determination whether a group of inventions is so linked as to form a single general inventive concept shall be made without regard to whether the inventions are claimed in separate claims or as alternatives within a single claim. See 37 CFR 1.475(e).

When Claims Are Directed to Multiple Categories of Inventions:

As provided in 37 CFR 1.475 (b), a national stage application containing claims to different categories of invention will be considered to have unity of invention if the claims are drawn only to one of the following combinations of categories:

(1) A product and a process specially adapted for the manufacture of said product; or

(2) A product and a process of use of said product; or

(3) A product, a process specially adapted for the manufacture of the said product, and a use of the said product; or

(4) A process and an apparatus or means specifically designed for carrying out the said process; or

(5) A product, a process specially adapted for the manufacture of the said product, and an apparatus or means specifically designed for carrying out the said process.

Otherwise, unity of invention might not be present. See 37 CFR 1.475 (c).

2. Restriction is required under 35 U.S.C. 121 and 372.

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

In accordance with 37 CFR 1.499, applicant is required, in reply to this action, to elect a single invention to which the claims must be restricted.

Group I, claim(s) 163-165, drawn to a genetically modified microorganism, comprising: one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides, wherein expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide: wherein the one or more heterologous nucleic acids that encode one or more terpene biosynthesis polypeptides is chosen from : terpene synthase, phytoene synthase, geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin

synthase, zeaxanthin glucosyltransferase, valencene synthase, cytochrome P450 reductase.

Group II, claim(s) 166-169, drawn to a genetically modified microorganism, comprising one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides, and a genetic modification that alters the expression of a polypeptide providing for transport of acetyl-carnitine in the microorganism, wherein the expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for glucose, fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide.

Group III, claim(s) 170-172, drawn to a genetically modified *Candida viswanathii* yeast, comprising one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides, wherein expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for glucose, fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide and wherein the one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides is selected from terpene synthase, phytoene synthase, geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, zeaxanthin glucosyltransferase and valencene synthase, or wherein the one or more heterologous nucleic acids is a cytochrome P450 reductase and is regulated by a nucleic acid that provides for glucose or alkane induction of expression of the cytochrome P450 reductase.

Group IV, claim(s) 173, 176-180, drawn to method for producing a terpene comprising: contacting a genetically modified microorganism, comprising: one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides, wherein expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide: wherein the one or more heterologous nucleic acids that encode one or more terpene biosynthesis polypeptides is chosen from : terpene synthase, phytoene synthase, geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, zeaxanthin glucosyltransferase, valencene synthase, cytochrome P450 reductase with a feedstock comprising a carbon source, and culturing the microorganism under conditions in which the terpenes are produced from the feedstock.

Group V, claim(s) 174, drawn to a method for producing a terpene comprising: contacting a genetically modified microorganism, comprising one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides, and a genetic modification that alters the expression of a polypeptide providing for transport of acetyl-carnitine in the microorganism, wherein the expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for glucose, fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide with a feedstock comprising a carbon source, and culturing the microorganism under conditions in which the terpenes are produced from the feedstock.

Group VI, claim(s) 175, drawn to a method for producing a terpene comprising: contacting a genetically modified *Candida viswanathii* yeast, comprising one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides, wherein expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for glucose, fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide and wherein the one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides is selected from terpene synthase, phytoene synthase, geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, zeaxanthin glucosyltransferase and valencene synthase, or wherein the one or more heterologous nucleic acids is a cytochrome P450 reductase and is regulated by a nucleic acid that provides for glucose or alkane induction of expression of the cytochrome P450 reductase with a feedstock comprising a carbon source, and culturing the microorganism under conditions in which the terpenes are produced from the feedstock.

Group VII, claim(s) 181-182, drawn to a genetically modified yeast comprising one or more heterologous nucleic acids encoding phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase, wherein the amount and/or activity of a Ras2 protein has been decreased.

Group VIII, claim(s) 183, drawn to a genetically modified yeast comprising one or more heterologous nucleic acids encoding geranylgeranyl diphosphate synthase, phytoene desaturase, bifunctional lycopene cyclase/phytoene synthase, β-carotene

ketolase, β-carotene hydroxylase, astaxanthin synthase, and cytochrome p450 reductase, wherein the amount and/or activity of a Ras2 protein has been decreased.

***Election of Species***

3. This application contains claims directed to more than one species of the generic invention. These species are deemed to lack unity of invention because they are not so linked as to form a single general inventive concept under PCT Rule 13.1.

The species are as follows:

A heterologous polypeptide selected from: terpene synthase, phytoene synthase, geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, zeaxanthin glucosyltransferase, valencene synthase, cytochrome P450 reductase; or a single combination thereof.

Applicant is required, in reply to this action, to elect a single species to which the claims shall be restricted if no generic claim is finally held to be allowable. The reply must also identify the claims readable on the elected species, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered non-responsive unless accompanied by an election.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which are written in dependent form or otherwise require all the limitations of an allowed generic claim. Currently, no claim(s) are generic.

4. The groups of inventions listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

I-VIII lack unity of invention because even though the inventions of these groups require the technical feature of a recombinant yeast comprising a heterologous nucleic acid encoding one or more phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase, cytochrome P450 reductase, this technical feature is not a special technical feature as it does not make a contribution over the prior art in view of Zhang et al. (US 2004/0265980 – cited herein).

Zhang et al. teach a recombinant yeast which has been transformed to encode a polypeptide of interest, e.g. a cytochrome P450 reductase (referred to as NCP or CRP – See paragraph 0004) which is under the control of the promoter POX4, which is a promoter induced by alkane – See Claim 10. This necessarily minimally anticipates instant claim 163.

***Inventorship***

5. Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be corrected in compliance with 37 CFR 1.48(a) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. A request to correct inventorship under 37 CFR 1.48(a) must be accompanied by an application data sheet in accordance with 37 CFR 1.76 that identifies each inventor by his or her legal name and by the processing fee required under 37 CFR 1.17(i).

***Rejoinder***

6. The examiner has required restriction between product or apparatus claims and process claims. Where applicant elects claims directed to the product/apparatus, and all product/apparatus claims are subsequently found allowable, withdrawn process claims that include all the limitations of the allowable product/apparatus claims should be considered for rejoinder. All claims directed to a nonelected process invention must include all the limitations of an allowable product/apparatus claim for that process invention to be rejoined.

In the event of rejoinder, the requirement for restriction between the product/apparatus claims and the rejoined process claims will be withdrawn, and the rejoined process claims will be fully examined for patentability in accordance with 37 CFR 1.104. Thus, to be allowable, the rejoined claims must meet all criteria for patentability including the requirements of 35 U.S.C. 101, 102, 103 and 112. Until all claims to the elected product/apparatus are found allowable, an otherwise proper restriction requirement between product/apparatus claims and process claims may be maintained. Withdrawn process claims that are not commensurate in scope with an allowable product/apparatus claim will not be rejoined. See MPEP § 821.04. Additionally, in order for rejoinder to occur, applicant is advised that the process claims should be amended during prosecution to require the limitations of the product/apparatus claims. **Failure to do so may result in no rejoinder.** Further, note that the prohibition against double patenting rejections of 35 U.S.C. 121 does not apply where the restriction requirement is withdrawn by the examiner before the patent issues. See MPEP § 804.01.

***Correspondence***

7. Any inquiry concerning this communication or earlier communications from the examiner should be directed to SUZANNE M NOAKES whose telephone number is (571)272-2924. The examiner can normally be reached M-F (7-4).

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Manjunath Rao can be reached on 571-272-0939. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of published or unpublished applications may be obtained from Patent Center. Unpublished application information in Patent Center is available to registered users. To file and manage patent submissions in Patent Center, visit: https://patentcenter.uspto.gov. Visit https://www.uspto.gov/patents/apply/patent-center for more information about Patent Center and https://www.uspto.gov/patents/docx for information about filing in DOCX format. For additional questions, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/SUZANNE M NOAKES/
Primary Examiner, Art Unit 1656
22 October 2021

| Notice of References Cited | Application/Control No. 16/630,567 | Applicant(s)/Patent Under Reexamination Aeling et al. | |
|---|---|---|---|
| | Examiner SUZANNE M NOAKES | Art Unit 1656 | Page 1 of 1 |

**U.S. PATENT DOCUMENTS**

| * | | Document Number Country Code-Number-Kind Code | Date MM-YYYY | Name | CPC Classification | US Classification |
|---|---|---|---|---|---|---|
| * | A | US-20040265980-A1 | 12-2004 | Zhang, Yeyan | C12P7/44 | 435/134 |
| | B | | | | | |
| | C | | | | | |
| | D | | | | | |
| | E | | | | | |
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**FOREIGN PATENT DOCUMENTS**

| * | | Document Number Country Code-Number-Kind Code | Date MM-YYYY | Country | Name | CPC Classification |
|---|---|---|---|---|---|---|
| | N | | | | | |
| | O | | | | | |
| | P | | | | | |
| | Q | | | | | |
| | R | | | | | |
| | S | | | | | |
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**NON-PATENT DOCUMENTS**

| * | | Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages) |
|---|---|---|
| | U | |
| | V | |
| | W | |
| | X | |

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.



LCY Biotechnology Holding, Inc.
Ex. 1002
Page 2659 of 3077

# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 44265710 |
| **Application Number:** | 16630567 |
| **International Application Number:** | |
| **Confirmation Number:** | 5356 |
| **Title of Invention:** | BIOLOGICAL METHODS FOR PREPARING TERPENES |
| **First Named Inventor/Applicant Name:** | Kimberly Ann Aeling |
| **Customer Number:** | 122515 |
| **Filer:** | Silvia Salvadori |
| **Filer Authorized By:** | |
| **Attorney Docket Number:** | SS118-1026US |
| **Receipt Date:** | 12-NOV-2021 |
| **Filing Date:** | 13-JAN-2020 |
| **Time Stamp:** | 09:49:46 |
| **Application Type:** | U.S. National Stage under 35 USC 371 |

## Payment information:

| | |
|---|---|
| Submitted with Payment | no |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Response to Election / Restriction Filed | Response.pdf | 73510<br>bd2b74e09a10c7e4aaf70114b2507332f65e1285 | no | 2 |

**Warnings:**

| Information: | |
|---|---|
| Total Files Size (in bytes): | 73510 |

**This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.**

**New Applications Under 35 U.S.C. 111**

**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**

**National Stage of an International Application under 35 U.S.C. 371**

**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**

**New International Application Filed with the USPTO as a Receiving Office**

**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

Atty Docket No.: SS118-1026US

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

| | | | |
|---|---|---|---|
| In re Application | : | Aeling et al. | Confirmation No. 5356 |
| Serial No. | : | 16/630,567 | |
| Filing Date | : | January 13, 2020 | |
| Group Art Unit | : | 1656 | |
| Examiner | : | Noakes, S.M. | |
| For | : | **BIOLOGICAL METHODS FOR PREPARING TERPENES** | |

Mail Stop Amendments
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

**RESPONSE TO REQUIREMENT FOR RESTRICTION/ELECTION**

Sir:

This Response to Requirement for Restriction/Election is filed in response to the Office Communication of October 27, 2021 issued in connection with the above-identified matter. Thus, this Response is timely filed.

**Remarks** begin on page 2 of this paper.



LCY Biotechnology Holding, Inc.
Ex. 1002
Page 2662 of 3077

**REMARKS**

Applicants' undersigned attorney thanks the Examiner for the clarification obtained during the telephonic communication held on November 12, 2021.

In Response to the Requirement for Restriction/Election Applicants elect to prosecute Group I, claims 163-165, drawn to a genetically modified microorganism.

Moreover, Applicants elect to prosecute the following combination of species:

phytoene synthase, geranyl geranyl diphosphate synthase and phytoene desaturase.

This Response is filed within the shortened statutory period for reply. Thus, no fee is believed to be due. However, the Director is hereby authorized to charge any fee deemed necessary or credit any overpayments to Deposit Account No. 506423.

Respectfully submitted,

November 12, 2021 By: /Silvia Salvadori/

Reg. No. 48,265
SILVIA SALVADORI, P.C.
485 Central Park West Unit 5C
New York, NY 10025
Phone: (212) 897-1938
Fax: (646) 219-6519



LCY Biotechnology Holding, Inc.
Ex. 1002
Page 2663 of 3077

Atty Docket No.: SS118-1026US

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

| | | | |
|---|---|---|---|
| In re Application | : | Aeling et al. | Confirmation No. 5356 |
| Serial No. | : | 16/630,567 | |
| Filing Date | : | January 13, 2020 | |
| Group Art Unit | : | 1656 | |
| Examiner | : | Noakes, S.M. | |
| For | : | **BIOLOGICAL METHODS FOR PREPARING TERPENES** | |

Mail Stop Amendments
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

**COVER LETTER**

Sir:

The above-identified matter was filed as a 371 application on January 13, 2020 without any inventors' oath or declaration. On October 4, 2021, Applicants filed a corrected ADS to correct the inventorship under 37 C.F.R. § 1.48(a). On October 7, 2021, the request was denied for failure to submit the fee set forth in 37 C.F.R.1.17(i). The fee was paid on October 7, 2021 as shown in PAIR (see Fee Worksheet SB06) and as shown in the TAB Fee (Fee Code 1830, fee amount $140 Mailroom Date October 7, 2021).

However, no corrected filing receipt reflecting the inventorship was ever mailed.

Thus, Applicants submit herewith another copy of the updated ADS filed on October 4, 2021 reflecting the correct inventorship.

The issuance of an updated filing receipt is respectfully requested.



LCY Biotechnology Holding, Inc.
Ex. 1002
Page 2664 of 3077

No fees are believed to be due. However, the Director is hereby authorized to charge any fee deemed necessary or credit any overpayments to Deposit Account No. 506423.

Respectfully submitted,

November 12, 2021 By: /Silvia Salvadori/

Reg. No. 48,265
SILVIA SALVADORI, P.C.
485 Central Park West Unit 5C
New York, NY 10025
Phone: (212) 897-1938
Fax: (646) 219-6519

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| Application Data Sheet 37 CFR 1.76 | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

The application data sheet is part of the provisional or nonprovisional application for which it is being submitted. The following form contains the bibliographic data arranged in a format specified by the United States Patent and Trademark Office as outlined in 37 CFR 1.76.
This document may be completed electronically and submitted to the Office in electronic format using the Electronic Filing System (EFS) or the document may be printed and included in a paper filed application.

## Secrecy Order 37 CFR 5.2:

☐ Portions or all of the application associated with this Application Data Sheet may fall under a Secrecy Order pursuant to 37 CFR 5.2 (Paper filers only. Applications that fall under Secrecy Order may not be filed electronically.)

## Inventor Information:

**Inventor 1** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | Kimberly | Ann | Aeling | |

**Residence Information (Select One)** ◉ US Residency ○ Non US Residency ○ Active US Military Service

| City | San Clemente | State/Province | CA | Country of Residence | US |
|---|---|---|---|---|---|

**Mailing Address of Inventor:**

| Address 1 | 324 Plaza Estival, |
|---|---|
| Address 2 | |

| City | San Clemente | State/Province | CA |
|---|---|---|---|
| Postal Code | 92672 | Country | US |

**Inventor 2** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | Pierre-Yves | | De Wals | |

**Residence Information (Select One)** ◉ US Residency ○ Non US Residency ○ Active US Military Service

| City | Encinitas | State/Province | CA | Country of Residence | US |
|---|---|---|---|---|---|

**Mailing Address of Inventor:**

| Address 1 | 925 North Vulcan Ave. Apt. #205 |
|---|---|
| Address 2 | |

| City | Encinitas | State/Province | CA |
|---|---|---|---|
| Postal Code | 92024 | Country | US |

**Inventor 3** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | Eric | | Scott | |

**Residence Information (Select One)** ◉ US Residency ○ Non US Residency ○ Active US Military Service

| Application Data Sheet 37 CFR 1.76 | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

| City | Encinitas | State/Province | CA | Country of Residence | US |
|---|---|---|---|---|---|

**Mailing Address of Inventor:**

| Address 1 | 1579 Caudor Street | | |
|---|---|---|---|
| Address 2 | | | |
| City | Encinitas | State/Province | CA |
| Postal Code | 92024 | Country | US |

**Inventor 4** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | ~~Eric~~ | | ~~Knight~~ | |

**Residence Information (Select One)** (•) US Residency ( ) Non US Residency ( ) Active US Military Service

| City | ~~Carlsbad~~ | State/Province | ~~CA~~ | Country of Residence | ~~US~~ |
|---|---|---|---|---|---|

**Mailing Address of Inventor:**

| Address 1 | ~~2744 Loker Ave West, unit 100~~ | | |
|---|---|---|---|
| Address 2 | | | |
| City | ~~Carlsbad~~ | State/Province | ~~CA~~ |
| Postal Code | ~~92010~~ | Country | ~~US~~ |

**Inventor 5** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | ~~Jian~~ | | ~~Yi~~ | |

**Residence Information (Select One)** (•) US Residency ( ) Non US Residency ( ) Active US Military Service

| City | ~~San Diego~~ | State/Province | ~~CA~~ | Country of Residence | ~~US~~ |
|---|---|---|---|---|---|

**Mailing Address of Inventor:**

| Address 1 | ~~13313 Thunderhead Street~~ | | |
|---|---|---|---|
| Address 2 | | | |
| City | ~~San Diego~~ | State/Province | ~~CA~~ |
| Postal Code | ~~92129~~ | Country | ~~US~~ |

**Inventor 6** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | Michael | | Waldbridge | |

**Residence Information (Select One)** (•) US Residency ( ) Non US Residency ( ) Active US Military Service

| City | Saugus | State/Province | MA | Country of Residence | US |
|---|---|---|---|---|---|

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| Application Data Sheet 37 CFR 1.76 | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

| Mailing Address of Inventor: | | | |
|---|---|---|---|
| **Address 1** | 4 Diane Dr | | |
| **Address 2** | | | |
| **City** | Saugus | **State/Province** | MA |
| **Postal Code** | 01906 | **Country** | US |

**Inventor 7** Remove

**Legal Name**

| Prefix | Given Name | Middle Name | Family Name | Suffix |
|---|---|---|---|---|
| | Thomas | | Beardslee | |

**Residence Information (Select One)** (•) US Residency ( ) Non US Residency ( ) Active US Military Service

| City | Carlsbad | State/Province | CA | Country of Residence | US |
|---|---|---|---|---|---|

| Mailing Address of Inventor: | | | |
|---|---|---|---|
| **Address 1** | 6827 Carnation Drive | | |
| **Address 2** | | | |
| **City** | Carlsbad | **State/Province** | CA |
| **Postal Code** | 92011 | **Country** | US |

All Inventors Must Be Listed - Additional Inventor Information blocks may be generated within this form by selecting the **Add** button. Add

## Correspondence Information:

**Enter either Customer Number or complete the Correspondence Information section below.**
**For further information see 37 CFR 1.33(a).**

☐ **An Address is being provided for the correspondence Information of this application.**

| | | | |
|---|---|---|---|
| **Customer Number** | | | |
| **Email Address** | | Add Email | Remove Email |

## Application Information:

| | | | |
|---|---|---|---|
| **Title of the Invention** | | | |
| **Attorney Docket Number** | | **Small Entity Status Claimed** | ☐ |
| **Application Type** | | | |
| **Subject Matter** | | | |
| **Total Number of Drawing Sheets (if any)** | | **Suggested Figure for Publication (if any)** | |

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| **Application Data Sheet 37 CFR 1.76** | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

## Filing By Reference:

Only complete this section when filing an application by reference under 35 U.S.C. 111(c) and 37 CFR 1.57(a). Do not complete this section if application papers including a specification and any drawings are being filed. Any domestic benefit or foreign priority information must be provided in the appropriate section(s) below (i.e., "Domestic Benefit/National Stage Information" and "Foreign Priority Information").

For the purposes of a filing date under 37 CFR 1.53(b), the description and any drawings of the present application are replaced by this reference to the previously filed application, subject to conditions and requirements of 37 CFR 1.57(a).

| Application number of the previously filed application | Filing date (YYYY-MM-DD) | Intellectual Property Authority or Country |
|---|---|---|
| | | |

## Publication Information:

☐ Request Early Publication (Fee required at time of Request 37 CFR 1.219)

☐ **Request Not to Publish.** I hereby request that the attached application not be published under 35 U.S.C. 122(b) and certify that the invention disclosed in the attached application **has not and will not** be the subject of an application filed in another country, or under a multilateral international agreement, that requires publication at eighteen months after filing.

## Representative Information:

Representative information should be provided for all practitioners having a power of attorney in the application. Providing this information in the Application Data Sheet does not constitute a power of attorney in the application (see 37 CFR 1.32).
Either enter Customer Number or complete the Representative Name section below. If both sections are completed the customer Number will be used for the Representative Information during processing.

| Please Select One: | ◉ Customer Number | ○ US Patent Practitioner | ○ Limited Recognition (37 CFR 11.9) |
|---|---|---|---|
| Customer Number | | | |

## Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, 365(c), or 386(c) or indicate National Stage entry from a PCT application. Providing benefit claim information in the Application Data Sheet constitutes the specific reference required by 35 U.S.C. 119(e) or 120, and 37 CFR 1.78.
When referring to the current application, please leave the "Application Number" field blank.

| Prior Application Status | Pending | | Remove |
|---|---|---|---|
| Application Number | Continuity Type | Prior Application Number | Filing or 371(c) Date (YYYY-MM-DD) |
| | | | |

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| **Application Data Sheet 37 CFR 1.76** | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

| Prior Application Status | Expired | | Remove |
|---|---|---|---|
| Application Number | Continuity Type | Prior Application Number | Filing or 371(c) Date (YYYY-MM-DD) |
| | | | |
| Additional Domestic Benefit/National Stage Data may be generated within this form by selecting the **Add** button. | | | Add |

## Foreign Priority Information:

This section allows for the applicant to claim priority to a foreign application. Providing this information in the application data sheet constitutes the claim for priority as required by 35 U.S.C. 119(b) and 37 CFR 1.55. When priority is claimed to a foreign application that is eligible for retrieval under the priority document exchange program (PDX)[i] the information will be used by the Office to automatically attempt retrieval pursuant to 37 CFR 1.55(i)(1) and (2). Under the PDX program, applicant bears the ultimate responsibility for ensuring that a copy of the foreign application is received by the Office from the participating foreign intellectual property office, or a certified copy of the foreign priority application is filed, within the time period specified in 37 CFR 1.55(g)(1).

| | | | Remove |
|---|---|---|---|
| Application Number | Country[i] | Filing Date (YYYY-MM-DD) | Access Code[i] (if applicable) |
| | | | |
| Additional Foreign Priority Data may be generated within this form by selecting the **Add** button. | | | Add |

## Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications

☐ This application (1) claims priority to or the benefit of an application filed before March 16, 2013 and (2) also contains, or contained at any time, a claim to a claimed invention that has an effective filing date on or after March 16, 2013.
NOTE: By providing this statement under 37 CFR 1.55 or 1.78, this application, with a filing date on or after March 16, 2013, will be examined under the first inventor to file provisions of the AIA.

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| Application Data Sheet 37 CFR 1.76 | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

## Authorization or Opt-Out of Authorization to Permit Access:

When this Application Data Sheet is properly signed and filed with the application, applicant has provided written authority to permit a participating foreign intellectual property (IP) office access to the instant application-as-filed (see paragraph A in subsection 1 below) and the European Patent Office (EPO) access to any search results from the instant application (see paragraph B in subsection 1 below).

Should applicant choose not to provide an authorization identified in subsection 1 below, applicant **must opt-out** of the authorization by checking the corresponding box A or B or both in subsection 2 below.

**NOTE**: This section of the Application Data Sheet is **ONLY** reviewed and processed with the **INITIAL** filing of an application. After the initial filing of an application, an Application Data Sheet cannot be used to provide or rescind authorization for access by a foreign IP office(s). Instead, Form PTO/SB/39 or PTO/SB/69 must be used as appropriate.

**1. Authorization to Permit Access by a Foreign Intellectual Property Office(s)**

**A. Priority Document Exchange (PDX)** - Unless box A in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the European Patent Office (EPO), the Japan Patent Office (JPO), the Korean Intellectual Property Office (KIPO), the State Intellectual Property Office of the People's Republic of China (SIPO), the World Intellectual Property Organization (WIPO), and any other foreign intellectual property office participating with the USPTO in a bilateral or multilateral priority document exchange agreement in which a foreign application claiming priority to the instant patent application is filed, access to: (1) the instant patent application-as-filed and its related bibliographic data, (2) any foreign or domestic application to which priority or benefit is claimed by the instant application and its related bibliographic data, and (3) the date of filing of this Authorization. See 37 CFR 1.14(h)(1).

**B. Search Results from U.S. Application to EPO** - Unless box B in subsection 2 (opt-out of authorization) is checked, the undersigned hereby **grants the USPTO authority** to provide the EPO access to the bibliographic data and search results from the instant patent application when a European patent application claiming priority to the instant patent application is filed. See 37 CFR 1.14(h)(2).

The applicant is reminded that the EPO's Rule 141(1) EPC (European Patent Convention) requires applicants to submit a copy of search results from the instant application without delay in a European patent application that claims priority to the instant application.

**2. Opt-Out of Authorizations to Permit Access by a Foreign Intellectual Property Office(s)**

☐ A. Applicant **DOES NOT** authorize the USPTO to permit a participating foreign IP office access to the instant application-as-filed. If this box is checked, the USPTO will not be providing a participating foreign IP office with any documents and information identified in subsection 1A above.

☐ B. Applicant **DOES NOT** authorize the USPTO to transmit to the EPO any search results from the instant patent application. If this box is checked, the USPTO will not be providing the EPO with search results from the instant application.

**NOTE:** Once the application has published or is otherwise publicly available, the USPTO may provide access to the application in accordance with 37 CFR 1.14.

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| **Application Data Sheet 37 CFR 1.76** | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

## Applicant Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

**Applicant 1** Remove

If the applicant is the inventor (or the remaining joint inventor or inventors under 37 CFR 1.45), this section should not be completed. The information to be provided in this section is the name and address of the legal representative who is the applicant under 37 CFR 1.43; or the name and address of the assignee, person to whom the inventor is under an obligation to assign the invention, or person who otherwise shows sufficient proprietary interest in the matter who is the applicant under 37 CFR 1.46. If the applicant is an applicant under 37 CFR 1.46 (assignee, person to whom the inventor is obligated to assign, or person who otherwise shows sufficient proprietary interest) together with one or more joint inventors, then the joint inventor or inventors who are also the applicant should be identified in this section.

Clear

| ◉ Assignee | ○ Legal Representative under 35 U.S.C. 117 | ○ Joint Inventor |
|---|---|---|
| ○ Person to whom the inventor is obligated to assign. | ○ Person who shows sufficient proprietary interest | |

If applicant is the legal representative, indicate the authority to file the patent application, the inventor is:

Name of the Deceased or Legally Incapacitated Inventor:

If the Applicant is an Organization check here. ☒

| Organization Name | | | |
|---|---|---|---|
| **Mailing Address Information For Applicant:** | | | |
| **Address 1** | | | |
| Address 2 | | | |
| **City** | | **State/Province** | |
| **Country** | | Postal Code | |
| Phone Number | | Fax Number | |
| Email Address | | | |

Additional Applicant Data may be generated within this form by selecting the Add button. Add

## Assignee Information including Non-Applicant Assignee Information:

Providing assignment information in this section does not substitute for compliance with any requirement of part 3 of Title 37 of CFR to have an assignment recorded by the Office.

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| **Application Data Sheet 37 CFR 1.76** | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

**Assignee 1**

Complete this section if assignee information, including non-applicant assignee information, is desired to be included on the patent application publication. An assignee-applicant identified in the "Applicant Information" section will appear on the patent application publication as an applicant. For an assignee-applicant, complete this section only if identification as an assignee is also desired on the patent application publication.

Remove

If the Assignee or Non-Applicant Assignee is an Organization check here. ☒

| Organization Name | |
|---|---|

**Mailing Address Information For Assignee including Non-Applicant Assignee:**

| **Address 1** | | | |
|---|---|---|---|
| Address 2 | | | |
| **City** | | **State/Province** | |
| **Country** i | | Postal Code | |
| Phone Number | | Fax Number | |
| Email Address | | | |

Additional Assignee or Non-Applicant Assignee Data may be generated within this form by selecting the Add button. Add

## Signature:

Remove

**NOTE:** This Application Data Sheet must be signed in accordance with 37 CFR 1.33(b). **However, if this Application Data Sheet is submitted with the INITIAL filing of the application and either box A or B is not checked in subsection 2 of the "Authorization or Opt-Out of Authorization to Permit Access" section, then this form must also be signed in accordance with 37 CFR 1.14(c).**

This Application Data Sheet **must** be signed by a patent practitioner if one or more of the applicants is a **juristic entity** (e.g., corporation or association). If the applicant is two or more joint inventors, this form must be signed by a patent practitioner, **all** joint inventors who are the applicant, or one or more joint inventor-applicants who have been given power of attorney (e.g., see USPTO Form PTO/AIA/81) on behalf of **all** joint inventor-applicants.

See 37 CFR 1.4(d) for the manner of making signatures and certifications.

| **Signature** | /Silvia Salvadori/ | | | Date (YYYY-MM-DD) | 2021-10-04 |
|---|---|---|---|---|---|
| First Name | Silvia | Last Name | Salvadori | Registration Number | 48265 |

Additional Signature may be generated within this form by selecting the Add button. Add

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| **Application Data Sheet 37 CFR 1.76** | Attorney Docket Number | |
|---|---|---|
| | Application Number | |
| Title of Invention | | |

This collection of information is required by 37 CFR 1.76. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 23 minutes to complete, including gathering, preparing, and submitting the completed application data sheet form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

# Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1 The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3 A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent CooperationTreaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.



LCY Biotechnology Holding, Inc.
Ex. 1002
Page 2675 of 3077

# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 44266072 |
| **Application Number:** | 16630567 |
| **International Application Number:** | |
| **Confirmation Number:** | 5356 |
| **Title of Invention:** | BIOLOGICAL METHODS FOR PREPARING TERPENES |
| **First Named Inventor/Applicant Name:** | Kimberly Ann Aeling |
| **Customer Number:** | 122515 |
| **Filer:** | Silvia Salvadori |
| **Filer Authorized By:** | |
| **Attorney Docket Number:** | SS118-1026US |
| **Receipt Date:** | 12-NOV-2021 |
| **Filing Date:** | 13-JAN-2020 |
| **Time Stamp:** | 10:24:14 |
| **Application Type:** | U.S. National Stage under 35 USC 371 |

## Payment information:

| | |
|---|---|
| Submitted with Payment | no |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Transmittal Letter | Transmittal_letter.pdf | 71046<br>e8f2769f1150b059059aeb2866ac886bfcf0fdd5 | no | 2 |
| **Warnings:** | | | | | |

| Information: | | | | | |
|---|---|---|---|---|---|
| 2 | Application Data Sheet | ADS.pdf | 442169<br>6cabe2370c14be7ce6cfafe05e5a9dfeb02fd340 | no | 10 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| This is not an USPTO supplied ADS fillable form | | | | | |
| | | | **Total Files Size (in bytes):** | 513215 | |

**This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.**

**New Applications Under 35 U.S.C. 111**

**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**

**National Stage of an International Application under 35 U.S.C. 371**

**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**

**New International Application Filed with the USPTO as a Receiving Office**

**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NUMBER | FILING OR 371(C) DATE | FIRST NAMED APPLICANT | ATTY. DOCKET NO./TITLE |
|---|---|---|---|
| 16/630,567 | 01/13/2020 | Kimberly Ann Aeling | SS118-1026US |

**CONFIRMATION NO. 5356**

122515
Silvia Salvadori, P.C.
Silvia Salvadori
485 Central Park West Unit 5C
New York, NY 10025

**37 CFR 1.48 ACKNOWLEDGEMENT LETTER**

*OC000000131056071*

Date Mailed: 01/13/2022

## NOTICE OF ACCEPTANCE OF REQUEST UNDER 37 CFR 1.48(a)

This is in response to the applicant's request under 37 CFR 1.48(a) submitted on 10/07/2021.

The request under 37 CFR 1.48(a) to correct the inventorship, to correct or update the name of an inventor, or to correct the order of names of joint inventors is accepted.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at **(571) 272-4000** or **(571) 272-4200** or **1-888-786-0101**.

/byemane/

UNITED STATES PATENT AND TRADEMARK OFFICE

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United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NUMBER | FILING or 371(c) DATE | GRP ART UNIT | FIL FEE REC'D | ATTY.DOCKET.NO | TOT CLAIMS | IND CLAIMS |
|---|---|---|---|---|---|---|
| 16/630,567 | 01/13/2020 | 1656 | 8600 | SS118-1026US | 21 | 6 |

**CONFIRMATION NO. 5356**

**UPDATED FILING RECEIPT**

122515
Silvia Salvadori, P.C.
Silvia Salvadori
485 Central Park West Unit 5C
New York, NY 10025

OC000000131036070

Date Mailed: 01/13/2022

Receipt is acknowledged of this non-provisional utility patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF FIRST INVENTOR, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection.

**Please verify the accuracy of the data presented on this receipt.** If an error is noted on this Filing Receipt, please submit a written request for a corrected Filing Receipt, including a properly marked-up ADS showing the changes with strike-through for deletions and underlining for additions. If you received a "Notice to File Missing Parts" or other Notice requiring a response for this application, please submit any request for correction to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections provided that the request is grantable.

**Inventor(s)**

Kimberly Ann Aeling, San Clemente, CA;
Pierre-Yves De Wals, Encinitas, CA;
Eric Scott, Encinitas, CA;
Michael Waldbridge, Saugus, MA;
Thomas Beardslee, Carlsbad, CA;

**Applicant(s)**

Radici Chimica S.p.A., Bergamo, ITALY;

**Assignment For Published Patent Application**

Radici Chimica S.p.A., Bergamo, ITALY

**Power of Attorney:** The patent practitioners associated with Customer Number 122515

**Domestic Priority data as claimed by applicant**

This application is a 371 of PCT/US2018/041579 07/11/2018
which claims benefit of 62/532,297 07/13/2017

**Foreign Applications** for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
*Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.*

**Permission to Access Application via Priority Document Exchange:** Yes

**Permission to Access Search Results:** Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

**Projected Publication Date:** Not Applicable

**Non-Publication Request:** No

**Early Publication Request:** No

**Title**

BIOLOGICAL METHODS FOR PREPARING TERPENES

**Preliminary Class**

435

**Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications:** No

## PROTECTING YOUR INVENTION OUTSIDE THE UNITED STATES

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative, this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).



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Ex. 1002
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## LICENSE FOR FOREIGN FILING UNDER

## Title 35, United States Code, Section 184

## Title 37, Code of Federal Regulations, 5.11 & 5.15

**GRANTED**

The applicant has been granted a license under 35 U.S.C. 184, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" followed by a date appears on this form. Such licenses are issued in all applications where the conditions for issuance of a license have been met, regardless of whether or not a license may be required as set forth in 37 CFR 5.15. The scope and limitations of this license are set forth in 37 CFR 5.15(a) unless an earlier license has been issued under 37 CFR 5.15(b). The license is subject to revocation upon written notification. The date indicated is the effective date of the license, unless an earlier license of similar scope has been granted under 37 CFR 5.13 or 5.14.

This license is to be retained by the licensee and may be used at any time on or after the effective date thereof unless it is revoked. This license is automatically transferred to any related applications(s) filed under 37 CFR 1.53(d). This license is not retroactive.

The grant of a license does not in any way lessen the responsibility of a licensee for the security of the subject matter as imposed by any Government contract or the provisions of existing laws relating to espionage and the national security or the export of technical data. Licensees should apprise themselves of current regulations especially with respect to certain countries, of other agencies, particularly the Office of Defense Trade Controls, Department of State (with respect to Arms, Munitions and Implements of War (22 CFR 121-128)); the Bureau of Industry and Security, Department of Commerce (15 CFR parts 730-774); the Office of Foreign AssetsControl, Department of Treasury (31 CFR Parts 500+) and the Department of Energy.

**NOT GRANTED**

No license under 35 U.S.C. 184 has been granted at this time, if the phrase "IF REQUIRED, FOREIGN FILING LICENSE GRANTED" DOES NOT appear on this form. Applicant may still petition for a license under 37 CFR 5.12, if a license is desired before the expiration of 6 months from the filing date of the application. If 6 months has lapsed from the filing date of this application and the licensee has not received any indication of a secrecy order under 35 U.S.C. 181, the licensee may foreign file the application pursuant to 37 CFR 5.15(b).

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The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

UNITED STATES PATENT AND TRADEMARK OFFICE

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United States Patent and Trademark Office
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P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 16/630,567 | 01/13/2020 | Kimberly Ann Aeling | SS118-1026US | 5356 |

122515 7590 02/18/2022

Silvia Salvadori, P.C.
Silvia Salvadori
485 Central Park West Unit 5C
New York, NY 10025

| EXAMINER | |
|---|---|
| NOAKES, SUZANNE MARIE | |

| ART UNIT | PAPER NUMBER |
|---|---|
| 1656 | |

| NOTIFICATION DATE | DELIVERY MODE |
|---|---|
| 02/18/2022 | ELECTRONIC |

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

eofficeaction@appcoll.com
silvia30121@me.com
silvia@salvadorilaw.com

| *Office Action Summary* | **Application No.** 16/630,567 | **Applicant(s)** Aeling et al. | |
|---|---|---|---|
| | **Examiner** SUZANNE M NOAKES | **Art Unit** 1656 | **AIA (FITF) Status** Yes |

*-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --*

**Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

1)☑ Responsive to communication(s) filed on 11/12/2021.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims***

5) ☑ Claim(s) 163-183 is/are pending in the application.

5a) Of the above claim(s) 166-183 is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 163-165 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

**Application Papers**

10)☑ The specification is objected to by the Examiner.

11)☑ The drawing(s) filed on 01/13/2020 is/are: a)☑ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

**Priority under 35 U.S.C. § 119**

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

**Certified copies:**

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

1) ☑ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

## DETAILED ACTION

### *Notice of AIA Status*

1. The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

### *Election/Restrictions*

2. Applicant's election of Group I, claims 163-165; and the species combination of phytoene synthase, geranylgeranyl diphosphate synthase and phytoene desaturase, in the reply filed on 12 November 2021 is acknowledged. Because applicant did not distinctly and specifically point out the supposed errors in the restriction requirement, the election has been treated as an election without traverse (MPEP § 818.01(a)).

The requirement is deemed proper and therefore made Final.

### *Status of Application*

3. Claims 163-183 are pending; Claims 166-183 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected subject matter, there being no allowable generic or linking claim. Thus, claims 163-165 are subject to examination on the merits.

### *Priority*

4. The instant application is a 371 of PCT/US2018/041579 filed 11 July 2018 which claims benefit of US Provisional 62/532,297 filed 13 July 2017 is acknowledged.

***Information Disclosure Statement***

5. The information disclosure statement (IDS) submitted on 13 January 2020 has been considered by the examiner. See initialed and signed PTO/SB/08.

***Compliance with Sequence Rules***

6. The sequence listing, filed in computer readable form (CRF) and paper copy on July 15, 2002, has been received and entered. This application contains sequence disclosures that are encompassed by the definitions for nucleotide and/or amino acid sequences set forth in 37 C.F.R. § 1.821(a)(1) and (a)(2). However, this application fails to **fully** comply with the requirements of 37 C.F.R. § 1.821 through 1.825; Applicants' attention is directed to the final rulemaking notice published at 55 FR 18230 (May 1, 1990), and 1114 OG 29 (May 15, 1990).

The following Figures contain sequences that contain four or more defined amino acids without any corresponding SEQ ID NO: in the drawing OR no reference to the sequences by SEQ ID NO: in the Brief Description of the Drawings (e.g. the sequences can be defined by SEQ ID NO: either in the Brief Description of Drawings or in the Figures themselves, but does not have to be both).

a) In Figure 25A and 25B, four different amino acid sequences of acetyltransferases.

b) In Figure 27, four different N-terminal sequences of carnitine acetyltransferases.

c) In Figure 38, partial sequence of Pox5p acyl-CoA oxidase.

* If the noted sequences are in the sequence listing as filed, Applicants must amend the specification to identify the sequences appropriately by SEQ ID NO:. If the noted sequences are not in the sequence listing as filed, Applicants must provide (1) an updated copy of the sequence listing containing the requisite sequences in computer readable form (CRF), (2) an amendment directing its entry into the specification, (3) a statement that no new matter has been added and (4) an amendment to the

specification to identify the identified sequences by SEQ ID NO:, which can be in the Brief Description of the Drawings section of the specification. – See also MPEP 2422.

***Claim Rejections - 35 USC § 103***

7. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

8. The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

9. Claims 163-165 are rejected under 35 U.S.C. 103 as being unpatentable over Sharpe et al. (WO 2008/073367 – cited herein) in view of Juretzek et al. (Biotechnol. Bioprocess. Eng., 2000 – cited herein).

Sharpe et al. teach:

Regarding claims 163-165, a recombinantly engineered oleaginous yeast is taught wherein said yeast has been engineered to express the heterologous genes: CrtE of geranyl geranyl pyrophosphate synthase (wherein in the instant specification at paragraph 0158 of PG-Pub, CrtE is defined as a geranyl geranyl diphosphate synthase), a CrtB phytoene synthase, and a CrtI phytoene desaturase as well as (See Claims 1 and 2; p. 4, line 32 to p. 5, line 10; p. 13, line 31 to p. 14, line 4; p. 34, line 17

to p. 26, line 18); additional genes are lycopene cyclase (CrtY), carotenoid hydroxylase (CrtZ) and carotenoid ketolase (CrtW).

Regarding claim 164, the term "oleaginous yeast" is to mean yeast selected from *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon* and *Lipomyces* – See p. 29, lines 12-27. It further taught, *Yarrowia lipolytica* is the host cell utilized in all working examples.

Regarding claims 163 and 165, it is taught: "Virtually any promoter capable of directing expression of these genes in the selected host cell is suitable for the present invention." – p. 59, lines 5-7. It is taught inducible promoters or constitutive promoters are suitable for expression of the genes (p. 59).

Sharpe et al., however, do not teach wherein the promoter is one that is responsive to fatty acid or alkane induction.

Juretzek et al. characterize five different promoters for the expression of foreign proteins in the yeast *Yarrowia lipolytica*. Up until that point in time, said yeast had few promoters available for use in the system, despite said yeast being known for several decades in the biotechnology industries for being a good host for expression of foreign genes because it can do so in considerable amounts (See Introduction). It was established that the promoters POX2, POT1 and ICL1 (inducible by fatty acids, fatty acid derivates, alkanes and/or acetate/ethanol, respectively) which all offer the advantage of being inexpensive carbon sources (See last paragraph, p. 325).

Therefore it would have been obvious to one of ordinary skill in the art prior to the effective filing date of the claimed invention to utilize any of the *Yarrowia lipolytica* strong promoters POX2, POT1 and ICL1 as taught by Juretzek et al. and to utilize these

in the recombinant *Yarrowia lipolytica* strains expressing the heterologous genes: CrtE of geranyl geranyl pyrophosphate synthase (wherein in the instant specification at paragraph 0158 of PG-Pub, CrtE is defined as a geranyl geranyl diphosphate synthase), a CrtB phytoene synthase, and a CrtI phytoene desaturase as well as (See Claims 1 and 2; p. 4, line 32 to p. 5, line 10; p. 13, line 31 to p. 14, line 4; p. 34, line 17 to p. 26, line 18); additional genes are lycopene cyclase (CrtY), carotenoid hydroxylase (CrtZ) and carotenoid ketolase (CrtW) as taught by Sharpe et al., because Sharpe et al. literally state: "Virtually any promoter capable of directing expression of these genes in the selected host cell is suitable for the present invention." – p. 59, lines 5-7. This would provide motivation in and of itself to utilize any of the known promoters, including POX2, POT1, ICL1, G3P, XRP2, for the species *Yarrowia lipolytica* for directing expression of the enzymes geranyl geranyl pyrophosphate synthase (wherein in the instant specification at paragraph 0158 of PG-Pub, CrtE is defined as a geranyl geranyl diphosphate synthase), a CrtB phytoene synthase, and a CrtI phytoene desaturase. One skilled in the art would have a reasonable expectation of success that the promoters as taught by Juretzek et al. would work well in the recombinant strains of Sharpe et al. because Juretzek et al. establishes them to be operable strong inducible promoters.

As such, the references when combined render the instant claims as *prima facie* obvious.

***Conclusion***

10. No claim is allowed.

11. Any inquiry concerning this communication or earlier communications from the examiner should be directed to SUZANNE M NOAKES whose telephone number is (571)272-2924. The examiner can normally be reached M-F (7-4).

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Manjunath Rao can be reached on 571-272-0939. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of published or unpublished applications may be obtained from Patent Center. Unpublished application information in Patent Center is available to registered users. To file and manage patent submissions in Patent Center, visit: https://patentcenter.uspto.gov. Visit https://www.uspto.gov/patents/apply/patent-center for more information about Patent Center and https://www.uspto.gov/patents/docx for information about filing in DOCX format. For additional questions, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/SUZANNE M NOAKES/
Primary Examiner, Art Unit 1656
15 February 2022

| Notice of References Cited | Application/Control No. 16/630,567 | Applicant(s)/Patent Under Reexamination Aeling et al. | |
|---|---|---|---|
| | Examiner SUZANNE M NOAKES | Art Unit 1656 | Page 1 of 1 |

**U.S. PATENT DOCUMENTS**

| * | | Document Number Country Code-Number-Kind Code | Date MM-YYYY | Name | CPC Classification | US Classification |
|---|---|---|---|---|---|---|
| | A | | | | | |
| | B | | | | | |
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| | K | | | | | |
| | L | | | | | |
| | M | | | | | |

**FOREIGN PATENT DOCUMENTS**

| * | | Document Number Country Code-Number-Kind Code | Date MM-YYYY | Country | Name | CPC Classification |
|---|---|---|---|---|---|---|
| | N | WO 2008/073367 | 06-2008 | WO | SHARPE et al. | |
| | O | | | | | |
| | P | | | | | |
| | Q | | | | | |
| | R | | | | | |
| | S | | | | | |
| | T | | | | | |

**NON-PATENT DOCUMENTS**

| * | | Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages) |
|---|---|---|
| | U | Juretzek et al., "Comparison of Promoters Suitable for Regulated Overexpression of β-Galactosidase in the Alkane-Utilizing Yeast Yarrowia lipolytica", Biotechnol. Bioprocess Eng. 2000, 5: 320-326. |
| | V | |
| | W | |
| | X | |

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

| Search Notes | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 16/630,567 | Aeling et al. |
| | Examiner | Art Unit |
| | SUZANNE M NOAKES | 1656 |

| CPC - Searched* | | |
|---|---|---|
| Symbol | Date | Examiner |
| C12N 15/815 w/text in EAST | 02/15/2022 | SMN |

| CPC Combination Sets - Searched* | | |
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| Symbol | Date | Examiner |
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| US Classification - Searched* | | | |
|---|---|---|---|
| Class | Subclass | Date | Examiner |
| | | | |

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

| Search Notes | | |
|---|---|---|
| Search Notes | Date | Examiner |
| Inventors names (all) in EAST and PE2E | 02/15/2022 | SMN |
| EAST: See attached | 02/15/2022 | SMN |
| ProQuest Dialog: see attached | 02/15/2022 | SMN |

| Interference Search | | | |
|---|---|---|---|
| US Class/CPC Symbol | US Subclass/CPC Group | Date | Examiner |
| | | | |



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Ex. 1002
Page 2691 of 3077

Doc code: IDS
Doc description: Information Disclosure Statement (IDS) Filed

PTO/SB/08a (02-18)
Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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| **INFORMATION DISCLOSURE STATEMENT BY APPLICANT** ( **Not for submission under 37 CFR 1.99**) | | |
|---|---|---|
| | Application Number | |
| | Filing Date | |
| | First Named Inventor | Kimberly Ann Aeling |
| | Art Unit | |
| | Examiner Name | |
| | Attorney Docket Number | SS118-1026US |

| **U.S.PATENTS** | | | | | | Remove |
|---|---|---|---|---|---|---|
| Examiner Initial* | Cite No | Patent Number | Kind Code[1] | Issue Date | Name of Patentee or Applicant of cited Document | Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear |
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| | 1 | 20030044946 | A1 | 2003-03-06 | Longo | Entire Document |
| | 2 | 20130066035 | A1 | 2013-03-14 | Burgard et al. | Entire Document |
| | 3 | 20130302861 | A1 | 2013-11-14 | Vainstein et al. | Entire Document |
| | 4 | 20140113343 | A1 | 2014-04-24 | Daviet et al. | Entire Document |
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| INFORMATION DISCLOSURE STATEMENT BY APPLICANT ( Not for submission under 37 CFR 1.99) | | |
|---|---|---|
| | Application Number | |
| | Filing Date | |
| | First Named Inventor | Kimberly Ann Aeling |
| | Art Unit | |
| | Examiner Name | |
| | Attorney Docket Number | SS118-1026US |

| Examiner Initial* | Cite No | Foreign Document Number[3] | Country Code[2] i | Kind Code[4] | Publication Date | Name of Patentee or Applicant of cited Document | Pages,Columns,Lines where Relevant Passages or Relevant Figures Appear | T[5] |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2012254044 | JP | A | 2012-12-27 | Tamagawa et al. | Entire Document | |
| | 2 | 2014100504 | WO | A2 | 2014-06-26 | Beardslee et al. | Entire Document | |
| | 3 | 2018094110 | WO | A2 | 2018-05-24 | Naughton et al. | Entire Document | |

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| Examiner Initials* | Cite No | Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc), date, pages(s), volume-issue number(s), publisher, city and/or country where published. | T[5] |
|---|---|---|---|
| | 1 | International Preliminary Report on Patentability of PCT/US2018/041579 of 2 December 2019. | |
| | 2 | Search Report and Written Opinion of PCT/US18/41579 of 13 September 2018. | |
| | 3 | Written Opinion of the International Preliminary Examining Authority of PCT/US2018/041579 of 13 September 2019. | |

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**EXAMINER SIGNATURE**

| Examiner Signature | /SUZANNE M NOAKES/ | Date Considered | 02/15/2022 |
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*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through a citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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| INFORMATION DISCLOSURE STATEMENT BY APPLICANT ( Not for submission under 37 CFR 1.99) | | |
|---|---|---|
| | Application Number | |
| | Filing Date | |
| | First Named Inventor | Kimberly Ann Aeling |
| | Art Unit | |
| | Examiner Name | |
| | Attorney Docket Number | SS118-1026US |

[1] See Kind Codes of USPTO Patent Documents at www.USPTO.GOV or MPEP 901.04. [2] Enter office that issued the document, by the two-letter code (WIPO Standard ST.3). [3] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [4] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [5] Applicant is to place a check mark here if English language translation is attached.

| INFORMATION DISCLOSURE STATEMENT BY APPLICANT ( Not for submission under 37 CFR 1.99) | | |
|---|---|---|
| | Application Number | |
| | Filing Date | |
| | First Named Inventor | Kimberly Ann Aeling |
| | Art Unit | |
| | Examiner Name | |
| | Attorney Docket Number | SS118-1026US |

**CERTIFICATION STATEMENT**

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

**OR**

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

See attached certification statement.

The fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

**SIGNATURE**

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

| Signature | /Silvia Salvadori/ | Date (YYYY-MM-DD) | 2020-01-13 |
|---|---|---|---|
| Name/Print | Silvia Salvadori | Registration Number | 48265 |

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Page 2696 of 3077

# EAST Search History (Prior Art)

| Ref # | Hits | Search Query | DBs | Default Operator | Plurals | Time Stamp |
|---|---|---|---|---|---|---|
| S64 | 1,046 | ((("AELING") near3 ("Kimberly")) OR (("DE") near3 ("WALS") near3 ("Pierre-Yves")) OR (("SCOTT") near3 ("Eric")) OR (("WALDBRIDGE") near3 ("Michael")) OR (("BEARDSLEE") near3 ("Thomas"))).INV. | US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT | OR | OFF | 2022/02/15 07:59 |
| S65 | 36 | S64 and terpene | US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT | OR | ON | 2022/02/15 08:00 |
| S66 | 6 | S64 and terpene and phytoene | US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT | OR | ON | 2022/02/15 08:00 |
| S67 | 1,648 | ("phytoene synthase" or CftB or "prephytoene-diphosphate synthase" or "15-cis-phytoene synthase" or PSase or "geranylgeranyl-diphosphate geranylgeranyltransferase" or "2.5.1.32") | US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT | OR | ON | 2022/02/15 08:09 |
| S68 | 6,520 | ("phytoene desaturase" or "3, 4-didehydrolycopene-forming phytoene desaturase" or "1.3.99.28" or CrtI or "1.3. 99.30" or CsCrtI or XdCrtI or PaCrtI) | US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT | OR | ON | 2022/02/15 08:12 |
| S69 | 4,105 | ("geranylgeranyl diphosphate synthase" or BTS1 or CrtE or GGPS1 or GGPPS or GGPPS1 or "geranylgeranyl diphosphate synthase 1" or "2.5.1.29") | US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT | OR | ON | 2022/02/15 08:14 |
| S70 | 528 | S67 and S68 and S69 | US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT | OR | ON | 2022/02/15 08:14 |
| S71 | 415 | S67 same3 S68 same3 S69 | US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT | OR | ON | 2022/02/15 08:14 |
| S72 | 360 | S67 same S68 same S69 | US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT | OR | ON | 2022/02/15 08:14 |



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Ex. 1002
Page 2697 of 3077

## EAST Search History (Prior Art)

| | | | | | | |
|---|---|---|---|---|---|---|
| S73 | 38 | S72 and (POX4 or POT or PEX or POX18 or ANT1 or DHE or ARE1) | US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT | OR | ON | 2022/02/15 08:15 |
| S74 | 5 | ("20030044946"\|"20130066035"\|"20130302861"\|"20140113343"\|"20150010978").PN. | US-PGPUB; USPAT | OR | OFF | 2022/02/15 09:01 |
| S75 | 171 | S72 and (Candida or Yarrowia) | US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT | OR | ON | 2022/02/15 09:10 |
| S76 | 21 | S72 same (Candida or Yarrowia) | US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT | OR | ON | 2022/02/15 09:10 |
| S77 | 1,648 | ("phytoene synthase" or CftB or "prephytoene-diphosphate synthase" or "15-cis-phytoene synthase" or PSase or "geranylgeranyl-diphosphate geranylgeranyltransferase" or "2.5.1.32") | US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT | OR | ON | 2022/02/15 10:57 |
| S78 | 6,520 | ("phytoene desaturase" or "3, 4-didehydrolycopene-forming phytoene desaturase" or "1.3.99.28" or CrtI or "1.3. 99.30" or CsCrtI or XdCrtI or PaCrtI) | US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT | OR | ON | 2022/02/15 10:57 |
| S79 | 4,105 | ("geranylgeranyl diphosphate synthase" or BTS1 or CrtE or GGPS1 or GGPPS or GGPPS1 or "geranylgeranyl diphosphate synthase 1" or "2.5.1.29") | US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT | OR | ON | 2022/02/15 10:57 |
| S80 | 360 | S77 same S78 same S79 | US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT | OR | ON | 2022/02/15 10:57 |
| S81 | 16 | S80 and C12N15/815.cpc. | US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT | OR | ON | 2022/02/15 10:57 |

## EAST Search History (Interference)

| | | | | | | |
|---|---|---|---|---|---|---|
| | | <This search history is empty> | | | | |



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Ex. 1002
Page 2698 of 3077

## Bibliographic Data

Application No: 16/630,567

Foreign Priority claimed: ○ Yes ◉ No

35 USC 119 (a-d) conditions met: ☐ Yes ☐ No ☐ Met After Allowance

Verified and Acknowledged: /SUZANNE M NOAKES/ (Examiner's Signature) | (Initials)

Title: BIOLOGICAL METHODS FOR PREPARING TERPENES

| FILING or 371(c) DATE | CLASS | GROUP ART UNIT | ATTORNEY DOCKET NO. |
|---|---|---|---|
| 01/13/2020 | 435 | 1656 | SS118-1026US |
| RULE | | | |

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**CONTINUING DATA**

This application is a 371 of PCT/US2018/041579 07/11/2018

PCT/US2018/041579 has PRO of 62532297 07/13/2017

**FOREIGN APPLICATIONS**

**IF REQUIRED, FOREIGN LICENSE GRANTED****

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**FILING FEE RECEIVED**

$8,600

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
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19 June 2008 (19.06.2008)

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(10) International Publication Number
WO 2008/073367 A1

(51) International Patent Classification:
*C12N 1/16* (2006.01) *C07C 403/24* (2006.01)
*C12N 1/15* (2006.01)

(21) International Application Number:
PCT/US2007/025222

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10 December 2007 (10.12.2007)

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| | | |
|---|---|---|
| 60/869,576 | 12 December 2006 (12.12.2006) | US |
| 60/869,574 | 12 December 2006 (12.12.2006) | US |
| 60/869,591 | 12 December 2006 (12.12.2006) | US |
| 60/869,582 | 12 December 2006 (12.12.2006) | US |
| 60/869,580 | 12 December 2006 (12.12.2006) | US |

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(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SHARPE, Pamela, L.** [US/US]; 19 King Circle, Apartment C, Newark, Delaware 19702 (US). **YE, Rick, W.** [US/US]; 27 Piersons Ridge, Hockessin, Delaware 19707 (US). **ZHU, Quinn, Qun** [US/US]; 544 Revere Road, West Chester, Pennsylvania 19381 (US).

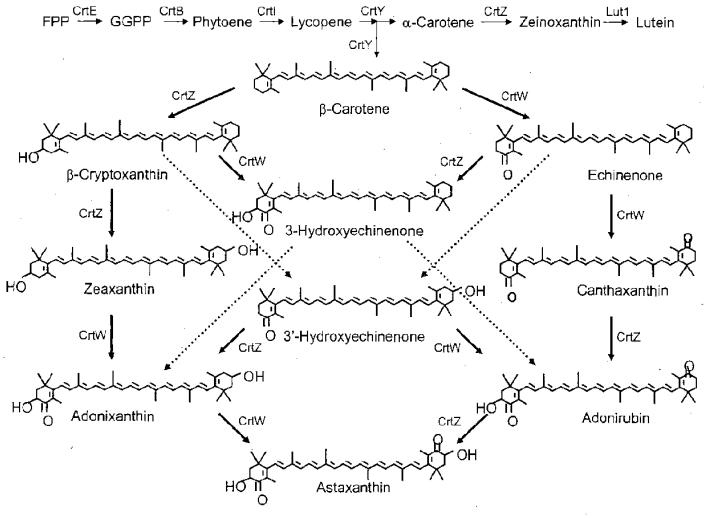
(74) Agent: **FELTHAM, S., Neil**; E. I. du Pont de Nemours and Company, Legal Patent Records Center, 4417 Lancaster Pike, Wilmington, Delaware 19805 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

*[Continued on next page]*

(54) Title: CAROTENOID PRODUCTION IN A RECOMBINANT OLEAGINOUS YEAST




(57) Abstract: Engineered strains of the oleaginous yeast *Yarrowia lipolytica* capable of producing carotenoids (e.g., ß-carotene, lycopene, lutein, zeaxanthin, canthaxanthin, astaxanthin) are provided. The strains may also be engineered to co-produce at least one ω-3/ω-6 polyunsaturated fatty acid and/or at least one additional antioxidant. Methods of using the carotenoid products obtained (e.g., biomass and/or pigmented oils) in food and feed applications are also provided.

WO 2008/073367 A1

**Published:**

- *with international search report*
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*
- *with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description*
- *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

# TITLE

# CAROTENOID PRODUCTION IN A RECOMBINANT OLEAGINOUS YEAST

This application claims the benefit of U.S. Provisional Applications No. 60/869,576, No. 60/869,591, No. 60/869,574, No. 60/869,582 and No. 60/869,580, each filed December 12, 2006.

## FIELD OF THE INVENTION

This invention is in the field of biotechnology. More specifically, this invention pertains to a process of producing carotenoids in oleaginous yeast, pigmented yeast biomass produced by the process, and pigmented oil obtained from the yeast biomass. The carotenoid-producing oleaginous yeast may also be engineered to produce one or more $\omega$-3/$\omega$-6 polyunsaturated fatty acids and/or antioxidants (e.g., coenzyme Q and/or resveratrol).

## BACKGROUND OF THE INVENTION

Carotenoids (e.g., lycopene, β-carotene, zeaxanthin, canthaxanthin and astaxanthin) represent one of the most widely distributed and structurally diverse classes of natural pigments, producing pigment colors of light yellow to orange to deep red color. Eye-catching examples of carotenogenic tissues include carrots, tomatoes, red peppers, and the petals of daffodils and marigolds. Carotenoids are synthesized by all photosynthetic organisms, as well as some bacteria and fungi. These pigments have important functions in photosynthesis, nutrition, and protection against photooxidative damage; as such, they are used today in e.g., food ingredients/colors, animal feed ingredients, pharmaceuticals, cosmetics and as nutritional supplements.

Animals do not have the ability to synthesize carotenoids but must obtain these nutritionally important compounds through their dietary sources. Many animals exhibit an increase in tissue pigmentation when carotenoids are included in their diets, a characteristic often valued by consumers. For example, canthaxanthin and astaxanthin are commonly used in commercial aquaculture industries to pigment shrimp and salmonid fish. It has also been reported that astaxanthin may be a dietary



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requirement for the growth and survival of some salmonid species (Christiansen et al., *Aquaculture Nutrition*, 1:189-198 (1995)). Similarly, lutein, canthaxanthin and astaxanthin are commonly used as pigments in poultry feeds to increase the pigmentation of chicken skin and egg yolks.

Industrially, only a few carotenoids are used, despite the existence of more than 600 different carotenoids identified in nature. This is largely due to difficulties in production and high associated costs. For example, the predominant source of aquaculture pigments used in the market today are produced synthetically and are sold under such trade names as Carophyll® Red (canthaxanthin; DSM Nutritional Products, Heerlen, NL) and Carophyll® Pink (astaxanthin; DSM Nutritional Products); however, the cost of utilizing the synthetically produced pigments is quite high even though the amount of pigment incorporated into the fishmeal is typically less than 100 ppm.

A further concern associated with the use of synthetically produced carotenoids for feed formulations is the common addition of synthetic antioxidants to act as a preservative and to help protect the pigments from oxidation. Many of these synthetic antioxidants are facing significant questions concerning their safety; for example, many adverse health effects (e.g., carcinogenesis, cytotoxicity, etc.) have been reported with the use of ethoxyquin [6-ethoxy-2,2,4-trimethyl-1H-quinoline] (Blaszczyk et. al., *Cell Mol. Biol. Lett.*, 10(1):15-21 (2005); Blaszczyk et al., *Mutat. Res.*, 542:117-128 (2003); Little, A.D., Chemical Evaluation Committee Draft Report, Ethoxyquin, CAS Number 91-53-2, submitted to National Toxicology Program, Executive Summary of Safety and Toxicity Information, U.S. Department of Health and Human Services, (1990)). As such, there is a need for a source of natural carotenoids that optionally comprise natural antioxidants suitable for their stabilization.

Natural carotenoids can either be obtained by extraction of plant material or by microbial synthesis; but, only a few plants are widely used for commercial carotenoid production and the productivity of carotenoid synthesis in these plants is relatively low. Microbial production of carotenoids is a more attractive production route. Examples of carotenoid-



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producing microorganisms include: algae (*Haematococcus pluvialis*, sold under the tradename NatuRose™ (Cyanotech Corp., Kailua-Kona, HI; *Dunaliella sp.*), yeast (*Phaffia rhodozyma,* recently renamed as *Xanthophyllomyces dendrorhous; Thraustochytrium sp.*; *Labyrinthula sp.*; and *Saccharomyces cerevisiae*), and bacteria (*Paracoccus marcusii*, *Bradyrhizobium*, *Rhodobacter sp.*, *Brevibacterium*, *Escherichia coli* and *Methylomonas sp.*). Additionally, recombinant production of carotenoids is also possible, since the genes involved in carotenoid biosynthesis are well-known and have been heterologously expressed in a variety of host cells (e.g., *E. coli*, *Candida utilis, Saccharomyces cerevisiae, Methylomonas* sp.). Thus far, few of these demonstrations are suitable to produce a carotenoid product in significant quantities in a cost-effective manner for industrial use.

Many commercial products are formulated to contain a mixture of pigments and fats/lipids and/or natural antioxidants. For example, animal feeds, dietary supplements, and personal care products are often formulated to contain carotenoids, polyunsaturated fatty acids (PUFAs) and antioxidants (e.g., $CoQ_{10}$). For fish feed formulations, fish oil is often supplemented to the feed to provide the necessary caloric intake and to provide essential fatty acids such as the $\omega$-3/$\omega$-6 PUFAs. Typically, for example, a commercial product formulator will obtain these compounds from a variety of sources and formulate them into a final product that contains an effective amount of each ingredient. The composition, purity and source of each ingredient may vary, resulting in a final product formulation that may require significant monitoring and/or processing to obtain the desired product specifications.

Engineering a microorganism to simultaneously produce carotenoids and fats/lipids and/or an additional antioxidant could create a higher value product and prove advantageous for commercial production economics (and therefore availability) to consumers. One class of organisms that are especially suitable as a production platform for synthesis of pigmented microbial oils (optionally comprising PUFAs and/or additional antioxidants) are the oleaginous yeast. Oleaginous yeast are



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defined as those yeast that are naturally capable of oil synthesis and accumulation, wherein oil accumulation is at least 25% of the cellular dry weight. In particular, *Yarrowia lipolytica* is an oleaginous yeast that has a number of characteristics that make it particularly useful for the production of ω-3/ω-6 PUFAs (see for example commonly owned U.S. Patent 7,238,482 and U.S. Patent Applications No. 11/198975, No. 11/265761, No. 11/264784 and No. 11/264737, corresponding to PCT Publication Nos. WO 2006/033723, WO 2006/052870, WO 2006/055322 and WO 2006/052871, respectively; see also U.S. Patent Application No. 60/977177).

PCT Publication No. WO 2006/102342 (Microbia, Inc.) has engineered an oleaginous yeast to produce carotenoids, thereby resulting in a pigmented microbial product. In particular, they demonstrated greater than 4 mg carotene per gram dry cell weight in *Yarrowia lipolytica* (see Example 2 therein). However, there have been no previous reports of an oleaginous microbial host cell that can co-produce carotenoids and ω-3/ω-6 PUFAs, to thereby result in a single product comprising both ingredients. This is particularly attractive when the recombinant cell biomass is used directly in the formulation (e.g., as an animal feed).

The problem to be solved therefore, is to provide a recombinant oleaginous yeast capable of producing at least one carotenoid. A further problem to be solved is to provide an oleaginous yeast capable of producing at least one carotenoid in combination with an ω-3/ω-6 PUFA and/or at least one additional antioxidant.

## SUMMARY OF THE INVENTION

The stated problem has been solved by providing a recombinant oleaginous yeast capable of producing at least one carotenoid. In a preferred embodiment, the carotenoid is selected from the group consisting of lycopene, β-carotene, zeaxanthin, lutein, canthaxanthin and astaxanthin. In another preferred embodiment, the carotenoid is astaxanthin.

Accordingly the invention provides a recombinant oleaginous yeast production host for the production of astaxanthin comprising:



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a.) at least one copy of a *crtE* gene encoding a geranyl geranyl pyrophosphate synthase;

b.) at least one copy of a *crtB* gene encoding a phytoene synthase;

c.) at least one copy of a *crtI* gene encoding a phytoene desaturase;

d.) at least one copy of a *crtY* gene encoding a lycopene cyclase;

e.) at least one copy of a *crtZ* gene encoding a carotenoid hydroxylase; and,

f.) at least one copy of a *crtW* gene encoding a carotenoid ketolase;

wherein said oleaginous yeast produces at least about 25 weight percent (wt%) of its dry cell weight as oil.

In another embodiment the invention provides a method to produce a pigmented oleaginous yeast biomass comprising a carotenoid compound, comprising:

a.) providing the recombinant production host of claim 1 wherein said production host accumulates at least 25 wt% of its dry cell weight as oil; and,

b.) culturing the recombinant production host under suitable conditions whereby pigmented oleaginous yeast biomass comprising a carotenoid compound is produced.

In another embodiment the invention provides a method to produce pigmented oleaginous yeast biomass comprising a recombinant oleaginous yeast which accumulates at least 25 wt% of its dry cell weight as oil and produces a carotenoid compound selected from the group consisting of astaxanthin, β-carotene, lycopene, lutein, zeaxanthin and canthaxanthin.

In another embodiment the invention provides a method to produce pigmented oil comprising:

a.) an $\omega$-6 polyunsaturated fatty acid or an $\omega$-3 polyunsaturated fatty acid; and,



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b.) a carotenoid compound selected from the group consisting of astaxanthin, β-carotene, lycopene, lutein, zeaxanthin and canthaxanthin.

In another embodiment the invention provides a pigmentation product comprising:

a.) the pigmented oleaginous yeast biomass of claim 11;

b.) the pigmented oil of claim 10; or,

c.) mixtures thereof.

In another embodiment the invention provides a method to produce a recombinant oleaginous yeast cell having at least about 25 wt% of its dry cell weight as oil and which accumulates a carotenoid compound selected from the group consisting of: astaxanthin, β-carotene, lycopene, lutein, zeaxanthin and canthaxanthin.

In another embodiment the invention provides a method of pigmenting an animal comprising the steps of:

a.) providing a pigmented animal feed comprising an effective amount of astaxanthin and an effective amount of a microbially-produced oil; and,

b.) feeding an animal the pigmented animal feed of step (a) whereby at least one tissue in said animal or a product produced by said animal, is pigmented.

## BRIEF DESCRIPTION OF THE BIOLOGICAL DEPOSITS, FIGURES, AND SEQUENCE DESCRIPTIONS

The invention can be more fully understood from the following biological deposits, figures, sequence descriptions, and the detailed description.

## BIOLOGICAL DEPOSITS

The following biological materials were made under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure:

| Biological Material | Accession Number | Date of Deposit |
|---|---|---|
| *Yarrowia lipolytica* Y2047 | ATCC PTA-7186 | October 26, 2005 |



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| | | |
|---|---|---|
| *Yarrowia lipolytica* Y2201 | ATCC PTA-7185 | October 26, 2005 |
| *Yarrowia lipolytica* Y2096 | ATCC PTA-7184 | October 26, 2005 |
| *Yarrowia lipolytica* Y3000 | ATCC PTA-7187 | October 26, 2005 |
| *Yarrowia lipolytica* Y4128 | ATCC PTA-8614 | August 23, 2007 |
| *Yarrowia lipolytica* Y4127 | ATCC PTA-___ | November X, 2007 |

As used herein, "ATCC" refers to the American Type Culture Collection International Depository Authority located at ATCC, 10801 University Blvd., Manassas, VA 20110-2209, U.S.A. The listed deposit will be maintained in the indicated international depository for at least 30 years and will be made available to the public upon the grant of a patent disclosing it. The availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by government action.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates the carotenoid biosynthetic pathway from farnesyl pyrophosphate (FPP) to astaxanthin.

Figure 2 illustrates the $\omega$-3/$\omega$-6 fatty acid biosynthetic pathway.

Figures 3A and 3B illustrate the CoQ biosynthetic pathway.

Figure 4 illustrates the resveratrol biosynthetic pathway.

Figure 5 illustrates the construction of plasmid pYCRTEBI.

Figure 6 provides plasmid maps for the following: (A) pEXPGUS1-P; and, (B) pZP34R.

Figure 7A is a HPLC profile of *Yarrowia lipolytica* strain YL5 illustrating the production of a carotenoid having the same retention time as lycopene; Figure 7B is the absorbance spectrum of the carotenoid produced by *Y. lipolytica* YL5 that is identical to the absorbance profile of the lycopene standard.

Figure 8 diagrams the development of some *Yarrowia lipolytica* strains producing various carotenoids.

Figure 9 provides plasmid maps for the following: (A) pY79; and, (B) pZKSL555.

Figure 10 provides a plasmid map for pYPS108.

Figure 11 provides plasmid maps for the following: (A) pYPS127; and, (B) pYPS128.



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Figure 12 diagrams the development of *Yarrowia lipolytica* strain Y4184U, producing about 31% EPA in the total lipid fraction.

Figure 13 provides plasmid maps for the following: (A) pZKLeuN-29E3; and, (B) pY116.

Figure 14 provides plasmid maps for the following: (A) pKO2UF8289; and, (B) pZKSL-555R.

Figure 15 provides plasmid maps for the following: (A) pZP3-Pa777U; and, (B) pY117.

Figure 16 provides plasmid maps for the following: (A) pZP2-2988; and, (B) pZKUE3S.

Figure 17 provides plasmid maps for the following: (A) pZKL1-2SP98C; and, (B) pZKL2-5U89GC.

Figure 18 diagrams the development of some *Yarrowia lipolytica* strains producing various carotenoids and ω-3 PUFAs.

Figure 19 provides plasmid maps for the following: (A) pYPS147; and, (B) pYPS162.

Figure 20 provides plasmid maps for the following: (A) pYPS152; and, (B) pZKUM.

BRIEF DESCRIPTION OF THE BIOLOGICAL SEQUENCES

The following sequences comply with 37 C.F.R. §1.821-1.825 ("Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures - the Sequence Rules") and are consistent with World Intellectual Property Organization (WIPO) Standard ST.25 (1998) and the sequence listing requirements of the EPO and PCT (Rules 5.2 and 49.5(a-bis), and Section 208 and Annex C of the Administrative Instructions). The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

Table 1

Summary Of Nucleic Acid And Protein SEQ ID Numbers

| Description and Abbreviation | Nucleic acid | Protein SEQ ID |
|---|---|---|



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| | SEQ ID NO. | NO. |
|---|---|---|
| *Yarrowia lipolytica* gene encoding a mutant acetohydroxyacid synthase (AHAS) comprising a W497L mutation (CDS corresponds to bases 146-2556, with a 461 bp intron between bases 268-732) | 1 (2987 bp) | -- |
| Synthetic geranylgeranyl pyrophosphate synthase derived from *Pantoea stewartii* DC413, codon-optimized for expression in *Yarrowia lipolytica* ("$crtE_{syn}$") | 2 (911 bp) | 3 (302 AA) |
| Synthetic phytoene synthase derived from *Pantoea stewartii* DC413, codon-optimized for expression in *Yarrowia lipolytica* ("$crtB_{syn}$") | 4 (936 bp) | 5 (309 AA) |
| Synthetic phytoene desaturase gene derived from *Pantoea stewartii* DC413, codon-optimized for expression in *Yarrowia lipolytica* ("$crtI_{syn}$") | 6 (1484 bp) | 7 (493 AA) |
| Plasmid pZKleuN-6EP | 8 (11,337 bp) | -- |
| *Escherichia coli* LoxP recombination site, recognized by a Cre recombinase enzyme | 9 (34 bp) | -- |
| Plasmid pEXPGUS1-P | 10 (6874 bp) | -- |
| Plasmid pZP34R | 11 (11,444 bp) | -- |
| Plasmid pYCRTEBI | 12 (13,489 bp) | -- |
| Plasmid pY79 | 13 (8982 bp) | -- |
| Plasmid pZKSL555 | 14 (14,256 bp) | -- |
| Plasmid pDCQ392 | 15 (12,210 bp) | -- |
| *Brevundimonas vesicularis* DC263 β-carotene ketolase ("$crtW_{392}$") | 16 (780 bp) | 17 (259 AA) |
| *Brevundimonas vesicularis* DC263 β-carotene hydroxylase ("$crtZ_{392}$") | 18 (486 bp) | 19 (161 AA) |
| *Pantoea stewartii* DC413 lycopene cyclase ("$crtY_{392}$") | 20 (1161 bp) | 21 (387 AA) |
| Plasmid pYPS106 | 30 (14,079 bp) | -- |
| Plasmid pYPS107 | 33 (13,239 bp) | -- |
| Plasmid pYPS108 | 36 (12,681 bp) | -- |



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| | | |
|---|---|---|
| Synthetic lycopene cyclase derived from *Pantoea stewartii* DC413, codon-optimized for expression in *Yarrowia lipolytica* ("*crtY*$392_{syn}$") | 37 (1161 bp) | 38 (387 AA) |
| Synthetic β-carotene ketolase derived from *Brevundimonas vesicularis* DC263, codon-optimized for expression in *Yarrowia lipolytica* ("*crtW*$392_{syn}$") | 39 (780 bp) | 40 (259 AA) |
| Synthetic β-carotene hydroxylase derived from *Brevundimonas vesicularis* DC263, codon-optimized for expression in *Yarrowia lipolytica* ("*crtZ*$392_{syn}$") | 41 (486 bp) | 42 (161 AA) |
| Plasmid pYPS127 | 43 (8252 bp) | -- |
| Plasmid pYPS128 | 44 (7958 bp) | -- |
| Plasmid pZKLeuN-29E3 | 45 (14,688 bp) | -- |
| *Fusarium moniliforme* Δ12 desaturase ("FmD12") | 46 (1434 bp) | 47 (477 AA) |
| *Euglena gracilis* Δ9 elongase ("EgD9e") | 48 (777 bp) | 49 (258 AA) |
| Synthetic Δ9 elongase derived from *Euglena gracilis*, codon-optimized for expression in *Yarrowia lipolytica* ("EgD9eS") | 50 (777 bp) | 51 (258 AA) |
| Synthetic $C_{16/18}$ elongase derived from *Mortierella alpina* ELO3, codon-optimized for expression in *Yarrowia lipolytica* ("ME3S") | 52 (828 bp) | 53 (275 AA) |
| Plasmid pY116 | 54 (8739 bp) | -- |
| Plasmid pKO2UF8289 | 55 (15,337 bp) | -- |
| *Yarrowia lipolytica* Δ12 desaturase ("YlD12") | 56 (1936 bp) | 57 (419 AA) |
| Synthetic mutant Δ8 desaturase ("EgD8M"; U.S. Patent Application No. 11/635258), derived from *Euglena gracilis* ("EgD8S"; PCT Publication No. WO 2006/012326) | 58 (1272 bp) | 59 (422 AA) |
| Synthetic Δ8 desaturase derived from *Euglena gracilis*, codon-optimized for expression in *Yarrowia lipolytica* ("D8SF" or "EgD8S") | 60 (1272 bp) | 61 (422 AA) |
| Plasmid pZKSL-555R | 62 (13,707 bp) | -- |
| Synthetic Δ5 desaturase derived from *Euglena gracilis* (U.S. Patent Application No. 11/748629), codon-optimized for expression in *Yarrowia lipolytica* ("EgD5S") | 63 (1350 bp) | 64 (449 AA) |



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| | | |
|---|---|---|
| Synthetic Δ5 desaturase derived from *Peridinium sp.* CCMP626 (U.S. Patent Application No. 11/748637), codon-optimized for expression in *Yarrowia lipolytica* ("RD5S") | 65 (1392 bp) | 66 (463 AA) |
| *Euglena gracilis* Δ5 desaturase (U.S. Patent Application No. 11/748629) ("EgD5") | 67 (1350 bp) | 68 (449 AA) |
| Plasmid pZP3-Pa777U | 69 (13,066 bp) | -- |
| Synthetic Δ17 desaturase derived from *Pythium aphanidermatum,* codon-optimized for expression in *Yarrowia lipolytica* (U.S. Patent Application No. 11/779915) ("PaD17S") | 70 (1080 bp) | 71 (359 AA) |
| *Pythium aphanidermatum* Δ17 desaturase (U.S. Patent Application No. 11/779915) ("PaD17") | 72 (1080 bp) | 73 (359 AA) |
| Plasmid pY117 | 74 (9570 bp) | -- |
| Plasmid pZP2-2988 | 75 (15,743 bp) | -- |
| Synthetic Δ12 desaturase derived from *Fusarium moniliforme,* codon-optimized for expression in *Yarrowia lipolytica* ("FmD12S") | 76 (1434 bp) | 77 (477 AA) |
| Plasmid pZKUE3S | 78 (6303 bp) | -- |
| Plasmid pZKL1-2SP98C | 79 (15,877 bp) | -- |
| *Yarrowia lipolytica* diacylglycerol cholinephosphotransferase gene ("YlCPT1") | 80 (1185 bp) | 81 (394 AA) |
| Plasmid pZKL2-5U89GC | 82 (15,812 bp) | -- |
| Synthetic lycopene cyclase derived from *Enterobacteriaceae* DC260, codon-optimized for expression in *Yarrowia lipolytica* ("*crtY*343$_{syn}$") | 83 (1164 bp) | 84 (388 AA) |
| Synthetic lycopene cyclase derived from *Pantoea agglomerans* DC404, codon-optimized for expression in *Yarrowia lipolytica* ("*crtY*334$_{syn}$") | 85 (1167 bp) | 86 (389 AA) |
| Synthetic β-carotene ketolase derived from *Sphingomonas melonis* DC18, codon-optimized for expression in *Yarrowia lipolytica* ("*crtW*341$_{syn}$") | 87 (744 bp) | 88 (248 AA) |
| Synthetic β-carotene ketolase derived from *Agrobacterium aurantiacum*, codon-optimized for expression in *Yarrowia lipolytica* ("*crtW*333$_{syn}$") | 89 (726 bp) | 90 (242 AA) |
| Synthetic β-carotene hydroxylase derived from *Brevundimonas vesicularis* DC263, codon-optimized for expression in *Yarrowia lipolytica* ("*crtZ*343$_{syn}$") | 91 (486 bp) | 92 (161 AA) |



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| | | |
|---|---|---|
| Synthetic β–carotene hydroxylase derived from *Agrobacterium aurantiacum*, codon-optimized for expression in *Yarrowia lipolytica* ("*crtZ*334$_{syn}$") | 93 (486 bp) | 94 (162 AA) |
| Plasmid pYPS147 | 95 (13,625 bp) | -- |
| Plasmid pYPS162 | 96 (12,829 bp) | -- |
| Plasmid pYPS152 | 97 (12,380 bp) | -- |
| Plasmid pZKUM | 98 (4313 bp) | -- |
| Synthetic mutant Ura3 gene comprising a 33 bp deletion from +21 to +53, a 1 bp deletion at +376 and a 3 bp deletion from +400 to +403 of the *Yarrowia* Ura3 coding region (GenBank Accession No. AJ306421) | 99 (1459 bp) | -- |

SEQ ID NOs:22 and 23 correspond to the forward and reverse primers, respectively, used to PCR amplify *crtY*$_{392}$ from the DCQ392 carotenoid gene cluster.

SEQ ID NOs:24 and 25 correspond to the forward and reverse primers, respectively, used to PCR amplify *crtZ*$_{392}$ from the DCQ392 carotenoid gene cluster.

SEQ ID NOs:26 and 27 correspond to the forward and reverse primers, respectively, used to PCR amplify *crtW*$_{392}$ from the DCQ392 carotenoid gene cluster.

SEQ ID NOs:28 and 29 are the nucleotide sequences of the Exp-forward primer and HY-339 reverse primer, respectively, used to verify construction of plasmid pYPS106.

SEQ ID NOs:31 and 32 are the nucleotide sequences of the NT forward primer and the Lip1-3' reverse primer, respectively, used to verify construction of plasmid pYPS107.

SEQ ID NOs:34 and 35 are the nucleotide sequences of the GPAT forward primer and the Pex20 reverse primer, respectively, used to verify construction of plasmid pYPS108.



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## DETAILED DESCRIPTION OF THE INVENTION

The following patents, patent applications, and publications may be referred to for the interpretation of the specification and the claims. This includes the following commonly owned patent applications and/or patents relating to carotenoids: U.S. Patent 6,929,928; U.S. Patent 7,064,196; U.S. Patent No. 7,091,031; U.S. Patent 7,217,537; U.S. Patent 7,232,666; U.S. Patent 7,252,985; U.S. Patent 7,288,387; U.S. Patent Application No. 11/230161 (filed September 19, 2005).

This also includes the following commonly owned patent applications relating to antioxidants in oleaginous yeast: U.S. Patent Application No. 11/436182 (filed May 17, 2006) and U.S. Patent Application No. 60/991266 (filed November 30, 2007).

Similarly, this specifically includes the following commonly owned and co-pending patent applications and/or patents related to polyunsaturated fatty acids and oleaginous yeast: U.S. Patent 7,125,672, U.S. Patent 7,189,559, U.S. Patent 7,192,762, U.S. Patent 7,198,937, U.S. Patent 7,202,356, U.S. Patent 7,214,491, U.S. Patent 7,238,482, U.S. Patent 7,256,033, U.S. Patent 7,259,255, U.S. Patent 7,264,949, U.S. Patent 7,267,976, U.S. Patent Applications No. 10/985254 and No. 10/985691, U.S. Patent Application No. 11/024544, U.S. Patent Application No. 11/183664, U.S. Patent Application No. 11/185301, U.S. Patent Application No. 11/190750, U.S. Patent Application No. 11/198975, U.S. Patent Application No. 11/253882, U.S. Patent Applications No. 11/264784 and No. 11/264737, U.S. Patent Application No. 11/265761, U.S. Patent Applications No. 11/601563 and No. 11/601564, U.S. Patent Application No. 11/635258, U.S. Patent Application No. 11/613420, U.S. Patent Application No. 11/787772, U.S. Patent Application No. 11/737772, U.S. Patent Application No. 11/740298, U.S. Patent Applications No. 11/748629 and No. 11/748637, and U.S. Patent Application No. 11/779915.

The invention provides a recombinant oleaginous yeast production host for the production of $C_{40}$ carotenoids, wherein said host comprises at least one *crtE* gene, at least one *crtB* gene, at least one *crtI* gene, and



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optionally at least one additional gene selected from the group consisting of: *crtY*, *crtW*, *crtZ* and *lut1*; wherein expression of the enzyme(s) results in the production of $C_{40}$ carotenoids (e.g., lycopene, β-carotene, lutein, zeaxanthin, canthaxanthin and astaxanthin). In additional embodiments, the recombinant oleaginous yeast production host can co-produce $C_{40}$ carotenoids and at least one ω-3/ω-6 PUFA (e.g., linoleic acid, γ-linolenic acid, eicosadienoic acid, dihomo-γ linolenic acid, arachidonic acid, α-linolenic acid, stearidonic acid, eicosatrienoic acid, eicosatetraenoic acid, eicosapentaenoic acid, docosapentaenoic acid, docosahexaenoic acid) and/or at least one antioxidant (e.g., coenzyme $Q_6$, coenzyme $Q_7$, coenzyme $Q_8$, coenzyme $Q_9$, coenzyme $Q_{10}$, resevatrol).

In all of the above aspects of the present invention, a recombinant oleaginous yeast is used as the production platform. In a preferred aspect, the oleaginous yeast host cell is *Yarrowia lipolytica*.

Definitions

In this disclosure, a number of terms and abbreviations are used. The following definitions are provided.

"Open reading frame" is abbreviated ORF.

"Polymerase chain reaction" is abbreviated PCR.

"American Type Culture Collection" is abbreviated ATCC.

"Isopentenyl pyrophosphate" is abbreviated IPP.

"Geranyl pyrophosphate" is abbreviated GPP.

"Farnesyl pyrophosphate " is abbreviated FPP.

"Polyunsaturated fatty acid(s)" is abbreviated PUFA(s).

"Triacylglycerols" are abbreviated TAGs.

"Coenzyme Q" is abbreviated CoQ.

"Weight percent" is abbreviated wt%.

"Dry cell weight" is abbreviated dcw.

The term "invention" or "present invention" as used herein is not meant to be limiting to any one specific embodiment of the invention but applies generally to any and all embodiments of the invention as described in the claims and specification.



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As used herein, the term "comprising" means the presence of the stated features, integers, steps, or components as referred to in the claims, but it does not preclude the presence or addition of one or more other features, integers, steps, components or groups thereof.

As used herein, the term "about" modifying the quantity of an ingredient or reactant of the invention employed refers to variation in the numerical quantity that can occur, for example, through: typical measuring and liquid handling procedures used for making concentrates or use solutions in the real world; inadvertent error in these procedures; differences in the manufacture, source, or purity of the ingredients employed to make the compositions or carry out the methods; and the like. The term "about" also encompasses amounts that differ due to different equilibrium conditions for a composition resulting from a particular initial mixture. Whether or not modified by the term "about", the claims include equivalents to the quantities. In one aspect, the term "about" means within 20% of the recited numerical value, preferably within 10%, and most preferably within 5%.

A metabolic pathway, or biosynthetic pathway, in a biochemical sense, can be regarded as a series of chemical reactions occurring within a cell, catalyzed by enzymes, to achieve either the formation of a metabolic product to be used or stored by the cell, or the initiation of another metabolic pathway (then called a flux generating step). Many of these pathways are elaborate, and involve a step by step modification of the initial substance to shape it into a product having the exact chemical structure desired. The subject Appliction describes carotenoid biosynthetic pathways, PUFA biosynthetic pathways and specific antioxidant pathways.

The term "isoprenoid compound" refers to compounds formally derived from isoprene (2-methylbuta-1,3-diene; $CH_2=C(CH_3)CH=CH_2$), the skeleton of which can generally be discerned in repeated occurrence in the molecule. These compounds are produced biosynthetically via the isoprenoid pathway beginning with isopentenyl pyrophosphate (IPP) and formed by the head-to-tail condensation of isoprene units, leading to



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molecules which may be, for example, of 5, 10, 15, 20, 30, or 40 carbons in length.

As used herein, the term "carotenoid" refers to a class of hydrocarbons having a conjugated polyene carbon skeleton formally derived from isoprene. This class of molecules is composed of triterpenes ($C_{30}$ diapocarotenoids) and tetraterpenes ($C_{40}$ carotenoids) and their oxygenated derivatives; and, these molecules typically have strong light absorbing properties and may range in length in excess of $C_{200}$. Such carotenoids react destructively with oxygen and hence may require additional antioxidant compounds to act as preservatives. Other "carotenoid compounds" are known which are $C_{35}$, $C_{50}$, $C_{60}$, $C_{70}$ and $C_{80}$ in length, for example.

All "tetraterpenes" or "$C_{40}$ carotenoids" consist of eight isoprenoid units joined in such a manner that the arrangement of isoprenoid units is reversed at the center of the molecule so that the two central methyl groups are in a 1,6-positional relationship and the remaining nonterminal methyl groups are in a 1,5-positional relationship. All $C_{40}$ carotenoids may be formally derived from the acyclic $C_{40}H_{56}$ structure, having a long central chain of conjugated double bonds that is subjected to various funcationalizations.

The term "functionalized" or "functionalization" refers to the (i) hydrogenation, (ii) dehydrogenation, (iii) cyclization, (iv) oxidation, or (v) esterification/glycosylation of any portion of the carotenoid backbone. This backbone is defined as the long central chain of conjugated double bonds. Functionalization may also occur by any combination of the above processes, to thereby result in creation of an acyclic carotenoid or a cartenoid terminated with one (monocyclic) or two (bicyclic) cyclic end groups. Additionally, some carotenoids arise from rearrangements of the carbon skeleton, or by the (formal) removal of part of the backbone structure.

The term "carotenoid" may include both carotenes and xanthophylls. A "carotene" refers to a hydrocarbon carotenoid (e.g., phytoene, β-carotene and lycopene). In contrast, the term "xanthophyll"



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refers to a $C_{40}$ carotenoid that contains one or more oxygen atoms in the form of hydroxy-, methoxy-, oxo-, epoxy-, carboxy-, or aldehydic functional groups. Examples of xanthophylls include, but are not limited to antheraxanthin, adonixanthin, astaxanthin (i.e., 3,3′-dihydroxy-β,β-carotene-4,4′-dione), canthaxanthin (i.e., β,β-carotene-4,4′-dione), β-cryptoxanthin, keto–γ–carotene, echinenone, 3-hydroxyechinenone, 3'-hydroxyechinenone, zeaxanthin, adonirubin, tetrahydroxy-β,β'-caroten-4,4'-dione, tetrahydroxy-β,β'-caroten-4-one, caloxanthin, erythroxanthin, nostoxanthin, flexixanthin, 3-hydroxy-γ-carotene, 3-hydroxy-4-keto-γ-carotene, bacteriorubixanthin, bacteriorubixanthinal and lutein. Xanthophylls are more polar than carotenes and this property dramatically reduces their solubility in fats and lipids.

As used herein, the terms "carotenoid biosynthetic pathway" and "carotenoid pathway" will be used interchangeably and refer to those enzymes which convert farnesyl pyrophosphate (FPP) to a suite of carotenoids. These include those genes and gene products that are involved in the immediate synthesis of phytoene (whose synthesis represents the first step unique to biosynthesis of $C_{40}$ carotenoids). All subsequent reactions leading to the production of various $C_{40}$ carotenoids are included within the carotenoid biosynthetic pathway. These genes and gene products comprise all of the "*crt*" genes including, but not limited to: *crtE, crtX, crtY, crtI, crtB, crtZ, crtW, crtO, crtA, crtC, crtD, crtF* and *crtU,* as well as the *lut1* gene. Finally, the term "carotenoid biosynthetic enzyme" is an inclusive term referring to any and all of the enzymes in the carotenoid pathway including, but not limited to: CrtE, CrtX, CrtY, CrtI, CrtB, CrtZ, CrtW, CrtO, CrtA, CrtC, CrtD, CrtF, CrtU and Lut1.

As used herein, the term "functional" in context with the carotenoid biosynthetic pathway means that some (or all of) the genes in the pathway express active enzymes, resulting in *in vivo* catalysis or substrate conversion that results in production of at least one carotenoid. It should be understood that "carotenoid biosynthetic pathway" or "functional carotenoid biosynthetic pathway" does not imply that all the genes listed in



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the above paragraph are required, as a number of carotenoid products will only require the expression of a subset of the genes of this pathway.

The term "CrtE" refers to a geranylgeranyl pyrophosphate synthase enzyme encoded by the *crtE* gene and which converts trans-trans-farnesyl diphosphate and IPP to pyrophosphate and geranylgeranyl diphosphate.

The term "CrtB" refers to a phytoene synthase enzyme encoded by the *crtB* gene which catalyzes the reaction from prephytoene diphosphate to phytoene.

The term "CrtI" refers to a phytoene desaturase enzyme encoded by the *crtI* gene. CrtI converts phytoene into lycopene via the intermediaries of phytofluene, ζ-carotene and neurosporene by the introduction of 4 double bonds.

The term "CrtY" refers to a lycopene cyclase enzyme encoded by the *crtY* gene that converts lycopene to β-carotene.

The term "CrtZ" refers to a carotenoid hydroxylase enzyme (also referred to herein as a "β-carotene hydroxylase") encoded by the *crtZ* gene that catalyzes a hydroxylation reaction. The oxidation reaction adds a hydroxyl group to cyclic carotenoids having a β-ionone type ring. It is known that CrtZ hydroxylases typically exhibit substrate flexibility, enabling production of a variety of hydroxylated carotenoids depending upon the available substrates; for example, CrtZ catalyzes the hydroxylation reaction from β-carotene to zeaxanthin.

The term "CrtX" refers to a zeaxanthin glucosyl transferase enzyme encoded by the *crtX* gene and which converts zeaxanthin to zeaxanthin-β-diglucoside.

The term "CrtW" refers to a β-carotene ketolase (also referred to herein as a "carotenoid ketolase") enzyme encoded by the *crtW* gene that catalyzes an oxidation reaction where a keto group is introduced on the β-ionone type ring of cyclic carotenoids. This reaction converts cyclic carotenoids, such as β-carotene or zeaxanthin, into the ketocarotenoids canthaxanthin or astaxanthin, respectively. Intermediates in the process typically include echinenone and adonixanthin. It is known that CrtW



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ketolases typically exhibit substrate flexibility, enabling production of a variety of ketocarotenoids depending upon the available substrates.

The term "CrtO" refers to a β-carotene ketolase (also referred to herein as a "carotenoid ketolase") enzyme encoded by the *crtO* gene that catalyzes an oxidation reaction where a keto group is introduced on the β-ionone type ring of cyclic carotenoids. The CrtO-type carotenoid ketolases are structurally unrelated to the CrtW-type ketolases, despite behaving with similar functionality and substrate flexibility as that of the CrtW-type ketolases (*supra*). In one aspect, a mixture of CrtW-type and CrtO-type carotenoid ketolases may be used to produce the desired ketocarotenoids.

The term "Lut1" refers to a ε-hydroxylase enzyme encoded by the *lut1* gene that catalyzes ε-ring hydroxylation. LUT1 defines a class of carotenoid hydroxylases that has evolved independently from and uses a different mechanism than nonheme diiron β-carotene hydroxylases (Tian, Li et al., *Proc. Natl. Acad. Sci. U.S.A.,* 101(1):402–407 (2004)).

"Pigment" refers to a substance used for coloring another material. With respect to the present invention, the pigments described herein are carotenoids produced by a recombinant oleaginous yeast. These caretenoids can be used for coloring, for example, animal tissues (e.g., shrimp, salmonid fish, chicken skin, egg yolks).

"Antioxidants" are described simplistically as compounds (e.g., enzymes, organic molecules) that slow the rate of oxidation reactions or that can counteract the damaging effects of oxygen. Although the term technically applies to molecules reacting with oxygen, it is often applied to molecules that protect from any free radical (i.e., a molecule with an unpaired electron, such as hydroxyl radicals, lipid oxyl or peroxyl radicals, singlet oxygen, and peroxinitrite formed from nitrogen oxide (NO)). Free radicals are natural by-products of cellular processes in an organism or are created by exposure to environmental factors. Within cellular organisms, free radicals can cause cellular and tissue damage, which can ultimately lead to disease. Antioxidants neutralize free radicals by donating one of their own electrons to the free radical, since the



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radicalized antioxidant molecule is more stable as a free-radical than the original free-radical.

As used herein, "coenzyme Q" or "CoQ" and "ubiquinone" will be used interchangeably and will generically refer to a series of lipophillic redox-active molecules comprised of a redox active quinone structure (CAS Registry No. 1339-63-5), including $CoQ_6$, $CoQ_7$, $CoQ_8$, $CoQ_9$ and $CoQ_{10}$. In its reduced state, coenzyme Q acts as an antioxidant; in its oxidized state, it can undergo a redox cycle in the presence of an electron donor and oxygen such that the electron donor is oxidized, the oxygen is reduced and the CoQ is available to undergo another redox cycle. The compound occurs in the majority of aerobic organisms, from bacteria to higher plants and animals.

The term coenzyme $Q_{10}$ ("$CoQ_{10}$") refers to 2,3-dimethoxy-dimethyl-6-decaprenyl-1,4-benzoquinone, also known as ubiquinone-10 (CAS Registry No. 303-98-0). This coenzyme has a hydrocarbon tail that is 50 carbon atoms in length, comprised of ten 5-carbon isoprene units. Synthesis of $CoQ_{10}$ requires a prenyl diphosphate synthase, known as decaprenyl diphosphate synthase (DecPP; catalyzing a $C_{15} \rightarrow C_{50}$ reaction). Means to genetically engineer production of $CoQ_{10}$ in oleaginous yeast is described in U.S. Provisional Patent Application No. 60/991266.

The term coenzyme $Q_9$ ("$CoQ_9$") refers to ubiquinone-9. This coenzyme has a hydrocarbon tail that is 45 carbon atoms in length, comprised of nine 5-carbon isoprene units. Synthesis of $CoQ_9$ requires a prenyl diphosphate synthase, known as solanesyl [or nonaprenyl] diphosphate synthase (SPP or NonPP; catalyzing a $C_{15} \rightarrow C_{45}$ reaction). Many oleaginous yeast, such as *Yarrowia lipolytica,* natural produce significant concentrations of the $CoQ_9$ antioxidant (at least about 2000 ppm).

As used herein, the term "resveratrol" is used to describe the compound *trans*-3,4',5-trihydroxystilbene. Synthesis of this potent antioxidant in oleaginous yeast is described in PCT Publication No. WO 2006/125000.



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"Microbial oils" or "single cell oils" are those oils naturally produced by microorganisms (e.g., algae, oleaginous yeasts and filamentous fungi) during their lifespan. As used herein, the term "oil" refers to a lipid substance that is liquid at 25 °C and usually polyunsaturated. In contrast, the term "fat" refers to a lipid substance that is solid at 25 °C and usually saturated.

"Lipid bodies" refer to lipid droplets that usually are bounded by specific proteins and a monolayer of phospholipid. These organelles are sites where most organisms transport/store neutral lipids. Lipid bodies are thought to arise from microdomains of the endoplasmic reticulum that contain TAG-biosynthesis enzymes and, their synthesis and size appear to be controlled by specific protein components.

"Neutral lipids" refer to those lipids commonly found in cells in lipid bodies as storage fats and oils and are so called because at cellular pH, the lipids bear no charged groups. Generally, they are completely non-polar with no affinity for water. Neutral lipids generally refer to mono-, di-, and/or triesters of glycerol with fatty acids, also called monoacylglycerol, diacylglycerol or TAG, respectively (or collectively, acylglycerols). A hydrolysis reaction must occur to release free fatty acids from acylglycerols.

The term "fatty acids" refers to long chain aliphatic acids (alkanoic acids) of varying chain lengths, from about $C_{12}$ to $C_{22}$ (although both longer and shorter chain-length acids are known). The predominant chain lengths are between $C_{16}$ and $C_{22}$. The structure of a fatty acid is represented by a simple notation system of "X:Y", where X is the total number of carbon (C) atoms in the particular fatty acid and Y is the number of double bonds. Additional details concerning the differentiation between "saturated fatty acids" versus "unsaturated fatty acids", "monounsaturated fatty acids" versus "polyunsaturated fatty acids" (or "PUFAs"), and "omega-6 fatty acids" (ω-6 or *n*-6) versus "omega-3 fatty acids" (ω-3 or *n*-3) are provided in U.S. Patent 7,238,482.

The term "oleaginous" refers to those organisms that tend to store their energy source in the form of lipid (Weete, In: Fungal Lipid



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Biochemistry, 2nd Ed., Plenum, 1980). The term "oleaginous yeast" refers to those microorganisms classified as yeasts that can make oil. Generally, the cellular oil or TAG content of oleaginous microorganisms follows a sigmoid curve, wherein the concentration of lipid increases until it reaches a maximum at the late logarithmic or early stationary growth phase and then gradually decreases during the late stationary and death phases (Yongmanitchai and Ward, *Appl. Environ. Microbiol.*, 57:419-25 (1991)). It is not uncommon for oleaginous microorganisms to accumulate in excess of about 25% of their dry cell weight as oil. Examples of oleaginous yeast include, but are no means limited to, the following genera: *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon* and *Lipomyces*.

As used herein the term "biomass" refers specifically to spent or used yeast cellular material from the fermentation of a recombinant production host producing at least one carotenoid (and optionally at least one PUFA and/or at least one additional antioxidant) in commercially significant amounts, wherein the preferred production host is a recombinant strain of the oleaginous yeast, *Yarrowia lipolytica*. The biomass may be in the form of whole cells, whole cell lysates, homogenized cells, partially hydrolyzed cellular material, and/or partially purified cellular material (e.g., microbially produced oil).

The term "fermentable carbon source" means a carbon source that a microorganism will metabolize to derive energy. Typical carbon sources of the invention include, but are not limited to: monosaccharides, oligosaccharides, polysaccharides, alkanes, fatty acids, esters of fatty acids, monoglycerides, diglycerides, triglycerides, carbon dioxide, methanol, formaldehyde, formate and carbon-containing amines.

As used herein, an "isolated nucleic acid fragment" or "genetic construct" is a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. An isolated nucleic acid fragment in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.



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The term "complementary" is used to describe the relationship between nucleotide bases that are capable of hybridizing to one another. For example, with respect to DNA, adenosine is complementary to thymine and cytosine is complementary to guanine.

"Codon degeneracy" refers to the nature in the genetic code permitting variation of the nucleotide sequence without effecting the amino acid sequence of an encoded polypeptide. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a gene for improved expression in a host cell, it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

"Chemically synthesized", as related to a sequence of DNA, means that the component nucleotides were assembled *in vitro*. Manual chemical synthesis of DNA may be accomplished using well-established procedures or, automated chemical synthesis can be performed using one of a number of commercially available machines. "Synthetic genes" can be assembled from oligonucleotide building blocks that are chemically synthesized using procedures known to those skilled in the art. These building blocks are ligated and annealed to form gene segments that are then enzymatically assembled to construct the entire gene. Accordingly, the genes can be tailored for optimal gene expression based on optimization of nucleotide sequence to reflect the codon bias of the host cell. The skilled artisan appreciates the likelihood of successful gene expression if codon usage is biased towards those codons favored by the host. Determination of preferred codons can be based on a survey of genes derived from the host cell, where sequence information is available. For example, the codon usage profile for *Yarrowia lipolytica* is provided in U.S. Patent 7,125,672.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, and that may refer to the coding region alone or may include regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a



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gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, native genes introduced into a new location within the native host, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure. A "codon-optimized gene" is a gene having its frequency of codon usage designed to mimic the frequency of preferred codon usage of the host cell.

"Coding sequence" refers to a DNA sequence that codes for a specific amino acid sequence. "Suitable regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence (or located within an intron thereof) , and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing sites, effector binding sites and stem-loop structures.

"Promoter" refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental or physiological conditions. Promoters that



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cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

The terms "3' non-coding sequences" and "transcription terminator" refer to DNA sequences located downstream of a coding sequence. This includes polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The 3' region can influence the transcription, RNA processing or stability, or translation of the associated coding sequence.

"RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript or it may be a RNA sequence derived from post-transcriptional processing of the primary transcript and is referred to as the mature RNA. "Messenger RNA" or "mRNA" refers to the RNA that is without introns and that can be translated into protein by the cell. "cDNA" refers to a double-stranded DNA that is complementary to, and derived from, mRNA. "Sense" RNA refers to RNA transcript that includes the mRNA and so can be translated into protein by the cell. "Antisense RNA" refers to a RNA transcript that is complementary to all or part of a target primary transcript or mRNA and that blocks the expression of a target gene (U.S. Patent No. 5,107,065; PCT Publication No. WO 99/28508). The complementarity of an antisense RNA may be with any part of the specific gene transcript, i.e., at the 5' non-coding sequence, 3' non-coding sequence, or the coding sequence. "Functional RNA" refers to antisense RNA, ribozyme RNA, or other RNA that is not translated and yet has an effect on cellular processes.

The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is



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affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragments of the invention. Expression may also refer to translation of mRNA into a polypeptide.

"Mature" protein refers to a post-translationally processed polypeptide, i.e., one from which any pre- or propeptides present in the primary translation product have been removed. "Precursor" protein refers to the primary product of translation of mRNA, i.e., with pre- and propeptides still present. Pre- and propeptides may be (but are not limited to) intracellular localization signals.

The term "recombinase" refers to an enzyme(s) that carries out site-specific recombination to alter the DNA structure and includes transposases, lambda integration/excision enzymes, as well as site-specific recombinases.

"Recombinase site" or "site-specific recombinase sequence" means a DNA sequence that a recombinase will recognize and bind to. It will be appreciated that this may be a wild type or mutant recombinase site, as long as functionality is maintained and the recombinase enzyme may still recognize the site, bind to the DNA sequence, and catalyze recombination between two adjacent recombinase sites.

"Transformation" refers to the transfer of a nucleic acid molecule into a host organism, resulting in genetically stable inheritance. The nucleic acid molecule may be a plasmid that replicates autonomously, for example, or, it may integrate into the genome of the host organism. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" or "recombinant" or "transformed" organisms.

The terms "plasmid" and "vector" refer to an extra chromosomal element often carrying genes that are not part of the central metabolism of



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the cell, and usually in the form of circular double-stranded DNA fragments. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing an expression cassette(s) into a cell.

The term "expression cassette" refers to a fragment of DNA comprising the coding sequence of a selected gene and regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence that are required for expression of the selected gene product. Thus, an expression cassette is typically composed of: (1) a promoter sequence; (2) a coding sequence (i.e., ORF); and, (3) a 3' untranslated region (i.e., a terminator) that, in eukaryotes, usually contains a polyadenylation site. The expression cassette(s) is usually included within a vector, to facilitate cloning and transformation. Different expression cassettes can be transformed into different organisms including bacteria, yeast, plants and mammalian cells, as long as the correct regulatory sequences are used for each host.

The term "homologous recombination" refers to the exchange of DNA fragments between two DNA molecules (during cross over). The fragments that are exchanged are flanked by sites of identical nucleotide sequences between the two DNA molecules (i.e., "regions of homology"). The term "regions of homology" refer to stretches of nucleotide sequence on nucleic acid fragments that participate in homologous recombination that have homology to each other. Effective homologous recombination will generally take place where these regions of homology are at least about 10 bp in length, where at least about 50 bp in length is preferred. Typically fragments that are intended for recombination contain at least two regions of homology where targeted gene disruption or replacement is desired.

As used herein, the term "chromosomal integration" means that a chromosomal integration vector becomes congruent with the chromosome



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of a microorganism through recombination between homologous DNA regions on the chromosomal integration vector and within the chromosome. Many of the modifications to the oleaginous yeast *Yarrowia lipolytica* were introduced by chromosomal integration.

As used herein, the term "chromosomal integration vector" means an extra-chromosomal vector that is capable of integrating into the host's genome through homologous recombination.

The term "sequence analysis software" refers to any computer algorithm or software program that is useful for the analysis of nucleotide or amino acid sequences. "Sequence analysis software" may be commercially available or independently developed. Typical sequence analysis software will include, but is not limited to: 1.) the GCG suite of programs (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI); 2.) BLASTP, BLASTN, BLASTX (Altschul et al., *J. Mol. Biol.*, 215:403-410 (1990)); 3.) DNASTAR (DNASTAR, Inc. Madison, WI); 4.) Sequencher (Gene Codes Corporation, Ann Arbor, MI); and, 5.) the FASTA program incorporating the Smith-Waterman algorithm (W. R. Pearson, *Comput. Methods Genome Res.,* [Proc. Int. Symp.] (1994), Meeting Date 1992, 111-20. Editor(s): Suhai, Sandor. Plenum: New York, NY). Within the context of this application it will be understood that where sequence analysis software is used for analysis, that the results of the analysis will be based on the "default values" of the program referenced, unless otherwise specified. As used herein, "default values" will mean any set of values or parameters (as set by the software manufacturer) which originally load with the software when first initialized.

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (2001) (hereinafter "Maniatis"); by Silhavy, T. J., Bennan, M. L. and Enquist, L. W., Experiments with Gene Fusions, Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1984); and by Ausubel, F. M. et al., Current Protocols



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in Molecular Biology, published by Greene Publishing Assoc. and Wiley-Interscience, Hoboken, NJ (1987).

Microbial Hosts For Carotenoid Production: Oleaginous Yeast

Oleaginous organisms are those organisms that tend to store their energy source in the form of lipid (Weete, In: Fungal Lipid Biochemistry, 2nd Ed., Plenum, 1980). Generally, the cellular oil content of these microorganisms follows a sigmoid curve, wherein the concentration of lipid increases until it reaches a maximum at the late logarithmic or early stationary growth phase and then gradually decreases during the late stationary and death phases (Yongmanitchai and Ward, *Appl. Environ. Microbiol.*, 57:419-25 (1991)).

The term "oleaginous yeast" refers to those microorganisms classified as yeasts that can accumulate in excess of about 25% of their dcw as oil, more preferably greater than about 30% of the dcw, and most preferably greater than about 40% of the dcw under oleaginous conditions. Examples of oleaginous yeast include, but are no means limited to, the following genera: *Yarrowia, Candida, Rhodotorula, Rhodosporidium*, *Cryptococcus, Trichosporon* and *Lipomyces*. More specifically, illustrative oil-synthesizing yeasts include: *Rhodosporidium toruloides, Liopmyces starkeyii, L. lipoferus*, *Candida revkaufi, C. pulcherrima, C. tropicalis, C. utilis, Trichosporon pullans, T. cutaneum, Rhodotorula glutinus, R. graminis* and *Yarrowia lipolytica* (formerly classified as *Candida lipolytica*). The technology for growing oleaginous yeast with high oil content is well developed (for example, see EP 0 005 277B1; Ratledge, C., *Prog. Ind. Microbiol.*, 16:119-206 (1982)); and, these organisms have been commercially used for a variety of purposes in the past.

Of those organisms classified as oleaginous yeast, *Yarrowia lipolytica* was selected as a preferred microbial host for the purposes herein. This selection was based on previous genetic engineering by the Applicants' Assignee resulting in significant production of various ω-3/ω-6 PUFAs (thereby demonstrating ease in genertic manipulation within the organism) and confirmation that the organism naturally produces high



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levels (at least about 2000 ppm) of the natural antioxidant $CoQ_9$. In preferred embodiments, the *Y. lipolytica* strains designated as ATCC #20362, ATCC #8862, ATCC #18944, ATCC #76982 and/or LGAM S(7)1 (Papanikolaou S., and Aggelis G., *Bioresour. Technol.*, 82(1):43-9 (2002)) are preferred wildtype hosts, due to preliminary studies targeted toward identification of wildtype strains having high lipid content (measured as a percent DCW) and high volumetric productivity (measured as g/L $h^{-1}$).

Carotenoid Production In Oleaginous Yeast

The genetics of carotenoid biosynthesis are well known (Armstrong, G., in Comprehensive Natural Products Chemistry, Elsevier, v.2, pp 321-352 (1999)); Lee, P. and Schmidt-Dannert, C., *Appl. Microbiol. Biotechnol.*, 60:1-11 (2002); Lee et al., *Chem. Biol.*, 10:453-462 (2003); Fraser, P. and Bramley, P., *Progress in Lipid Research*, 43:228-265 (2004)). This pathway is extremely well studied in the Gram-negative, pigmented bacteria of the genera *Pantoea*, formerly known as *Erwinia*. Of particular interest are the genes responsible for the production of $C_{40}$ carotenoids used as pigments in animal feeds (e.g., zeaxanthin, lutein, canthaxanthin and astaxanthin).

The enzymatic pathway involved in the biosynthesis of carotenoid compounds can be conveniently viewed in two parts: the upper isoprenoid pathway (isoprenoid biosynthesis is found in all organisms) providing farnesyl pyrophosphate (FPP); and, the lower carotenoid biosynthetic pathway (found in a subset of organisms), which converts FPP to $C_{40}$ carotenoids.

Farnesyl Pyrophosphate Synthesis Via The Mevalonate Pathway:

The upper isoprenoid biosynthetic pathway leads to the production of the $C_5$ isoprene subunit, isopentenyl pyrophosphate (IPP). This biosynthetic process may occur through the mevalonate pathway (from acetyl CoA) or the non-mevalonate pathway (from pyruvate and glyceraldehyde-3-phosphate). The non-mevalonate pathway has been characterized in bacteria, green algae and higher plants, but not in yeast and animals (Horbach *et al., FEMS Microbiol. Lett.*, 111:135-140 (1993); Rohmer *et al., Biochem.*, 295:517-524 (1993); Schwender *et al., Biochem.*, 316:73-80



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(1996); and, Eisenreich *et al., Proc. Natl. Acad. Sci. U.S.A.,* 93:6431-6436 (1996)).

Yeasts and animals typically use the mevalonate pathway to produce IPP, which is subsequently converted to FPP ($C_{15}$). In this pathway, 2 molecules of acetyl-CoA are condensed by thiolase to yield acetoacetyl-CoA, which is subsequently converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by the action of 3-hydroxymethyl-3-glutaryl-CoA synthase (HMG-CoA synthase). Next, 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase; the rate controlling step in the mevalonate pathway) converts HMG-CoA to mevalonate, to which 2 molecules of phosphate residues are then added by the action of 2 kinases (i.e., mevalonate kinase and phosphomevalonate kinase, respectively). Mevalonate pyrophosphate is then decarboxylated by the action of mevalonate pyrophosphate decarboxylase to yield IPP, which becomes the building unit for a wide variety of isoprene molecules necessary in living organisms.

IPP is isomerized to dimethylaryl pyrophosphate (DMAPP) by the action of IPP isomerase. IPP and DMAPP are then converted to the $C_{10}$ unit geranyl pyrophosphate (GPP) by a head to tail condensation. In a similar condensation reaction between GPP and IPP, GPP is converted to the $C_{15}$ unit FPP, an important substrate in ergosterol biosynthesis in yeast. The biosynthesis of GPP and FPP from IPP and DMAPP is catalyzed by the enzyme FPP synthase.

Carotenoid Biosynthesis From Farnesyl Pyrophosphate: Although the enzymatic pathway involved in the biosynthesis of carotenoid compounds converts FPP to a suite of carotenoids, the $C_{40}$ pathway can be subdivided into two parts comprising: (1) the $C_{40}$ backbone genes (i.e., *crtE*, *crtB*, *crtI*, and *crtY*) encoding enzymes responsible for converting FPP to β-carotene; and, (2) subsequent functionalization genes (e.g., *crtW, crtO, crtR, crtX* and *crtZ,* responsible for adding various functional groups to the β-ionone rings of β-carotene; and, *Lut1*, responsible for adding a hydroxyl group to α-carotene) (Figure 1).



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More specifically, the carotenoid biosynthetic pathway begins with the conversion of FPP to geranylgeranyl pyrophosphate (GGPP). In this first step, the enzyme geranylgeranyl pyrophosphate synthase (encoded by the *crtE* gene) condenses the $C_{15}$ FPP with IPP, creating the $C_{20}$ compound GGPP. Next, a phytoene synthase (encoded by the gene *crtB*) condenses two GGPP molecules to form phytoene, the first $C_{40}$ carotenoid compound in the pathway. Subsequently, a series of sequential desaturations (i.e., producing the intermediaries of phytofluene, ζ-carotene and neurosporene) occur, catalyzed by the enzyme phytoene desaturase (encoded by the gene *crtI*) and resulting in production of lycopene. Finally, the enzyme lycopene cyclase (encoded by the gene *crtY*) forms β-ionone rings on each end of lycopene, forming the bicyclic carotenoid β-carotene.

The rings of β-carotene can subsequently be functionalized by a carotenoid ketolase (encoded by the genes *crtW*, *crtO* or *bkt*) and/or carotenoid hydroxylase (encoded by the genes *crtZ* or *crtR*) forming commercially important xanthophyll pigments such as canthaxanthin, astaxanthin and zeaxanthin. In terms of the ketolation and hydroxylation reactions, the CrtW-type ketolases and the CrtZ-type hydroxylases are preferred in the present invention. The pathway from β-carotene to astaxanthin is somewhat non-linear in nature as a variety of intermediates can be formed (Figure 1).

In alternate emodiments, the enzyme lycopene cyclase (encoded by the gene *crtY*) catalyzes formation of the bicyclic carotenoid α-carotene (α-carotene and *β*-carotene differ in the position of double bonds within their cyclic end groups). The rings of α-carotene can then be functionalized by a carotenoid hydroxylase (i.e., a *crtZ* or *crtR* gene) forming zeinoxanthin. This carotenoid is further modified by hydroxylation by an ε-hydroxylase (encoded by the gene *lut1*) to result in production of lutein.

Genetically Engineered Oleaginous Yeast For Carotenoid Production: As is demonstrated in the present Application, oleaginous



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yeast can be engineered to produce various $C_{40}$ carotenoids by integration of appropriate heterologous genes encoding *crtE, crtY, crtI, crtB, crtZ, crtW* and *lut1* into the oleaginous host organism for production of any particular $C_{40}$ carotenoid of interest using FPP as the substrate, according to the general guidelines below in Table 2.

Table 2

Genes Required For Synthesis Of Various Carotenoids

| | *crtE* | *crtB* | *crtI* | *crtY* | *crtW* or *crtO* | *crtZ* or *crtR* | *lut1* |
|---|---|---|---|---|---|---|---|
| Lycopene | 1 (+) | 1 (+) | 1 (+) | -- | -- | -- | -- |
| β-Carotene | 1 (+) | 1 (+) | 1 (+) | 1 (+) | -- | -- | -- |
| Canthaxanthin | 1 (+) | 1 (+) | 1 (+) | 1 (+) | 1 (+) | -- | -- |
| Zeaxanthin | 1 (+) | 1 (+) | 1 (+) | 1 (+) | -- | 1 (+) | -- |
| Astaxanthin | 1 (+) | 1 (+) | 1 (+) | 1 (+) | 1 (+) | 1 (+) | -- |
| Lutein | 1 (+) | 1 (+) | 1 (+) | 1 (+) | -- | 1 (+) | 1 (+) |

Note: The designation 1 (+) represents "one or more".

Thus, for example, production of a ketocarotenoid, such as canthaxanthin, will require expression of the $C_{40}$ backbone genes (i.e., *crtE*, *crtB*, *crtI* and *crtY*) and at least one carotenoid ketolase gene (i.e., *crtW* and/or *crtO*). Production of hydroxylated carotenoids, such as zeaxanthin and astaxanthin, require the expression of the $C_{40}$ backbone genes (i.e., *crtE*, *crtB*, *crtI* and *crtY*) and at least one carotenoid hydroxylating gene (i.e., *crtZ*).

It is contemplated that the particular functionalities required to be expressed in a specific oleaginous host organism for production of carotenoids will depend on the host cell (and its native isoprenoid and/or carotenoid pathway), the availability of substrate, and the desired end product(s). One skilled in the art will be able to identify various candidate genes encoding each of the enzymes desired for carotenoid biosynthesis. Useful *crt* gene sequences may be derived from any source, e.g., isolated from a natural source (from bacteria, algae, fungi, plants, animals, etc.), produced via a semi-synthetic route or synthesized *de novo.* Although the particular source of the *crt* genes introduced into the host is not critical,



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considerations for choosing a specific polypeptide having carotenogenic activity include: 1.) the substrate specificity and activity of the polypeptide; 2.) whether the polypeptide or a component thereof is a rate-limiting enzyme; 3.) whether the *crt* gene is essential for synthesis of a desired carotenoid; and/or, 4.) co-factors required by the polypeptide. The expressed polypeptide preferably has parameters compatible with the biochemical environment of its location in the host cell.

In additional embodiments, it will also be useful to consider the conversion efficiency of each particular Crt enzyme. More specifically, since each enzyme rarely functions with 100% efficiency to convert substrate to product, the final carotene profile of un-purified oils produced in a host cell will typically be a mixture of various carotenoids consisting of the desired carotenoid, as well as various upstream intermediary carotenoid. Thus, each enzyme's conversion efficiency is also a variable to consider, when optimizing biosynthesis of a particular carotenoid (e.g., astaxanthin).

With each of the considerations above in mind, candidate genes having the appropriate *crtE, crtY, crtI, crtB, crtZ, crtR, crtO, crtW* and *lut1* activities can be identified according to publicly available literature (e.g., GenBank), the patent literature, and experimental analysis of organisms having the ability to produce carotenoids. These genes will be suitable for introduction into a specific host organism, to enable or enhance the organism's synthesis of carotenoids. Some non-limiting examples of suitable carotenoid biosynthesis genes include, but are not limited to the GenBank® Accession Number references provided below:

- ***crtE:*** AB000835, AB016043, AB019036, AB016044, AB027705, AB027706, AB034249, AB034250, AF020041, AF049658, AF049659, AF139916, AF279807, AF279808, AJ010302, AJ133724, AJ276129, D85029, L25813, L37405, U15778, U44876, X92893, X95596, X98795 and Y15112;
- ***crtB:*** AB001284, AB032797, AB034704, AB037975, AF009954, AF139916, AF152892, AF218415, AF220218, AJ010302, AJ133724, AJ278287, AJ304825 and AJ308385, D58420,



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L23424, L25812, L37405, M38424, M87280, S71770, U32636, U62808, U87626, U91900, X52291, X60441, X63873, X68017, X69172 and X78814;

- ***crtI:*** AB046992, AF039585, AF049356, AF139916, AF218415, AF251014, AF364515, D58420, D83514, L16237, L37405, L39266, M64704, M88683, S71770, U37285, U46919, U62808, X55289, X59948, X62574, X68058, X71023, X78271, X78434, X78815, X86783, Y14807, Y15007, Y15112, Y15114 and Z11165;
- ***crtY:*** AF139916, AF152246, AF218415, AF272737, AJ133724, AJ250827, AJ276965, D58420, D83513, L40176, M87280, U50738, U50739, U62808, X74599, X81787, X86221, X86452, X95596 and X98796;
- ***crtZ:*** D58420, D58422, D90087, M87280, U62808 and Y15112;
- ***crtX:*** D90087, M87280 and M90698;
- ***crtW:*** AF218415, D45881, D58420, D58422, X86782 and Y15112; and,
- ***lut1:*** AAR83120, EDO99174 and NP_564384.

Preferred sources of carotenoid biosynthesis genes are from *Pantoea stewartii* (ATCC #8199; PCT Publication No. WO 2002/079395), *Pantoea stewartii* DC413 (U.S. Patent 7,288,387), *Pantoea agglomerans* DC404 (U.S. Patent 6,929,928), *Enterobacteriaceae* DC260 (U.S. Patent 7,064,196), *Brevundimonas vesicularis* DC263 (U.S. Patent 7,252,985 and U.S. Patent 7,091,031), *Sphingomonas melonis* DC18 (U.S. Patent 7,252,985), *Novosphingobium aromaticivorans* ATCC #700278 (U.S. Patent 7,091,031) and *Agrobacterium aurantiacum* (U.S. Patent 5,811,273, U.S. Patent 5,972,690 and U.S. Patent 6,150,130).

Depending upon the source of carotenoid biosynthesis gene(s), it may be necessary to codon-optimize at least a portion (up to the entire length) of the gene targeted for heterologous expression using the preferred codon usage within the host cell. The preferred codon usage for *Yarrowia lipolytica* has previously been reported (see U.S. Patent 7,125,672).



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Thus, in one embodiment the present invention provides a recombinant oleaginous yeast production host for the production of astaxanthin comprising:

a.) at least one copy of a *crtE* gene encoding a GGPP synthase;

b.) at least one copy of a *crtB* gene encoding a phytoene synthase;

c.) at least one copy of a *crtI* gene encoding a phytoene desaturase;

d.) at least one copy of a *crtY* gene encoding a lycopene cyclase;

e.) at least one copy of a *crtZ* gene encoding a carotenoid hydroxylase; and,

f.) at least one copy of a *crtW* gene encoding a carotenoid ketolase;

wherein said oleaginous yeast produces at least about 25 wt% of its dcw as oil.

Alternate embodiments of the present invention are drawn to a recombinant oleaginous yeast production host for the production of lycopene, comprising at least one copy each of *crtE, crtB* and *crtI* genes.

Similarly, alternate embodiments of the present invention are drawn to a recombinant oleaginous yeast production host for the production of β-carotene, comprising at least one copy each of *crtE, crtB*, *crtI* and *crtY* genes.

In additional embodiments of the present invention, a recombinant oleaginous yeast production host for the production of zeaxanthin is claimed, wherein the host comprises at least one copy each of *crtE, crtB*, *crtI*, *crtY* and *crtZ* genes.

Alternate embodiments of the present invention are drawn to a recombinant oleaginous yeast production host for the production of lutein, comprising at least one copy each of *crtE, crtB*, *crtI crtY, crtZ* and *lut1* genes.

Furthermore, the present invention provides a recombinant oleaginous yeast production host for the production of canthaxanthin, wherein the host comprises at least one copy each of *crtE, crtB*, *crtI*, *crtY* and *crtW* genes.



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In preferred embodiments, the recombinant oleaginous yeast production host in any of the above embodiments is a recombinant strain of *Yarrowia lipolytica*. In a further aspect, the recombinant oleaginous yeast production host produces at least about 30-40 wt%, and most preferably at least about 40-50 wt% microbially-produced oil, in addition to carotenoids.

Relatedly, the invention provides a method to produce a pigmented oleaginous yeast biomass comprising a carotenoid compound, comprising:

a.) providing any of the recombinant production hosts of the present invention expressing at least one copy each of *crtE, crtB* and *crtI* genes (and optionally expressing at least one copy each of *crtY, crtZ, crtW* and/or *lut1* genes, according to the requirements of Table 2), wherein said production host accumulates at least 25 wt% of its dcw as oil; and,

b.) culturing the recombinant production host under suitable conditions whereby pigmented oleaginous yeast biomass comprising a carotenoid compound is produced (wherein the preferred carotenoid compound is selected from the group consisting of: astaxanthin, β-carotene, lycopene, lutein, zeaxanthin and canthaxanthin).

Of course, a variety of means are available to increase the total amount of carotenoid that is produced in the above recombinant oleaginous yeast. For example, previous work in *Yarrowia lipolytica* has demonstrated that use of strong promoters, expression in multicopy, and/or codon-optimization of heterologous genes can very successfully be used as a means to increase expression. These tools should be equally applicable with respect to expression of carotenoid biosynthetic genes (e.g., encoded by *crtE, crtB*, *crtI*, *crtY, crtW, crtZ* and/or *lut1* genes). Thus, in preferred embodiments, the recombinant oleaginous yeast comprises a plurality of each codon-optimized *crt* gene required to product the desired carotenoid. For clarity, for example, if 2 copies of a *crtY* are required, this can refer to: (1) two copies of an identical coding sequence for a particular *crtY* isolated from a single species; or, (2) one coding sequence for *crtY*



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isolated from a species "A" and one coding sequence for a *crtY* isolated from a species "B", thus collectively resulting in two *crtY* genes. This strategy is exemplified in Example 10 in the β-carotene-producing *Yarrowia lipolytica* strain series YCS8700. Specifically, a single copy each of synthetic *crtE, crtB* and *crtI* genes (each codon-optimized for expression in *Y. lipolytica*) was expressed, in addition to 3 copies of different synthetic *crtY* genes (i.e., $crtY392_{syn}$, derived from *Pantoea stewartii* DC413; $crtY343_{syn}$, derived from *Enterobacteriaceae* DC260; and, $crtY334_{syn}$, derived from *Pantoea agglomerans* DC404), wherein each *crtY* was codon-optimized for expression in *Y. lipolytica*.

Manipulation of pathways and global regulators that affect production of carotenoids and/or down-regulate expression of specific genes within the carotenoid biosynthetic pathway that diminish overall accumulation of carotenoids are also contemplated as a means to increase carotenoid production. For example, it may be useful to increase the production of FPP to thereby enable increased production of carotenoids. Introducing and/or amplifying the *idi* gene (responsible for isomerization of IPP to dimethyl allyl diphosphate, a rate limiting step in the biosynthesis of carotenoids genes [Wang et al., *Biotechnol. Bioeng.,* 62:235-241 (1999)]) may accomplish this.

In certain embodiments of the invention, it will be desirable to accumulate carotenoids in the recombinant oleaginous host to levels (i.e., considering the total amount of all produced carotenoids together) that are greater than at least about 100 ppm, preferably at least about 500 ppm, more preferably at least about 1000 ppm, and even more preferably at least about 10,000 ppm. It should be noted that, for those recombinant oleaginous yeast hosts that produce more than one carotenoid, it will sometimes be possible to adjust the relative amounts of individual carotenoids produced by adjusting growth conditions. For example, it has been reported that controlling the concentration of dissolved oxygen in a culture during cultivation can regulate relative production levels of certain carotenoids such as β-carotene, echinenone, β-cryptoxanthin, 3-hydroxyechinenone, asteroidenone, canthaxanthin, zeaxanthin,



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adonirubin, adonixanthin and astaxanthin (see, for example, U.S. Patent 6,825,002).

Production Of Carotenoids And PUFAs In Oleaginous Yeast

In certain embodiments, the oleaginous yeast co-produces carotenoids and PUFAs. The importance of PUFAs is well understood. For example, certain PUFAs are important biological components of healthy cells and are recognized as: "essential" fatty acids that cannot be synthesized *de novo* in mammals and instead must be obtained either in the diet or derived by further desaturation and elongation of linoleic acid (LA; 18:2 ω-6) or α-linolenic acid (ALA; 18:3 ω-3); constituents of plasma membranes of cells, where they may be found in such forms as phospholipids or TAGs; necessary for proper development (particularly in the developing infant brain) and for tissue formation and repair; and, precursors to several biologically active eicosanoids of importance in mammals (e.g., prostacyclins, eicosanoids, leukotrienes, prostaglandins). Additionally, a high intake of long-chain ω-3 PUFAs has cardiovascular protective effects (Dyerberg, J. et al., *Amer. J. Clin. Nutr.*, 28:958-966 (1975); Dyerberg, J. et al., *Lancet,* 2(8081):117-119 (1978); Shimokawa, H., *World Rev. Nutr. Diet*, 88:100-108 (2001); von Schacky, C. and Dyerberg, J., *World Rev. Nutr. Diet*, 88:90-99 (2001)). Numerous other studies document wide-ranging health benefits conferred by administration of *ω*-3/*ω*-6 PUFAs against a variety of symptoms and diseases (e.g., asthma, psoriasis, eczema, diabetes, cancer).

Polyunsaturated Fatty Acid Definitions And Biosynthetic Pathway:

Nomenclature used to describe PUFAs is shown below in Table 3. In the column titled "Shorthand Notation", the omega-reference system is used to indicate the number of carbons, the number of double bonds and the position of the double bond closest to the omega carbon, counting from the omega carbon (which is numbered 1 for this purpose). The remainder of the Table summarizes the common names of ω-3 and ω-6 fatty acids and their precursors, the abbreviations that will be used throughout the specification and each compounds' chemical name.



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Table 3

Nomenclature of Various PUFAs And Precursors

| Common Name | Abbreviation | Chemical Name | Shorthand Notation |
|---|---|---|---|
| Myristic | -- | tetradecanoic | 14:0 |
| Palmitic | Palmitate | hexadecanoic | 16:0 |
| Palmitoleic | -- | 9-hexadecenoic | 16:1 |
| Stearic | -- | octadecanoic | 18:0 |
| Oleic | -- | *cis*-9-octadecenoic | 18:1 |
| Linoleic | LA | *cis*-9, 12-octadecadienoic | 18:2 ω-6 |
| γ-Linolenic | GLA | *cis*-6, 9, 12-octadecatrienoic | 18:3 ω-6 |
| Eicosadienoic | EDA | *cis*-11, 14- eicosadienoic | 20:2 ω-6 |
| Dihomo-γ Linolenic | DGLA | *cis*-8, 11, 14-eicosatrienoic | 20:3 -ω6 |
| Arachidonic | ARA | *cis*-5, 8, 11, 14-eicosatetraenoic | 20:4 ω–6 |
| α-Linolenic | ALA | *cis*-9, 12, 15-octadecatrienoic | 18:3 ω-3 |
| Stearidonic | STA | *cis*-6, 9, 12, 15-octadecatetraenoic | 18:4 ω-3 |
| Eicosatrienoic | ETrA | *cis*-11, 14, 17-eicosatrienoic | 20:3 ω-3 |
| Eicosa-tetraenoic | ETA | *cis*-8, 11, 14, 17-eicosatetraenoic | 20:4 ω-3 |
| Eicosa-pentaenoic | EPA | *cis*-5, 8, 11, 14, 17-eicosapentaenoic | 20:5 ω-3 |
| Docosa-pentaenoic | DPA | *cis*-7, 10, 13, 16, 19-docosapentaenoic | 22:5 ω-3 |
| Docosa-hexaenoic | DHA | *cis*-4, 7, 10, 13, 16, 19-docosahexaenoic | 22:6 ω-3 |

"Percent (%) PUFAs in the total lipid" refers to the percent of PUFAs relative to the total fatty acids in those fractions. As used herein, the term "total lipid fraction" or "lipid fraction" both refer to the sum of all lipids (i.e., neutral and polar) within an oleaginous organism, thus including those lipids that are located in the phosphatidylcholine fraction, phosphatidyletanolamine fraction and TAG (or oil) fraction. However, as used herein, the terms "lipid" and "oil" will be used interchangeably throughout the specification.



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The term "PUFA biosynthetic pathway" refers to a metabolic process that converts oleic acid to LA, EDA, GLA, DGLA, ARA, ALA, STA, ETrA, ETA, EPA, DPA and DHA. This process is well described in the literature (e.g., see PCT Publication No. WO 2006/052870). Simplistically, this process involves elongation of the carbon chain through the addition of carbon atoms and desaturation of the molecule through the addition of double bonds, via a series of special elongation and desaturation enzymes, respectively, (i.e., "PUFA biosynthetic pathway enzymes") present in the endoplasmic reticulim membrane. More specifically, "PUFA biosynthetic pathway enzyme" refers to any of the following enzymes (and genes which encode said enzymes) associated with the biosynthesis of a PUFA, including: a Δ9 elongase, a $C_{14/16}$ elongase, a $C_{16/18}$ elongase, a $C_{18/20}$ elongase, a $C_{20/22}$ elongase. a Δ4 desaturase, a Δ5 desaturase, a Δ6 desaturase, a Δ8 desaturase, Δ9 desaturase, a Δ12 desaturase, a Δ15 desaturase, and/or Δ17 desaturase.

The term "ω-3/ω-6 fatty acid biosynthetic pathway" refers to a set of genes which, when expressed under the appropriate conditions encode enzymes that catalyze the production of either or both ω-3 and ω-6 fatty acids. Typically the genes involved in the ω-3/ω-6 fatty acid biosynthetic pathway encode PUFA biosynthetic pathway enzymes.

More specifically, a representative pathway is illustrated in Figure 2, providing for the conversion of myristic acid through various intermediates to DHA, which demonstrates how both ω-3 and ω–6 fatty acids may be produced from a common source. The pathway is naturally divided into two portions wherein one portion will generate ω-3 fatty acids and the other portion, only ω-6 fatty acids. However, as seen in Figure 2 and as described below, there are often multiple alternate pathways for production of a specific PUFA.

All pathways require the initial conversion of oleic acid to LA, the first of the ω-6 fatty acids, by a Δ12 desaturase. Then, using the "Δ9 elongase/ Δ8 desaturase pathway" and LA as substrate, long-chain ω-6 and ω-3 fatty acids are formed as follows: (1) LA is converted to EDA by a



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Δ9 elongase; (2) EDA is converted to DGLA by a Δ8 desaturase; (3) DGLA is converted to ARA by a Δ5 desaturase; and, (4) ARA is converted to EPA by Δ17 desaturase. Alternatively, the "Δ9 elongase/ Δ8 desaturase pathway" can use ALA as substrate to produce long-chain ω-3 fatty acids as follows: (1) LA is converted to ALA, the first of the ω-3 fatty acids, by a Δ15 desaturase; (2) ALA is converted to ETrA by a Δ9 elongase; (3) ETrA is converted to ETA by a Δ8 desaturase; (4) ETA is converted to EPA by a Δ5 desaturase; (5) EPA is converted to DPA by a $C_{20/22}$ elongase; and, (6) DPA is converted to DHA by a Δ4 desaturase. Optionally, ω-6 fatty acids may be converted to ω-3 fatty acids; for example, ETA and EPA are produced from DGLA and ARA, respectively, by Δ17 desaturase activity.

Alternate pathways for the biosynthesis of ω-3/ω-6 fatty acids utilize a Δ6 desaturase and $C_{18/20}$ elongase (i.e., the "Δ6 desaturase/ Δ6 elongase pathway"). More specifically, LA and ALA may be converted to GLA and STA, respectively, by a Δ6 desaturase; then, a $C_{18/20}$ elongase converts GLA to DGLA and/or STA to ETA. Downstream PUFAs are subsequently formed as described above.

As used herein, the term "functional" in context with the ω-3/ω-6 fatty acid biosynthetic pathway means that some (or all of) the genes in the pathway express active enzymes, resulting in *in vivo* catalysis or substrate conversion. It should be understood that "ω-3/ω-6 fatty acid biosynthetic pathway" or "functional ω-3/ω-6 fatty acid biosynthetic pathway" does not imply that all the genes listed in the above paragraphs are required, as a number of fatty acid products will only require the expression of a subset of the genes of this pathway.

As used herein, the term "desaturase" refers to a polypeptide that can desaturate, i.e., introduce a double bond, in one or more fatty acids to produce a fatty acid or precursor of interest. Despite use of the omega-reference system throughout the specification to refer to specific fatty acids, it is more convenient to indicate the activity of a desaturase by counting from the carboxyl end of the substrate using the delta-system. Of



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particular interest herein are: 1) Δ5 desaturases that catalyze the conversion of DGLA to ARA and/or ETA to EPA; 2.) Δ17 desaturases that desaturate a fatty acid between the 17th and 18th carbon atom numbered from the carboxyl-terminal end of the molecule and which, for example, catalyze the conversion of ARA to EPA and/or DGLA to ETA; 3.) Δ6 desaturases that catalyze the conversion of LA to GLA and/or ALA to STA; 4.) Δ12 desaturases that catalyze the conversion of oleic acid to LA; 5.) Δ15 desaturases that catalyze the conversion of LA to ALA and/or GLA to STA; 6.) Δ4 desaturases that catalyze the conversion of DPA to DHA; 7.) Δ8 desaturases that catalyze the conversion of EDA to DGLA and/or ETrA to ETA; and, 8.) Δ9 desaturases that catalyze the conversion of palmitate to palmitoleic acid (16:1) and/or stearate to oleic acid. In the art, Δ15 and Δ17 desaturases are also occasionally referred to as "omega-3 desaturases", "ω-3 desaturases", and/or "Δ-3 desaturases", based on their ability to convert ω-6 fatty acids into their ω-3 counterparts (e.g., conversion of LA into ALA and ARA into EPA, respectively). In some embodiments, it is most desirable to empirically determine the specificity of a particular fatty acid desaturase by transforming a suitable host with the gene for the fatty acid desaturase and determining its effect on the fatty acid profile of the host.

As used herein, the term "elongase" refers to a polypeptide that can elongate a fatty acid carbon chain to produce an acid that is 2 carbons longer than the fatty acid substrate that the elongase acts upon. This process of elongation occurs in a multi-step mechanism in association with fatty acid synthase, as described in PCT Publication No. WO 2005/047480. Examples of reactions catalyzed by elongase systems are the conversion of GLA to DGLA, STA to ETA and EPA to DPA. In general, the substrate selectivity of elongases is somewhat broad but segregated by both chain length and the degree and type of unsaturation. For example, a $C_{14/16}$ elongase will utilize a $C_{14}$ substrate (e.g., myristic acid), a $C_{16/18}$ elongase will utilize a $C_{16}$ substrate (e.g., palmitate), a $C_{18/20}$ elongase will utilize a $C_{18}$ substrate (e.g., GLA, STA) and a $C_{20/22}$ elongase



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will utilize a $C_{20}$ substrate (e.g., EPA). In like manner, a Δ9 elongase is able to catalyze the conversion of LA and ALA to EDA and ETrA, respectively. It is important to note that some elongases have broad specificity and thus a single enzyme may be capable of catalyzing several elongase reactions (e.g., thereby acting as both a $C_{16/18}$ elongase and a $C_{18/20}$ elongase).

Genetically Engineered Oleaginous Yeast For PUFA Production:

As described above, oleaginous yeast can be engineered to produce ω-3/ω-6 PUFAs by integration of appropriate heterologous genes encoding desaturases and elongases of the Δ6 desaturase/Δ6 elongase pathway or the Δ9 elongase/Δ8 desaturase pathway into the host organism for production of any particular PUFA of interest. Preferred genes and considerations for choosing a specific polypeptide having desaturase or elongase activity are detailed in U.S. Patent Application No. 11/198975, and Publication No. US-2006-0110806, as are details concerning additional modifications that may be required to enable high level production of a particular PUFA, including: (1) manipulation of the activity of acyltransferases that allow for the transfer of omega fatty acids into storage lipid pools (i.e., the TAG fraction); (2) over-expression of desaturases, elongases and diacylglycerol cholinephosphotransferases by use of strong promoters, expression in multicopy, and/or codon-optimization; (3) down-regulation of the expression of specific genes that diminish overall accumulation of the desired PUFA; (4) manipulation of pathways and global regulators that affect production of the desired PUFA; and, (5) "pushing/pulling" within the PUFA biosynthetic pathway. The term "high-level production" refers to production of at least about 5-10% of the desired PUFA (i.e., LA, ALA, EDA, GLA, STA, ETrA, DGLA, ETA, ARA, EPA, DPA and/or DHA) in the total lipids of the microbial host, preferably at least about 10-25% of the desired PUFA in the total lipids, more preferably at least about 25-35% of the desired PUFA in the total lipids, more preferably at least about 35-45% of the desired PUFA in the total lipids, and most preferably at least about 45-55% of the desired PUFA in the total lipids. The structural form of the PUFA is not limiting;



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thus, for example, the EPA may exist in the total lipids as free fatty acids or in esterified forms such as acylglycerols, phospholipids, sulfolipids or glycolipids.

Although numerous oleaginous yeast could be engineered for production of preferred ω-3/ ω-6 PUFAs based on the teachings in the present invention and in co-pending U.S. Patent Applications No. 11/198975, No. 11/265761, No. 11/264784, and No. 11/264737, representative strains of the oleaginous yeast *Yarrowia lipolytica* are described in Table 4. These include the following strains that have been deposited with the ATCC: *Y. lipolytica* strain Y2047 (producing ARA; ATCC Accession No. PTA-7186); *Y. lipolytica* strain Y2096 (producing EPA; ATCC Accession No. PTA-7184); *Y. lipolytica* strain Y2201 (producing EPA; ATCC Accession No. PTA-7185); and, *Y. lipolytica* strain Y3000 (producing DHA ATCC Accession No. PTA-7187). Additionally, *Y. lipolytica* strain Y4128; ATCC Accession No. PTA-8614) is described in U.S. Patent Application No. 60/977177 (filed October 3, 2007). Example 7 herein describes *Y. lipolytica* strain Y4127 (producing EPA; ATCC Accession No. PTA-[XXXX]).



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Table 4

Lipid Profile Of Representative *Yarrowia lipolytica* Strains Engineered To Produce ω-3/ω-6 PUFAs

| Strain | Reference | ATCC Deposit No. | Fatty Acid Content (As A Percent [%] of Total Fatty Acids) | | | | | | | | | | | | | Lipid % dcw |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 16:00 | 16:01 | 18:00 | 18:01 | 18:02 | GLA | 20:02 | DGLA | ARA | ETA | EPA | DPA | DHA | |
| Wildtype | US 2006-0035351-A1; WO2006/033723 | #76982 | 14 | 11 | 3.5 | 34.8 | 31 | 0 | -- | -- | -- | -- | -- | -- | -- | -- |
| pDMW208 | | -- | 11.9 | 8.6 | 1.5 | 24.4 | 17.8 | 25.9 | -- | -- | -- | -- | -- | -- | -- | -- |
| pDMW208D62 | | -- | 16.2 | 1.5 | 0.1 | 17.8 | 22.2 | 34 | -- | -- | -- | -- | -- | -- | -- | -- |
| M4 | US 2006-0115881-A1; WO2006/052870 | -- | 15 | 4 | 2 | 5 | 27 | 35 | -- | 8 | 0 | 0 | 0 | -- | -- | -- |
| Y2034 | US 2006-0094092-A1; WO2006/055322 | -- | 13.1 | 8.1 | 1.7 | 7.4 | 14.8 | 25.2 | -- | 8.3 | 11.2 | -- | -- | -- | -- | -- |
| Y2047 | | PTA-7186 | 15.9 | 6.6 | 0.7 | 8.9 | 16.6 | 29.7 | -- | 0 | 10.9 | -- | -- | -- | -- | -- |
| Y2214 | | -- | 7.9 | 15.3 | 0 | 13.7 | 37.5 | 0 | -- | 7.9 | 14 | -- | -- | -- | -- | -- |
| EU | US 2006-0115881-A1; WO2006/052870 | -- | 19 | 10.3 | 2.3 | 15.8 | 12 | 18.7 | -- | 5.7 | 0.2 | 3 | 10.3 | -- | -- | 36 |
| Y2072 | | -- | 7.6 | 4.1 | 2.2 | 16.8 | 13.9 | 27.8 | -- | 3.7 | 1.7 | 2.2 | 15 | -- | -- | -- |
| Y2102 | | -- | 9 | 3 | 3.5 | 5.6 | 18.6 | 29.6 | -- | 3.8 | 2.8 | 2.3 | 18.4 | -- | -- | -- |
| Y2088 | | -- | 17 | 4.5 | 3 | 2.5 | 10 | 20 | -- | 3 | 2.8 | 1.7 | 20 | -- | -- | -- |
| Y2089 | | -- | 7.9 | 3.4 | 2.5 | 9.9 | 14.3 | 37.5 | -- | 2.5 | 1.8 | 1.6 | 17.6 | -- | -- | -- |
| Y2095 | | -- | 13 | 0 | 2.6 | 5.1 | 16 | 29.1 | -- | 3.1 | 1.9 | 2.7 | 19.3 | -- | -- | -- |
| Y2090 | | -- | 6 | 1 | 6.1 | 7.7 | 12.6 | 26.4 | -- | 6.7 | 2.4 | 3.6 | 26.6 | -- | -- | 22.9 |
| Y2096 | | PTA-7184 | 8.1 | 1 | 6.3 | 8.5 | 11.5 | 25 | -- | 5.8 | 2.1 | 2.5 | 28.1 | -- | -- | 20.8 |
| Y2201 | | PTA-7185 | 11 | 16.1 | 0.7 | 18.4 | 27 | -- | 3.3 | 3.3 | 1 | 3.8 | 9 | -- | -- | -- |
| Y3000 | US 2006-0110806-A1; WO2006/052871 | PTA-7187 | 5.9 | 1.2 | 5.5 | 7.7 | 11.7 | 30.1 | -- | 2.6 | 1.2 | 1.2 | 4.7 | 18.3 | 5.6 | -- |



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Genetically Engineered Oleaginous Yeast For Carotenoid Production And PUFA Production:

Lipid peroxidation, which leads to rancidity in oils, most often affects PUFAs, because they contain multiple double bonds in between which lie methylene (i.e., $-CH_2$) groups that are especially reactive to hydrogen. The oxygen-dependent deterioration can occur non-enzymatically via a free radical chain reaction mechanism (i.e., autoxidation) or via photo-oxygenation; additionally, fatty acids may also be peroxidized via enzymatic peroxidation (i.e., via lipoxygenase enzymes). Furthermore, fatty acids may be autoxidized either in free form or combined into glycerolipids or glycolipids. Thus, oxidized triacylglycerol monomers include molecules containing different oxygenated groups, mainly hydroxyl-, keto- and epoxy-, as well as short-chain fatty acyl and short-chain n-oxo fatty acyl groups as the main products (Chang, S.S. et al., *JOACS,* 55:718 (1978); Velasco, J. et al., *Eur. J. Lipid Sci. Technol.,* 106:728 (2004)). Autoxidation of fatty acids with more than 3 double bonds leads to even more complex mixtures of product (e.g., see Porter, N.A. et al., *J. Am. Chem. Soc.,* 103:6447 (1981) and Bruna, E. et al., *Lipids*, 24:970 (1990) concerning autoxidation of arachidonic, pentaenoic and hexaenoic acids).

Based on the above, a recombinant oleaginous yeast engineered to co-produce both carotenoids and PUFAs should prove advantageous. Carotenoids are themselves generally classified as antioxidants; and, in certain product applications the carotenoid is not used as a pigment but is used as a natural antioxidant (e.g., use of lycopene as an antioxidant in food products and/or animal feeds). Thus, it is expected that the presence of the carotenoids should minimize overall lipid peroxidation and thus stabilize the PUFAs during production and/or storage.

In alternate embodiments wherein the utility of the carotenoid is as a pigment versus as an antioxidant, it may be advantageous to produce a single product that comprises both carotenoids and PUFAs to simplify subsequent formulations and manufacture (e.g., aquaculture feeds often



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require at least carotenoid pigment [i.e., canthaxanthin and/or astaxanthin] and ω-3 PUFAs).

One embodiment of the present invention encompasses a recombinant oleaginous yeast production host for the production of carotenoids and PUFAs comprising:

a) a functional carotenoid biosynthetic pathway, wherein expression of said carotenoid biosynthetic pathway results in the production of at least one carotenoid selected from the group consisting of: lycopene, β-carotene, canthaxanthin, zeaxanthin, lutein and astaxanthin; and,

b) a functional ω-3/ω-6 PUFA biosynthetic pathway, wherein expression of said ω-3/ω-6 PUFA biosynthetic pathway results in the production of at least one PUFA selected from the group consisting of: an ω-6 PUFA and an ω-3 PUFA;

wherein said oleaginous yeast produces at least about 25 wt% of its dcw as oil.

Similarly, the invention comprises a method to produce a pigmented oleaginous yeast biomass comprising a carotenoid compound and a PUFA, comprising: a.) providing the recombinant production host described above, wherein said production host accumulates at least 25 wt% of its dcw as oil and wherein said product host comprises a functional carotenoid biosynthetic pathway and a functional ω-3/ω-6 PUFA biosynthetic pathway; and, b.) culturing the recombinant production host under suitable conditions whereby pigmented oleaginous yeast biomass is produced comprising a carotenoid and an ω-3 and/or an ω-6 PUFA (preferably selected from the group consisting of LA, ALA, EDA, GLA, STA, ETrA, DGLA, ETA, ARA, EPA, DPA and DHA).

In preferred embodiments, the oleaginous yeast is a recombinant strain of *Yarrowia lipolytica* that has been previously engineered for high-level production of a preferred ω-3/ω-6 PUFA. Upon transformation with a functional carotenoid biosynthetic pathway using the methodology described previously, the resultant recombinant yeast will co-produce carotenoids and PUFAs.



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Production Of Carotenoids And Additional Antioxidants In Oleaginous Yeast

In some embodiments, the oleaginous yeast co-produces carotenoids and at least one additional antioxidant (i.e., wherein the additional antioxidant is not a carotenoid). Antioxidants slow the rate of oxidation reactions or that can counteract the damaging effects of oxygen. A variety of nutrients or dietary components have antioxidant properties and thus can function to decrease the tissue content of reactive oxygen. Common antioxidants include vitamins C and E, β-carotene, proanthocyanidin, the minerals selenium and zinc, and coenzyme Q. Similarly, resveratrol is a potent antioxidant reported to have numerous beneficial effects.

The antioxidant properties of CoQ are significant. In addition to quenching free radicals that threaten cellular components (e.g., nucleic acids, proteins) in the mitochondria, ubiquinol also inhibits lipid peroxidation (i.e., degradation of lipids) in biological membranes and in low-density lipoprotein (LDL). As such, the coenzyme has become widely used as a nutritional supplement and as a pharmacological active agent. It has wide use and acceptance in the treatment of: mitochondrial disorders, cardiovascular disease processes, atherosclerosis, slow muscle degeneration (dystrophy or atrophy), neurodegenerative diseases (e.g., Parkinson's disease, Huntington's disease, Alzheimer's, amyotrophic lateral sclerosis (ALS)), periodontal disease, diabetes and CoQ deficiencies. $CoQ_{10}$ is also believed to strengthen the immune system, act as an anticancer agent and help counteract the aging processes.

The antioxidant properties of resveratrol decrease LDL oxidation, a factor associated with the development of atherosclerosis (Manna *et al.*, *J. Immunol.*, 164:6509-6519 (2000)). It is also reported to lower serum cholesterol levels and the incidents of heart disease. This effect as been attributed to a phenomenon known and the "French Paradox". French citizens that regularly consume red wine tend to have lower incidents of heart disease and serum cholesterol levels even though this same group tends to consume foods high in both fat and cholesterol. There is also



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evidence that resveratrol may have other cardiovascular protective effects including modulation of vascular cell function, suppression of platelet aggregation, and reduction of myocardial damage during ischemia-reperfusion (Bradamante *et al.*, *Cardiovasc. Drug. Rev.*, 22(3):169-188 (2004)). Resveratrol is reported to have anti-inflammatory effects associated with the inhibition of the cyclooxygenase-1 (Cox-1), an enzyme associated with the conversion of ARA to pro-inflammatory mediators. It may also aid in the inhibition of carcinogenesis (Schultz, J., *J Natl Cancer Inst.*, 96(20):1497-1498 (2004); Scifo *et al.*, *Oncol Res.*, 14(9):415-426 (2004); and Kundu, J. and Surh, Y., *Mutat Res.*, 555(1-2):65-80 (2004)).

Resveratrol is classified as a phytoalexin due to its antifungal properties. It appears that some plants (e.g., red grapes) produce resveratrol as natural defense mechanism against fungal infections. Transgenic plants modified to express the resveratrol synthase gene exhibit improved resistance to fungal infections. Furthermore, it has been reported that treatment of fresh fruits and vegetables with an effective amount of resveratrol will significantly increase shelf life (Gonzalez-Urena et al., *J. Agric. Food Chem.*, 51:82-89 (2003)).

Although $CoQ_{10}$ is currently available via chemical synthesis, semi-chemical synthesis and microbial conversion (Choi, Jin-Ho et al., *Appl. Microbiol. Biotechnol.*, 68:9-15 (2005)), use of resveratrol in commercial products is limited due to the current market price of the compound. Engineering an oleaginous microorganism to simultaneously produce both carotenoids and at least one additional antioxidant (e.g., CoQ, resveratrol, or mixtures thereof) could create a higher value product or reduce production costs, if a synthetic antioxidant was no longer required, safety concerns with the synthetic antioxidant were avoided, or difficult co-formulations from individual ingredients were unnecessary.

Coenzyme Q Definitions And Biosynthetic Pathway: The term coenzyme Q refers to a series of related 2-3-dimethoxy-5-methyl-benzoquinones (i.e., known as $CoQ_6$, $CoQ_7$, $CoQ_8$, $CoQ_9$ and $CoQ_{10}$) with a polyisoprenoid side chain in the 6-position that are widely distributed in animals, plants and microorganisms. The quinones of the CoQ series



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found in various biological species differ only slightly in chemical structure based on the length of the hydrocarbon tail and which facilitates CoQ's localization in mitochondrial or cytoplasmic membranes. Differences in properties are due to the differences in length of the side chain.

The biochemical reactions that lead to production of $CoQ_6$, $CoQ_7$, $CoQ_8$, $CoQ_9$ and $CoQ_{10}$ are well-described in the minireview of R. Meganathan (*FEMS Microbiol. Lett.*, 203:131-139 (2001)) for *Escherichia coli* and *Saccharomyces cerevisiae* and are summarized in Figures 3A and 3B herein. In brief, the quinonoid nucleus is derived from the shikimate pathway via chorismate or tyrosine. Formation of 4-hydroxybenzoate is the first committed step in the biosynthesis of CoQ. This molecule then undergoes prenylation, wherein the polyprenyl side chain of the final CoQ enzyme is added.

*Prenylation*: The polyprenyl side chain itself is formed via the mevalonate pathway, which results in the formation of the $C_{15}$ unit FPP from the starting precursor, acetyl-CoA (as previously described *supra*). FPP then reacts with a prenyl diphosphate synthase, to produce the final polyprenyl side chain having from 30-50 carbon atoms (corresponding to 6, 7, 8, 9 or 10 isoprenoid units in the side chain). The precise length of the polyprenyl side chain present in a particular organism is constant and is determined by the nature of the prenyl diphosphate synthase.

More specifically, the term "prenyl diphosphate synthase" is used as a general term for those enzymes which catalyze a condensation reaction that polymerizes prenyl diphosphate (an allylic primer) and IPP to produce polyprenyl diphosphate. This class of genes is divided into 2 types, based on whether the condensation reaction of IPP is *cis*- [or (Z)-chain] elongation or *trans*- [or (E)-chain] elongation. Bacterial prenyl diphosphate synthases are classified into the following four groups: (1) Prenyl diphosphate synthase I comprising E type, short chain prenyl diphosphate synthases such as geranyl diphosphate synthase (catalyzing a $C_5 \rightarrow C_{10}$ reaction), farnesyl diphosphate synthase (catalyzing a $C_5 \rightarrow C_{15}$ reaction) and geranylgeranyl diphosphate synthase (catalyzing a $C_5 \rightarrow C_{20}$ reaction); (2) Prenyl diphosphate synthase II comprising E type, medium



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chain prenyl diphosphate synthases such as hexaprenyl diphosphate synthase (HexPP; catalyzing a $C_{15} \rightarrow C_{30}$ reaction) and heptaprenyl diphosphate synthase (HepPP; catalyzing a $C_{15} \rightarrow C_{35}$ reaction); (3) Prenyl diphosphate synthase III comprising E type, long chain prenyl diphosphate synthases such as octaprenyl diphosphate synthase (OctPP; catalyzing a $C_{15} \rightarrow C_{40}$ reaction), solanesyl [or nonaprenyl] diphosphate synthase (SPP or NonPP; catalyzing a $C_{15} \rightarrow C_{45}$ reaction) and decaprenyl diphosphate synthase (DecPP; catalyzing a $C_{15} \rightarrow C_{50}$ reaction); and (4) Prenyl diphosphate synthase IV comprising Z type, long chain prenyl diphosphate synthases such as Z-nonaprenyl diphosphate synthase (catalyzing a $C_{15} \rightarrow C_{45}$ reaction), undecaprenyl diphosphate synthase (UPP; catalyzing a $C_{15} \rightarrow C_{55}$ reaction) and dehydrodolichyl diphosphate synthase (deDolPP; catalyzing a $C_{15} \rightarrow C_{85\text{-}105}$ reaction) (reviewed in Szkopińska, A., *Acta Biochimica Polonica*, 47(2):469-480 (2000)).

*Subsequent Ring Modifications:* Prenylation results in the formation of 3-polyprenyl-4-hydroxybenzoate. This molecule then undergoes multiple ring modifications (including hydroxylation, methylation and decarboxylation) to result in formation of ubiquinol, which is non-enzymatically converted to ubiquinone (i.e., CoQ).

A "functional" CoQ biosynthetic pathway thus necessarily requires at least one gene encoding a phenyl diphosphate synthase that is expressed as an active enzyme and which therefore results in production of $CoQ_6$, $CoQ_7$, $CoQ_8$, $CoQ_9$ and/or $CoQ_{10}$.

Genetically Engineered Oleaginous Yeast For CoQ Production:

The wildtype oleaginous yeast *Yarrowia lipolytica* contains all of the genes required for $CoQ_9$ production (producing at least about 2000 ppm under typical growth conditions) (Example 15). Analysis of the microbial biomass indicates that the concentration of $CoQ_9$ therein may be sufficient to reduce and/or eliminate the need to the microbial biomass/oils (and any products contained therein e.g., carotenoids, PUFAs) with additional antioxidants.

Means to increase production of $CoQ_9$ in this organism would likely require genetic manipulation. However, the genes required for CoQ



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biosynthesis in *Yarrowia lipolytica* have not been extensively characterized. Despite this, based on the complete sequencing of the entire genome (Dujon, B. et al., *Nature,* 430 (6995):35-44 (2004)) and the public *Y. lipolytica* protein database of the "Yeast project *Genolevures*" (Center for Bioinformatics, LaBRI, Talence Cedex, France), one skilled in the art would readily be able to identify homologous genes corresponding to the *ubiC, ubiA, ubiD, ubiX, ubiB, ubiG, ubiH, ubiE* and *ubiF* genes of *Escherichia coli* and/or the *coq2, coq3, coq6, coq5* and *coq7* genes of *Saccharomyces cerevisiae,* which are known to catalyze the reactions diagrammed in Figures 3A and 3B. Similarly, the initial and intermediate genes of the mevalonate pathway responsible for the biosynthesis of the polyprenyl side chain of $CoQ_9$ should also easily be identified (up to and including FPP synthase). The last gene required for synthesis of the polyprenyl side chain of *Yarrowia lipolytica*'s $CoQ_9$ is a SPP, which has not yet been identified or characterized. One of skill in the art would be able to devise a means to up-regulate genes that enable $CoQ_9$ production, following identification of the appropriate genes.

Production of alternate molecules of the CoQ series (i.e., $CoQ_6$, $CoQ_7$, $CoQ_8$ or $CoQ_{10}$) would require disruption of the native SPP and expression of a heterologous HexPP, HepPP, OctPP or DecPP (see, for example, U.S. Patent Application No 60/991266 (filed November 30, 2007), which describes means to engineer $CoQ_{10}$ production in *Yarrowia lipolytica*). Studies summarized in Meganathan (*supra*) have demonstrated that mutant bacteria and yeast expressing a non-native prenyl diphosphate synthase that results in a side chain of different length than in the parent do not affect the mutant strain.

Genetically Engineered Oleaginous Yeast For Carotenoid And CoQ Production: In one embodiment the present invention encompasses a recombinant oleaginous yeast production host for the production of carotenoids and an additional antioxidant comprising:

a.) a functional carotenoid biosynthetic pathway, wherein expression of said carotenoid biosynthetic pathway results in the production of at least one carotenoid selected from the group consisting of:



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lycopene, β-carotene, canthaxanthin, zeaxanthin, lutein and astaxanthin; and,

b.) a functional CoQ biosynthetic pathway, wherein expression of said CoQ biosynthetic pathway results in the production of a CoQ selected from the group consisting of $CoQ_6$, $CoQ_7$, $CoQ_8$, $CoQ_9$ and $CoQ_{10}$;

wherein said oleaginous yeast produces at least about 25 wt% of its dcw as oil.

The CoQ produced by *Yarrowia lipolytica* and derivatives thereof during fermentation is typically in a substantially reduced state. As used herein, "substantially reduced form of CoQ" or "substantially reduced" refers a condition where the majority of the CoQ present in a composition is in the reduced form. In one aspect, substantially reduced refers to a condition where at least about 50% of the CoQ (percentage based on total CoQ present) in a composition is in the reduced state, preferably at least about 70-80% is in the reduced form, more preferably at least about 80-90% is in the reduced form and even more preferably at least about 90-98% is in the reduced form.

Similarly, the invention comprises a method to produce a pigmented oleaginous yeast biomass comprising a carotenoid and an additional antioxidant, comprising: a.) providing the recombinant production host described above, wherein said production host accumulates at least 25 wt% of its dcw as oil and wherein said product host comprises a functional carotenoid biosynthetic pathway and a functional CoQ biosynthetic pathway; and, b.) culturing the recombinant production host under suitable conditions whereby pigmented oleaginous yeast biomass is produced comprising a carotenoid and an additional antioxidant selected from the group consisting of $CoQ_6$, $CoQ_7$, $CoQ_8$, $CoQ_9$ and $CoQ_{10}$.

In preferred embodiments, the oleaginous yeast is a recombinant strain of *Yarrowia lipolytica* that has the natural ability to produce $CoQ_9$ (i.e., the pathway is not genetically engineered). Upon transformation with a functional carotenoid biosynthetic pathway using the methodology



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described previously, the resultant recombinant yeast will co-produce carotenoids and $CoQ_9$.

In alternate preferred embodiments, the oleaginous yeast is engineered to produce $CoQ_{10}$ (preferably at least about 0.0001%-0.01% of the total dcw, and more preferably at least about 0.01%-1% of the total dcw), prior to transformation with a functional carotenoid biosynthetic pathway.

Resveratrol Definitions And Biosynthetic Pathway: Production of resveratrol (*trans*-3,4',5-trihydroxystilbene) in *Yarrowia lipolytica* has been reported (U.S. Patent Application No. 11/436182. Specifically, a recombinant host cell producing significant quantities of the antioxidant was created by introducing several genes from the phenylpropanoid pathway in combination with a suitable gene encoding resveratrol synthase (Figure 4). A resveratrol synthase is a type III polyketide synthase (E. C. 2.3.1.95) that condenses one molecule of *p*-coumaroyl CoA with 3 molecules of malonyl CoA to produce 1 molecule of resveratrol. This, in combination with the natural ability of the oleaginous host cell to produce a suitable amount of malonyl CoA, enabled production of resveratrol.

More specifically, suitable combinations of genes from the phenylpropanoid pathway were selected from the following: 1.) at least one gene encoding a "tyrosine ammonia lyase" or "TAL" (EC 4.3.1.-) that catalyzes the direct conversion of tyrosine to *p*-hydroxycinnamic acid (pHCA) and at least one gene encoding a "coumaroyl CoA ligase" (E.C. 6.2.1.12), to enable conversion of pHCA into *p*-coumaroyl CoA; 2.) at least one gene encoding a "phenylalanine ammonia-lyase" or "PAL" (E.C. 4.3.1.5) that catalyzes the conversion of phenylalanine to *trans*-cinnamic acid, at least one gene encoding a "cinnamate 4-hydroxylase" (E.C. 1.14.13.11) that converts *trans*-cinnamic acid to pHCA, and at least one gene encoding a coumaroyl CoA ligase; 3.) at least one gene encoding a "phenylalanine/tyrosine ammonia lyase" or "PAL/TAL" (EC 4.3.1.-), wherein the enzyme has both PAL activity and TAL activity to thereby convert phenylalanine to *trans*-cinnamic acid and tyrosine to pHCA, at



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least one gene encoding a coumaroyl CoA ligase, and optionally at least one gene encoding a cinnamate 4-hydroxylase.

Although there are many sources for each of the genes in the resveratrol biosynthesis pathway, as exemplified in PCT Publication No. WO 2006/125000, a resveratrol synthase from *Vitis sp.*, a coumaroyl CoA ligase from *Streptomyces coelicolor* (GenBank® Accession No. AL939119), and a PAL (having some TAL activity) from *Rhodotorula glutinis* (GenBank® Accession No. X12702) were each codon optimized and recombinantly expressed in *Yarrowia lipolytica*, resulting in the production of resveratrol. Genes encoding the enzymes phenylalanine hydroxylase, acetyl CoA carboxylase, and β-glucosidase could also be engineered into the recombinant host for increased resveratrol production.

Genetically Engineered Oleaginous Yeast For Carotenoid And Resveratrol Production: In one embodiment the present invention encompasses a recombinant oleaginous yeast production host for the production of carotenoids and resveratrol comprising:

a.) a functional carotenoid biosynthetic pathway, wherein expression of said carotenoid biosynthetic pathway results in the production of at least one carotenoid selected from the group consisting of: lycopene, β-carotene, canthaxanthin, zeaxanthin, lutein and astaxanthin; and,

b.) a functional phenylpropanoid biosynthetic pathway, wherein expression of said phenylpropanoid biosynthetic pathway results in the production of coumaroyl CoA; and,

c.) a functional resveratrol synthase, wherein expression of said gene results in the conversion of coumaroyl CoA to resveratrol;

wherein said oleaginous yeast produces at least about 25 wt% of its dcw as oil.

Similarly, the invention comprises a method to produce a pigmented oleaginous yeast biomass comprising a carotenoid compound and resveratrol, comprising: a.) providing the recombinant production host described above, wherein said production host accumulates at least 25 wt% of its dcw as oil and wherein said production host comprises a



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functional carotenoid biosynthetic pathway and a functional biosynthetic pathway for resveratrol production (i.e., comprising a functional phenylpropanoid biosynthetic pathway as described above and a resveratrol synthase); and, b.) culturing the recombinant production host under suitable conditions whereby pigmented oleaginous yeast biomass is produced comprising a carotenoid and resveratrol.

In preferred embodiments, the oleaginous yeast is a recombinant strain of *Yarrowia lipolytica* that has been previously engineered for high-level production of resveratrol, via expression of a PAL/TAL, a coumaroyl CoA ligase, and a resveratrol synthase. Upon transformation with a functional carotenoid biosynthetic pathway using the methodology described previously, the resultant recombinant yeast will co-produce carotenoids and at least 0.01 wt% resveratrol and/or resveratrol glucoside.

Genetically Engineered Oleaginous Yeast For Carotenoid, PUFA And Antioxidant Production

The invention provides recombinant oleaginous yeast able to produce carotenoids, and $C_{40}$ carotenoids in particular. The yeast is effectively transformed with the genetic elements of the $C_{40}$ carotenoid biosynthetic pathway as described herein, wherein Crt enzymes of particular usefulness include, but are not limited to phytoene synthase (*crtB),* phytoene desaturase (*crtI)*, lycopene cyclase (*crtY*), carotenoid hydroxylase (*crtZ*), a carotenoid ketolase (*crtW*) and a carotenoid $\epsilon$–hydroxylase (*lut1*). Carotenoids of particular relevance in the present invention include but are not limited to: lycopene, β-carotene, zeaxanthin, lutein, canthaxanthin and astaxanthin.

Similarly, the same host expressing various *crt* genes may also be engineered to express PUFA biosynthetic pathways as described herein. PUFAs of particular interest in the present invention are $\omega$-3 PUFAs including ALA, STA, ETrA, ETA, EPA, DPA and DHA and $\omega$-6 PUFAs including LA, GLA, EDA, DGLA and ARA. PUFA synthesis may take many paths but generally utilizes enzymes selected from the group consisting of desaturases (e.g., Δ4, Δ5, Δ6, Δ8, Δ9, Δ12, Δ15, Δ17



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desaturases) and elongases (e.g., Δ9 elongases and $C_{14/16}$, $C_{16/18}$, $C_{18/20}$ and $C_{20/22}$ elongases).

Similarly, the same host expressing various *crt* genes and PUFA biosynthetic genes may also be engineered to express additional antioxidant biosynthetic pathways as described herein. Antioxidants of particular interest in the present invention are those of the CoQ series and resveratrol. Resveratrol biosynthesis preferentially requires expression of a PAL/TAL, a coumaroyl CoA ligase, and a resveratrol synthase, while coenzyme Q biosynthesis requires expression of a prenyl diphosphate synthase (e.g., HexPP, HepPP, OctPP, SPP or DecPP).

These host may be genetically engineered using methods well known in the art.

Microbial Expression Systems, Cassettes & Vectors, And Transformation

Microbial expression systems and expression vectors containing regulatory sequences that direct high level expression of foreign proteins are well known to those skilled in the art. Any of these could be used to construct chimeric genes for production of the desired compound(s) (i.e., carotenoids and optionally PUFAs and/or additional antioxidants). These chimeric genes could then be introduced into appropriate microorganisms via transformation to allow for high level expression of the enzymes.

Vectors (e.g., constructs, plasmids) and DNA expression cassettes useful for the transformation of suitable host cells are well known in the art. The specific choice of sequences present in the construct is dependent upon the desired expression products (*supra*), the nature of the host cell, and the proposed means of separating transformed cells versus non-transformed cells. Typically, however, the vector contains at least one expression cassette, a selectable marker and sequences allowing autonomous replication or chromosomal integration. Suitable expression cassettes comprise a region 5' of the gene that controls transcriptional initiation (e.g., a promoter), the gene coding sequence, and a region 3' of the DNA fragment that controls transcriptional termination (i.e., a terminator). It is most preferred when both control regions are derived from genes from the transformed host cell, although it is to be understood



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that such control regions need not be derived from the genes native to the specific species chosen as a production host.

Initiation control regions or promoters, which are useful to drive expression of the relevant genes in the desired yeast host cell are numerous and familiar to those skilled in the art. Virtually any promoter capable of directing expression of these genes in the selected host cell is suitable for the present invention. Expression in a host cell can be accomplished in a transient or stable fashion. Transient expression can be accomplished by inducing the activity of a regulatable promoter operably linked to the gene of interest. Stable expression can be achieved by the use of a constitutive promoter operably linked to the gene of interest. As an example, when the host cell is yeast, transcriptional and translational regions functional in yeast cells are provided, particularly from the host species (e.g., see U.S. Patent 7,238,482 and PCT Publication No. WO 2006/052870 [U.S. Publication 2006-0115881-A1] for preferred transcriptional initiation regulatory regions for use in *Yarrowia lipolytica*). Any one of a number of regulatory sequences can be used, depending upon whether constitutive or induced transcription is desired, the efficiency of the promoter in expressing the ORF of interest, the ease of construction and the like.

Nucleotide sequences surrounding the translational initiation codon 'ATG' have been found to affect expression in yeast cells. If the desired polypeptide is poorly expressed in yeast, the nucleotide sequences of exogenous genes can be modified to include an efficient yeast translation initiation sequence to obtain optimal gene expression. For expression in yeast, this can be done by site-directed mutagenesis of an inefficiently expressed gene by fusing it in-frame to an endogenous yeast gene, preferably a highly expressed gene. Alternatively, as demonstrated in *Yarrowia lipolytica*, one can determine the consensus translation initiation sequence in the host and engineer this sequence into heterologous genes for their optimal expression in the host of interest (U.S. Patent 7,125,672).

Termination control regions may be derived from various genes native to the preferred hosts. Optionally, a termination site may be



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unnecessary; however, it is most preferred if included. As used herein, the termination region can be derived from the 3' region of the gene from which the initiation region was obtained or from a different gene. A large number of termination regions are known and function satisfactorily in a variety of hosts (when utilized both in the same and different genera and species from where they were derived). Typically, the termination region usually is selected more as a matter of convenience rather than because of any particular property. For the purposes herein, wherein the host cell is an oleaginous yeast, the termination region is preferably derived from a yeast gene, particularly *Saccharomyces, Schizosaccharomyces, Candida*, *Yarrowia* or *Kluyveromyces*. The 3'-regions of mammalian genes encoding γ-interferon and α–2 interferon are also known to function in yeast. Although not intended to be limiting, preferred termination regions useful in the disclosure herein include: ~100 bp of the 3' region of the *Yarrowia lipolytica* extracellular protease (*Xpr*; GenBank® Accession No. M17741); the acyl-CoA oxidase (*Aco3*: GenBank® Accession No. AJ001301 and No. CAA04661; *Pox3*: GenBank® Accession No. XP_503244) terminators; the *Pex20* (GenBank® Accession No. AF054613) terminator; the *Pex16* (GenBank® Accession No. U75433) terminator; the *Lip1* (GenBank® Accession No. Z50020) terminator; the *Lip2* (GenBank® Accession No. AJ012632) terminator; and the 3-oxoacyl-coA thiolase (*Oct*; GenBank® Accession No. X69988) terminator.

Merely inserting a gene into a cloning vector does not ensure that it will be successfully expressed at the level needed. In response to the need for a high expression rate, many specialized expression vectors have been created by manipulating a number of different genetic elements that control aspects of transcription, translation, protein stability, oxygen limitation and secretion from the microbial host cell. More specifically, some of the molecular features that have been manipulated to control gene expression include: 1.) the nature of the relevant transcriptional promoter and terminator sequences; 2.) the number of copies of the cloned gene and whether the gene is plasmid-borne or integrated into the



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genome of the host cell; 3.) the final cellular location of the synthesized foreign protein; 4.) the efficiency of translation and correct folding of the protein in the host organism; 5.) the intrinsic stability of the mRNA and protein of the cloned gene within the host cell; and, 6.) the codon usage within the cloned gene, such that its frequency approaches the frequency of preferred codon usage of the host cell. Each of these types of modifications are encompassed in the present invention, as means to further optimize expression of the *crt* genes (and optionally the PUFA biosynthetic pathway genes and/or antioxidant biosynthetic pathway genes) required herein. Methods of codon-optimizing foreign genes for optimal expression in *Yarrowia lipolytica* are set forth in U.S. Patent No. 7,125,672.

Once the DNA encoding a polypeptide suitable for expression in an appropriate microbial host cell (e.g., oleaginous yeast) has been obtained (e.g., a chimeric gene comprising a promoter, ORF and terminator), it is placed in a plasmid vector capable of autonomous replication in a host cell, or it is directly integrated into the genome of the host cell. Integration of expression cassettes can occur randomly within the host genome or can be targeted through the use of constructs containing regions of homology with the host genome sufficient to target recombination within the host locus. Where constructs are targeted to an endogenous locus, all or some of the transcriptional and translational regulatory regions can be provided by the endogenous locus.

Constructs comprising a coding region of interest may be introduced into a host cell by any standard technique. These techniques include transformation (e.g., lithium acetate transformation [Guthrie, C., *Methods in Enzymology*, 194:186-187 (1991)]), protoplast fusion, biolistic impact, electroporation, microinjection, or any other method that introduces the gene of interest into the host cell. More specific teachings applicable for oleaginous yeast (i.e., *Yarrowia lipolytica*) include U.S. Patents 4,880,741 and 5,071,764 and Chen, D. C. et al. (*Appl. Microbiol. Biotechnol.*, 48(2):232-235 (1997)).



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In the present invention, the preferred method of expressing genes in *Yarrowia lipolytica* is by integration of linear DNA into the genome of the host; and, integration into multiple locations within the genome can be particularly useful when high level expression of genes are desired. For example, preferred loci for integration include: the *Ura3* locus (GenBank® Accession No. AJ306421), the *Leu2* gene locus (GenBank® Accession No. AF260230), the *Lys5* gene (GenBank® Accession No. M34929), the *Aco2* gene locus (GenBank® Accession No. AJ001300), the *Pox3* gene locus (Pox3: GenBank® Accession No. XP_503244; or, Aco3: GenBank® Accession No. AJ001301), the Δ12 desaturase gene locus (U.S. Patent 7,214,491), the *Lip1* gene locus (GenBank® Accession No. Z50020), the *Lip2* gene locus (GenBank® Accession No. AJ012632) and/or the Pex10 gene locus (GenBank® Accession No. CAG81606).

Where two or more genes are expressed from separate replicating vectors, it is desirable that each vector has a different means of selection and should lack homology to the other construct(s) to maintain stable expression and prevent reassortment of elements among constructs. Judicious choice of regulatory regions, selection means and method of propagation of the introduced construct(s) can be experimentally determined so that all introduced genes are expressed at the necessary levels to provide for synthesis of the desired products.

For convenience, a host cell that has been manipulated by any method to take up a DNA sequence (e.g., an expression cassette) will be referred to as "transformed" or "recombinant" herein. The transformed host will have at least one copy of the expression construct and may have two or more, depending upon whether the gene is integrated into the genome, amplified, or is present on an extrachromosomal element having multiple copy numbers.

The transformed host cell can be identified by various selection techniques, as described in U.S. Patent 7,238,482 and PCT Publication No. WO 2006/052870 [U.S. Publication 2006-0115881-A1]. Preferred selection methods for use herein are resistance to kanamycin, hygromycin



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and the amino glycoside G418, as well as ability to grow on media lacking uracil, leucine, lysine, tryptophan or histidine. In alternate embodiments, 5-fluoroorotic acid (5-fluorouracil-6-carboxylic acid monohydrate; "5-FOA") is used for selection of yeast Ura$^-$ mutants. The compound is toxic to yeast cells that possess a functioning URA3 gene encoding orotidine 5'-monophosphate decarboxylase (OMP decarboxylase); thus, based on this toxicity, 5-FOA is especially useful for the selection and identification of Ura$^-$ mutant yeast strains (Bartel, P.L. and Fields, S., Yeast 2-Hybrid System, Oxford University: New York, v. 7, pp 109-147, 1997). More specifically, one can first knockout the native Ura3 gene to produce a strain having a Ura- phenotype, wherein selection occurs based on 5-FOA resistance. Then, a cluster of multiple chimeric genes and a new Ura3 gene can be integrated into a different locus of the *Yarrowia* genome to thereby produce a new strain having a Ura+ phenotype. Subsequent integration produces a new Ura3- strain (again identified using 5-FOA selection), when the introduced Ura3 gene is knocked out. Thus, the Ura3 gene (in combination with 5-FOA selection) can be used as a selection marker in multiple rounds of transformation.

An alternate preferred selection method utilized herein relies on a dominant, non antibiotic marker for *Yarrowia lipolytica* based on sulfonylurea resistance (see PCT Publication No. WO 2006/052870 for additional details). The technique is also generally applicable to other industrial yeast strains that may be haploid, diploid, aneuploid or heterozygous. Specifically, the sulfonylurea resistance selection marker utilized herein for transforming *Y. lipolytica* does not rely on a foreign gene but on a mutant native gene. Thus, it neither requires auxotrophy nor results in auxotrophy and allows transformation of wild type strains. More specifically, the marker gene (SEQ ID NO:1) is a native acetohydroxyacid synthase (AHAS or acetolactate synthase; E.C. 4.1.3.18) that has a single amino acid change (W497L) that confers sulfonyl urea herbicide resistance.

An additional method for recycling a selection marker relies on site-specific recombinase systems. Briefly, the site-specific recombination



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system consists of two elements: (1) a recombination site having a characteristic DNA sequence [e.g., LoxP]; and, (2) a recombinase enzyme that binds to the DNA sequence specifically and catalyzes recombination (i.e., excision) between DNA sequences when two or more of the recombination sites are oriented in the same direction at a given interval on the same DNA molecule [e.g., Cre]. This methodology has utility as a means of selection, since it is possible to "recycle" a pair of preferred selection markers for their use in multiple sequential transformations.

Specifically, an integration construct is created comprising a target gene that is desirable to insert into the host genome (e.g., a *crt* gene), as well as a first selection marker (e.g., *ura*) that is flanked by recombination sites. Following transformation and selection of the transformants, the first selection marker is excised from the chromosome by the introduction of a replicating plasmid carrying a second selection marker (e.g., sulfonylurea resistance [AHAS]) and a recombinase suitable to recognize the site-specific recombination sites introduced into the genome. Upon selection of those transformants carrying the second marker and confirmation of excision of the first selection marker from the host genome, the replicating plasmid is then cured from the host in the absence of selection. This produces a transformant that possesses neither the first nor second selection marker, and thus the cured strain is available for another round of transformation. One skilled in the art will recognize that the methodology is not limited to the particular selection markers or site-specific recombination system used in the present invention.

Methods for manipulating biochemical pathways are well known to those skilled in the art; and, it is expected that numerous manipulations will be possible to maximize carotenoid production (and optionally PUFAs and/or additional antioxidants) in oleaginous yeasts, and particularly, in *Yarrowia lipolytica*. This may require metabolic engineering directly within the $C_{40}$ carotenoid, PUFA and/or CoQ/resveratrol biosynthetic pathways or additional coordinated manipulation of various other metabolic pathways.



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Microbial Fermentation Processes

The transformed microbial host cell is grown under conditions that optimize expression of chimeric genes and produce the greatest and the most economical yield of desired carotenoids (and optionally PUFAs and/or additional antioxidants). In general, media conditions that may be optimized include the type and amount of carbon source, the type and amount of nitrogen source, the carbon-to-nitrogen ratio, the oxygen level, growth temperature, pH, length of the biomass production phase, length of the oil accumulation phase and the time and method of cell harvest. Microorganisms of interest, such as oleaginous yeast (e.g., *Yarrowia lipolytica*) are generally grown in complex media (e.g., yeast extract-peptone-dextrose broth (YPD)) or a defined minimal media that lacks a component necessary for growth and thereby forces selection of the desired expression cassettes (e.g., Yeast Nitrogen Base (DIFCO Laboratories, Detroit, MI)).

Fermentation media in the present invention must contain a suitable carbon source. Suitable carbon sources are taught in U.S. Patent 7,238,482. Although it is contemplated that the source of carbon utilized in the present invention may encompass a wide variety of carbon-containing sources, preferred carbon sources are sugars, glycerol and/or fatty acids. Most preferred is glucose and/or fatty acids containing between 10-22 carbons.

Nitrogen may be supplied from an inorganic (e.g., $(NH_4)_2SO_4$) or organic (e.g., urea or glutamate) source. In addition to appropriate carbon and nitrogen sources, the fermentation media must also contain suitable minerals, salts, cofactors, buffers, vitamins and other components known to those skilled in the art suitable for the growth of the oleaginous host and promotion of the enzymatic pathways necessary for carotenoid production (and optionally PUFAs and/or CoQ/resveratrol). Particular attention is given to several metal ions (e.g., $Mn^{+2}$, $Co^{+2}$, $Zn^{+2}$, $Mg^{+2}$) that promote synthesis of lipids and PUFAs (Nakahara, T. et al., *Ind. Appl. Single Cell Oils,* D. J. Kyle and R. Colin, eds. pp 61-97 (1992)).



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Preferred growth media in the present invention are common commercially prepared media, such as Yeast Nitrogen Base (DIFCO Laboratories, Detroit, MI). Other defined or synthetic growth media may also be used and the appropriate medium for growth of the transformant host cells will be known by one skilled in the art of microbiology or fermentation science. A suitable pH range for the fermentation is typically between about pH 4.0 to pH 8.0, wherein pH 5.5 to pH 7.5 is preferred as the range for the initial growth conditions. The fermentation may be conducted under aerobic or anaerobic conditions, wherein microaerobic conditions are preferred.

Typically, accumulation of high levels of carotenoids (and optionally PUFAs and/or additional antioxidants) in oleaginous yeast cells requires a two-stage process, since the metabolic state must be "balanced" between growth and synthesis/storage of fats. Thus, for example, a two-stage fermentation process is preferred for the production of EPA in *Yarrowia lipolytica*. This approach is described in U.S. Patent 7,238,482, as are various suitable fermentation process designs (i.e., batch, fed-batch and continuous) and considerations during growth.

Purification And Processing Of Carotenoids, PUFAs And/Or Antioxidants

In one embodiment, the primary product is oleaginous yeast biomass. As such, isolation and purification of the carotenoid-containing oils (including those comprising PUFAs and antioxidants) from the biomass may not be necessary (i.e., wherein the biomass is the product).

However, certain end uses and/or product forms may require partial and/or complete isolation/purification of the carotenoid-containing oil (optionally comprising PUFAs and antioxidants) from the biomass, to result in partially purified biomass, purified oil, and/or purified carotenoids. Given the lipophilic/hydrophobic nature of carotenoids (which are therefore expected to accumulate in oil bodies within the recombinant oleaginous yeast), many techniques applied to isolate/purify microbially produced oils/PUFAs should work to isolate carotenoids as well, especially when the desired product is a pigmented oil. As such, any number of well known techniques can be used to isolate the compounds from the biomass



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including, but not limited to: extraction (e.g., U.S. Patents No. 6,797,303 and No. 5,648,564) with organic solvents, sonication, supercritical fluid extraction (e.g., using carbon dioxide), saponification and physical means such as presses, or combinations thereof. One is referred to the teachings of U.S. Patent 7,238,482 for additional details.

One review of PUFA extraction techniques, quality analysis and acceptability standards for yeast lipids is that of Z. Jacobs (*Critical Reviews in Biotechnology,* 12(5/6):463-491 (1992)). A brief review of downstream processing is also available by A. Singh and O. Ward (*Adv. Appl. Microbiol.*, 45:271-312 (1997)).

Finally, one skilled in the art will be aware of the appropriate means to selectively purify a specific carotenoid from a carotenoid-containing mixture comprising various carotenoid intermediates in addition to the desired carotenoid.

Use Of Compositions Comprising Carotenoids

The carotenoids produced by the present processes may be used as pigments, antioxidants, or as both in various commercial products (described *infra*).

In alternate embodiments, it is useful to engineer an oleaginous microorganism to simultaneously produce one of the following: 1.) both carotenoids and at least one $\omega$-3/$\omega$-6 PUFA [preferably selected from the group consisting of LA, EDA, GLA, DGLA, ARA, ALA, STA, ETrA, ETA, EPA, DPA and DHA]; 2.) both carotenoids and at least one additional antioxidant [preferably selected from the group consisting of $CoQ_6$, $CoQ_7$, $CoQ_8$, $CoQ_9$, $CoQ_{10}$ and resveratrol]; and, 3.) carotenoids and at least one $\omega$-3/$\omega$-6 PUFA and at least one additional antioxidant. Any of the above combinations of products are expected to create a higher value product or reduce production costs. This would thereby avoid the situation that a commercial product formulator must typically face, wherein carotenoids and PUFAs and/or additional antioxidants are obtained from a variety of sources and then must be formulated into a final product that contains an effective amount of each ingredient ( as the composition, purity and source of each ingredient may vary, the final product formulation may require



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significant monitoring and/or processing to obtain the desired product specifications).

The present oleaginous yeast biomass comprising at least one carotenoid (and optionally at least one ω-3/ω-6 PUFA and/or at least one additional antioxidant) can be prepared and sold in a variety of product forms including, but not limited to whole cell biomass, partially purified biomass, purified oil comprising carotenoid, and purified carotenoid. The product form will depend upon the targeted end use. For ease of discussion, the term "microbial biomass/oils" will refer to any of the above product forms.

In some embodiments, the present invention is drawn to "pigmented microbial biomass/oils", wherein the term pigmented microbial biomass/oils refers to a microbial biomass/oil of the invention comprising at least one carotenoid (and optionally at least one PUFA and/or at least one additional antioxidant), wherein the carotenoid is present in an "effective" amount such that the final product and/or product formulation within which the pigmented microbial biomass/oil is incorporated becomes effectively pigmented. One of skill in the art of processing and formulation will understand how the amount and composition of the pigmented microbial biomass/oils may be added to the product and/or product formulation and how the "effective" amount will depend according to target species and/or end use (e.g., the food or feed product, cosmetic or personal care product, supplement, etc.). For example, an "effective amount of pigment" with respect to an animal feed refers to an amount that effectively pigments at least one animal tissue (e.g., chicken products such as egg yolks; crustacean muscle tissue and/or shell tissue; fish muscle tissue and/or skin tissue, etc.) under feeding conditions considered suitable for growth of the target animal species. The amount of pigment incorporated into the animal feed may vary according to target species. Typically, the amount of pigment product incorporated into the feed product takes into account pigmentation losses associated with feed processing conditions, typical handling and storage conditions, the stability of the pigment in the feed, the bioavailability/bioabsorption efficiency of the



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particular species, the pigmentation rate of the animal tissue targeted for pigmentation, and the overall profile of pigment isomers (wherein some are preferentially absorbed over others), to name a few.

In alternate embodiments, the present invention is drawn to stabilized microbial biomass/oils, wherein the term "stabilized microbial biomass/oils" refers herein to a microbial biomass/oil comprising carotenoids (and optionally comprising at least one ω-3/ω-6 PUFA and/or at least one additional antioxidant [e.g., a CoQ , resveratrol]), wherein the carotenoids (and optional additional antioxidant) are present in an "effective amount" such that the rate of oxidation of the microbial biomass/oil (or any other compound that is subject to oxidation therein, e.g., *ω*-3 and/or *ω*-6 PUFAs) is reduced. One of skill in the art will understand that an effective amount of carotenoid antioxidant (and optional additional antioxidant) varies according to the product and/or product formulation according to target species and/or end use. Typically, the amount of antioxidant incorporated into the product takes into account losses associated with processing conditions, typical handling and storage conditions and the stability of the antioxidant in the product, to name a few.

In further preferred embodiments, the amount of PUFA present in the pigmented and/or stabilized microbial biomass/oil of the invention is an "effective" amount wherein the term "effective amount of PUFA" refers to the amount of a microbially produced *ω*-3/*ω*-6 PUFA incorporated into a product formulation that is sufficient to provide the desirable health characteristics associated with *ω*-3/*ω*-6 PUFA consumption. One of skill in the art can vary the amount and type of PUFA incorporated into the product formulation according to target species and/or end use. Typically, the amount of PUFA incorporated into the product takes into account losses associated with processing conditions, typical handling and storage conditions, the stability of the PUFA in the product, and the bioavailability/ bioabsorption efficiency with the target species, to name a few.

In alternate embodiments, the invention provides an animal feed, food product, dietary supplement, pharmaceutical composition, infant



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formula, or personal care product comprising oleaginous yeast biomass/oil comprising at least one carotenoid (and optionally at least one $\omega$-3/$\omega$-6 PUFA and/or at least one additional antioxidant [e.g., $CoQ_6$, $CoQ_7$, $CoQ_8$, $CoQ_9$, $CoQ_{10}$, resveratrol]). In other words, the carotenoid product of the present invention is used as an ingredient in the final formulation of an animal feed, food product, dietary supplement, pharmaceutical composition, infant formula, or personal care product. It is contemplated that the pigmented and/or stabilized microbial biomass/oils of the invention comprising carotenoids will function in each of these applications to impart the health benefits of current formulations using more traditional sources of carotenoids. In preferred embodiments, yeast biomass comprises at least about 25 wt% oil, preferably at least about 30-40 wt%, and most preferably at least about 40-50 wt% microbially-produced oil.

Food Products And Infant Formulas

Pigmented and/or stabilized microbial biomass/oils of the invention comprising at least one carotenoid will be suitable for use in a variety of food and feed products including, but not limited to food analogs, meat products, cereal products, baked foods, snack foods and dairy products. Alternatively, the pigmented and/or stabilized biomass/oils (or derivatives thereof) may be incorporated into cooking oils, fats or margarines formulated so that in normal use the recipient would receive the desired amount for dietary supplementation. The pigmented biomass/oils may also be incorporated into infant formulas, nutritional supplements or other food products and may find use as anti-inflammatory or cholesterol lowering agents.

The term "food product" refers to any food generally suitable for human consumption. Typical food products include but are not limited to meat products, cereal products, baked foods, snack foods, dairy products and the like.

Meat products encompass a broad variety of products. In the United States "meat" includes "red meats" produced from cattle, hogs and sheep. In addition to the red meats there are poultry items which include chickens, turkeys, geese, guineas and ducks and the fish and shellfish.



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There is a wide assortment of seasoned and processed meat products: fresh, cured and fried, and cured and cooked. Sausages and hot dogs are examples of processed meat products. Thus, the term "meat products" as used herein includes, but is not limited to, processed meat products.

A cereal food product is a food product derived from the processing of a cereal grain. A cereal grain includes any plant from the grass family that yields an edible grain (seed). The most popular grains are barley, corn, millet, oats, quinoa, rice, rye, sorghum, triticale, wheat and wild rice. Examples of a cereal food product include, but are not limited to: whole grain, crushed grain, grits, flour, bran, germ, breakfast cereals, extruded foods, pastas and the like.

A baked goods product comprises any of the cereal food products mentioned above and has been baked or processed in a manner comparable to baking, i.e., to dry or harden by subjecting to heat. Examples of a baked good product include, but are not limited to: bread, cakes, doughnuts, bars, pastas, bread crumbs, baked snacks, mini-biscuits, mini-crackers, mini-cookies and mini-pretzels. As was mentioned above, pigmented and/or stabilized microbial biomass/oils of the invention can be used as an ingredient.

A snack food product comprises any of the above or below described food products.

A fried food product comprises any of the above or below described food products that has been fried.

A beverage can be in a liquid or in a dry powdered form. For example, there can be mentioned non-carbonated drinks; fruit juices, fresh, frozen, canned or concentrate; flavored or plain milk drinks, etc. Adult and infant nutritional formulas are well known in the art and commercially available (e.g., Similac®, Ensure®, Jevity®, and Alimentum® from Ross Products Division, Abbott Laboratories).

A dairy product is a product derived from milk. A milk analog or nondairy product is derived from a source other than milk, for example, soymilk. These products include, but are not limited to: whole milk, skim milk, fermented milk products such as yogurt or sour milk, cream, butter,



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condensed milk, dehydrated milk, coffee whitener, coffee creamer, ice cream, cheese, etc.

Additional food products into which the pigmented and/or stabilized microbial biomass/oils could be included are, for example: chewing gums, confections and frostings, gelatins and puddings, hard and soft candies, jams and jellies, white granulated sugar, sugar substitutes, sweet sauces, toppings and syrups, and dry-blended powder mixes.

As used herein, a "food analog" is a food-like product manufactured to resemble its food counterpart, whether meat, cheese, milk or the like, and is intended to have the appearance, taste, and texture of its counterpart. Thus, as used herein, the term "food product" also encompasses food analogs.

Food analogs can be made using processes well known to those skilled in the art. There can be mentioned meat analogs, cheese analogs, milk analogs and the like. Meat analogs made from soybeans contain soy protein or tofu and other ingredients mixed together to simulate various kinds of meats. These meat alternatives are sold as frozen, canned or dried foods. Usually, they can be used the same way as the foods they replace. Examples of meat analogs include, but are not limited to: ham analogs, sausage analogs, bacon analogs, and the like.

Food analogs can be classified as imitation or substitutes depending on their functional and compositional characteristics. For example, an imitation cheese need only resemble the cheese it is designed to replace. However, a product can generally be called a substitute cheese only if it is nutritionally equivalent to the cheese it is replacing and meets the minimum compositional requirements for that cheese. Thus, substitute cheese will often have higher protein levels than imitation cheeses and be fortified with vitamins and minerals.

Infant formulas are liquids or reconstituted powders fed to infants and young children. "Infant formula" is defined herein as an enteral nutritional product which can be substituted for human breast milk in feeding infants and typically is composed of a desired percentage of fat mixed with desired percentages of carbohydrates and proteins in an



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aqueous solution (e.g., see U.S. Patent 4,670,285). Based on the worldwide composition studies, as well as levels specified by expert groups, average human breast milk typically contains about 0.20% to 0.40% of total fatty acids (assuming about 50% of calories from fat); and, generally the ratio of DHA to ARA would range from about 1:1 to 1:2 (see, e.g., formulations of Enfamil LIPIL™ [Mead Johnson & Company] and Similac Advance™ [Ross Products Division, Abbott Laboratories]). Infant formulas have a special role to play in the diets of infants because they are often the only source of nutrients for infants; and, although breast-feeding is still the best nourishment for infants, infant formula is a close enough second that babies not only survive but thrive.

Health Food Products And Pharmaceuticals

A health food product is any food product that imparts a health benefit and includes functional foods, medical foods, medical nutritionals and dietary supplements. Pigmented and/or stabilized microbial biomass/oils of the invention may also be used in standard pharmaceutical compositions.

As used herein, the term "functional food" refers to those foods that encompass potentially healthful products including any modified food or ingredient that may provide a health benefit beyond the traditional nutrients it contains. Functional foods can include foods like cereals, breads and beverages that are fortified with vitamins, herbs and nutraceuticals. Functional foods contain a substance that provides health benefits beyond its nutritional value, wherein the substance either is naturally present in the food or is deliberately added. As used herein, the term "nutraceutical" refers to any substance that may be considered a food or part of a food and provides medical or health benefits, including the prevention and treatment of disease.

As used herein, the term "medical food" refers to a food which is formulated to be consumed or administered enterally under the supervision of a physician and which is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognized scientific principles, are established by



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medical evaluation [see section 5(b) of the Orphan Drug Act (21 U.S.C. 360ee(b)(3))]. A food is a "medical food" only if: (i) It is a specially formulated and processed product (as opposed to a naturally occurring foodstuff used in its natural state) for the partial or exclusive feeding of a patient by means of oral intake or enteral feeding by tube; (ii) It is intended for the dietary management of a patient who, because of therapeutic or chronic medical needs, has limited or impaired capacity to ingest, digest, absorb, or metabolize ordinary foodstuffs or certain nutrients, or who has other special medically determined nutrient requirements, the dietary management of which cannot be achieved by the modification of the normal diet alone; (iii) It provides nutritional support specifically modified for the management of the unique nutrient needs that result from the specific disease or condition, as determined by medical evaluation; (iv) It is intended to be used under medical supervision; and, (v) It is intended only for a patient receiving active and ongoing medical supervision wherein the patient requires medical care on a recurring basis for, among other things, instructions on the use of the medical food. Thus, unlike dietary supplements or conventional foods, a medical food that is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements have been established, may bear scientifically valid claims relating to providing distinctive nutritional support for a specific disease or condition. Medical foods are distinguished from the broader category of foods for special dietary use (e.g., hypoallergenic foods) and from foods that make health claims (e.g., dietary supplements) by the requirement that medical foods be used under medical supervision.

As used herein, the term "medical nutritional" is a medical food as defined herein and typically refers to a fortified beverage that is specifically designed for special dietary needs. The medical nutritional generally comprises a dietary composition focused at a specific medical or dietary condition. Examples of commercial medical nutritionals include, but are not limited to Ensure® and Boost®.

As used herein, the term "dietary supplement" refers to a product that: (i) is intended to supplement the diet and thus is not represented for



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use as a conventional food or as a sole item of a meal or the diet; (ii) contains one or more dietary ingredients (including, e.g., vitamins, minerals, herbs or other botanicals, amino acids, enzymes and glandulars) or their constituents; (iii) is intended to be taken by mouth as a pill, capsule, tablet, or liquid; and, (iv) is labeled as being a dietary supplement.

As used herein, the term "pharmaceutical" means a compound or substance which if sold in the United States would be controlled by e.g., Section 505 of the Federal Food, Drug and Cosmetic Act.

Engineered carotenoid-producing strains of oleaginous yeast of the present invention or pigmented and/or stabilized microbial biomass/oils produced therefrom could readily be incorporated into the any of the above mentioned health food products, to thereby produce e.g., a functional or medical food. For example more concentrated formulations comprising carotenoids and $\omega$-3/$\omega$-6 PUFAs (e.g., ARA or EPA) include capsules, powders, tablets, softgels, gelcaps, liquid concentrates and emulsions which can be used as a dietary supplement in humans or animals other than humans. Similarly, compositions comprising carotenoids and optionally at least one $\omega$-3/$\omega$-6 PUFA and/or at least one additional antioxidant can be used as dietary substitutes, or supplements, particularly infant formulas, for patients undergoing intravenous feeding or for preventing or treating malnutrition. Optionally, the compositions may be used for pharmaceutical use (human or veterinary). In this case, the pigmented PUFAs are generally administered orally but can be administered by any route by which they may be successfully absorbed, e.g., parenterally (e.g., subcutaneously, intramuscularly or intravenously), rectally, vaginally or topically (e.g., as a skin ointment or lotion).

Animal Feed Products

Animal feeds are generically defined herein as products intended for use as feed or for mixing in feed for animals other than humans. More specifically, the term "animal feed" refers to feeds intended exclusively for consumption by animals, including domestic animals (e.g., pets, farm



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animals, home aquarium fish, etc.) or for animals raised for the production of food (e.g., poultry, eggs, fish, crustacea, etc.).

More specifically, although not limited therein, it is expected that the pigmented and/or stabilized microbial biomass/oils of the present invention can be used within pet food products, ruminant and poultry food products and aquacultural food products. Pet food products are those products intended to be fed to a pet (e.g., a dog, cat, bird, reptile, rodent); these products can include the cereal and health food products above, as well as meat and meat byproducts, soy protein products, grass and hay products (e.g., alfalfa, timothy, oat or brome grass, vegetables). Ruminant and poultry food products are those wherein the product is intended to be fed to e.g., turkeys, chickens, cattle and swine. As with the pet foods above, these products can include cereal and health food products, soy protein products, meat and meat byproducts, and grass and hay products as listed above. Aquacultural food products (or "aquafeeds") are those products intended to be used in aquafarming, which concerns the propagation, cultivation or farming of aquatic organisms and/or animals in fresh or marine waters. More specifically, the term "aquaculture" refers to the production and sale of farm raised aquatic plants and animals. Typical examples of animals produced through aquaculture include, but are not limited to: lobsters, shrimp, prawns, and fish (i.e., ornamental and/or food fish).

The pigmented and/or stabilized microbial biomass/oils of the present invention (comprising at least one carotenoid [and optionally PUFAs and/or additional antioxidants]) can be used as an ingredient in any of the animal feeds described above. In addition to providing necessary carotenoid pigments, the oleaginous yeast itself is a useful source of protein and other nutrients (e.g., vitamins, minerals, nucleic acids, complex carbohydrates, etc.) that can contribute to overall animal health and nutrition, as well as increase a formulation's palatability. Accordingly it is contemplated that the addition of yeast biomass comprising the recombinant production hosts of the invention will be an excellent additional source of feed nutrients in animal feed formulations,



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wherein the term "feed nutrient" means nutrients such as proteins, lipids, carbohydrates, vitamins, minerals and nucleic acids that may be derived from the yeast biomass comprising the recombinant production hosts of the invention.

More specifically, for example, *Yarrowia lipolytica* (ATCC #20362) has the following approximate chemical composition, as a percent relative to the dcw: 35% protein, 40% lipid, 10% carbohydrate, 5% nucleic acids, 5% ash and 5% moisture. Furthermore, within the carbohydrate fraction, β-glucans comprise approximately 45.6 mg/g, mannans comprise approximately 11.4 mg/g, and chitin comprises approximately 52.6 mg/g (while trehalose is a minor component [approximately 0.7 mg/g]).

A considerable body of literature has examined the immuno-modulating effects of yeast β-glucans, mannans and chitin in both traditional animal husbandry and within the aquacultural sector. The means by which β-glucans, the primary constituents of bacterial and fungal cell walls, stimulate non-specific immunity (i.e., "immunostimulant effects") to thereby improve health of aquaculture species, pets and farm animals and humans are best studied, although both chitin and mannans are similarly recognized as useful immunostimulants (see PCT Publication No. WO 2006/052870).

Based on the unique protein:lipid:carbohydrate composition of *Yarrowia lipolytica*, as well as the unique complex carbohydrate profile (comprising an approximate 1:4:4.6 ratio of mannan:β-glucans:chitin), it is contemplated that the genetically engineered yeast cells of the present invention (or portions thereof) would be useful additives to animal feed formulations (e.g., as whole [lyophilized] yeast cells, as purified cells walls, as purified yeast carbohydrates or within various other fractionated forms).

In some embodiments, wherein the pigmented and/or stabilized microbial biomass/oils of the present invention comprise an effective amount of at least one carotenoid (preferably lutein, lycopene, β-carotene, canthaxanthin, astaxanthin and/or zeaxanthin), the biomass/oil is suitable as an ingredient in a "pigmented feed product" or "pigmented animal feed".



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Many animals have been reported to acquire tissue pigmentation by absorbing xanthophylls in their feed. In one embodiment, the pigmented animal feed is an animal feed selected from the group consisting of: fish feed, crustacea feed, shrimp feed, crab feed, lobster feed, and chicken feed. The nutritional requirements and feed forms for each animal feed are well known in the art (for example, see Nutrient Requirements of Fish, published by the Board of Agriculture's Committee on Animal Nutrition, National Research Council, National Academy: Washington, D.C. 1993; and Nutrient Requirements of Poultry, published by the Board of Agriculture's Committee on Animal Nutrition, National Research Council, National Academy: Washington, D.C. 1994).

Various means are available to incorporate the pigmented and/or stabilized microbial biomass/oils of the present invention into animal feed (typically in the form of feed pellets). For example, the biomass/oils can be incorporated into the feed mash prior to extrusion or after the extrusion process ("post-extrusion applied") by mixing and dispersing the biomass/oils in a suitable oil that is subsequently applied to the pellet. Typically a "suitable oil" is fish oil (e.g., Capelin oil) or a vegetable oil (e.g., corn oil, sunflower oil, soybean oil, etc.), although in preferred embodiments of the presention invention the "suitable oil" is microbially produced. In preferred embodiments of the present invention, an effective amount of recombinantly produced carotenoid is supplied within an effective amount of microbial oil produced from recombinant *Yarrowia lipolytica,* wherein the carotenoid and oil are co-produced. In alternate embodiments, however, microbially produced oil may be admixed with the pigmented or stabilized microbial biomass/oils of the present invention or purified carotenoids of the present invention to result in the appropriate concentration of each.

Although the amount of total carotenoid incorporated into the post-extrusion prepared pigmented animal feed may be less than that found in pre-extrusion supplemented feed, the resulting preferential isomer content may be higher (e.g., the heat of the extrusion process may isomerize some pigments). It should be noted that many extrusion processes run at



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elevated temperatures sufficient to possibly degrade and/or alter carotenoids supplemented to the feed mash prior to extrusion. It is possible to use a cold extrusion process to circumvent this problem; however, the physical stability of the cold-extruded pellets tends to be inferior in comparison to the "hot-extruded" feed pellets.

The size and shape of the feed pellets may vary according to the target species and developmental stage. The amount of pigmented biomass product formulated into feed pellets can be adjusted and/or optimized for the particular application. Factors to consider include, but are not limited to: the concentration of the pigment in the biomass, the concentration of the pigment in the pigmentation product, the target species, the age and/or growth rate of the selected species, the type of carotenoid used, the bioabsorption characteristics of the chosen pigment in the context of the species to be pigmented, the feeding schedule, the cost of the pigment, and the palatability of the resulting feed. One of skill in the art can adjust the amount of pigmented and/or stabilized microbial biomass/oil incorporated into the feed so that adequate levels of carotenoid are present while balancing the nutritional requirements of the species. Typical concentrations of the carotenoid pigment incorporated into, for example, fish feed range from about 10 to about 200 mg/kg of fish feed, wherein a preferred range is from about 10 mg/kg to about 100 mg/kg, a more preferred range is from about 10 mg/kg to about 80 mg/kg and a most preferred range is from about 20 mg/kg to about 60 mg/kg, depending on the specific product.

Although numerous commercial products comprising the pigmented and/or stabilized microbial biomass/oils of the invention (or isolated carotenoids thereof) are contemplated by the Applicants herein, applications of the products of the invention are described below in additional detail in reference to various specific animal feeds. Generally, the recombinant oleaginous yeast biomass comprises about 0.1 wt% to about 50 wt% of the animal feed, preferably about 0.5 wt% to about 20 wt%, more preferably about 1 wt% to about 10 wt%, most preferably about



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1 wt% to about 5 wt%. These examples are not intended to be limiting to the invention herein.

*Chicken Feed – Nutritional Requirements:* The dietary requirements of poultry are well known (see Nutrient Requirements of Poultry, published by the Board of Agriculture's Committee on Animal Nutrition, National Research Council, National Academy: Washington, D.C. 1994). Typical feeds are comprised of crude protein (including essentially amino acids), carbohydrates, fats/lipids (e.g., $\omega$-3/$\omega$-6 PUFAs), vitamins (i.e., fat and water soluble), minerals (i.e., both macrominerals and trace minerals) and water. Additional feed ingredients may include antioxidants (e.g., $CoQ_9$, $CoQ_{10}$, resveratrol; typically included to prevent oxidation of lipids and/or pigments), hormones, antibiotics and pigments (i.e., carotenoids), to name of few. The source of the various components is typically chosen based on cost, availability and quality of the nutrients they contain. Typically, components include, but are not limited to: cereal grains (primarily carbohydrates and protein), animal protein meal, animal or vegetable fats/lipids, and isolates from various plants including corn, rice, alfalfa and soybean, to name a few.

When producing a "poultry pigmentation product", referring to pigmented feed additives used to color poultry via commercial farming techniques, preferred carotenoids are lutein, canthaxanthin, astaxanthin and/or zeaxanthin. Typically, the poultry is a chicken and the pigmentation product is used to pigment chicken tissue (e.g., skin) and/or egg yolks.

*Crustacea Feed – Nutritional Requirements:* The dietary requirements of crustacea are well-known (see "Training Manual on Shrimp and Fish Nutrition and Feed Management", CIBA Special Bulletin No. 15; October 22-31, 2002; Ali, Amanad, ed.; published by the Central Institute of Brackishwater Aquaculture (CIBA), Chennai, India, hereinafter referred to as "Amanad"). Typical feeds are comprised of crude protein (including essentially amino acids), carbohydrates, fats/lipids (e.g., $\omega$-3/$\omega$-6 PUFAs), vitamins (i.e., fat and water soluble), minerals (i.e., both macrominerals and trace minerals) and water. Additional feed ingredients may include antioxidants (e.g., $CoQ_9$, $CoQ_{10}$, resveratrol; typically included



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to prevent oxidation of lipids and/or pigments), hormones, antibiotics and pigments (i.e., carotenoids), to name of few. The source of the various components is typically chosen based on cost, availability and quality of the nutrients they contain. Typically, components include, but are not limited to: cereal grains (primarily carbohydrates and protein), animal protein meal (e.g., fish, prawn, squid, and clam meat meal), animal or vegetable fats/lipids, and isolates from various plants including corn, rice, alfalfa, soybean, groundnut cake (i.e., peanuts), sunflower cake, and gingelly (sesame) cake, to name a few. Examples of the moisture, protein, fat, fiber, carbohydrate, and ash content of various raw feed materials typically used in feed is shown in Tables 5 and 6 (see Amanad).

Table 5

Composition Of Various Raw Feed Materials Used In Preparing Shrimp Feed

| Ingredient | Moisture | Protein | Fat | Fiber | Carbohydrate | Ash |
|---|---|---|---|---|---|---|
| Fish Meal | 10.8 | 55.0 | 5.4 | 1.73 | 3.27 | 23.78 |
| Prawn Head | 9.91 | 39.8 | 9.6 | 16.3 | 4.0 | 20.18 |
| Squid Meal | 8.4 | 66.5 | 4.4 | 3.98 | 5.9 | 10.8 |
| Clam meat meal | 10.1 | 49.9 | 8.66 | - | 28.3 | 7.75 |
| Soybean Meal | 10.45 | 51.5 | 1.00 | 8.85 | 19.7 | 8.5 |
| Groundnut Cake | 13.05 | 46.93 | 5.0 | 8.9 | 18.03 | 8.9 |
| Sunflower Cake | 7.0 | 26.69 | 2.04 | 30.13 | 26.37 | 7.7 |
| Gingelly Cake | 9.76 | 38.71 | 6.00 | 10.96 | 15.8 | 19.02 |

* Note: All values reported as weight percent (wt %) of ingredient.

Table 6

Typical Composition Of Feed Formulation For Shrimp

| Ingredient | Starter Feed | Grower Feed | Finishing Feed |
|---|---|---|---|
| Crude protein | 40-45 | 38-40 | 35-38 |
| Lipid | 6-8 | 8-10 | 8-10 |
| Carbohydrate | 10-16 | 15-20 | 20-25 |
| Crude fiber | 1-2 | 1-3 | 2-4 |



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| | | | |
|---|---|---|---|
| Ash | 10-12 | 10-15 | 12-18 |
| Mineral mix | 2-5 | 2-5 | 2-5 |

* Note: All values reported as weight percent (wt %) of ingredient.

Mixtures comprising these compositions are blended and then pelleted and dried with 16-17% moisture.

When producing a "crustacean pigmentation product", referring to pigmented feed additives used to color crustaceans via commercial aquacultural techniques, preferred carotenoids are canthaxanthin and/or astaxanthin. Typically, the crustaceans are shrimp or lobsters and the pigmentation product is used to pigment tissue and shells.

*Fish Feed-- Xanthophylls Supplementation:* Many commercially important fish have pigmented pink muscle tissue. Fish cannot synthesize xanthophylls; therefore, they must obtain them from there diet. Wild fish obtain the necessary carotenoids responsible for flesh pigmentation from their natural diet (i.e., from zooplankton that naturally produce xanthophylls). However, farm raised fish (i.e., aquaculture) need to obtain the pigments by supplementing standard fish feed formulations with one or more pigments. It is important to include pigment in the diet of farm-raised fish as consumers generally prefer pigmented muscle tissue, especially when purchasing salmonid fish products (e.g., pink salmon fillets).

Many fish preferentially bioabsorb certain isomers of the present carotenoids (i.e., 3S,3'S-astaxanthin or all-*E*-canthaxanthin). Enzymatic synthesis typically produces more of the desired isomer when compared to chemically synthesized xanthophylls. In one aspect, the amount of preferentially bioabsorbed isomer is at least about 40% of the total xanthophyll content, preferably at least about 50% of the total xanthophyll content, even more preferable at least about 70%, and most preferably at least about 85% of the total amount of the respective xanthophyll in the resulting pigmented feed material.

Astaxanthin is the major carotenoid of wild salmonid fishes (Khare et al., *Comp. Biochem. Physiol.*, 45B:971-973 (1973); Schiedt et al., *Helv. Chim. Acta.*, 64:449-457 (1981), Schiedt et al., *Comp. Biochem. Physiol.*, 83B:9-12 (1986)). In farmed Atlantic salmon, the characteristic flesh



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colour is caused by carotenoids, usually astaxanthin or canthaxanthin, which are supplemented to the diet (reviewed by Torrissen et al., *CRC Crit. Rev. Aquat. Sci.*, 1:209-225 (1989); Storebakken, T. and No, H., *Aquaculture*, 100:209-229 (1992)). Whereas astaxanthin is more efficiently accumulated in the muscle of rainbow trout than canthaxanthin (reviewed by Storebakken, T. and No, H., *supra*, 1992), the opposite appears to be true for Atlantic salmon (Buttle et al., *L. Aquacult. Res.*, 32:103-111 (2001); Baker et al., *Anim. Feed. Sci. Technol.*, 99:97-106 (2002)).

Canthaxanthin consists of 272 possible geometrical *E/Z* isomers of which the quantitatively most important are all-*E*-, 9*Z*-, 13*Z*-, and 15*Z*-astaxanthin. With respect to astaxanthin, the all-*E*-astaxanthin isomer is more efficiently utilized for pigmentation of rainbow trout muscle than the *Z*-isomers (Bjerkeng *et al.*, *Aquaculture*, 157:63-82 (1997); Østerlie *et al.*, *J. Nutr.*, 129:391-398 (1999)).

The efficiency of dietary canthaxanthin or astaxanthin utilisation for flesh pigmentation in Atlantic salmon and rainbow trout rarely exceeds 10-15% (Torrissen *et al.*, *supra*, 1989; Storebakken, T. and No, H., *supra*, 1992). This has been explained in general terms by poor uptake from the intestinal tract (faecal losses normally account for 2/3 of the dietary astaxanthin) and by poor retention (less than 1/2) of the absorbed astaxanthin. Carotenoids are poorly absorbed in young salmon (Schiedt et al., *Pure Appl. Chem.*, 57:685-692 (1985)). Astaxanthin deposition has been shown to vary in different life stages of rainbow trout, and in the muscle a theoretical saturation level of 100 mg/kg has been estimated (*Bjerkeng et al., Aquaculture*, 108:333-346 (1992)). This is far from the levels (up to 15 mg/kg) experienced in commercial farming of Atlantic salmon.

*Fish Feed--Nutritional Requirements:* An increased understanding of the nutritional requirements for various fish species and technological advances in feed manufacturing have allowed the development and use of manufactured or artificial diets (formulated feeds) to supplement or to replace natural feeds in the aquaculture industry. The dietary



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requirements of fish are well known (see Nutrient Requirements of Fish, published by the Board of Agriculture's Committee on Animal Nutrition, National Research Council, National Academy:Washington, D.C. 1993). The dietary requirements of fish feed may vary according to species, aquatic environment, temperature and age. However, the general nutrition requirements for most aquatic species produced via aquaculture are known and are generally comprised of about 25 wt% to about 55 wt% crude protein (including essentially amino acids), up to about 30 wt% lipids (e.g., ω-3/ω-6 PUFAs), vitamins (i.e., fat and water soluble), minerals (i.e., including essential minerals), carbohydrates (sometimes included as an economical energy source although not required) and water (typically about 6-40 wt% depending upon the desired product form). Additional feed ingredients may include: (1) antioxidants (i.e., $CoQ_9$, $CoQ_{10}$, resveratrol), to prevent oxidation of lipids and/or pigments and to extend product shelf-life; (2) carotenoids, particularly for salmonid and ornamental "aquarium" fishes, to enhance tissue coloration of e.g., muscle, skin, shell, etc.; (3) binding agents, to provide stability to the pellet and reduce leaching of nutrients into the water (e.g., beef heart, starch, cellulose, pectin, gelatin, gum arabic, locust bean, agar, carageenin and other alginates); (4) chemoattractants and flavorings, to enhance feed palatability and its intake; and, (5) other feedstuffs. These other feedstuffs can include such materials as hormones, antibiotics, fiber and ash (for use as a filler and as a source of calcium and phosphorus, respectively), vegetable matter and/or fish or squid meal (e.g., live, frozen or dried algae, brine shrimp, rotifers or other zooplankton) to enhance the nutritional value of the diet and increase its acceptance by the fish.

When producing a "fish pigmentation product", referring to pigmented feed additives used to color fish via commercial aquacultural techniques, preferred carotenoids are canthaxanthin and/or astaxanthin. Fish pigmented by absorbing carotenoids include, but are not limited to salmonids, carp (*Cyprinus* sp.), red sea bream, tilapia, goldfish, Cichlidae, and yellowtail (*Serola lalandi*). In one aspect, fish pigmented using the



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present materials are members of the family Salmonidae, whose typical members include salmon, trout and chars.

The source of the various components is typically chosen based on cost relative to growth performance. Typically, components include but are not limited to: fish meal, fish oil (typically marine fish oil), meat meal, shrimp meal, and isolates from various plants including corn, rice, and soybean, to name a few. In one aspect, the majority of the crude protein and crude lipids in the fish feed is preferably provided by fish meal and fish oil. In more preferred embodiments, the fish oil is replaced with microbially produced oil, preferably extracted from an oleaginous yeast.

The manufacture of aquafeed formulations requires consideration of a variety of factors, since a complete diet must be nutritionally balanced, palatable, water stable and have the proper size and texture. With regard to nutrient composition of aquafeeds, one is referred to: *Handbook on Ingredients for Aquaculture Feeds* (Hertrampf, J. W. and F. Piedad-Pascual. Kluwer Academic: Dordrecht, The Netherlands, 2000) and *Standard Methods for the Nutrition and Feeding of Farmed Fish and Shrimp* (Tacon, A. G. J. Argent Laboratories: Redmond, 1990). In general, feeds are formulated to be dry (i.e., final moisture content of 6-10%), semi-moist (i.e., 35-40% water content) or wet (i.e., 50-70% water content). Dry feeds include the following: simple loose mixtures of dry ingredients (i.e., "mash" or "meals"); compressed pellets, crumbles or granules; and flakes. Depending on the feeding requirements of the fish, pellets can be made to sink or float. Semi-moist and wet feeds are made from single or mixed ingredients (e.g., trash fish or cooked legumes) and can be shaped into cakes or balls.

Based on the above discussions relating to the nutritional requirements for chicken feeds, crustacean feeds and fish feeds, it will be clear that the present invention also includes a method of pigmenting an animal, wherein a pigmented animal feed is provided (comprising an effective amount of pigmented microbial biomass/oils of the present invention comprising at least one caretenoid) and said animal is fed said pigmented animal feed, thereby resulting in pigmentation of at least one



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tissue in said animal or an animal product produced thereof. Similarly, in yet another aspect, the invention provides an animal tissue (or animal product produced thereof) pigmented by feeding an animal an animal feed comprising a carotenoid produced by the present process.

## EXAMPLES

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

### GENERAL METHODS

Standard recombinant DNA and molecular cloning techniques used in the Examples are well known in the art and are described by: 1.) Sambrook, J., Fritsch, E. F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1989) (Maniatis); 2.) T. J. Silhavy, M. L. Bennan, and L. W. Enquist, Experiments with Gene Fusions; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1984); and, 3.) Ausubel, F. M. et al., Current Protocols in Molecular Biology, published by Greene Publishing Assoc. and Wiley-Interscience, Hoboken, NJ (1987).

Materials and methods suitable for the maintenance and growth of microbial cultures are well known in the art. Techniques suitable for use in the following examples may be found as set out in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, Eds), American Society for Microbiology: Washington, D.C. (1994)); or by Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, 2nd ed., Sinauer Associates: Sunderland, MA (1989). All reagents, restriction enzymes and materials used for the growth and maintenance of microbial cells were obtained from Aldrich Chemicals



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(Milwaukee, WI), DIFCO Laboratories (Detroit, MI), New England Biolabs, Inc. (Beverly, MA), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO), unless otherwise specified. *E. coli* strains were typically grown at 37 °C on Luria Bertani (LB) plates.

General molecular cloning was performed according to standard methods (Sambrook *et al.*, *supra*). Oligonucleotides were synthesized by Sigma-Genosys (Spring, TX). Individual PCR amplification reactions were carried out in a 50 µl total volume, comprising: PCR buffer (containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.75), 2 mM $MgSO_4$, 0.1% Triton X-100), 100 µg/mL BSA (final concentration), 200 µM each deoxyribonucleotide triphosphate, 10 pmole of each primer and 1 µl of Pfu DNA polymerase (Stratagene, San Diego, CA), unless otherwise specified. Alternatively, PCR amplification reactions were carried out in a 50 µl total volume, comprising: 19.75 µL sterile $dH_2O$, 5 µL MasterAmp™ Taq 10X PCR Buffer, 4 µL $MgCl_2$ (25mM), 15 µL MasterAmp™ 10X PCR Enhancer, 1 µL each (10 mM) of dATP, dCTP, dGTP and dTTP, 1 µL each of Forward and Reverse primers, 0.25 µL MasterAmp™ Taq DNA polymerase (Epicentre® Biotechnologies, Madison, WI), unless otherwise specified.

The meaning of abbreviations is as follows: "sec" means second(s), "min" means minute(s), "h" means hour(s), "d" means day(s), "µL" means microliter(s), "mL" means milliliter(s), "L" means liter(s), "µM" means micromolar, "mM" means millimolar, "M" means molar, "mmol" means millimole(s), "µmol" mean micromole(s), "g" means gram(s), "µg" means microgram(s), "ng" means nanogram(s), "U" means unit(s), "bp" means base pair(s), "dcw" means dry cell weight, and "kB" means kilobase(s).

The structure of an expression cassette will be represented by a simple notation system of "X::Y::Z", wherein X describes the promoter fragment, Y describes the gene fragment, and Z describes the terminator fragment, which are all operably linked to one another.



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Cultivation Of *Yarrowia lipolytica*

*Yarrowia lipolytica* strain ATCC #20362 was purchased from the American Type Culture Collection (Rockville, MD). *Yarrowia lipolytica* strains were routinely grown at 28-30 °C in several media, according to the recipes shown below. Agar plates were prepared as required by addition of 15-20 g/L agar to each liquid media, according to standard methodology.

YPD agar medium (per liter): 10 g of yeast extract [Difco], 20 g of Bacto peptone [Difco]; and 20 g of glucose.

Basic Minimal Media (MM) (per liter): 20 g glucose; 1.7 g yeast nitrogen base without amino acids; 1.0 g proline; and pH 6.1 (not adjusted).

Minimal Media + Uracil (MM+uracil or MMU) (per liter): Prepare MM media as above and add 0.1 g uracil and 0.1 g uridine.

Minimal Media + Uracil + Sulfonylurea (MMU+SU) (per liter): Prepare MMU media as above and add 280 mg sulfonylurea.

Minimal Media + Leucine (MM+leucine or MMLeu) (per liter): Prepare MM media as above and add 0.1 g leucine.

Minimal Media + Leucine + Uracil (MMLeuUra) (per liter): Prepare MMU media as above and add 0.1 g leucine.

Minimal Media + Lysine (MM+lysine or MMLys) (per liter): Prepare MM media as above and add 0.1 g lysine.

Minimal Media + Leucine + Lysine (MMLeuLys) (per liter): Prepare MMLeu media as above and add 0.1 g lysine.

Minimal Media + Leucine + Lysine + Uracil (MMLeuLysUra) (per liter): Prepare MMLeuLys media as above and add 0.1 g uracil and 0.1 g uridine.

Minimal Media + 5-Fluoroorotic Acid (MM + 5-FOA) (per liter): 20 g glucose, 6.7 g Yeast Nitrogen base without amino acids, 75 mg uracil, 75 mg uridine and appropriate amount of FOA (Zymo



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Research Corp., Orange, CA), based on FOA activity testing against a range of concentrations from 100 mg/L to 1000 mg/L (since variation occurs within each batch received from the supplier).

High Glucose Media (HGM) (per liter): 80 glucose, 2.58 g $KH_2PO_4$ and 5.36 g $K_2HPO_4$, pH 7.5 (do not need to adjust).

Fermentation medium* without Yeast Extract (FM* without YE) (per liter): 6.70 g/L Yeast nitrogen base (YNB, without amino acids and without ammonium sulfate); 6.00 g $KH_2PO_4$; 2.00 g $K_2HPO_4$; 1.50 g $MgSO_4*7H_2O$; 1.5 mg thiamine hydrochloride; and 20 g glucose.

Fermentation medium* (FM*) (per liter): Prepare FM* without YE media as above and add 5.00 g Yeast extract (BBL).

The methodology used to create various *Ura-* strains of *Yarrowia lipolytica* (a "recycling" protocol that resulted in removal of the *Ura* marker) relied on site-specific recombinase systems. Briefly, the site-specific recombination system consists of two elements: (1) a recombination site having a characteristic DNA sequence [e.g., LoxP]; and, (2) a recombinase enzyme that binds to the DNA sequence specifically and catalyzes recombination (i.e., excision) between DNA sequences when two or more of the recombination sites are oriented in the same direction at a given interval on the same DNA molecule [e.g., Cre]. For the purposes herein, an integration construct was created comprising a target gene that was desirable to insert into the host genome (i.e., a first selection marker [i.e., Ura3]) that was flanked by recombination sites. Following transformation and selection of the transformants, the first selection marker was excised from the chromosome by the introduction of a replicating plasmid carrying a second selection marker (i.e., Leu2 or sulfonylurea resistance [AHAS]) and a recombinase suitable to recognize the site-specific recombination sites introduced into the genome (i.e., Cre). Upon selection of those transformants carrying the second marker, the replicating plasmid was then cured from the host in the absence of



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selection and excision of the first selection marker from the cured strain's host genome was confirmed by loss of *Ura* prototrophy. This produced a transformant that possessed neither the first nor second selection marker, and thus the cured strain was available for another round of transformation using the first selection marker. Additional details concerning site-specific recombinase based methodology for use in *Yarrowia lipolytica* is described in PCT Publication No. WO 2006/052870.

The second selection marker gene occasionally utilized in *Yarrowia lipolytica* is a native *Y. lipolytica* acetohydroxyacid synthase (AHAS or acetolactate synthase; E.C. 4.1.3.18; GenBank Accession No. XM_501277) containing a single amino acid change (W497L) that confers sulfonyl urea herbicide resistance ($SU^R$; described in PCT Publication No. WO 2006/052870) (SEQ ID NO:1). AHAS is the first common enzyme in the pathway for the biosynthesis of branched-chain amino acids and it is the target of the sulfonylurea and imidazolinone herbicides.

Fatty Acid Analysis of *Yarrowia lipolytica*

For fatty acid analysis, cells were collected by centrifugation and lipids were extracted as described in Bligh, E. G. and Dyer, W. J. (*Can. J. Biochem. Physiol.*, 37:911-917 (1959)). Fatty acid methyl esters were prepared by transesterification of the lipid extract with sodium methoxide (Roughan, G., and Nishida I., *Arch. Biochem. Biophys.*, 276(1):38-46 (1990)) and subsequently analyzed with a Hewlett-Packard 6890 GC fitted with a 30-m X 0.25 mm (i.d.) HP-INNOWAX (Hewlett-Packard) column. The oven temperature was from 170 °C (25 min hold) to 185 °C at 3.5 °C/min.

For direct base transesterification, *Yarrowia* culture (3 mL) was harvested, washed once in distilled water, and dried under vacuum in a Speed-Vac for 5-10 min. Sodium methoxide (100 μl of 1 %) was added to the sample, and then the sample was vortexed and rocked for 20 min. After adding 3 drops of 1 M NaCl and 400 μl hexane, the sample was vortexed and spun. The upper layer was removed and analyzed by GC as described above.



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EXAMPLE 1

DNA Transformation In *Yarrowia lipolytica*

The following general procedure was used to make *Yarrowia lipolytica* chemically competent for DNA transformation (Chen, D. C. *et al.*, *Appl. Microbiol Biotechnol.*, 48:232-235 (1997)).

*Yarrowia* cells were streaked on YPD medium plates 1 day prior to transformation. The cells were incubated at 30 °C. Several large loopfuls (~3) of cells from the YPD plate were resuspended in 1 mL of transformation medium [comprising 2.25 mL 50% PEG (average MW 3350); 0.125 mL 2 M lithium acetate pH 6.0; 0.125 mL 2 M dithiothreitol (DTT; prepared fresh prior to each use); and optionally 50 μL salmon sperm DNA (10 mg/mL)]. Approximately 100 μL of the *Yarrowia* cells suspended in the transformation medium were mixed with 100 to 500 ng of the desired plasmid (in linearized form when transforming integration plasmids). The cells were subsequently incubated at 39 °C for 1 hr and mixed using a Vortex mixer about every 15 min. After incubation, the cells were streaked on MM agar plates with appropriate selection and incubated at 30 °C for a defined period of time (typically up to 4 days) depending upon the specific plasmid. Alternatively, transformants were selected on MM + 5-FOA agar plates. Transformants containing self-replicating plasmid were visible within about 2-6 days depending upon the strain. Transformants containing an integrative plasmid were typically visible within 3-4 days.

EXAMPLE 2

Construction Of *Yarrowia lipolytica* Strain YL5, Producing Lycopene

The present Example describes the construction of lycopene-producing *Yarrowia lipolytica* strain YL5. This strain was prepared as a means to initially demonstrate the feasibility of $C_{40}$ carotenoid biosynthesis in an oleaginous yeast.

After construction of integration vector pYCRTEBI (comprising *crtE*, *crtB* and *crtI* genes for lycopene synthesis) and transformation of the vector into *Yarrowia lipolytica* Y2224 (i.e., a *Ura*⁻ derivative of *Y. lipolytica* ATCC# 20362 [isolation described in Example 7]), lycopene production



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was characterized in resulting strain YL5 under growth and oleaginous conditions.

Construction Of Integration Vector For Lycopene Biosynthesis

Production of lycopene requires *crtE, crtB* and *crtI* genes, which encode GGPP synthase (CrtE), phytoene synthase (CrtB) and phytoene desaturase (CrtI), respectively. The *crtE*, *crtB* and *crtI* genes from *Pantoea stewartii* DC413 (U.S. Patent 7,288,387) were selected (Table 7). In order to optimize the expression of these 3 genes, they were codon-optimized based on the codon usage preference for *Yarrowia lipolytica* (U.S. Patent 7,125,672). The synthetic genes were produced by GenScript Corp. (Piscataway, NJ) and provided in the high-copy vector pUC57 (GenBank® Accession No. Y14837).

Table 7

Codon-Optimized Carotenoid Biosynthesis Genes Derived From *Pantoea stewartii* DC413

| Carotenoid Gene | Codon Optimized Nucleotide Sequence (SEQ ID NO) | Amino Acid Sequence (SEQ ID NO) |
|---|---|---|
| $crtE_{syn}$ | 2 | 3 |
| $crtB_{syn}$ | 4 | 5 |
| $crtI_{syn}$ | 6 | 7 |

During synthesis of these genes, a *Nco*I restriction site was introduced at the ATG start site and a *Not*I site was introduced after the translational stop codon. These two sites were used to facilitate the assembly of the genes with different promoters and terminators.

The construction of vector pYCRTEBI, comprising the codon-optimized $crtE_{syn}$, $crtB_{syn}$ and $crtI_{syn}$ genes, is summarized in Figure 5. Integration vector pZKleuN-6EP (SEQ ID NO:8) was used as the vector backbone.



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Table 8

Components Of Plasmid pZKLeuN-6EP (SEQ ID NO:8)

| RE Sites And Nucleotides Within SEQ ID NO:8 | Description Of Fragment And Chimeric Gene Components |
|---|---|
| *BsiWI/EcoRI* (11314-9601) | LoxP::*Ura3*::LoxP, comprising:<br>• LoxP sequence (SEQ ID NO:9);<br>• *Yarrowia* Ura3 gene (GenBank Accession No. AJ306421);<br>• LoxP sequence (SEQ ID NO:9) |
| *BglII/SwaI* (2522-5220)<br><br>[wherein bp 3511-4888, corresponding to D6S, can be excised by a *NcoI/NotI* digestion] | FBAIN::D6S::Lip1, comprising:<br>• FBAIN: *Yarrowia lipolytica* FBAIN promoter (PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356);<br>• D6S: codon-optimized Δ6 desaturase gene (PCT Publication No. WO 2004/101753; U.S. Patent No. 7,125,672), derived from *Mortierella alpina* (GenBank® Accession No. AF465281);<br>• Lip1: Lip1 terminator sequence from *Yarrowia Lip1* gene (GenBank® Accession No. Z50020) |
| *PmeI/ClaI* (2478-1)<br><br>[wherein bp 1301-341, corresponding to EL1S, can be excised by a *NcoI/NotI* digestion] | GPDIN::EL1S::Lip2, comprising:<br>• GPDIN: *Yarrowia lipolytica* GPDIN promoter (Patent Publication US 2006/0019297-A1; labeled as "GPD Pro +Intron" in Figure);<br>• EL1S: codon-optimized elongase 1 gene (PCT Publication No. WO 2004/101753; U.S. Patent No. 7,125,672), derived from *Mortierella alpina* (GenBank® Accession No. AX464731);<br>• Lip2: Lip2 terminator sequence from *Yarrowia Lip2* gene (GenBank® Accession No. AJ012632) |
| *AscI/BsiWI* (6171-5376) | 5' region of *Yarrowia Leu2* gene (GenBank® Accession No. AF260230) |
| *PacI/SphI* (9588-8879) | 3' region of *Yarrowia Leu2* gene (GenBank® Accession No. AF260230) |

Vector pZKleuN-6EP contains DNA regions from the *Yarrowia Leu2* gene that are involved in leucine biosynthesis. Integration of the DNA fragments in this region will result in a phenotype that requires the presence of the amino acid leucine in the minimal medium for growth. In addition, the plasmid contains a *Ura3* gene as a selection marker for growth requirement with uracil. The plasmid also has different promoter-terminator modules.



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In the first step of the construction of vector pYCRTEBI, the *crtE*$_{syn}$ gene (SEQ ID NO:2) was digested with *Nco*I and *Not*I. The promoter region, FBAIN was isolated from pZKleuN-6EP as a *Bgl*II/*Nco*I fragment; similarly, the terminator *Lip1* was isolated as a *Not*I/*Swa*I fragment. The integration vector was digested with enzymes *Bgl*II and *Swa*I. All DNA fragments were gel purified and then recovered using a BIO101® Geneclean® II kit (QBiogene, Irvine, CA) or Zymoclean™ Gel DNA recovery kit (Zymo Research Corp., Orange, CA). A four-way ligation procedure was used to assemble these fragments. The ligation reaction had a final volume of 11 μL that contained 5.25 μL of 2X rapid ligation buffer (Promega, Madison, WI), 0.75 μL of vector, 1.33 μL of each insert, and 1 μL of enzyme (T4 DNA ligase, Promega). The reaction was allowed to proceed for 2 hr at room temperature. After ligation, 2 μL of the mixture was transformed into *E. coli* XL2-Blue ultracompetent cells (Stratagene, La Jolla, CA).

The resulting vector, pYCRTE, was then used to clone the next gene (i.e., *crtB*$_{syn}$). The GPDIN promoter and the *Lip2* terminator were used for expression of *crtB*$_{syn}$ (SEQ ID NO:4). The GPDIN promoter from pYCRTE was isolated as a *Pme*I/*Nco*I fragment, while the *Lip2* terminator was isolated as a *Cla*I/*Not*I fragment. The vector pYCRTE was isolated as a *Cla*I/*Pme*I fragment. A 4-way ligation and transformation, similar to that described above for synthesis of pYCRTE, was used to introduce the chimeric *crtB*$_{syn}$ gene (i.e., *GPDIN*::*crtB*$_{syn}$::*Lip2*).

The resulting plasmid, pYCRTEB, was used to clone the last gene (i.e., *crtI*$_{syn}$; SEQ ID NO:6). The *Yarrowia lipolytica* export protein (EXP1) promoter (PCT Publication No. WO 2006/052870 and U.S. Patent Application No. 11/265761) was isolated from vector pEXPGUS1-P (SEQ ID NO:10; Figure 6A) as a *Pme*I/*Nco*I fragment, while the OCT terminator of the *Yarrowia OCT* gene (GenBank Accession No. X69988) was isolated from vector pZP34R (SEQ ID NO:11; Figure 6B) as a *Bgl*II/*Not*I fragment. The vector pYCRTEB was digested with *Pme*I and *Bgl*II and gel-purified with the BIO101® Kit (QBiogene). Again, a 4-way ligation and



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transformation procedure was utilized to introduce the chimeric *EXP1*::*crtI*$_{syn}$::*Oct* gene. Thus, the final plasmid construct pYCRTEBI (SEQ ID NO:12; Figure 5), was created to thereby contain the following chimeric carotenoid biosynthesis genes: *FBAIN*::*crtE*$_{syn}$::*Lip1*, *GPDIN*::*crtB*$_{syn}$::*Lip2* and *EXP1*::*crtI*$_{syn}$::*Oct*.

For integration into *Yarrowia lipolytica* strain Y2224, the pYCRTEBI plasmid was linearized (digested) with restriction enzymes *Sph*I and *Asc*I before transformation, according to the methodology of Example 1. After transformation, the cells were spread and grown on minimal plates without uracil and several colonies having red or orange color were selected for analysis. Red and orange transformants were streaked onto minimal plates and checked for stability and color intensity. One stable strain was designated as *Yarrowia lipolytica* strain YL5.

Characterization Of Lycopene Production In *Y. lipolytica* Strain YL5

*Yarrowia lipolytica* strain YL5 was grown in 100 mL FM* in a 500 mL flask in a rotary shaker at 250 rpm. After 2 days of growth, 1 mL of cell culture was harvested by centrifugation at 12,000 rpm using a microcentrifuge. The cell pellet was extracted using the method described below for lycopene analysis. At the same time, 10 mL culture was collected in a 47 mm Nuclepore® Track-Etch polycarbonate membrane (0.2 μM, Whatman, Florham Park, NJ) under vacuum for dry cell weight measurement.

To extract the pigments, the cell pellet was first resuspended by vortexing with a small amount of liquid remaining in the pellets. Next, 0.1 mm glass beads were added, followed by 2 mL of ethanol. The mixture was vortexed until the entire pellet dissolved. Next, 3 mL of dichloromethane was added and the samples were vortexed again for about 2 min. After centrifugation at 6600 x *g* (room temperature) with a table top centrifuge for 10 min, the supernatant was transferred to a new 50 mL Corning® polypropylene centrifuge tube (Corning Inc., Corning, NY) and was dried under a stream of nitrogen. The residue was dissolved completely in 90 μL chloroform followed by the addition of 1910 μL n-hexane (HPLC grade). Before injection into the HPLC, the samples were



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filtered with a 0.2 µm Gelman Teflon® filter (Pall Life Sciences, Ann Arbor, MI). The extract (20 µL) was analyzed using a HPLC system (Beckman, Fullerton, CA) equipped with a 250 x 4.6 mm Brownlee™, Spheri-5 silica, 5 µm, normal phase HPLC column (PerkinElmer, Norwalk, CT). The mobile phase consisted of 14% acetone and 86% *n*-hexane under isocratic conditions. The flow rate was 1.5 mL per min.

Figure 7A shows the HPLC profile of the carotenoid product of strain YL5, comprising pYCRTEBI. The pigment from YL5 had the same retention time as the lycopene standard purchased from CaroteNature GmbH (Lupsingen, Switzerland). Furthermore, the pigment had the same absorbance profile characteristics of lycopene (Figure 7B). The molecular weight of the extracted lycopene was confirmed by mass spectroscopy.

Co-Production Of Oil And Lycopene In *Yarrowia lipolytica* Strain YL5

It is known that the oleaginous yeast *Yarrowia lipolytica* can accumulate significant amounts of oil, especially under nitrogen-limiting conditions. In order to investigate whether lycopene and oil could be co-produced in the same cell, strain YL5 was first grown for 2 days in a 500 mL flask with 100 mL in FM*. A portion of the culture (30 mL) was harvested by centrifugation at 4,000 x g at room temperature and resuspended with 30 mL of oil induction medium ("OI" medium: 100 mM glucose, 50 mM potassium phosphate [pH 6.5]) without yeast nitrogen base (YNB) added. Another 30 mL of the culture was resuspended in OI medium with 6.7 g/L YNB added. The cultures were placed in 250 mL flasks and allowed to incubate at 30 °C with constant shaking at 225 rpm. Analytical samples were taken for lycopene and total oil analysis was conducted after 2 and 4 days of incubation. Results are shown in Table 9.

Table 9

Production Of Lycopene And Oil In Strain YL5 Under Different Growth Conditions

| Growth conditions | Total oil (% DCW) | Lycopene (mg $g^{-1}$ DCW) |
|---|---|---|
| *Before induction* | 4.0 | 374 |
| *2 days in OI medium* | | |



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| | | |
|---|---|---|
| OI with YNB | 3.6 | 1.92 |
| OI without YNB | 7.5 | 1.60 |
| *4 days in OI medium* | | |
| OI with YNB | 7.2 | 1.63 |
| OI without YNB | 12.5 | 1.33 |

Note: Dry cell weight is abbreviated "DCW".

Strain YL5 accumulated more oil in OI medium without the YNB supplement. The amount of lycopene increased after 2 days of induction in OI medium with or without YNB added. After 4 days, the lycopene level dropped slightly, but it was still much higher than the level observed before the induction. The overall observation indicated that oil and lycopene could be co-produced in a recombinant oleaginous yeast.

EXAMPLE 3

Synthesis Of *Yarrowia lipolytica* Strains YCS1001-YCS1026, Producing Lycopene

The present Example describes the construction of lycopene-producing *Yarrowia lipolytica* strains YCS1001-YSC1026 (Figure 8), by transformation and expression of plasmid pYCRTEBI (Example 2, *supra*) in *Y. lipolytica* strain Y2224 (i.e., a *Ura*⁻ derivative of *Y. lipolytica* ATCC #20362 [isolation described in Example 7]). Strain YCS1002 served as the host strain in Example 5, *infra*, for creation of the YCS1200 series of carotenoid-producing *Y. lipolytica* transformants, while strain YCS1013 served as the host strain in Example 5, *infra*, for creation of the YCS1300 series of carotenoid-producing *Y. lipolytica* transformants.

Specifically, additional pigmented, lycopene-producing *Y. lipolytica* strains were generated by transforming plasmid pYCRTEBI into *Y. lipolytica* strain Y2224, according to the methodology of Example 1. Strain Y2224 was separately transformed with pYCRTEBI two times (i.e., the "first" or "second" transformation, respectively herein). Following transformation, cells were plated onto MM + leucine (Leu) and colonies were visible after 2 days.

In the first transformation, 50 colonies were patched onto MM and MM + Leu agar plates. Twenty-two of the 50 patches from the first transformation were only able to grow on the MM + Leu plates, indicating



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that the cells were Leu$^-$. In a second transformation, 11 of the 50 patches were only able to grow on the MM + Leu agar plates.

Ten colonies from each transformation were re-streaked onto the MM and MM + Leu agar plates. Leu$^-$ cells were unable to grow on the MM plates and cells growing on the MM+ Leu plates had very similar orange hues. The Leu+ cells appeared darker in color than the Leu$^-$ cells.

From the first transformation, the 10 Leu$^-$ transformants were named YCS1001 through YCS1010 and 2 Leu+ transformants were named YCS1021 and YCS1022.

From the second transformation, the 10 Leu$^-$ transformants were named YCS1011 through YCS1020 and the 4 Leu+ transformants were named YCS1023 through YCS1026.

The *Ura* marker was removed from 6 of the dark orange *Yarrowia* strains (i.e., YCS1021, YCS1022, YCS1023, YCS1024, YCS1025 and YCS1026) containing the $crtE_{syn}$, $crtB_{syn}$ and $crtI_{syn}$ carotenoid genes, as described below. Specifically, *Yarrowia* cells were streaked onto fresh YPD plates and incubated at 30 °C for 1 day. Then, cells were transformed using the methodology of Example 1 with 1 μL of replicating plasmid pY79. Plasmid pY79 (Figure 9A; SEQ ID NO:13) contained the following components:

Table 10

Components of Plasmid pY79 (SEQ ID NO:13)

| RE Sites And Nucleotides Within SEQ ID NO:13 | Description Of Fragment And Chimeric Gene Components |
|---|---|
| 4329-7315 | *Yarrowia lipolytica* AHAS gene comprising a W497L mutation (confers sulfonylurea resistance) (SEQ ID NO:1; GenBank® Accession No. XM_501277; PCT Publication No. WO 2006/052870) |
| 7362-1 | *TEF*::*Cre*::*XPR*, comprising:<br>• *TEF*: *Yarrowia* TEF promoter (GenBank® Accession No. AF054508);<br>• *Cre*: Enterobacteria phage P1 *Cre* gene for recombinase protein (GenBank® Accession No. X03453); |



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| | |
|---|---|
| | • *XPR*: XPR terminator sequence comprising ~100 bp of the 3' region of the *Yarrowia Xpr* gene (GenBank® Accession No. M17741) |

For transformation, the cells and pY79 were incubated at 39 °C for 1 hr, with vortexing every 15 min. Cells were then plated onto MMU + SU agar plates and incubated at 30 °C for 2-3 days.

It was observed that the color intensity of the colonies decreased when the *Ura* marker was removed. It was also observed that colonies from strain YCS1026 were white upon *Ura* marker removal, suggesting that the integration of the carotenoid genes in this strain was unstable. The lycopene-producing strains that were evaluated via HPLC analysis for their carotenoid profile were YCS1007 ($Leu^-$) and YCS1021, YCS1023, YCS1024 and YCS1025. All strains were confirmed to produce lycopene.

## EXAMPLE 4

## Preparation Of Promoter And Terminator Fragments For Construction Of *Yarrowia lipolytica* Integration Vectors Comprising Carotenoid Biosynthetic Genes

The present Example describes the preparation of various *Yarrowia lipolytica* promoters and terminators, that would subsequently be used in Examples 5, 6, 10, 11 and 12 for construction of various integration vectors comprising carotenoid biosynthetic genes.

Vector pZKSL555 (Figure 9B; SEQ ID NO:14) was previously constructed for expression of 3 heterologous Δ5 desaturases in *Yarrowia lipolytica*, and contained the components listed below in Table 11.

### Table 11

### Components Of Plasmid pZKSL555 (SEQ ID NO:14)

| RE Sites And Nucleotides Within SEQ ID NO:14 | Description of Fragment and Chimeric Gene Components |
|---|---|



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| | |
|---|---|
| *PacI/ClaI* (11735-1) | EXP1::M.Ad5::Pex16, comprising<br>• EXP1: *Yarrowia lipolytica* export protein (EXP1) promoter (labeled as "Exp" in Figure; PCT Publication No. WO 2006/052870 and U.S. Patent Application No. 11/265761);<br>• M.Ad5: codon-optimized Δ5 desaturase, derived from *Mortierella alpina* (GenBank® Accession No. AF067654);<br>• Pex16: Pex16 terminator sequence from *Yarrowia Pex16* gene (GenBank® Accession No. U75433) |
| *SalI/BsiWI* (2468-5081) | GPAT::M.Ad5::Pex20, comprising<br>• GPAT: *Yarrowia lipolytica* GPAT promoter (PCT Publication No. WO 2006/031937 and U.S. Patent 7,264,949);<br>• M.Ad5: codon-optimized Δ5 desaturase, derived from *Mortierella alpina* (GenBank® Accession No. AF067654);<br>• Pex20: Pex20 terminator sequence of *Yarrowia lipolytica Pex20* gene (GenBank® Accession No. AF054613) |
| *BsiWI/SwaI* (5081-7504) | YAT1::I.g.D5::Lip1, comprising<br>• YAT1: *Yarrowia lipolytica YAT1* promoter (labeled as "NT" in Figure; Patent Publication US 2006/0094102-A1);<br>• I.g.D5: codon-optimized Δ5 desaturase gene, derived from *Isochrysis galbana* (PCT Publication No. WO 2002/ 081668);<br>• Lip1: Lip1 terminator sequence from *Yarrowia Lip1* gene (GenBank® Accession No. Z50020) |

Various promoter and terminator elements were isolated from pZKSL555 via enzymatic digestions. In the initial digestion, pZKSL555 was digested with *Pac*I and *Cla*I. The 2.5 kb *PacI/ClaI* fragment was digested with *Nco*I or *Not*I to prepare fragments comprising either: (a) the *EXP1* promoter; or, (b) the *Pex16* terminator. The *EXP1* promoter fragment was 839 bp and the *Pex16-3'* terminator fragment was 339 bp.

Plasmid pZKSL555 was also subjected to *BsiWI/Swa*I digestion, resulting in production of 11.7 kb and 2.4 kb DNA fragments. The 2.4 kb DNA fragment was excised from the agarose gel, purified and subsequently digested with *Nco*I, which produced two DNA fragments (i.e., 1.6 kb and 0.8 kb). The 0.8 kb DNA fragment contained the *YAT1* promoter. The 2.4 kb DNA fragment was also digested with *Not*I, thereby generating 2.1 kb and 0.3 kb DNA fragments. The 0.3 kb DNA fragment,



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which contained the *Lip1* terminator (332 bp), was excised from the agarose gel and purified.

Digestion of pZKSL555 with *BsiW*I and *Sal*I resulted in the production of four DNA fragments (i.e., 7.7 kb, 4.0 kb, 1.6 kb and 1.0 kb). The 1.6 kb and the 1.0 kb DNA fragments were excised from the agarose gel and purified. Further digestion of the 1.6 kb DNA fragment with *Nco*I generated two DNA fragments (i.e., ~1.0 kb and 0.7 kb). The ~1.0 kb fragment containing the *GPAT* promoter was excised from the agarose gel and purified. Further digestion of the 1.0 kb *BsiW*I/*Sal*I DNA fragment with *Not*I generated two more fragments (i.e., 0.7 kb and 0.3 kb). The 0.3 kb fragment, which contained the *Pex20* terminator, was excised from the agarose gel and purified. All DNA fragment purification from the agarose gels was done using the Geneclean® II kit.

EXAMPLE 5

Synthesis Of *Yarrowia lipolytica* Strain Series YCS1200 And Series YCS1300, Producing Primarily β-Carotene

The present Example describes the construction of β-carotene-producing *Yarrowia lipolytica* strains, identified herein as the YCS1200 series and the YCS1300 series (Figure 8). Strain YCS1205 served as the host strain in Example 6, *infra*, for creation of the YCS3700 series of carotenoid-producing *Y. lipolytica* transformants, while strain YCS1306 served as the host strain in Example 6, *infra*, for creation of the YCS4000 and YCS4100 series of carotenoid-producing *Y. lipolytica* transformants.

As described herein, construction of the *Y. lipolytica* YCS1200 series and the YCS1300 series required: (1) preparation of wildtype *crtY*, *crtZ* and *crtW* genes for subsequent cloning; (2) synthesis of pYPS106, comprising a *crtY* gene; (3) synthesis of pYPS107, comprising a *crtY* gene and a *crtZ* gene; (4) synthesis of pYPS108, comprising a *crtY* gene, a *crtZ* gene and a *crtW* gene; (5) transformation of lycopene-producing *Yarrowia* strains with integration plasmid pYPS108; and, (6) extraction and analysis of the carotenoids produced.



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Cloning Wildtype *crtY, crtZ* And *crtW* Genes

Production of astaxanthin requires *crtY, crtW,* and *crtZ* genes, which encode lycopene cyclase (CrtY), carotenoid ketolase (CrtW), and carotenoid hydroxylase (CrtZ), respectively.

Integration plasmid pYPS108 was constructed to contain wildtype *crtY*, *crtZ,* and *crtW* genes from plasmid pDCQ392 (SEQ ID NO:15). Plasmid pDCQ392 comprises the carotenoid gene cluster identified as "DCQ392", which comprises the gene cluster *crtWZEidiYIB*. The *crtW* gene in DCQ392 (herein referred to as $crtW_{392}$; SEQ ID NO:16) is from *Brevundimonas vesicularis* DC263 (U.S. Patent 7,252,985). The *crtZ* gene in DCQ392 (herein referred to as $crtZ_{392}$; SEQ ID NO:18) is from *Brevundimonas vesicularis* DC263 (U.S. Patent 7,091,031). The *crtEidiYIB* carotenoid biosynthesis "backbone" genes from DCQ392 are from the *Pantoea stewartii* DC413 (U.S. Patent 7,288,387). Hereinafter, the *crtY* gene from the DCQ392 carotenoid cluster will be referred to herein as $crtY_{392}$; SEQ ID NO:20).

Each of the *crt* genes described above was PCR amplified from pDCQ392 using primers that added *Nco*I and *Not*I sites to the ends of each gene. Specifically, $crtY_{392}$ was amplified with the forward and reverse primers set forth as SEQ ID NOs:22 and 23, $crtZ_{392}$ was amplified with the forward and reverse primers set forth as SEQ ID NOs:24 and 25, and $crtW_{392}$ was amplified with the forward and reverse primers set forth as SEQ ID NOs:26 and 27.

All three PCR products were ligated into pCR®2.1-TOPO® using the TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA). Each gene was subsequently excised from the pCR®2.1-TOPO® derived vector by digestion with *Nco*I and *Not*I. The TOPO plasmids containing $crtY_{392}$ had DNA fragments that were 2.3 kb, 1.6 kb and 1.2 kb. The $crtY_{392}$ gene was present on the 1.2 kb DNA fragment. The TOPO plasmids containing $crtW_{392}$ had DNA fragments that were 2.3 kb, 1.6 kb and 0.78 kb; the $crtW_{392}$ gene was located on the 0.78 kb DNA fragment. The TOPO plasmids containing $crtZ_{392}$ also had three fragments (i.e., 2.3 kb, 1.6 kb



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and 0.5 kb) when digested with *Nco*I and *Not*I; the 0.5 kb DNA fragment contained the *crtZ*$_{392}$ gene.

Synthesis Of pYPS106, Comprising A *crtY*$_{392}$ Gene

The *EXP1* promoter and the *Pex16* terminator were ligated to the 5' and 3' ends of the *crtY*$_{392}$ coding sequence (SEQ ID NO:20), respectively, in a four-way ligation reaction creating the chimeric *EXP1::crtY*$_{392}$*::Pex16* gene. The ligation reaction included an 11.7 kb *Pac*I/*Cla*I DNA fragment from pZKSL555, a *Cla*I/*Nco*I DNA fragment containing the *EXP1* promoter, a *Nco*I/*Not*I DNA fragment comprising the *crtY*$_{392}$ coding sequence and a *Not*I/*Pac*I DNA fragment including the *Pex16-3'* terminator. The ligation reaction was transformed into *E. coli* XL2 Blue cells and the transformation mixture was streaked onto LB agar plates containing 100 mg/L ampicillin (i.e., LB + Amp$^{100}$ agar plates).

Approximately 30 colonies were screened via PCR amplification to verify the construction of plasmid pYPS106, using PCR primers Exp-forward (SEQ ID NO:28) and HY-339 reverse (SEQ ID NO:29).

The PCR reaction comprised the MasterAmp™ Taq (Epicentre) components described in the General Methods and a single colony. The PCR reaction was run using the following PCR parameters: 94 °C for 5 min (1 cycle); 94 °C for 30 sec, 60 °C for 1 min, 72 °C for 1 min (30 cycles); and 72 °C for 6 min (1 cycle).

Seven of the samples produced a PCR fragment of the expected size of 1.4 kb. The plasmid DNA was extracted from cells and confirmed to be correct by digestion with *Mlu*I and *Cla*I. The vectors having the correct insert DNA fragment generated 3 bands upon digestion that were 9.6 kb, 3.6 kb and 1.0 kb in size. This plasmid was named pYPS106 (SEQ ID NO:30).

Synthesis Of pYPS107, Comprising A *crtY*$_{392}$ Gene And A *crtZ*$_{392}$ Gene

Plasmid pYPS106 was digested with *Bsi*WI and *Swa*I and the 11.7 kb DNA fragment was excised from the agarose gel. This 11.7 kb fragment served as the vector backbone in a four-way ligation reaction that created a chimeric *YAT1::crtZ*$_{392}$*::Lip1* gene in pYPS107. Specifically, the four-way ligation reaction comprised: the vector backbone, the



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*Bsi*WI/*Nco*I digested *YAT1* promoter, the *Nco*I/*Not*I digested *crtZ*$_{392}$ coding sequence (SEQ ID NO:18) and the *Not*I/*Swa*I digested *Lip1* terminator. The ligation mixture was used to transform *E. coli* XL2 Blue cells which were streaked onto LB + Amp$^{100}$ agar plates. Twenty-four colonies were evaluated for the correct insert DNA fragment using the PCR methodology described above for pYPS106 and PCR primers NT forward (SEQ ID NO:31) and Lip1-3' reverse (SEQ ID NO:32).

A 2.1 kb PCR fragment was generated if the cells contained the parental vector pYPS106. However, cells having plasmids with the correct insert DNA fragment generated PCR fragments that were 1.1 kb. The PCR results were confirmed via restriction digestion of plasmid DNA using *Bsi*WI and *Swa*I. This new vector, comprising a chimeric *EXP1*::*crtY*$_{392}$::*Pex16* gene and a chimeric *YAT1*::*crtZ*$_{392}$::*Lip1* gene, was named pYPS107 (SEQ ID NO:33).

Synthesis Of pYPS108, Comprising A *crtY*$_{392}$ Gene, A *crtZ*$_{392}$ Gene And A *crtW*$_{392}$ Gene

Plasmid pYPS107 was digested with *Bsi*WI and *Swa*I to prepare a vector backbone for the final four-way ligation reaction required to incorporate the third carotenoid gene, *crtW*$_{392}$ (SEQ ID NO:16), into the vector as a chimeric *GPAT*::*crtW*$_{392}$::*Pex20* gene. The other DNA fragments included in this four-way ligation reaction were: a *Sal*I/*Nco*I digested DNA fragment containing the *GPAT* promoter, the *Nco*I/*Not*I digested DNA fragment containing the *crtW*$_{392}$ coding sequence, and a *Not*I/*Bsi*WI digested DNA fragment containing the *Pex20* terminator. The ligation mixture was used to transform *E. coli* XL2 Blue cells, which were streaked onto LB + Amp$^{100}$ agar plates.

Several of the colonies were screened to identify those having the correct insert DNA fragment via a modified PCR methodology with respect to that used for pYPS106. Specifically, the components of the PCR reaction were the same as described for pYPS106, with the exception that DNA primers GPAT forward (SEQ ID NO:34) and Pex20 reverse (SEQ ID NO:35) were utilized. The PCR parameters were: 94°C for 5 min (1 cycle); 94°C for 30 sec, 60°C for 1 min, 72 °C for 90 sec (30 cycles); and 72°C for 6 min (1 cycle).



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The PCR products were evaluated on an agarose gel and a 1.6 kb band was detected for samples having the correct insert DNA fragment. These plasmid vectors were confirmed to have the correct insert DNA fragments by restriction digests. Specifically, the plasmid containing the chimeric *GPAT::crtW$_{392}$::Pex20* gene plus the pYPS107 vector backbone generated two fragments (i.e., 10.6 kb and 2.1 kb) upon digestion with *BsiW*I and *Sal*I. This plasmid was named pYPS108 (SEQ ID NO:36; Figure 10) and thereby comprised the following chimeric carotenoid biosynthetic genes: *EXP1::crtY$_{392}$::Pex16*, *YAT1::crtZ$_{392}$::Lip1* and *GPAT::crtW$_{392}$::Pex20*.

Transformation Of A Lycopene-Producing *Yarrowia* Strain With Integration Plasmid pYPS108

To prepare pYPS108 (SEQ ID NO:36) for transformation into *Yarrowia*, the plasmid DNA was linearized by digestion with *Aat*II and *Asc*I. The linearized plasmid DNA was transformed into YCS1002 and YCS1013, both strains (isolates) comprising the *crtE$_{syn}$*, *crtB$_{syn}$* and *crtI$_{syn}$* genes integrated at the leucin chromosomal site (Example 3). The transformation was carried out according to Example 1. The transformation mixture was streaked onto MMLys agar plates. Colonies resulting from this transformation were a part of the YCS1200 series (if derived from YCS1002) and the YCS1300 series (if derived from YCS1013). Eight colonies were patched onto MM and MMLys to determine if the plasmid had integrated at the lysine site or elsewhere within the host chromosome. All 8 colonies were able to grow on both mediums, indicating that integration had not occurred at the lysine loci.

The *ura* marker was removed from YCS1201 and YCS1302 transformant via transformation with pY79 (Example 3). Four colonies from each transformation were grown in MMLeuLysUra medium and streaked onto MMU agar plates. Forty colonies were patched onto MM, MMU+SU, and MMU. Some of the colonies were unable to grow on the MM agar plates, suggesting that the *ura* marker had been removed (cells were thus auxotrophs for uracil). Approximately 25% of the colonies were unable to grow on plates containing SU suggesting that cells had lost the plasmid expressing the Cre recombinase. One YCS1200 series colony selected for further analysis was named YCS1205 and



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its phenotype was *ura*$^-$ Leu$^+$ Lys$^+$. Similarly, one YCS1300 colony that was *ura*$^-$ Leu$^+$ Lys$^+$ was chosen for further analysis and was named YCS1306.

Carotenoid Extraction And Analysis

Individual colonies were inoculated into a 24-well block containing 700 μL of MMLeuLysUra medium and were grown overnight with aeration. From these seed cultures, 20 mL of YPD medium was inoculated to a starting $OD_{600}$ of 0.05. Following ~40 hr of growth, 2 mL was removed and the cells were harvested by centrifugation. After disrupting the cell pellets by dragging the microcentrifuge tube across a rack, 400 μL of a 1% sodium methoxide solution and 100 μL of 0.5 mm glass beads were added to the cells. The mixture was agitated in a BeadBeater™ apparatus (BioSpec Products, Bartlesville, OK) for 5 min at high speed, followed by mixing on a rocking platform for 20 min. Afterwards, 50 μL of a 1.0 M NaCl solution was added to the disrupted cells. In addition, 200 μL of chloroform and 400 μL of hexane were added and were mixed together by vortexing. The solution was separated into layers by a 3 min centrifugation. The upper organic layer was removed using a glass Pasteur pipette. The extract was subsequently placed into a syringe and was filtered into a small glass vial containing an insert for small samples using a 0.2 μm Teflon® filter (Pall Corp., Ann Arbor, MI). The carotenoid extracts were analyzed via HPLC using the following conditions. Specifically, each carotenoid extract (20 μL) was analyzed using a HPLC system (Agilent Technologies, Palo Alto, CA) equipped with an Agilent Technologies Zorbax $C_{18}$ reverse phase column. The mobile phase consisted of 2 solvents in an isocratic method: 95% acetonitrile/5% water and 100% tetrahydrofuran (THF). The flow rate was 1.0 mL per min. The column was run at room temperature for 20 min. Results are shown below in Table 12.

Table 12

Carotenoid Analysis Of Selected Carotenoid-Producing Isolates

| Strain Designation | Carotenoid Produced | Percentage of Total Carotenoids Produced |
|---|---|---|
| YCS1201 | lycopene | 15% |



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| | | |
|---|---|---|
| | β-carotene<br>zeaxanthin | 72%<br>5% |
| YCS1302 | lycopene<br>β-carotene | 40%<br>53% |

Results showed that the primary carotenoid product in strains of the YSC1200 and YSC1300 series was β-carotene, with the secondary product as primarily lycopene. This observation was despite the presence of the *crtY*$_{392}$, *crtZ*$_{392}$ and *crtW*$_{392}$ genes, which could theoretically enable astaxanthin production. It was assumed that the conversion efficiency of the *crtZ*$_{392}$ and *crtW*$_{392}$ genes was extremely limited.

EXAMPLE 6

Synthesis Of *Yarrowia lipolytica* Strain Series YCS3700, Series YCS4000 And Series YCS4100, Producing Primarily Canthaxanthin And Zeaxanthin

The present Example describes the construction of canthaxanthin- and zeaxanthin-producing *Yarrowia lipolytica* strains, identified herein as the YCS3700 series, the YCS4000 series and the YCS4100 series (Figure 8). These strains were derived from strains of the YSC1200 and YSC1300 series, following transformation with additional copies of *crtZ* and *crtW* genes.

As described herein, construction of the *Yarrowia lipolytica* YCS3700 series, the YCS4000 series and the YCS4100 series required: (1) synthesis of codon-optimized *crtW*$_{syn}$ and *crtZ*$_{syn}$ genes; (2) synthesis of pYPS127, comprising a codon-optimized *crtW*$_{syn}$ gene; (3) synthesis of pYPS128, comprising a codon-optimized *crtZ*$_{syn}$ gene; (4) transformation of primarily β-carotene-producing *Yarrowia* strains; and, (5) extraction and analysis of the carotenoids produced.

Synthesis of Synthetic Codon-Optimized *crtY*, *crtZ* And *crtW* Genes

The wildtype *crtY*$_{392}$, *crtZ*$_{392}$ and *crtW*$_{392}$ genes (Example 5) were codon-optimized for expression in *Yarrowia*, thus resulting in creation of the synthetic codon-optimized *crtY392*$_{syn}$, *crtW392*$_{syn}$ and *crtZ392*$_{syn}$ genes, as described below in Table 13. The codon-optimized genes were synthesized by GenScript Corp. (Piscataway, NJ) and each provided in the high-copy vector pUC57 (GenBank® Accession No. Y14837).



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Table 13

Codon-Optimized Carotenoid Biosynthesis Genes

| Carotenoid Gene (Original Source) | Nucleotide SEQ ID NO | Amino Acid SEQ ID NO |
|---|---|---|
| *crtY392*$_{syn}$ (*Pantoea stewartii* DC413; U.S. Patent 7,288,387) | 37 | 38 |
| *crtW392*$_{syn}$ (*Brevundimonas vesicularis* DC263; U.S. Patent 7,252,985) | 39 | 40 |
| *crtZ392*$_{syn}$ (*Brevundimonas vesicularis* DC263; U.S. Patent 7,091,031) | 41 | 42 |

Synthesis Of Vector pYPS127 And pYPS128 For Expression Of *crtZ392*$_{syn}$ And *crtW392*$_{syn}$ Genes

Integration plasmids pYPS127 and pYPS128 were constructed to contain the synthetic *crtZ392*$_{syn}$ or *crtW392*$_{syn}$ genes. In preparation for the ligation reaction, both of the genes were excised from the pUC57 derived vector by digestion with *Nco*I and *Not*I. The DNA fragments were run on a 0.8% agarose gel. A 0.5 kb DNA fragment was excised for the *crtZ392*$_{syn}$ reaction; and, a 0.8 kb DNA fragment was excised for the *crtW392*$_{syn}$ reaction. The DNA was extracted and purified from the agarose using the Qbiogene Geneclean® kit.

The promoter *FBAIN* used in the construction of pYPS127 and pYPS128 was taken from plasmid pZKLeuN-6EP (SEQ ID NO:8; Example 2), which was digested with *Nco*I and *Bgl*II. An ~1.0 kb DNA fragment was excised from the 0.8% agarose gel containing the *FBAIN* promoter fragment. The vector backbone corresponded substantially to the *Swa*I/*Not*I portion of pZKLeuN-6EP; however, intervening cloning (not described herein) had added additional nucleotides upstream of the *Swa*I restriction site resulting in a *Bgl*II site. Thus, the vector backbone was a *Bgl*II/*Not*I fragment. The *Lip2* terminator was present on the 6.5 kb DNA fragment comprising the vector backbone.

Two three-way ligation reactions were set-up. In one reaction, the DNA fragments were: the *Bgl*II/*Not*I-digested vector backbone and the *Lip2* terminator, the *Bgl*II/*Not*I *FBAIN* promoter, and the *Nco*I/*Not*I



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*crtW392*$_{syn}$ gene. In another reaction, the DNA fragments were: the *Bgl*II/*Not*I-digested vector backbone and the *Lip2* terminator, the *Bgl*II/*Not*I *FBAIN* promoter, and the *Nco*I/*Not*I *crtZ392*$_{syn}$ gene.

The ligation reactions were carried out at room temperature for 3 hr. The ligation mixtures were used to transform *E. coli* XL2 Blue cells (Stratagene). The transformation mixtures were streaked onto LB +Amp$^{100}$ agar plates. Sixteen colonies were picked from each transformation and were checked by colony PCR to identify colonies containing plasmids with the correct insert DNA fragments. The PCR results for all 16 colonies were positive for the expected DNA fragments. Four colonies from each transformation were grown overnight in 4 mL of LB broth at 37 °C with aeration. The plasmid DNA was purified from cells and was confirmed to have the correct configuration by digestion with *BamH*I and *Not*I. The digestion reactions were evaluated on a 0.8% agarose gel.

For plasmids containing the vector backbone with the newly constructed chimeric *FBAIN*::*crtW392*$_{syn}$::*Lip2* gene, 2 DNA fragments were expected having sizes of 7.7 kB and 0.5 kB. Plasmids having this configuration were named pYPS127 (SEQ ID NO:43; Figure 11A).

Those plasmids containing the vector backbone and the newly constructed chimeric *FBAIN*::*crtZ392*$_{syn}$::*Lip2* gene were expected to yield a single DNA fragment that was 7.9 kB upon digestion with *BamH*I and *Not*I. The plasmid having this configuration was named pYPS128 (SEQ ID NO:44; Figure 11B).

Transformation Of β-Carotene-Producing *Yarrowia* Strains With Integration Plasmids pYPS127 and pYPS128

Two *Ura-*, β-carotene-producing strains from the YCS1200 and YCS1300 series of transformants (i.e., YCS1205 and YCS1306, respectively) were selected as hosts for transformations with the *crtW392*$_{syn}$ and *crtZ392*$_{syn}$ genes to result in canthaxanthin and zeaxanthin producing strains.

To facilitate the integration of the carotenoid genes into the *Yarrowia* chromosome, pYPS127 and pYPS128 were linearized by



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digestion with *Asc*I and *Sph*I. The *Yarrowia* strains were transformed as described in Example 1 and several colonies were selected and re-streaked onto MMLeu agar plates. Strain YCS1205 was transformed with linearized pYPS128 to generate the YCS3700 series; strain YCS1306 was transformed with linearized pYPS127 to generate the YCS4000 series; and, strain YCS1306 was transformed with linearized pYPS128 to generate the YCS4100 series.

For the YCS3700 series, 4 orange and 5 yellow colonies were patched onto MM and MMLeu agar plates to determine if the $crtW392_{syn}$ gene had integrated at the leucine site. In the case of the YCS4000 series, 5 orange and 4 yellow colonies were evaluated. Two orange and 7 yellow colonies from the YCS4100 series were also evaluated on the MM and MMLeu agar plates.

Select strains from each series were selected for further analysis. YCS3701 and YCS3702 grew on both the MM and MMLeu plates, suggesting that the $crtZ392_{syn}$ gene did not integrate at the leucine site. Strain YCS4002 was unable to grow on the MM agar plates, suggesting that the $crtW392_{syn}$ gene had integrated at the leucine site and thereby inactivated the leucine gene added during the second transformation with pYPS108. Strain YCS4101, transformed with the $crtZ392_{syn}$ gene, was also the result of random integration as indicated by its ability to grow on both the MM and MMLeu agar plates. These 4 strains (i.e.,YCS3701, YCS3702, YCS4002 and YCS4101) were evaluated by HPLC analysis.

Carotenoid Extraction And Analysis

*Yarrowia* strains engineered to produce canthaxanthin and zeaxanthin were analyzed using the carotenoid extraction protocol described above (Example 5). Using HPLC analysis, carotenoids that absorb at 450 nm were evaluated. The elution time, mass of carotenoid and UV absorption spectrum corresponded to the canthaxanthin and zeaxanthin standards (CaroteNature GmbH). Results are shown below in Table 14.



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Table 14

Carotenoid Analysis Of Selected Strains Of The YCS3700, YCS4000 And YCS4100 Series

| Strain | Plasmid Used to Transform (*crt* biosynthesis gene) | Xanthophyll Produced (% of total carotenoid) |
|---|---|---|
| YCS3701 | pYPS128 ($crtZ392_{syn}$) | 6% zeaxanthin |
| YCS3702 | pYPS128 ($crtZ392_{syn}$) | 4% zeaxanthin |
| YCS4002 | pYPS127 ($crtW392_{syn}$) | 5% canthaxanthin |
| YCS4101 | pYPS128 ($crtZ392_{syn}$) | 6% zeaxanthin |

## EXAMPLE 7

### Generation Of *Yarrowia lipolytica* Strain Y4184U To Produce About 31% EPA Of Total Lipids *Via* The Δ9 Elongase/Δ8 Desaturase Pathway

The present Example describes the construction of strain Y4184U, derived from *Yarrowia lipolytica* ATCC #20362, capable of producing about 31% EPA relative to the total lipids via expression of a Δ9 elongase/Δ8 desaturase pathway (Figure 12). The strain has a *Ura-* phenotype.

The development of strain Y4184U required the construction of strain Y2224 (a FOA resistant mutant from an autonomous mutation of the *Ura3* gene of wildtype *Yarrowia* strain ATCC #20362), strain Y4001 (producing EDA with a *Leu-* phenotype), strain Y4001U (*Leu-*, *Ura-*), strain Y4036 (producing DGLA with a *Leu-* phenotype), strain Y4036U (*Leu-*, *Ura-*), strain Y4069 (producing ARA with a *Ura-* phenotype), strain Y4084 (producing EPA), strain Y4084U1 (*Ura-*), strain Y4127 (producing EPA), strain Y4127U2 (*Ura-*), strain Y4158 (producing EPA), strain Y4158U1 (*Ura-*) and strain 4184 (producing 30.7% EPA).

Generation Of Strain Y2224

Strain Y2224 was isolated in the following manner: *Yarrowia lipolytica* ATCC #20362 cells from a YPD agar plate were streaked onto a MM plate (75 mg/L each of uracil and uridine, 6.7 g/L YNB with ammonia sulfate, without amino acids, and 20 g/L glucose) containing 250 mg/L 5-FOA (Zymo Research, Orange, CA). Plates were incubated at 28 °C and four of the resulting colonies were patched separately onto MM plates



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containing 200 mg/mL 5-FOA and MM plates lacking uracil and uridine to confirm uracil *Ura3* auxotrophy.

Generation Of Strain Y4001 To Produce About 17% EDA Of Total Lipids

Strain Y4001 was created via integration of construct pZKLeuN-29E3 (Figure 13A). This construct, comprising four chimeric genes (i.e., a Δ12 desaturase, a $C_{16/18}$ elongase and two Δ9 elongases), was integrated into the *Leu2* loci of strain Y2224 to thereby enable production of EDA.

Construct pZKLeuN-29E3 contained the components shown below:

Table 15

Description of Plasmid pZKLeuN-29E3 (SEQ ID NO:45)

| RE Sites And Nucleotides Within SEQ ID NO:45 | Description Of Fragment And Chimeric Gene Components |
|---|---|
| *BsiW* I/*Asc* I (7797-7002) | 788 bp 3' portion of *Yarrowia Leu2* gene (GenBank Accession No. AF260230) |
| *Sph* I/*Pac* I (4302-3591) | 703 bp 5' portion of *Yarrowia Leu2* gene (GenBank Accession No. AF260230) |
| *Swa* I/*BsiW* I (10533-7797) | GPD::FmD12::Pex20, comprising:<br>• GPD: *Yarrowia lipolytica* GPD promoter (PCT Publication No. WO 2005/003310);<br>• FmD12: *Fusarium moniliforme* Δ12 desaturase gene (SEQ ID NO:46) (labeled as "F.D12" in Figure; PCT Publication No. WO 2005/047485);<br>• Pex20: Pex20 terminator sequence from *Yarrowia* Pex20 gene (GenBank Accession No. AF054613) |
| *Bgl* II/*Swa* I (12559-10533) | EXP1::EgD9e::Lip1, comprising:<br>• EXP1: *Yarrowia lipolytica* export protein (EXP1) promoter (labeled as "Exp pro" in Figure; PCT Publication No. WO 2006/052870 and U.S. Patent Application No. 11/265761);<br>• EgD9e: *Euglena gracilis* Δ9 elon®gase (SEQ ID NO:48) (labeled as "EgD9E" in Figure; PCT Publication No. WO 2007/061742);<br>• Lip1: Lip1 terminator sequence from *Yarrowia* Lip1 gene (GenBank Accession No. Z50020) |
| *Pme* I/*Cla* I (12577-1) | FBAINm::EgD9eS::Lip2, comprising:<br>• FBAINm: *Yarrowia lipolytica* FBAINm promoter (PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356);<br>• EgD9eS: codon-optimized Δ9 elongase gene (SEQ ID NO:50), derived from *Euglena gracilis* (labeled as |



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| | |
|---|---|
| | "EgD9ES" in Figure; PCT Publication No. WO 2007/061742);<br>• Lip2: Lip2 terminator sequence from *Yarrowia* Lip2 gene (GenBank Accession No. AJ012632) |
| *Cla* I/*EcoR* I (1-1736) | LoxP::*Ura3*::LoxP, comprising:<br>• LoxP sequence (SEQ ID NO:9);<br>• *Yarrowia* Ura3 gene (GenBank Accession No. AJ306421);<br>• LoxP sequence (SEQ ID NO:9) |
| *EcoR* I/*Pac* I (1736-3591) | YAT1::ME3S::Pex16, comprising:<br>• YAT1: *Yarrowia lipolytica* YAT1 promoter (labeled as "YAT" in Figure; Patent Publication No. U.S. 2006/0094102-A1);<br>• ME3S: codon-optimized $C_{16/18}$ elongase gene (SEQ ID NO:52), derived from *Mortierella alpina* (PCT Publication No. WO 2007/046817);<br>• Pex16: Pex16 terminator sequence of *Yarrowia Pex 16* gene (GenBank Accession No. U75433) |

Plasmid pZKLeuN-29E3 was digested with *Asc*I/*Sph*I, and then used for transformation of *Y. lipolytica* strain Y2224 (i.e., ATCC #20362 *Ura3*-) according to the General Methods. The transformant cells were plated onto MMLeu media plates and maintained at 30 °C for 2 to 3 days. The colonies were picked and streaked onto MM and MMLeu selection plates. The colonies that could grow on MMLeu plates but not on MM plates were selected as *Leu*- strains. Single colonies of *Leu*- strains were then inoculated into liquid MMLeu at 30 °C and shaken at 250 rpm/min for 2 days. The cells were collected by centrifugation, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed the presence of EDA in the transformants containing the 4 chimeric genes of pZKLeuN-29E3, but not in the *Yarrowia* Y2224 control strain. Most of the selected 36 *Leu*- strains produced about 12 to 16.9% EDA of total lipids. There were 3 strains (i.e., strains #11, #30 and #34) that produced about 17.4%, 17% and 17.5% EDA of total lipids; they were designated as strains Y4001, Y4002 and Y4003, respectively.



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Single colonies of Y4001, Y4002 and Y4003 strains were inoculated in liquid MMLeu at 30 °C and shaken at 250 rpm/min for 2 days. The cells were collected by centrifugation, resuspended in High Glucose Media and then shaken at 250 rpm/min for 5 days. The cells were collected by centrifugation, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC. GC analyses showed that the Y4001, Y4002 and Y4003 strains produced about 24% EDA of total lipids.

Generation Of Strain Y4001U (*Leu-, Ura-*)

Strain Y4001U was created via temporary expression of the *Cre* recombinase enzyme in plasmid pY116 (Figure 13B) within strain Y4001 to produce a *Leu-* and *Ura-* phenotype. Construct pY116 contained the following components:

Table 16

Description of Plasmid pY116 (SEQ ID NO:54)

| RE Sites And Nucleotides Within SEQ ID NO:54 | Description Of Fragment And Chimeric Gene Components |
|---|---|
| 1328-448 | ColE1 plasmid origin of replication |
| 2258-1398 | Ampicillin-resistance gene ($Amp^R$) for selection in *E. coli* |
| 3157-4461 | *Yarrowia* autonomous replication sequence (ARS18; GenBank Accession No. A17608) |
| *SwaI/PacI* 6667-4504 | *Yarrowia* Leu2 gene (GenBank Accession No. AF260230) |
| *Swa I/Pme I* (6667-218) | GPAT::Cre::XPR2, comprising:<br>• GPAT: *Yarrowia lipolytica* GPAT promoter (PCT Publication No. WO 2006/031937 and U.S. Patent 7,264,949);<br>• Cre: Enterobacteria phage P1 Cre gene for recombinase protein (GenBank Accession No. X03453);<br>• XPR2: ~100 bp of the 3' region of the *Yarrowia Xpr* gene (GenBank Accession No. M17741) |

Plasmid pY116 was used for transformation of freshly grown Y4001 cells according to the General Methods. The transformant cells were plated onto MMLeuUra plates containing 280 μg/mL sulfonylurea



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(chlorimuron ethyl, E. I. duPont de Nemours & Co., Inc., Wilmington, DE) and maintained at 30 °C for 3 to 4 days. Four colonies were picked, inoculated into 3 mL liquid YPD media at 30 °C and shaken at 250 rpm/min for 1 day. The cultures were diluted to 1:50,000 with liquid MMLeuUra media, and 100 µL was plated onto new YPD plates and maintained at 30 °C for 2 days. Colonies were picked and streaked onto MMLeu and MMLeuUra selection plates. The colonies that could grow on MMLeuUra plates but not on MMLeu plates were selected and analyzed by GC to confirm the presence of C20:2 (EDA). One strain, having a *Leu-* and *Ura-* phenotype, produced about 17% EDA of total lipids and was designated as Y4001U.

Generation Of Strain Y4036 To Produce About 18% DGLA Of Total Lipids

Construct pKO2UF8289 (Figure 14A; SEQ ID NO:55) was generated to integrate four chimeric genes (comprising a Δ12 desaturase, one Δ9 elongase and two mutant Δ8 desaturases) into the Δ12 loci of strain Y4001U1, to thereby enable production of DGLA. Construct pKO2UF8289 contained the following components:

Table 17

Description of Plasmid pKO2UF8289 (SEQ ID NO:55)

| RE Sites And Nucleotides Within SEQ ID NO:55 | Description Of Fragment And Chimeric Gene Components |
|---|---|
| *Asc*I/*Bsi*WI (10337-9600) | 5' portion of *Yarrowia* Δ12 desaturase gene (SEQ ID NO:56) (labeled as "YLD12-N" in Figure; PCT Publication No. WO 2004/104167; U.S. Patent 7,214,491) |
| *Eco*RI/*Sph*I (13601-13045) | 3' portion of *Yarrowia* Δ12 desaturase gene (SEQ ID NO:56) (labeled as "YL12-C" in Figure; PCT Publication No. WO 2004/104167; U.S. Patent 7,214,491) |



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| | |
|---|---|
| *SwaI/BsiWI* (7088-9600) | FBAINm::EgD8M::Pex20, comprising:<br>• FBAINm: *Yarrowia lipolytica* FBAINm promoter (PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356);<br>• EgD8M: Synthetic mutant Δ8 desaturase (SEQ ID NO:58) (labeled as "D8S-23" in Figure; U.S. Patent Application No. 11/635258), derived from *Euglena gracilis* ("EgD8S"; PCT Publication No. WO 2006/012326);<br>• Pex20: Pex20 terminator sequence from *Yarrowia Pex20* gene (GenBank Accession No. AF054613) |
| *SwaI/PmeI* (7088-4581) | YAT1::FmD12::OCT, comprising:<br>• YAT1: *Yarrowia lipolytica* YAT1 promoter (labeled as "YAT" in Figure; Patent Publication No. US 2006/0094102-A1);<br>• FmD12: *Fusarium moniliforme* Δ12 desaturase gene (SEQ ID NO:46) (labeled as "F.D12" in Figure; PCT Publication No. WO 2005/047485);<br>• OCT: OCT terminator sequence of *Yarrowia OCT* gene (GenBank Accession No. X69988) |
| *PmeI/PacI* (4581-2124) | EXP1:: EgD8M::Pex16, comprising:<br>• EXP1: *Yarrowia lipolytica* export protein (EXP1) promoter (PCT Publication No. WO 2006/052870 and U.S. Patent Application No. 11/265761);<br>• EgD8M: Synthetic mutant Δ8 desaturase (SEQ ID NO:58) (labeled as "D8-23" in Figure; U.S. Patent Application No. 11/635258), derived from *Euglena gracilis* ("EgD8S"; PCT Publication No. WO 2006/012326);<br>• Pex16: Pex16 terminator sequence from *Yarrowia Pex16* gene (GenBank Accession No. U75433) |
| *PmeI/ClaI* (2038-1) | GPAT::EgD9e::Lip2, comprising:<br>• GPAT: *Yarrowia lipolytica* GPAT promoter (PCT Publication No. WO 2006/031937 and U.S. Patent 7,264,949);<br>• EgD9e: *Euglena gracilis* Δ9 elongase gene (SEQ ID NO:48) (labeled as "EgD9E" in Figure; PCT Publication No. WO 2007/061742);<br>• Lip2: Lip2 terminator sequence from *Yarrowia Lip2* gene (GenBank Accession No. AJ012632) |
| *ClaI/Eco*RI (13601-1) | LoxP::*Ura3*::LoxP, comprising:<br>• LoxP sequence (SEQ ID NO:9);<br>• *Yarrowia* Ura3 gene (GenBank Accession No. AJ306421);<br>• LoxP sequence (SEQ ID NO:9) |



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The pKO2UF8289 plasmid was digested with *Asc*I/*Sph*I, and then used for transformation of strain Y4001U1 according to the General Methods. The transformant cells were plated onto MMLeu plates and maintained at 30 °C for 2 to 3 days. The colonies were picked and streaked onto MMLeu selection plates at 30 °C for 2 days. These cells were then inoculated into liquid MMLeu at 30 °C and shaken at 250 rpm/min for 2 days. The cells were collected by centrifugation, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed the presence of DGLA in the transformants containing the 4 chimeric genes of pKO2UF8289, but not in the parent Y4001U1 strain. Most of the selected 96 strains produced between 7% and 13% DGLA of total lipids. There were 6 strains (i.e., #32, #42, #60, #68, #72 and #94) that produced about 15%, 13.8%, 18.2%, 13.1%, 15.6% and 13.9% DGLA of total lipids. These six strains were designated as Y4034, Y4035, Y4036, Y4037, Y4038 and Y4039, respectively.

Generation Of Strain Y4036U (*Leu-, Ura3-*)

Construct pY116 (Figure 13B; SEQ ID NO:54 was utilized to temporarily express a *Cre* recombinase enzyme in strain Y4036. This released the LoxP sandwiched *Ura3* gene from the genome.

Plasmid pY116 was used to transform strain Y4036 according to the General Methods. Following transformation, the cells were plated onto MMLeuUra plates and maintained at 30 °C for 2 to 3 days. The individual colonies grown on MMLeuUra plates were picked, and streaked into YPD liquid media at 30 °C and shaken at 250 rpm/min for 1 day to cure the pY116 plasmid. The grown cultures were streaked on MMLeuUra plates. After two days at 30 °C, the individual colonies were re-streaked on MMLeuUra, MMU and MMLeu plates. Those colonies that could grow on MMLeuUra, but not on MMU or MMLeu plates were selected. One of these strains with *Leu-* and *Ura-* phenotypes was designated as Y4036U (*Ura-, Leu-*).



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Generation Of Strain Y4069 To Produce About 12% ARA Of Total Lipids

Construct pZKSL-555R (Figure 14B; SEQ ID NO:62) was generated to integrate three Δ5 desaturase genes into the *Lys* loci of strain Y4036U, to thereby enable production of ARA. The pZKSL-555R plasmid contained the following components:

Table 18

Description of Plasmid pZKSL-555R (SEQ ID NO:62)

| RE Sites And Nucleotides Within SEQ ID NO:62 | Description Of Fragment And Chimeric Gene Components |
|---|---|
| *Asc*I/*Bsi*WI (3321-2601) | 720 bp 5' portion of *Yarrowia Lys5* gene (GenBank Accession No. M34929) |
| *Pac*I/*Sph*I (6716-6029) | 687 bp 3' portion of *Yarrowia Lys5* gene (GenBank Accession No. M34929) |
| *Bgl*II/*Bsi*WI (15-2601) | EXP1::EgD5S::Pex20, comprising:<br>• EXP1: *Yarrowia lipolytica* export protein (EXP1) promoter (labeled as "EXP" in Figure; PCT Publication No. WO 2006/052870 and U.S. Patent Application No. 11/265761);<br>• EgD5S: codon-optimized Δ5 desaturase (SEQ ID NO:63), derived from *Euglena gracilis* (U.S. Patent Application No. 11/748629);<br>• Pex20: Pex20 terminator sequence from *Yarrowia Pex20* gene (GenBank Accession No. AF054613) |
| *Cla*I/*Pme*I (11243-1) | YAT1::RD5S::OCT, comprising:<br>• YAT1: *Yarrowia lipolytica* YAT1 promoter (labeled as "YAT" in Figure; Patent Publication US 2006/0094102-A1);<br>• RD5S: codon-optimized Δ5 desaturase (SEQ ID NO:65), derived from *Peridinium* sp. CCMP626 (labeled as "RD5S(626)" in Figure; U.S. Patent Application No. 11/748637);<br>• OCT: OCT terminator sequence of *Yarrowia OCT* gene (GenBank Accession No. X69988) |
| *Eco*RI/*Pac*I (9500-6716) | FBAIN::EgD5::Aco, comprising:<br>• FBAIN: *Yarrowia lipolytica* FBAIN promoter (PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356);<br>• EgD5: *Euglena gracilis* Δ5 desaturase (SEQ ID NO:67) (labeled as "EgD5WT" in Figure; U.S. Patent Application No. 11/748629) with elimination of internal *Eco*RI, *Bgl*II, *Hind*III and *Nco*I restriction enzyme sites [mutations |



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| | |
|---|---|
| | labeled as "M.EI", "M.BII", "M.H" and "M.N", respectively];<br>• Aco: Aco terminator sequence from *Yarrowia Aco* gene (GenBank Accession No. AJ001300) |
| *Eco*RI/*Cla*I (9500-11243) | *Yarrowia Leu2* gene (GenBank Accession No. M37309) |

The pZKSL-555R plasmid was digested with *Asc*I/*Sph*I, and then used for transformation of strain Y4036U according to the General Methods. The transformant cells were plated onto MMLeuLys plates and maintained at 30 °C for 2 to 3 days. Single colonies were then re-streaked onto MMLeuLys plates, and then inoculated into liquid MMLeuLys at 30 °C and shaken at 250 rpm/min for 2 days. The cells were collected by centrifugation, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed the presence of ARA in the transformants containing the 3 chimeric genes of pZKSL-555R, but not in the parent Y4036U strain. Most of the selected 96 strains produced ~10% ARA of total lipids. There were 4 strains (i.e., #57, #58, #69 and #75) that produced about 11.7%, 11.8%, 11.9% and 11.7% ARA of total lipids. These four strains were designated as Y4068, Y4069, Y4070 and Y4071, respectively. Further analyses showed that the three chimeric genes of pZKSL-555R were not integrated into the *Lys5* site in the Y4068, Y4069, Y4070 and Y4071 strains. All strains possessed a *Lys+* phenotype.

Generation Of Strain Y4084 To Produce About 14% EPA Of Total Lipids

Construct pZP3-Pa777U (Figure 15A; SEQ ID NO:69) was generated to integrate three Δ17 desaturase genes into the *Pox3* loci (GenBank Accession No. AJ001301) of strain Y4069, to thereby enable production of EPA. The pZP3-Pa777U plasmid contained the following components:



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Table 19

Description of Plasmid pZP3-Pa777U (SEQ ID NO:69)

| RE Sites And Nucleotides Within SEQ ID NO:69 | Description Of Fragment And Chimeric Gene Components |
|---|---|
| *AscI*/*BsiWI* (3527-4297) | 770 bp 5' portion of *Yarrowia Pox3* gene (GenBank Accession No. AJ001301) |
| *PacI*/*SphI* (1-827) | 827 bp 3' portion of *Yarrowia Pox3* gene (GenBank Accession No. AJ001301) |
| *ClaI*/*SwaWI* (6624-4457) | YAT1::PaD17S::Lip1, comprising:<br>• YAT1: *Yarrowia lipolytica* YAT1 promoter (labeled as "YAT" in Figure; Patent Publication US 2006/0094102-A1);<br>• PaD17S: codon-optimized Δ17 desaturase (SEQ ID NO:70), derived from *Pythium aphanidermatum* (U.S. Patent Application No. 11/779915);<br>• Lip1: Lip1 terminator sequence from *Yarrowia Lip1* gene (GenBank Accession No. Z50020) |
| *EcoRI*/*PmeI* (8359-10611) | EXP1::PaD17::Pex16, comprising:<br>• EXP1: *Yarrowia lipolytica* export protein (EXP1) promoter (labeled as "Exp" in Figure; PCT Publication No. WO 2006/052870 and U.S. Patent Application No. 11/265761);<br>• PaD17: *Pythium aphanidermatum* Δ17 desaturase gene (SEQ ID NO:72) (labeled as "PaD17WT" in Figure; U.S. Patent Application No. 11/779915);<br>• Pex16: Pex16 terminator sequence from *Yarrowia Pex16* gene (GenBank Accession No. U75433) |
| *PmeI*/*PacI* (10611-1) | FBAINm::PaD17::Aco, comprising:<br>• FBAINm: *Yarrowia lipolytica* FBAINm promoter (PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356);<br>• PaD17: *Pythium aphanidermatum* Δ17 desaturase gene (SEQ ID NO:72) (labeled as "PaD17WT" in Figure; U.S. Patent Application No. 11/779915);<br>• Aco: Aco terminator sequence from *Yarrowia Aco* gene (GenBank Accession No. AJ001300) |
| *ClaI*/*EcoRI* (6624-8359) | LoxP::*Ura3*::LoxP, comprising:<br>• LoxP sequence (SEQ ID NO:9);<br>• *Yarrowia* Ura3 gene (GenBank Accession No. AJ306421);<br>• LoxP sequence (SEQ ID NO:9) |

The pZP3-Pa777U plasmid was digested with *AscI*/*SphI*, and then used for transformation of strain Y4069 according to the General Methods.



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The transformant cells were plated onto MM plates and maintained at 30 °C for 2 to 3 days. Single colonies were then re-streaked onto MM plates, and inoculated into liquid MMLeuLys at 30 °C and shaken at 250 rpm/min for 2 days. The cells were collected by centrifugation, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed the presence of EPA in the transformants containing the 3 chimeric genes of pZP3-Pa777U, but not in the parent Y4069 strain. Most of the selected 96 strains produced 10-13.5% EPA of total lipids. There was one strain (i.e., #83) that produced about 13.7% EPA of total lipids. This strain was designated as Y4084.

The final genotype of strain Y4084 with respect to wildtype *Yarrowia lipolytica* ATCC #20362 was *Ura3+, Leu+, Lys+, unknown 1-, unknown 2-, unknown 3-*, GPD::FmD12::Pex20, YAT1::FmD12::OCT, YAT1::ME3S::Pex16, GPAT::EgD9e::Lip2, EXP1::EgD9eS::Lip1, FBAINm::EgD9eS::Lip2, FBAINm::EgD8M::Pex20, EXP1::EgD8M::Pex16, FBAIN::EgD5::Aco, EXP1::EgD5S::Pex20, YAT1::RD5S::OCT, YAT1::PaD17S::Lip1, EXP1::PaD17::Pex16, FBAINm::PaD17::Aco.

Generation Of Y4084U1 Strain (*Ura3-*)

Strain Y4084U1 was created via temporary expression of the *Cre* recombinase enzyme in construct pY117 (Figure 15B; SEQ ID NO:74) within strain Y4084 to produce a *Ura-* phenotype. This released the LoxP sandwiched *Ura3* gene from the genome. The mutated *Yarrowia* AHAS enzyme (SEQ ID NO:1) in plasmid pY117 conferred $SU^R$, which was used as a positive screening marker.

Plasmid pY117 was derived from plasmid pY116 (*supra,* and in U.S. Patent Application No. 11/635258) by inserting the mutant AHAS gene flanked by *PacI-SwaI* sites into *PacI-SwaI* digested pY116, thereby replacing the LEU selectable marker with the sulfonylurea marker. Construct pY117 thereby contained the following components:



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Table 20

Description of Plasmid pY117 (SEQ ID NO:74)

| RE Sites And Nucleotides Within SEQ ID NO:74 | Description Of Fragment And Chimeric Gene Components |
|---|---|
| 1328-448 | ColE1 plasmid origin of replication |
| 2258-1398 | Ampicillin-resistance gene ($Amp^R$) for selection in *E. coli* |
| 2438-2838 | *E. coli* f1 origin of replication |
| 3157-4461 | *Yarrowia* autonomous replication sequence (ARS18; GenBank Accession No. A17608) |
| *PacI/SwaI* 4504-7498 | *Yarrowia lipolytica* AHAS gene (GenBank Accession No. XP_501277) comprising a W497L mutation (SEQ ID NO:1; P Publication No. WO 2006/052870) |
| *SwaI/PmeI* 7498-218 | GPAT::Cre::XPR, comprising:<br>• GPAT: *Yarrowia lipolytica* GPAT promoter (PCT Publication No. WO 2006/031937 and U.S. Patent 7,264,949);<br>• Cre: Enterobacteria phage P1 Cre gene for recombinase protein (GenBank Accession No. X03453) except for single base change (T4G) resulting in a single amino acid change (S2A) to create a *NcoI* site for cloning convenience;<br>• XPR: ~100 bp of the 3' region of the *Yarrowia* Xpr gene (GenBank Accession No. M17741) |

Plasmid pY117 was used to transform strain Y4084 according to the General Methods. Following transformation, the cells were plated onto MMU+SU (280 μg/mL sulfonylurea; also known as chlorimuron ethyl, E. I. duPont de Nemours & Co., Inc., Wilmington, DE) plates and maintained at 30 °C for 2 to 3 days. The individual $SU^R$ colonies grown on MMU+SU plates were picked, and streaked into YPD liquid media at 30 °C and shaken at 250 rpm/min for 1 day to cure the pY117 plasmid. The grown cultures were streaked onto MMU plates. After two days at 30 °C, the individual colonies were re-streaked onto MM and MMU plates. Those colonies that could grow on MMU, but not on MM plates were selected. Two of these strains with *Ura-* phenotypes were designated as Y4084U1 and Y4084U2 (*Ura-*).



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Generation Of Strain Y4127 To Produce About 18% EPA Of Total Lipids

Construct pZP2-2988 (Figure 16A; SEQ ID NO:75) was generated to integrate one Δ12 desaturase gene, two Δ8 desaturase genes and one Δ9 elongase gene into the *Pox2* loci (GenBank Accession No. AJ001300) of strain Y4084U1, to thereby enable higher level production of EPA. The pZP2-2988 plasmid contained the following components:

Table 21

Description of Plasmid pZP2-2988 (SEQ ID NO:75)

| RE Sites And Nucleotides Within SEQ ID NO:75 | Description Of Fragment And Chimeric Gene Components |
|---|---|
| *AscI*/*BsiWI* (3083-2273) | 803 bp 5' portion of *Yarrowia Pox2* gene (GenBank Accession No. AJ001300) |
| *PacI*/*SphI* (6446-5791) | 649 bp 3' portion of *Yarrowia Pox2* gene (GenBank Accession No. AJ001300) |
| *PmeI*/*BsiWI* (347-2273) | FBA::EgD9eS::Pex20, comprising:<br>• FBA: *Yarrowia lipolytica* FBA promoter (PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356);<br>• EgD9eS: codon-optimized Δ9 elongase (SEQ ID NO:50), derived from *Euglena gracilis* (PCT Publication No. WO 2007/061742);<br>• Pex20: Pex20 terminator sequence from *Yarrowia Pex20* gene (GenBank Accession No. AF054613) |
| *ClaI*/*PmeI* (13318-347) | GPM/FBAIN::FmD12S::OCT, comprising:<br>• GPM/FBAIN: chimeric *Yarrowia lipolytica* GPM/FBAIN promoter (separately labeled as "GPM" and "FBA intron" in Figure) (PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356);<br>• FmD12S: codon-optimized Δ12 desaturase (SEQ ID NO:76), derived from *Fusarium moniliforme* (labeled as "F.D12S" in Figure; PCT Publication No. WO 2005/047485);<br>• OCT: OCT terminator sequence of *Yarrowia OCT* gene (GenBank Accession No. X69988) |
| *ClaI*/*EcoRI* (13318-11581) | LoxP::*Ura3*::LoxP, comprising:<br>• LoxP sequence (SEQ ID NO:9);<br>• *Yarrowia* Ura3 gene (GenBank Accession No. AJ306421);<br>• LoxP sequence (SEQ ID NO:9) |
| *EcoRII*/*SwaI* (11581-8884) | GPDIN::EgD8M::Lip1, comprising:<br>• GPDIN: *Yarrowia lipolytica* GPDIN promoter (Patent |



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| | |
|---|---|
| | Publication US 2006/0019297-A1);<br>• EgD8M: Synthetic mutant Δ8 desaturase (SEQ ID NO:58; U.S. Patent Application No. 11/635258), derived from *Euglena gracilis* ("EgD8S"; PCT Publication No. WO 2006/012326);<br>• Lip1: Lip1 terminator sequence from *Yarrowia Lip1* gene (GenBank Accession No. Z50020) |
| *SwaI/PacI* (8884-6446) | YAT1::EgD8M::ACO, comprising:<br>• YAT1: *Yarrowia lipolytica* YAT1 promoter (labeled as "YAT" in Figure; Patent Publication US 2006/0094102-A1);<br>• EgD8M: Synthetic mutant Δ8 desaturase (SEQ ID NO:58; U.S. Patent Application No. 11/635258), derived from *Euglena gracilis* ("EgD8S"; PCT Publication No. WO 2006/012326);<br>• Aco: Aco terminator sequence from *Yarrowia Aco* gene (GenBank Accession No. AJ001300) |

The pZP2-2988 plasmid was digested with *AscI/SphI*, and then used for transformation of strain Y4084U1 according to the General Methods. The transformant cells were plated onto MM plates and maintained at 30 °C for 2 to 3 days. Single colonies were re-streaked onto MM plates, and then inoculated into liquid MMLeuLys at 30 °C and shaken at 250 rpm/min for 2 days. The cells were collected by centrifugation, resuspended in High Glucose Media and then shaken at 250 rpm/min for 5 days. The cells were collected by centrifugation, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed that most of the selected 96 strains produced12-16.4% EPA of total lipids. There were 6 strains (i.e., #5, #12, #15, #17, #74 and #80) that produced about 16.6%, 16.9%, 17%, 16.7% 16.5% and 18.1% EPA of total lipids. These six strains were designated as Y4122, Y4123, Y4124, Y4125, Y4126 and Y4127, respectively.

The final genotype of strain Y4127 with respect to wildtype *Yarrowia lipolytica* ATCC #20362 was: *unknown 1-, unknown 2-, unknown 3-, unknown 4-,* GPD::FmD12::Pex20, YAT1::FmD12::OCT, GPM/FBAIN::FmD12S::OCT, YAT1::ME3S::Pex16, GPAT::EgD9e::Lip2, EXP1::EgD9e::Lip1, FBAINm::EgD9eS::Lip2, FBA::EgD9eS::Pex20,



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FBAINm::EgD8M::Pex20, EXP1::EgD8M::Pex16, GPDIN::EgD8M::Lip1, YAT1::EgD8M::Aco, FBAIN::EgD5::Aco, EXP1::EgD5S::Pex20, YAT1::RD5S::OCT, YAT1::PaD17S::Lip1, EXP1::PaD17::Pex16, FBAINm::PaD17::Aco.

Generation Of Strain Y4127U2 (*Ura3-*)

In order to disrupt the Ura3 gene in strain Y4127, construct pZKUE3S (Figure 16B; SEQ ID NO:78) was created to integrate a EXP1::ME3S::Pex20 chimeric gene into the *Ura3* gene of strain Y4127. Plasmid pZKUE3S contained the following components:

Table 22

Description of Plasmid pZKUE3S (SEQ ID NO:78)

| RE Sites And Nucleotides Within SEQ ID NO:78 | Description Of Fragment And Chimeric Gene Components |
|---|---|
| *BsiWI/PacI* (318-1038) | 721 bp 5' portion of *Yarrowia* Ura3 gene (GenBank Accession No. AJ306421) |
| *SphI/PmeI* (3915-4594) | 729 bp 3' portion of *Yarrowia* Ura3 gene (GenBank Accession No. AJ306421) |
| *EcoRI/BsiWI* (4628-318) | EXP1::ME3S::Pex20, comprising:<br>• EXP1: *Yarrowia lipolytica* export protein (EXP1) promoter (labeled as "Exp" in Figure; PCT Publication No. WO 2006/052870 and U.S. Patent Application No. 11/265761);<br>• ME3S: codon-optimized $C_{16/18}$ elongase gene (SEQ ID NO:52), derived from *Mortierella alpina* (PCT Publication No. WO 2007/046817);<br>• Pex20: Pex20 terminator sequence of *Yarrowia Pex20* gene (GenBank Accession No. AF054613) |
| 2149-1269 | *ColE1* plasmid origin of replication |
| 3079-2219 | Ampicillin-resistance gene ($Amp^R$) for selection in *E. coli* |
| 3659-3259 | *E. coli* f1 origin of replication |

Plasmid pZKUE3S was digested with *SphI/PacI*, and then used to transform strain Y4127 according to the General Methods. Following transformation, cells were plated onto MM + 5-FOA selection plates and maintained at 30 °C for 2 to 3 days.



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Two transformants grown on MM + 5-FOA selection plates were picked and re-streaked onto fresh MM + 5-FOA plates. The cells were stripped from the plates, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed that there were about 10.4% and 11.3% EPA of total lipids in these two transformants with pZKUE3S from plates. These two strains were designated as Y4127U1 and Y4127U2.

Generation Of Strain Y4158 To Produce About 25% EPA Of Total Lipids

Construct pZKL1-2SP98C (Figure 17A; SEQ ID NO:79) was generated to integrate one Δ9 elongase gene, one Δ8 desaturase gene, one Δ12 desaturase gene and one *Yarrowia lipolytica* diacylglycerol cholinephosphotransferase (CPT1) gene into the *Lip1* loci (GenBank Accession No. Z50020) of strain Y4127U2, thereby resulting in isolation of strain Y4158 (producing 25% EPA). The pZKL1-2SP98C plasmid contained the following components:

Table 23

Description of Plasmid pZKL1-2SP98C (SEQ ID NO:79)

| RE Sites And Nucleotides Within SEQ ID NO:79 | Description Of Fragment And Chimeric Gene Components |
|---|---|
| *Ascl*/*BsiW*I (3474-2658) | 809 bp 5' portion of *Yarrowia Lip1* gene (labeled as "Lip1-5'N" in Figure; GenBank Accession No. Z50020) |
| *Pacl*/*Sph*I (6951-6182) | 763 bp 3' portion of *Yarrowia Lip1* gene (labeled as "Lip1.3N" in Figure; GenBank Accession No. Z50020) |
| *Swa*I/*BsiW*I (1-2658) | GPD::YlCPT1::Aco, comprising:<br>• GPD: *Yarrowia lipolytica* GPD promoter (PCT Publication No. WO 2005/003310);<br>• YlCPT1: *Yarrowia lipolytica* diacylglycerol cholinephosphotransferase gene (SEQ ID NO:80) (labeled as "CPT1" in Figure; PCT Publication No. WO 2006/052870);<br>• Aco: Aco terminator sequence from *Yarrowia Aco* gene (GenBank Accession No. AJ001300) |
| *Pme*I/*Swa*I (13241-1) | FBAIN::EgD8M::Lip1 comprising:<br>• FBAIN: *Yarrowia lipolytica* FBAIN promoter (PCT Publication No. WO 2005/049805; U.S. Patent |



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| | |
|---|---|
| | 7,202,356);<br>• EgD8M: Synthetic mutant Δ8 desaturase (SEQ ID NO:58; U.S. Patent Application No. 11/635258), derived from *Euglena gracilis* ("EgD8S"; PCT Publication No. WO 2006/012326);<br>• Lip1: Lip1 terminator sequence from *Yarrowia Lip1* gene (GenBank Accession No. Z50020) |
| *Pme*I/*Cla*I (13241-11385) | YAT1::EgD9eS::Lip2, comprising:<br>• YAT1: *Yarrowia lipolytica* YAT1 promoter (labeled as "YAT" in Figure; Patent Publication US 2006/0094102-A1);<br>• EgD9eS: codon-optimized Δ9 elongase gene (SEQ ID NO:50), derived from *Euglena gracilis* (labeled as "EgD9ES" in Figure; PCT Publication No. WO 2007/061742);<br>• Lip2: Lip2 terminator sequence from *Yarrowia Lip2* gene (GenBank Accession No. AJ012632) |
| *Cla*I/*Eco*RI (11385-9648) | LoxP::*Ura3*::LoxP, comprising:<br>• LoxP sequence (SEQ ID NO:9);<br>• *Yarrowia* Ura3 gene (GenBank Accession No. AJ306421);<br>• LoxP sequence (SEQ ID NO:9) |
| *Eco*RI/*Pac*I (9648-6951) | EXP1::FmD12S::ACO, comprising:<br>• EXP1: *Yarrowia lipolytica* export protein (EXP1) promoter (labeled as "Exp" in Figure; PCT Publication No. WO 2006/052870 and U.S. Patent Application No. 11/265761);<br>• FmD12S: codon-optimized Δ12 elongase (SEQ ID NO:76), derived from *Fusarium moniliforme* (labeled as "FD12S" in Figure; PCT Publication No. WO 2005/047485);<br>• Aco: Aco terminator sequence from *Yarrowia Aco* gene (GenBank Accession No. AJ001300) |

The pZKL1-2SP98C plasmid was digested with *Asc*I/*Sph*I, and then used for transformation of strain Y4127U2 according to the General Methods. The transformant cells were plated onto MM plates and maintained at 30 °C for 3 to 4 days. Single colonies were re-streaked onto MM plates, and then inoculated into liquid MM at 30 °C and shaken at 250 rpm/min for 2 days. The cells were collected by centrifugation, resuspended in High Glucose Media and then shaken at 250 rpm/min for 5 days. The cells were collected by centrifugation, lipids were extracted,



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and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed that most of the selected 96 strains produced 18-23.9% EPA of total lipids. There were 11 strains (i.e.,#23, #61, #63, #67, #68, #73, #75, #85, #90, #91 and #95) that produced about 25.2%, 24.2%, 24%, 24.2%, 24%, 24%, 24.4%, 24.3%, 24.6%, 24.4% and 25.4% EPA of total lipids. These eleven strains were designated as Y4148, Y4149, Y4150, Y4151, Y4152, Y4153, Y4154, Y4155, Y4156, Y4157 and Y4158, respectively.

The final genotype of strain Y4158 with respect to wildtype *Yarrowia lipolytica* ATCC #20362 was: *unknown 1-, unknown 2-, unknown 3-, unknown 4-, unknown 5-,* GPD::FmD12::Pex20, YAT1::FmD12::OCT, GPM/FBAIN::FmD12S::OCT, EXP1::FmD12S::ACO, EXP1::ME3S::Pex20, YAT1::ME3S::Pex16, GPAT::EgD9e::Lip2, EXP1::EgD9e::Lip1, FBAINm::EgD9eS::Lip2, FBA::EgD9eS::Pex20, YAT1::EgD9eS::Lip2, FBAIN::EgD8M::Lip1, FBAINm::EgD8M::Pex20, EXP1::EgD8M::Pex16, GPDIN::EgD8M::Lip1, YAT1::EgD8M::Aco, FBAIN::EgD5::Aco, EXP1::EgD5S::Pex20, YAT1::RD5S::OCT, YAT1::PaD17S::Lip1, EXP1::PaD17::Pex16, FBAINm::PaD17::Aco, GPD::YlCPT1::Aco.

Generation Of Strain Y4158U1 (*Ura3-*)

A *Ura-* derivative (i.e., strain Y4158U1) was then created, via transformation with construct pZKUE3S (Figure 16B; SEQ ID NO:78), comprising a chimeric EXP1::ME3S::Pex20 gene targeted for the *Ura3* gene. Following transformation, cells were plated onto MM + 5-FOA selection plates and maintained at 30 °C for 3 to 4 days.

A total of 6 transformants grown on MM + 5-FOA plates were picked and re-streaked onto MM plates and MM + 5-FOA plates, separately. All 6 strains had a *Ura-* phenotype (i.e., cells could grow on MM + 5-FOA plates, but not on MM plates). The cells were scraped from the MM + 5-FOA plates, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.



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GC analyses showed the presence of 6.4% to 10.2% EPA in all of the transformants with pZKUE3S grown on MM + 5-FOA plates. Two strains (i.e., # and #5) that produced 10.2% and 10.1% EPA were designated as Y4158U1 and Y4158U2, respectively.

Generation Of Strain Y4184 To Produce About 30.7% EPA Of Total Lipids

Construct pZKL2-5U89GC (Figure 17B; SEQ ID NO:82) was generated to integrate one Δ9 elongase gene, one Δ8 desaturase gene, one Δ5 desaturase gene and one *Yarrowia lipolytica* CPT1 into the *Lip2* loci (GenBank Accession No. AJ012632) of Y4158U1, thereby resulting in isolation of strain Y4184. The pZKL2-5U89GC plasmid contained the following components:

Table 24

Description of Plasmid pZKL2-5U89GC (SEQ ID NO:82)

| RE Sites And Nucleotides Within SEQ ID NO:82 | Description Of Fragment And Chimeric Gene Components |
|---|---|
| *AscI*/*BsiWI* (730-1) | 722 bp 5' portion of *Yarrowia Lip2* gene (labeled as "Lip2.5N" in Figure; GenBank Accession No. AJ012632) |
| *PacI*/*SphI* (4141-3438) | 697 bp 3' portion of *Yarrowia Lip2* gene (labeled as "Lip2.3N" in Figure; GenBank Accession No. AJ012632) |
| *SwaI*/*BsiWI* (13382-1) | YAT1::YlCPT1::Aco, comprising:<br>• YAT1: *Yarrowia lipolytica* YAT1 promoter (labeled as "YAT" in Figure; Patent Publication US 2006/0094102-A1);<br>• YlCPT1: *Yarrowia lipolytica* diacylglycerol cholinephosphotransferase gene (SEQ ID NO:80) (labeled as "CPT1" in Figure; PCT Publication No. WO 2006/052870);<br>• Aco: Aco terminator sequence from *Yarrowia Aco* gene (GenBank Accession No. AJ001300) |
| *PmeI*/*SwaI* (10745-13382) | FBAIN::EgD8M::Lip1 comprising:<br>• FBAIN: *Yarrowia lipolytica* FBAIN promoter (PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356);<br>• EgD8M: Synthetic mutant Δ8 desaturase (SEQ ID NO:58) (labeled as "D8S-23" in Figure; U.S. Patent Application No. 11/635258), derived from *Euglena gracilis* ("EgD8S"; PCT Publication No. WO 2006/012326); |



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| | |
|---|---|
| | • Lip1: Lip1 terminator sequence from *Yarrowia Lip1* gene (GenBank Accession No. Z50020) |
| *Pme*I/*Cla*I (10745-8650) | GPD::EgD9eS::Lip2, comprising:<br>• GPD: *Yarrowia lipolytica* GPD promoter (labeled as "GPD Pro" in Figure; PCT Publication No. WO 2005/003310);<br>• EgD9eS: codon-optimized D9 elongase gene (SEQ ID NO:50), derived from *Euglena gracilis* (labeled as "EgD9ES" in Figure; PCT Publication No. WO 2007/061742);<br>• Lip2: Lip2 terminator sequence from *Yarrowia Lip2* gene (GenBank Accession No. AJ012632) |
| *Cla*I/*Eco*RI (8650-6581) | *Yarrowia* Ura3 gene (GenBank Accession No. AJ306421) |
| *Eco*RI/*Pac*I (6581-4141) | YAT1::EgD5S::ACO, comprising:<br>• YAT1: *Yarrowia lipolytica* YAT1 promoter (labeled as "YAT" in Figure; Patent Publication US 2006/0094102-A1);<br>• EgD5S: codon-optimized Δ5 desaturase (SEQ ID NO:63), derived from *Euglena gracilis* (U.S. Patent Application No. 11/748629);<br>• Aco: Aco terminator sequence from *Yarrowia Aco* gene (GenBank Accession No. AJ001300) |

The pZKL2-5U89GC plasmid was digested with *Asc*I/*Sph*I, and then used for transformation of strain Y4158U1 according to the General Methods. The transformant cells were plated onto MM plates and maintained at 30 °C for 3 to 4 days. Single colonies were re-streaked onto MM plates, and then inoculated into liquid MM at 30 °C and shaken at 250 rpm/min for 2 days. The cells were collected by centrifugation, resuspended in High Glucose Media and then shaken at 250 rpm/min for 5 days. The cells were collected by centrifugation, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed that most of the selected 96 strains produced 22.5-26.8% EPA of total lipids. There were three strains (i.e., #36, #47 and #65) that produced about 30.7%, 29.1% and 29.2% EPA of total lipids. These three strains were designated as Y4184, Y4185 and Y4186, respectively.



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The final genotype of strain Y4184 with respect to wildtype *Yarrowia lipolytica* ATCC #20362 was *unknown 1-, unknown 2-, unknown 3-, unknown 4-, unknown 5-, unknown 6-,* GPD::FmD12::Pex20, YAT1::FmD12::Oct, GPM/FBAIN::FmD12S::Oct, EXP1::FmD12S::Aco, YAT1::ME3S::Pex16, EXP1::ME3S::Pex20 (2 copies), GPAT::EgD9e::Lip2, EXP1::EgD9e::Lip1, FBAINm::EgD9eS::Lip2, FBA::EgD9eS::Pex20, YAT1::EgD9eS::Lip2, GPD::EgD9eS::Lip2, GPDIN::EgD8M::Lip1, YAT1::EgD8M::Aco, EXP1::EgD8M::Pex16, FBAINm::EgD8M::Pex20, FBAIN::EgD8M::Lip1 (2 copies), EXP1::EgD5S::Pex20, YAT1::EgD5S::Aco, YAT1::RD5S::Oct, FBAIN::EgD5::Aco, FBAINm::PaD17::Aco, EXP1::PaD17::Pex16, YAT1::PaD17S::Lip1, YAT1::YlCPT1::Aco, GPD::YlCPT1::Aco.

Generation Of Strain Y4184U (*Ura3-*)

In order to disrupt the Ura3 gene in strain Y4184, construct pZKUE3S (Figure 16B; SEQ ID NO:78) was used to integrate an EXP1::ME3S::Pex20 chimeric gene into the *Ura3* gene of strain Y4184.

A total of 11 transformants grown on MM + 5-FOA plates were picked and re-streaked onto MM plates and MM + 5-FOA plates, separately. All 11 strains had a *Ura-* phenotype (i.e., cells could grow on MM + 5-FOA plates, but not on MM plates). The cells were scraped from the MM + 5-FOA plates, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed the presence of 11.2%, 10.6% and 15.5% EPA in the #7, #8 and #10 transformants with pZKUE3S grown on MM + 5-FOA plates. These three strains were designated as strains Y4184U1, Y4184U2 and Y4184U4, respectively.

The discrepancy in the % EPA quantified in Y4184 (30.7%) versus Y4184U (average 12.4%) is based on differing growth conditions. Specifically, the former culture was analyzed following two days of growth in liquid culture, while the latter culture was analyzed after growth on an agar plate. The Applicants have observed a 2-3 fold increase in % EPA, when comparing results from agar plates to those in liquid culture. Thus,



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although results are not directly comparable, both Y4184 and Y4184U strains demonstrate high production of EPA.

EXAMPLE 8

Synthesis Of *Yarrowia lipolytica* Strain Series YCS8600, Producing Lycopene And Omega-3 PUFAs

The present Example describes the construction of the *Yarrowia lipolytica* YCS8600 strain series, co-producing lycopene and ω-3 PUFAs (Figure 18). Strain YCS8601U served as the host strain in Example 10, *infra*, for creation of the YCS8700 series of carotenoid-producing *Y. lipolytica* transformants.

As described herein, construction of the *Y. lipolytica* YCS8600 series required: (1) transformation of *Yarrowia lipolytica* strain Y4184U4 with plasmid pYCRTEBI, comprising chimeric $crtE_{syn}$,$crtB_{syn}$ and $crtI_{syn}$ genes; and, (2) extraction and analysis of the carotenoids produced.

Transformation Of *Yarrowia* Strain Y4184U4 With Integration Plasmid pYCRTEBI

The ω-3 producing strain, *Yarrowia lipolytica* strain Y4184U4 (Example 7), was transformed with the carotenoid integration plasmid pYCRTEBI (Example 2; SEQ ID NO:12; Figure 5), comprising chimeric *FBAIN::*$crtE_{syn}$*::Lip1, GPDIN::*$crtB_{syn}$*::Lip2* and *EXP1::*$crtI_{syn}$*::Oct* genes. Plasmid DNA was prepared using the Qiagen Spin Miniprep kit according to the manufacturer's protocol (Qiagen, Valencia, CA). In preparation for the transformation, pYCRTEBI was linearized by digestion with *Asc*I and *Sph*I. The digestion reaction was separated on an 0.8% agarose gel and the 10.8 kb DNA fragment was excised and gel purified using the Qbiogene Geneclean® II kit according to the manufacturer's protocol (Qbiogene, Carlsbad, CA). The transformations were performed as described in Example 1 with the exception that salmon sperm DNA was not added to the transformation buffer. The transformation plates were incubated at 30 °C for approximately 6 days before orange colonies were picked and streaked onto fresh MMLeu agar plates. The transformants were re-streaked onto MM and MMLeu agar plates to determine if integration in the *Yarrowia* Y4184U4 genome was at the leucine site or



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elsewhere in the chromosome. Growth on the MM and the MMLeu agar plates indicated that none of the transformants had integrated at the leucine site. The 15 lycopene-producing strains (i.e., YCS8601- YCS8611 and YCS8612- YCS8616) were named as the YCS8600 series.

Carotenoid Extraction And Analysis

To determine lycopene titers, each strain in the YCS8600 series was inoculated into 20 mL of FM* without YE media; strains YCS8601 and YCS8602 were additionally inoculated into 20 mL of FM* media. The cultures were grown in 125 mL shake flasks at 30 °C with aeration until they were turbid (approximate $OD_{600}$ was 12 - 17). In contrast, the YCS8601 and YCS8602 strains grown in FM* were cultured to an approximate $OD_{600}$ of 20 -25.

To determine the dry cell weight (DCW) of each culture, 10 mL of each culture was filtered using a magnetic filter funnel (300 mL capacity, 47 mm) (Pal Gelman, Ann Arbor, MI). The cells were filtered onto a 47 mm, 0.2 μm polycarbonate Whatman Nuclepore Track-Etch membrane (Whatman Inc., Florham Park, NJ) to remove the liquid. A 47 mm, 10.0 mm polypropylene separator (Pall Gelman) was also used to enhance the filtration process and concentrate the cells in the middle of the membrane.

For carotenoid extractions, 5 mL of culture was centrifuged (8,000 rpm and 4 °C) in a 50 mL polypropylene tube. To the cell pellet, glass beads (approximately 0.5 mL, 0.5 mm diameter), 2 mL of ethanol and 3 mL of dichloromethane were added and the mixture was vigorously mixed using a Vortex mixer before centrifugation for 10 min at 8,000 rpm and 4 °C. The supernatant was transferred to a new 50 mL polypropylene tube and taken to dryness under a stream of nitrogen. The dried carotenoids were re-suspended in 135 μL chloroform, mixed using a Vortex mixer when 2865 μL hexane was added prior to further mixing as described above. Prior to HPLC (Beckman Beckman Coulter, Fullerton, CA) analysis, samples were filtered through a 0.2 μm Teflon® filter. The lycopene titers (mg of lycopene/kg of cells) for each strain are listed below in Table 25.



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Table 25

Lycopene Titers Of The YCS8600 Strain Series

| Strain | Lycopene Titer (mg lycopene/ kg cells) | | Strain | Lycopene Titer (mg lycopene/ kg cells) |
|---|---|---|---|---|
| YCS8601 | 2119 * | | YCS8608 | 594 |
| YCS8602 | 1264 * | | YCS8609 | 400 |
| **YCS8601** | **1594** | | YCS8610 | 596 |
| YCS8602 | 655 | | YCS8611 | 708 |
| YCS8603 | 625 | | **YCS8613** | **1063** |
| YCS8604 | 948 | | YCS8614 | 522 |
| YCS8605 | 792 | | YCS8615 | 769 |
| YCS8606 | 426 | | YCS8616 | 601 |
| **YCS8607** | **1165** | | | |

* Lycopene titer obtained following extended growth in FM* media, as opposed to in FM* without YE media.

The *ura* marker was removed from *Yarrowia* strains YCS8601, YCS8607 and YCS8613 (which had the highest lycopene titers when grown in FM* without YE media) via transformation with pY79, as described in Example 3. For each strain, ~150 colonies were patched onto MM and MMU agar plates. One colony from strain YCS8601 grew on the MMU medium containing plate. This colony was re-streaked onto MM, MMU and YPD agar plates to reconfirm its growth characteristic and was given the new name YCS8601U.

Colonies from strains YCS8607 and YCS8613 grew on both media, therefore suggesting that the Ura3 gene was still present in the genomes of these two strains. The cells were confirmed to have lost the mutant AHAS-containing plasmid (i.e., pY79) by their ability to grow only on the MMU agar plates and not on the MMU+SU agar plates.

## EXAMPLE 9

## Construction Of Additional Synthetic Codon-Optimized *crtY*, *crtW* And *crtZ* Genes For Overexpression In *Yarrowia lipolytica*

Although the construction of the β-carotene-producing YSC1200 and YSC1300 series (Example 5), zeaxanthin-producing YSC3700 and YSC4100 series (Example 6) and canthaxanthin-producing YSC4000



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series (Example 6) was suitable to demonstrate feasibility of engineering carotenoid production in *Yarrowia lipolytica,* it is well known that gene expression can be increased by increasing the number of copies of the cloned gene(s).

The present Example describes the construction of various synthetic $crtY_{syn}$, $crtW_{syn}$ and $crtZ_{syn}$ genes suitable for expression in the *Yarrowia lipolytica* YCS8600 strain series (Example 8), to thereby enable higher-level co-production of β-carotene, zeaxanthin, canthaxanthin and/or astaxanthin in conjunction with ω-3 PUFAs. These synthetic genes were then incorporated into genetic constructs to produce expression plasmids pYPS147, pYPS152 and pYPS162 (described and expressed in Examples 10-13, *infra*).

Selected wildtype and codon-optimized *crtY, crtW* and *crtZ* genes contained within expression plasmids pDCQ333, pDCQ334, pDCQ341 and pDCQ343 (described below in Table 26) were codon-optimized based on the codon usage preference for *Yarrowia lipolytica* (see U.S. Patent 7,125,672). Nomenclature is similar to that utilized to describe the DCQ392 gene cluster comprising *crtWZEidiYIB* in plasmid pDCQ392 (Example 5).



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Table 26

Sources Of Carotenoid Biosynthetic Genes

| **Plasmid** (Reference) | **Complete Crt Cluster Within Plasmid; Product** | **Origin of *crtY* Within Plasmid** (Reference) | **Origin of *crtW* Within Plasmid** (Reference) | **Origin of *crtZ* Within Plasmid** (Reference) |
|---|---|---|---|---|
| pDCQ333 (U.S. 7,232,666) | *crtWEidiYIB;* canthaxanthin | *Pantoea agglomerans* DC404 (U.S. 6,929,928) | *Agrobacterium aurantiacum** (U.S. 5,972,690, U.S. 6,150,130; also U.S. 7,232,666*) | -- |
| pDCQ334 (U.S. 7,232,666) | *crtWZEidiYIB;* astaxanthin | *Pantoea agglomerans* DC404 (U.S. 6,929,928) | *Agrobacterium aurantiacum** (U.S. 5,972,690, U.S. 6,150,130; also U.S. 7,232,666*) | *Agrobacterium aurantiacum** (U.S. 5,811,273; also U.S. 7,232,666*) |
| pDCQ341 (U.S. 7,252,985;) | *crtWEYIB;* canthaxanthin | *Enterobacteriaceae* DC260 (U.S. 7,064,196; Sedkova, N., et al., *Appl. Environ. Microbiol.*, 71 (12):8141-8146 (2005)) | *Sphingomonas melonis* DC18 (U.S. 7,252,985) | -- |
| pDCQ343 (U.S. 7,217,537) | *crtWZEYIB;* astaxanthin | *Enterobacteriaceae* DC260 (U.S. 7,064,196; Sedkova, N., et al., *supra*) | *Sphingomonas melonis* DC18 (U.S. 7,252,985) | *Brevundimonas vesicularis* DC263 (U.S. 7,091,031; Tao, L., et al., *Gene,* 379:101-108 (2006)) |

Gene in plasmid was subjected to codon-optimization for the pink-pigmented methanotrophic bacterial strain, *Methylomonas* sp. 16a, as described in U.S. Patent 7,232,666.



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The synthetic genes were produced by GenScript Corp. (Piscataway, NJ) and provided in the high-copy vector pUC57 (GenBank® Accession No. Y14837). This resulted in creation of the synthetic codon-optimized *crtY*$_{syn}$, *crtW*$_{syn}$ and *crtZ*$_{syn}$ genes, as described below in Table 27.

Table 27

Codon-Optimized Carotenoid Biosynthesis Genes

| Carotenoid Gene | Plasmid Source | Organism Source | Nucleotide SEQ ID NO | Amino Acid SEQ ID NO |
|---|---|---|---|---|
| *crtY*343$_{syn}$ | pDCQ343 | *Enterobacteriaceae* DC260 | 83 | 84 |
| *crtY*334$_{syn}$ | pDCQ334 | *Pantoea agglomerans* DC404 | 85 | 86 |
| *crtW*341$_{syn}$ | pDCQ341 | *Sphingomonas melonis* DC18 | 87 | 88 |
| *crtW*333$_{syn}$ | pDCQ333 | *Agrobacterium aurantiacum* | 89 | 90 |
| *crtZ*343$_{syn}$ | pDCQ343 | *Brevundimonas vesicularis* DC263 | 91 * | 92 |
| *crtZ*334$_{syn}$ | pDCQ334 | *Agrobacterium aurantiacum* | 93 | 94 |

* Note: Both SEQ ID NO:91 (*crtZ*343$_{syn}$) and SEQ ID NO:41 (*crtZ*392$_{syn}$) were codon-optimized for expression in *Yarrowia lipolytica*, based on the same wildtype *Brevundimonas vesicularis* DC263 gene (i.e., SEQ ID NO:18; *crtZ*$_{392}$); however, codon degeneracy resulted in two different sequences that share only ~85% identity with one another.

All genes were excised from the pUC57 derived vectors by digestion with *Nco*I/*Not*I, fragments were run on 0.8% agarose gels, and the appropriate sized fragments were excised from the gel. In this way, DNA encoding *crtY*343$_{syn}$, *crtY*334 $_{syn}$, *crtW*341$_{syn,}$, *crtW*333$_{syn}$, *crtZ*343$_{syn}$, and *crtZ*334$_{syn}$ were extracted and then purified with the Qbiogene Geneclean® kit, in preparation for construction of integration vectors pYPS147, pYPS152 and pYPS162. The genes were used in conjuction with *crtY*392$_{syn}$, *crtZ*392 $_{syn}$ and *crtW*392$_{syn,}$ (Table 13 of Example 6).



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EXAMPLE 10

Synthesis Of *Yarrowia lipolytica* Strain Series YCS8700, Producing β-Carotene And Omega-3 PUFAs

The present Example describes the construction of the *Yarrowia lipolytica* YCS8700 strain series, co-producing β-carotene and ω-3 PUFAs (Figure 18). Strains YCS8734, YSC8740 and YCS8747 served as the host strains in Examples 11 and 12, *infra*, for creation of the YCS9100, YCS9400, YCS9500 and YCS9600 series of carotenoid-producing *Y. lipolytica* transformants.

As described herein, construction of the *Y. lipolytica* YCS8700 series required: (1) construction of plasmid pYPS147, comprising three different chimeric *crtY* genes; (2) transformation of *Ura-* strain YSC8601U (producing lycopene) with plasmid pYPS147; and, (3) extraction and analysis of the carotenoids produced.

Construction Of pYPS147, Comprising Three *crtY* Genes

Plasmid pYPS147 (Figure 19A; SEQ ID NO:95) was constructed to contain three different chimeric *crtY* genes (i.e., FBAINm::*crtY*$392_{syn}$::Lip1, GDPIN::*crtY*$343_{syn}$::Lip2 and EXP1::*crtY*$334_{syn}$::OCT), using a series of 4-way ligations to consecutively create each expression cassette (i.e., comprising a promoter, *crt* gene, and terminator) within an existing vector backbone. After each ligation, the reaction was transformed into *E. coli* XL2 Blue cells (Stratagene) and transformants were screened via colony PCR using the MasterAmp™ Taq protocol of the General Methods, to identify the plasmid having the correct configuration.

The pYPS147 plasmid contained the following components:

Table 28

Description of Plasmid pYPS147 (SEQ ID NO:95)

| RE Sites And Nucleotides Within SEQ ID NO:95 | Description Of Fragment And Chimeric Gene Components |
|---|---|
| *Asc*I/*Bsi*WI (3395-2601) | 795 bp 5' portion of *Yarrowia Leu2* gene (GenBank Accession No. AF260230) |
| *Pac*I/*Sph*I | 709 bp 3' portion of *Yarrowia Leu2* gene (GenBank |



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| | |
|---|---|
| (6812-6104) | Accession No. AF260230) |
| SwaI/*SwaI* (23-2444) | FBAINm::crtY392$_{syn}$::Lip1, comprising:<br>• FBAINm: *Yarrowia lipolytica* FBAIN promoter (PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356);<br>• crtY392$_{syn}$: codon-optimized *crtY* (SEQ ID NO:37), derived from *Pantoea stewartii* DC413 (labeled as "crtY-syn" in Figure; U.S. Patent 7,288,387);<br>• Lip1: Lip1 terminator sequence from *Yarrowia Lip1* gene (GenBank Accession No. Z50020) |
| *PmeI*/*ClaI* (11249-8563) | GPDIN::crtY343$_{syn}$::Lip2, comprising:<br>• GPDIN: *Yarrowia lipolytica* GPDIN promoter (labeled as "GPD Pro + Intron" in Figure; Patent Publication US 2006/0019297-A1);<br>• crtY343$_{syn}$; codon-optimized *crtY* (SEQ ID NO:83), derived from *Enterobacteriaceae* DC260 (U.S. Patent 7,064,196);<br>• Lip2: Lip2 terminator sequence from *Yarrowia Lip2* gene (GenBank Accession No. AJ012632) |
| *PmeI*/*BglII* (11250--13625) | EXP1::crtY334$_{syn}$::OCT, comprising:<br>• EXP1: *Yarrowia lipolytica* export protein (EXP1) promoter (labeled as "EXP" in Figure; PCT Publication No. WO 2006/052870 and U.S. Patent Application No. 11/265761);<br>• crtY334$_{syn}$: codon-optimized *crtY* (SEQ ID NO:85), derived from *Pantoea agglomerans* DC404 (U.S. Patent 6,929,928);<br>• OCT: OCT terminator sequence of *Yarrowia OCT* gene (GenBank Accession No. X69988) |
| 8537-6844 | LoxP::*Ura3*::LoxP, comprising:<br>• LoxP sequence (SEQ ID NO:9);<br>• *Yarrowia* Ura3 gene (GenBank Accession No. AJ306421);<br>• LoxP sequence (SEQ ID NO:9) |
| 5244-4384 | Ampicillin-resistance gene (Amp$^R$) for selection in *E. coli* |

Generation And Analysis Of β-Carotene-Producing Strains

The pYPS147 plasmid was digested *DrdI*, separated on an 0.8% agarose gel and purified using the Qbiogene Geneclean II kit. The resulting pYPS147 fragment was then used for transformation of lycopene-producing *Yarrowia* strain YCS8601U (Example 8), according to the methodology of Example 1.

The transformant cells were plated onto MMLeu agar plates. Colonies present after 4 days of incubation at 30 °C were pink and yellow



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in color. The pink colonies were similar to cells present on the cells alone control plate, which suggested that they were background colonies and not real transformants. The yellowish and orange colonies were re-streaked and the new strains were given names as a part of the YCS8700 series.

To determine whether the strains were able to convert lycopene into β-carotene due to the expression of the β-carotene cyclase (i.e., *crtY*) genes, 20 mL cultures were grown in FM* for 48 hrs with aeration in 125 mL shake flasks. All of the cultures grew to similar ODs. Two milliliters of each culture were harvested in tubes used for bead beating. To each tube ~100 μL of 0.5 mm glass beads were added along with 400 μL of methanol. The tubes were put into the BeadBeater™ apparatus (BioSpec Products, Inc., Bartlesville, OK) for 5 min at high speed. The samples were mixed by rocking for 20 min on an oscillator. To each tube, 50 μL of NaCl (1.0 M), 200 μL of chloroform and 400 μL hexane were added, samples were mixed using a Vortex mixer and carotenoids were partitioned into the organic layer following centrifugation for 10 min in a microfuge. The organic top layer, containing the carotenoids, was transferred to a new microfuge tube using a glass Pasteur pipette. Samples were passed through a Teflon® filter (0.2 μm) prior to HPLC analysis using a reverse-phase $C_{18}$ column as described in Example 5.

As shown in Table 29, the level of β-carotene produced by various strains in the YCS8700 series ranged from 22% to 66% (of total carotenoids). The amount of β-carotene made by the strains, as determined by the "area of the β-carotene peaks", ranged from ~1150 for strain YCS8704 to ~5690 for strain YCS8740. The color of the pellet was not indicative of the relative amount of β-carotene produced by the strains. Strain YCS8601 (row shaded in gray) was the control strain, which did not contain the *crtY* genes and thus could not produce β-carotene.



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Table 29

β-carotene Production In The YCS8700 Strain Series

| Strain* | Lycopene (% of total carotenoid) | β-carotene (% of total carotenoid) | Pellet Color | Area of β-carotene Peaks |
|---|---|---|---|---|
| YCS8601 | 71 | --- | Red | --- |
| YCS8701 | 4 | 45 | Yellow | 2380 |
| YCS8702 | 4 | 46 | Yellow | 2500 |
| YCS8703 | 22 | 29 | Orange | 1580 |
| YCS8704 | 30 | 22 | Orange | 1150 |
| YCS8705 | 24 | 28 | Orange | 1440 |
| YCS8706 | 7 | 48 | Yellow | 2950 |
| YCS8707 | 25 | 27 | Orange | 1310 |
| YCS8708 | 4 | 51 | Yellow | 3020 |
| YCS8709 | 5 | 50 | Orange | 2680 |
| YCS8710 | 17 | 38 | Orange | 2015 |
| YCS8711 | 4 | 54 | Yellow | 3450 |
| YCS8712 | 4 | 53 | Yellow | 3800 |
| YCS8713 | 4 | 51 | Yellow | 3000 |
| YCS8714 | 19 | 35 | Orange | 2060 |
| YCS8715 | 4 | 53 | Yellow | 3330 |
| YCS8716 | 18 | 45 | Orange | 5060 |
| YCS8717 | 5 | 61 | Yellow | 4370 |
| YCS8718 | 4 | 60 | Yellow | 4240 |
| YCS8719 | 22 | 40 | Orange | 2070 |
| YCS8720 | 24 | 36 | Orange | 2180 |
| YCS8721 | 25 | 37 | Orange | 2530 |
| YCS8722 | 4 | 63 | Yellow | 4610 |
| YCS8723 | 22 | 40 | Orange | 2400 |
| YCS8724 | 4 | 63 | Yellow | 4770 |
| **YCS8725** | **3** | **66** | **Yellow** | **4890** |
| YCS8726 | 26 | 38 | Orange | 2500 |
| YCS8727 | 22 | 42 | Orange | 2680 |
| YCS8728 | 20 | 45 | Orange | 3010 |
| YCS8729 | 4 | 65 | Yellow | 2730 |
| YCS8730 | 5 | 62 | Yellow | 4550 |
| YCS8731 | 31 | 34 | Orange | 2850 |
| YCS8732 | 6 | 61 | Yellow | 1930 |
| YCS8733 | 7 | 61 | Yellow | 2020 |
| **YCS8734** | **5** | **63** | **Orange** | **5080** |
| YCS8735 | 5 | 62 | Orange | 4630 |
| YCS8736 | 24 | 39 | Orange | 2580 |
| YCS8737 | 4 | 63 | Orange | 4690 |
| YCS8738 | 4 | 63 | Yellow | 4630 |
| YCS8739 | 22 | 43 | Orange | 2700 |
| **YCS8740** | **5** | **64** | **Orange** | **5690** |



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| | | | | |
|---|---|---|---|---|
| YCS8741 | 6 | 63 | Orange | 4670 |
| YCS8742 | 7 | 62 | Orange | 4614 |
| YCS8743 | 22 | 43 | Orange | 2870 |
| YCS8744 | 4 | 65 | Yellow | 4410 |
| YCS8745 | 27 | 38 | Orange | 2350 |
| YCS8746 | 6 | 62 | Orange | 4580 |
| **YCS8747** | **6** | **66** | **Orange** | **4410** |

* Strains selected for further evaluation are highlighted in bold.

## EXAMPLE 11

## Synthesis Of *Yarrowia lipolytica* Strain Series YCS9400, YCS9500 And YCS9600, Producing Zeaxanthin And Omega-3 PUFAs

The present Example describes the construction of the *Yarrowia lipolytica* YCS9400, YCS9500 and YCS9600 strain series, co-producing zeaxanthin and ω-3 PUFAs (Figure 18).

As described herein, construction of the *Y. lipolytica* YCS9400, YCS9500 and YCS9600 series required: (1) construction of plasmid pYPS162, comprising three different chimeric *crtZ* genes; (2) transformation of *Yarrowia lipolytica* strains YCS8734, YCS8740 and YCS8747 (producing β-carotene), with plasmid pYPS162; and, (3) extraction and analysis of the carotenoids produced.

### Construction Of pYPS162, Comprising Three *crtZ* Genes

Plasmid pYPS162 (Figure 19B; SEQ ID NO:96) was constructed to contain three different chimeric *crtZ* genes (i.e., *FBAIN::crtZ334$_{syn}$::Lip, GDPIN::crtZ392$_{syn}$::Lip2* and *EXP1::crtZ343$_{syn}$::OCT*), using methodology similar to that described for construction of pYPS147 (Example 10). The pYPS162 plasmid contained the following components:

Table 30

Description of Plasmid pYPS162 (SEQ ID NO:96)

| RE Sites And Nucleotides Within SEQ ID NO:96 | Description Of Fragment And Chimeric Gene Components |
|---|---|
| *AscI/BsiWI* 6418-5624 | 795 bp 5' portion of *Yarrowia Leu2* gene (GenBank Accession No. AF260230) |
| *PacI/SphI* | 709 bp 3' portion of *Yarrowia Leu2* gene (GenBank |



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| | |
|---|---|
| 9835-9127 | Accession No. AF260230) |
| *BglII/SwaI* 3699-5467 | FBAIN::crtZ334$_{syn}$::Lip1, comprising:<br>• FBAINm: *Yarrowia lipolytica* FBAIN promoter (PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356);<br>• crtZ334$_{syn}$: codon-optimized *crtZ* (SEQ ID NO:93), derived from *Agrobacterium aurantiacum* (U.S. Patent 7,232,666);<br>• Lip1: Lip1 terminator sequence from *Yarrowia Lip1* gene (GenBank Accession No. Z50020) |
| 2006-5 | GPDIN::crtZ392$_{syn}$::Lip2, comprising:<br>• GPDIN: *Yarrowia lipolytica* GPDIN promoter (labeled as "GPD Pro + Intron" in Figure; Patent Publication US 2006/0019297-A1);<br>• crtZ392$_{syn}$; codon-optimized *crtZ* (SEQ ID NO:41), derived from *Brevundimonas vesicularis* DC263 (labeled as "crtZ" in Figure; U.S. Patent 7,091,031);<br>• Lip2: Lip2 terminator sequence from *Yarrowia Lip2* gene (GenBank Accession No. AJ012632) |
| *PmeI/BglII* 2007-3698 | EXP1::crtZ343$_{syn}$::OCT, comprising:<br>• EXP1: *Yarrowia lipolytica* export protein (EXP1) promoter (labeled as "EXP" in Figure; PCT Publication No. WO 2006/052870 and U.S. Patent Application No. 11/265761);<br>• crtZ343$_{syn}$: codon-optimized *crtZ* (SEQ ID NO:91), derived from *Brevundimonas vesicularis* DC263 (U.S. Patent 7,091,031);<br>• OCT: OCT terminator sequence of *Yarrowia OCT* gene (GenBank Accession No. X69988) |
| 9836-12825 | *Yarrowia lipolytica* AHAS gene (GenBank Accession No. XP_501277) comprising a W497L mutation (SEQ ID NO:1) (labeled as "SUR" in Figure; PCT Publication No. WO 2006/052870) |
| 8267-7407 | Ampicillin-resistance gene (Amp$^R$) for selection in *E. coli* |

Generation And Analysis Of Zeaxanthin-Producing Strains

The pYPS162 plasmid was digested *Fsp*I and then used for transformation of the β-carotene-producing *Yarrowia* strains YCS8734, YCS8740 and YCS8747 (Example 10), according to the methodology of Example 1.

The transformant cells were plated onto MM+SU agar plates and maintained at 30 °C for 3 to 4 days. The transformants obtained from the YCS8734, YCS8740 and YCS8747 transformations were designated as a part of the YCS9400, YCS9500 and YCS9600 series, respectively. Single



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colonies of each were re-streaked onto MM plates and isolated colonies were inoculated into FM*.

To determine the ability of the strains to convert β-carotene into zeaxanthin due to the expression of the β-carotene hydroxylase (i.e., *crtZ*) genes, 20 mL cultures were grown for 48 hr in 125 mL capacity shake flasks with aeration. The cultures had similar ODs (approximate $OD_{600}$ was 20-25) at end of the incubation. Carotenoids were extracted as described above in Example 10.

The percent of lycopene, β-carotene and zeaxanthin accumulated in individual strains within the YCS9400, YCS9500 and YCS9600 series are shown in Table 31. Strains YCS8734, YCS8740 and YCS8747 (rows shaded in gray) are the control strains, which do not contain the β-carotene hydroxylase and thus can not produce zeaxanthin. The strains having the highest levels of zeaxanthin are highlighted in bold font. As expected, the zeaxanthin-producing strains showed a decrease in their percentages of lycopene and β-carotene.

Table 31

Lycopene, β-Carotene And Zeaxanthin Production In The YCS9400, YCS9500 And YCS9600 Strain Series

| Strain | Lycopene/ β-Carotene (% of total carotenoid) | Zeaxanthin (% of total carotenoid) | | Strain | Lycopene/ β-Carotene (% of total carotenoid) | Zeaxanthin (% of total carotenoid) |
|---|---|---|---|---|---|---|
| YCS8734 | 94 | --- | | YCS9508 | 81 | 12 |
| YCS9401 | 64 | 28 | | YCS9509 | 94 | --- |
| YCS9402 | 62 | 31 | | YCS9510 | 94 | --- |
| YCS9403 | 62 | 32 | | YCS9511 | 92 | --- |
| YCS9404 | 94 | --- | | YCS9512 | 93 | --- |
| YCS9405 | 94 | --- | | YCS9513 | 93 | --- |
| YCS9406 | 61 | 32 | | YCS9514 | 68 | 24 |
| YCS9407 | 72 | 22 | | **YCS9515** | **38** | **55** |
| YCS9408 | 63 | 28 | | YCS8747 | 93 | --- |
| YCS9409 | 94 | --- | | YCS9601 | 57 | 37 |
| **YCS9410** | **54** | **40** | | YCS9602 | 57 | 36 |
| YCS9411 | 94 | --- | | YCS9603 | 79 | 13 |
| YCS9412 | 95 | --- | | YCS9604 | 94 | --- |



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| | | | | | |
|---|---|---|---|---|---|
| YCS9413 | 94 | --- | YCS9605 | 61 | 32 |
| YCS9414 | 93 | --- | YCS9606 | 59 | 35 |
| YCS9415 | 68 | 26 | **YCS9607** | **48** | **47** |
| YCS8740 | 93 | --- | YCS9608 | 63 | 30 |
| YCS9501 | 72 | 20 | YCS9609 | 85 | 7 |
| YCS9502 | 93 | --- | YCS9610 | 67 | 26 |
| YCS9503 | 67 | 27 | YCS9611 | 94 | --- |
| YCS9504 | 60 | 33 | YCS9612 | 77 | 16 |
| YCS9505 | 72 | 21 | YCS9613 | 56 | 38 |
| YCS9506 | 94 | --- | YCS9614 | 56 | 38 |
| YCS9507 | 94 | --- | YCS9615 | 82 | 12 |

* Strains selected for further evaluation are highlighted in bold.

## EXAMPLE 12

## Synthesis Of *Yarrowia lipolytica* Strain Series YCS9100, Producing Canthaxantin And Omega-3 PUFAs

The present Example describes the construction of the *Yarrowia lipolytica* YCS9100 strain series, co-producing canthaxanthin and ω-3 PUFAs (Figure 18). Strains YCS8158, YCS9165 and YCS9169 served as the host strains in Example 13, *infra*, for creation of the YCS9700, YCS9800 and YCS9900 series of carotenoid-producing *Y. lipolytica* transformants.

As described herein, construction of the *Y. lipolytica* YCS9100 series required: (1) construction of plasmid pYPS152, comprising three different chimeric *crtW* genes; (2) generation of *Ura3*- strains derived from *Yarrowia lipolytica* strains YCS8734, YCS8740 and YCS8747 (producing β-carotene); (3) transformation of the *Ura3*-, β-carotene-producing strains with plasmid pYPS152; and, (4) extraction and analysis of the carotenoids produced.

### Construction Of pYPS152, Comprising Three *crtW* Genes

Plasmid pYPS152 (Figure 20A; SEQ ID NO:97) was constructed to contain three different chimeric *crtY* genes (i.e., FBAIN::*crtW*$333_{syn}$::Lip1, GDPIN::*crtW*$341_{syn}$::Lip2 and EXP1::*crtW*$392_{syn}$::OCT), using methodology similar to that described for construction of pYPS147 (Example 10). The pYPS152 plasmid contained the following components:



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Table 32

Description of Plasmid pYPS152 (SEQ ID NO:97)

| RE Sites And Nucleotides Within SEQ ID NO:97 | Description Of Fragment And Chimeric Gene Components |
|---|---|
| *AscI/SphI* 3004-2210 | 795 bp 5' portion of *Yarrowia Leu2* gene (GenBank Accession No. AF260230) |
| *PacI/SphI* 6421-5713 | 709 bp 3' portion of *Yarrowia Leu2* gene (GenBank Accession No. AF260230) |
| *BglII/SwaI* 45-2053 | FBAINm::crtW333$_{syn}$::Lip1, comprising:<br>• FBAINm: *Yarrowia lipolytica* FBAIN promoter (PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356);<br>• crtW333$_{syn}$: codon-optimized *crtW* (SEQ ID NO:89), derived from *Agrobabacterium aurantiacum* (U.S. Patent 7,232,666);<br>• Lip1: Lip1 terminator sequence from *Yarrowia Lip1* gene (GenBank Accession No. Z50020) |
| *PmeI/ClaI* 10438-8172 | GPDIN::crtW341$_{syn}$::Lip2, comprising:<br>• GPDIN: *Yarrowia lipolytica* GPDIN promoter (labeled as "GPD Pro + Intron" in Figure; Patent Publication US 2006/0019297-A1);<br>• crtW341$_{syn}$; codon-optimized *crtW* (SEQ ID NO:87), derived from *Sphingomonas melonis* DC18 (U.S. Patent 7,252,985);<br>• Lip2: Lip2 terminator sequence from *Yarrowia Lip2* gene (GenBank Accession No. AJ012632) |
| *PmeI/BglII* 10439-44 | EXP1::crtW392$_{syn}$::OCT, comprising:<br>• EXP1: *Yarrowia lipolytica* export protein (EXP1) promoter (labeled as "EXP" in Figure; PCT Publication No. WO 2006/052870 and U.S. Patent Application No. 11/265761);<br>• crtW392$_{syn}$: codon-optimized *crtW* (SEQ ID NO:39), derived from *Brevundimonas vesicularis* DC263 (U.S. Patent 7,252,985);<br>• OCT: OCT terminator sequence of *Yarrowia OCT* gene (GenBank Accession No. X69988) |
| *ClaI/PacI* 8171-6422 | LoxP::*Ura3*::LoxP, comprising:<br>• LoxP sequence (SEQ ID NO:9);<br>• *Yarrowia* Ura3 gene (GenBank Accession No. AJ306421);<br>• LoxP sequence (SEQ ID NO:9) |
| 4853-3993 | Ampicillin-resistance gene (Amp$^R$) for selection in *E. coli* |



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Generation Of *Ura3*$^-$, β-Carotene-Producing *Yarrowia lipolytica* Strains

The β-carotene producing strains YCS8734, YCS8740 and YCS8747 (Example 10) were *Ura3*$^+$. In order to disrupt the Ura3 gene in each of these strains (to thereby enable the Ura3 selectable marker to be "re-used" during subsequent transformations), construct pZKUM (Figure 20B; SEQ ID NO:98) was used to integrate a *Ura3* mutant gene into the *Ura3* gene. The plasmid pZKUM contained the following components:

Table 33

Description of Plasmid pZKUM (SEQ ID NO:98)

| RE Sites And Nucleotides Within SEQ ID NO:98 | Description Of Fragment And Chimeric Gene Components |
|---|---|
| *SalI*/*PacI* (32845-1) | Synthetic mutant Ura3 gene (SEQ ID NO:99, wherein the 1459 bp DNA fragment contains a 33 bp deletion from +21 to +53, a 1 bp deletion at +376 and a 3 bp deletion from +400 to +403 of the *Yarrowia* Ura3 coding region (GenBank Accession No. AJ306421)) |
| 1112-232 | ColE1 plasmid origin of replication |
| 2042-1182 | Ampicillin-resistance gene (Amp$^R$) for selection in *E. coli* |

Following transformations using the linearized pZKUM DNA fragment containing the mutated Ura3 gene into strains YCS8734, YCS8740 and YCS8747, cells were streaked onto MM + 5-FOA agar plates. This selection technique allowed identification of transformants having a *Ura*- phenotype. The phenotype was confirmed after streaking isolated colonies onto MMU agar plates (i.e., cells that had lost the *Ura*3 gene required the presence of uracil in the growth medium and thus could grow) and MM agar plates (i.e., cells that had lost the *Ura*3 gene could not grow). Several isolates that were *Ura*$^-$ were given the following new designations: YCS8734U1, YCS8734U2, YCS8734U3, YCS8734U4, YCS8734U6, YCS8740U1, YCS8740U2 and YCS8747U2.



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Generation And Analysis Of Canthaxanthin-Producing Strains

Plasmid pYPS152 (SEQ ID NO:97) was digested with *Fsp*I and then used for transformation of the *Ura*$^-$, β-carotene-producing *Yarrowia* strains YCS8734U1, YCS8734U2, YCS8734U3, YCS8734U4, YCS8740U1 and YCS8747U2 (*supra*), according to the methodology of Example 1.

The transformant cells were plated onto MM agar plates and maintained at 30 °C for 3 to 4 days. The resulting orange colonies were re-streaked and the new strains were given designations as a part of the YCS9100 series, according to Table 34 below.

Table 34

Parentage Of Canthaxanthin-Producing Strains (YCS9100 Series)

| Parent Host | Number Of Colonies Following pYPS152 Transformation | New Strain Designations |
|---|---|---|
| YCS8734U1 | 26 | YCS9101 - YCS9126 |
| YCS8734U2 | 22 | YCS9127 - YCS9148 |
| YCS8734U3 | 9 | YCS9149 - YCS9158 |
| YCS8734U4 | 12 | YCS9159 - YCS9171 |

To determine the ability of the YCS9100 series of strains to convert β-carotene into canthaxanthin due to the expression of the β-carotene ketolase (i.e., *crtW*) genes, 20 mL cultures were grow for 48 hrs in 125 mL capacity shake flasks with aeration. The cultures were grown to similar ODs (approximate $OD_{600}$ was 20-25). Carotenoids were extracted as described in Example 10.

Table 35 shows the percentages of lycopene, β-carotene, echinone and canthaxanthin produced within individual strains of the YCS9100 series. Strain YCS8734 (row shaded in gray) is the control strain, which did not contain the β-carotene ketolase (i.e., *crtW*) and thus was unable to make canthaxanthin. The amount of canthaxanthin made by the strains is also provided, as determined by the "area of the canthaxanthin peaks".



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Table 35

% Canthaxanthin Produced By The YCS9100 Series Strains

| Strain | Lycopene (% of total carotenoid) | β-carotene (% of total carotenoid) | Echinone (% of total carotenoid) | Canthaxanthin (% of total carotenoid) | Canthaxanthin + Echinone (% of total carotenoid) | Area of Canthaxanthin Peaks |
|---|---|---|---|---|---|---|
| YCS8734 | 14 | 63 | --- | --- | --- | --- |
| YCS9101 | 23 | 16 | 13 | 34 | 47 | 1810 |
| YCS9102 | 22 | 40 | 23 | 2 | 26 | 140 |
| YCS9103 | 20 | 31 | 25 | 10 | 35 | 580 |
| YCS9104 | 19 | 32 | 26 | 9 | 36 | 510 |
| YCS9105 | 20 | 28 | 29 | 7 | 36 | 400 |
| YCS9106 | 25 | 54 | --- | --- | --- | --- |
| YCS9107 | 27 | 9 | 11 | 39 | 50 | 2300 |
| YCS9108 | 19 | 39 | 24 | --- | 24 | --- |
| YCS9109 | 30 | 49 | --- | --- | --- | --- |
| YCS9110 | 26 | 56 | --- | --- | --- | --- |
| YCS9111 | 29 | 54 | --- | --- | --- | --- |
| YCS9112 | 29 | 10 | 11 | 35 | 45 | 2270 |
| YCS9113 | 26 | 10 | 11 | 38 | 49 | 2190 |
| YCS9114 | 20 | 40 | 24 | 2 | 26 | 140 |
| YCS9115 | 28 | 55 | --- | --- | --- | --- |
| YCS9116 | 20 | 26 | 30 | 7 | 36 | 400 |
| YCS9117 | 19 | 30 | 26 | 9 | 34 | 530 |
| YCS9118 | 27 | 55 | --- | --- | --- | --- |
| YCS9119 | 30 | 48 | --- | --- | --- | --- |
| YCS9120 | 36 | 46 | --- | --- | --- | --- |



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| | | | | | | |
|---|---|---|---|---|---|---|
| YCS9121 | 36 | 8 | 9 | 30 | 39 | 1820 |
| YCS9122 | 35 | 51 | --- | --- | --- | --- |
| YCS9123 | 31 | 9 | 10 | 34 | 44 | 2070 |
| YCS9124 | 33 | 52 | --- | --- | --- | --- |
| YCS9125 | 31 | 8 | 13 | 34 | 48 | 1970 |
| YCS9126 | 17 | 39 | 25 | 4 | 29 | 210 |
| YCS9127 | 34 | 7 | 9 | 35 | 44 | 2030 |
| YCS9128 | 36 | 7 | 8 | 34 | 42 | 1950 |
| YCS9129 | 79 | --- | --- | --- | --- | --- |
| YCS9130 | 32 | 21 | 26 | 6 | 32 | 380 |
| YCS9131 | 41 | 5 | 7 | 32 | 39 | 2370 |
| YCS9132 | 39 | 6 | 7 | 31 | 39 | 2040 |
| YCS9133 | 34 | 7 | 8 | 34 | 43 | 2000 |
| YCS9134 | 28 | 21 | 24 | 12 | 36 | 680 |
| YCS9135 | 35 | 7 | 8 | 36 | 44 | 2090 |
| YCS9136 | 32 | 7 | 9 | 37 | 46 | 2170 |
| YCS9137 | 33 | 7 | 9 | 35 | 44 | 4280 |
| YCS9138 | 37 | 49 | --- | --- | --- | --- |
| YCS9139 | 29 | 7 | 13 | 37 | 50 | 1890 |
| YCS9140 | 32 | 7 | 9 | 36 | 45 | 2100 |
| YCS9141 | 23 | 61 | --- | --- | --- | --- |
| YCS9142 | 33 | 52 | --- | --- | --- | --- |
| YCS9143 | 33 | 7 | 9 | 34 | 43 | 1630 |
| YCS9144 | 39 | 51 | --- | --- | --- | --- |
| YCS9145 | 34 | 54 | --- | --- | --- | --- |
| YCS 9146 | 46 | 42 | --- | --- | --- | --- |
| YCS9147 | 43 | 44 | --- | --- | --- | --- |

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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| YCS9148 | 19 | 42 | 23 | 4 | 27 | 240 |
| YCS9149 | 17 | 44 | 24 | 4 | 28 | 260 |
| YCS9150 | 24 | 10 | 19 | 32 | 51 | 2050 |
| YCS9151 | 24 | 9 | 17 | 35 | 52 | 2280 |
| YCS9152 | 26 | 7 | 15 | 34 | 49 | 2100 |
| YCS9153 | 17 | 43 | 21 | 4 | 25 | 270 |
| YCS9154 | 29 | 25 | 21 | 13 | 34 | 500 |
| YCS9155 | 29 | 8 | 16 | 33 | 49 | 1850 |
| YCS9156 | 26 | 59 | --- | --- | --- | --- |
| YCS9157 | 14 | 50 | 23 | 3 | 27 | 198 |
| YCS9158 | 23 | 7 | 12 | 43 | 55 | 2664 |
| YCS9159 | 30 | 30 | 23 | 7 | 30 | 363 |
| YCS9160 | 17 | 37 | 27 | 5 | 32 | 352 |
| YCS9161 | 26 | 7 | 12 | 40 | 52 | 2837 |
| YCS9162 | 18 | 8 | 20 | 39 | 59 | 2649 |
| YCS9163 | 20 | 8 | 19 | 40 | 59 | 2950 |
| YCS9164 | 19 | 7 | 16 | 43 | 59 | 3004 |
| YCS9165 | 22 | 5 | 11 | 46 | 56 | 3436 |
| YCS9166 | 18 | 31 | 29 | 11 | 40 | 701 |
| YCS9167 | 21 | 8 | 10 | 44 | 54 | 2665 |
| YCS9168 | 25 | 62 | --- | --- | --- | --- |
| YCS9169 | 22 | 9 | 10 | 43 | 53 | 2414 |
| YCS9170 | 27 | 56 | --- | --- | --- | --- |
| YCS9171 | 19 | 24 | 25 | 14 | 39 | 659 |



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EXAMPLE 13

Synthesis Of *Yarrowia lipolytica* Strain Series YCS9700, YCS9800 and YCS9900, Producing Astaxantin And Omega-3 PUFAs

The present Example describes the construction of the *Yarrowia lipolytica* YCS9700, YSC9800 and YCS9900 strain series, co-producing astaxanthin and ω-3 PUFAs (Figure 18).

As described herein, construction of the *Y. lipolytica* YCS9700, YSC9800 and YCS9900 series required: (1) transformation of *Yarrowia lipolytica* strains YCS9158, YCS9165 and YCS9169 (producing canthaxantin) with plasmid pYPS162; and, (2) extraction and analysis of the carotenoids produced.

Generation And Analysis Of Astaxanthin-Producing Strains

Plasmid pYPS162 (SEQ ID NO:96) was digested with *Fsp*I and then used for transformation of the canthaxanthin-producing *Yarrowia* strains YCS9158, YCS9165 and YCS9169 (Example 12), according to the methodology of Example 1.

The transformant cells were plated onto MM+SU agar plates and maintained at 30 °C for 3 to 4 days. The resulting red colonies were re-streaked and the new strains were given designations as part of the YCS9700, YSC9800 or YCS9900 series, according to Table 36 below.

Table 36

Parentage Of Astaxanthin-Producing Strains (YCS9700, YCS9800 And YCS9900 Series)

| Parent Host | Number Of Colonies Following pYPS162 Transformation | New Strain Designations |
|---|---|---|
| YCS9158 | 15 | YCS9701 - YCS9715 |
| YCS9165 | 15 | YCS9801 - YCS9815 |
| YCS9169 | 15 | YCS9901 - YCS9915 |

To determine the ability of the strains to convert canthaxanthin into astaxanthin due to the expression of the β-carotene hydroxylase (i.e., *crtZ*) genes, 20 mL cultures were grown for 48 hrs with aeration in 125 mL



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shake flasks. The cultures had similar ODs at end of the incubation. Carotenoids were extracted as described in Example 10.

The percentage of lycopene, β-carotene, echinone, canthaxanthin and astaxanthin (relative to the total carotenoids) within individual strains of the YCS9700, YSC9800 and YCS9900 series are shown in Table 37. Strains YCS9158, YCS9165 and YCS9169 (rows shaded in gray) are the control strains, which did not contain the β-carotene hydroxylase (i.e., *crtZ*) and thus were unable to make astaxanthin or adonixanthin. The strains having the highest levels of astaxanthin are highlighted in bold font. As expected, the astaxanthin-producing strains showed a decrease in the percentage of lycopene plus β-carotene that was accumulated.



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Table 37

% Astaxanthin And Adonixanthin Produced By The YCS9700, YSC9800 and YCS9900 Series Strains

| Strain | Lycopene + β-carotene (%) | Canthaxanthin (%) | Adonirubin (%) | Astaxanthin (%) | Adonixanthin (%) | Carotenoids past β-carotene (%) |
|---|---|---|---|---|---|---|
| YCS9158 | 54 | 36 | --- | --- | --- | 36 |
| YCS9701 | 63 | 1 | 1 | 13 | 14 | 28 |
| YCS9702 | 65 | 13 | --- | 8 | 5 | 26 |
| **YCS9703** | **47** | **14** | **6** | **25** | **1** | **46** |
| YCS9704 | 77 | --- | --- | 10 | 4 | 14 |
| YCS9705 | 67 | 1 | <1 | 13 | 12 | 26 |
| YCS9706 | 61 | 34 | --- | --- | --- | 34 |
| YCS9707 | 63 | 8 | --- | 9 | 11 | 29 |
| YCS9708 | 61 | 6 | 2 | 22 | 1 | 32 |
| YCS9709 | 66 | 1 | --- | 12 | 13 | 25 |
| YCS9710 | 65 | 1 | 1 | 13 | 12 | 26 |
| YCS9711 | 60 | 35 | <1 | --- | --- | 35 |
| YCS9712 | 55 | 35 | --- | --- | --- | 35 |
| YCS9713 | 59 | 36 | --- | --- | --- | 36 |
| YCS9714 | 59 | 34 | --- | --- | --- | 34 |
| YCS9715 | 59 | 34 | --- | --- | --- | 36 |
| YCS9165 | 54 | 37 | --- | --- | --- | 37 |
| YCS9801 | 58 | 37 | --- | --- | --- | 36 |
| YCS9802 | 63 | 29 | --- | --- | --- | 29 |
| YCS9803 | 64 | 29 | --- | --- | --- | 29 |
| YCS9804 | 64 | 29 | --- | --- | --- | 29 |



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| | | | | | | |
|---|---|---|---|---|---|---|
| YCS9805 | 64 | 28 | --- | --- | --- | 28 |
| YCS9806 | 57 | 1 | 1 | 14 | 18 | 34 |
| YCS9807 | 61 | 5 | 2 | 23 | 2 | 32 |
| YCS9808 | 59 | 3 | 1 | 19 | 9 | 32 |
| YCS9809 | 59 | 2 | 1 | 18 | 11 | 32 |
| YCS9810 | 61 | 30 | --- | --- | --- | 30 |
| YCS9811 | 58 | 1 | <1 | 15 | 16 | 33 |
| **YCS9812** | **46** | **13** | **5** | **27** | **1** | **45** |
| YCS9813 | 61 | 30 | --- | --- | --- | 30 |
| YCS9814 | 57 | 1 | 1 | 15 | 17 | 34 |
| YCS9815 | 58 | 33 | --- | --- | --- | 33 |
| YCS9169 | 56 | 35 | --- | --- | --- | 35 |
| YCS9901 | 49 | 42 | --- | --- | --- | 42 |
| YCS9902 | 52 | 40 | --- | --- | --- | 40 |
| YCS9903 | 54 | 38 | --- | --- | --- | 38 |
| YCS9904 | 66 | 1 | 8 | 16 | --- | 24 |
| YCS9905 | 55 | 15 | 6 | 15 | --- | 36 |
| **YCS9906** | **38** | **14** | **6** | **30** | **4** | **54** |
| YCS9907 | 57 | 35 | --- | --- | --- | 35 |
| YCS9908 | 51 | 11 | 5 | 23 | 3 | 42 |
| YCS9909 | 67 | 1 | --- | 8 | 16 | 24 |
| YCS9910 | 57 | 10 | 1 | 15 | 8 | 34 |
| YCS9911 | 70 | 1 | 8 | 11 | --- | 20 |
| YCS9912 | 56 | 35 | --- | --- | --- | 35 |
| YCS9913 | 55 | 37 | --- | --- | --- | 37 |
| YCS9914 | 52 | 39 | --- | <1 | --- | 40 |
| YCS9915 | 60 | 10 | --- | 9 | 11 | 31 |



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EXAMPLE 14

Quantification Of Carotenoids And PUFAs In *Yarrowia lipolytica* Strains Of The YCS8600, YCS8700 And YCS9100 Series

Although carotenoids were previously quantified upon isolation of strain YCS8601 (of the YCS8600 strain series; Example 8), strains YCS8734, YCS8740 and YCS8747 (of the YCS8700 strain series; Example 10) and strain YCS9165 and YCS9169 (of the YCS9100 strain series; Example 12), based on the % lycopene, β–carotene, or canthaxanthin produced (with respect to the total carotenoids), more qualitative quantification of carotenoid production had not been performed. Nor had the ω-3 and/or ω-6 PUFA content of the strains been measured, although it was assumed that PUFAs were being produced based on the PUFA biosynthetic pathway that had been engineered in the parent strain of the lineage, Y4184 (31% EPA; Example 7).

In the present Example, each of the aforementioned strains was subjected to analysis, using methodology previously described in the General Methods and Example 2, respectively, to enable quantification of: 1.) the total fatty acid methyl esters (FAME), measured as a percent of the dcw; and, 2.) the lycopene/β–carotene/canthaxanthin concentration, measured in parts per million (ppm). The following exceptions were made to the Example 2 protocol for determination of carotenoid titers in the selected *Yarrowia lipolytica* strains listed in Table 38. Specifically, the size of the glass beads used were 0.5 mm; and, prior to loading the samples onto the HPLC, the samples were dissolved completely into 135 μL chloroform followed by the addition of 2855 μL n-hexane (HPLC-grade) for a final sample volume of 3 mL. Data are summarized below.



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Table 38

Quantification Of PUFAs And Carotenoid Titer In Select *Yarrowia lipolytica* Strains

| Strain | FAME as % DCW | Lycopene (ppm) | Lycopene/ β-carotene (ppm) | Canthaxanthin (ppm) | Total carotenoid (ppm) |
|---|---|---|---|---|---|
| Y4184 | 15.3% | -- | -- | -- | 0 |
| YCS8601 | 6.4% | 1088 | -- | -- | 1088 |
| YCS8734 | 10.1% | -- | 528 | -- | 528 |
| YCS8740 | 10.1% | -- | 521 | -- | 521 |
| YCS8747 | 10.0% | -- | 608 | -- | 608 |
| YCS9165 | 7.0% | -- | -- | 293 | 479 |
| YCS9169 | 9.8% | -- | -- | 340 | 494 |

## EXAMPLE 15

### Coenzyme Q Pathway And Profile In Wildtype *Yarrowia lipolytica*

The present Example describes the quantification of $CoQ_9$ in wiildtype *Yarrowia lipolytica*. Specifically, determination of the amount of $CoQ_9$ in *Yarrowia lipolytica* strain ATCC #20362 was made using an Agilent series 1100 HPLC equipped with a DAD detector. The sample was generated by extracting the oil in 3:1 hexane:methanol overnight, partitioning the solvents using a 1 N NaCl solution and stripping the hexane with a Büchi® Rotavapor® (New Castle, DE).

Three samples of approximately 15, 38, and 86 mg were added to a 4 mL amber vial. Then, 100 μL of glass beads and 1.0 mL of a previously sparged 1:1 tetrahydrofuran(THF)/methanol (MeOH) solution were added and shaken for 15 min at 200 rpm. The sample was then transferred to a Whatman uni-prep vial and analyzed.

A 1 mg standard sample of $CoQ_9$ received from Fluka Chemical Corp. (Milwaukee, WI; Catalog #27597, lot #378472) was diluted to 0.1 mg using 1:1 THF/MeOH and analyzed. The standard received was assumed to be exactly 1 mg.

The HPLC conditions used were as follows: Zorbax SB-C18 4.6 x 250 mm column, 5 micron; 1.0 mL/min flow rate; 35 °C column temperature; 5 μL injection volume; UV detection at 270 and 450 nm wavelengths.



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Table 39

HPLC Solvents/Gradients For $CoQ_9$ Extraction

| Time (min) | Solvent A: Acetonitrile | Solvent B: Isopropanol |
|---|---|---|
| 0.0 | 95% | 5% |
| 30.0 | 0% | 100% |
| 35.0 | 0% | 100% |
| 36.0 | 95% | 5% |
| 45.0 | 95% | 5% |

Calculations were performed as follows. Specifically, the standard reference factor was equivalent to the milligrams (mg) of standard/area. The percent (%) sample amount was determined according to the following formula: standard reference factor * (sample peak area/ sample concentration) * 100. The ratio of the sample against the standard was calculated and the sample weight taken into account. The sample used was 26.64 mg and diluted with 1 mL of MeOH/THF that had been sparged of air. This was run against the existing standard of 0.1 mg/mL of $CoQ_9$. The area counts for the standard were 18.9 at 450 nm and 711 at 270 nm, while for the sample, the area counts were 13 at 450 nm and 569 at 270 nm. Based on these results, the $CoQ_9$ calculated in the oil was in the range of 0.2 to 0.3%.



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## CLAIMS

What is claimed is:

1. A recombinant oleaginous yeast production host for the production of astaxanthin comprising:

a.) at least one copy of a *crtE* gene encoding a geranyl geranyl pyrophosphate synthase;

b.) at least one copy of a *crtB* gene encoding a phytoene synthase;

c.) at least one copy of a *crtI* gene encoding a phytoene desaturase;

d.) at least one copy of a *crtY* gene encoding a lycopene cyclase;

e.) at least one copy of a *crtZ* gene encoding a carotenoid hydroxylase; and,

f.) at least one copy of a *crtW* gene encoding a carotenoid ketolase;

wherein said oleaginous yeast produces at least about 25 weight percent of its dry cell weight as oil.

2. The recombinant production host of claim 1 wherein the yeast is a recombinant strain of *Yarrowia lipolytica*.

3. The recombinant production host of claim 1 wherein the host cell accumulates a carotenoid compound selected from the group consisting of: astaxanthin, β-carotene, lycopene, zeaxanthin, lutein and canthaxanthin.

4. The recombinant production host of claim 1 where said oil comprises at least one polyunsaturated fatty acid selected from the group consisting of an $\omega$-6 polyunsaturated fatty acid and an $\omega$-3 polyunsaturated fatty acid.

5. The recombinant production host of claim 4 wherein the at least one polyunsaturated fatty acid is selected from the group consisting of: arachidonic acid, eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid.



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6. The recombinant production of host of claim 1 comprising all of the following:

a.) at least one gene encoding a resveratrol synthase;

b.) at least one gene encoding a coumaroyl-CoA ligase; and,

c.) at least one gene encoding a polypeptide having both phenylalanine ammonia lyase activity and tyrosine ammonia lyase activity.

7. A method to produce a pigmented oleaginous yeast biomass comprising a carotenoid compound, comprising:

a.) providing the recombinant production host of claim 1 wherein said production host accumulates at least 25 wt% of its dry cell weight as oil; and,

b.) culturing the recombinant production host under suitable conditions whereby pigmented oleaginous yeast biomass comprising a carotenoid compound is produced.

8. The method of claim 7 wherein the oil comprises an $\omega$-3 or an $\omega$-6 polyunsaturated fatty acid selected from the group consisting of: arachidonic acid, eicosapentaenoic acid, docosapentaenoic acid, and docosahexaenoic acid.

9. A pigmented oleaginous yeast biomass comprising a recombinant oleaginous yeast which accumulates at least 25 weight percent of its dry cell weight as oil and produces a carotenoid compound selected from the group consisting of: astaxanthin, β-carotene, lycopene, lutein, zeaxanthin and canthaxanthin.

10. A pigmented oil comprising:

a.) an $\omega$-6 polyunsaturated fatty acid or an $\omega$-3 polyunsaturated fatty acid; and,

b.) a carotenoid compound selected from the group consisting of: astaxanthin, β-carotene, lycopene, zeaxanthin, lutein and canthaxanthin.

11. A pigmentation product comprising:

a.) the pigmented oleaginous yeast biomass of claim 9;

b.) the pigmented oil of claim 10; or,



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c.) mixtures thereof.

12. An animal feed comprising the pigmentation product of claim 11.

13. A food product comprising the pigmentation product of claim 11.

14. A recombinant oleaginous yeast cell having at least about 25 weight percent of its dry cell weight as oil and which accumulates a carotenoid compound selected from the group consisting of: astaxanthin, β-carotene, lycopene, lutein, zeaxanthin and canthaxanthin.

15. The recombinant oleaginous yeast cell of claim 14 wherein the oil comprises an $\omega$-3 or an $\omega$-6 polyunsaturated fatty acid.

16. The recombinant oleaginous yeast cell of claim 15 further comprising an antioxidant selected from the group consisting of: coenzyme Q6, coenzyme Q7, coenzyme Q8, coenzyme Q9, coenzyme Q10, resveratrol and mixtures thereof.

17. The animal feed of claim 16 wherein said animal feed is selected from the group consisting of: fish feed, crustacean feed, shrimp feed, poultry feed and pet feed.

18. A method of pigmenting an animal comprising the steps of:

a.) providing a pigmented animal feed comprising an effective amount of astaxanthin and an effective amount of a microbially-produced oil; and,

b.) feeding an animal the pigmented animal feed of step (a) whereby at least one tissue in said animal or a product produced by said animal, is pigmented.



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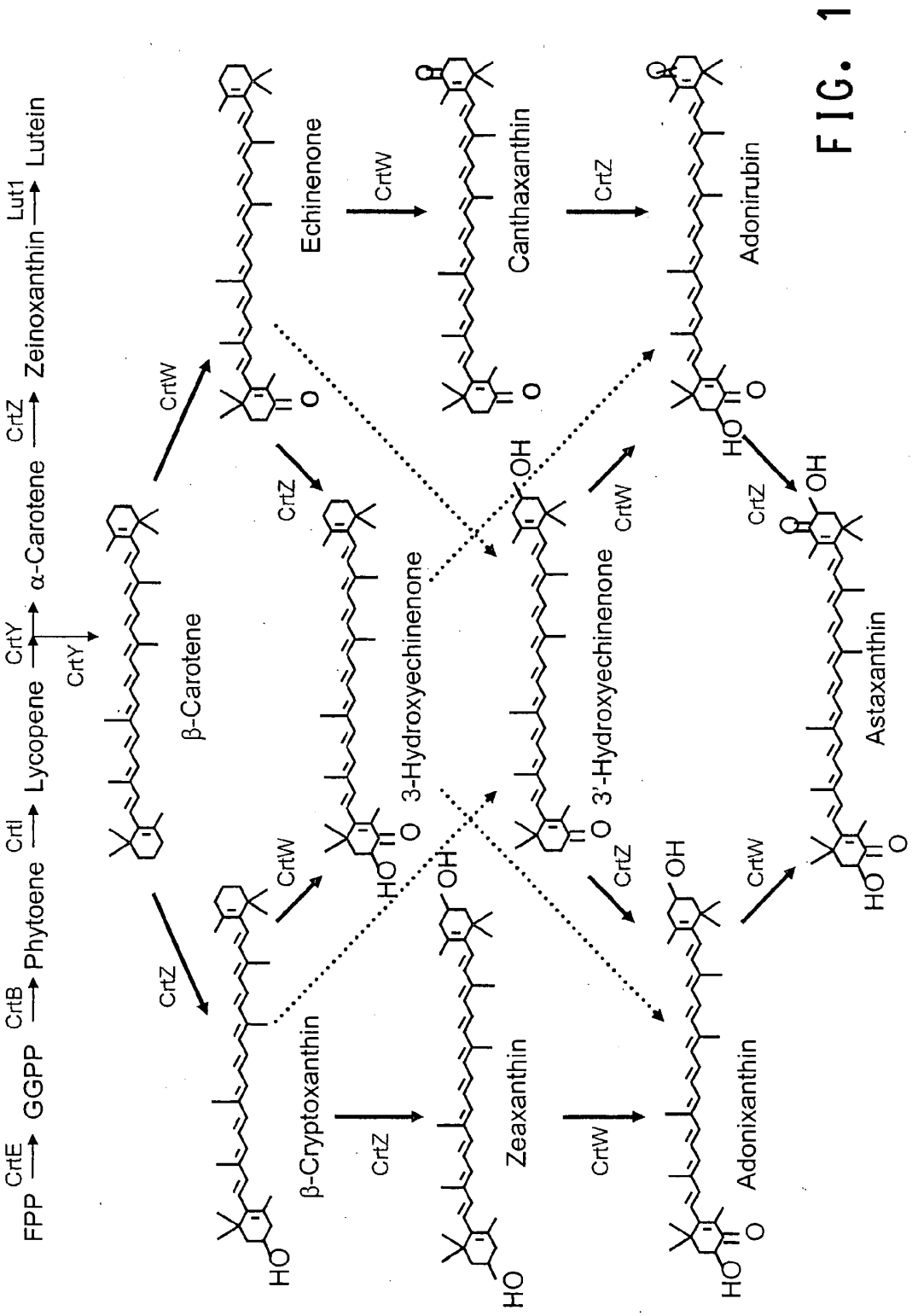



FIG. 1



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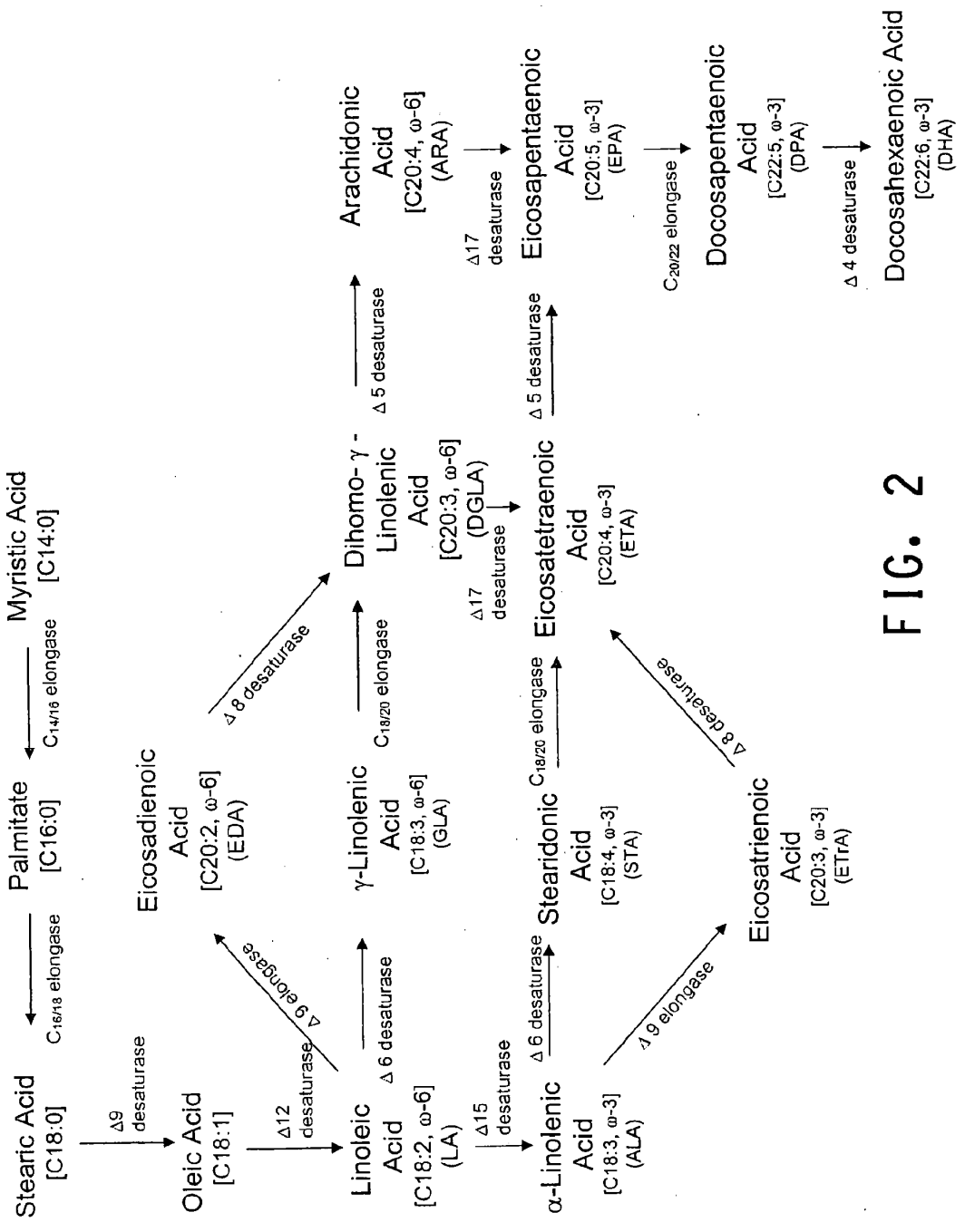
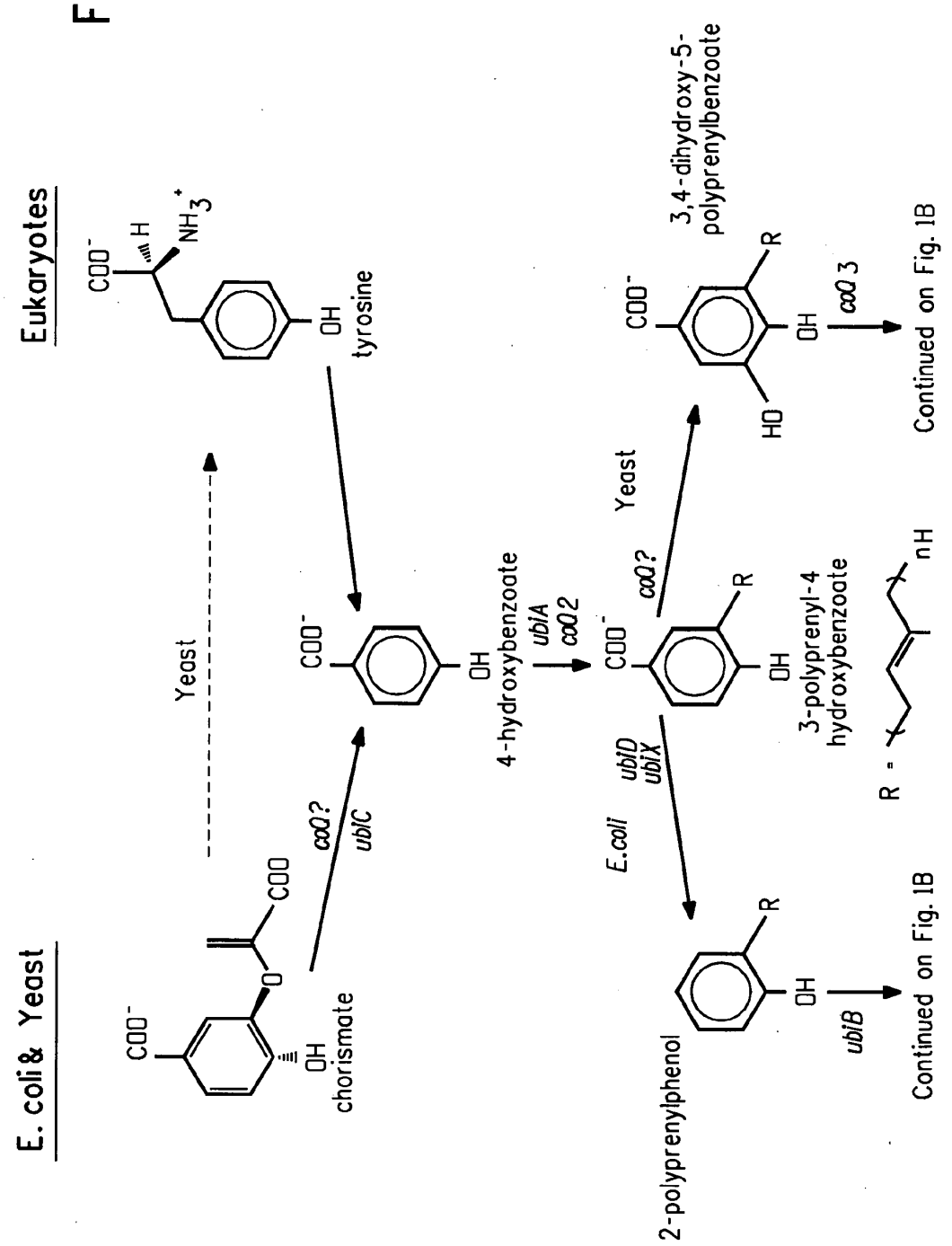



FIG. 2



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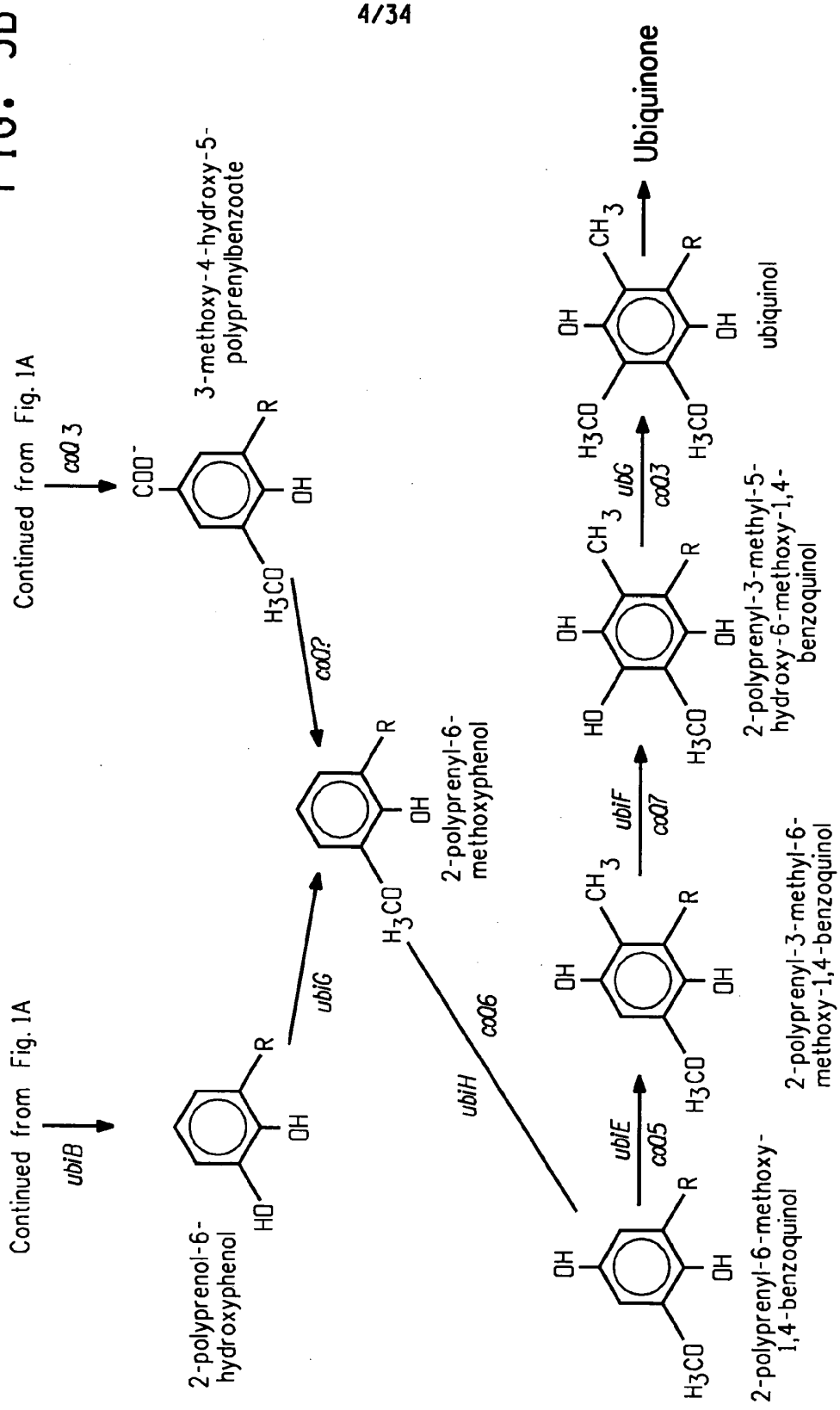
FIG. 3A






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FIG. 3B






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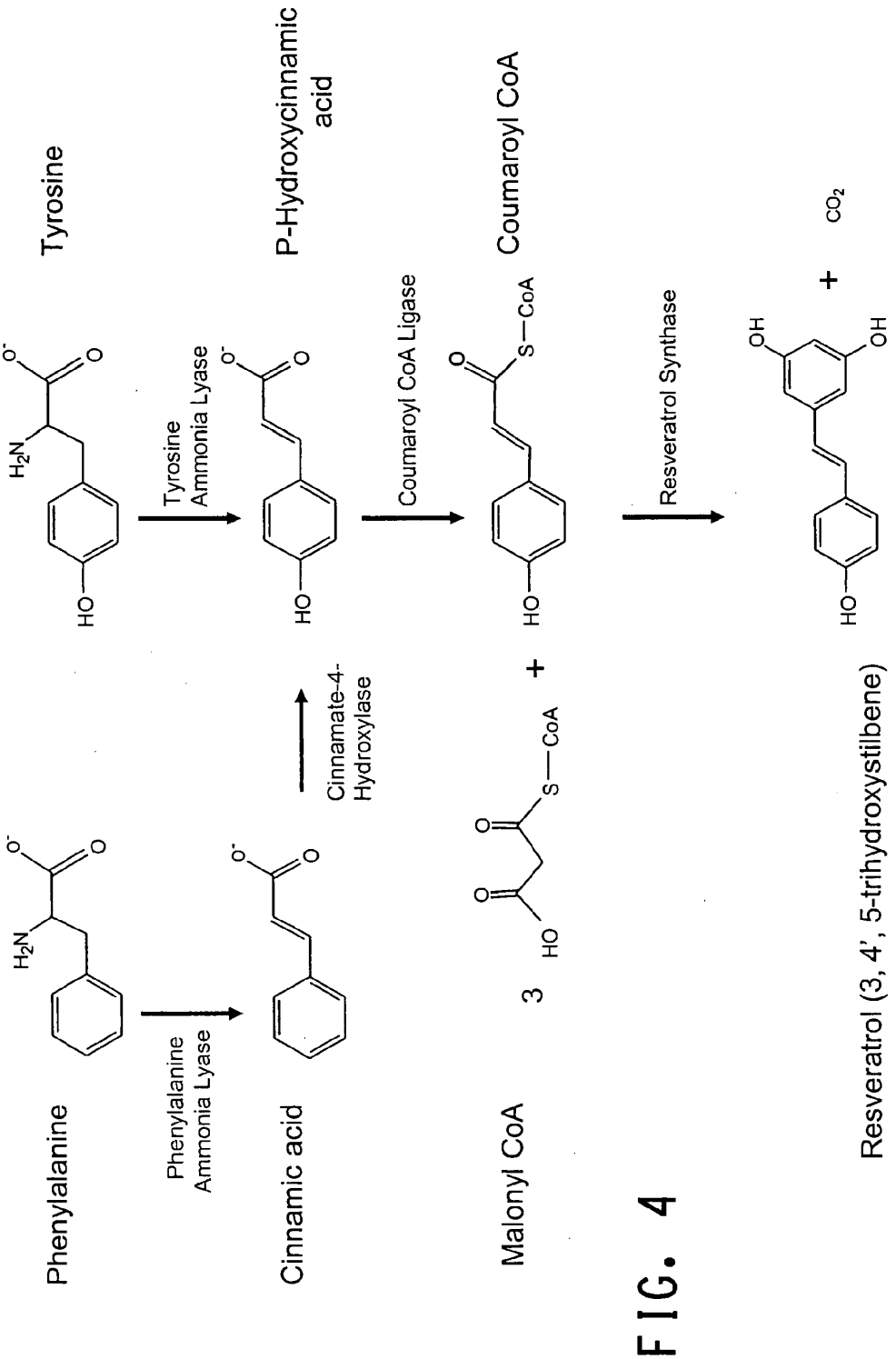



FIG. 4



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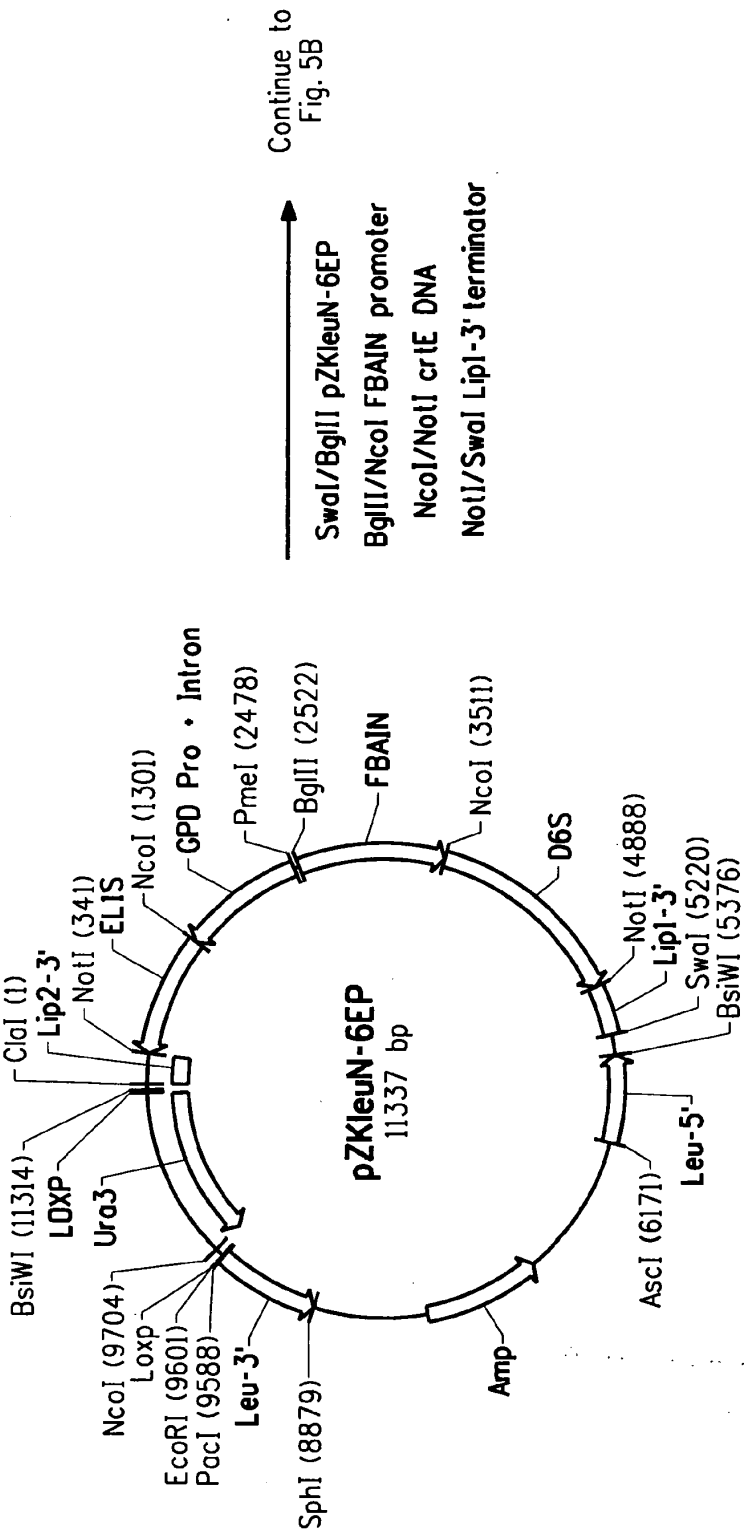



FIG. 5A



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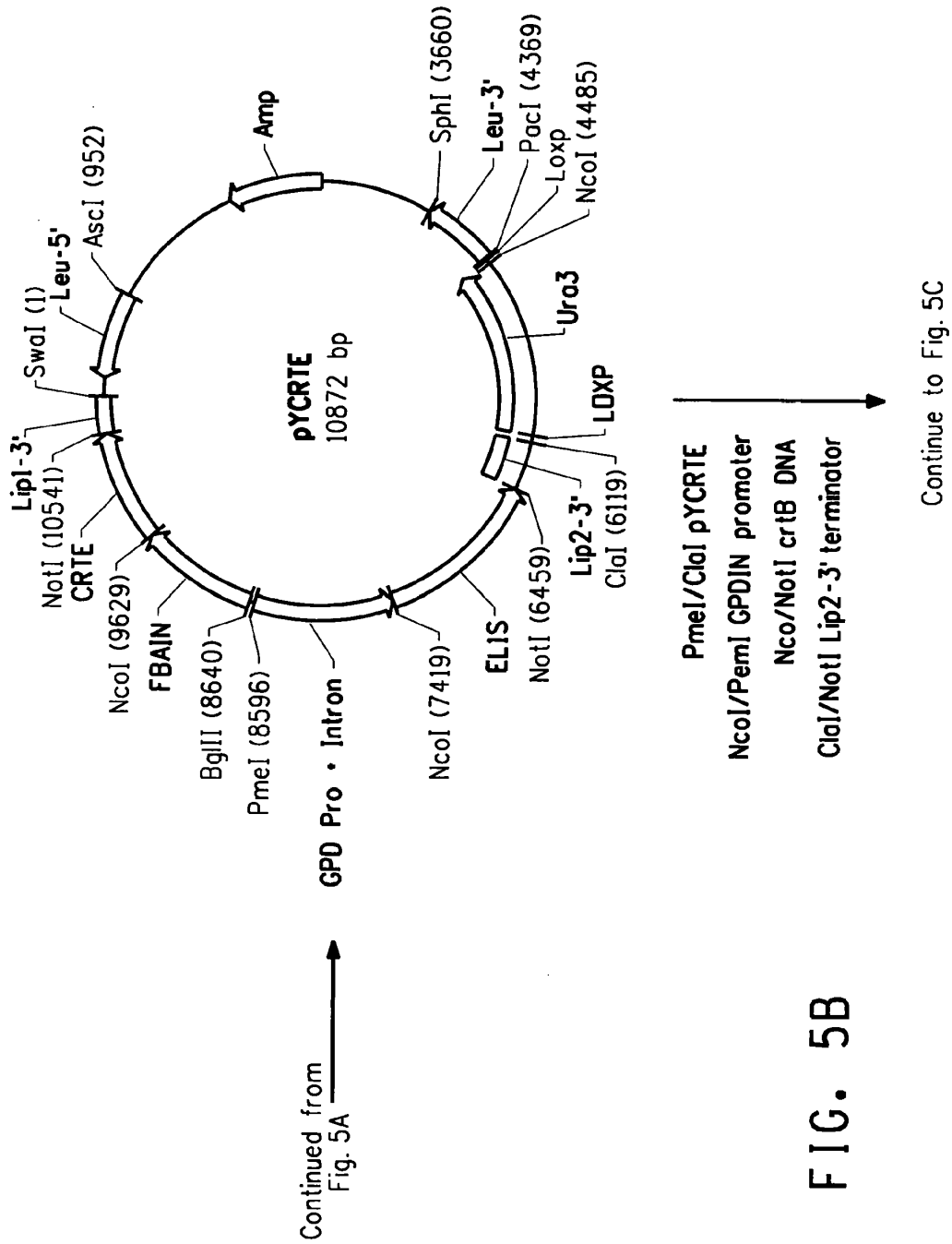



FIG. 5B



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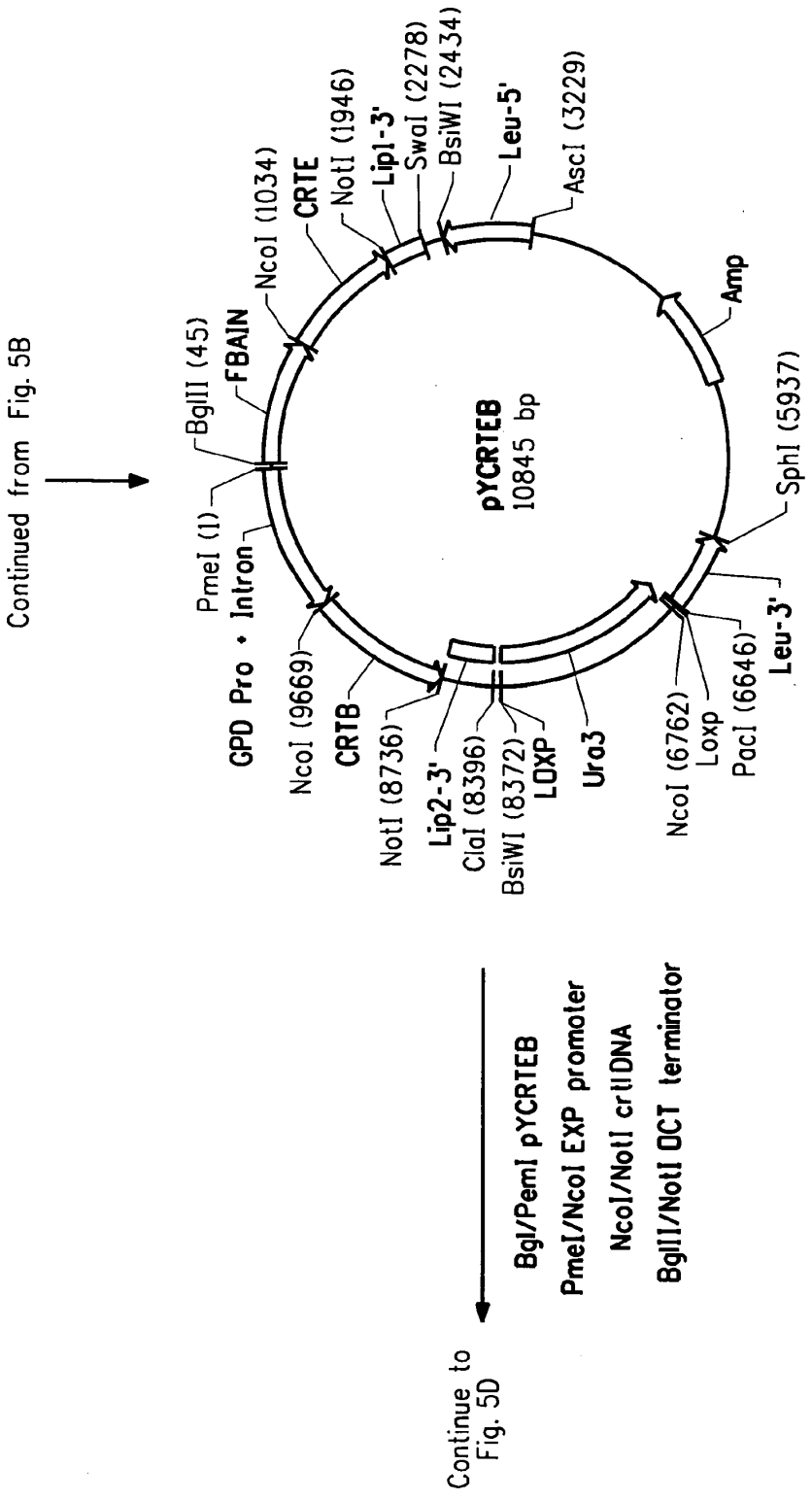



FIG. 5C



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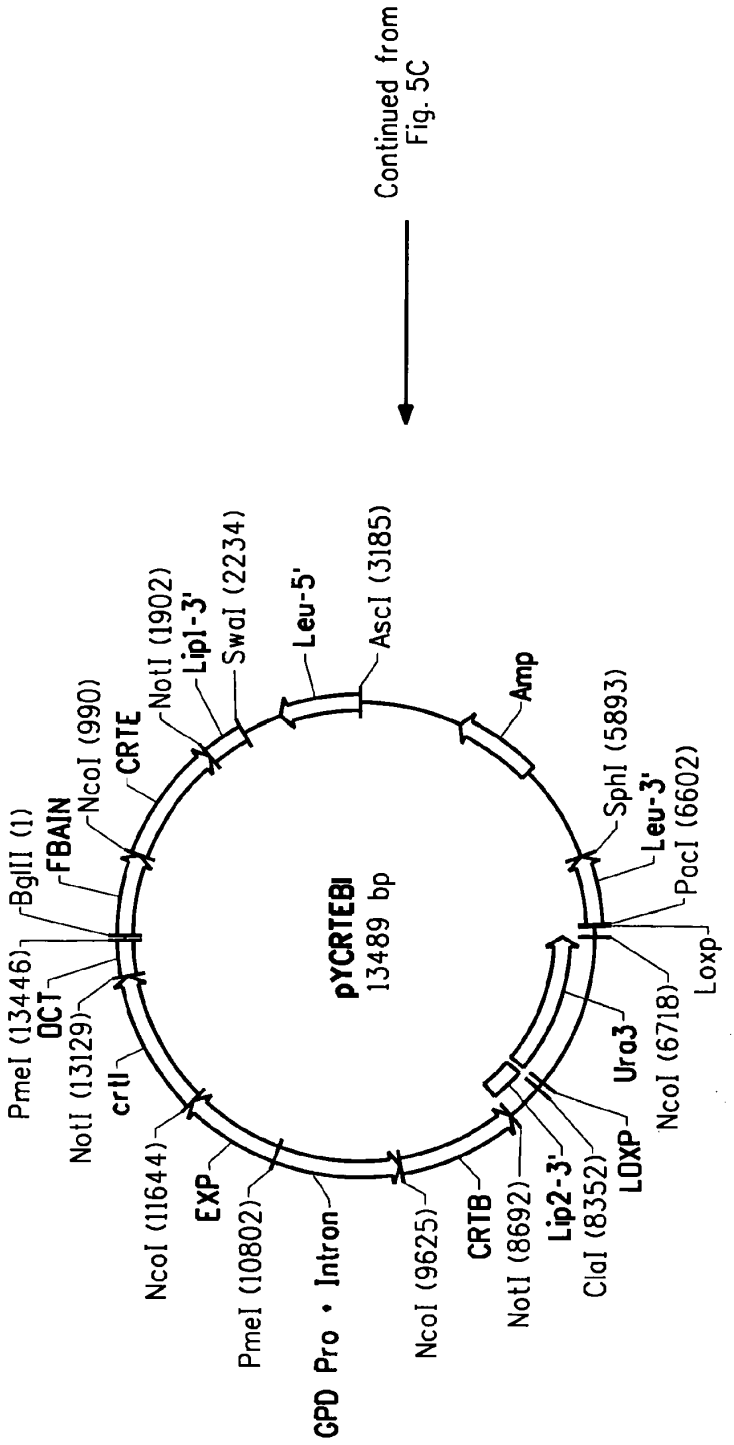



FIG. 5D



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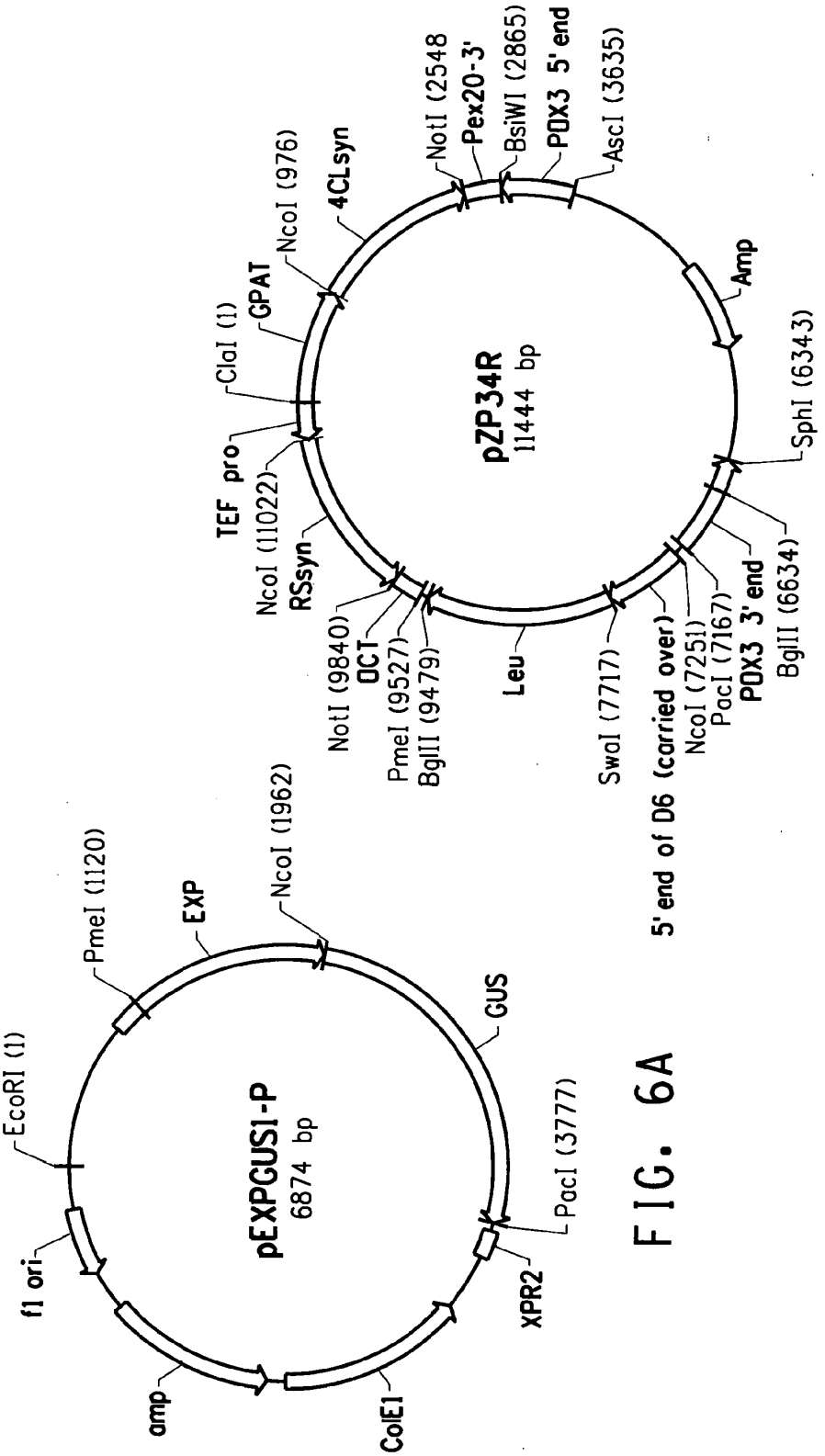



FIG. 6A




FIG. 6B



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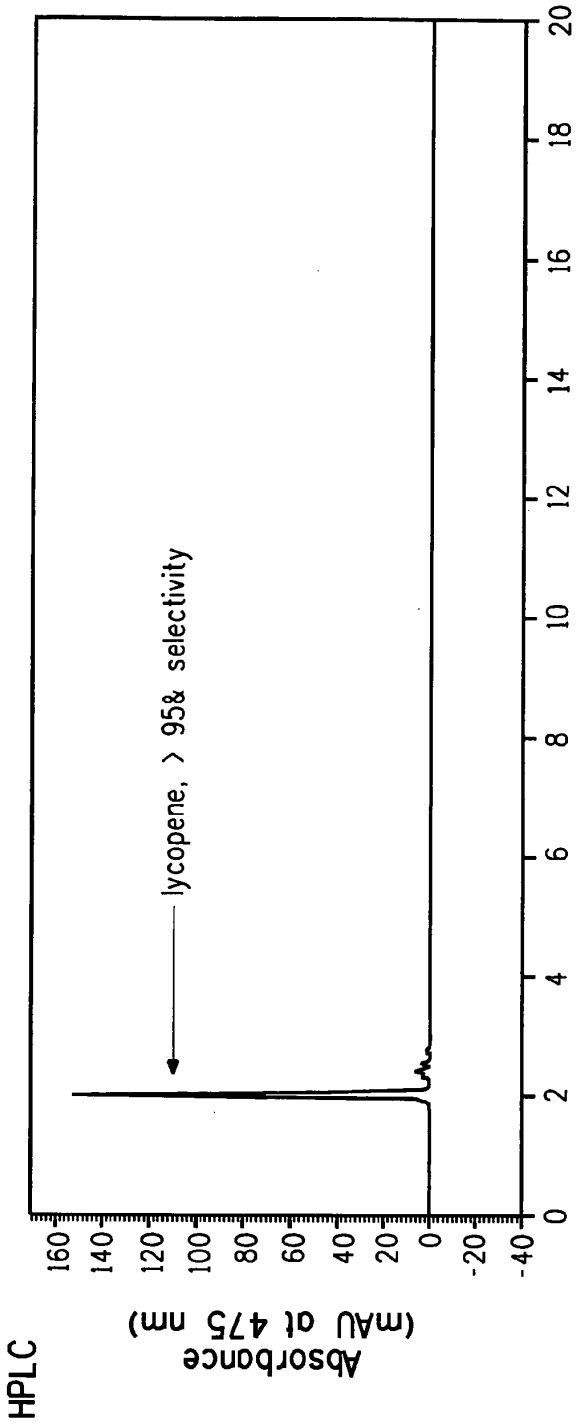



FIG. 7A



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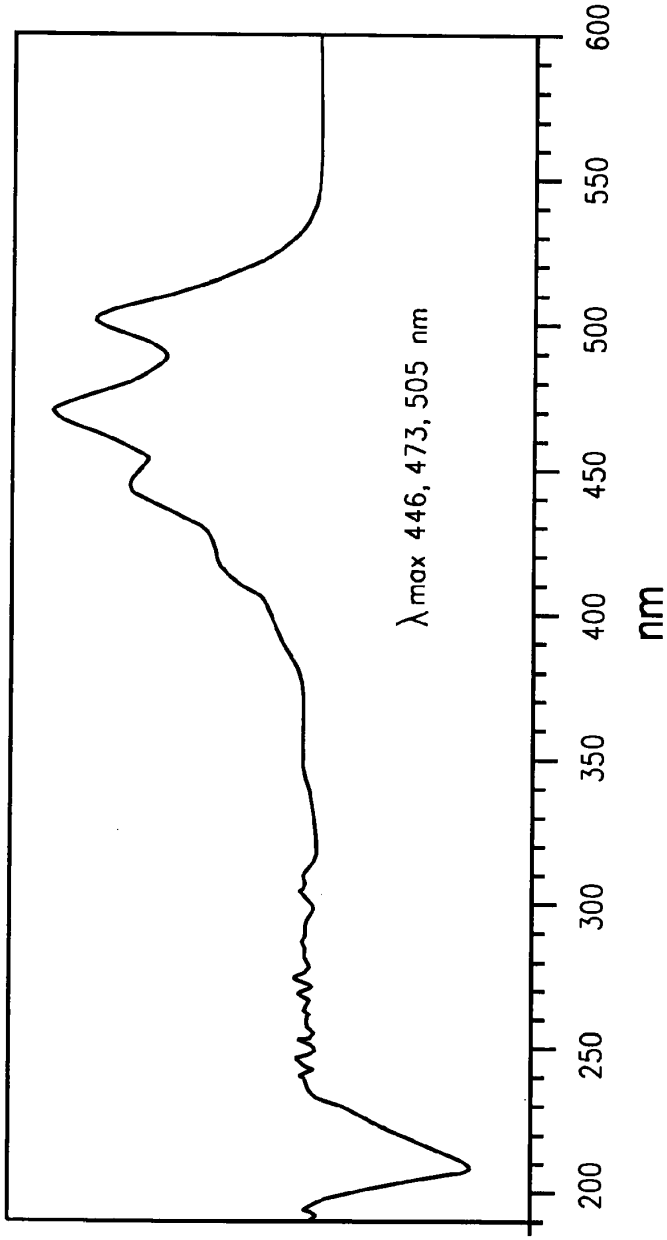



FIG. 7B



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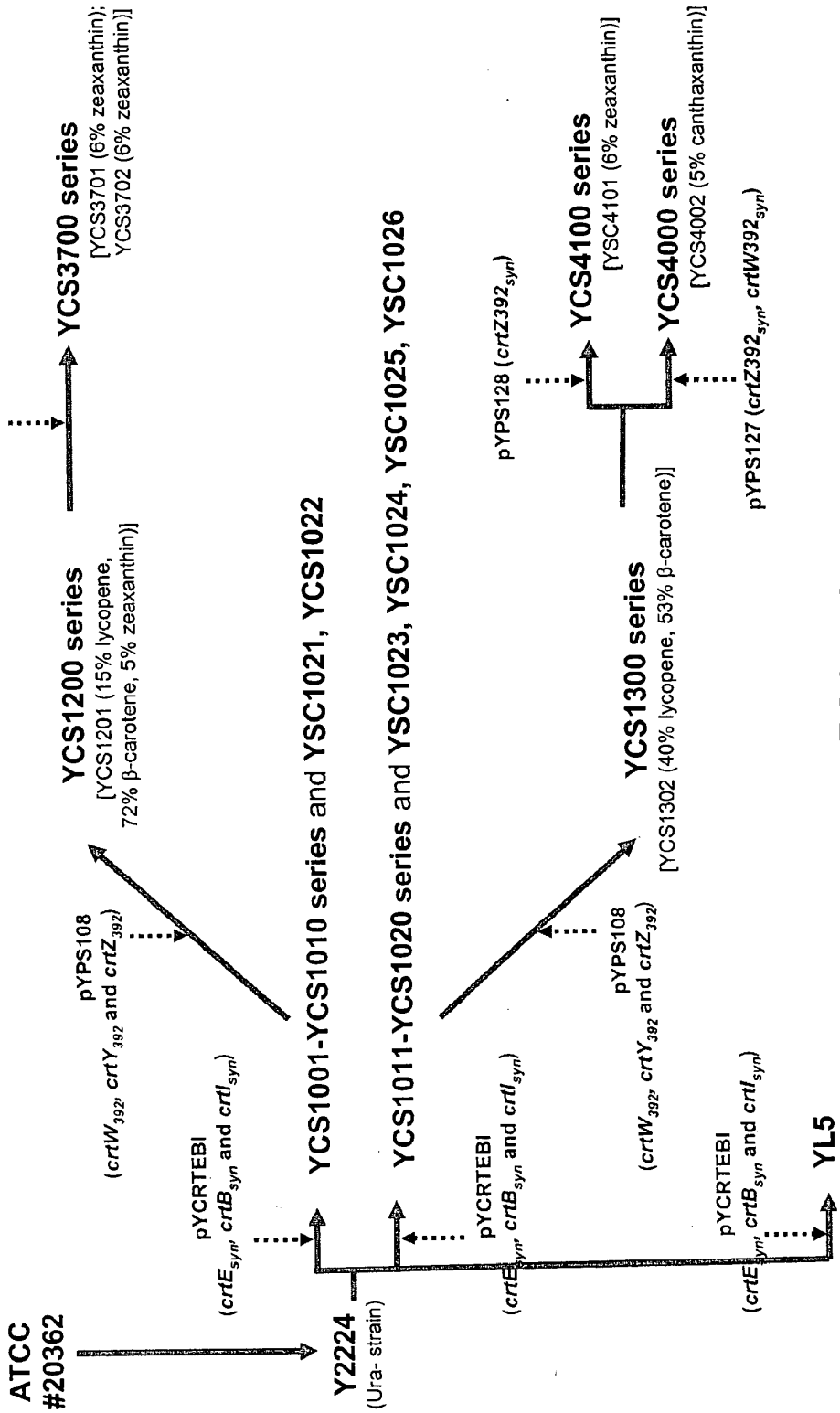



FIG. 8



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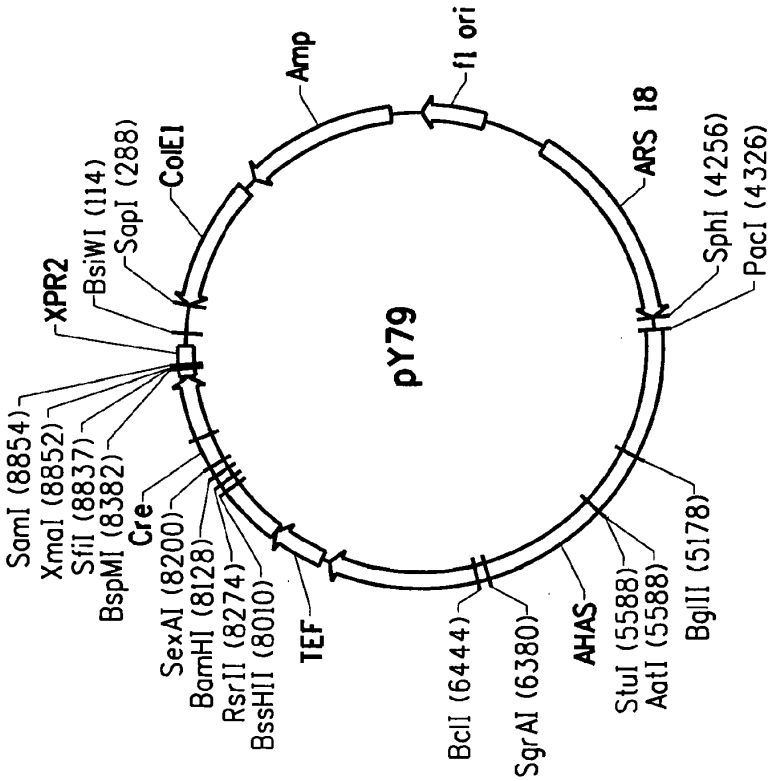



FIG. 9A



LCY Biotechnology Holding, Inc.
Ex. 1002
Page 2876 of 3077

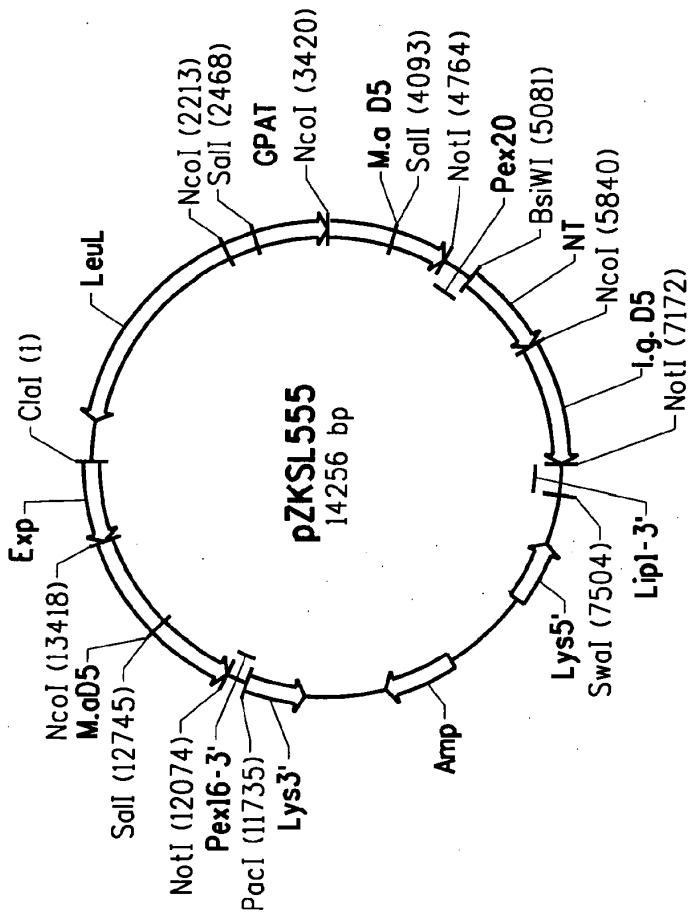



FIG. 9B



LCY Biotechnology Holding, Inc.
Ex. 1002
Page 2877 of 3077

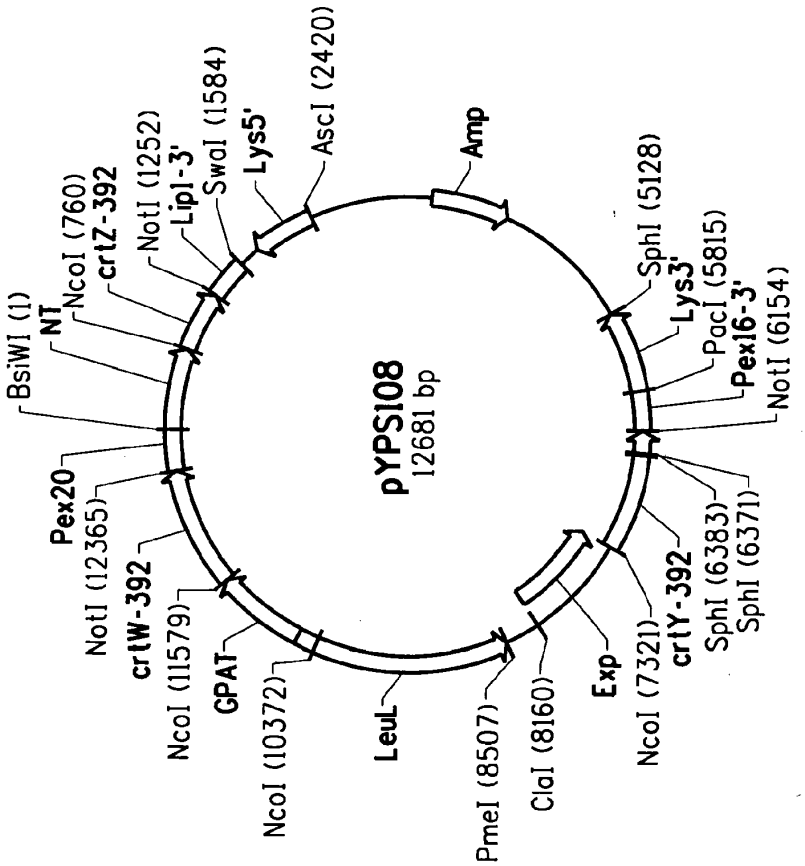



FIG. 10



LCY Biotechnology Holding, Inc.
Ex. 1002
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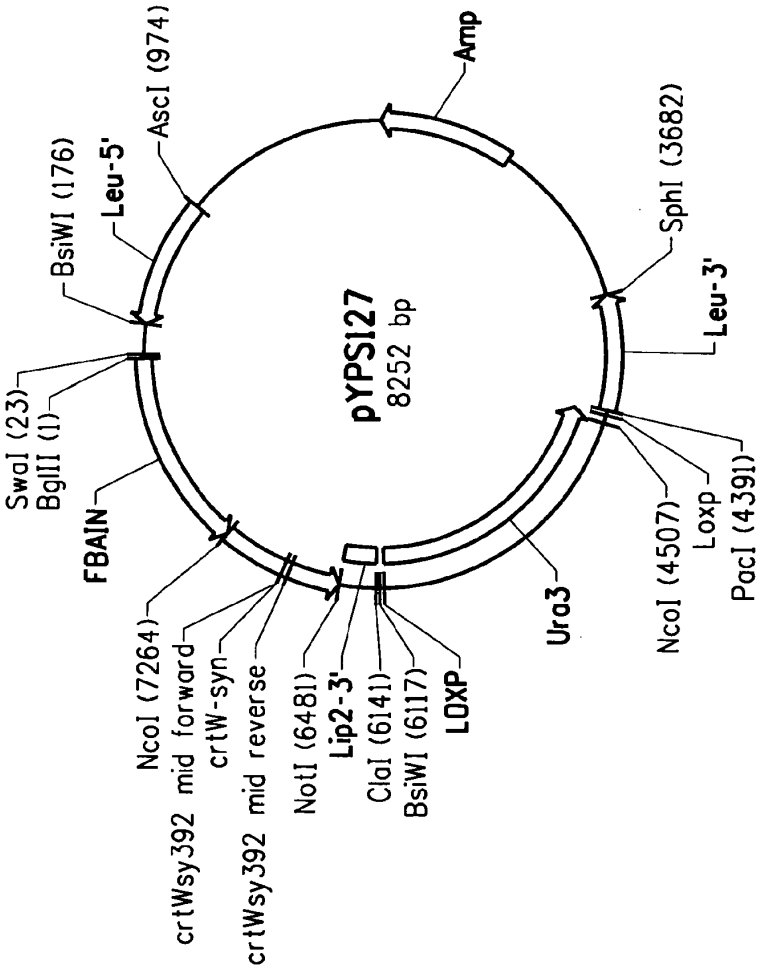



FIG. 11A



LCY Biotechnology Holding, Inc.
Ex. 1002
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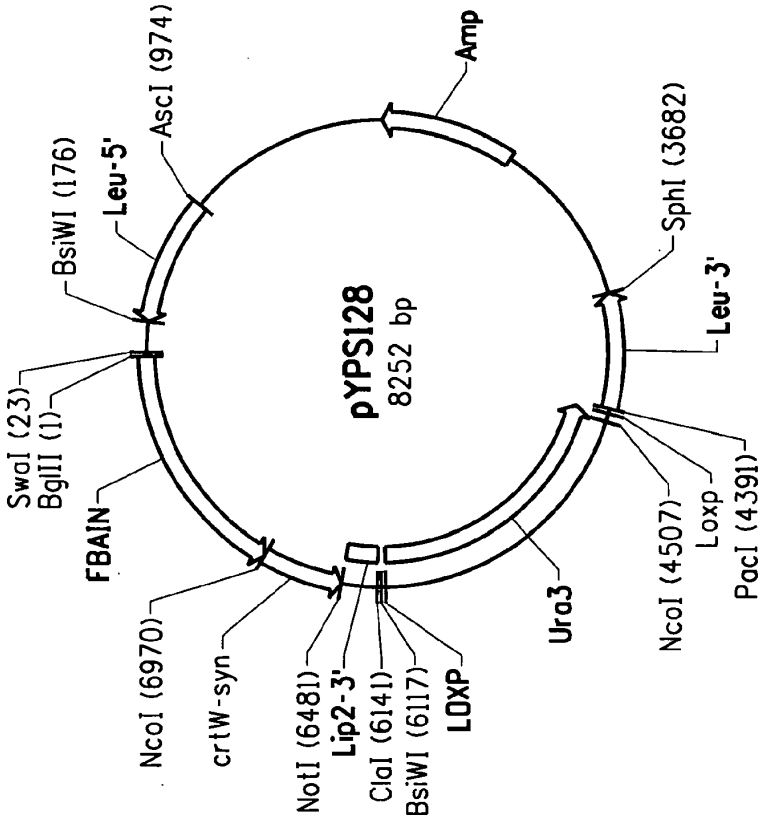



FIG. 11B



LCY Biotechnology Holding, Inc.
Ex. 1002
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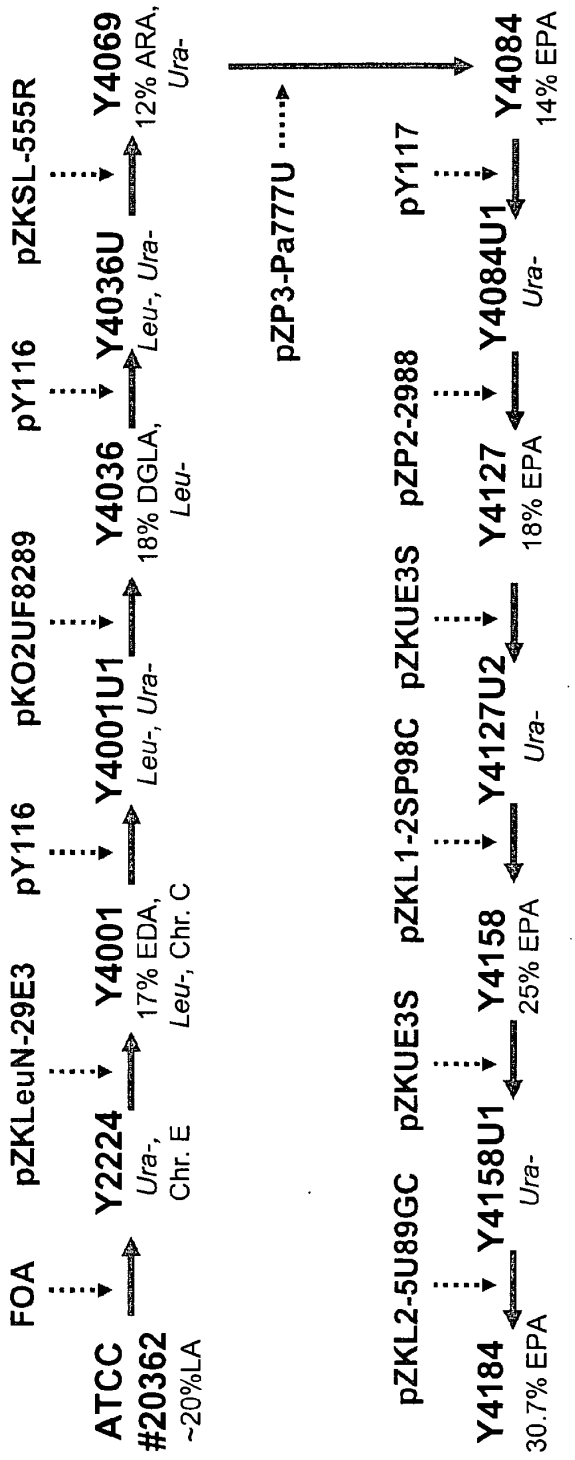



FIG. 12



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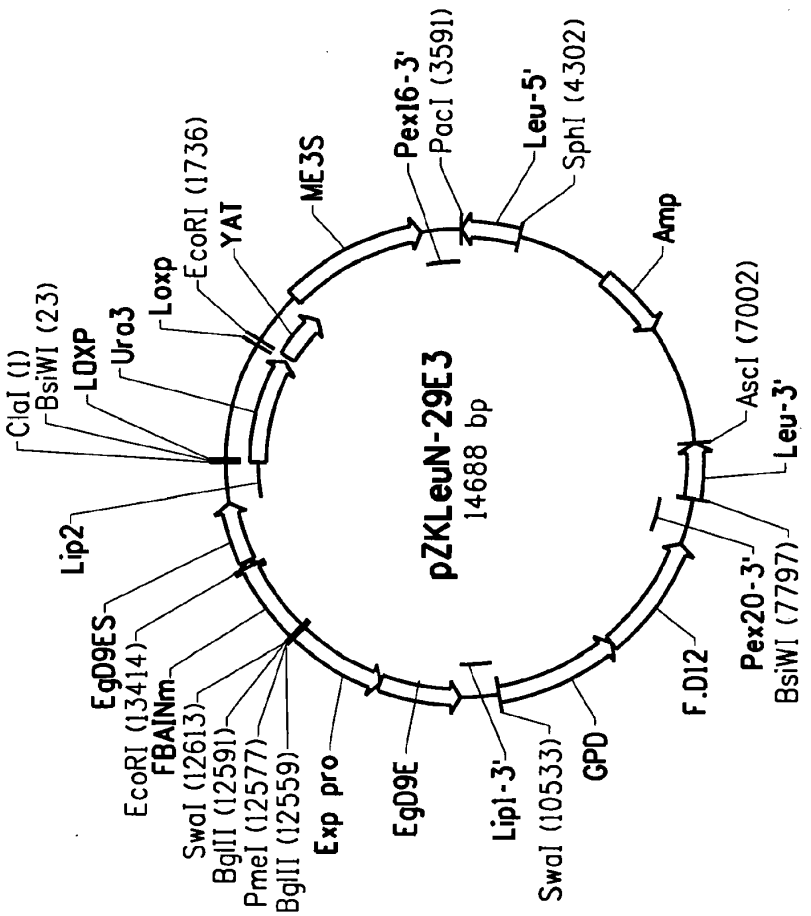



FIG. 13A



LCY Biotechnology Holding, Inc.
Ex. 1002
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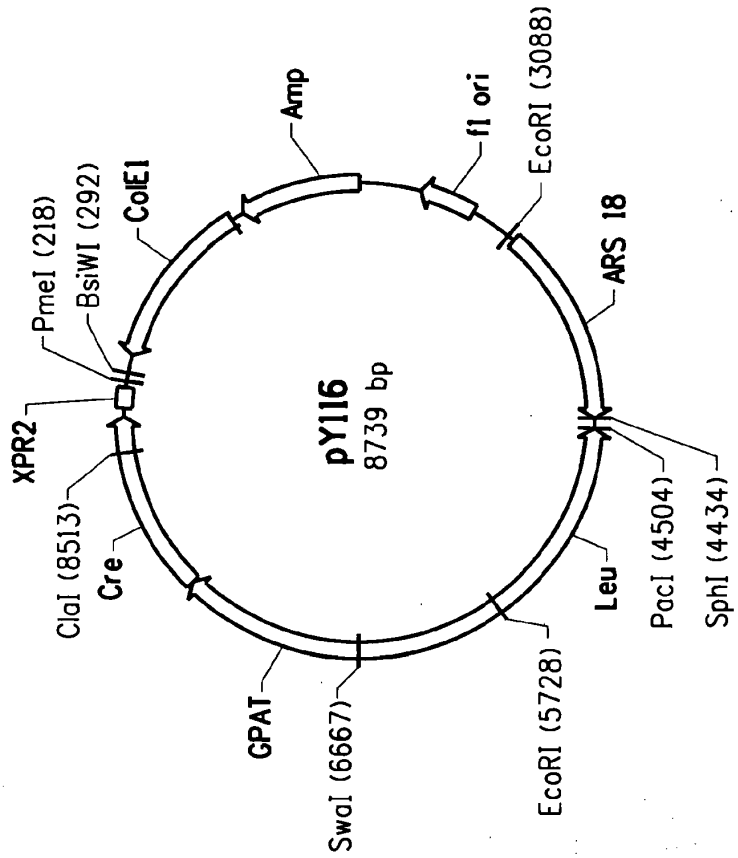



FIG. 13B



LCY Biotechnology Holding, Inc.
Ex. 1002
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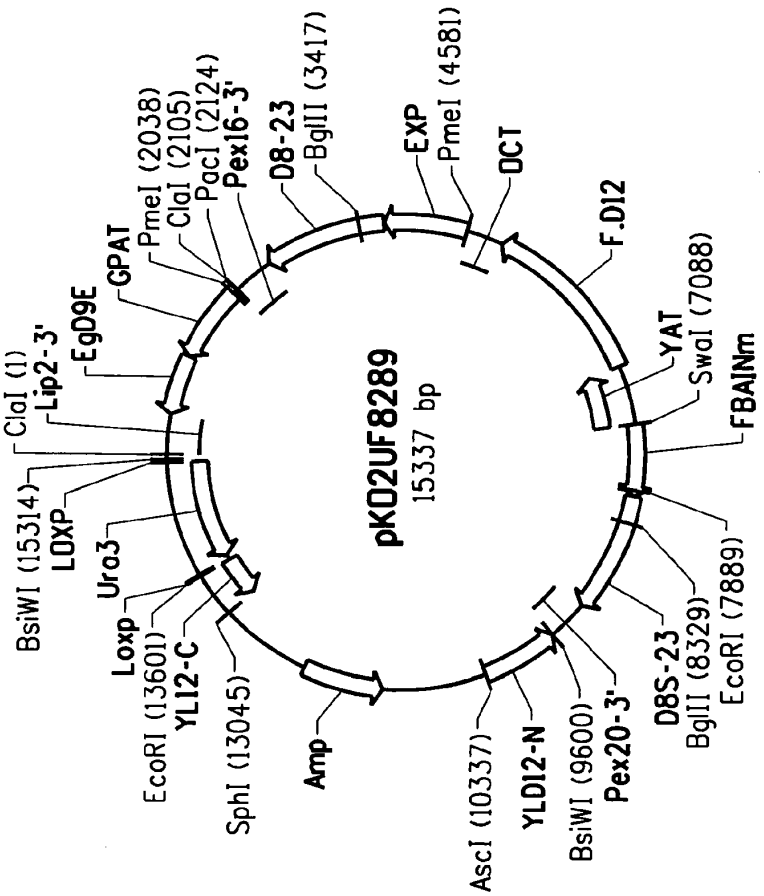



FIG. 14A



LCY Biotechnology Holding, Inc.
Ex. 1002
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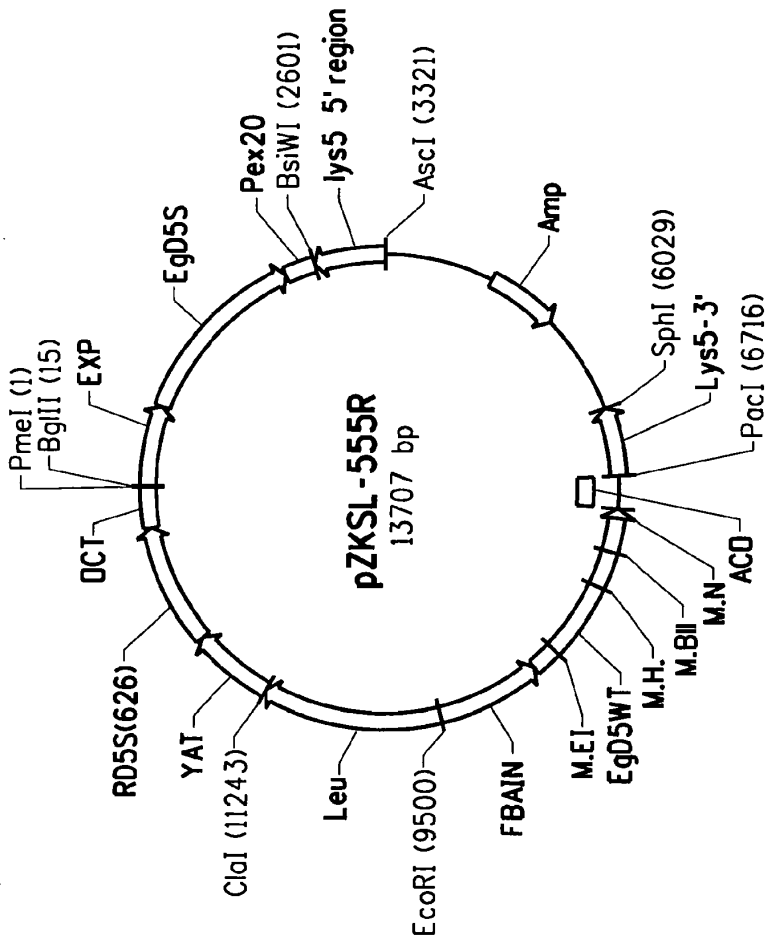



FIG. 14B



LCY Biotechnology Holding, Inc.
Ex. 1002
Page 2885 of 3077

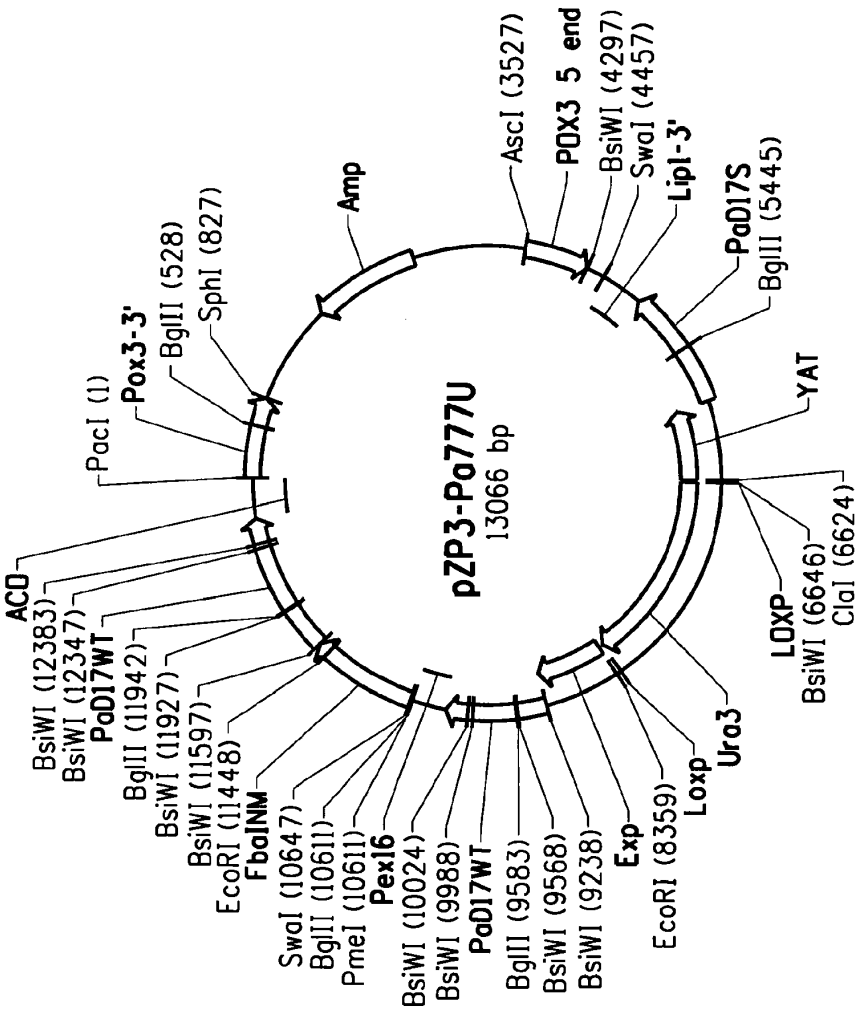



FIG. 15A



LCY Biotechnology Holding, Inc.
Ex. 1002
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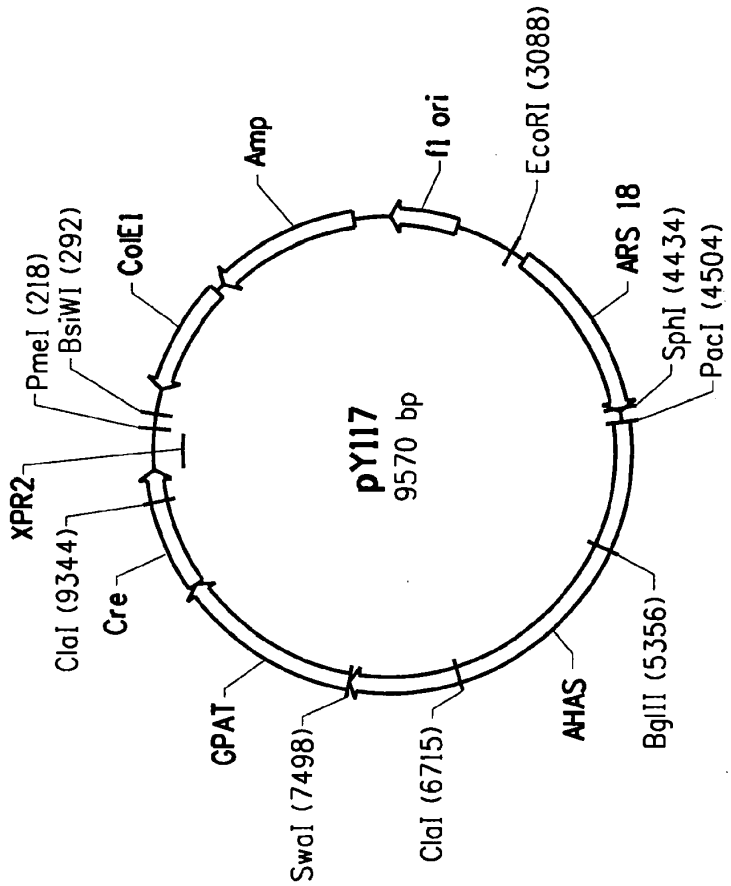



FIG. 15B



LCY Biotechnology Holding, Inc.
Ex. 1002
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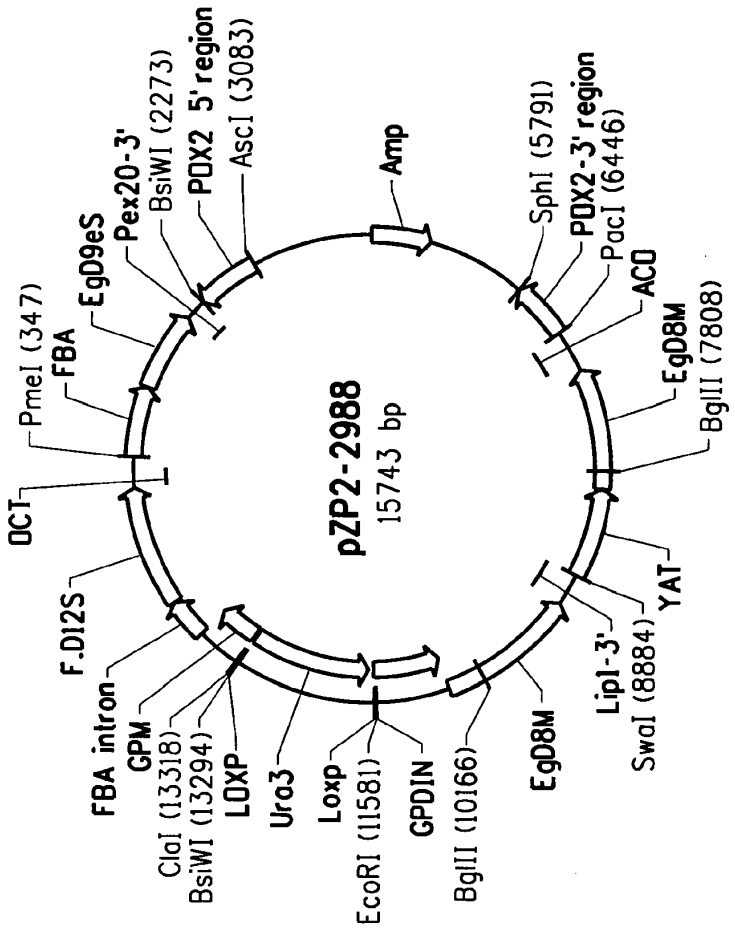



FIG. 16A



LCY Biotechnology Holding, Inc.
Ex. 1002
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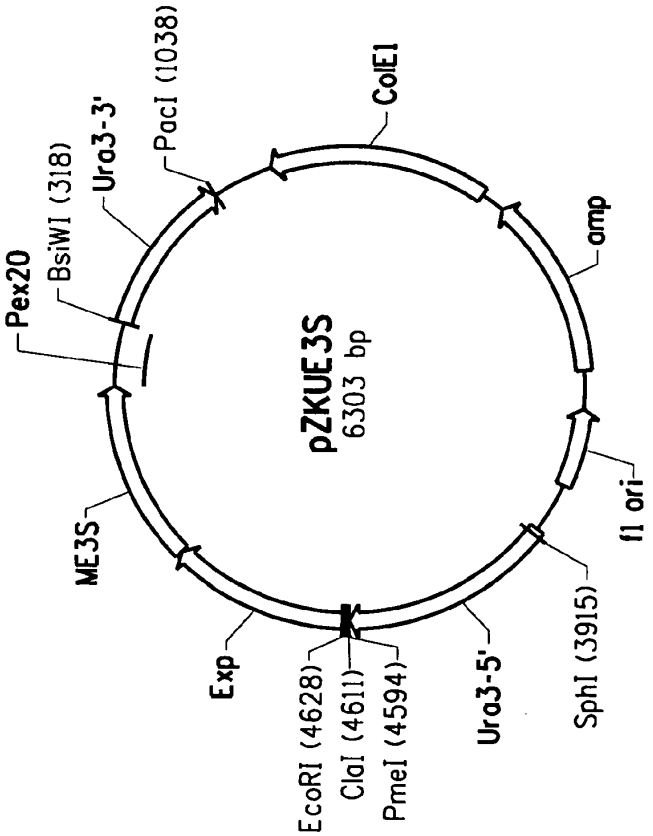



FIG. 16B



LCY Biotechnology Holding, Inc.
Ex. 1002
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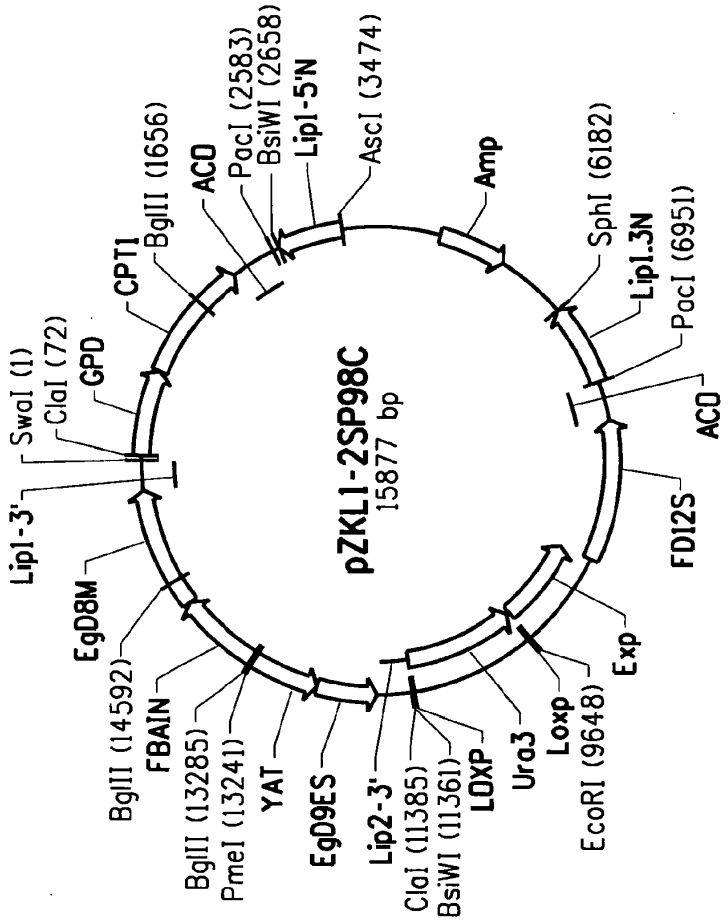



FIG. 17A



LCY Biotechnology Holding, Inc.
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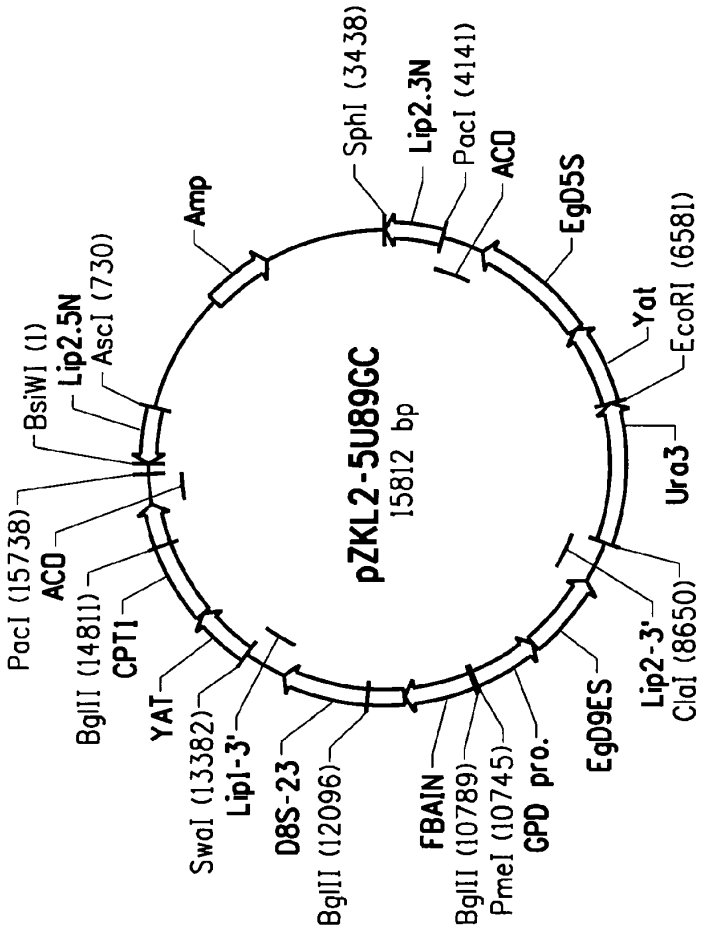



FIG. 17B



LCY Biotechnology Holding, Inc.
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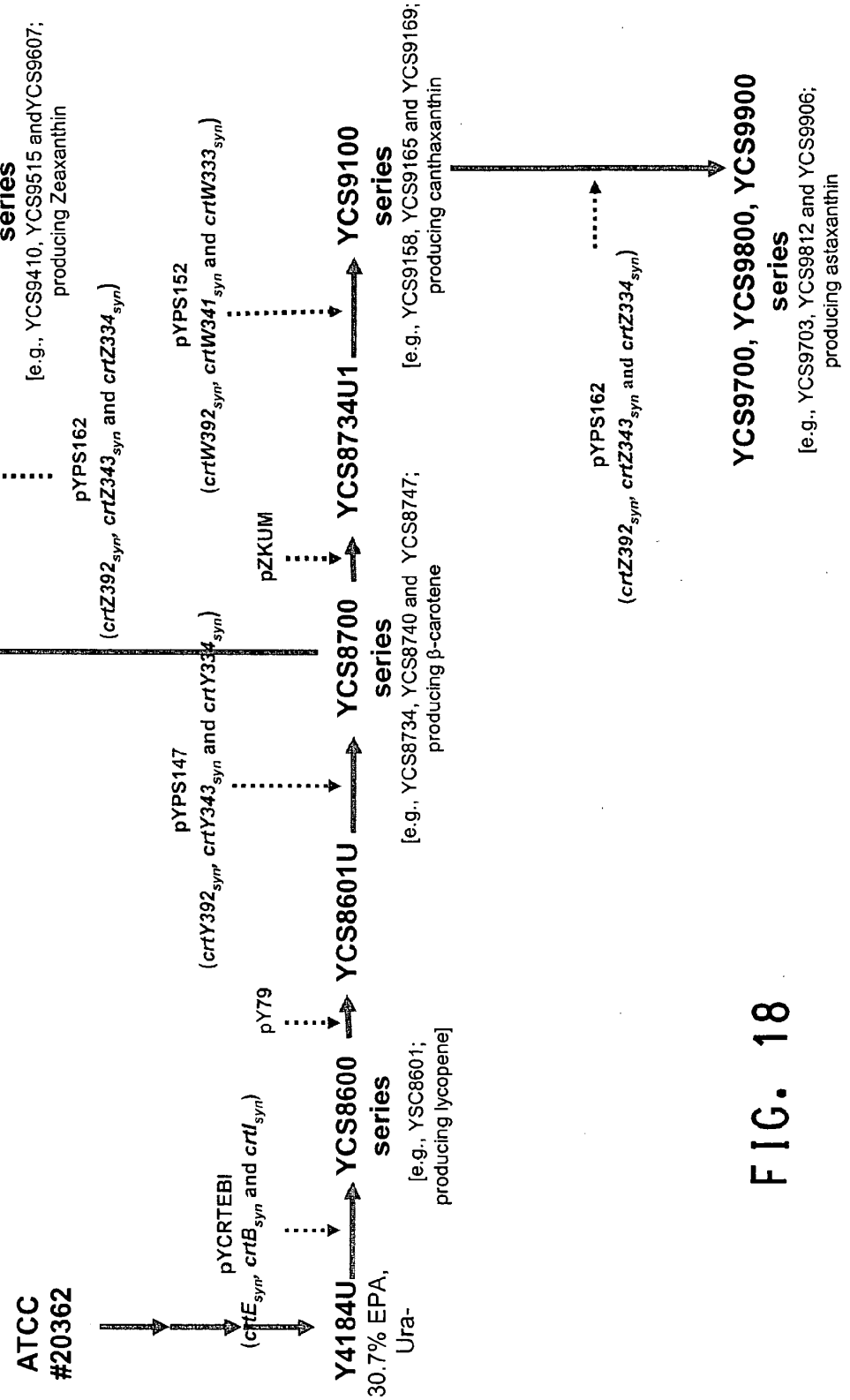



FIG. 18



LCY Biotechnology Holding, Inc.
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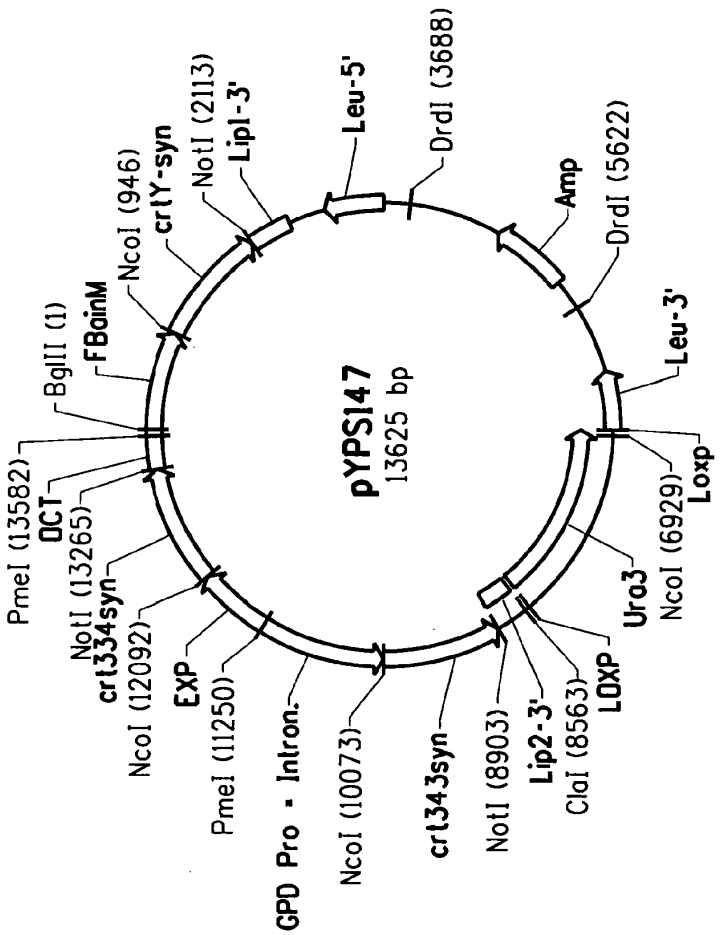



FIG. 19A



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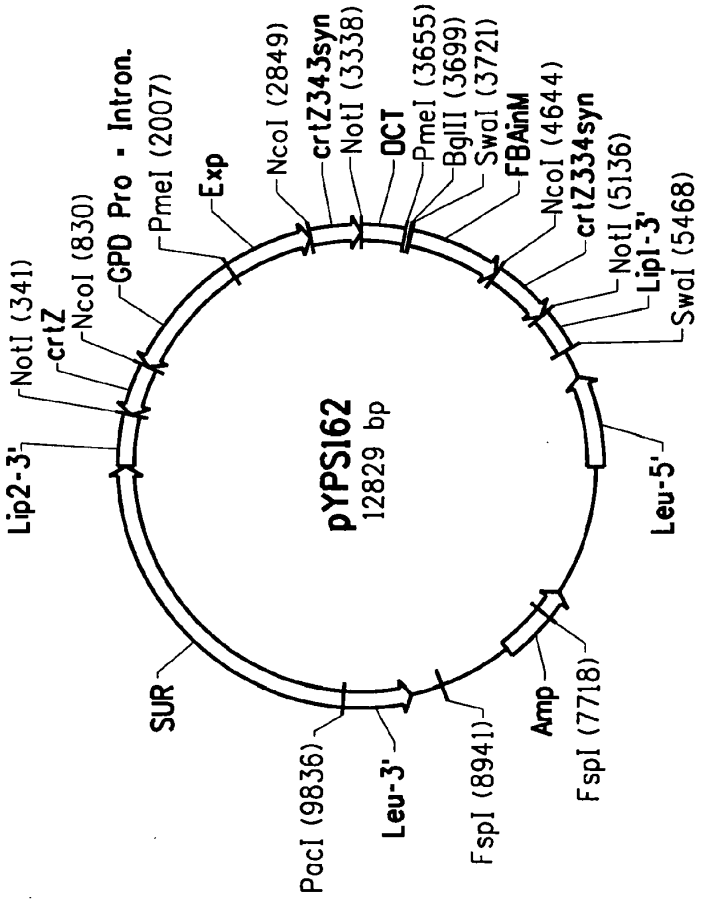



FIG. 19B



LCY Biotechnology Holding, Inc.
Ex. 1002
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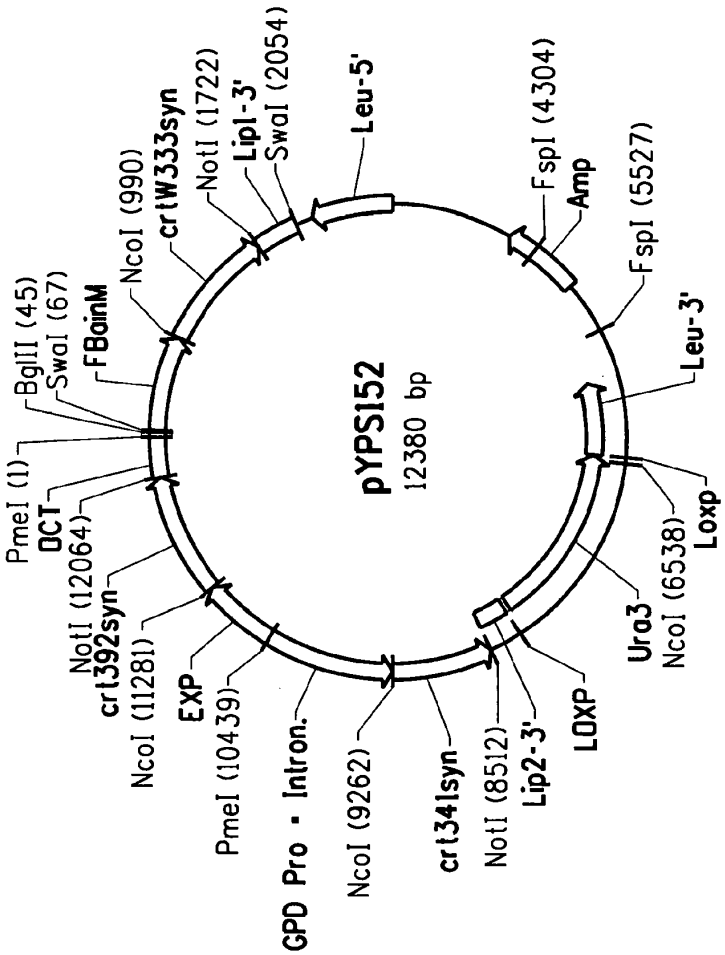



FIG. 20A



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Ex. 1002
Page 2895 of 3077

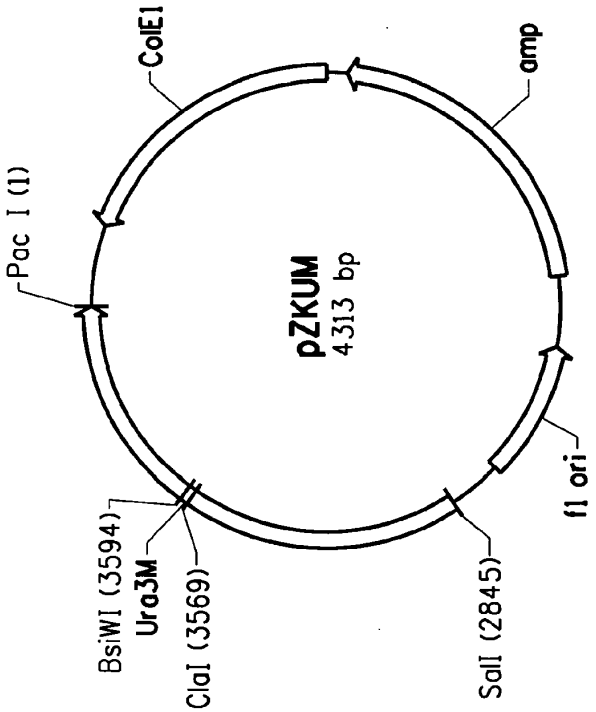



FIG. 20B



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Ex. 1002
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CL3517PCT

1/1

**PCT**

Original (for **SUBMISSION** )

| | | |
|---|---|---|
| 0-1 | **Form PCT/RO/134 (SAFE) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)** | |
| 0-1-1 | Prepared Using | PCT-SAFE [EASY mode]<br>Version 3.51.022.197 MT/FOP<br>20071101/0.20.5.10 |
| 0-2 | **International Application No.** | |
| 0-3 | **Applicant's or agent's file reference** | CL3517PCT |

| | | |
|---|---|---|
| 1 | **The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:** | |
| 1-1 | **page** | 6 |
| 1-2 | **line** | 30 |
| 1-3 | **Identification of deposit** | |
| 1-3-1 | Name of depositary institution | ATCC American Type Culture Collection |
| 1-3-2 | Address of depositary institution | 10801 University Blvd., Manassas, Virginia 20110-2209United States of America |
| 1-3-3 | Date of deposit | 26 October 2005 (26.10.2005) |
| 1-3-4 | Accession Number | ATCC PTA-7186 |
| 1-4 | **Additional Indications** | PTA-7185, PTA-7184, PTA-7186 on 26 October 2005; PTA-8614 on 23 August 2007; PTA-____ on X November 2007 |
| 1-5 | **Designated States for Which Indications are Made** | all designations |

**FOR RECEIVING OFFICE USE ONLY**

| | | |
|---|---|---|
| 0-4 | **This form was received with the international application:** (yes or no) | Rec'd PCT/PTO 10 DEC 2007 (10.12.07) |
| 0-4-1 | Authorized officer | |

**FOR INTERNATIONAL BUREAU USE ONLY**

| | | |
|---|---|---|
| 0-5 | **This form was received by the international Bureau on:** | |
| 0-5-1 | Authorized officer | |



LCY Biotechnology Holding, Inc.
Ex. 1002
Page 2897 of 3077

**PATENT COOPERATION TREATY**

IN THE APPLICATION OF:

E. I. DUPONT DE NEMOURS AND COMPANY CASE NO.: CL3517PCT

APPLICATION NO.: UNKNOWN GROUP ART UNIT: UNKNOWN

FILED: CONCURRENTLY HEREWITH EXAMINER: UNKNOWN

FOR: CAROTENOID PRODUCTION IN A RECOMBINANT OLEAGINOUS YEAST

## DECLARATION OF BIOLOGICAL CULTURE DEPOSIT

Mail Stop PCT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, S. NEIL FELTHAM, declare that:

I am an attorney/agent of record for the owner of the above-identified application.

Cultures of the following biological materials have been deposited with the following international depository(ies):

American Type Culture Collection (ATCC)
10801 University Blvd., Manassas, VA 20110-2209, U.S.A.

under conditions that satisfy the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. A copy of the receipt issued pursuant to Rules 7.3 and 10.2 (stating the term of the deposit) is attached.

| Depositor's Identification of Organism | International Depository Accession Number | Date of Deposit |
|---|---|---|
| *Yarrowia lipolytica* Y2047 | ATCC PTA-7186 | October 26, 2005 |
| *Yarrowia lipolytica* Y2201 | ATCC PTA-7185 | October 26, 2005 |
| *Yarrowia lipolytica* Y2096 | ATCC PTA-7184 | October 26, 2005 |
| *Yarrowia lipolytica* Y3000 | ATCC PTA-7187 | October 26, 2005 |
| *Yarrowia lipolytica* Y4128 | ATCC PTA-8614 | August 23, 2007 |
| *Yarrowia lipolytica* Y4127 | ATCC PTA-____ | November X, 2007 |

I further aver that all restrictions on the availability to the public of the culture will be irrevocably removed upon the granting of a U.S. patent on the above-identified application.

Respectfully submitted,

S. NEIL FELTHAM
ATTORNEY FOR APPLICANT
REGISTRATION NO. 36,506
TELEPHONE: (302) 992-6460
FACSIMILE: (302) 992-5374

Dated: 12/7/07

Enclosure: Deposit Receipts



LCY Biotechnology Holding, Inc.
Ex. 1002
Page 2899 of 3077

ATCC

10801 University Blvd • Manassas, VA 20110-2209 • Telephone: 703-365-2700 • FAX: 703-365-2745

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

*INTERNATIONAL FORM*

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.

**To: (Name and Address of Depositor or Attorney)**

DuPont Experimental Station, E301/317
Attn: Robert S. Scott
200 Powder Mill Road
PO BOX 80301
Wilmington, DE 19880-0301

**Deposited on Behalf of:** E. I. du Pont de Nemours and Company

| **Identification Reference by Depositor:** | **Patent Deposit Designation** |
|---|---|
| Yarrowia lipolytica: Y2096 | PTA-7184 |
| Yarrowia lipolytica: Y2201 | PTA-7185 |
| Yarrowia lipolytica: Y2047 | PTA-7186 |
| Yarrowia lipolytica: Y3000 | PTA-7187 |

**The deposits were accompanied by: __ a scientific description _ a proposed taxonomic description indicated above. The deposits were received October 26, 2005 by this International Depository Authority and have been accepted.**

**AT YOUR REQUEST: X We will inform you of requests for the strains for 30 years.**

**The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strains.**

**If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.**

**The strains will be maintained for a period of at least 30 years from date of deposit, or five years after the most recent request for a sample, whichever is longer. The United States and many other countries are signatory to the Budapest Treaty.**

**The viability of the cultures cited above was tested January 13, 2006. On that date, the cultures were viable.**

**International Depository Authority: American Type Culture Collection, Manassas, VA 20110-2209 USA.**

**Signature of person having authority to represent ATCC:**

Marie Harris

**Marie Harris, Patent Specialist, ATCC Patent Depository**

**Date: February 18, 2006**

cc: S. Neil Feltham
CL2698 and CL-3160



LCY Biotechnology Holding, Inc.
Ex. 1002
Page 2900 of 3077

# ATCC®

10801 University Boulevard • Manassas, VA 20110-2209 • Telephone: 703-365-2700 • FAX: 703-334-2932

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

*INTERNATIONAL FORM*

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address if Depositor or Attorney)

DuPont Experimental Station
ATTN: Robert S. Scott
E301-317, 200 Powder Mill Road
P.O.Box 80301
Wilmington, DE 19880-0301

Deposited on Behalf of: E.I.du Pont de Nemours and Company

Date of Receipt of Culture by the ATCC®: August 23, 2007

| Identification Reference by Depositor: | | ATCC® Patent Deposit Designation: |
|---|---|---|
| Yarrowia lipolytica | Y4128 | PTA-8614 |

The deposit was received by this International Depository Authority and has been accepted.

The strain will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strain, and ATCC® is instructed by the United States Patent & Trademark Office or the depositor to release said strain.

If the deposit should die or be destroyed during the effective term of the patent, it shall be your responsibility to replace it with viable material. It is also your responsibility to supply a sufficient quantity for distribution for the deposit term. ATCC® will distribute the material for 30 years or 5 years following the most recent request for the deposit, whichever is longer. The United States and many other countries are signatory to the Budapest Treaty.

We will inform you of requests for the strain for 30 years from date of deposit.

The deposit was tested September 10, 2007 and on that date, the culture was viable.

International Depository Authority: American Type Culture Collection, Manassas, VA, USA

Signature of person having authority to represent ATCC®:

*Latha Ramakrishnan*

Digitally signed by Latha Ramakrishnan
DN: cn=Latha Ramakrishnan, o=ATCC, ou=IP,Licensing and Services, email=lramakrishnan@atcc.org, c=US
Date: 2007.09.12 10:02:14 -04'00'

ATCC® Patent Depository

September 12, 2007
Date

cc: S. Neil Feltham
Ref: Docket or Case No. CL 3847 and CL3941



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# INTERNATIONAL SEARCH REPORT

International application No
PCT/US2007/025222

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N1/16 C12N1/15 C07C403/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 2004/104180 A (DU PONT [US]; CHENG QIONG [US]; TAO LUAN [US]) 2 December 2004 (2004-12-02) cited in the application | 1-3,7,9, 11-14,18 |
| Y | the whole document | 5,6,8, 10,15-17 |
| X | WO 2006/102342 A (MICROBIA INC [US]; BAILEY RICHARD [US]; MADDEN KEVIN T [US]; TRUEHEART) 28 September 2006 (2006-09-28) cited in the application | 1-3,7,9, 11-14,18 |
| Y | the whole document | 5,6,8, 10,15-17 |
| Y | US 2006/115881 A1 (DAMUDE HOWARD G [US] ET AL) 1 June 2006 (2006-06-01) cited in the application the whole document | 5,6,8, 10,15-17 |

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 23 April 2008 | 02/05/2008 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Bilang, Jürg |

Form PCT/ISA/210 (second sheet) (April 2005)

## INTERNATIONAL SEARCH REPORT

International application No
PCT/US2007/025222

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US 2006/110806 A1 (DAMUDE HOWARD G [US] ET AL) 25 May 2006 (2006-05-25)<br>cited in the application<br>the whole document<br>----- | 5,6,8,<br>10,15-17 |
| Y | CANNIZZARO CHRISTOPHER ET AL: "Metabolic network analysis on Phaffia rhodozyma yeast using 13C-labeled glucose and gas chromatography-mass spectrometry."<br>METABOLIC ENGINEERING OCT 2004,<br>vol. 6, no. 4, October 2004 (2004-10),<br>pages 340-351, XP009099270<br>ISSN: 1096-7176<br>the whole document<br>----- | 5,6,8,<br>10,15-17 |

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

## INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2007/025222

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 2004104180 A | 02-12-2004 | AU 2004242132 A1 | 02-12-2004 |
| | | CA 2523789 A1 | 02-12-2004 |
| | | EP 1625204 A2 | 15-02-2006 |
| | | JP 2006528885 T | 28-12-2006 |
| | | US 2004268439 A1 | 30-12-2004 |
| WO 2006102342 A | 28-09-2006 | AU 2006227165 A1 | 28-09-2006 |
| | | CA 2602183 A1 | 28-09-2006 |
| | | EP 1866428 A2 | 19-12-2007 |
| | | KR 20070121679 A | 27-12-2007 |
| | | NO 20075200 B | 11-10-2007 |
| US 2006115881 A1 | 01-06-2006 | NONE | |
| US 2006110806 A1 | 25-05-2006 | NONE | |

Form PCT/ISA/210 (patent family annex) (April 2005)

Atty Docket No.: SS118-1026US

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

| | | | |
|---|---|---|---|
| In re Application | : | Aeling et al. | Confirmation No. 5356 |
| Serial No. | : | 16/630,567 | |
| Filing Date | : | January 13, 2020 | |
| Group Art Unit | : | 1656 | |
| Examiner | : | Noakes, S.M. | |
| For | : | **BIOLOGICAL METHODS FOR PREPARING TERPENES** | |

Mail Stop Amendments
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

**AMENDMENT AND RESPONSE**

Sir:

This Amendment and Response is filed in response to the Non-Final Office Action ("Office Action") of February 18, 2022 issued in connection with the above-identified matter. Thus, this Amendment and Response is timely filed.

**Amendments to the Specification** begin on page 2 of this paper.

**Remarks** begin on page 3 of this paper.



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**AMENDMENTS TO THE SPECIFICATION**

Please substitute drawing sheets 25 (Figures 25A, 25B), 27 (Figure 27) and 38 (Figure 38) with the replacement drawing sheets attached hereto. No new matter has been added.



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Page 2906 of 3077

## REMARKS

Claims 163-183 are pending and 163-165 have been examined on the merits. In the Office Action, the specification is objected to and the claims are rejected as follows:

1. The specification is objected to for allegedly failing to comply with the requirements of the Sequence Rules; and

2. Claims 163-165 are rejected under 35 U.S.C. § 103 for allegedly being unpatentable over Sharpe et al. (WO2008/073367, hereinafter "Sharpe") in view of Juretzek et al. (Biotechnol. Bioprocess. Eng. 2000, hereinafter "Juretzek").

**Objection to the Specification**

Replacement drawing sheets are filed herein, rendering this objection moot. Thus, reconsideration and withdrawal of this objection is respectfully requested.

**Rejection under 35 U.S.C. § 103**

The presently claimed invention is directed to a genetically modified microorganism, comprising:

one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides, wherein expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide:

- wherein the one or more heterologous nucleic acids that encode one or more terpene biosynthesis polypeptides is chosen from terpene synthase, phytoene synthase, geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, zeaxanthin glucosyltransferase and valencene synthase, or



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- wherein the one or more heterologous nucleic acids is a cytochrome P450 reductase and is regulated by a nucleic acid that provides for alkane induction of expression of the cytochrome P450 reductase.

In the Office Action, the Examiner has taken the position that Sharpe describes the features of claims 163-165.

Specifically, according to the Examiner, Sharpe describes a recombinantly engineered oleaginous yeast wherein said yeast has been engineered to express the heterologous genes CrtE of geranyl geranyl pyrophosphate synthase, a CrtB phytoene synthase and a Crtl phytoene desaturase.

Further, according to the Examiner Sharpe describes that virtually any promoter capable of directing expression of these genes in the selected host cells is suitable for the present invention.

The Examiner, however, has admitted that Sharpe is completely silent with regard to a promoter being responsive to fatty acid or alkane induction.

Juretzek does not correct Share's deficiencies nor can it provide for the missing link.

But the Examiner has taken the position that Juretzek, describes a comparison of different promoters suitable for regulating the overexpression of beta-galactosidase in the yeast Yarrowia lipolytica (Title and Abstract of Juretzek).

Thus, it is the Examiner's opinion that the person skilled in the art would have easily combined the teachings regarding the promoters of the yeast Yarrowia lipolytica, and more specifically POX2, POT1 and ICL1, described in Juretzek, and used them in order to express the heterologous genes of terpenes.



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Applicants respectfully disagrees and respectfully submit that for the foregoing reasons the combination of the cited references would not have rendered obvious the presently claimed invention to one skilled in the art.

As an initial matter Sharpe is only a generic document regarding engineered strains of the oleaginous yeasts capable of producing carotenoids (*e.g.,* the Abstract).

In addition, although Sharpe describes several recombinant yeasts, among which the yeasts presently claimed (*e.g.*, page 29) as mentioned by the Examiner, only Yarrowia is effectively tested and none of the others, namely Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon and Lipomyces are explicitly and univocally suggested to the person skilled in the art.

The Examiner recognizes (*e.g.*, page 5 of the Office Action) that, apart from the use of Yarrowia as recombinant yeast, Sharpe never discloses or suggests any promoter of said yeast that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide.

It is important to highlight that this promoter is not only mandatory and critical in the presently claimed invention, but it is extremely important to achieve the unexpected and surprising results obtainable with the present invention.

Second, Juretzek does not describes a real expression of genes.

Contrarily, and more generically, Juretzek is a comparison of possible available promoters, specifically POX2, POT1 and ICL1, tested via the reaction of a very easily activated gene, namely the beta-galactosidase, which in fact cannot be a representative gene.

Beta-galactosidase is always used as gene-test for such studies, precisely due to its very easy activation.



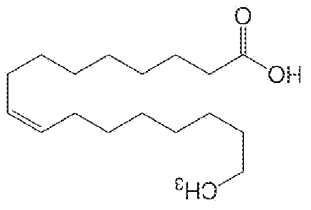
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Applicants hence underline that the person skilled in the art, by reading the study of Juretzek and having in mind its teachings, cannot find any teachings or motivation to support the notion that promoters capable to activate beta-galactosidase will certainly activate much more complexes genes, such as the ones needed in order to achieve the objective technical problem, as above exposed.

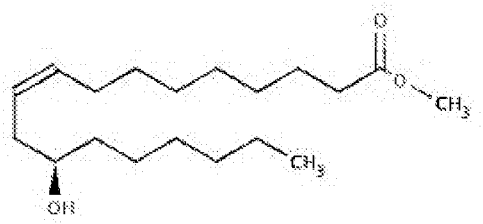
The person skilled in art, which in this case in a biologist, surely knows that he/she has to consider much more complex genes, and that beta-galactosidase is only a sort of control, even though surely it is a starting point.

Hence, starting from the study on beta-galactosidase, the person skilled in the art would have surely noticed that Fig.2 (b) of Juretzek shows that the best promoters (listed and described in the left column of page 324 of Juretzek), namely POX2, ICL1 and POT1, have very different behaviors depending on the fatty acid considered.

Specifically, in the case of oleic acid (1), POX2, ICL1 and POT1 have surely and evidently a much lower power of promoting beta-galactosidase with respect to ricinoleic acid methyl ester (2), even though from the chemical point of view the two structures are very similar:




(1)




(2)



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This demonstrates that the behavior cannot be representative for all fatty acid and surely the trends of the graphs are not really representative of the biological behavior of such complex interactions happening between long chain fatty acids and promoters in biological alive systems.

Moreover, it is worthy to underline that none of the promoters specifically listed, described, tested and clearly suggested as the best one in Juretzek, specifically POX2, ICL1 and POT1, are the ones used in present Application, which instead describes for the same yeast, namely Yarrowia lipolytica: ACO1 (Genbank accession no. AJ001299), ACO2 (Genbank accession no. A001300), ACO3 (Genbank accession no. AJ001301), ACO4 (Genbank accession no. AJ001302), and ACO5 (Genbank accession no. AJ001303) (page 141, lines 17-21 of present Application).

Last but not least, even if the promoters specifically suggested in Juretzek, specifically POX2, ICL1 and POT1, were the same of the ones of the presently claimed invention, the article itself discourages their use, when it describes that "the POX2 and POT1 promoters are only inducible by fatty acid, what may be a disadvantage for biotechnological applications" (page 324, left column of Juretzek).

Hence the person skilled in the art would have understood their limitations and looked for an alternative.

But even trying to find an alternative, he/she would have been discouraged of even using fatty acid promoters in general since Juretzek explains that "These results present ricinoleic acid methyl ester as the strongest inducer of pPOX2 and pPOT1 among the tested carbon sources, in spite of the disadvantage that this carbon source inhibits growth of Y. lipolytica" (page 324, left column of Juretzek).



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The person skilled in the art reading the article would have surely consider that, by using source of carbon as long chain fatty acid, this could lead to damage of the microorganism, that instead is the real protagonist, playing the main role in the biosynthesis. He/she would have surely been directed in the direction of finding another solution which replaces the use of fatty acid as promoters, in order to avoid any damage on the Y. lipolytica.

In view of the above, the person skilled in the art, while combining the teachings of the two documents, namely Sharpe and Juretzek, would have considered different promoters, with respect to those presently claimed, to begin with, and afterwards would have been discouraged in using them in the biotechnology field and for the yeast described, since the promoters shown in Juretzek show to damage the yeast itself.

Moreover, with the presently claimed invention it is possible to provide a biosynthesis of terpene polypeptides and the result is brilliantly achieved via the proviso of a specific genetically modified microorganism, which comprises a promoter modified by replacing the endogenous promoters with a promoters comprising an alkane response element (ARE1) (*e.g.*, page 30 lines 19-20, page 33, lines 10-12, page 35 lines 25-27, page 38, lines 1-3, page 40, lines 15-18, etc of present Application) or a fatty acid-inducible promoters (*e.g.*, page 48, line 10 of present Application) directing the flux of carbons from fatty acids toward a particular target product pathway (*e.g.*, mevalonate production, isopentenyl diphosphate production, geranyl diphosphate production, farnesyl diphosphate production, geranylgeranyl diphosphate production, terpene production, carotenoid production, beta carotene production, lycopene production, astaxanthin production) and away from other cellular metabolic pathways not involved in target molecule production (*e.g.*, page 29, lines 29-34 and page 46, lines 26-34 of present Application).



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None of these advantages can be obtained or can be extrapolated by combining and modifying the two cited references as suggested by the Examiner.

Thus, for all of the reasons discussed above, Applicants respectfully submit that the combination of the cited references would not have rendered obvious the presently claimed invention to one skilled in the art.

Accordingly, reconsideration and withdrawal of this rejection is respectfully requested.

**Conclusions**

This Amendment and Response is filed within the shortened statutory period for reply. Thus, no fee is believed to be due. However, the Director is hereby authorized to charge any fee deemed necessary or credit any overpayments to Deposit Account No. 506423.

Respectfully submitted,

May 16, 2022 By: /Silvia Salvadori/

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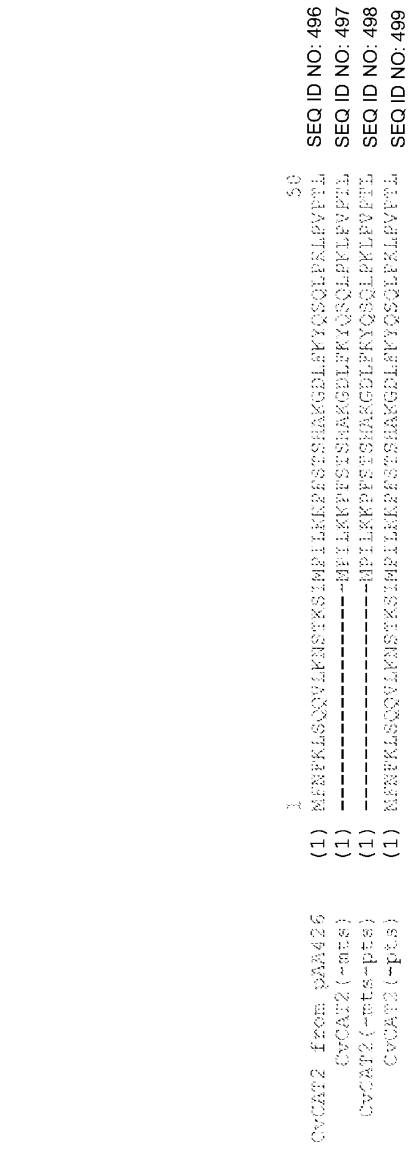



FIG. 25A




FIG. 25B

```
                                1                                                  50
CvYat1p                   (1)  ----------------------------------------MSTYQFQETLEKLPIPD  SEQ ID NO: 504
CvYat1p+CAT2mts           (1)  MFNFKLSQQVLKNSTKSIMPILKKP-FSTSHAKGSTYQFQETLEKLPIPD  SEQ ID NO: 505
CvYAT1p+CIT1mts           (1)  ----------MSALRSFQRSSSNVAKST----LKNSVRTYSTYQFQETLEKLPIPD  SEQ ID NO: 506
CvYat1p+COX4mts           (1)  ----------MLSRTTLRVARQQTRLLSTSRILFNSRTDQSTYQFQETLENLPIPD  SEQ ID NO: 507
```

FIG. 27

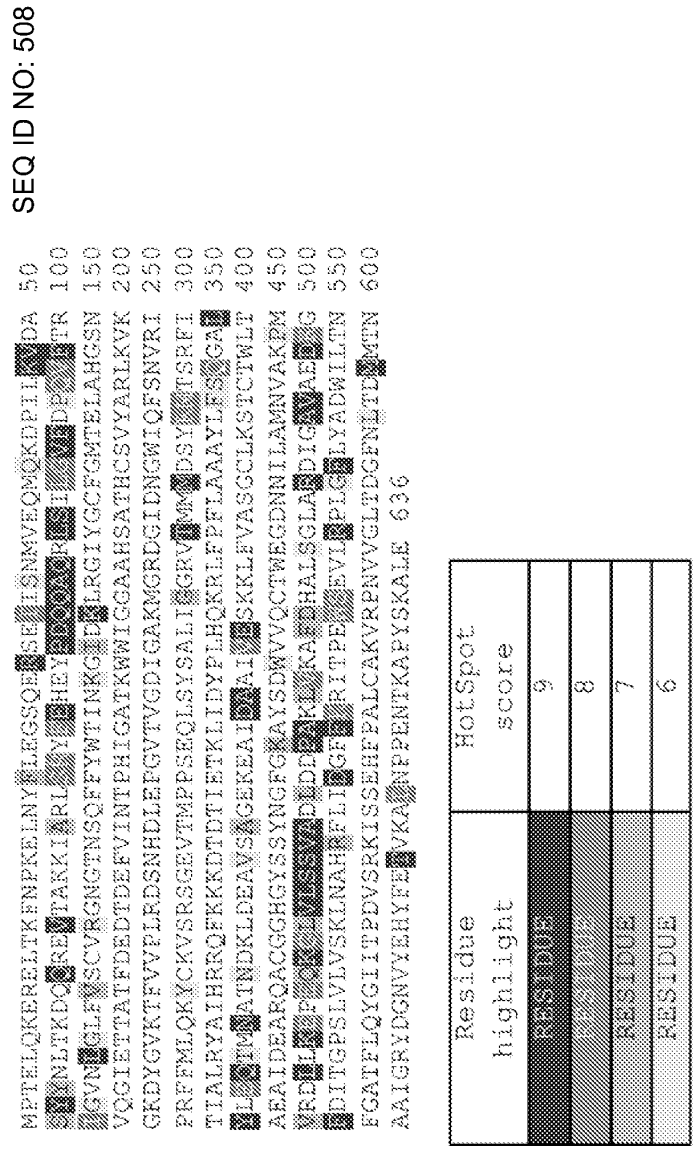

SEQ ID NO: 508
Residue highlight
HotSpot score
9
8
7
6
RESIDUE
RESIDUE
RESIDUE

FIG. 38

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# ELECTRONIC ACKNOWLEDGEMENT RECEIPT

| APPLICATION # | RECEIPT DATE / TIME | ATTORNEY DOCKET # |
|---|---|---|
| **16/630,567** | **05/16/2022 03:11:03 AM ET** | **SS118-1026US** |

## Title of Invention

BIOLOGICAL METHODS FOR PREPARING TERPENES

## Application Information

| | | | |
|---|---|---|---|
| APPLICATION TYPE | Utility - U.S. National Stage under 35 USC 371 | PATENT # | - |
| CONFIRMATION # | 5356 | FILED BY | Silvia Salvadori |
| PATENT CENTER # | 60669379 | FILING DATE | 01/13/2020 |
| CUSTOMER # | 122515 | FIRST NAMED INVENTOR | Kimberly Ann Aeling |
| INTL. APPLICATION # | - | INTL. FILING DATE | - |
| CORRESPONDENCE ADDRESS | - | AUTHORIZED BY | - |

## Documents

**TOTAL DOCUMENTS: 4**

| DOCUMENT | | PAGES | DESCRIPTION | SIZE (KB) |
|---|---|---|---|---|
| Response.pdf | | 12 | - | 574 KB |
| Response-A...pdf | (1-1) | 1 | Amendment/Request for Reconsideration-After Non-Final Rejection | 76 KB |
| Response-SPEC.pdf | (2-2) | 1 | Specification | 74 KB |
| Response-REM.pdf | (3-9) | 7 | Applicant Arguments/Remarks Made in an Amendment | 123 KB |
| Response-DRW.pdf | (10-12) | 3 | Drawings-only black and white line drawings | 441 KB |

## Digest

| DOCUMENT | MESSAGE DIGEST(SHA-512) |
|---|---|
| Response.pdf | E43588A8CC08DF6161C2D33B13587B5512D1C523661073A3E AA4DEB48ACF4A265CAF58491FDB990E618F73016E301D1680 6CD967EF6AF5E4D8F2EB012CB2345D |
| Response-A....pdf | 2082EF817322190C5CD01A35F5367C08CEA508C0E0FC8849F A16FE5F8BF3478ED9AAE779CF7DCBB7346FE64079EB67BF4 F1F45CE75C4B94D9079ED6BFDF517BC |
| Response-SPEC.pdf | 390188C16861DD2F530E31C8A52F4AAB625DE7D4615FEB952 BE1DC9580EFDB91CA210B428FA41F478A2C4A65634FE5ACA 86DE9E968A2DD5DFD3BC87D1D973C3F |
| Response-REM.pdf | 6B51E3BBCBEAF46E8DB4FE4BBEA3F4A1966D22CF13F08422 A19D8F79B70E9774C60F720BB8DEB04D054BD863FC380A81D 617B1F34FE7608B0B32703D00ADD183 |
| Response-DRW.pdf | 6228318C586D9E0EFFDF7C1864958B0B7AB630ABDC4A69195 EE01104B04C2EDB8DCFE53EC2143367319DAEE5284B8CC93 C2869A46D9EA3B73425D452FB41E5A9 |

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

**New Applications Under 35 U.S.C. 111**

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

**National Stage of an International Application under 35 U.S.C. 371**

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

**New International Application Filed with the USPTO as a Receiving Office**

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

DocCode - SCORE

# SCORE Placeholder Sheet for IFW Content

Application Number: 16630567 Document Date: 05/16/2022

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

- Drawing

At the time of document entry (noted above):

- USPTO employees may access SCORE content via DAV or via the SCORE web page.
- External customers may access SCORE content via PAIR using the Supplemental Content tab.

Form Revision Date: March 1, 2019

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| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 16/630,567 | 01/13/2020 | Kimberly Ann Aeling | SS118-1026US | 5356 |

122515 7590 07/29/2022

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New York, NY 10025

| EXAMINER | |
|---|---|
| NOAKES, SUZANNE MARIE | |

| ART UNIT | PAPER NUMBER |
|---|---|
| 1656 | |

| NOTIFICATION DATE | DELIVERY MODE |
|---|---|
| 07/29/2022 | ELECTRONIC |

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

eofficeaction@appcoll.com
silvia30121@me.com
silvia@salvadorilaw.com

| *Office Action Summary* | **Application No.** 16/630,567 | **Applicant(s)** Aeling et al. | |
|---|---|---|---|
| | **Examiner** SUZANNE M NOAKES | **Art Unit** 1656 | **AIA (FITF) Status** Yes |

*-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --*

**Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

1)☑ Responsive to communication(s) filed on 05/16/2022.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☑ This action is **FINAL.** 2b) ☐ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims***

5) ☑ Claim(s) 163-183 is/are pending in the application.

5a) Of the above claim(s) 166-183 is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 163-165 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

**Application Papers**

10)☐ The specification is objected to by the Examiner.

11)☑ The drawing(s) filed on 05/16/2022 is/are: a)☑ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

**Priority under 35 U.S.C. § 119**

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

**Certified copies:**

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.



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## DETAILED ACTION

### *Status of Application*

1. The response filed 16 May 2022 is acknowledged and has been considered in its entirety. Claims 163-183 are pending; Claims 166-183 remain withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected subject matter, there being no allowable generic or linking claim. Thus, claims 163-165 remain subject to examination on the merits.

### *Withdrawal of Previous Objection*

2. The objection to the specification or drawings is withdrawn in view of the filing of replacement drawings for Figures 25A, 25B, Figure 27, and Figure 38, which all incorporate the requisite sequence identifiers therein.

### *Maintained Rejection*

### *Claim Rejections - 35 USC § 103*

3. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

4. The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have

> been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

5. Claims 163-165 are rejected under 35 U.S.C. 103 as being unpatentable over Sharpe et al. (WO 2008/073367 – cited herein) in view of Juretzek et al. (Biotechnol. Bioprocess. Eng., 2000 – cited herein).

Sharpe et al. teach:

Regarding claims 163-165, a recombinantly engineered oleaginous yeast is taught wherein said yeast has been engineered to express the heterologous genes: CrtE of geranyl geranyl pyrophosphate synthase (wherein in the instant specification at paragraph 0158 of PG-Pub, CrtE is defined as a geranyl geranyl diphosphate synthase), a CrtB phytoene synthase, and a CrtI phytoene desaturase as well as (See Claims 1 and 2; p. 4, line 32 to p. 5, line 10; p. 13, line 31 to p. 14, line 4; p. 34, line 17 to p. 26, line 18); additional genes are lycopene cyclase (CrtY), carotenoid hydroxylase (CrtZ) and carotenoid ketolase (CrtW).

Regarding claim 164, the term "oleaginous yeast" is to mean yeast selected from *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon* and *Lipomyces* – See p. 29, lines 12-27. It further taught, *Yarrowia lipolytica* is the host cell utilized in all working examples.

Regarding claims 163 and 165, it is taught: "Virtually any promoter capable of directing expression of these genes in the selected host cell is suitable for the present invention." – p. 59, lines 5-7. It is taught inducible promoters or constitutive promoters are suitable for expression of the genes (p. 59).

Sharpe et al., however, do not teach wherein the promoter is one that is responsive to fatty acid or alkane induction.

Juretzek et al. characterize five different promoters for the expression of foreign proteins in the yeast *Yarrowia lipolytica*. Up until that point in time, said yeast had few promoters available for use in the system, despite said yeast being known for several decades in the biotechnology industries for being a good host for expression of foreign genes because it can do so in considerable amounts (See Introduction). It was established that the promoters POX2, POT1 and ICL1 (inducible by fatty acids, fatty acid derivates, alkanes and/or acetate/ethanol, respectively) which all offer the advantage of being inexpensive carbon sources (See last paragraph, p. 325).

Therefore it would have been obvious to one of ordinary skill in the art prior to the effective filing date of the claimed invention to utilize any of the *Yarrowia lipolytica* strong promoters POX2, POT1 and ICL1 as taught by Juretzek et al. and to utilize these in the recombinant *Yarrowia lipolytica* strains expressing the heterologous genes: CrtE of geranyl geranyl pyrophosphate synthase (wherein in the instant specification at paragraph 0158 of PG-Pub, CrtE is defined as a geranyl geranyl diphosphate synthase), a CrtB phytoene synthase, and a CrtI phytoene desaturase as well as (See Claims 1 and 2; p. 4, line 32 to p. 5, line 10; p. 13, line 31 to p. 14, line 4; p. 34, line 17 to p. 26, line 18); additional genes are lycopene cyclase (CrtY), carotenoid hydroxylase (CrtZ) and carotenoid ketolase (CrtW) as taught by Sharpe et al., because Sharpe et al. literally state: "Virtually any promoter capable of directing expression of these genes in the selected host cell is suitable for the present invention." – p. 59, lines 5-7. This would provide motivation in and of itself to utilize any of the known promoters, including POX2, POT1, ICL1, G3P, XRP2, for the species *Yarrowia lipolytica* for directing expression of the enzymes geranyl geranyl pyrophosphate synthase (wherein in the instant

specification at paragraph 0158 of PG-Pub, CrtE is defined as a geranyl geranyl diphosphate synthase), a CrtB phytoene synthase, and a CrtI phytoene desaturase. One skilled in the art would have a reasonable expectation of success that the promoters as taught by Juretzek et al. would work well in the recombinant strains of Sharpe et al. because Juretzek et al. establishes them to be operable strong inducible promoters.

As such, the references when combined render the instant claims as *prima facie* obvious.

**Applicant's Response and Examiner's Rebuttal**:

Applicant's traverse the rejection of record and state the combined references do not render obvious the present claims.

It is first stated that Sharpe as the primary reference is merely a generic document and point to the abstract as providing evidence of this (See Remarks, p. 5, 2nd paragraph).

However, the Examiner points Applicant's specifically to the working examples to demonstrate that Sharpe is anything but a generic document. Specifically expressing a CrtE geranyl geranyl pyrophosphate synthase as well as two other genes crtB and crtI to thus produce the terpenoid lycopene, e.g. one of the same enzymes of claim 163, in the specifies *Yarrowis lipolytica*, e.g. one of the specific genera claimed in instant claim 164. As such, it is entirely unclear how this would be considered by anyone a "generic" disclosure.

With regard to Applicant's assertions that Sharpe describing only *Yarrowia* and not teaching anything regarding any of the other microorganisms as described in claim 164 (See Remarks, p. 5, 3rd paragraph), given that *Yarrowia* is one of the options in the Markush group, teachings of it will necessarily meet the limitations of the claim(s) – MPEP 2131.02(II).

With regard to Applicant's assertion that the promoter is critical to the instant invention and its surprising and unexpected results (See Remarks, p. 5, 5th paragraph), Applicant's never disclose what said results actually might be and why they would be unexpected. As such, without such a showing, merely stating this on the record is not sufficient – See MPEP 716.02(b).

With regard to Applicant's assertions that the secondary reference does not describe "real expression of genes" because the representative gene used for analyzing promoters is beta-galactosidase, which is commonly utilized and easily activated, would not find the entire reference of any use because they do not describe any more "complex genes" is not also not convincing (See Remarks, p.6). This is because Applicant's have not demonstrated other than mere conjecture that beta-galactosidase is not a "complex gene" (although what Applicant's even mean by this is entirely unclear to the Examiner). "Activation" of a gene as indicated by Applicant's remarks completely misses the point that said enzyme, any enzyme or protein recombinantly produced, need a promoter for the production of said foreign gene. Thus, activation of an enzyme (e.g. perhaps it needs a cofactor to function, or it needs to fold correctly so it is not in inclusion bodies) as understood by any enzymologist and molecular biologist is not the

same thing as producing said enzyme when said enzyme is under the control of promoter.

The comparison of the different chemical structures of ricinoleic acid methyl ester and oleic acid, and because they are asserted to be chemically similar is not convincing (See Remarks, p.6). One skilled in the art of both chemistry and structural biology will understand the considerable size difference between the two would likely be the contributing factor in the difference of its activation.

With regard to Applicant's remarks on p. 7-8 that the promoters of Juretzek, specifically POX2, ICL1 and POT1 are not the same ones utilized in the instant examples, this is not convincing because it is noted that the features upon which applicant relies (e.g. the promoters that Applicant's utilize) are not recited in the rejected claim(s). Although the claims are interpreted in light of the specification, limitations from the specification are not read into the claims. See *In re Van Geuns*, 988 F.2d 1181, 26 USPQ2d 1057 (Fed. Cir. 1993).

Finally with regard to Applicant's assertions that one skilled in the art would be directed away from using any of the fatty acid inducible promoters of POX1, ICL1 and POT1 as taught by Juretzek (See Remarks, p. 7, 3rd paragraph) because they state "The POX2 and POT1 promoters are only inducible by fatty acid, what may be a disadvantage for biotechnological applications" (at p. 324, last paragraph of Juretzek) is also not convincing. The rest of the paragraph goes on to state:

> Further studies using these promoters inserted in the newly developed multiple copy integration vectors [12,13] have already been started to elucidate the potential of *Y. lipolytica* for heterologous gene expression. First results of successful high expression of the bovine cytochrome P45017α cDNA (CYP17) and homologous NADPH-cytochrome P450 reductase under the control of the ICL1 promoter [28,34] as well as of the homologous lipase gene LIP2 with the

POX2 promoter (Nicaud, unpublished) demonstrate that these promoters are very useful tools for induction of high expression levels in *Y. lipolytica*.

In this regard, these studies seem to use "complex genes" which are not beta-galactosidase, in *Y. lipolytica,* and are quite successful in achieving high levels of expression.

For these reasons, the rejection of record is maintained.

***Conclusion***

6. No claim is allowed.

7. **THIS ACTION IS MADE FINAL.** Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

8. Any inquiry concerning this communication or earlier communications from the examiner should be directed to SUZANNE M NOAKES whose telephone number is (571)272-2924. The examiner can normally be reached M-F (7-4).

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Manjunath Rao can be reached on 571-272-0939. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of published or unpublished applications may be obtained from Patent Center. Unpublished application information in Patent Center is available to registered users. To file and manage patent submissions in Patent Center, visit: https://patentcenter.uspto.gov. Visit https://www.uspto.gov/patents/apply/patent-center for more information about Patent Center and https://www.uspto.gov/patents/docx for information about filing in DOCX format. For additional questions, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/SUZANNE M NOAKES/
Primary Examiner, Art Unit 1656
25 July 2022

| Search Notes | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 16/630,567 | Aeling et al. |
| | Examiner | Art Unit |
| | SUZANNE M NOAKES | 1656 |

| CPC - Searched* | | |
|---|---|---|
| Symbol | Date | Examiner |
| C12N 15/815 w/text in EAST | 02/15/2022 | SMN |

| CPC Combination Sets - Searched* | | |
|---|---|---|
| Symbol | Date | Examiner |
| | | |

| US Classification - Searched* | | | |
|---|---|---|---|
| Class | Subclass | Date | Examiner |
| | | | |

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

| Search Notes | | |
|---|---|---|
| Search Notes | Date | Examiner |
| Inventors names (all) in EAST and PE2E | 02/15/2022 | SMN |
| EAST: See attached | 02/15/2022 | SMN |
| ProQuest Dialog: see attached | 02/15/2022 | SMN |
| Updated in SEARCH: See attached | 07/25/2022 | SMN |

| Interference Search | | | |
|---|---|---|---|
| US Class/CPC Symbol | US Subclass/CPC Group | Date | Examiner |
| | | | |



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Page 2930 of 3077

## PE2E SEARCH - Search History (Prior Art)

| Ref # | Hits | Search Query | DBs | Default Operator | Plurals | British Equivalents | Time Stamp |
|---|---|---|---|---|---|---|---|
| L64 | 1046 | ((("AELING") near3 ("Kimberly")) OR (("DE") near3 ("WALS") near3 ("Pierre-Yves")) OR (("SCOTT") near3 ("Eric")) OR (("WALDBRIDGE") near3 ("Michael")) OR (("BEARDSLEE") near3 ("Thomas"))).INV. | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | OFF | OFF | 2022/02/15 07:59 AM |
| L65 | 36 | L64 and terpene | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:00 AM |
| L66 | 6 | L64 and terpene and phytoene | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:00 AM |
| L67 | 1648 | ("phytoene synthase" or CftB or "prephytoene-diphosphate synthase" or "15-cis-phytoene synthase" or PSase or "geranylgeranyl-diphosphate geranylgeranyltransferase" or "2.5.1.32") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:09 AM |
| L68 | 6520 | ("phytoene desaturase" or "3,4-didehydrolycopene-forming phytoene desaturase" or "1.3.99.28" or CrtI or "1.3.99.30" or CsCrtI or XdCrtI or PaCrtI) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:12 AM |
| L69 | 4105 | ("geranylgeranyl diphosphate synthase" or BTS1 or CrtE or GGPS1 or GGPPS or GGPPS1 or "geranylgeranyl diphosphate synthase 1" or "2.5.1.29") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:14 AM |
| L70 | 528 | L67 and L68 and L69 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:14 AM |
| L71 | 415 | L67 same3 L68 same3 L69 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:14 AM |
| L72 | 360 | L67 same L68 same L69 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:14 AM |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| L73 | 38 | L72 and (POX4 or POT or PEX or POX18 or ANT1 or DHE or ARE1) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:15 AM |
| L74 | 5 | ("20030044946"\|"20130066035"\|"20130302861"\|"20140113343"\|"20150010978").PN. | (US-PGPUB; USPAT) | OR | OFF | OFF | 2022/02/15 09:01 AM |
| L75 | 171 | L72 and (Candida or Yarrowia) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 09:10 AM |
| L76 | 21 | L72 same (Candida or Yarrowia) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 09:10 AM |
| L77 | 1648 | ("phytoene synthase" or CftB or "prephytoene-diphosphate synthase" or "15-cis-phytoene synthase" or PSase or "geranylgeranyl-diphosphate geranylgeranyltransferase" or "2.5.1.32") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 10:57 AM |
| L78 | 6520 | ("phytoene desaturase" or "3,4-didehydrolycopene-forming phytoene desaturase" or "1.3.99.28" or CrtI or "1.3.99.30" or CsCrtI or XdCrtI or PaCrtI) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 10:57 AM |
| L79 | 4105 | ("geranylgeranyl diphosphate synthase" or BTS1 or CrtE or GGPS1 or GGPPS or GGPPS1 or "geranylgeranyl diphosphate synthase 1" or "2.5.1.29") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 10:57 AM |
| L80 | 360 | L77 same L78 same L79 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 10:57 AM |
| L81 | 16 | L80 and C12N15/815.cpc. | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 10:57 AM |
| L82 | 1102 | ((("AELING") near3 ("Kimberly")) OR (("DE") near3 ("WALS") near3 ("Pierre-Yves")) OR (("SCOTT") near3 ("Eric")) OR (("WALDBRIDGE") near3 ("Michael")) OR (("BEARDSLEE") near3 ("Thomas"))).INV. | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | OFF | OFF | 2022/07/25 12:07 PM |
| L83 | 37 | L64 and terpene | (US-PGPUB; USPAT; | OR | ON | ON | 2022/07/25 |



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Page 2932 of 3077

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | USOCR; EPO; JPO; DERWENT) | | | | 12:07 PM |
| L84 | 6 | L64 and terpene and phytoene | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L85 | 1713 | ("phytoene synthase" or CftB or "prephytoene-diphosphate synthase" or "15-cis-phytoene synthase" or PSase or "geranylgeranyl-diphosphate geranylgeranyltransferase" or "2.5.1.32") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L86 | 6381 | ("phytoene desaturase" or "3,4-didehydrolycopene-forming phytoene desaturase" or "1.3.99.28" or CrtI or "1.3.99.30" or CsCrtI or XdCrtI or PaCrtI) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L87 | 4424 | ("geranylgeranyl diphosphate synthase" or BTS1 or CrtE or GGPS1 or GGPPS or GGPPS1 or "geranylgeranyl diphosphate synthase 1" or "2.5.1.29") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L88 | 538 | L67 and L68 and L69 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L89 | 430 | L67 same3 L68 same3 L69 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L90 | 368 | L67 same L68 same L69 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L91 | 42 | L72 and (POX4 or POT or PEX or POX18 or ANT1 or DHE or ARE1) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L92 | 5 | ("20030044946"\|"20130066035"\|"20130302861"\|"20140113343"\|"20150010978").PN. | (US-PGPUB; USPAT) | OR | OFF | OFF | 2022/07/25 12:07 PM |
| L93 | 176 | L72 and (Candida or Yarrowia) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L94 | 21 | L72 same (Candida or Yarrowia) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L95 | 1713 | ("phytoene synthase" or CftB or "prephytoene- | (US-PGPUB; USPAT; USOCR; EPO; JPO; | OR | ON | ON | 2022/07/25 12:07 PM |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | diphosphate synthase" or "15-cis-phytoene synthase" or PSase or "geranylgeranyl-diphosphate geranylgeranyltransferase" or "2.5.1.32") | DERWENT) | | | | |
| L96 | 6381 | ("phytoene desaturase" or "3,4-didehydrolycopene-forming phytoene desaturase" or "1.3.99.28" or CrtI or "1.3.99.30" or CsCrtI or XdCrtI or PaCrtI) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L97 | 4424 | ("geranylgeranyl diphosphate synthase" or BTS1 or CrtE or GGPS1 or GGPPS or GGPPS1 or "geranylgeranyl diphosphate synthase 1" or "2.5.1.29") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L98 | 368 | L77 same L78 same L79 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L99 | 16 | L80 and C12N15/815.cpc. | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |

## PE2E SEARCH - Search History (Interference)

There are no Interference searches to show.

Doc code: RCEX
Doc description: Request for Continued Examination (RCE)

PTO/SB/30EFS (01-22)
Approved for use through 05/31/2024. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

# REQUEST FOR CONTINUED EXAMINATION(RCE)TRANSMITTAL
## (Submitted Only via EFS-Web)

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Application Number | 16/630,567 | Filing Date | 2020-01-13 | Docket Number (if applicable) | SS118-1026US | Art Unit | 1656 |
| First Named Inventor | Kimberly Ann Aeling | | | Examiner Name | Suzanne Marie NOAKES | | |

**This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.**
Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. The Instruction Sheet for this form is located at WWW.USPTO.GOV

### SUBMISSION REQUIRED UNDER 37 CFR 1.114

Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

- ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____
- ☐ Other _____

☒ Enclosed

- ☒ Amendment/Reply
- ☐ Information Disclosure Statement (IDS)
- ☒ Affidavit(s)/ Declaration(s)
- ☐ Other _____

### MISCELLANEOUS

☐ Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of months _____ (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

☐ Other _____

### FEES

☒ **The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.**
The Director is hereby authorized to charge any underpayment of fees, or credit any overpayments, to Deposit Account No 506423

### SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

☒ Patent Practitioner Signature

Applicant Signature

PTO/SB/30EFS (01-22)
Approved for use through 05/31/2024. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

| Signature of Registered U.S. Patent Practitioner | | | |
|---|---|---|---|
| Signature | /Silvia Salvadori/ | Date (YYYY-MM-DD) | 2022-10-20 |
| Name | Silvia Salvadori | Registration Number | 48265 |

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450.

*If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.*

## Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether the Freedom of Information Act requires disclosure of these records.

2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.

3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.

4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).

5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.

6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).

7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.

8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspections or an issued patent.

9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.



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Ex. 1002
Page 2937 of 3077

UNITED STATES
PATENT AND TRADEMARK OFFICE

P.O. Box 1450
Alexandria, VA 22313 - 1450
www.uspto.gov

# ELECTRONIC ACKNOWLEDGEMENT RECEIPT

| APPLICATION # | RECEIPT DATE / TIME | ATTORNEY DOCKET # |
|---|---|---|
| **16/630,567** | **10/20/2022 09:54:20 AM ET** | **SS118-1026US** |

## Title of Invention

BIOLOGICAL METHODS FOR PREPARING TERPENES

## Application Information

| | | | |
|---|---|---|---|
| APPLICATION TYPE | Utility - U.S. National Stage under 35 USC 371 | PATENT # | - |
| CONFIRMATION # | 5356 | FILED BY | Silvia Salvadori |
| PATENT CENTER # | 61086167 | FILING DATE | 01/13/2020 |
| CUSTOMER # | 122515 | FIRST NAMED INVENTOR | Kimberly Ann Aeling |
| INTL. APPLICATION # | - | INTL. FILING DATE | - |
| CORRESPONDENCE ADDRESS | - | AUTHORIZED BY | - |

## Documents

**TOTAL DOCUMENTS: 5**

| DOCUMENT | | PAGES | DESCRIPTION | SIZE (KB) |
|---|---|---|---|---|
| RCE.pdf | | 3 | Request for Continued Examination (RCE) | 1642 KB |
| Response.pdf | | 14 | - | 86 KB |
| Response-AMSB.pdf | (1-1) | 1 | Amendment Submitted/Entered with Filing of Continued Prosecution Application (CPA)/Request for Continued Examination(RCE) | 30 KB |
| Response-CLM.pdf | (2-3) | 2 | Claims | 41 KB |

| | | | | |
|---|---|---|---|---|
| Response-REM.pdf | (4-14) | 11 | Applicant Arguments/Remarks Made in an Amendment | 75 KB |
| Declaration.pdf | | 12 | Affidavit-traversing rejections or objections rule 132 | 944 KB |

## Digest

| DOCUMENT | MESSAGE DIGEST(SHA-512) |
|---|---|
| RCE.pdf | ED444DE25418EB662807DEF8E36F505F037AB5997ED80D1B19BFCD603639E9A727D8E87280865DC1C3E94DB8F74BB51E49E7C907D9E8B14C99895BE6BA574184 |
| Response.pdf | 8F59468AB0C20DF289EFF8CB10777AF339D891CF8CF071F693643AE951F98DEFB986606E4C2B0FBD70438C29B98E0F3925B20BF327D84C4525A462B1D8D0F15F |
| Response-AMSB.pdf | 9FCE057FC55A77DF615BD568BB27FB70D0FDA3EB6B4265E13138ACC08F4C37E0A063BFEA86086CEC62384BC4473384D42B8A502C2526E7041FC6C13FAD052FA6 |
| Response-CLM.pdf | 74AA1F20717343FE6FF5338EC6E35339B80058B4938DFE1ECB0282923E8CE7335BFA35EA0EC5F409B504ADAF5EDB6699351776D01C9C49C058C1DABD02FD2A3B |
| Response-REM.pdf | 6E9AF7F883142112A512499640E22B3484CB28A63DBFF663CC345810C1F2E40A01A3463EB1EC38ACD829CAAB67CEA626D020A9DB9CBE3D68A66FC25BB7F3EE4D |
| Declaration.pdf | C948F3B4D0CF0899FAC44309E7A712CEA4610E4F049EAD6203D2AC364E3DB11305F4143C2D3DB2DD6282E6FF670B99FCBCFEDD9A390185B36D2B635A6A78B9D7 |

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

**New Applications Under 35 U.S.C. 111**

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

**National Stage of an International Application under 35 U.S.C. 371**

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

**New International Application Filed with the USPTO as a Receiving Office**

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

| | | |
|---|---|---|
| In re Application of | : | |
| App. No | : | 16/630,567 |
| Filed | : | January 13, 2020 |
| For | : | BIOLOGICAL METHODS FOR PREPARING TERPENES |
| Examiner | : | Suzanne Marie Noakes |
| Art Unit | : | 1656 |
| Conf No. | : | 5356 |

**DECLARATION UNDER 37 C.F.R §1.132**

Mail Stop Amendments
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. I, Thomas Beardslee, am an inventor of the present application.

2. I duly sworn depose and say that:

- I am a citizen of the U.S.A. residing at 6827 Carnation Drive, Carlsbad CA 92011
- I am fluent in the English language.
- I graduated with a Ph.D. in Biochemistry in the academic year 1999-2000.

3. I have extensive experience in the fields of synthetic biology, metabolic engineering, and fermentation process development. I was the Vice President of Research and Development at Verdezyne from 2012 to 2018 and currently I work at Rynetech Bio as the Founder and CEO. Rynetech Bio has been developing technology for the fermentation-based manufacturing of renewable chemicals for over 3 years. In my capacity as VP of R&D at Verdezyne in the past and in my current position as CEO of Rynetech Bio, I have been and am currently involved in the development of engineered yeast for the production of renewable chemicals by fermentation from fatty acid

feedstocks. This work has resulted in several patented inventions and publications. My *Curriculum vitae* is attached hereto.

4. I have reviewed the office action dated July 29th, 2022 issued for US patent application 16/630,567, including the two references cited therein, namely WO 2008/073367 A1 (hereinafter "Sharpe") in combination with Juretzek et al., Biotechnol. Bioprocess. Eng., 2000, (hereinafter "Juretzek").

5. I understand that the United States Patent and Trademark Office ("USPTO") has taken the position that Claims 163-165 are not inventive under 35 U.S.C. 103, in the light of "Sharpe" in combination with "Juretzek". Specifically, the USPTO has taken the position that the combination of the teachings of Sharpe and Juretzek renders the invention obvious.

6. I am presenting this declaration to show that Claim 163, and its dependent claims, of the above-referenced US patent application are inventive over the combination of the teachings of Sharpe and Juretzek.

7. Moreover, I am presenting this declaration to show the technical background behind any inventive choice leading to the unexpected result of providing a specific genetically modified microorganism able to unexpectedly and suprisingly achieve the fatty acid-induced expression of one or more terpene biosynthesis polypeptides. In fact, microorganic industrial production can minimize the use of caustic chemicals and the production of toxic byproducts, thus providing a "clean" source for certain compounds. The use of appropriate plant-derived feedstocks allows production of "green" compounds while further minimizing the need for and use of petroleum-derived compounds (page 4, lines 25-29 of the present Application).

8. I note that the specific genetically modified microorganism of the present invention specifically comprises a promoter modified by replacing the endogenous promoters with a promoter comprising an alkane response element (ARE1) (page 30 lines 19-20, page 33, lines 10-12, page 35 lines 25-27, page 38, lines 1-3, page 40, lines 15-18, etc of present Application) or a fatty acid-inducible promoter (page 48, line 10 of present Application) directing the flux of carbons from fatty acids toward a particular target product pathway (e.g., mevalonate production, isopentenyl diphosphate production, geranyl diphosphate production, farnesyl diphosphate production, geranylgeranyl diphosphate production, terpene production, carotenoid production, beta carotene production, lycopene production, astaxanthin production) and away from other cellular metabolic pathways not involved in target molecule production (page 29, lines 29-34 and page 46, lines 26-34 of present Application).



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9. I note that the Examiner is of the opinion that the assertion that the promoter is critical to the instant invention and its surprising and unexpected results are never disclosed and the surprising technical improvements are, thus, not clear (page 6 of the Office Action).

10. In view of this objection, I herein declare that the mandatory nature of the nucleic acid that provides for fatty acid induction of expression of a terpene biosynthesis polypeptide being a promoter, as recited in new claim 163, is extremely important and critical to achieve the technical problem underlined by the present Application.

11. In fact, it is to be highlighted that in any fermentation system, promoters should be not only present but also, carefully selected based upon the production organism and the fermentation conditions present during production. If the promoters selected are effectively "off" in the conditions present during fermentation, then no gene product (enzyme) will be made, rendering the intended engineered biochemical pathway useless for production.

12. I note that the fermentation system the presently claimed invention, i.e., the genetically modified microorganism, as described in claim 163, comprises a fatty acid as desired carbon source. The technical reason behind this choice is of biochemical efficiency, letting the yeast save the biochemical energy that it takes for de novo fatty acid synthesis. The fats used as feedstock can be used by the yeast through a salvage pathway (instead of de novo synthesis) to produce lipid bodies. Lipid bodies provide the intracellular storage compartment for hydrophobic products such as the 40-carbon carotenoids (and can be used for other hydrophobic terpenes). With the choice of fatty acid as carbon source, the nucleic acid that provides for fatty acid induction of expression of a terpene biosynthesis polypeptide must be carefully chosen and in the presently claimed invention, it must be a promoter that induces the expression of enzymes in the desired biochemical pathway(s).

13. I note that the Examples of the present Application, which show the use of several promoters, such as hydratase-dehydrogenase-epimerase (HDE), peroxisomal 18-kDa protein (POX18), 2,4-dienoyl-CoA reductase (SPS19), acyl-CoA oxidase 4 (POX4), dienoyl CoA Isomerase (DCI) and acyl-CoA oxidase (POX5), demonstrate the achievement of the result.

14. Moreover, I note that all these promoters have in common the ability of being a nucleic acid, which provides for fatty acid induction of expression of a terpene biosynthesis polypeptide in a genetically modified microorganism.

15. I observe that the Examiner is of the opinion that the invention as disclosed in Claims 163-165 is not inventive over the combination of Sharpe and Juretzek.





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16. I have carefully read Sharpe and I note that it is a document regarding engineered strains of the oleaginous yeasts capable of producing carotenoids (Abstract of Sharpe) and, more importantly, that said document does not teach nor mentions any promoter of said yeast that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide, as also recognized by the Examiner (page 3 of the Office Action).

17. I note that the Examiner is of the opinion that Sharpe would have suggested a promoter, since it mentions that "Virtually any promoter capable of directing expression of these genes in the selected host cell is suitable for the present invention".

18. I respectfully disagree with the opinion of the Examiner, and I do not consider this sentence as a teaching that would allow the person skilled in the art to find suitable promoters for the specific biological system herein proposed.

19. In fact, I declare that the specific choice of a promoter for any biological system will surely require an extensive trial and error and a deep understanding of the processes and the parameters to be checked and selected. In fact, the necessity of the use of a promoter, its precise choice and its specific modifications, depend on several crucial parameters, such as: - the specific induction upon exposure to the nutrients (which only CAN be but surely not mandatorily be fatty acids), - the specific fermentation system used (which in the present Application is a specific genetically modified microorganism, while in the prior arts cited are either generic oleaginous yeasts structurally different or promoters suitable for regulating the overexpression of beta-galactosidase in the yeast *Yarrowia lipolytica*), - the biochemical pathways desired, and - the overall level of expression of the targeted genes.

20. In view of the above, I note that Sharpe, when considered as a whole, teaches, and suggests only a way to synthesize generic oleaginous yeast for the production of carotenoids and polyunsaturated fatty acids (PUFA's), without taking into account any fatty acid-inducible promoter, thus not solving the technical problem addressed by the present Application. Hence, Sharpe would have not been useful for the person skilled in the art in reaching the solution, since Sharpe does not even mention any promoter of the yeast that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide.

21. I note that the Examiner further combines a second document Juretzek, which presents a comparison of different promoters suitable for regulating the overexpression of beta-galactosidase in the yeast *Yarrowia lipolytica* (Title and Abstract of Juretzek).



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22. I observe that the Examiner is of the opinion that the person skilled in the art would have easily combined the teachings regarding the promoters of the yeast *Yarrowia lipolytica,* and more specifically POX2, POT1 and ICL1, described in Juretzek, and used them in order to express the heterologous genes of terpene biosynthesis polypeptides.

23. Furthermore, the Examiner is of the opinion that beta-galactosidase can be used as example of an expression of genes.

24. In view of the above, I declare that beta-galactosidase is a single gene product that is easily expressed in many microbiological systems as an enzyme with an easy activity assay, and, precisely due to its easy expression and activity assay, beta-galactosidase is mostly used as gene-test or control for studies regarding the expression regulated by promoters, and it is a sort of protocol, which cannot be considered as representative of all promoter-gene combinations and all situations.

25. I carefully read Juretzek and I note that, not only does it not list, nor suggest any of the promoters useful for the biological system of the present Application, but also discourages the use of fatty acid promoters in general, since Juretzek explains that "*These results present ricinoleic acid methyl ester as the strongest inducer of pPOX2 and pPOT1 among the tested carbon sources, in spite of the disadvantage that this carbon source inhibits growth of Y. lipolytica*" (page 324, right column of Juretzek).

26. Moreover, I note that Juretzek clearly lists some specific promoters, able to achieve the result claimed in the present Application (and thus listed and described in the present Application), such as POX2 and POT1, stating that "*the POX2 and POT1 promoters are only inducible by fatty acid, fatty acid derivatives or alkanes, what may be, at least in some respect, a disadvantage for biotechnological applications*" (page 324, right column of Juretzek).

27. Therefore, I declare that the promoters used to drive the genes in the terpene biochemical pathways were chosen based on mRNA sequencing data. The samples were taken from a two-stage fed-batch fermentation using glucose as the carbon source for the growth phase and oleic acid as the feedstock for the subsequent production phase (once glucose was exhausted). Samples were taken in a time-course before and after the switch to oleic acid. Promoters were chosen based not only on being induced upon exposure to fatty acids, but also on having an overall high level of expression. The mRNA sequencing data revealed that 6 of the top 10 promoters in terms of overall expression level are those recited in new claim 184 and described in the Application.



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28. I note that the promoters suggested by Juretzek were selected for a specific biotechnological system, namely *Yarrowia* on oleic acid. However, in the case of different systems, such as for example a *Candida viswanathii* system, these promoters (POX2, POT1, and ICL1) showed in the mRNA sequencing data very poor overall expression levels on oleic acid. Hence, even if the person skilled in the art reading Juretzek would have taken into account the teachings contained thereof, he/she would have chosen poorly working promoters, hence he/she would have never reached the solution for the technical problem above described.

29. In view of the above, I declare that the person skilled in the art, while combining the teachings of Sharpe and Juretzek, would have surely considered that long chain fatty acid feedstocks could lead to damage of the microorganism or at least a disadvantage in biotechnological production, instead of considering fatty acids the real protagonist, playing the main role in the biosynthesis. To avoid any damage to the *Y. lipolytica,* he/she would have surely been directed to finding another solution which removes the use of fatty acid-inducible promoters. If fatty acids were still used, he/she would at least consider different promoters, specifically POX2, ICL1 and POT1 indicated as the most promising in Juretzek, with respect to the ones chosen and tested in present Application, hence reaching a different solution, with respect to the invention presently claimed.

30. In conclusion, the microorganism of new claim 163 and its dependent claims represent a new and inventive alternative to known microorganism, due to the specific and non-obvious choice of each of its mandatory technical features, not present nor suggested by the combination of the documents cited by the Examiner.

31. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated October 12, 2022 Signed 



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**Thomas A. Beardslee, Ph.D.**
6827 Carnation Drive, Carlsbad CA 92011 • (858) 336-2457 • thomasabeardslee@gmail.com

**Summary**

Biotechnology professional and commercially focused leader with experience leading multi-disciplinary scientific teams and managing multimillion dollar R&D programs. Led teams of up to 50 scientists with specialties in molecular biology, metabolic engineering, fermentation, protein engineering, proteomics, and analytical chemistry. Experienced program manager leading new product development from ideation to commercial launch and inventor on over twenty patents and patent applications. International experience with technology transfer and scale-up in India, Europe, and Southeast Asia.

**Professional Experience**

Rynetech Bio, Inc., Carlsbad CA
October 2018 – Present: Founder/CEO

- Negotiated IP purchase and licensing terms for yeast synthetic biology platform from the sale of assets from predecessor company
- Executed a successful JDA with Radici Chimica (Italy) to improve yields and scale up fermentation production resulting in multi-ton quantities of adipic acid for renewable nylon synthesis
- Executed a successful JDA with a US-based biomanufacturer hitting production level targets for three terpene molecules
- Sublicensed the production of eight products to two companies

Verdezyne, Inc., Carlsbad CA
October 2012 – May 2018: Vice President, Research and Development
July 2011 – October 2012: Senior Director, Renewable Chemicals
November 2008 – July 2011: Director, Renewable Chemicals

- Led a team of 50 scientists comprising metabolic engineering, fermentation process development, bioinformatics, protein engineering, analytical chemistry and downstream product isolation.
- Developed a non-traditional yeast fermentation technology platform for the conversion of renewable fatty acid feedstocks to high value target chemicals. Demonstrated the proof of concept for the production of sixteen different chemicals achieving commercially relevant production for five (adipic acid, dodecanedioic acid, lycopene, beta-carotene, and canthaxanthin).
- Advised the CEO and Board of Directors on the progress of R&D programs towards timeline goals as well as the strategic direction of the company.
- Supervised the scale-up of two fermentation processes to commercial scale (up to 180 $m^3$) working with external partners.
- Advised internal and external engineering teams towards the construction of the commercial-scale (6 kta capacity) VerdePalm facility located in Nusajaya, Malaysia for the production of renewable DDDA.



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- Led the regulatory process for importation of the Verdezyne GMO yeast into four different countries including for commercial use in Malaysia.
- Program manager for the metabolic engineering of the diploid yeast *Candida viswanathii* to produce adipic acid from fatty acid feedstocks. Demonstrated feedstock flexibility in the fermentation process by using over 50 different feedstocks considered low value co-products or waste products.
- Developed a product pipeline that leveraged the metabolic engineering of previous programs to shorten product development timelines.
- Presented at numerous industry conferences and university lectures around the world on topics ranging from the highly technical to the general state of the bio-based industry.
- Worked with human resources in the recruitment and development of scientific staff including yearly personnel evaluations, 360 reviews, and management training for scientific managers.

CODA Genomics, Laguna Hills CA (Predecessor Company to Verdezyne, Inc.)
October 2007 – November 2008: Director of Project Design

- Worked directly with customers to design custom projects solving protein expression problems. Services managed include gene sequence optimization, gene variant library design, custom cloning, expression testing (*E. coli, S. cerevisiae, and P. pastoris*), small-scale protein expression, pilot protein expression, and protein purification.
- Developed strategic corporate partnerships to broaden the company's product offering.
- Evaluated new technology and R&D programs, providing guidance on research direction for the company as a whole.

Invitrogen Corporation, Carlsbad CA (now Thermo Fisher Scientific)
October 2006 – October 2007: Senior Scientist
July 2002 – October 2006: Scientist

- Program manager, Human Proteome Organization (HUPO) proteomic standards for mass spectrometry. Coordinated all aspects of new product development with team members from marketing, engineering, intellectual property, regulatory and manufacturing.
- Collaborated with HUPO leadership in the development and testing of a panel of highly purified recombinant human proteins. Twenty tagless recombinant human proteins were expressed in *E. coli*, purified to >95%, formulated at 5 picomoles per protein, and sent to HUPO test labs around the world for validation.
- Coordinated the transfer of recombinant protein production and purification manufacturing processes to Invitrogen Bioservices India (Bangalore).
- Contributed scientific evaluation of technologies in strategic planning for future directions of new product development.
- IDEO University, trained in the process used by one of the world-leading industrial engineering firms, IDEO, for product and process innovation.
- Development of new electrophoresis technologies for proteomic analysis: IPG strips, solution phase IEF, and precast mini-gels for denaturing and native electrophoresis.
- Program manager, Native Complex Purification & Analysis program. Products launched Q1, 2006 include NativePAGE™ gels for blue-native electrophoresis, NativePAGE™ sample preparation kit, NativeMark™ unstained markers, and NativePure™ vectors for



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mammalian expression (pcDNA and lentiviral based) and purification of native protein complexes.
- Presented proteomics sample preparation seminars to customers at research centers across the U.S.
- R&D contact for the Technical Services department to support detailed technical resolution of customer issues with all proteomics products in the ZOOM® product line. Technical assistance included direct customer interaction via email, phone, and in person for customers at academic, research institution, and industrial accounts.

University of Nebraska-Lincoln, Lincoln NE
August 2000 – June 2002: Postdoctoral Research Associate in the lab of Dr. Lori Allison, Biochemistry Department
- Expressed four newly discovered maize multi-subunit RNA polymerase sigma factor proteins in *E. coli*. Optimization of expression included employing rare tRNA expressing strain of *E. coli* and thioredoxin fusion partner to enhance solubility during refolding. Purified recombinant proteins were used for antibody production and *in vitro* activity assays.
- Determined the subcellular localization of maize sigma factors by immunoblotting, and by confocal microscopy *in situ* immunolocalization.
- Demonstrated the activity of maize sigma factors using an electrophoretic mobility shift assay showing protein-protein and protein-DNA interactions.
- Lab manager: ordered supplies, organized equipment and storage of chemicals, supervised the activities of two graduate and three undergraduate students.

Fall semester 2001: Instructor, Biochemistry 431/831, Biochemistry Department
- Taught the first semester of Senior and Graduate student level Biochemistry for a class of approximately seventy students.
- Prepared and delivered lectures using traditional, multi-media, and on-line resources.
- Hosted an on-line discussion board for student assistance outside of office hours.
- Composed and administered examinations for evaluation of student performance.

August 1996-August 2000: Graduate Research Assistant in the labs of Dr. Mike Zeece (Food Science Department) and Dr. John Markwell (Biochemistry Department)
- Identified IgE epitopes of a major soybean storage protein using soy-sensitive human serum in immunoblotting each of the following: proteolytic fragments of native protein, recombinantly expressed (*E. coli*) purified protein fragments, and overlapping synthetic peptides.
- Assay development of an ELISA using purified fusion proteins for the identification and quantification of the interaction between soy-sensitive serum IgE and protein allergens.
- Collaborated with physicians for the collection of human sera. In charge of the storage, cataloging, use, and disposal of human sera.
- Lab manager: ordered supplies, organized equipment and storage of chemicals, supervised the activities of three undergraduate students.

Pius X High School, Lincoln NE
August 1993 – May 1995: Chemistry/Advanced Placement Chemistry Teacher
- Taught five sections of Chemistry and one section of A.P. Chemistry



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- Head Coach girls soccer, Assistant Coach wrestling, Assistant Coach volleyball

**Education**

- University of Nebraska-Lincoln, PhD, Biochemistry, August, 2000. Dissertation: IgE Epitope Mapping of Soybean Glycinin G1 Acidic Chain
- Creighton University, Omaha, NE, Secondary Education Teaching Certification, May 1993
- Creighton University, Omaha, NE, BS, Chemistry, May 1992

**Honors/Awards**

- E.P.A. Presidential Green Chemistry Challenge Award Winner for Commercialization of Renewable Dodecanedioic Acid, 2016.
- Bloomberg New Energy Pioneers Award Winner for Commercialization of Renewable Dodecanedioic Acid, 2016.
- UCSD Connect, Most Innovative New Product, Finalist 2002. Team member for the development of the Invitrogen ZOOM® IPG System.
- University of Nebraska-Lincoln Teaching Council/Parents Association Recognition Award, fall 2001. For effective teaching and nurturing of students, as voted on by University students.
- Milton E. Mohr Fellowship, academic year 1999-2000. Recognizing outstanding students performing research in the sciences of biotechnology and engineering.
- Farmers National Company Fellowship, academic year 1998-1999. Recognizing excellence in teaching.

**Publications/Patents/Patent Applications**

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**Beardslee T** (2019) Biological methods for modifying cellular carbon flux. Subject of patent application WO/2019/014309.

**Beardslee T**, Picataggio S, Hutagalung A, Fahland T (2018) Biological methods for preparing a fatty dicarboxylic acid. Subject of U.S. Patent 9,957,512.

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Diller T, **Beardslee T**, Rooney R, Amshey J. (2005) Isoelectric focusing gels and methods of use thereof. Subject of U.S. Patent Application #US20050103629A1.

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**Beardslee TA**, Zeece MG, Sarath G, and Markwell JP (2003) Method for allergen characterization. Subject of U.S. Patent Application #US20030166518A1.

**Beardslee TA**, Roy-Chowdury S, Jaiswal P, Buhot L, Lerbs-Mache S, Stern DB, Allison LA (2002) A nucleus-encoded maize protein with sigma factor activity accumulates in mitochondria and chloroplasts. Plant J. 31: 199-209.

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UNITED STATES
PATENT AND TRADEMARK OFFICE

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# ELECTRONIC PAYMENT RECEIPT

| APPLICATION # | RECEIPT DATE / TIME | ATTORNEY DOCKET # |
|---|---|---|
| 16/630,567 | 10/20/2022 09:54:20 AM ET | SS118-1026US |

## Title of Invention

BIOLOGICAL METHODS FOR PREPARING TERPENES

## Application Information

| | | | |
|---|---|---|---|
| APPLICATION TYPE | Utility - U.S. National Stage under 35 USC 371 | PATENT # | - |
| CONFIRMATION # | 5356 | FILED BY | Silvia Salvadori |
| PATENT CENTER # | 61086167 | AUTHORIZED BY | - |
| CUSTOMER # | 122515 | FILING DATE | 01/13/2020 |
| INTL. APPLICATION # | - | INTL. FILING DATE | - |
| CORRESPONDENCE ADDRESS | - | FIRST NAMED INVENTOR | Kimberly Ann Aeling |

## Payment Information

| PAYMENT METHOD | PAYMENT TRANSACTION ID | PAYMENT AUTHORIZED BY |
|---|---|---|
| CARD / 4002 | E20220J055410834 | Silvia Salvadori |

| PRE-AUTHORIZED ACCOUNT | PRE-AUTHORIZED CATEGORY |
|---|---|
| 506423 | 37 CFR 1.21 (Miscellaneous fees and charges) |

| FEE CODE | DESCRIPTION | ITEM PRICE($) | QUANTITY | ITEM TOTAL($) |
|---|---|---|---|---|
| 1801 | REQUEST FOR CONTINUED EXAMINATION (RCE) - 1ST REQUEST (SEE 37 CFR 1.114) | 1360.00 | 1 | 1360.00 |

| | |
|---|---|
| TOTAL AMOUNT: | $1,360.00 |

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

**New Applications Under 35 U.S.C. 111**

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

**National Stage of an International Application under 35 U.S.C. 371**

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

**New International Application Filed with the USPTO as a Receiving Office**

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Atty Docket No.: SS118-1026US

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

| | | | |
|---|---|---|---|
| In re Application | : | Aeling et al. | Confirmation No. 5356 |
| Serial No. | : | 16/630,567 | |
| Filing Date | : | January 13, 2020 | |
| Group Art Unit | : | 1656 | |
| Examiner | : | Noakes, S.M. | |
| For | : | **BIOLOGICAL METHODS FOR PREPARING TERPENES** | |

Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

**37 C.F.R. § 1.114 AMENDMENT AND RESPONSE**

Sir:

This *37 C.F.R. § 1.114 Amendment and Response* is filed concurrently with a Request for Continued Examination (RCE) in response to the Final Office Action of July 29, 2022 issued in connection with the above-identified matter. Thus, this *37 C.F.R. § 1.114 Amendment and Response* is timely filed.

**Amendments to the Claims** begin on page 2 of this paper.

**Remarks** begin on page 4 of this paper.



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**AMENDMENTS TO THE CLAIMS**

This listing of claims will replace all prior versions, and listings, of the claims in this application.

1-162. (Cancelled).

163. (Currently Amended) A genetically modified microorganism, comprising:

one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides, wherein expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide:

- wherein the one or more heterologous nucleic acids that encode one or more terpene biosynthesis polypeptides is chosen from terpene synthase, phytoene synthase, geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, zeaxanthin glucosyltransferase and valencene synthase,

wherein the nucleic acid that provides for said fatty acid induction of expression of the terpene biosynthesis polypeptide is a promoter, or

- wherein the one or more heterologous nucleic acids is a cytochrome P450 reductase and is regulated by a nucleic acid that provides for alkane induction of expression of the cytochrome P450 reductase.

164. (Previously Presented) The microorganism of claim 163, wherein the microorganism is a yeast selected from *Candida spp*, *Yarrowia spp*, *Rhodotorula spp*, *Rhodosporidium spp*, *Cryptococcus spp*, *Trichosporon spp*, *Lipomyces spp*, and *Blastobotrys spp*.



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165. (Previously Presented) The microorganism of claims 164, wherein the fatty acid is a saturated fatty acid or an unsaturated fatty acid selected from one or more of oleic acid, palmitoleic acid, erucic acid, linoleic acid, palmitic acid, caproic acid, enanthic acid, caprylic acid pelargonic acid, capric acid, undecylic acid, lauric acid, myristic acid, pentadecanoic acid, margaric acid, stearic acid arachidic acid, behenic acid, tridecylic acid, and linolenic acid, and wherein the alkane is selected from one or more of hexane, heptane, nonane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, hexadecane, heptadecane, and octadecane.

166-183 (Cancelled).

184. (New) The microorganism of claim 163, wherein the promoter of the nucleic acid that provides for said fatty acid induction of expression of said terpene biosynthesis polypeptide is selected from the group consisting of hydratase-dehydrogenase-epimerase (HDE), peroxisomal 18-kDa protein (POX18), 2,4-dienoyl-CoA reductase (SPS19), acyl-CoA oxidase 4 (POX4), dienoyl CoA Isomerase (DCI) and acyl-CoA oxidase (POX5).



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## REMARKS

Claims 163-183 are pending and 163-165 have been examined on the merits. Claim 163 is amended hereinabove, claims 166-183 are cancelled and claim 184 is added. Support for amended claim 163 can be found in the specification on page 26, lines 1-11, 23-24 and on page 27, lines 3-5, 21-23 and 25-27. The subject matter of new claim 184 find support in the specification (*e.g.*, page 43, lines 1-6, page 45, lines 27-28, page 48, lines 5-10 for hydratase-dehydrogenase-epimerase (HDE), page 43, lines 1-6 and page 45, lines 26-29 for peroxisomal 18-kDa protein (POX18), page 159, lines 8-10 and page 205, lines 18-22 for 2,4-dienoyl-CoA reductase (SPS19), page 10, line 35, page 11, line 14 and 22 and page 146, lines 26-30 for acyl-CoA oxidase 4 (POX4), - page 160, lines 5-16 and page 161, lines 1- 12 for dienoyl CoA Isomerase (DCI), page 143, lines 24-35 and page 252, lines 1-25 for acyl-CoA oxidase (POX5).

In the Final Office Action, claims 163-165 are still rejected under 35 U.S.C. § 103 for allegedly being unpatentable over Sharpe et al. (WO2008/073367, hereinafter "Sharpe") in view of Juretzek et al. (Biotechnol. Bioprocess. Eng. 2000, hereinafter "Juretzek").

The presently claimed invention is directed to a genetically modified microorganism, comprising:

one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides, wherein expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide:

- wherein the one or more heterologous nucleic acids that encode one or more terpene biosynthesis polypeptides is chosen from terpene synthase, phytoene synthase, geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, bifunctional lycopene



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cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, zeaxanthin glucosyltransferase and valencene synthase,

wherein the nucleic acid that provides for said fatty acid induction of said expression of the terpene biosynthesis polypeptide is a promoter, or

- wherein the one or more heterologous nucleic acids is a cytochrome P450 reductase and is regulated by a nucleic acid that provides for alkane induction of expression of the cytochrome P450 reductase.

In the Final Office Action, the Examiner cites Sharpe, a generic document regarding engineered strains of the oleaginous yeasts capable of producing carotenoids (Abstract of Sharpe).

The Examiner recognizes (page 3 of the Final Office Action) that, apart from the use of Yarrowia as recombinant yeast, Sharpe never mentions nor suggests any promoter of said yeast that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide.

Sharpe only mentions that "Virtually any promoter capable of directing expression of these genes in the selected host cell is suitable for the present invention", but as extensively explained in the expert's declaration attached herein, this will surely require more than a routinary trial from a person skilled in the art.

Thus, to allegedly correct Sharpe's deficiencies and to provide for the missing link, the Examiner cites Juretzek which allegedly describes five different promoters for the expression of foreign proteins in the yeast *Yarrowia lipolytica*.

Accordingly, it is the Examiner's conclusion that it would have been obvious to one of ordinary skill in the art to utilize any of the *Yarrowia lipolytica* strong promoters POX2, POT1



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and ICL1 as described by Juretzek and to utilize these in the recombinant *Yarrowia lipolytica* strains expressing the heterologous genes as described by Sharpe, because, as discussed above, Sharpe describes that "Virtually any promoter capable of directing expression of these genes in the selected host cell is suitable for the present invention". This, according to the Examiner, would provide motivation in and of itself to utilize any of the known promoters and with a reasonable expectation of success.

Applicants respectfully disagree.

As an initial matter, even just the choice of a suitable promoter requires an extensive trial and error and a deep understanding of the processes and the parameters to be checked and selected.

In other words, the necessity of the use of a promoter, its precise choice and its specific modifications, depend on several crucial parameters, such as the specific induction upon exposure to the nutrients (which only can be, albeit surely not mandatorily, fatty acids), the specific fermentation system used (which in the presently claimed invention is a specific genetically modified microorganism, while in the cited documents are either generic oleaginous yeasts structurally different or promoters suitable for regulating the overexpression of beta-galactosidase in the yeast Yarrowia lipolytica), the biochemical pathways desired, and the overall level of expression of the targeted genes.

For all of the reasons discussed hereinbelow, Applicants have demonstrated that taking for granted all these premises, could only be based on the impermissible hindsight.

The amendments and the arguments provided herein clarify the mandatory biological nature of the nucleic acid that provides for fatty acid induction of expression of a terpene



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biosynthesis polypeptide, namely a promoter, and the importance of the presence of said promoter.

Thus, it is clear that the person skilled in the art, by reading Sharpe, would not have considered using anything more than a generic promoter and more specifically, would not have considered using a promoter responsive to fatty acid or alkane induction as presently claimed, since Sharpe is completely silent in this regard.

As stated in the Expert's Declaration attached herein, the promoters used to drive the genes in the terpene biochemical pathways were chosen based on mRNA sequencing data.

The 37 C.F.R. § 1.132 Expert's Declaration of Dr. Beardslee (hereinafter "the 'Beardslee Declaration"), shows that the technical background behind the importance of the presence of a promoter, the mechanisms taking place in the genetically modified microorganism, and the specific choice of promoters, is neither obvious or routinary for a person skilled in the art.

Specifically, this declaration shows the technical and scientific reasons behind the presently inventive selection leading to the unexpected and surprising result of providing a specific genetically modified microorganism capable of unexpectedly and surprisingly achieve the fatty acid-induced expression of one or more terpene biosynthesis polypeptides.

In fact, micro-organic industrial production can minimize the use of caustic chemicals and the production of toxic byproducts, thus providing a "clean" source for certain compounds.

The use of appropriate plant-derived feedstocks allows production of "green" compounds while further minimizing the need for and use of petroleum-derived compounds (page 4, lines 25-29 of the present Application). ¶ 7 Beardslee Declaration.

Applicants submit that the specific genetically modified microorganism of the present invention specifically comprises a promoter modified by replacing the endogenous promoters



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with a promoter comprising an alkane response element (ARE1) (*e.g.*, specification, page 30 lines 19-20, page 33, lines 10-12, page 35 lines 25-27, page 38, lines 1-3, page 40, lines 15-18, et sequitur) or a fatty acid-inducible promoter (*e.g.*, specification page 48, line 10) directing the flux of carbons from fatty acids toward a particular target product pathway (e.g., mevalonate production, isopentenyl diphosphate production, geranyl diphosphate production, farnesyl diphosphate production, geranylgeranyl diphosphate production, terpene production, carotenoid production, beta carotene production, lycopene production, astaxanthin production) and away from other cellular metabolic pathways not involved in target molecule production (*e.g.*, specification, page 29, lines 29-34 and page 46, lines 26-34). ¶ 8, Beardslee Declaration.

However, the Examiner is of the opinion that the assertion that the promoter is critical to the instant invention and that its surprising and unexpected results are never disclosed and thus, that the surprising technical improvements are not clear (page 6 of the Office Action). ¶ 9, Beardslee Declaration.

In view of this objection, it is respectfully submitted that the mandatory nature of the nucleic acid that provides for fatty acid induction of expression of a terpene biosynthesis polypeptide being a promoter, as presently claimed, is extremely important and critical to achieve the surprising and unexpected results obtainable with the presently claimed invention. ¶ 10, Beardslee Declaration.

In fact, in any fermentation system, promoters should be not only present but also should be carefully selected based upon the production organism and the fermentation conditions present during production. If the promoters selected are effectively "off" in the conditions of fermentation, then no gene product (enzyme) will be made, rendering the intended engineered biochemical pathway useless for production. ¶ 11, Beardslee Declaration.



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The fermentation system the presently claimed invention, i.e., the genetically modified microorganism, as presently claimed, comprises a fatty acid as desired carbon source. The technical reason behind this selection is of biochemical efficiency, letting the yeast save the biochemical energy that it takes for de novo fatty acid synthesis. The fats used as feedstock can be used by the yeast through a salvage pathway (instead of de novo synthesis) to produce lipid bodies. Lipid bodies provide the intracellular storage compartment for hydrophobic products such as the 40-carbon carotenoids (and can be used for other hydrophobic terpenes). With the choice of fatty acid as carbon source, the nucleic acid that provides for fatty acid induction of expression of a terpene biosynthesis polypeptide must be carefully chosen and in the presently claimed invention, it must be a promoter that induces the expression of enzymes in the desired biochemical pathway(s). ¶ 12, Beardslee Declaration.

The examples of the present specification, which show the use of several promoters such as hydratase-dehydrogenase-epimerase (HDE), peroxisomal 18-kDa protein (POX18), 2,4-dienoyl-CoA reductase (SPS19), acyl-CoA oxidase 4 (POX4), dienoyl CoA Isomerase (DCI) and acyl-CoA oxidase (POX5), demonstrate the achievement of the results discussed above. ¶ 13, Beardslee Declaration.

Moreover, all these promoters have in common the ability of being a nucleic acid, which provides for fatty acid induction of expression of a terpene biosynthesis polypeptide in a genetically modified microorganism. ¶ 14, Beardslee Declaration.

But in the Final Office Action, the Examiner has taken the position that the invention presently claimed is not inventive over the combination of Sharpe and Juretzek. ¶ 15, Beardslee Declaration.

Applicants respectfully disagree.



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Sharpe is a document regarding engineered strains of the oleaginous yeasts capable of producing carotenoids (Abstract of Sharpe) and, more importantly, it does not teach nor does it mention any promoter of said yeast that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide, as also recognized by the Examiner (page 3 of the Office Action). ¶ 16, Beardslee Declaration

But the Examiner has taken the position that Sharpe would have suggested a promoter, since it mentions that "Virtually any promoter capable of directing expression of these genes in the selected host cell is suitable for the present invention". ¶ 17, Beardslee Declaration.

Applicants respectfully disagree with the Examiner and respectfully submit this sentence is not sufficient to provide a teaching that would allow the person skilled in the art to find suitable promoters for the specific biological system herein proposed. ¶ 18, Beardslee Declaration.

In fact, the specific choice of a promoter for any biological system requires an extensive trial and error and a deep understanding of the processes and the parameters to be checked and selected. In other words, the necessity of using a promoter, its precise choice and its specific modifications, depend on several crucial parameters, such as: - the specific induction upon exposure to the nutrients (which only CAN be but surely not mandatorily be fatty acids), - the specific fermentation system used (which in the present Application is a specific genetically modified microorganism, while in the prior arts cited are either generic oleaginous yeasts structurally different or promoters suitable for regulating the overexpression of beta-galactosidase in the yeast *Yarrowia lipolytica*), - the biochemical pathways desired, and - the overall level of expression of the targeted genes. ¶ 19, Beardslee Declaration.



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In view of the above, Sharpe, when considered as a whole, teaches and suggests only a way to synthesize generic oleaginous yeast for the production of carotenoids and polyunsaturated fatty acids (PUFA's), without taking into account any fatty acid-inducible promoter, thus not providing any teachings or suggestions to arrive at the results obtainable with the presently claimed invention. Hence, Sharpe would have not been useful for the person skilled in the art, since Sharpe does not even mention any promoter of the yeast that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide. ¶ 20, Beardslee Declaration.

Further, the Examiner combines a second document Juretzek, which presents a comparison of different promoters suitable for regulating the overexpression of beta-galactosidase in the yeast *Yarrowia lipolytica* (Title and Abstract of Juretzek). ¶ 21, Beardslee Declaration.

In the Final Office Action, the Examiner is of the opinion that the person skilled in the art would have easily combined the teachings regarding the promoters of the yeast *Yarrowia lipolytica*, and more specifically POX2, POT1 and ICL1, described in Juretzek, and used them in order to express the heterologous genes of terpene biosynthesis polypeptides. ¶ 22, Beardslee Declaration.

Furthermore, the Examiner is of the opinion that beta-galactosidase can be used as example of an expression of genes. ¶ 23, Beardslee Declaration.

In view of the above, it is respectfully submitted that beta-galactosidase is a single gene product that is easily expressed in many microbiological systems as an enzyme with an easy activity assay, and, precisely due to its easy expression and activity assay, beta-galactosidase is mostly used as gene-test or control for studies regarding the expression regulated by promoters,



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and it is a sort of protocol, which cannot be considered as representative of all promoter-gene combinations and all situations. ¶ 24, Beardslee Declaration.

Juretzek, on the other hand, not only does it not list, nor suggest any of the promoters useful for the biological system presently claimed, but it also discourages the use of fatty acid promoters in general, since Juretzek explains that "*These results present ricinoleic acid methyl ester as the strongest inducer of pPOX2 and pPOT1 among the tested carbon sources, in spite of the disadvantage that this carbon source inhibits growth of Y. lipolytica*" (page 324, right column of Juretzek). ¶ 25, Beardslee Declaration.

Moreover, Juretzek clearly lists some specific promoters, capable of achieving the result presently claimed, such as POX2 and POT1, stating that "*the POX2 and POT1 promoters are only inducible by fatty acid, fatty acid derivatives or alkanes, what may be, at least in some respect, a disadvantage for biotechnological applications*" (page 324, right column of Juretzek). ¶ 26, Beardslee Declaration.

Therefore, the promoters used to drive the genes in the terpene biochemical pathways were chosen based on mRNA sequencing data. The samples were taken from a two-stage fed-batch fermentation using glucose as the carbon source for the growth phase and oleic acid as the feedstock for the subsequent production phase (once glucose was exhausted). Samples were taken in a time-course before and after the switch to oleic acid. Promoters were chosen based not only on being induced upon exposure to fatty acids, but also on having an overall high level of expression. The mRNA sequencing data revealed that 6 of the top 10 promoters in terms of overall expression level are those recited in new claim 184 and described in the present specification. ¶ 27, Beardslee Declaration.



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On the other hand, the promoters suggested by Juretzek were selected for a specific biotechnological system, namely *Yarrowia* on oleic acid. However, in the case of different systems, such as for example a *Candida viswanathii* system, these promoters (POX2, POT1, and ICL1) showed in the mRNA sequencing data very poor overall expression levels on oleic acid. Hence, even if the person skilled in the art reading Juretzek would have taken into account the teachings contained thereof, he/she would have chosen poorly working promoters, hence he/she would have never reached the results discussed above. ¶ 28, Beardslee Declaration.

In view of the above, it is respectfully submitted that the person skilled in the art, while combining the teachings of Sharpe and Juretzek, would have considered that long chain fatty acid feedstocks could lead to damage of the microorganism or at least a disadvantage in biotechnological production, instead of considering fatty acids the real protagonist, playing the main role in the biosynthesis. To avoid any damage to the *Y. lipolytica,* the skilled artisan would have been directed to finding another solution which removes the use of fatty acid-inducible promoters. If fatty acids were still used, the skillful person would at least considered different promoters, specifically POX2, ICL1 and POT1, indicated as the most promising in Juretzek, with respect to those selected and tested in the presently claimed invention, hence reaching a different solution, with respect to the invention presently claimed. ¶ 29, Beardslee Declaration.

In conclusion, the microorganism presently claimed represents a new and inventive alternative to known microorganisms, due to the specific and non-obvious choice of each of its mandatory technical features, not present or suggested by the combination of the documents cited by the Examiner. ¶ 30, Beardslee Declaration.

Accordingly, for all of the reasons discussed above, reconsideration and withdrawal of this rejection is respectfully requested.



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This Amendment and Response is filed concurrently with an RCE and within the shortened statutory period for reply. Thus, no extra-fees are believed to be due. However, the Director is hereby authorized to charge any fee deemed necessary or credit any overpayments to Deposit Account No. 506423.

Respectfully submitted,

October 20, 2022 By: /Silvia Salvadori/

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| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 16/630,567 | 01/13/2020 | Kimberly Ann Aeling | SS118-1026US | 5356 |

122515 7590 12/02/2022

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| EXAMINER | |
|---|---|
| NOAKES, SUZANNE MARIE | |

| ART UNIT | PAPER NUMBER |
|---|---|
| 1656 | |

| NOTIFICATION DATE | DELIVERY MODE |
|---|---|
| 12/02/2022 | ELECTRONIC |

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

eofficeaction@appcoll.com
silvia30121@me.com
silvia@salvadorilaw.com

| *Office Action Summary* | **Application No.** 16/630,567 | **Applicant(s)** Aeling et al. | |
|---|---|---|---|
| | **Examiner** SUZANNE M NOAKES | **Art Unit** 1656 | **AIA (FITF) Status** Yes |

*-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --*

**Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

1)☑ Responsive to communication(s) filed on 05/16/2022.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims***

5) ☑ Claim(s) 163-165 and 184 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 163-165 and 184 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

**Application Papers**

10)☐ The specification is objected to by the Examiner.

11)☑ The drawing(s) filed on 05/16/2022 is/are: a)☑ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

**Priority under 35 U.S.C. § 119**

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

**Certified copies:**

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

1) ☐ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.



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## DETAILED ACTION

### *Continued Examination Under 37 CFR 1.114*

1. A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 20 October 2022 has been entered.

### *Status of Application*

2. The response, amendments and Declaration filed under 37 C.F.R. 1.132, all filed 16 May 2022 are acknowledged and have been considered in their entireties. Claims 166-183 and cancelled and claim 184 is new. Thus, claims 163-165, 184 are pending and subject to examination on the merits.

3. It is noted, the search of the terpene biosynthesis polypeptide species has been extended to cytochrome P450.

### *New Rejection*

### *Claim Rejections - 35 USC § 102*

4. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of

rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (a)(1) the claimed invention was patented, described in a printed publication, or in public use, on sale, or otherwise available to the public before the effective filing date of the claimed invention.
>
> (a)(2) the claimed invention was described in a patent issued under section 151, or in an application for patent published or deemed published under section 122(b), in which the patent or application, as the case may be, names another inventor and was effectively filed before the effective filing date of the claimed invention.

5. Claim(s) 163-165 and 184 are rejected under 35 U.S.C. 102(a)(1) and 102(a)(2) as being anticipated by Zhang et al. (US 2004/0265980 – cited previously).

Zhang et al. teach:

Regarding claim 163-164, a recombinant host cell of *Candida* which has a cytochrome P450 under the control of a POX4 promoter for expression in said *Candida* (See Examples). Paragraph 0035, stipulates that POX4 is a promoter that is induced by either fatty acids or alkanes. It is further taught, the host cell where POX4 promoter is utilized for heterologous expression of an enzyme of interest involved in dicarboxylic acid production can be any yeast host such as *Yarrowia*, *Bebaromyces, Saccharomyces, Schizosaccharomyces, Pichia* and *Candida* (See paragraph 0042).

Regarding claim 165, saturated fatty acids that induce POX4 are selected from caproic, enanthic, caprylic, pelargonic, capric, undecylic, lauric, myristic, pentadecanoic, palmitic, margaric, stearic, arachidic and behenic acids; unsaturated fatty acids for

induction are selected from palmitoleic, oleic, erucic, linoleic and linolenic acids; and alkanes for induction are selected from C12-C24 alkanes (See paragraph 0053).

Regarding claim 184, paragraph 0035, stipulates that POX4 is a promoter that is induced by either fatty acids or alkanes.

***Maintained Rejection***

***Claim Rejections - 35 USC § 103***

6. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

7. The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

8. Claims 163-165 are rejected under 35 U.S.C. 103 as being unpatentable over Sharpe et al. (WO 2008/073367 – cited herein) in view of Juretzek et al. (Biotechnol. Bioprocess. Eng., 2000 – cited herein).

Sharpe et al. teach:

Regarding claims 163-165, a recombinantly engineered oleaginous yeast is taught wherein said yeast has been engineered to express the heterologous genes:

CrtE of geranyl geranyl pyrophosphate synthase (wherein in the instant specification at paragraph 0158 of PG-Pub, CrtE is defined as a geranyl geranyl diphosphate synthase), a CrtB phytoene synthase, and a CrtI phytoene desaturase as well as (See Claims 1 and 2; p. 4, line 32 to p. 5, line 10; p. 13, line 31 to p. 14, line 4; p. 34, line 17 to p. 26, line 18); additional genes are lycopene cyclase (CrtY), carotenoid hydroxylase (CrtZ) and carotenoid ketolase (CrtW).

Regarding claim 164, the term "oleaginous yeast" is to mean yeast selected from *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon* and *Lipomyces* – See p. 29, lines 12-27. It further taught, *Yarrowia lipolytica* is the host cell utilized in all working examples.

Regarding claims 163 and 165, it is taught: "Virtually any promoter capable of directing expression of these genes in the selected host cell is suitable for the present invention." – p. 59, lines 5-7. It is taught inducible promoters or constitutive promoters are suitable for expression of the genes (p. 59).

Sharpe et al., however, do not teach wherein the promoter is one that is responsive to fatty acid or alkane induction.

Juretzek et al. characterize five different promoters for the expression of foreign proteins in the yeast *Yarrowia lipolytica*. Up until that point in time, said yeast had few promoters available for use in the system, despite said yeast being known for several decades in the biotechnology industries for being a good host for expression of foreign genes because it can do so in considerable amounts (See Introduction). It was established that the promoters POX5, POX2, POT1 and ICL1 (inducible by fatty acids,

fatty acid derivates, alkanes and/or acetate/ethanol, respectively) all offer the advantage of being inexpensive carbon sources (See Figure 2 and last paragraph, p. 325).

Therefore it would have been obvious to one of ordinary skill in the art prior to the effective filing date of the claimed invention to utilize any of the *Yarrowia lipolytica* strong promoters POX5, POX2, POT1 and ICL1 as taught by Juretzek et al. and to utilize these in the recombinant *Yarrowia lipolytica* strains expressing the heterologous genes: CrtE of geranyl geranyl pyrophosphate synthase (wherein in the instant specification at paragraph 0158 of PG-Pub, CrtE is defined as a geranyl geranyl diphosphate synthase), a CrtB phytoene synthase, and a CrtI phytoene desaturase as well as (See Claims 1 and 2; p. 4, line 32 to p. 5, line 10; p. 13, line 31 to p. 14, line 4; p. 34, line 17 to p. 26, line 18); additional genes are lycopene cyclase (CrtY), carotenoid hydroxylase (CrtZ) and carotenoid ketolase (CrtW) as taught by Sharpe et al., because Sharpe et al. literally state: "Virtually any promoter capable of directing expression of these genes in the selected host cell is suitable for the present invention." – p. 59, lines 5-7. This would provide motivation in and of itself to utilize any of the known promoters, including POX2, POT1, ICL1, G3P, XRP2, for the species *Yarrowia lipolytica* for directing expression of the enzymes geranyl geranyl pyrophosphate synthase (wherein in the instant specification at paragraph 0158 of PG-Pub, CrtE is defined as a geranyl geranyl diphosphate synthase), a CrtB phytoene synthase, and a CrtI phytoene desaturase. One skilled in the art would have a reasonable expectation of success that the promoters as taught by Juretzek et al. would work well in the recombinant strains of Sharpe et al. because Juretzek et al. establishes them to be operable strong inducible promoters.

As such, the references when combined render the instant claims as *prima facie* obvious.

**Applicant's Response and Examiner's Rebuttal**:

Applicant's traverse the rejection of record and state the combined references do not render obvious the present claims.

Applicant's state it is highly unpredictable to choose a promoter for expression of an enzyme in a host. Specifically the unpredictability of choosing a promoter induced by fatty acids or alkenes for expression of an enzyme that is involved in terpene biosynthesis. Both Applicant's and the Declaration from Dr. Beardslee assert that the promoter must be able to direct flux of the carbon source from fatty acids to a particular target product pathway (See Remarks, p. 8, and Decl. paragraph 0008).

However, this argument is not convincing given the microorganism as recited in the claims need not produce any sort of target product. It just needs to express a single enzyme under the control of promoter induced by fatty acids and/or alkanes. That is, Applicant's and Dr. Beardslee are arguing limitations simply not present in the claims.

In this regard, Applicant's and Dr. Beardslee state it is highly unpredictable to choose a promoter that will work for one skilled in the art. This is because many factors must be considered and if the wrong promoter is chosen, then it will provide no expression for the desired enzyme. These include the fermentation conditions, the selection of the host and the "*extensive trial and error* and a deep understanding of the processes and parameters to be checked and selected." – Remarks, p. 8-10, last paragraph and Dr. Beardslee Declaration at paragraph 11-12, 19.

Here this is not found convincing because this begs the question, given the breadth and scope of the current claims, Applicant's seemingly are arguing their own claims are not enabled? They state the cited prior art are generic to *Yarrowia* but they themselves have demonstrated specific genetically modified microorganisms capable of expressing a terpene enzyme under the control of a promoter included by fatty acids or alkanes *only in Candida*. But given the breadth of the claims inclusive of *any genetically modified microorganism* and any promoter with the exception of claim 184, then seemingly Applicant's are intimating that the current scope and breadth of claims cannot be enabled because of the *extensive trial and error* required by a skilled artisan.

Nonetheless, the prior art does direct those skilled in the art to be able to utilize most any promoter for expression. Again, there is no requirement in the claims that dictates the genetically modified microorganism produces any product whatsoever. It just has to direct expression of a single enzyme or combination of enzymes. To this extent, the prior art of Juretzek et al. is pretty clear the promoters that are best available for expression of any enzyme in *Yarrowia*. In addition, the standard for *prima facie* obviousness is that of a reasonable expectation of success (MPEP 2143). Might some tweaking need to be done? In all likelihood, but that is well within the knowledge and capabilities of one skilled in the art. Applicant's and the Declaration by Dr. Beardslee, however, argue on the lines that absolutely predictability of success is required before choosing which promoter or promoters to utilize.

Applicants also assert that Sharpe et al. is only generic in its teaching because it teaches a way to synthesis oleaginous yeast for the production of carotenoids and PUFA's without taking into account any fatty-acid inducible promoter and thus it is of no

use to one skilled in the art wanting to produce the same things with a fatty acid inducible promoter. Remarks, p. 11 and Dr. Beardslee at paragraph 20.

However, this is not convincing because Sharpe et al. the teachings are directed to making *terpenes* (not just carotenoids and PUFA's) utilizing any promoter. Furthermore, it is of such little consequence as to the choice of the promoter that they leave it up to the skilled artisan to choose any which they desire. This is because those skilled in the art are highly skilled and can appropriately choose the ones they know will work, or those taken or suggested from the prior art, such as those suggested by Juretzek et al., wherein both teach utilizing *Yarrowia* as the specific host (although Sharp et al. does suggest other yeast can be utilized).

In this regard, Applicant's argue that Juretzek is of little use because the representative gene used for analyzing promoters is beta-galactosidase, which is commonly utilized and easily assesses with a common activity assay but says nothing about enzymes involved in terpene biosynthesis. Thus, it cannot be considered representative of all promoter-gene combinations and all situations (See p. 11-12, and Dr. Beardslee at paragraphs 21-24).

This is not found unconvincing because both Applicant's and Dr. Beardslee again seem to be of the opinion that absolute predictability of success if required, before any sort of substitution of the promoters of Juretzek et al. with those of Sharp et al. occurs (although, as noted, Sharp leaves it up to the skilled artisan). Again, however, this is not the standard by which *prima facie* obviousness is assessed (MPEP 2143).

With regard to Applicant's assertions that it is taught that ricinoleic acid methyl esters strongly activated the promoters pPOT1 and pPOX2, but that it inhibited growth

and is a major disadvantage (See p. 12 and Dr. Beardslee paragraph 25). This aspect is acknowledged. However, consistently Applicant's take sections out of context and ignore the rest/overall teachings of the document. The rest of the section recites:

> "Further studies will elucidate whether ricinoleic acid methyl ester can be used as strong inducer for heterologous gene expression under control of the POT1 or the POX2 promoters in mixed substrate fermentations or whether any effects will inhibit enzyme overproduction under such conditions."

In addition, the following sentence from that directly above would direct one skilled in the art to utilize the fatty acid oleic acid (See p. 324, 2nd col.).

"Oleic acid is the carbon source without any growth inhibitory effect among the tested ones, which induced the highest activation of the promoters of the ICL1 and POX2 genes and to a little less extend of the POT1 promoter (Fig. 2(b))."

With regard to Applicant's assertions that one skilled in the art would be directed away from using any of the fatty acid inducible promoters of POX1, ICL1 and POT1 as taught by Juretzek (See Remarks, p. 12, 3rd paragraph and Dr. Beardslee paragraph 0026) because they state "*The POX2 and POT1 promoters are only inducible by fatty acid, what may be a disadvantage for biotechnological applications*" (at p. 324, last paragraph of Juretzek) is also not convincing. The rest of the paragraph goes on to state:

> Further studies using these promoters inserted in the newly developed multiple copy integration vectors [12,13] have already been started to elucidate the potential of *Y. lipolytica* for heterologous gene expression. First results of successful high expression of the bovine cytochrome P45017α cDNA (CYP17) and homologous NADPH-cytochrome P450 reductase under the control of the ICL1 promoter [28,34] as well as of the homologous lipase gene LIP2 with the POX2 promoter (Nicaud, unpublished) demonstrate that these promoters are very useful tools for induction of high expression levels in *Y. lipolytica*.

Notably, cytochrome P450 reductase is a terpene biosynthesis enzyme and this establishes the successful expression of it in *Yarrowia* using a fatty acid promoter, e.g. not just a generic beta-galactosidase as contended by Applicant's.

It is asserted the promoters of the present invention/claims were based upon mRNA sequencing and upon performing fed-batch fermentation growth in two stages, the first being glucose and the second being oleic acid, and the promoters with the highest expression levels were chosen and are reflected in claim 184.

On the other hand, it is asserted the promoters of Juretzek et al. were selected for *Yarrowia* using oleic acid. However, when these were utilized in *Candida* said promoters were highly unsuccessful. Thus, one skilled in the art reading Juretzek et al. would have chosen very poor promoters (See pp. 12-13 and Dr. Beardslee at paragraphs 27-28).

The Examiner has considered this but finds the two statements conflicting. The mRNA sequence and fed-batch experiments with different promoters were *not* performed in all microorganisms. Just in *Candida*. Thus, just as Applicant's are stating that the teachings of Juretzek et al. are only relevant, essentially, to *Yarrowia* then the same holds true for the Applicant's assertions and experiments in *Candida*. Based upon Applicant's own arguments, especially about the unpredictability and extensive trial and error required to find a suitable promoter for expression of an enzyme, they then cannot be relevant to any other microorganism. But yet the claims and breadth of the claims are to any or all microorganisms and in the case of claim 164 to eight different yeast genera. Furthermore, the rejection of record makes no assertions of

strains other than *Yarrowia*. Sharpe et al. teach utilizing *Yarrowia* and so does Juretzek et al. All of the claims are inclusive of *Yarrowia*.

With respect to Applicant's arguments that one skilled in the art would not utilize any of the promoters from Juretzek et al. or try to find ones that have fewer disadvantages and look to those that do not utilize fatty acid-inducible promoters (See p. 13, Dr. Breadslee at paragraph 29).

The Examiner does not find this convincing either. First, Juretzek et al. do teach that POX2, ICL1 and POT1 are very useful tools in expression of heterologous genes in *Yarrowia*, and contrary to Applicant's assertions of disadvantages teach (p. 324, 2nd col.):

"Oleic acid is the carbon source without any growth inhibitory effect among the tested ones, which induced the highest activation of the promoters of the ICL1 and POX2 genes and to a little less extend of the POT1 promoter."

Thus, the assertions of disadvantages at most can be leveled at ricinoleic acid methyl ester, although even Juretzek et al. assert more testing is necessary (See p. 324, 2nd col.).

Applicant's then assert if, however, fatty acid promoters were still utilized the skilled artisan would at the very least look to utilize POX2, ICL1 and POT1 indicated as the most promising, which are different from those indicated as most promising as indicated in the claimed invention (See p. 13 and Dr. Beardslee at paragraphs 28-30).

However, the claims are so broad so as to encompass the promoters POX2, ICL1 and POT1, with the exception of claim 184, and in any microorganism, with the exception of claim 164. Thus, yes one skilled in the art would likely be motivated to utilize those considered the most advantageous such as POX2, ICL1 and POT1 but

likely would not rule out any of those tested in Juretzek et al., including POX5, which is still inducible by oleic acid (See Figure 2).

For these reasons, the rejection of record is maintained.

***Conclusion***

9. No claim is allowed.

10. Any inquiry concerning this communication or earlier communications from the examiner should be directed to SUZANNE M NOAKES whose telephone number is (571)272-2924. The examiner can normally be reached M-F (7-4).

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Manjunath Rao can be reached on 571-272-0939. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of published or unpublished applications may be obtained from Patent Center. Unpublished application information in Patent Center is available to registered users. To file and manage patent submissions in Patent Center, visit: https://patentcenter.uspto.gov. Visit https://www.uspto.gov/patents/apply/patent-center for more information about Patent Center and https://www.uspto.gov/patents/docx for information about filing in DOCX format. For additional questions, contact the Electronic Business Center (EBC) at 866-217-9197

(toll-free). If you would like assistance from a USPTO Customer Service

Representative, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/SUZANNE M NOAKES/
Primary Examiner, Art Unit 1656
29 November 2022

| Search Notes | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 16/630,567 | Aeling et al. |
| | Examiner | Art Unit |
| | SUZANNE M NOAKES | 1656 |

| CPC - Searched* | | |
|---|---|---|
| **Symbol** | **Date** | **Examiner** |
| C12N 15/815 w/text in EAST | 02/15/2022 | SMN |
| Updated in SEARCH | 11/29/2022 | SMN |

| CPC Combination Sets - Searched* | | |
|---|---|---|
| **Symbol** | **Date** | **Examiner** |
| | | |

| US Classification - Searched* | | | |
|---|---|---|---|
| **Class** | **Subclass** | **Date** | **Examiner** |
| | | | |

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

| Search Notes | | |
|---|---|---|
| **Search Notes** | **Date** | **Examiner** |
| Inventors names (all) in EAST and PE2E | 02/15/2022 | SMN |
| EAST: See attached | 02/15/2022 | SMN |
| ProQuest Dialog: see attached | 02/15/2022 | SMN |
| Updated in SEARCH: See attached | 07/25/2022 | SMN |
| Updated SEARCH: See attaced | 11/28/2022 | SMN |
| Updated ProQuest Dialog: See attached | 11/28/2022 | SMN |



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| Search Notes | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 16/630,567 | Aeling et al. |
| | Examiner | Art Unit |
| | SUZANNE M NOAKES | 1656 |

| Interference Search | | | |
|---|---|---|---|
| US Class/CPC Symbol | US Subclass/CPC Group | Date | Examiner |
| | | | |



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## PE2E SEARCH - Search History (Prior Art)

| Ref # | Hits | Search Query | DBs | Default Operator | Plurals | British Equivalents | Time Stamp |
|---|---|---|---|---|---|---|---|
| L64 | 1046 | ((("AELING") near3 ("Kimberly")) OR (("DE") near3 ("WALS") near3 ("Pierre-Yves")) OR (("SCOTT") near3 ("Eric")) OR (("WALDBRIDGE") near3 ("Michael")) OR (("BEARDSLEE") near3 ("Thomas"))).INV. | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | OFF | OFF | 2022/02/15 07:59 AM |
| L65 | 36 | L64 and terpene | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:00 AM |
| L66 | 6 | L64 and terpene and phytoene | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:00 AM |
| L67 | 1648 | ("phytoene synthase" or CftB or "prephytoene-diphosphate synthase" or "15-cis-phytoene synthase" or PSase or "geranylgeranyl-diphosphate geranylgeranyltransferase" or "2.5.1.32") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:09 AM |
| L68 | 6520 | ("phytoene desaturase" or "3,4-didehydrolycopene-forming phytoene desaturase" or "1.3.99.28" or CrtI or "1.3.99.30" or CsCrtI or XdCrtI or PaCrtI) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:12 AM |
| L69 | 4105 | ("geranylgeranyl diphosphate synthase" or BTS1 or CrtE or GGPS1 or GGPPS or GGPPS1 or "geranylgeranyl diphosphate synthase 1" or "2.5.1.29") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:14 AM |
| L70 | 528 | L67 and L68 and L69 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:14 AM |
| L71 | 415 | L67 same3 L68 same3 L69 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:14 AM |
| L72 | 360 | L67 same L68 same L69 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:14 AM |

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|---|---|---|---|---|---|---|---|
| L73 | 38 | L72 and (POX4 or POT or PEX or POX18 or ANT1 or DHE or ARE1) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:15 AM |
| L74 | 5 | ("20030044946"\|"20130066035"\|"20130302861"\|"20140113343"\|"20150010978").PN. | (US-PGPUB; USPAT) | OR | OFF | OFF | 2022/02/15 09:01 AM |
| L75 | 171 | L72 and (Candida or Yarrowia) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 09:10 AM |
| L76 | 21 | L72 same (Candida or Yarrowia) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 09:10 AM |
| L77 | 1648 | ("phytoene synthase" or CftB or "prephytoene-diphosphate synthase" or "15-cis-phytoene synthase" or PSase or "geranylgeranyl-diphosphate geranylgeranyltransferase" or "2.5.1.32") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 10:57 AM |
| L78 | 6520 | ("phytoene desaturase" or "3,4-didehydrolycopene-forming phytoene desaturase" or "1.3.99.28" or CrtI or "1.3.99.30" or CsCrtI or XdCrtI or PaCrtI) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 10:57 AM |
| L79 | 4105 | ("geranylgeranyl diphosphate synthase" or BTS1 or CrtE or GGPS1 or GGPPS or GGPPS1 or "geranylgeranyl diphosphate synthase 1" or "2.5.1.29") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 10:57 AM |
| L80 | 360 | L77 same L78 same L79 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 10:57 AM |
| L81 | 16 | L80 and C12N15/815.cpc. | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 10:57 AM |
| L82 | 1102 | ((("AELING") near3 ("Kimberly")) OR (("DE") near3 ("WALS") near3 ("Pierre-Yves")) OR (("SCOTT") near3 ("Eric")) OR (("WALDBRIDGE") near3 ("Michael")) OR (("BEARDSLEE") near3 ("Thomas"))).INV. | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | OFF | OFF | 2022/07/25 12:07 PM |
| L83 | 37 | L64 and terpene | (US-PGPUB; USPAT; | OR | ON | ON | 2022/07/25 |



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|---|---|---|---|---|---|---|---|
| | | | USOCR; EPO; JPO; DERWENT) | | | | 12:07 PM |
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| L85 | 1713 | ("phytoene synthase" or CftB or "prephytoene-diphosphate synthase" or "15-cis-phytoene synthase" or PSase or "geranylgeranyl-diphosphate geranylgeranyltransferase" or "2.5.1.32") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L86 | 6381 | ("phytoene desaturase" or "3,4-didehydrolycopene-forming phytoene desaturase" or "1.3.99.28" or CrtI or "1.3.99.30" or CsCrtI or XdCrtI or PaCrtI) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L87 | 4424 | ("geranylgeranyl diphosphate synthase" or BTS1 or CrtE or GGPS1 or GGPPS or GGPPS1 or "geranylgeranyl diphosphate synthase 1" or "2.5.1.29") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L88 | 538 | L67 and L68 and L69 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L89 | 430 | L67 same3 L68 same3 L69 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L90 | 368 | L67 same L68 same L69 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L91 | 42 | L72 and (POX4 or POT or PEX or POX18 or ANT1 or DHE or ARE1) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
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| L93 | 176 | L72 and (Candida or Yarrowia) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L94 | 21 | L72 same (Candida or Yarrowia) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L95 | 1713 | ("phytoene synthase" or CftB or "prephytoene- | (US-PGPUB; USPAT; USOCR; EPO; JPO; | OR | ON | ON | 2022/07/25 12:07 PM |

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|---|---|---|---|---|---|---|---|
| | | diphosphate synthase" or "15-cis-phytoene synthase" or PSase or "geranylgeranyl-diphosphate geranylgeranyltransferase" or "2.5.1.32") | DERWENT) | | | | |
| L96 | 6381 | ("phytoene desaturase" or "3,4-didehydrolycopene-forming phytoene desaturase" or "1.3.99.28" or CrtI or "1.3.99.30" or CsCrtI or XdCrtI or PaCrtI) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L97 | 4424 | ("geranylgeranyl diphosphate synthase" or BTS1 or CrtE or GGPS1 or GGPPS or GGPPS1 or "geranylgeranyl diphosphate synthase 1" or "2.5.1.29") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L98 | 368 | L77 same L78 same L79 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L99 | 16 | L80 and C12N15/815.cpc. | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L100 | 153 | promoter WITH (POX4 OR SPS19 OR POX5 OR POX18 OR DCI) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/11/28 11:09 AM |
| L101 | 101 | (promoter WITH (POX4 OR SPS19 OR POX5 OR POX18 OR DCI)) AND yarrowia | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/11/28 11:10 AM |
| L102 | 36 | (promoter WITH (POX4 OR SPS19 OR POX5 OR POX18 OR DCI)) SAME yarrowia | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/11/28 11:10 AM |
| L103 | 4 | L102 AND terpene | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/11/28 11:27 AM |

## PE2E SEARCH - Search History (Interference)

There are no Interference searches to show.

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## Report Information from Dialog

November 29 2022 12:47



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# Search Strategy

**Databases:** ABI/INFORM® Professional Advanced, Abstracts in New Technology & Engineering, AdisInsight: Drugs, AdisInsight: Trials, Adis Pharmacoeconomics & Outcomes News, AGRICOLA, AGRIS, Allied & Complementary Medicine™, Analytical Abstracts, APA PsycInfo®, Aqualine, Aquatic Science & Fisheries Abstracts (ASFA), Australian Education Index, BIOSIS® Toxicology, BIOSIS Previews®, British Library Inside Conferences, British Nursing Index, Business & Industry, CAB ABSTRACTS, Chemical Business Newsbase, Chemical Engineering & Biotechnology Abstracts, Chemical Safety Newsbase, Civil Engineering Abstracts, ClinicalTrials.gov, Current Contents® Search, Derwent Drug File, Derwent Drug Registry, DH-DATA: Health Administration, Medical Toxicology & Environmental Health, DIOGENES® FDA Regulatory Updates, Drug Information Fulltext, Earthquake Engineering Abstracts, EconLit, Ei Compendex®, Ei EnCompassLIT, Embase®, Embase® French Local Literature, Embase Preprints, EMCare®, ERIC, ESPICOM Pharmaceutical & Medical Device News, FDAnews, FLUIDEX (Fluid Engineering Abstracts), Foodline®: MARKET, Foodline®: PRODUCT, Foodline®: SCIENCE, FSTA®, Gale Group Computer Database™, Gale Group Health Periodicals Database, Gale Group New Product Announcements / Plus®, Gale Group Newsletter Database™, Gale Group PharmaBiomed Business Journals, Gale Group PROMT®, Gale Group Trade & Industry Database™, GEOBASE, GeoRef, Global Health, HSELINE: Health and Safety, ICONDA - International Construction Database, IMS Company Profiles, IMS New Product Focus, IMS Pharma Trademarks, IMS R&D Focus, IMS R&D Focus Drug News, Inspec®, International Pharmaceutical Abstracts, Jane's Defense & Aerospace News, King's Fund, KOSMET: Cosmetic Science, Lancet Titles, Mechanical & Transportation Engineering Abstracts, MEDLINE®, Meteorological & Geoastrophysical Abstracts, Morressier Life Science Conference Abstracts and Posters, New England Journal of Medicine, Northern Light Life Sciences Conference Abstracts, NTIS: National Technical Information Service, Oceanic Abstracts, PAIS International, Paperbase, PAPERCHEM, ProQuest Advanced Tech & Aerospace Professional, ProQuest Biological & Health Science Professional, ProQuest Dissertations and Theses Professional, ProQuest Environmental Science Professional, ProQuest Materials Research Professional, ProQuest Newsstand Professional, ProQuest Technology Research Professional, Prous Science Daily Essentials, Prous Science Drug Data Report, Prous Science Drugs Of The Future™, Publicly Available Content, Registry of Toxic Effects of Chemical Substances (RTECS®), SciSearch®: a Cited Reference Science Database, Social SciSearch®, ToxFile®, Transport Research International Documentation, TULSA™ (Petroleum Abstracts), UBM Computer Full Text, Weldasearch®, Zoological Record

| Set# | Searched for | Results |
|---|---|---|
| S1 | (((POX4 OR SPS19 OR POX5 OR POX18 OR DCI) near/2 promoter)) | 16° |
| S2 | (((POX4 OR SPS19 OR POX5 OR POX18 OR DCI) near/10 promoter)) | 48° |
| S3 | S2 not S1 | 35° |
| S4 | (S2 not S1) and (pd(19000101-20170713)) | 29° |

° Duplicates are removed from the search and from the result count.



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## Meiotic interactors of a mitotic gene TAO3 revealed by functional analysis of its rare variant

**Author:** Gupta, Saumya; Radhakrishnan, Aparna; Rachana Nitin; Raharja-Liu, Pandu; Lin, Gen; Steinmetz, Lars M; Gagneur, Julien; Sinha, Himanshu

**Publication info:** bioRxiv Cold Spring Harbor: Cold Spring Harbor Laboratory Press. (Apr 3, 2016)
ProQuest document link

**Abstract (English):** Studying the molecular consequences of rare genetic variants has the potential of identifying novel and hereto uncharacterized pathways causally contributing to phenotypic variation. Here we characterize the functional consequences of a rare coding variant of TAO3, previously reported to significantly contribute to sporulation efficiency variation in Saccharomyces cerevisiae. During mitosis TAO3 interacts with CBK1, a conserved NDR kinase and a component of RAM network. The RAM network genes are involved in regulation cell separation and polarization. We demonstrate that the role of the rare allele TAO3(4477C) in meiosis is distinct from its role in mitosis by being independent of ACE2, which is a RAM network target gene. By quantitatively measuring cell morphological dynamics and conditionally expressing TAO3(4477C) allele during sporulation, we show that TAO3 has an early role in meiosis. This early role of TAO3 coincides with entry of cells into meiotic division. Time-resolved transcriptome analyses during early sporulation phase identified regulators of carbon and lipid metabolic pathways as candidate mediators. We experimentally show that during sporulation the TAO3 allele genetically interacts with ERT1 and PIP2, the regulators of tricarboxylic acid cycle and gluconeogenic enzymes, respectively. We thus uncover meiotic functions of TAO3, a mitotic gene and propose ERT1 and PIP2 as novel regulators of sporulation efficiency. Our results demonstrate that study of causal effects of genetic variation on the underlying molecular network has the potential to provide more extensive comprehension of the pathways driving a complex trait. This can help identify prospective personalized targets for intervention in complex diseases.

**Links:** Check USPTO-STIC for Availability

**Language:** English

**Language of abstract:** English

**Publication title:** bioRxiv

**DOI:** http://dx.doi.org/10.1101/033167

**Document type:** Working Paper

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**Updates:** 2018-07-172019-09-202021-12-07

**Database:** Publicly Available Content (1970 - current)



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## Promoter Methylation Status Modulate the Expression of Tumor Suppressor (RbL2/p130) Gene in Breast Cancer

**Author:** Ullah, Farman; Khan, Taimoor; Ali, Nawab; Malik, Faraz Arshad; Kayani, Mahmood Akhtar; Shah, Syed Tahir Abbas; Saeed, Muhammad

**Publication info:** PLoS ONE 10.8: NA. Public Library of Science. (Aug 13, 2015)

ProQuest document link

**Abstract (English):** Background

Aberrant expression of tumor suppressor genes may correspond to the abnormal cell development and tumorigenesis. Rbl2/p130, a member of retinoblastoma family of proteins, has growth suppressive properties. Numerous studies reported de-regulation of Rbl2/p130 in various types of cancer as a consequence of a number of genetic alterations. However, role of epigenetic mechanisms like DNA methylation in Rbl2/p130 expression remains elusive.

Methods

In the current study, 76 breast cancer tumors along with normal tissues (n = 76), blood (n = 76) of respective individuals and control blood (n = 50) were analyzed. Rbl2/p130 expression was analyzed by quantitative real time PCR (syber green method). Promoter methylation status was studied through methylation specific PCR of bisulfite converted genomic DNA. Data was analyzed using various statistical tests.

Results

We report significantly reduced Rbl2/p130 expression (P = 0.001) in tumors tissues as compared to control samples. Similarly, Rbl2/p130 expression varies with age and disease stages (P = 0.022), which suggest its involvement in tumor progression. Aberrant promoter methylation ([DELTA]meth) was found in almost all the diseased samples and that was significantly different (P0.001) with control samples. Similarly, methylation status varies significantly with tumor progression stages (P = 0.022). Hyper-methylation was observed at -1, +3, +15 and +75 of Rbl2/p130 promoter flanking around the TSS. Statistical analysis revealed that Rbl2/p130 expression negatively correlates to its promoter methylation (r = -0.412) in tumor tissues. Our results reflect an epigenetic regulation of Rbl2/p130 expression in breast cancer. This highlights the importance of Rbl2/p130 promoter methylation in breast cancer pathogenesis.

Author(s): Farman Ullah [sup.1], Taimoor Khan [sup.1], Nawab Ali [sup.2], Faraz Arshad Malik [sup.1], Mahmood Akhtar Kayani [sup.1], Syed Tahir Abbas Shah [sup.1], Muhammad Saeed [sup.1]*

Introduction

The eukaryotic cell cycle is a precisely regulated set of events, which ensures cell growth and precise transmission of genetic information to daughter cells. Alterations in the activity of genes regulating cell cycle, leads to abnormal cells division and proliferation [1]. Such deregulations are common in cancer pathogenesis[2].

Members of retinoblastoma (Rb) family of proteins play pivotal role in cell cycle regulation. Rb family of proteins comprises of three members namely retinoblastoma protein (pRb/p105), retinoblastoma like protein-1 (Rbl1/p107) and retinoblastoma like protein-2 (Rbl2/p130). These three members of the family are collectively referred to as "pocket proteins"[3-5].

**Links:** Check USPTO-STIC for Availability

**Language:** English

**Publication title:** PLoS ONE



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**Volume:** 10

**Issue:** 8

**Pages:** NA

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**ISSN:** 1932-6203

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**First available:** 2015-08-15

**Updates:** 2015-08-152015-08-21

**Database:** Gale Group Health Periodicals Database (1983 - current)



## Gene-based polymorphisms, genomic organization of interferon-β promoter stimulator 1 (IPS-1) gene and association study with the natural resistance to grass carp reovirus in grass carp Ctenopharyngodon idella

**Author:** Wan, Quanyuan; Su, Jianguo; Chen, Xiaohui; Yang, Chunrong

**Publication info:** Developmental and comparative immunology : 756-765. Elsevier Ltd. (2013)
ProQuest document link

**Abstract (summary):** Interferon-β promoter stimulator 1 (IPS-1) plays a pivotal role in the production of type I interferon (IFN) and pro-inflammatory cytokines. Though its function in innate immunity was widely studied, its genetic polymorphisms and evolution information were rarely known. Herein, the present study firstly identified and characterized the genomic organization of CiIPS-1 (IPS-1 gene of grass carp, Ctenopharyngodon idella) and its nucleotide polymorphisms. CiIPS-1 consists of 8789bp, with a 314bp 5′-flanking region, 6 exons and 5 introns. After a challenge experiment, statistical analysis was performed to assess the association between the 17 polymorphisms of CiIPS-1 and the natural resistance of C. idella to grass carp reovirus (GCRV), which revealed that −3741 C/T in the intron 1, 933 C/G in the coding sequence and 2299 G/T in the last intron were significant ($P<0.05$). The subsequent secondary challenge test further confirmed that −3741TT group were more resistant than −3741CC group to GCRV infection, while 933GG and 2299GG stocks were respectively more susceptible than 933CC and 2299TT stocks ($P<0.05$). In addition, haplotype and polymorphism–polymorphism interaction analyses uncovered four significant haplotypes ($P<0.05$) and two notable polymorphisms. These findings may provide basic data for the further functional research of CiIPS-1,



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and genetic markers for molecular and transgenic breeding of C. idella.

**Links:** Check USPTO-STIC for Availability

**Language:** English

**Publication title:** Developmental and comparative immunology

**Pages:** 756-765.

**Publication type:** Journal

**ISSN:** 0145-305X

**Publisher:** Elsevier Ltd

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**Updates:** 2016-05-04

**Database:** AGRIS (1975 - 2016)

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## Signal Transducer and Activator of Transcription 2 (STAT2) Metabolism Coupling Postmitotic Outgrowth to Visual and Sound Perception Network in Human Left Cerebrum by Biocomputation

**Author:** Wang, Lin; Huang, Juxiang; Jiang, Minghu; Lin, Hong
**Publication info:** Journal of Molecular Neuroscience 47.3: 649-58. Springer Nature B.V. (Jul 2012)
ProQuest document link

**Abstract (summary):** We constructed the high-expression signal transducer and activator of transcription 2 (STAT2) metabolism coupling postmitotic outgrowth to visual and sound perception network in human left cerebrum compared with low-expression (fold change ≥2) chimpanzee left cerebrum in GEO data set by using integration of gene regulatory network inference method with gene ontology (GO) analysis of STAT2-activated up- and downstream network. Our result showed that upstream RECQL, PDIA2, ENOSF1, THBS4, RASGRP1, PER2, PDE8A, ORC2L, DCI, OGG1_2, SMA4, GPD1, etc. activated STAT2, and downstream STAT2-activated GSTM3_1, GOSR1, SH3BGR, OSBPL8, PHYH, SAPS2, C21orf33, PDIA2, TRAPPC6A, ENOSF1, CAMTA1, GTF2I_2, etc. in human left cerebrum. STAT2-activated network enhanced regulation of small GTPase-mediated signal transduction, regulation of transcription, regulation of mitosis, regulation of cell growth, positive regulation of phosphoinositide 3-kinase cascade, positive regulation of fat cell differentiation, negative regulation of DNA replication, negative regulation of progression through cell cycle, cyclic nucleotide metabolism, lipid metabolism, carbohydrate metabolism, vitamin A metabolism, N-acetylglucosamine metabolism, UDP-N-acetylgalactosamine metabolism, fatty acid transport, intracellular protein transport, vesicle-mediated transport, lipid transport, retrograde transport, Ras protein signal transduction, Wnt receptor signaling pathway, nervous system development, cell extension, cell adhesion, cell differentiation, circadian rhythm, generation of precursor



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metabolites and energy, establishment of blood-nerve barrier, visual perception, sensory perception of sound, and poly-N-acetyllactosamine biosynthesis, as a result of inducing metabolism coupling postmitotic outgrowth to visual and sound perception in human left cerebrum.[PUBLICATION ABSTRACT]

**Links:** Check USPTO-STIC for Availability

**Language:** English

**Publication title:** Journal of Molecular Neuroscience

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**DOI:** http://dx.doi.org/10.1007/s12031-011-9702-4

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**Copyright:** Springer Science+Business Media, LLC 2012

**First available:** 2012-06-07

**Updates:** 2012-06-072012-06-082012-08-012013-01-022013-04-122014-03-072014-03-072014-08-30

**Database:** 2 databasesView listHide listProQuest Biological &Health Science Professional, ProQuest Environmental Science Professional

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Document 5 of 21

## Defense response signaling for disease resistance in rice

**Author:** Bruce, Myron Anthony

**Publication info:** Colorado State University. ProQuest Dissertations Publishing, 2011. 3468751.

ProQuest document link

**Abstract (English):** Plant disease resistance is often associated with a type of programmed cell death (PCD) called the hypersensitive response (HR). Upon recognition of pathogen proteins or their activity, the affected cell and surrounding cells commit to the HR to limit pathogen spread throughout the plant. This mechanism of plant disease resistance renders a pathogen avirulent on the host plant. Lesion mimics (LM) are a class of mutant or transgenic plants that spontaneously show lesions resembling the HR in the absence of biotic stress. Based on the association of the LM phenotype to cell death and its similarity to disease symptoms and the HR, this phenotype is a useful tool to dissect and understand the plant defense response.

To identify genes that when mutated result in the LM phenotype in rice, we used a microarray approach. By hybridizing labeled genomic DNA from an allelic series of deletion mutants to an oligonucleotide microarray, we identified candidate genes and genic regions that were deleted in a set of mutants. For one mutant, *spl1*, mutations in a cytochrome P450 gene were confirmed to confer the LM phenotype. A genome browser



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developed to handle these microarray data is a community resource that enables researchers to rapidly identify untagged deletion mutations in rice.

Members of the 14-3-3 protein family were recently shown to be positive regulators of cell death and the HR in Arabidopsis. In contrast, the work herein shows that a rice 14-3-3 protein is a negative regulator of cell death and resistance. Transgenic plants carrying a construct that silences the rice 14-3-3 gene *GF14e* exhibit a LM phenotype and enhanced resistance to two distinct rice pathogens, *Xanthomonas oryzae* pv. *oryzae* and *Rhizoctonia solani*. These *GF14e*-silenced plants also showed enhanced expression of genes associated with salicylic acid (SA) mediated defense responses, including members of the peroxidase gene family. The *GF14e*-silenced plants did not show enhanced expression of marker genes associated with the ethylene response pathway, indicating that *GF14e* may negatively regulate SA mediated defense responses, but does not affect ethylene regulated responses.

Silencing *GF14e* results in the upregulation of several defense responsive peroxidases. cDNA from *GF14e* silenced plants was used in quantitative PCR (qPCR) to assay expression of four peroxidase genes. Of these, three showed significant upregulation in 2 weeks after sowing (WAS) and 5 WAS *GF14e*-silenced plants. The promoters of the three upregulated genes (*PO-C1*, *Pox8.1*, *Pox22.3*) contain at least one W-box element. In contrast, the peroxidase (*Pox5.1*) that did not show upregulation and is not upregulated in R gene mediated responses had no W-box elements. W-box elements are binding sites for the WRKY class of transcription factors. This result, coupled with bioinformatic predictions of potential rice 14-3-3 clients, and the observation that some WRKY genes are upregulated in *GF14e* silenced lines indicates that *GF14e* may negatively regulate WRKY transcription factors related to cell death and defense responses.

Based on the implication that one 14-3-3 protein negatively regulates defense responses related cell death, provides a framework to develop a model for how this protein might function in the plant disease resistance response.

**Links:** Check USPTO-STIC for Availability

**Subject:** Molecular biology;Plant pathology

**Classification:** 0307: Molecular biology0480: Plant Pathology

**Identifier / keyword:** Biological sciences, Disease resistance, Lesion mimics, Rice pathogens

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**Advisor:** Leach, Jan E.

**Committee member:** Bush, Daniel R., Chisholm, Stephen, Ranu, Rajinder

**University/institution:** Colorado State University



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**Department:** Bioagricultural Sciences &Pest Management

**University location:** United States -- Colorado

**Degree:** Ph.D.

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## FEMS YEAST RESEARCH SEP 2009 Volume: 9 Issue: 6

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**First available:** 2010-11-17

**Database:** Current Contents® Search (1998 - current)

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Document 7 of 21

## Avoiding unscheduled transcription in shared promoters: Saccharomyces cerevisiae Sum1p represses the divergent gene pair SPS18-SPS19 through a midsporulation element (MSE)

**Author:** Gurvitz, Aner 1 ; Suomi, Fumi; Rottensteiner, Hanspeter; Hiltunen, J Kalervo; Dawes, Ian W; Guenther, Daum 1 Center for Physiology, Pathophysiology and Immunology, Institute of Physiology, Section of Physiology of Lipid Metabolism, Medical University of Vienna Vienna, Austria

**Publication info:** FEMS Yeast Research 9.6: 821-831. Wiley-Blackwell, 111 River Street Hoboken NJ 07030-5774 USA. (Sep 2009)

ProQuest document link

**Abstract (English):** The sporulation-specific gene SPS18 shares a common promoter region with the oleic acid-inducible gene SPS19. Both genes are transcribed in sporulating diploid cells, albeit unevenly in favour of SPS18, whereas in haploid cells grown on fatty acids only SPS19 is highly activated. Here, SPS19 oleate-response element (ORE) conferred activation on a basal CYC1-lacZ reporter gene equally in both orientations, but promoter analysis using SPS18-lacZ reporter constructs with deletions identified a repressing fragment containing a midsporulation element (MSE) that could be involved in imposing directionality towards SPS19 in oleic acid-induced cells. In sporulating diploids, MSEs recruit the Ndt80p transcription factor for activation, whereas under vegetative conditions, certain MSEs are targeted by the Sum1p repressor in association with Hst1p and Rfm1p. Quantitative real-time PCR demonstrated that in haploid sum1l, hst1l, or rfm1l cells, oleic acid-dependent expression of SPS18 was higher compared with the situation in wild-type cells, but in the sum1l mutant, this effect was diminished in the absence of Oaf1p or Pip2p. We conclude that SPS18 MSE is a functional element repressing the expression of both SPS18 and SPS19, and is a component of a stricture mechanism shielding SPS18 from the dramatic increase in ORE-dependent transcription of SPS19 in oleic acid-grown cells.

**Links:** Check USPTO-STIC for Availability

**Language:** English

**Publication title:** FEMS Yeast Research

**Volume:** 9

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**First available:** 2010-04-29

**Updates:** 2015-03-31

**Database:** ProQuest Biological &Health Science Professional (1971 - current)



## Promoter motifs in Candida tropicalis

**Author:** Anonymous

**Publication info:** Official Gazette of the United States Patent and Trademark Office Patents (Jun 17, 2008)
ProQuest document link

**Abstract (English):** The present invention provides modified promoters from Candida troplicalis CYP and POX4 genes. The modified promoters have various sequence motifs added, deleted, or altered in order to modulate expression of a coding sequence operably linked thereto. The sequence motifs comprise repressors of gene induction (URS sequences) and activators of gene induction (UAS sequences) as well as oleic acid response elements (ORE sequences). Yeast host cells comprising such modified promoters are also provided. Methods of altering expression of a protein of the beta or omega oxidation pathways using a subject modified promoter are also provided.

**Links:** Check USPTO-STIC for Availability

**Author affiliation:** Loveland, OH USA

**Patent information:** Publication number: US 07388084; Patent assignee: Cognis Corporation; Patent publication date: 17 June 2008 (20080617); Patent classification: 536/231; Publication country: US; Patent inventor: Wilson, C. Ron; ; Craft, David L.; ; Zhang, Yeyan; ; Stavenhagen, Jeffrey B.;

**Language:** English

**Language of abstract:** English

**Publication title:** Official Gazette of the United States Patent and Trademark Office Patents



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**Publication type:** Patent

**ISSN:** 0098-1133

**Date revised:** 2013-06-21: Correction to source data

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**Document type:** Patent

**Accession number:** PREV200800551723

**Document URL:** https://dialog.proquest.com/professional/docview/636427786?accountid=131444

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**First available:** 2010-07-22

**Updates:** 2013-07-03

**Database:** BIOSIS Previews® (1926 - current)

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Document 9 of 21

## Comparing and contrasting cis-regulatory sequences to identify functional noncoding sequence variation

**Author:** Doniger, Scott William

**Publication info:** Washington University in St. Louis. ProQuest Dissertations Publishing, 2008. 3316613.
ProQuest document link

**Abstract (English):** Cis-regulatory sequences play a key role in gene regulation, and are expected to be an important component of the genetic variation within and between species. While several studies have demonstrated that there is extensive gene expression variation within and between species, and that a large portion of this variation is due to cis-acting variation, identifying the molecular basis of this variation remains a substantial challenge. Here, we use comparative genomics to address this problem. We developed multiple evolutionary models specific to cis-regulatory motifs that enable us to identify conserved transcription factor binding sites and single nucleotide substitutions that are likely to disrupt binding. We applied these models to *Saccharomyces cerevisiae* and show that it is possible to identify functional binding sites by their conservation in three closely related yeast species, demonstrating that conservation is a good indicator of biological function. However, we also find that not all functional binding sites are conserved across these species, and in fact, the gain and loss of transcription factor binding sites appears to be common in the yeast genome. Furthermore, we find that even within the *S. cerevisiae* population there is sequence variation within binding sites that leads to significant allele-specific expression differences. Finally, we introduce additional evolutionary models that will identify all sequences showing lineage specific constraints and apply these models to identify sequences that are rapidly evolving along the human lineage. Our results show that comparative genomics is a powerful method for identifying functional noncoding sequences and noncoding variation, however, the data also show that the relationship between conservation and function is complex and much remains to be learned about the functional constraint in noncoding DNA.



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**Links:** Check USPTO-STIC for Availability

**Subject:** Genetics;Gynecology;Bioinformatics

**Classification:** 0369: Genetics0715: Bioinformatics

**Identifier / keyword:** Health and environmental sciences, Biological sciences, Comparative genomics, Computational biology, Preterm birth, Transcription factor binding sites, cis-regulatory

**Number of pages:** 226

**Degree date:** 2008

**School code:** 0252

**Source:** DAI-B 69/05, Dissertation Abstracts International

**Place of publication:** Ann Arbor

**Country of publication:** United States

**ISBN:** 978-0-549-64678-5

**Advisor:** Fay, Justin

**University/institution:** Washington University in St. Louis

**University location:** United States -- Missouri

**Degree:** Ph.D.

**Source type:** Dissertations &Theses

**Language:** English

**Document type:** Dissertation/Thesis

**Dissertation/thesis number:** 3316613

**ProQuest document ID:** 304442059

**Document URL:** https://dialog.proquest.com/professional/docview/304442059?accountid=131444

**Copyright:** Database copyright ProQuest LLC; ProQuest does not claim copyright in the individual underlying works.

**First available:** 2010-06-17

**Updates:** 2012-07-102014-01-132014-01-232015-08-272016-05-282020-10-082020-11-182020-11-302021-10-262022-10-29

**Database:** ProQuest Dissertations and Theses Professional

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## Mathematical modeling of signaling pathway dynamics and *stochastic gene expression

**Author:** Wang, Xiao

**Publication info:** The University of North Carolina at Chapel Hill. ProQuest Dissertations Publishing, 2006. 3219438.

ProQuest document link

**Abstract (English):** This thesis presents the development and analysis of stochastic and deterministic models of cellular biochemical networks, such as signaling pathways and gene regulatory networks. First, the model of the yeast pheromone response pathway is constructed. Stochastic modeling reveals that the biochemical steps that regulate activation of the mitogen-activated protein kinase Fus3 can account for the graded-to-binary conversion. The model is also used to investigate the effects of protein turnover on the response of the pathway. It is demonstrated that the inclusion of protein turnover can lead to sustained oscillations of protein concentration in the absence of feedback regulation, which indicates protein turnover as an important signaling regulation mechanism. Second, an engineered promoter that allowed the simultaneous repression and activation of gene expression in *Escherichia coli* was constructed and used to construct a stochastic model to study synthetic gene networks under increasingly complex conditions: unregulated, repressed, activated and simultaneously repressed and activated, and in the presence of positive feedback. The stochastic model quantitatively captures the means and distributions of the expression from the engineered promoter of this modular system and accurately predict the *in vivo* behavior of an expanded network that includes positive feedback. The model also reveals the counterintuitive prediction that noise in protein expression levels can increase upon arrest of cell division, which was confirmed experimentally.

**Links:** Check USPTO-STIC for Availability

**Subject:** Mathematics;Pharmacology;Operations research

**Classification:** 0796: Operations research0405: Mathematics0419: Pharmacology

**Identifier / keyword:** Health and environmental sciences, Applied sciences, Pure sciences, Gene expression, Signaling pathway, Stochastic

**Number of pages:** 113

**Degree date:** 2006

**School code:** 0153

**Source:** DAI-B 67/05, Dissertation Abstracts International

**Place of publication:** Ann Arbor

**Country of publication:** United States

**ISBN:** 978-0-542-68935-2

**Advisor:** Elston, Timothy C.

**University/institution:** The University of North Carolina at Chapel Hill

**University location:** United States -- North Carolina



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**Degree:** Ph.D.

**Source type:** Dissertations &Theses

**Language:** English

**Document type:** Dissertation/Thesis

**Dissertation/thesis number:** 3219438

**ProQuest document ID:** 305279903

**Document URL:** https://dialog.proquest.com/professional/docview/305279903?accountid=131444

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**First available:** 2010-06-17

**Updates:** 2013-05-012014-01-162014-01-202015-08-262016-05-252020-10-072020-11-202020-11-222021-04-222021-10-262022-10-31

**Database:** ProQuest Dissertations and Theses Professional

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## A bottom-up approach to gene regulation

**Author:** Guido, Nicholas James

**Publication info:** Boston University. ProQuest Dissertations Publishing, 2006. 3214940.

ProQuest document link

**Abstract (English):** The ability to construct synthetic gene networks enables detailed experimental investigations of simplified systems that can be compared to qualitative and quantitative models. If simple, well-characterized modules can be coupled together into more complex networks whose behavior is then predictable from that of the components, we may begin to build an understanding of cellular regulatory processes from the bottom up. Here, we engineered a promoter to allow simultaneous repression and activation of gene expression in *Escherichia coli,* and studied its behavior, in synthetic gene networks, under increasingly complex conditions: unregulated, repressed, activated, and simultaneously repressed and activated. We developed a stochastic model that quantitatively captures the means and distributions of the expression from the engineered promoter of this modular system, and showed that the model can accurately predict the in vivo behavior of the network when it is expanded to include positive feedback. The model also reveals the counterintuitive prediction that noise in protein expression levels can increase upon arrest of cell growth and division, which we confirmed experimentally. This work shows that one can indeed use the properties of regulatory subsystems to predict the behavior of larger, more complex regulatory networks, and that this bottom-up approach can provide novel insights into gene regulation.

**Links:** Check USPTO-STIC for Availability

**Subject:** Genetics;Molecular biology



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**Classification:** 0369: Genetics0307: Molecular biology

**Identifier / keyword:** Biological sciences, Gene regulation, Genetic networks, Synthetic biology

**Number of pages:** 73

**Degree date:** 2006

**School code:** 0017

**Source:** DAI-B 67/04, Dissertation Abstracts International

**Place of publication:** Ann Arbor

**Country of publication:** United States

**ISBN:** 978-0-542-66129-7

**Advisor:** Collins, James J.

**University/institution:** Boston University

**University location:** United States -- Massachusetts

**Degree:** Ph.D.

**Source type:** Dissertations &Theses

**Language:** English

**Document type:** Dissertation/Thesis

**Dissertation/thesis number:** 3214940

**ProQuest document ID:** 305363667

**Document URL:** https://dialog.proquest.com/professional/docview/305363667?accountid=131444

**Copyright:** Database copyright ProQuest LLC; ProQuest does not claim copyright in the individual underlying works.

**First available:** 2010-06-17

**Updates:** 2013-04-082014-01-162014-01-202015-08-232016-03-142020-10-052020-11-202020-11-202021-11-182022-11-01

**Database:** ProQuest Dissertations and Theses Professional

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## Identification of a gene induced in conjugation-promoted cells of toxic marine dinoflagellates *Alexandrium tamarense* and *Alexandrium catenella* using differential display analysis

**Author:** Hosoi-Tanabe, Shoko 1 ; Tomishima, Sayaka 2 ; Nagai, Satoshi 3 ; Sako, Yoshihiko 2 1 Department of Ecosystem Studies, School of Environmental Science, The University of Shiga Prefecture, 2500 Hassaka-cho,



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Hikone, Shiga 522-8533, Japan 2 Laboratory of Marine Microbiology, Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan 3 Fisheries Research Agency of Japan, National Research Institute of Fisheries and Environment of Inland Sea, Harmful Algal Bloom Division, Toxic Phytoplankton Section, Ohno, Saeki, Hiroshima 739-0452, Japan
**Publication info:** FEMS Microbiology Letters 251.1: 161-168. Oxford University Press. (Oct 2005)
ProQuest document link

**Abstract (English):** Marine dinoflagellates *Alexandrium tamarense* and *Alexandrium catenella* produce toxins that cause paralytic shellfish poisoning (PSP). A detailed mechanism of encystment is necessary for a better understanding of bloom dynamics and the toxic effect of these organisms. In this study, a cDNA that was up-regulated in conjugation-promoted cells at encystment was identified using differential display. It encoded a polypeptide of 195 amino acids with a molecular weight of 20,900 Da. The deduced amino acid sequence of this cDNA showed 62% similarity with the polypeptide encoded by *SPS19*, a gene that is activated specifically during spore maturation and spore wall formation in *Saccharomyces cerevisiae*. Therefore, the cDNA obtained was termed an *SPS19* homolog in this study. The expression levels of the *SPS19* homolog were highest immediately after the promotion of conjugation and decreased sequentially later, a pattern similar to that of *SPS19* in the sporulation of *S. cerevisiae* in terms of the time of induction and the duration of expression. These similarities between the *SPS19* homolog and *SPS19* suggested that the putative function of the *SPS19* homolog might be an involvement in encystment. RT-PCR showed that the expression of the *SPS19* homolog was highest in conjugation-promoted cells but low in vegetative cells. The *SPS19* homolog was believed to be expressed constantly in order for cells to respond rapidly to environmental changes and ensure encystment. Characterization of the identified gene might help in understanding the mechanism of encystment.

**Links:** Check USPTO-STIC for Availability

**Language:** English

**Language of abstract:** English

**Publication title:** FEMS Microbiology Letters

**Volume:** 251

**Issue:** 1

**Pages:** 161-168

**Publisher location:** Oxford

**DOI:** http://dx.doi.org/10.1016/j.femsle.2005.07.046

**Document type:** Journal Article

**Document URL:** https://dialog.proquest.com/professional/docview/23065555777?accountid=131444

**Copyright:** © 2005 Federation of European Microbiological Societies

**First available:** 2019-10-18

**Updates:** 2019-10-182019-12-11

**Database:** 2 databasesView listHide listProQuest Biological &Health Science Professional, ProQuest Environmental Science Professional



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## Functional analysis of the *Saccharomyces cerevisiae* Arf -GAPs in sporulation

**Author:** Connolly Rohrbach, Jaime Elizabeth

**Publication info:** State University of New York at Stony Brook. ProQuest Dissertations Publishing, 2005. 3206493.

ProQuest document link

**Abstract (English):** *SPO14*, the major yeast phospholipase D (PLD), is essential for sporulation and mediates synthesis of the prospore membrane that encompasses the haploid nuclei generated through the meiotic divisions. PLD catalyzes the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) and choline. PA stimulates Arf-GTPase activating proteins (Arf-GAPs), key components in membrane trafficking events and actin cytoskeletal function. To determine if Spo14p-generated PA mediates its biological response through Arf-GAPs, sporulation was analyzed in cells deleted for each of the five yeast Arf-GAPs. Only *gcs1Δ* mutants displayed a sporulation defect similar to *spo14* mutants: cells deleted for *GCS1* initiate the sporulation program but are defective in the synthesis of the prospore membrane. Arf-GAP catalytic activity, but not the pleckstrin homology (PH) domain, is required for prospore membrane formation. Examination of Gcs1p-GFP revealed that it is a soluble protein and is phosphorylated during sporulation. Interestingly, cells deleted for *GCS1* have reduced levels of Spo14p-generated PA.

In addition to a defect in prospore membrane formation, morphological studies revealed unusual structures reminiscent of the class E compartment, in a small number of cells, which results from an inability of the late endosome to invaginate intralumenal vesicles. FM4-64 analysis revealed that endosome to vacuole transport is impaired in *gcs1* mutants during sporulation. This suggests that Gcs1p plays a minor role in a recycling step between the endosome and the prospore membrane.

To further delineate the genetic network that *GCS1* operates in during sporulation, an unbiased screen and a candidate approach were undertaken. Given the requirement for Arf-GAP activity, the relationship between Arf1p and Gcs1p was probed in sporulation. Arf1p plays an important role in sporulation and appears to be the major Arf functioning with Gcs1p during this process. A unique genetic relationship between *GCS1* and *SWH1* , which encodes for an oxysterol binding (OSB) protein, has additionally been uncovered. The OSB domain of Swh1p is not required for genetic suppression of *gcs1Δ*. Furthermore, overexpression of other yeast Arf-GAPs was unable to suppress *gcs1Δ*. Taken together, these results indicate that *GCS1* is uniquely essential for sporulation and suggests that *GCS1* and the Arf cycle positively regulate *SPO14*.

**Links:** Check USPTO-STIC for Availability

**Subject:** Molecular biology;Cellular biology;Genetics

**Classification:** 0307: Molecular biology0379: Cellular biology0369: Genetics

**Identifier / keyword:** Biological sciences, Arf-GAPs, Phospholipase D, Sporulation

**Number of pages:** 157

**Degree date:** 2005

**School code:** 0771



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**Source:** DAI-B 67/02, Dissertation Abstracts International

**Place of publication:** Ann Arbor

**Country of publication:** United States

**ISBN:** 978-0-542-55266-3

**Advisor:** Engebrecht, JoAnne

**University/institution:** State University of New York at Stony Brook

**University location:** United States -- New York

**Degree:** Ph.D.

**Source type:** Dissertations &Theses

**Language:** English

**Document type:** Dissertation/Thesis

**Dissertation/thesis number:** 3206493

**ProQuest document ID:** 305363427

**Document URL:** https://dialog.proquest.com/professional/docview/305363427?accountid=131444

**Copyright:** Database copyright ProQuest LLC; ProQuest does not claim copyright in the individual underlying works.

**First available:** 2010-06-17

**Updates:** 2013-05-132014-01-212015-08-272016-03-142020-10-082020-11-112020-11-182022-10-31

**Database:** ProQuest Dissertations and Theses Professional

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## Distinct functions for yeast Arf-GAPs in development

**Author:** Adams, Cherie-Lee A.

**Publication info:** Dalhousie University (Canada). ProQuest Dissertations Publishing, 2004. MR00810.
ProQuest document link

**Abstract (English):** Development is a programmed sequence of events in cells involving regulated growth resulting from interaction between the genome, cytoplasm, and extracellular environment. Stationary phase is a developmental state characterized by a cessation of cell proliferation in response to starvation. Sporulation in yeast is a developmental process encompassing the two meiotic divisions and the subsequent packaging of the four haploid progeny as spores within the maternal cytoplasm. I have investigated the yeast Gcs1 protein that has been shown to be essential to (1) cells exiting stationary phase and (2) completion of sporulation. My data demonstrate that the catalytic Arf-GAP activity is the function of Gcs1 required during exit from stationary phase, but not for sporulation. Furthermore, I have shown that the characterized capacity for Gcs1 to stimulate actin polymerization is independent of its Arf-GAP activity. Thus, I have identified distinct and separable



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functions in the defining member of the yeast Arf-GAP family.

**Links:** Check USPTO-STIC for Availability

**Subject:** Cellular biology;Molecular biology

**Classification:** 0379: Cellular biology0307: Molecular biology

**Identifier / keyword:** Biological sciences

**Number of pages:** 114

**Degree date:** 2004

**School code:** 0328

**Source:** MAI 43/06M, Masters Abstracts International

**Place of publication:** Ann Arbor

**Country of publication:** United States

**ISBN:** 978-0-494-00810-2

**Advisor:** Murray, Lois E.

**University/institution:** Dalhousie University (Canada)

**University location:** Canada -- Nova Scotia, CA

**Degree:** M.Sc.

**Source type:** Dissertations &Theses

**Language:** English

**Document type:** Dissertation/Thesis

**Dissertation/thesis number:** MR00810

**ProQuest document ID:** 305100455

**Document URL:** https://dialog.proquest.com/professional/docview/305100455?accountid=131444

**Copyright:** Database copyright ProQuest LLC; ProQuest does not claim copyright in the individual underlying works.

**First available:** 2010-06-17

**Updates:** 2022-10-142022-10-152022-10-152022-10-152022-10-152022-10-162022-10-162022-10-162022-10-162022-10-162022-10-172022-10-172022-10-172022-10-172022-10-182022-10-182022-10-192022-10-19 2022-10-202022-10-30

**Database:** ProQuest Dissertations and Theses Professional



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### Promoter Motifs in Candida Tropicalis

**Author:** Wilson, R C; Craft, D L; Zhang, Y; Stavenhagen, J B

**Publication info:** National Technical Information Service (Aug 14, 2003)

ProQuest document link

**Abstract (English):** The present invention provides modified promoters from Candida troplicalis CYP and POX4 genes. The modified promoters have various sequence motifs added, deleted, or altered in order to modulate expression of a coding sequence operably linked thereto. The sequence motifs comprise repressors of gene induction (URS sequences) and activators of gene induction (UAS sequences) as well as oleic acid response elements (ORE sequences). Yeast host cells comprising such modified promoters are also provided. Methods of altering expression of a protein of the beta or omega oxidation pathways using a subject modified promoter are also provided.

**Links:** Check USPTO-STIC for Availability

**Corporate/institutional author:** Cognis Corporation, Ambler, PA

**Language:** English

**Language of abstract:** English

**Publication title:** National Technical Information Service

**Publication type:** Government &Official Publications

**Report number:** PAT-APPL-10 640 962

**Contract number:** 70NANB8H4033

**Publisher location:** United States

**Document type:** Patent

**Accession number:** PB2007-110042

**Document URL:** https://dialog.proquest.com/professional/docview/806491168?accountid=131444

**First available:** 2010-11-19

**Database:** NTIS: National Technical Information Service (1964 - current)

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### The biochemistry of peroxisomal β-oxidation in the yeast *Saccharomyces cerevisiae*

**Author:** Hiltunen, J Kalervo 1 ; Mursula, Anu M 1 ; Rottensteiner, Hanspeter 2 ; Wierenga, Rik K 1 ; Kastaniotis, Alexander J 1 ; Gurvitz, Aner 3 1 Biocenter Oulu and Department of Biochemistry, P.O. Box 3000, FIN-90014 University of Oulu, Oulu, Finland 2 Institut für Physiologische Chemie, Ruhr Universität Bochum, D-44780 Bochum, Germany 3 Biocenter Oulu and Department of Biochemistry, P.O. Box 3000, FIN-90014 University of Oulu, Oulu, Finland; Institut für Biochemie und Molekulare Zellbiochemie der Universität Wien and Ludwig Boltzmann-Forschungstelle für Biochemie, Vienna Biocenter, Dr. Bohrgasse 9, A-1030 Vienna, Austria



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**Publication info:** FEMS Microbiology Reviews 27.1: 35-64. Oxford University Press. (Apr 2003)
ProQuest document link

**Abstract (English):** Peroxisomal fatty acid degradation in the yeast *Saccharomyces cerevisiae* requires an array of β-oxidation enzyme activities as well as a set of auxiliary activities to provide the β-oxidation machinery with the proper substrates. The corresponding classical and auxiliary enzymes of β-oxidation have been completely characterized, many at the structural level with the identification of catalytic residues. Import of fatty acids from the growth medium involves passive diffusion in combination with an active, protein-mediated component that includes acyl-CoA ligases, illustrating the intimate linkage between fatty acid import and activation. The main factors involved in protein import into peroxisomes are also known, but only one peroxisomal metabolite transporter has been characterized in detail, Ant1p, which exchanges intraperoxisomal AMP with cytosolic ATP. The other known transporter is Pxa1p–Pxa2p, which bears similarity to the human adrenoleukodystrophy protein ALDP. The major players in the regulation of fatty acid-induced gene expression are Pip2p and Oaf1p, which unite to form a transcription factor that binds to oleate response elements in the promoter regions of genes encoding peroxisomal proteins. Adr1p, a transcription factor, binding upstream activating sequence 1, also regulates key genes involved in β-oxidation. The development of new, postgenomic-era tools allows for the characterization of the entire transcriptome involved in β-oxidation and will facilitate the identification of novel proteins as well as the characterization of protein families involved in this process.

**Links:** Check USPTO-STIC for Availability

**Language:** English

**Language of abstract:** English

**Publication title:** FEMS Microbiology Reviews

**Volume:** 27

**Issue:** 1

**Pages:** 35-64

**Publisher location:** Delft

**DOI:** http://dx.doi.org/10.1016/S0168-6445(03)00017-2

**Document type:** Journal Article

**Document URL:** https://dialog.proquest.com/professional/docview/2476548518?accountid=131444

**Copyright:** © 2003 Federation of European Microbiological Societies.

**First available:** 2021-01-11

**Updates:** 2021-01-112021-06-212022-08-22

**Database:** 2 databasesView listHide listProQuest Biological &Health Science Professional, ProQuest Environmental Science Professional

**A novel element in the promoter of the Saccharomyces cerevisiae gene SPS19 enhances ORE-dependent up-regulation in oleic acid and is essential for de-repression**

**Publication info:** Molecular and General Genetics 262.3: 0481-0492. Springer-VerlagLOC: BerlinLOC: HeidelbergJID: 438. (Nov 4, 1999)
ProQuest document link

**Links:** Check USPTO-STIC for Availability

**Language:** English

**Publication title:** Molecular and General Genetics

**Volume:** 262

**Issue:** 3

**Pages:** 0481-0492

**ISSN:** 0026-8925

**Publisher:** Springer-VerlagLOC: BerlinLOC: HeidelbergJID: 438

**Subfile:** Genetics Abstracts; Microbiology Abstracts C: Algology, Mycology &Protozoology

**Date revised:** 2006-11-01

**Document type:** Journal Article

**Accession number:** 4613206

**Document URL:** https://dialog.proquest.com/professional/docview/17383897?accountid=131444

**First available:** 2010-04-29

**Database:** ProQuest Biological &Health Science Professional (1971 - current)

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**Characterisation and transcriptional regulation of the gene SPS19 encoding the peroxisomal 2,4-dienoyl-CoA reductase of Saccharomyces cerevisiae, and the fate and role of peroxisomes during yeast sporulation**

**Author:** Aner, Gurvitz
**Publication info:** University of New South Wales (Australia). ProQuest Dissertations Publishing, 1998. 0598986.
ProQuest document link

**Abstract (English):** In the course of this study, the Saccharomyces cerevisiae SPS19 gene was shown to encode the $\beta$-oxidation auxiliary enzyme 2,4-dienoyl-CoA reductase that is required for the degradation of fatty acids with double bonds at even-numbered positions such as petroselineate (cis-C$\sb{18:1(6)}).$ A $\Delta$sps19 strain was unable to grow or sporulate on this fatty acid as the sole carbon source. Sps19p was over-expressed in S. cerevisiae and purified to homogeneity, and antibodies raised against it decorated the peroxisomal matrix of oleate-induced cells. Mutant yeast cells expressing a modified human mitochondrial reductase that was directed to peroxisomes sporulated on petroselineate, and these may serve as a test



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organism for human 2,4-dienoyl-CoA reductase deficiency.

SPS19 diverges from a promoter region that regulates the sporulation-specific gene SPS18 which was previously cloned in a screen for promoters that were expressed during ascosporogenesis. SPS19 was shown here to be regulated by an oleate response element (ORE) that was devoid of essential sporulation-specific regulatory functions when tested in a promoterless CYC1-LacZ reporter construct. The SPS19 ORE as well as the 3$\sp\prime$-ORE half site bound the Pip2p-Oaf1p transcriptional activator protein, and a DNA fragment delimited by the half site and an adjacent GC-rich stretch may act as the target for a putative auxiliary factor that both enhances the oleate induction exerted by the Pip2p-Oaf1p protein, and also dictates that this up-regulation be solely directed towards SPS19.

Peroxisomes are a major class of organelles that in fungi are the exclusive site of $\beta$-oxidation, however, their role in S. cerevisiae sporulation, and their transmission to spores was not known. A green fluorescent protein probe targeted to peroxisomes was used to show that peroxisomes were heritable and not formed de novo either during sporulation or spore germination. S. cerevisiae $\Delta$pex6 mutants lacking peroxisomal structures, and a $\Delta$pox1 strain that was blocked at the first step of $\beta$-oxidation, were both proficient for sporulation in acetate medium, indicating that this metabolic process and the compartment containing it are dispensible for meiotic development.

**Links:** Check USPTO-STIC for Availability

**Subject:** Biochemistry;Molecular biology

**Classification:** 0487: Biochemistry0307: Molecular biology

**Identifier / keyword:** Pure sciences, Biological sciences, dienoyl-CoA reductase

**Number of pages:** 1

**Degree date:** 1998

**School code:** 0423

**Source:** DAI-B 59/03, Dissertation Abstracts International

**Place of publication:** Ann Arbor

**Country of publication:** United States

**University/institution:** University of New South Wales (Australia)

**University location:** Australia

**Degree:** Ph.D.

**Source type:** Dissertations &Theses

**Language:** English

**Document type:** Dissertation/Thesis

**Dissertation/thesis number:** 0598986

**ProQuest document ID:** 304476727

**Document URL:** https://dialog.proquest.com/professional/docview/304476727?accountid=131444



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**First available:** 2010-06-17

**Updates:** 2014-01-152014-01-152015-08-272016-05-132020-10-022020-11-232020-11-302020-11-302020-12-042021-11-182022-10-29

**Database:** ProQuest Dissertations and Theses Professional



## Regulation of the yeast SPS19 gene encoding peroxisomal 2,4-dienoyl-CoA reductase by the transcription factors Pip2p and Oaf1p: beta-oxidation is dispensable for Saccharomyces cerevisiae sporulation in acetate medium.

**Author:** Gurvitz, A 1 ; Rottensteiner, H; Hiltunen, J K; Binder, M; Dawes, I W; Ruis, H; Hamilton, B 1 School of Biochemistry and Molecular Genetics, University of New South Wales, Sydney, Australia.

**Publication info:** Molecular microbiology 26.4: 675-685. (November 1997)

ProQuest document link

**Abstract (summary):** The yeast SPS19 gene encoding the peroxisomally targeted 2,4-dienoyl-CoA reductase shares its promoter region (291 bp) with the sporulation-specific gene SPS18. SPS19 is induced during sporulation in diploids but to a lesser extent than SPS18; under oleate induction conditions, SPS19, but not SPS18, is transcribed via an oleate response element (ORE) independently of ploidy or sporulation. The SPS19 ORE is the binding target of the Pip2p and Oaf1p transcription factors, and an SPS19-lacZ reporter gene, which is highly expressed in oleate-induced cells, is not activated in haploids devoid of either protein. We examined the expression of CYC1-lacZ reporter constructs carrying the SPS19 and CTA1 OREs in diploids propagated under sporulation conditions and have shown that OREs are not sufficient for heterologous expression during yeast development. In addition, diploids deleted at either PIP2 or OAF1 demonstrated abundant ascosporogenesis, indicating that these genes are not essential for sporulation. A deltapex6 strain lacking peroxisomal structures and one devoid of fatty acyl-CoA oxidase (deltapox1), the first step in fungal beta-oxidation, were both proficient for sporulation and, hence, beta-oxidation and the peroxisomal compartment containing it are dispensable for meiotic development.

**Links:** Check USPTO-STIC for Availability

**Language:** English

**Publication title:** Molecular microbiology

**Volume:** 26

**Issue:** 4

**Pages:** 675-685

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Page 3015 of 3077

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## The Saccharomyces cerevisiae peroxisomal 2,4-dienoyl-CoA reductase is encoded by the oleate-inducible gene SPS19

**Author:** Gurvitz, A; Rottensteiner, H; Kilpelainen, S H; Hartig, A; Hiltunen, J K; Binder, M; Dawes, I W; Hamilton, B

**Publication info:** JOURNAL OF BIOLOGICAL CHEMISTRY 272.35: 22140-22147. AMER SOC BIOCHEMISTRY MOLECULAR BIOLOGY INC. (Aug 29, 1997)

ProQuest document link

**Abstract (English):** beta-Oxidation is compartmentalized in mammals into both mitochondria and peroxisomes. Fatty acids with double bonds at even-numbered positions require for their degradation the auxiliary enzyme 2,4-dienoyl-CoA reductase, and at least three isoforms, two mitochondrial and one peroxisomal, exist in the rat. The Saccharomyces cerevisiae Sps19p is 34% similar to the human and rat mitochondrial reductases, and an SPS19 deleted strain was unable to utilize petroselineate (cis-C-18:1(6)) as the sole carbon source, but remained viable on oleate (cis-C-18:1(9)). Sps19p was purified to homogeneity from oleate-induced cells and the homodimeric enzyme (native molecular weight 69,000) converted 2,4-hexadienoyl-CoA into 3-hexenoyl-CoA in an NADPH-dependent manner and therefore contained 2,4-dienoyl-CoA reductase activity, Antibodies raised against Sps19p decorated the peroxisomal matrix of oleate-induced cells. SPS19 shares with the sporulation-specific SPS18 a common promoter region that contains an oleate response element, This element unidirectionally regulates transcription of the reductase and is sufficient for oleate induction of a promoterless CYC1-lacZ reporter gene. SPS19 is dispensable for growth and sporulation on solid acetate and oleate media, but is essential for these processes to occur on petroselineate.

**Links:** Check USPTO-STIC for Availability

**Author affiliation:** UNIV NEW S WALES,SCH BIOCHEM &MOL GENET,SYDNEY,NSW 2052,AUSTRALIA, UNIV VIENNA,INST BIOCHEM &MOL ZELLBIOL,A-1030 VIENNA,AUSTRIA, VIENNA BIOCTR,LUDWIG BOLTZMANN FORSCHUNGSSTELLE BIOCHEM,A-1030 VIENNA,AUSTRIA, UNIV OULU,DEPT BIOCHEM,BIOCTR OULU,FIN-90570 OULU,FINLAND, UNIV VIENNA,INST TUMORBIOL KREBSFORSCH,A-



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Page 3016 of 3077

1090 VIENNA,AUSTRIA

**Collaborator:** Hartig, Andreas, Hiltunen, Kalervo

**Language:** English

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**Publication title:** JOURNAL OF BIOLOGICAL CHEMISTRY

**Volume:** 272

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**Pages:** 22140-22147

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**Publisher:** AMER SOC BIOCHEMISTRY MOLECULAR BIOLOGY INC; 9650 ROCKVILLE PIKE, BETHESDA, MD 20814

**Publisher location:** 9650 ROCKVILLE PIKE, BETHESDA, MD 20814

**Number of references:** 61

**Document type:** Article

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**First available:** 2010-07-27

**Database:** SciSearch®: a Cited Reference Science Database (1974 - current)

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Document 21 of 21

## Identification of a sporulation-specific promoter regulating divergent transcription of two novel sporulation genes in Saccharomyces cerevisiae

**Author:** Coe, J G 1 ; Murray, L E; Dawes, I W 1 School of Biochemistry and Molecular Genetics, University of New South Wales, Sydney, Australia, Australia

**Publication info:** Molecular & general genetics : MGG 244.6: 661-72. (Sep 28, 1994)

ProQuest document link



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**Abstract (English):** Promoters that control gene expression in Saccharomyces cerevisiae only in a sporulation-specific manner have previously been isolated from a genomic yeast DNA library fused to a promoterless Escherichia coli lacZ gene. Two novel sporulation-specific genes, SPS18 and SPS19, were isolated using this technique. These genes are divergently controlled by the same promoter but with SPS18 expressed at four times the level of SPS19. Deletion analysis has shown that the promoter elements that exert sporulation control on each of the genes overlap, having a common 25 bp sequence located within the intergenic region. SPS18 encodes a 34-KDa protein of 300 amino acids that contains a putative zinc-binding domain and a region of highly basic residues that could target the protein to the nucleus. SPS19 encodes a 31-KDa protein of 295 amino acids, which has a peroxisomal targeting signal (SKL) at its C terminus; this protein belongs to the family of non-metallo short-chain alcohol dehydrogenases. A null mutation deleting the intergenic promoter prevented expression of both genes, and when homozygous in diploids, reduced the extent of sporulation four-fold; the spores that did form were viable, but failed to become resistant to ether, and were more sensitive to lytic enzymes. This phenotype reflects a defect in spore wall maturation, indicating that the product of at least one of the genes functions during the process of spore wall formation. Therefore these genes belong to the class of late sporulation-specific genes that are sequentially activated during the process of meiosis and spore formation.

**Links:** Check USPTO-STIC for Availability

**Language:** English

**Language of abstract:** English

**Publication title:** Molecular &general genetics : MGG

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**Issue:** 6

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**Publication type:** Journal

**ISSN:** 0026-8925, 0026-8925 (ISSNLinking)

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Ex. 1002
Page 3018 of 3077

**Database:** ToxFile® (1900 - current)

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PTO/SB/06 (09-11)
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| **PATENT APPLICATION FEE DETERMINATION RECORD**<br>Substitute for Form PTO-875 | Application or Docket Number<br>16/630,567 | Filing Date<br>01/13/2020 | ☑ To be Mailed |
|---|---|---|---|

**ENTITY:** ☑ LARGE ☐ SMALL ☐ MICRO

**APPLICATION AS FILED - PART I**

| FOR | (Column 1)<br>NUMBER FILED | (Column 2)<br>NUMBER EXTRA | | RATE ($) | FEE ($) |
|---|---|---|---|---|---|
| ☐ BASIC FEE (37 CFR 1.16(a), (b), or (c)) | N/A | N/A | | N/A | |
| ☐ SEARCH FEE (37 CFR 1.16(k), (i), or (m)) | N/A | N/A | | N/A | |
| ☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q)) | N/A | N/A | | N/A | |
| TOTAL CLAIMS (37 CFR 1.16(i)) | minus 20 = | * | | x $100 = | |
| INDEPENDENT CLAIMS (37 CFR 1.16(h)) | minus 3 = | * | | x $460 = | |
| ☐ APPLICATION SIZE FEE (37 CFR 1.16(s)) | If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s). | | | | |
| ☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j)) | | | | | |
| * If the difference in column 1 is less than zero, enter "0" in column 2. | | | | TOTAL | |

**APPLICATION AS AMENDED - PART II**

| AMENDMENT | 03/22/2023 | (Column 1)<br>CLAIMS REMAINING AFTER AMENDMENT | | (Column 2)<br>HIGHEST NUMBER PREVIOUSLY PAID FOR | (Column 3)<br>PRESENT EXTRA | | RATE ($) | ADDITIONAL FEE ($) |
|---|---|---|---|---|---|---|---|---|
| | Total (37 CFR 1.16(i)) | * 3 | Minus | ** 21 | = 0 | | x $100 = | 0 |
| | Independent (37 CFR 1.16(h)) | * 1 | Minus | *** 6 | = 0 | | x $480 = | 0 |
| | ☐ Application Size Fee (37 CFR 1.16(s)) | | | | | | | |
| | ☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j)) | | | | | | | |
| | | | | | | | TOTAL ADD'L FEE | 0 |

| AMENDMENT | | (Column 1)<br>CLAIMS REMAINING AFTER AMENDMENT | | (Column 2)<br>HIGHEST NUMBER PREVIOUSLY PAID FOR | (Column 3)<br>PRESENT EXTRA | | RATE ($) | ADDITIONAL FEE ($) |
|---|---|---|---|---|---|---|---|---|
| | Total (37 CFR 1.16(i)) | * | Minus | ** | = | | x $0 = | |
| | Independent (37 CFR 1.16(h)) | * | Minus | *** | = | | x $0 = | |
| | ☐ Application Size Fee (37 CFR 1.16(s)) | | | | | | | |
| | ☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j)) | | | | | | | |
| | | | | | | | TOTAL ADD'L FEE | |

| | |
|---|---|
| * If the entry in column 1 is less than the entry in column 2, write "0" in column 3. | LIE |
| ** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20". | /ANGELA D JOHNSON/ |
| *** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3". | |
| The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1. | |

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

*If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.*

Atty Docket No.: SS118-1026US

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

| | | | |
|---|---|---|---|
| In re Application | : | Aeling et al. | Confirmation No. 5356 |
| Serial No. | : | 16/630,567 | |
| Filing Date | : | January 13, 2020 | |
| Group Art Unit | : | 1656 | |
| Examiner | : | Noakes, S.M. | |
| For | : | **BIOLOGICAL METHODS FOR PREPARING TERPENES** | |

Mail Stop Amendments
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

**AMENDMENT AND RESPONSE**

Sir:

This *Amendment and Response* is filed concurrently with a petition for a one month extension of time in response to the Non-Final Office Action ("Office Action") of December 2, 2022 issued in connection with the above-identified matter. Thus, this *Amendment and Response* is timely filed.

**Amendments to the Claims** begin on page 2 of this paper.

**Remarks** begin on page 4 of this paper.



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**AMENDMENTS TO THE CLAIMS**

This listing of claims will replace all prior versions, and listings, of the claims in this application.

1-162. (Cancelled).

163. (Currently Amended) A genetically modified microorganism, comprising:

one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides, wherein expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide:

- wherein the one or more heterologous nucleic acids that encode one or more terpene biosynthesis polypeptides is selected from the group consisting of ~~chosen from~~ terpene synthase, phytoene synthase, geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, zeaxanthin glucosyltransferase and valencene synthase,

-wherein the nucleic acid that provides for said fatty acid induction of expression of the terpene biosynthesis polypeptide is a promoter, ~~or~~

- wherein the genetically modified microorganism is a yeast selected from the group consisting of *Candida ssp* and *Blastobotrys ssp.*, and

-wherein said genetically modified microorganism provides for terpene biosynthesis

~~wherein the one or more heterologous nucleic acids is a cytochrome P450 reductase and is regulated by a nucleic acid that provides for alkane induction of expression of the cytochrome P450 reductase~~.



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164. (Cancelled).

165. (Currently Amended) The microorganism of claims 163 ~~164~~, wherein the fatty acid is a saturated fatty acid or an unsaturated fatty acid selected from one or more of oleic acid, palmitoleic acid, erucic acid, linoleic acid, palmitic acid, caproic acid, enanthic acid, caprylic acid, pelargonic acid, capric acid, undecylic acid, lauric acid, myristic acid, pentadecanoic acid, margaric acid, stearic acid, arachidic acid, behenic acid, tridecylic acid, methyl pentadecanoate, ethyl laurate, ethyl PFAD and linolenic acid, and wherein the alkane is selected from one or more of hexane, heptane, nonane, decane, undecane, dodecane, tridecane, tetradecane, pentadecane, hexadecane, heptadecane, and octadecane.

166-183, (Cancelled).

184. (Currently Amended) The microorganism of claim 163, wherein the nucleic acid promoter ~~of the nucleic acid~~ that provides for said fatty acid induction of expression of said terpene biosynthesis polypeptide is selected from the group consisting of hydratase-dehydrogenase-epimerase (HDE), peroxisomal 18-kDa protein (POX18), 2,4-dienoyl-CoA reductase (SPS19), acyl-CoA oxidase 4 (POX4), dienoyl CoA Isomerase (DCI) and acyl-CoA oxidase (POX5).



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## REMARKS

Claims 163-165 and 184 are pending and have been examined on the merits. Claims 163, 165 and 184 are amended hereinabove and claim 164 is cancelled. Support for amended claim 163 can be found in the specification and in the 37 C.F.R. § 1.112 expert's declaration attached hereto. No new matter has been added.

In the Office Action, the claims are rejected as follows:

1. Claims 163-165 and 184 are rejected under 35 U.S.C. § 102(a)(1) and 102(a)(2) for allegedly being anticipated by Zhang et al. (U.S. Patent Application Publication No. 2004/0265980, hereinafter "Zhang")

2. Claims 163-165 are still rejected under 35 U.S.C. § 103 for allegedly being unpatentable over Sharpe et al. (WO2008/073367, hereinafter "Sharpe") in view of Juretzek et al. (Biotechnol. Bioprocess. Eng. 2000, hereinafter "Juretzek").

**Rejection under 35 U.S.C. §§ 102(a)(1) and 102(a)(2)**

In the Office Action, the Examiner has taken the position that Zhang describes a recombinant host cell of *Candida* which has a cytochrome P450 under the control of a POX4 promoter for expression in said *Candida*.

It is settled law that a claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference. *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987).

The claims are amended hereinabove and no longer refer to cytochrome P450 reductase.

Thus, Zhang does not describe each and every element as set forth in the claims and therefore cannot anticipate the claimed invention.



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Accordingly, reconsideration and withdrawal of this rejection is respectfully requested.

**Rejection under 35 U.S.C. § 103**

The Examiner has again rejected the claims for allegedly being unpatentable over the combination of Sharpe with Juretzek.

Applicants respectfully traverse.

As an initial matter the Examiner based the rejection on the expression of foreign proteins in the yeast *Yarrowia lipolytica*. It should be noted, however, that the presently claimed invention is directed to gene expression on yeast selected from the group consisting of *Candida ssp and Blastobotrys ssp*.

Moreover, Applicants also point out that the target product of the genetically modified microorganism is terpene biosynthesis. Thus, unlike stated by the Examiner on page 7 of the Office Action, the target product and the host are clearly specified.

Further, on page 8 of the Office Action the Examiner has indicates that the breadth of the claims is "inclusive of any genetically modified microorganism and any promoter with the exception of claim 184".

Claim 163 now recites wherein the "*genetically modified microorganism is a yeast selected from the group consisting of Candida ssp and Blastobotrys ssp.*,". Claim 163 now is directed to two specific microorganisms and to terpene biosynthesis, and are believed to be enabled and no longer would require *extensive trial and error by a skilled artisan*.

Finally, Applicants submit herewith a 37 C.F.R. § 1.132 Declaration of Dr. Beardslee (hereinafter "the Beardslee Declaration") in support of the patentability of the claimed invention.

The presently claimed invention is directed to a genetically modified microorganism, comprising:



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one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides, wherein expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide:

- wherein the one or more heterologous nucleic acids that encode one or more terpene biosynthesis polypeptides is selected from the group consisting of terpene synthase, phytoene synthase, geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, zeaxanthin glucosyltransferase and valencene synthase,

-wherein the nucleic acid that provides for said fatty acid induction of expression of the terpene biosynthesis polypeptide is a promoter,

- wherein the genetically modified microorganism is a yeast selected from the group consisting of *Candida ssp* and *Blastobotrys ssp.*, and

-wherein said genetically modified microorganism provides for terpene biosynthesis.

On page 7 of the office action the Examiner states "Both Applicants' and the Declaration from Dr Beardslee assert that the promoter must be able to direct flux of the carbon source from fatty acids to a particular target product pathway" and that "this argument is not convincing given the microorganism as recited in the claims need not produce any sort of target product". ¶ 7, Beardslee Declaration.

Claim 163 has been amended to recite a genetically modified microorganism, "wherein said genetically modified microorganism provides for terpene biosynthesis". The microorganism recited in the claims is now directed to the mandatory production of the specific terpene target product. ¶ 9, Beardslee Declaration.



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On page 8 of the office action the Examiner further states "given the breadth of the claims inclusive of any genetically modified microorganism and any promoter with the exception of claim 184, then seemingly Applicants' are intimating that the current scope and breadth of claims cannot be enabled because of the extensive trial and error required by a skilled artisan". ¶ 10, Beardslee Declaration.

Amended claim 163 has also been limited to a genetically modified microorganism, wherein the "genetically modified microorganism is a yeast selected from the group consisting of Candida spp. and Blastobotrys spp.". ¶ Beardslee Declaration.

Claim 163 has been thus limited to two yeast genera, Candida and Blastobotrys. As indicated by the Examiner in the office action, the specification provides support for Candida. I am presenting this declaration to provide data and support for the Blastobotrys yeast genus, I declare that this data was available at the time of filing of the Application. ¶ 12, Beardslee Declaration.

An example of terpene biosynthesis from the Blastobotrys yeast is shown with the lycopene production from Blastobotrys (Arxula) adeninivorans, strain sAA9936. In order to integrate genes into the genome of Blastobotrys adeninivorans, genes were cloned into a plasmid that allows for single crossover recombination at the 25S rDNA loci. In addition, since oleic acid was the primary carbon source, all genes were expressed under a Blastobotrys adeninivorans POX promoter with a Blastobotrys adeninivorans POX terminator. The backbone for all plasmids was PCR amplified from pVZ4052 with primers oVZ466 and oVZ467. The CrtE gene from Archaeoglobus fulgidus (AfCRTE) was codon optimized for B. adeninivorans and synthesized. The AfCrtE synthesized DNA and backbone contained overlapping sequence at their termini allowing directional ligation, generating plasmid pVZ4199. The CrtB gene from



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Cronobacter sakazakii (CsCrtB) was codon optimized for B. adeninivorans and synthesized. The CsCrtB synthesized DNA and backbone contained overlapping sequence at their termini allowing directional ligation, generating plasmid pVZ4146. The CrtI gene from X. dendrorhous (XdCrtI) was codon optimized for B. adeninivorans and synthesized. The XdCrtI PCR product and backbone contained overlapping sequence at their termini allowing directional ligation, generating plasmid pVZ4119. Integration cassettes containing oleic acid inducible AfCrtE, CsCrtB, XdCrtI genes were then generated by PCR amplifying plasmids pVZ4199, pVZ4146, and pVZ4119 with primers oVZ176 and oVZ201. All three cassettes were purified and transformed into strain sAA9516 (B. adeninivorans NRRL-Y-17692 leu-), which was then plated onto SCD-leu plates. Yellow colored colonies were then streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of all three genes, and one verified isolate was saved as strain sAA9936. Strain sAA9936 was run in shake flask fermentation as described previously, and results are shown in Table 1, which shows that Blastobotrys strain sAA9936 produced approximately 0.15 g/L lycopene.

Table 1:

| Strain | Substrate | Lycopene (g/L) | β-carotene (g/L) | Astaxanthin (g/L) |
|---|---|---|---|---|
| Candida strain sVZ287 | Oleic acid | 0.283 | | |
| Blastobotrys strain sAA9936 | Oleic acid | 0.150 | | |

¶ 13, Beardslee Declaration.

14. I declare that the Blastobotrys yeast strain and the Candida yeast strains can both be considered microorganism strains that provide for terpene biosynthesis as presently claimed. ¶ 14, Beardslee Declaration.

In conclusion, the microorganism strains of amended claim 163 and its dependent claims represent a new and inventive alternative to known microorganism, due to the specific and non-obvious choice of each of its mandatory technical features, not present nor suggested by the combination of the documents cited by the Examiner. ¶ 15, Beardslee Declaration.

Accordingly, for all of the reasons discussed above, the combination of the cited references would not have rendered obvious the presently claimed invention to one skilled in the art.

Thus, reconsideration and withdrawal of this rejection is respectfully requested.

**Conclusions**

This *Amendment and Response* is filed concurrently with a petition for a one month extension of time. Thus, no extra-fees are believed to be due.

However, the Director is hereby authorized to charge any fee deemed necessary or credit any overpayments to Deposit Account No. 506423.

Respectfully submitted,

March 22, 2023 By: /Silvia Salvadori/

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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

| | | |
|---|---|---|
| In re Application of | : | |
| App. No | : | 16/630,567 |
| Filed | : | January 13, 2020 |
| For | : | BIOLOGICAL METHODS FOR PREPARING TERPENES |
| Examiner | : | Suzanne Marie Noakes |
| Art Unit | : | 1656 |
| Conf No. | : | 5356 |

**DECLARATION UNDER 37 C.F.R §1.132**

Mail Stop Amendments
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

1. I, Thomas Beardslee, am an inventor of the present application.

2. I duly sworn depose and say that:

- I am a citizen of the U.S.A. residing at 6827 Carnation Drive, Carlsbad CA 92011
- I am fluent in the English language.
- I graduated with a Ph.D. in Biochemistry in the academic year 1999-2000.

3. I have extensive experience in the fields of synthetic biology, metabolic engineering, and fermentation process development. I was the Vice President of Research and Development at Verdezyne from 2012 to 2018 and currently I work at Rynetech Bio as the Founder and CEO. Rynetech Bio has been developing technology for the fermentation-based manufacturing of renewable chemicals for over 3 years. In my capacity as VP of R&D at Verdezyne in the past and in my current position as CEO of Rynetech Bio, I have been and am currently involved in the development of engineered yeast for the production of renewable chemicals by fermentation from fatty acid

feedstocks. This work has resulted in several patented inventions and publications. My *Curriculum vitae* is attached hereto.

4. I have reviewed the office action dated December 2nd, 2022 issued for US patent application 16/630,567 (hereinafter "office action").

5. I understand that the United States Patent and Trademark Office ("USPTO") remains of the position that Claims 163-165 are rejected under 35 U.S.C. 102(a)(1) and 102(a)(2) and under 35 U.S.C. 103, in the light of WO 2008/073367 A1 (hereinafter "Sharpe") in combination with Juretzek et al., Biotechnol. Bioprocess. Eng., 2000, (hereinafter "Juretzek").

6. I am presenting this declaration to show that new Claim 163, and its dependent claims, of the above-referenced US patent application are inventive over the combination of the teachings of Sharpe and Juretzek.

7. On page 7 of the office action the Examiner states "*Both Applicant's and the Declaration from Dr Beardslee assert that the promoter must be able to direct flux of the carbon source from fatty acids to a particular target product pathway*" and that "*this argument is not convincing given the microorganism as recited in the claims need not produce any sort of target product*".

8. Claim 163 has been amended and now describes:

> "A genetically modified microorganism, comprising:
>
> one or more heterologous nucleic acids encoding one or more terpene biosynthesis polypeptides, wherein expression of at least one of the heterologous nucleic acids is regulated by a nucleic acid that provides for fatty acid or alkane induction of expression of the terpene biosynthesis polypeptide:
>
> - wherein the one or more heterologous nucleic acids that encode one or more terpene biosynthesis polypeptides is selected from the group consisting of terpene synthase, phytoene synthase, geranylgeranyl diphosphate synthase, phytoene desaturase, lycopene cyclase, bifunctional lycopene cyclase/phytoene synthase, β-carotene ketolase, β-carotene hydroxylase, astaxanthin synthase, zeaxanthin glucosyltransferase and valencene synthase,
>
> - wherein the nucleic acid that provides for fatty acid induction of expression of a terpene biosynthesis polypeptide is a promoter, or



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- wherein the genetically modified microorganism is a yeast selected from the group consisting of Candida spp. and Blastobotrys spp.; and

- wherein said genetically modified microorganism provides for terpene biosynthesis."

9. Claim 163 has been amended to recite a genetically modified microorganism, "wherein said genetically modified microorganism provides for terpene biosynthesis". The microorganism recited in the claims is now directed to the mandatory production of the specific terpene target product.

10. On page 8 of the office action the Examiner states "*given the breadth of the claims inclusive of any genetically modified microorganism and any promoter with the exception of claim 184, then seemingly Applicant's are intimating that the current scope and breadth of claims cannot be enabled because of the extensive trial and error required by a skilled artisan*".

11. Amended claim 163 has also been limited to a genetically modified microorganism, wherein the "genetically modified microorganism is a yeast selected from the group consisting of Candida spp. and Blastobotrys spp.".

12. Claim 163 has been thus limited to two yeast genera, Candida and Blastobotrys. As indicated by the Examiner in the office action, the specification provides support for Candida. I am presenting this declaration to provide data and support for the Blastobotrys yeast genus, I declare that this data was available at the time of filing of the Application.

13. An example of terpene biosynthesis from the *Blastobotrys* yeast is shown with the lycopene production from *Blastobotrys (Arxula) adeninivorans*, strain sAA9936. In order to integrate genes into the genome of *Blastobotrys adeninivorans*, genes were cloned into a plasmid that allows for single crossover recombination at the 25S rDNA loci. In addition, since oleic acid was the primary carbon source, all genes were expressed under a *Blastobotrys adeninivorans* POX promoter with a *Blastobotrys adeninivorans* POX terminator. The backbone for all plasmids was PCR amplified from pVZ4052 with primers oVZ466 and oVZ467. The CrtE gene from *Archaeoglobus fulgidus* (AfCRTE) was codon optimized for *B. adeninivorans* and synthesized. The AfCrtE synthesized DNA and backbone contained overlapping sequence at their termini allowing directional ligation, generating plasmid pVZ4199. The CrtB gene from *Cronobacter sakazakii* (CsCrtB) was codon optimized for *B. adeninivorans* and synthesized. The CsCrtB synthesized DNA and backbone contained overlapping sequence at their termini allowing directional ligation, generating plasmid pVZ4146. The CrtI gene from *X. dendrorhous* (XdCrtI) was codon optimized for *B. adeninivorans* and synthesized. The



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XdCrtI PCR product and backbone contained overlapping sequence at their termini allowing directional ligation, generating plasmid pVZ4119. Integration cassettes containing oleic acid inducible AfCrtE, CsCrtB, XdCrtI genes were then generated by PCR amplifying plasmids pVZ4199, pVZ4146, and pVZ4119 with primers oVZ176 and oVZ201. All three cassettes were purified and transformed into strain sAA9516 (*B. adeninivorans* NRRL-Y-17692 leu-), which was then plated onto SCD-leu plates. Yellow colored colonies were then streaked onto YPD for isolation and characterization. Colony PCR was performed to confirm the presence of all three genes, and one verified isolate was saved as strain sAA9936. Strain sAA9936 was run in shake flask fermentation as described previously, and results are shown in Table 1, which shows that Blastobotrys strain sAA9936 produced approximately 0.15 g/L lycopene.

Table 1:

| Strain | Substrate | Lycopene (g/L) | β-carotene (g/L) | Astaxanthin (g/L) |
|---|---|---|---|---|
| Candida strain sVZ287 | Oleic acid | 0.283 | | |
| Blastobotrys strain sAA9936 | Oleic acid | 0.150 | | |

14. I declare that the Blastobotrys yeast strain and the Candida yeast strains can both be considered microorganism strains that provide for terpene biosynthesis as presently claimed.

15. In conclusion, the microorganism strains of amended claim 163 and its dependent claims represent a new and inventive alternative to known microorganism, due to the specific and non-obvious choice of each of its mandatory technical features, not present nor suggested by the combination of the documents cited by the Examiner.

16. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issuing therefrom.

Dated March 14, 2023 Signed 



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Page 3033 of 3077

**Thomas A. Beardslee, Ph.D.**
6827 Carnation Drive, Carlsbad CA 92011 • (858) 336-2457 • thomasabeardslee@gmail.com

## Summary

Biotechnology professional and commercially focused leader with experience leading multi-disciplinary scientific teams and managing multimillion dollar R&D programs. Led teams of up to 50 scientists with specialties in molecular biology, metabolic engineering, fermentation, protein engineering, proteomics, and analytical chemistry. Experienced program manager leading new product development from ideation to commercial launch and inventor on over twenty patents and patent applications. International experience with technology transfer and scale-up in India, Europe, and Southeast Asia.

## Professional Experience

Rynetech Bio, Inc., Carlsbad CA
October 2018 – Present: Founder/CEO

- Negotiated IP purchase and licensing terms for yeast synthetic biology platform from the sale of assets from predecessor company
- Executed a successful JDA with Radici Chimica (Italy) to improve yields and scale up fermentation production resulting in multi-ton quantities of adipic acid for renewable nylon synthesis
- Executed a successful JDA with a US-based biomanufacturer hitting production level targets for three terpene molecules
- Sublicensed the production of eight products to two companies

Verdezyne, Inc., Carlsbad CA
October 2012 – May 2018: Vice President, Research and Development
July 2011 – October 2012: Senior Director, Renewable Chemicals
November 2008 – July 2011: Director, Renewable Chemicals

- Led a team of 50 scientists comprising metabolic engineering, fermentation process development, bioinformatics, protein engineering, analytical chemistry and downstream product isolation.
- Developed a non-traditional yeast fermentation technology platform for the conversion of renewable fatty acid feedstocks to high value target chemicals. Demonstrated the proof of concept for the production of sixteen different chemicals achieving commercially relevant production for five (adipic acid, dodecanedioic acid, lycopene, beta-carotene, and canthaxanthin).
- Advised the CEO and Board of Directors on the progress of R&D programs towards timeline goals as well as the strategic direction of the company.
- Supervised the scale-up of two fermentation processes to commercial scale (up to 180 $m^3$) working with external partners.
- Advised internal and external engineering teams towards the construction of the commercial-scale (6 kta capacity) VerdePalm facility located in Nusajaya, Malaysia for the production of renewable DDDA.



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- Led the regulatory process for importation of the Verdezyne GMO yeast into four different countries including for commercial use in Malaysia.
- Program manager for the metabolic engineering of the diploid yeast *Candida viswanathii* to produce adipic acid from fatty acid feedstocks. Demonstrated feedstock flexibility in the fermentation process by using over 50 different feedstocks considered low value co-products or waste products.
- Developed a product pipeline that leveraged the metabolic engineering of previous programs to shorten product development timelines.
- Presented at numerous industry conferences and university lectures around the world on topics ranging from the highly technical to the general state of the bio-based industry.
- Worked with human resources in the recruitment and development of scientific staff including yearly personnel evaluations, 360 reviews, and management training for scientific managers.

CODA Genomics, Laguna Hills CA (Predecessor Company to Verdezyne, Inc.)
October 2007 – November 2008: Director of Project Design

- Worked directly with customers to design custom projects solving protein expression problems. Services managed include gene sequence optimization, gene variant library design, custom cloning, expression testing (*E. coli, S. cerevisiae, and P. pastoris*), small-scale protein expression, pilot protein expression, and protein purification.
- Developed strategic corporate partnerships to broaden the company's product offering.
- Evaluated new technology and R&D programs, providing guidance on research direction for the company as a whole.

Invitrogen Corporation, Carlsbad CA (now Thermo Fisher Scientific)
October 2006 – October 2007: Senior Scientist
July 2002 – October 2006: Scientist

- Program manager, Human Proteome Organization (HUPO) proteomic standards for mass spectrometry. Coordinated all aspects of new product development with team members from marketing, engineering, intellectual property, regulatory and manufacturing.
- Collaborated with HUPO leadership in the development and testing of a panel of highly purified recombinant human proteins. Twenty tagless recombinant human proteins were expressed in *E. coli*, purified to >95%, formulated at 5 picomoles per protein, and sent to HUPO test labs around the world for validation.
- Coordinated the transfer of recombinant protein production and purification manufacturing processes to Invitrogen Bioservices India (Bangalore).
- Contributed scientific evaluation of technologies in strategic planning for future directions of new product development.
- IDEO University, trained in the process used by one of the world-leading industrial engineering firms, IDEO, for product and process innovation.
- Development of new electrophoresis technologies for proteomic analysis: IPG strips, solution phase IEF, and precast mini-gels for denaturing and native electrophoresis.
- Program manager, Native Complex Purification & Analysis program. Products launched Q1, 2006 include NativePAGE™ gels for blue-native electrophoresis, NativePAGE™ sample preparation kit, NativeMark™ unstained markers, and NativePure™ vectors for



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mammalian expression (pcDNA and lentiviral based) and purification of native protein complexes.
- Presented proteomics sample preparation seminars to customers at research centers across the U.S.
- R&D contact for the Technical Services department to support detailed technical resolution of customer issues with all proteomics products in the ZOOM® product line. Technical assistance included direct customer interaction via email, phone, and in person for customers at academic, research institution, and industrial accounts.

University of Nebraska-Lincoln, Lincoln NE
August 2000 – June 2002: Postdoctoral Research Associate in the lab of Dr. Lori Allison, Biochemistry Department

- Expressed four newly discovered maize multi-subunit RNA polymerase sigma factor proteins in *E. coli*. Optimization of expression included employing rare tRNA expressing strain of *E. coli* and thioredoxin fusion partner to enhance solubility during refolding. Purified recombinant proteins were used for antibody production and *in vitro* activity assays.
- Determined the subcellular localization of maize sigma factors by immunoblotting, and by confocal microscopy *in situ* immunolocalization.
- Demonstrated the activity of maize sigma factors using an electrophoretic mobility shift assay showing protein-protein and protein-DNA interactions.
- Lab manager: ordered supplies, organized equipment and storage of chemicals, supervised the activities of two graduate and three undergraduate students.

Fall semester 2001: Instructor, Biochemistry 431/831, Biochemistry Department

- Taught the first semester of Senior and Graduate student level Biochemistry for a class of approximately seventy students.
- Prepared and delivered lectures using traditional, multi-media, and on-line resources.
- Hosted an on-line discussion board for student assistance outside of office hours.
- Composed and administered examinations for evaluation of student performance.

August 1996-August 2000: Graduate Research Assistant in the labs of Dr. Mike Zeece (Food Science Department) and Dr. John Markwell (Biochemistry Department)

- Identified IgE epitopes of a major soybean storage protein using soy-sensitive human serum in immunoblotting each of the following: proteolytic fragments of native protein, recombinantly expressed (*E. coli*) purified protein fragments, and overlapping synthetic peptides.
- Assay development of an ELISA using purified fusion proteins for the identification and quantification of the interaction between soy-sensitive serum IgE and protein allergens.
- Collaborated with physicians for the collection of human sera. In charge of the storage, cataloging, use, and disposal of human sera.
- Lab manager: ordered supplies, organized equipment and storage of chemicals, supervised the activities of three undergraduate students.

Pius X High School, Lincoln NE
August 1993 – May 1995: Chemistry/Advanced Placement Chemistry Teacher

- Taught five sections of Chemistry and one section of A.P. Chemistry



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- Head Coach girls soccer, Assistant Coach wrestling, Assistant Coach volleyball

**Education**

- University of Nebraska-Lincoln, PhD, Biochemistry, August, 2000. Dissertation: IgE Epitope Mapping of Soybean Glycinin G1 Acidic Chain
- Creighton University, Omaha, NE, Secondary Education Teaching Certification, May 1993
- Creighton University, Omaha, NE, BS, Chemistry, May 1992

**Honors/Awards**

- E.P.A. Presidential Green Chemistry Challenge Award Winner for Commercialization of Renewable Dodecanedioic Acid, 2016.
- Bloomberg New Energy Pioneers Award Winner for Commercialization of Renewable Dodecanedioic Acid, 2016.
- UCSD Connect, Most Innovative New Product, Finalist 2002. Team member for the development of the Invitrogen ZOOM® IPG System.
- University of Nebraska-Lincoln Teaching Council/Parents Association Recognition Award, fall 2001. For effective teaching and nurturing of students, as voted on by University students.
- Milton E. Mohr Fellowship, academic year 1999-2000. Recognizing outstanding students performing research in the sciences of biotechnology and engineering.
- Farmers National Company Fellowship, academic year 1998-1999. Recognizing excellence in teaching.

**Publications/Patents/Patent Applications**

**Beardslee T** (2020) Biosynthetic cannabinoid production in engineered microorganisms. Subject of patent application WO/2020/198679.

Aeling K, De Wals P, Scott E, Knight E, Yi J, Walbridge M, **Beardslee T** (2019) Biological methods for preparing terpenes. Subject of patent application WO/2019/014310.

**Beardslee T** (2019) Biological methods for modifying cellular carbon flux. Subject of patent application WO/2019/014309.

**Beardslee T**, Picataggio S, Hutagalung A, Fahland T (2018) Biological methods for preparing a fatty dicarboxylic acid. Subject of U.S. Patent 9,957,512.

**Beardslee T,** Picataggio S, Eirich D, Laplaza J (2018) Biological methods for preparing a fatty dicarboxylic acid. Subject of U.S. Patent 9,938,544.

**Beardslee T,** Hutagalung A, Picataggio S (2017) Biological methods for preparing a fatty dicarboxylic acid. Subject of U.S. Patent 9,850,493.

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Picataggio S and **Beardslee T** (2016) Biological methods for preparing adipic acid. Subject of U.S. Patent 9,434,966.

Updyke T and **Beardslee T** (2015) Compositions and methods for improving resolution of biomolecules separated on polyacrylamide gels. Subject of U.S. Patent 9,057,694.

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Picataggio S and **Beardslee T** (2014) Biological methods for preparing adipic acid. Subject of U.S. Patent 8,728.798.

**Beardslee T**, Picataggio S, Hutagalung A, Fahland T (2013) Biological methods for preparing a fatty dicarboxylic acid. Subject of WO Patent Application #2013006733

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Picataggio S and **Beardslee T** (2013) Biological methods for preparing adipic acid. Subject of U.S. Patent 8,343,752.

**Beardslee T** and Picataggio S (2012) Bio-based adipic acid from renewable oils. Lipid Tech. 24: 223-225.

Picataggio S and **Beardslee T** (2012) Biological methods for preparing adipic acid. Subject of U.S. Patent 8,241,879.

Picataggio S and **Beardslee T** (2011) Biological methods for preparing adipic acid. Subject of WO Patent Application #2011003034.

Bell AW, Deutsch EW, Au CE, Kearney RE, Beavis R, Sechi S, Nilsson T, Bergeron J, **Beardslee TA**, Chappell T, Meredith G, Sheffield P, Gray P, Hajivandi M, Pope M, Predki P, Kullolli M, Hincapie M, Hancock WS, Jia W, Song L, Li L, Wei J, Yang B, Wang J, Ying W, Zhang Y, Cai Y, Qian X, He F, Meyer HE, Stephan C, Eisenacher M, Marcus K, Langenfeld E, May C, Carr S, Ahmad R, Zhu W, Smith JW, Hanash SM, Struthers JJ, Wang H, Zhang Q, An Y, Goldman R, Carlsohn E, van der Post S, Hung KE, Sarracino DA, Parker K, Krastins B, Kucherlapati R, Bourassa S, Poirier GG, Kapp E, Patsiouras H, Moritz R, Simpson R, Houle B, LaBoissiere S, Metalnikov P, Nguyen V, Pawson T, Wong C, Cociorva D, Yates III JR, Ellison MJ, Lopez-Campistrous A, Semchuk P, Wang Y, Ping P, Elia G, Dunn MJ, Wynne K, Walker AK, Strahler JR, Andrews PC, Hood BL, Bigbee WL, Conrads TP, Smith D, Borchers CH, Lajoie GA, Bendall SC, Speicher KD, Speicher DW, Fujimoto M, Nakamura K, Paik YK, Cho SY, Kwon MS, Lee HJ, Seul-Ki Jeong SK, Chung AS, Miller CA, Grimm R, Williams K,



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Dorschel C, Falkner JA, Martens L, and Vizcaino JA (2009) A HUPO test sample study reveals common problems in mass spectrometry-based proteomics. Nature Methods 6: 423-430.

**Beardslee T**, Bolz C, Updyke T, Rooney R. (2007) Liquid protein markers for native gel electrophoresis. Subject of U.S. Patent Application #US20070026479A1.

Diller T, **Beardslee T**, Rooney R, Amshey J. (2005) Isoelectric focusing gels and methods of use thereof. Subject of U.S. Patent Application #US20050103629A1.

**Beardslee T** and Hajivandi M (2005) 2DE analysis of spinach chloroplast using the ZOOM® Benchtop Proteomics™ system. Quest 2.1: 64-68.

Suzuki JY, Ytterberg AJ, **Beardslee TA**, Allison LA, van Wijk KJ, Maliga P (2004) Affinity purification of the tobacco plastid RNA polymerase and in vitro reconstitution of the holoenzyme. Plant J. 40: 164-172.

**Beardslee TA**, Zeece MG, Sarath G, and Markwell JP (2003) Method for allergen characterization. Subject of U.S. Patent Application #US20030166518A1.

**Beardslee TA**, Roy-Chowdury S, Jaiswal P, Buhot L, Lerbs-Mache S, Stern DB, Allison LA (2002) A nucleus-encoded maize protein with sigma factor activity accumulates in mitochondria and chloroplasts. Plant J. 31: 199-209.

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Gu X, **Beardslee TA**, Zeece M, Sarath G, Markwell J (2001) Identification of IgE–binding proteins in soy lecithin. Int. Arch. Allergy Immunol. 126: 218-225.

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Zeece MG, **Beardslee TA**, Markwell JP, Sarath G (1999) Identification of an IgE–binding region in soybean acidic glycinin G1. Food Ag. Immunol. 11: 83-90.



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# NOTICE OF ALLOWANCE AND FEE(S) DUE

122515 7590 06/09/2023

Silvia Salvadori, P.C.
Silvia Salvadori
485 Central Park West Unit 5C
New York, NY 10025

| EXAMINER | |
|---|---|
| NOAKES, SUZANNE MARIE | |
| ART UNIT | PAPER NUMBER |
| 1656 | |

DATE MAILED: 06/09/2023

| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 16/630,567 | 01/13/2020 | Kimberly Ann Aeling | SS118-1026US | 5356 |

TITLE OF INVENTION: BIOLOGICAL METHODS FOR PREPARING TERPENES

| APPLN. TYPE | ENTITY STATUS | ISSUE FEE DUE | PUBLICATION FEE DUE | PREV. PAID ISSUE FEE | TOTAL FEE(S) DUE | DATE DUE |
|---|---|---|---|---|---|---|
| nonprovisional | UNDISCOUNTED | $1200 | $0.00 | $0.00 | $1200 | 09/11/2023 |

**THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.**

**THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.**

**HOW TO REPLY TO THIS NOTICE:**

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 40% the amount of undiscounted fees, and micro entity fees are 20% the amount of undiscounted fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

**IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.**



PTOL-85 (Rev. 02/11)

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## PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
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By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications. **Because electronic patent issuance may occur shortly after issue fee payment, any desired continuing application should preferably be filed prior to payment of this issue fee in order not to jeopardize copendency.**

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

122515 7590 06/09/2023

Silvia Salvadori, P.C.
Silvia Salvadori
485 Central Park West Unit 5C
New York, NY 10025

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

**Certificate of Mailing or Transmission**

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

| (Typed or printed name) |
|---|
| (Signature) |
| (Date) |

| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 16/630,567 | 01/13/2020 | Kimberly Ann Aeling | SS118-1026US | 5356 |

TITLE OF INVENTION: BIOLOGICAL METHODS FOR PREPARING TERPENES

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|---|---|---|---|---|---|---|
| nonprovisional | UNDISCOUNTED | $1200 | $0.00 | $0.00 | $1200 | 09/11/2023 |

| EXAMINER | ART UNIT | CLASS-SUBCLASS |
|---|---|---|
| NOAKES, SUZANNE MARIE | 1656 | 435-132000 |

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

❑ Change of correspondence address (or Change of Correspondence Address form PTO/AIA/122 or PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/AIA/47 or PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list
(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,
(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ❑ Individual ❑ Corporation or other private group entity ❑ Government

4a. Fees submitted: ❑Issue Fee ❑Publication Fee (if required)

4b. Method of Payment: *(Please first reapply any previously paid fee shown above)*

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❑ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. **Change in Entity Status** (from status indicated above)

❑ Applicant certifying micro entity status. See 37 CFR 1.29

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

❑ Applicant asserting small entity status. See 37 CFR 1.27

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

❑ Applicant changing to regular undiscounted fee status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____



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| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
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122515 7590 06/09/2023

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| EXAMINER | |
|---|---|
| NOAKES, SUZANNE MARIE | |
| ART UNIT | PAPER NUMBER |
| 1656 | |

DATE MAILED: 06/09/2023

**Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)**
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.



PTOL-85 (Rev. 02/11)

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Ex. 1002
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## OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

## Privacy Act Statement

**The Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

| Notice of Allowability | Application No. 16/630,567 | Applicant(s) Aeling et al. | |
|---|---|---|---|
| | Examiner SUZANNE M NOAKES | Art Unit 1656 | AIA (FITF) Status Yes |

*-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--*

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☑ This communication is responsive to the amendments and response filed 22 March 2023.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☑ The allowed claim(s) is/are 163,165 and 184 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information , please see **http://www.uspto.gov/patents/init_events/pph/index.jsp** or send an inquiry to **PPHfeedback@uspto.gov.**

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   **Certified copies:**

   a) ☐ All b) ☐ Some* c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____ .
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
**THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.**

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

   **Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).**

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

**Attachment(s)**

1. ☐ Notice of References Cited (PTO-892)
2. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____.
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.
5. ☐ Examiner's Amendment/Comment
6. ☑ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/SUZANNE M NOAKES/
Primary Examiner, Art Unit 1656



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## NOTICE OF ALLOWANCE

### *Status of Application*

1. The response, amendments and Declaration filed under 37 C.F.R. 1.132, all filed 22 March 2023 are acknowledged and have been considered in their entireties. Claim 164 is cancelled, thus, claims 163, 165, 184 are pending and subject to examination on the merits.

### *Withdrawal of Previous Rejections*

2. The rejection of claim(s) 163-165 and 184 are rejected under 35 U.S.C. 102(a)(1) and 102(a)(2) as being anticipated by Zhang et al. (US 2004/0265980 – cited previously) is withdrawn in view of the amendments to the claims to remove cytochrome P450 reductase from claim 164.

3. The rejection of Sharpe et al. (WO 2008/073367 – cited previously) in view of Juretzek et al. (Biotechnol. Bioprocess. Eng., 2000 – cited previously) is withdrawn in view of the amendments to the claims to limit the host cell to *Candida ssp.* and *Blastobotrys ssp.* (*Arxula spp.*). Juretzek et al. teach promoters for expression in *Yarrowia* but discuss nothing regarding other yeast species.

4. The Declaration filed under 37 CFR 1.132 is acknowledged. The declaration is mainly directed to the rejection of record above in Section 3, and also establishes that both *Candida ssp.* and *Blastobotrys ssp.* are capable of terpene biosynthesis with inducible promoters.

5. The prior art does not teach nor suggest a genetically modified *Candida ssp.* and *Blastobotrys ssp.* comprising heterologous expression of various terpene biosynthesis

enzymes as in claim 164, which are further induced by fatty acids or alkanes, and wherein *Candida ssp.* and *Blastobotrys ssp.* provide for terpene biosynthesis. Thus, **claims 163, 165 and 184 are allowed**.

***Correspondence***

6. Any inquiry concerning this communication or earlier communications from the examiner should be directed to SUZANNE M NOAKES whose telephone number is (571)272-2924. The examiner can normally be reached M-F (7-4).

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Manjunath Rao can be reached on 571-272-0939. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of published or unpublished applications may be obtained from Patent Center. Unpublished application information in Patent Center is available to registered users. To file and manage patent submissions in Patent Center, visit: https://patentcenter.uspto.gov. Visit https://www.uspto.gov/patents/apply/patent-center for more information about Patent Center and https://www.uspto.gov/patents/docx for information about filing in DOCX format. For additional questions, contact the Electronic Business Center (EBC) at 866-217-9197

(toll-free). If you would like assistance from a USPTO Customer Service

Representative, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/SUZANNE M NOAKES/
Primary Examiner, Art Unit 1656
30 May 2023

| Issue Classification | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 16/630,567 | Aeling et al. |
| | **Examiner** | **Art Unit** |
| | SUZANNE M NOAKES | 1656 |

| CPC | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Symbol** | | | | | | **Type** | **Version** |
| C12N | / | 15 | / | 815 | | F | 2013-01-01 |
| C12N | / | 1 | / | 16 | | I | 2013-01-01 |
| C12N | / | 15 | / | 52 | | I | 2013-01-01 |
| C12P | / | 7 | / | 44 | | I | 2013-01-01 |

| CPC Combination Sets | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Symbol** | | | | | | **Type** | **Set** | **Ranking** | **Version** |
| | / | | / | | | | | | |

| NONE | | **Total Claims Allowed:** | |
|---|---|---|---|
| (Assistant Examiner) | (Date) | 3 | |
| /SUZANNE M NOAKES/<br>Primary Examiner, Art Unit 1656 | 30 May 2023 | O.G. Print Claim(s) | O.G. Print Figure |
| (Primary Examiner) | (Date) | 1 | none |

U.S. Patent and Trademark Office — Part of Paper No.: 20230530



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| Issue Classification | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 16/630,567 | Aeling et al. |
| | **Examiner** | **Art Unit** |
| | SUZANNE M NOAKES | 1656 |

| INTERNATIONAL CLASSIFICATION | | | | |
|---|---|---|---|---|
| **CLAIMED** | | | | |
| C12N | / | 15 | / | 81 |
| C12N | / | 1 | / | 16 |
| C12N | / | 15 | / | 52 |
| C12P | / | 7 | / | 44 |

| NON-CLAIMED | | | | |
|---|---|---|---|---|
| | / | | / | |

| US ORIGINAL CLASSIFICATION | |
|---|---|
| **CLASS** | **SUBCLASS** |
| | |

| CROSS REFERENCES(S) | | | | | | |
|---|---|---|---|---|---|---|
| **CLASS** | **SUBCLASS (ONE SUBCLASS PER BLOCK)** | | | | | |
| | | | | | | |

| NONE | | **Total Claims Allowed:** | |
|---|---|---|---|
| (Assistant Examiner) | (Date) | 3 | |
| /SUZANNE M NOAKES/<br>Primary Examiner, Art Unit 1656 | 30 May 2023 | O.G. Print Claim(s) | O.G. Print Figure |
| (Primary Examiner) | (Date) | 1 | none |

U.S. Patent and Trademark Office — Part of Paper No.: 20230530



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| Issue Classification | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 16/630,567 | Aeling et al. |
| | Examiner | Art Unit |
| | SUZANNE M NOAKES | 1656 |

☑ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIMS

| Final | Original | Final | Original | Final | Original | Final | Original | Final | Original | Final | Original | Final | Original | Final | Original |
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| NONE | | Total Claims Allowed: | |
|---|---|---|---|
| (Assistant Examiner) | (Date) | 3 | |
| /SUZANNE M NOAKES/<br>Primary Examiner, Art Unit 1656 | 30 May 2023 | O.G. Print Claim(s) | O.G. Print Figure |
| (Primary Examiner) | (Date) | 1 | none |

U.S. Patent and Trademark Office — Part of Paper No.: 20230530



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| Search Notes | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 16/630,567 | Aeling et al. |
| | Examiner | Art Unit |
| | SUZANNE M NOAKES | 1656 |

| CPC - Searched* | | |
|---|---|---|
| **Symbol** | **Date** | **Examiner** |
| C12N 15/815 w/text in EAST | 02/15/2022 | SMN |
| Updated in SEARCH | 11/29/2022 | SMN |
| Updated in SEARCH | 05/30/2023 | SMN |

| CPC Combination Sets - Searched* | | |
|---|---|---|
| **Symbol** | **Date** | **Examiner** |
| | | |

| US Classification - Searched* | | | |
|---|---|---|---|
| **Class** | **Subclass** | **Date** | **Examiner** |
| | | | |

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

| Search Notes | | |
|---|---|---|
| **Search Notes** | **Date** | **Examiner** |
| Inventors names (all) in EAST and PE2E | 02/15/2022 | SMN |
| EAST: See attached | 02/15/2022 | SMN |
| ProQuest Dialog: see attached | 02/15/2022 | SMN |
| Updated in SEARCH: See attached | 07/25/2022 | SMN |
| Updated SEARCH: See attaced | 11/28/2022 | SMN |
| Updated ProQuest Dialog: See attached | 11/28/2022 | SMN |
| Updated SEARCH and ProQuest: See attached | 05/30/2023 | SMN |

| /SUZANNE M NOAKES/<br>Primary Examiner, Art Unit 1656 | |
|---|---|



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| Search Notes | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 16/630,567 | Aeling et al. |
| | Examiner | Art Unit |
| | SUZANNE M NOAKES | 1656 |

| Interference Search | | | |
|---|---|---|---|
| US Class/CPC Symbol | US Subclass/CPC Group | Date | Examiner |
| | Inventors names (all) DAV and SEARCH | 05/30/2023 | SMN |

| | |
|---|---|
| /SUZANNE M NOAKES/<br>Primary Examiner, Art Unit 1656 | |



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## PE2E SEARCH - Search History (Prior Art)

| Ref # | Hits | Search Query | DBs | Default Operator | Plurals | British Equivalents | Time Stamp |
|---|---|---|---|---|---|---|---|
| L64 | 1046 | ((("AELING") near3 ("Kimberly")) OR (("DE") near3 ("WALS") near3 ("Pierre-Yves")) OR (("SCOTT") near3 ("Eric")) OR (("WALDBRIDGE") near3 ("Michael")) OR (("BEARDSLEE") near3 ("Thomas"))).INV. | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | OFF | OFF | 2022/02/15 07:59 AM |
| L65 | 36 | L64 and terpene | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:00 AM |
| L66 | 6 | L64 and terpene and phytoene | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:00 AM |
| L67 | 1648 | ("phytoene synthase" or CftB or "prephytoene-diphosphate synthase" or "15-cis-phytoene synthase" or PSase or "geranylgeranyl-diphosphate geranylgeranyltransferase" or "2.5.1.32") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:09 AM |
| L68 | 6520 | ("phytoene desaturase" or "3,4-didehydrolycopene-forming phytoene desaturase" or "1.3.99.28" or CrtI or "1.3.99.30" or CsCrtI or XdCrtI or PaCrtI) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:12 AM |
| L69 | 4105 | ("geranylgeranyl diphosphate synthase" or BTS1 or CrtE or GGPS1 or GGPPS or GGPPS1 or "geranylgeranyl diphosphate synthase 1" or "2.5.1.29") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:14 AM |
| L70 | 528 | L67 and L68 and L69 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:14 AM |
| L71 | 415 | L67 same3 L68 same3 L69 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:14 AM |
| L72 | 360 | L67 same L68 same L69 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:14 AM |

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|---|---|---|---|---|---|---|---|
| L73 | 38 | L72 and (POX4 or POT or PEX or POX18 or ANT1 or DHE or ARE1) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 08:15 AM |
| L74 | 5 | ("20030044946"\|"20130066035"\|"20130302861"\|"20140113343"\|"20150010978").PN. | (US-PGPUB; USPAT) | OR | OFF | OFF | 2022/02/15 09:01 AM |
| L75 | 171 | L72 and (Candida or Yarrowia) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 09:10 AM |
| L76 | 21 | L72 same (Candida or Yarrowia) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 09:10 AM |
| L77 | 1648 | ("phytoene synthase" or CftB or "prephytoene-diphosphate synthase" or "15-cis-phytoene synthase" or PSase or "geranylgeranyl-diphosphate geranylgeranyltransferase" or "2.5.1.32") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 10:57 AM |
| L78 | 6520 | ("phytoene desaturase" or "3,4-didehydrolycopene-forming phytoene desaturase" or "1.3.99.28" or CrtI or "1.3.99.30" or CsCrtI or XdCrtI or PaCrtI) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 10:57 AM |
| L79 | 4105 | ("geranylgeranyl diphosphate synthase" or BTS1 or CrtE or GGPS1 or GGPPS or GGPPS1 or "geranylgeranyl diphosphate synthase 1" or "2.5.1.29") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 10:57 AM |
| L80 | 360 | L77 same L78 same L79 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 10:57 AM |
| L81 | 16 | L80 and C12N15/815.cpc. | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/02/15 10:57 AM |
| L82 | 1102 | ((("AELING") near3 ("Kimberly")) OR (("DE") near3 ("WALS") near3 ("Pierre-Yves")) OR (("SCOTT") near3 ("Eric")) OR (("WALDBRIDGE") near3 ("Michael")) OR (("BEARDSLEE") near3 ("Thomas"))).INV. | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | OFF | OFF | 2022/07/25 12:07 PM |
| L83 | 37 | L64 and terpene | (US-PGPUB; USPAT; | OR | ON | ON | 2022/07/25 |

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|---|---|---|---|---|---|---|---|
| | | | USOCR; EPO; JPO; DERWENT) | | | | 12:07 PM |
| L84 | 6 | L64 and terpene and phytoene | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L85 | 1713 | ("phytoene synthase" or CftB or "prephytoene-diphosphate synthase" or "15-cis-phytoene synthase" or PSase or "geranylgeranyl-diphosphate geranylgeranyltransferase" or "2.5.1.32") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L86 | 6381 | ("phytoene desaturase" or "3,4-didehydrolycopene-forming phytoene desaturase" or "1.3.99.28" or CrtI or "1.3.99.30" or CsCrtI or XdCrtI or PaCrtI) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L87 | 4424 | ("geranylgeranyl diphosphate synthase" or BTS1 or CrtE or GGPS1 or GGPPS or GGPPS1 or "geranylgeranyl diphosphate synthase 1" or "2.5.1.29") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L88 | 538 | L67 and L68 and L69 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L89 | 430 | L67 same3 L68 same3 L69 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L90 | 368 | L67 same L68 same L69 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L91 | 42 | L72 and (POX4 or POT or PEX or POX18 or ANT1 or DHE or ARE1) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L92 | 5 | ("20030044946"\|"20130066035"\|"20130302861"\|"20140113343"\|"20150010978").PN. | (US-PGPUB; USPAT) | OR | OFF | OFF | 2022/07/25 12:07 PM |
| L93 | 176 | L72 and (Candida or Yarrowia) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L94 | 21 | L72 same (Candida or Yarrowia) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L95 | 1713 | ("phytoene synthase" or CftB or "prephytoene- | (US-PGPUB; USPAT; USOCR; EPO; JPO; | OR | ON | ON | 2022/07/25 12:07 PM |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | diphosphate synthase" or "15-cis-phytoene synthase" or PSase or "geranylgeranyl-diphosphate geranylgeranyltransferase" or "2.5.1.32") | DERWENT) | | | | |
| L96 | 6381 | ("phytoene desaturase" or "3,4-didehydrolycopene-forming phytoene desaturase" or "1.3.99.28" or CrtI or "1.3.99.30" or CsCrtI or XdCrtI or PaCrtI) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L97 | 4424 | ("geranylgeranyl diphosphate synthase" or BTS1 or CrtE or GGPS1 or GGPPS or GGPPS1 or "geranylgeranyl diphosphate synthase 1" or "2.5.1.29") | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L98 | 368 | L77 same L78 same L79 | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L99 | 16 | L80 and C12N15/815.cpc. | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/07/25 12:07 PM |
| L100 | 153 | promoter WITH (POX4 OR SPS19 OR POX5 OR POX18 OR DCI) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/11/28 11:09 AM |
| L101 | 101 | (promoter WITH (POX4 OR SPS19 OR POX5 OR POX18 OR DCI)) AND yarrowia | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/11/28 11:10 AM |
| L102 | 36 | (promoter WITH (POX4 OR SPS19 OR POX5 OR POX18 OR DCI)) SAME yarrowia | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/11/28 11:10 AM |
| L103 | 4 | L102 AND terpene | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2022/11/28 11:27 AM |
| L104 | 38 | (Artificial Intelligence) Similar to: 16/630,567 with 0 CPC Selections and 0 Text Selections<br>---<br>Text: (EP-3652192-A1 OR WO-2019014310-A1 OR CA-3069708-A1 OR US-20210079408-A1 OR EP-2182072-B1 OR US-10059974-B2 OR | (US-PGPUB; USPAT; USOCR; FIT (AU, AP, AT, BE, BG, BR, BY, CA, CH, CN, CS, CU, CZ, DD, DE, DK, EA, EE, EP, ES, FI, FR, GB, HR, HU, ID, IE, IL, IS, IT, JP, KR, LT, LU, LV, MA, OA, RU, SU, WO, MC, MD, MY, NL, NO, NZ, PH, PL, PT, RO, RS, SE, SG, SI, SK, | OR | ON | ON | 2023/05/24 01:35 PM |



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| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | US-20150005534-A1 OR US-20200032314-A1 OR WO-2016154314-A1 OR WO-2022245988-A2 OR US-20200024607-A1 OR JP-2016010347-A OR WO-2015199043-A1 OR US-20060031963-A1 OR AU-2003258623-A1 OR CA-2496133-A1 OR US-20180080031-A1 OR JP-4685308-B2 OR US-7122341-B1 OR WO-2013119552-A2 OR DE-102004007622-A1 OR US-11124809-B2 OR JP-2018526027-A OR US-20060194274-A1 OR US-20020092039-A1 OR WO-2005019467-A1 OR CA-2535972-A1 OR US-20080060096-A1 OR DE-102013209950-A1 OR WO-2005019461-A2 OR EP-0925366-A1 OR EP-3574105-B1 OR ES-2880004-T3 OR EP-1532264-A2 OR WO-2008058946-A1 OR DE-102004007624-A1 OR JP-2007502605-A OR MX-PA06001620-A OR EP-1658377-A1 OR AU-2004267196-A1 OR US-20210115454-A1 OR EP-1658372-A2 OR US-20150353966-A1 OR DE-102004007623-A1 OR WO-2005019460-A2 OR JP-20070502605-A6 OR US-10364434-B2 OR WO-2007144342-A2 OR EP-2032705-A2 OR CA-3049126-A1).did. | TH, TN, TR, TW, UA, VN)) | | | | |
| L105 | 249 | (Blastobotrys OR Candida OR Arxula) SAME terpen$ | (US-PGPUB; USPAT) | OR | ON | ON | 2023/05/25 11:01 AM |
| L106 | 14 | (Blastobotrys OR Candida OR Arxula) SAME terpen$ SAME (synthesis OR | (US-PGPUB; USPAT) | OR | ON | ON | 2023/05/25 11:01 AM |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | biosynthesis OR "bio-synthesis") | | | | | |
| L107 | 31 | (Blastobotrys OR Candida OR Arxula) SAME lycopene SAME (synthesis OR biosynthesis OR "bio-synthesis") | (US-PGPUB; USPAT) | OR | ON | ON | 2023/05/25 11:17 AM |
| L108 | 1 | (Blastobotrys OR Candida OR Arxula) SAME lycopene SAME (HDE OR POX18 OR SPS19 OR POX4 OR DCI OR POX5) | (US-PGPUB; USPAT) | OR | ON | ON | 2023/05/25 11:26 AM |
| L109 | 19 | (Blastobotrys OR Candida OR Arxula) SAME (crtE OR crtB OR crtI OR crtY OR crtZ OR crtW) | (US-PGPUB; USPAT) | OR | ON | ON | 2023/05/25 11:29 AM |
| L110 | 4 | L104 AND (POX4 OR SPS19 OR POX5 OR POX18 OR DCI) | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2023/05/25 01:10 PM |
| L111 | 14 | (crtE OR crtB OR crtI OR crtY OR crtZ OR crtW) AND candida AND "POX4" | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2023/05/25 01:36 PM |
| L112 | 13 | (crtE OR crtB OR crtI OR crtY OR crtZ OR crtW) AND candida AND "POX5" | (US-PGPUB; USPAT; USOCR; EPO; JPO; DERWENT) | OR | ON | ON | 2023/05/25 01:46 PM |

## PE2E SEARCH - Search History (Interference)

There are no Interference searches to show.

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## Search Strategy from Dialog

May 25 2023 14:11



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# Search Strategy

**Databases:** ABI/INFORM® Professional Advanced, Abstracts in New Technology & Engineering, AdisInsight: Drugs, AdisInsight: Trials, Adis Pharmacoeconomics & Outcomes News, AGRICOLA, AGRIS, Allied & Complementary Medicine™, Analytical Abstracts, APA PsycInfo®, Aqualine, Aquatic Science & Fisheries Abstracts (ASFA), Australian Education Index, BIOSIS® Toxicology, BIOSIS Previews®, British Library Inside Conferences, British Nursing Index, Business & Industry, CAB ABSTRACTS, Chemical Business Newsbase, Chemical Engineering & Biotechnology Abstracts, Chemical Safety Newsbase, Civil Engineering Abstracts, ClinicalTrials.gov, Current Contents® Search, Derwent Drug File, Derwent Drug Registry, DH-DATA: Health Administration, Medical Toxicology & Environmental Health, DIOGENES® FDA Regulatory Updates, Drug Information Fulltext, Earthquake Engineering Abstracts, EconLit, Ei Compendex®, Ei EnCompassLIT, Embase®, Embase® French Local Literature, Embase Preprints, EMCare®, ERIC, ESPICOM Pharmaceutical & Medical Device News, FDAnews, FLUIDEX (Fluid Engineering Abstracts), Foodline®: MARKET, Foodline®: PRODUCT, Foodline®: SCIENCE, FSTA®, Gale Group Computer Database™, Gale Group Health Periodicals Database, Gale Group New Product Announcements / Plus®, Gale Group Newsletter Database™, Gale Group PharmaBiomed Business Journals, Gale Group PROMT®, Gale Group Trade & Industry Database™, GEOBASE, GeoRef, Global Health, HSELINE: Health and Safety, ICONDA - International Construction Database, IMS Company Profiles, IMS New Product Focus, IMS Pharma Trademarks, IMS R&D Focus, IMS R&D Focus Drug News, Inspec®, International Pharmaceutical Abstracts, Jane's Defense & Aerospace News, King's Fund, KOSMET: Cosmetic Science, Lancet Titles, Mechanical & Transportation Engineering Abstracts, MEDLINE®, Meteorological & Geoastrophysical Abstracts, Morressier Life Science Conference Abstracts and Posters, New England Journal of Medicine, Northern Light Life Sciences Conference Abstracts, NTIS: National Technical Information Service, Oceanic Abstracts, PAIS International, Paperbase, PAPERCHEM, ProQuest Advanced Tech & Aerospace Professional, ProQuest Biological & Health Science Professional, ProQuest Dissertations and Theses Professional, ProQuest Environmental Science Professional, ProQuest Materials Research Professional, ProQuest Newsstand Professional, ProQuest Technology Research Professional, Prous Science Daily Essentials, Prous Science Drug Data Report, Prous Science Drugs Of The Future™, Publicly Available Content, Registry of Toxic Effects of Chemical Substances (RTECS®), SciSearch®: a Cited Reference Science Database, Social SciSearch®, ToxFile®, Transport Research International Documentation, TULSA™ (Petroleum Abstracts), UBM Computer Full Text, Weldasearch®, Zoological Record

| Set# | Searched for | Results |
|---|---|---|
| S1 | ((crte or crtb or crti or crty or crtz or crtw) near/100 candida near/100 ("inducible promoter")) | 0° |
| S2 | ((crte or crtb or crti or crty or crtz or crtw) near/500 candida near/500 ("inducible promoter")) | 2° |
| S3 | (("terpene synthase") near/500 candida near/500 ("inducible promoter")) | 0° |
| S4 | ((("terpene synthase") near/500 blastobotyrs near/500 ("inducible promoter"))) | 0° |
| S5 | ((("phytoene synthase") near/500 candida near/500 ("inducible promoter"))) | 3° |
| S6 | ((("geranylgeranyl") near/500 candida near/500 ("inducible promoter"))) | 4° |
| S7 | ((("phytoene desaturase") near/500 candida near/500 ("inducible promoter"))) | 0° |
| S8 | (((("lycopene cyclase") near/500 candida near/500 ("inducible | 0° |



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| | | |
|---|---|---|
| | promoter")))) | |
| S9 | (((("valencene cyclase") near/500 candida near/500 ("inducible promoter")))) | 0° |
| S10 | (((("valencene synthase") near/500 candida near/500 ("inducible promoter")))) | 1° |
| S11 | (((("β-carotene ketolase") near/500 candida near/500 ("inducible promoter")))) | 0° |
| S12 | ((((("β-carotene hydroxylase") near/500 candida near/500 ("inducible promoter"))))) | 0° |
| S13 | ((((("astaxanthin synthase") near/500 candida near/500 ("inducible promoter"))))) | 0° |
| S14 | ((((("zeaxanthin glucosyltransferase") near/500 candida near/500 ("inducible promoter"))))) | 0° |
| S15 | (("zeaxanthin glucosyltransferase") near/500 candida near/500 ("inducible promoter")) | 0° |

° Duplicates are removed from the search and from the result count.



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| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 16/630,567 | 01/13/2020 | Kimberly Ann Aeling | SS118-1026US | 5356 |

7590 06/14/2023

Silvia Salvadori, P.C.
Silvia Salvadori
485 Central Park West Unit 5C
New York, NY 10025

| EXAMINER | |
|---|---|
| NOAKES, SUZANNE MARIE | |
| ART UNIT | PAPER NUMBER |
| 1656 | |
| NOTIFICATION DATE | DELIVERY MODE |
| 06/14/2023 | ELECTRONIC |

**NOTICE REQUIRING INVENTOR'S OATH OR DECLARATION**

❑ An inventor's oath or declaration in compliance with 37 CFR 1.63 or substitute statement in compliance with 37 CFR 1.64 executed by or with respect to each inventor has not yet been submitted.

The inventor's oath or declaration or substitute statement dated 10/4/21 is defective. The inventor's oath or declaration does not comply with 37 CFR 1.63 or the substitute statement does not comply with 37 CFR 1.64 because it:

❑ does not include a statement that the person executing the oath or declaration or substitute statement believes the named inventor or joint inventor to be the original inventor or an original joint inventor of a claimed invention in the application for which the oath or declaration is being submitted.

❑ does not state that the application was made or was authorized to be made by the person executing the oath or declaration or substitute statement.

❑ does not acknowledge that any willful false statement made in such oath or declaration or substitute statement is punishable under section 1001 of title 18 by fine or imprisonment of not more than 5 years, or both.

❑ does not identify the person executing the oath or declaration or substitute statement by his or her legal name.

❑ does not identify the application to which the oath or declaration or substitute statement is directed.

☑ Other: Inventor 7 first name don't match. Tom vs. Thomas

The substitute statement dated _____ does not comply with 37 CFR 1.64 because it:

❑ does not identify the inventor with respect to whom the statement applies.

❑ does not identify the relationship to the non-signing inventor.

❑ does not identify the circumstances permitting execution of the statement.

❑ was signed by someone other than the applicant.

❑ Other: _____

An oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each inventor MUST be filed **no later than the date on which the issue fee is paid.** See 35 U.S.C. 115(f). Failure to timely comply will result in ABANDONMENT of this application. This period for reply is not extendable under 37 CFR 1.136(a).

Questions relating to this Notice should be directed to the Application Assistance Unit at 571-272-4200.

(571)-272-4200 or 1(888)-786-0101
Patent Publication Branch
Office of Data Management

PTO/AIA/01 (06-12)
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# DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

| Title of Invention | BIOLOGICAL METHODS FOR PREPARING TERPENES |
|---|---|

As the below named inventor, I hereby declare that:

This declaration is directed to:

☐ The attached application, or

☒ United States application or PCT international application number PCT/US2018/041579 filed on 11 July 2018.

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

## WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Thomas Beardslee Date (Optional): 15-JUN-2023

Signature: Thomas Beardslee

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

*If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.*

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The **Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

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2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
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5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant ( *i.e.*, GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

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# ELECTRONIC ACKNOWLEDGEMENT RECEIPT

| APPLICATION # | RECEIPT DATE / TIME | ATTORNEY DOCKET # |
|---|---|---|
| **16/630,567** | **06/16/2023 05:34:04 AM ET** | **SS118-1026US** |

## Title of Invention

BIOLOGICAL METHODS FOR PREPARING TERPENES

## Application Information

| | | | |
|---|---|---|---|
| APPLICATION TYPE | Utility - U.S. National Stage under 35 USC 371 | PATENT # | - |
| CONFIRMATION # | 5356 | FILED BY | Silvia Salvadori |
| PATENT CENTER # | 62282866 | FILING DATE | 01/13/2020 |
| CUSTOMER # | 122515 | FIRST NAMED INVENTOR | Kimberly Ann Aeling |
| INTL. APPLICATION # | - | INTL. FILING DATE | - |
| CORRESPONDENCE ADDRESS | - | AUTHORIZED BY | - |

## Documents

**TOTAL DOCUMENTS: 1**

| DOCUMENT | PAGES | DESCRIPTION | SIZE (KB) |
|---|---|---|---|
| Beardslee_declaration.pdf | 2 | Oath or Declaration filed | 4093 KB |

## Digest

| DOCUMENT | MESSAGE DIGEST(SHA-512) |
|---|---|
| Beardslee_declaration.pdf | C1ADD8B73DED3805B4B5250A6A126F0D335A16E7D6BF31D0 A0323B839B7D71E3045F7E2567407BF4472EB22BCA7C75BC6 9BFABA6A959A4289A3CD04F02652248 |

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

**New Applications Under 35 U.S.C. 111**

If a new application is being filed and the application includes the necessary components for filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application

**National Stage of an International Application under 35 U.S.C. 371**

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

**New International Application Filed with the USPTO as a Receiving Office**

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Doc Code: IFEE PTOL/85B-EFS

Document Description: Issue Fee Payment (PTO-85B)

# Issue Fee Transmittal Form

| Application Number | Filing Date | First Named Inventor | Atty. Docket No. | Confirmation No. |
|---|---|---|---|---|
| 16630567 | 13-Jan-2020 | Kimberly Ann Aeling | SS118-1026US | 5356 |

**TITLE OF INVENTION :**

BIOLOGICAL METHODS FOR PREPARING TERPENES

| Entity Status | Application Type | Art Unit | Class - Subclass | EXAMINER |
|---|---|---|---|---|
| Regular Undiscounted | U.S. National Stage under 35 USC 371 | 1656 | 132000 | SUZANNE NOAKES |

| Issue Fee Due | Publication Due | Total Fee(s) Due | Date Due | Prev. Paid Fee |
|---|---|---|---|---|
| $1200 | $0 | $1200 | 11-Sep-2023 | $0 |

## 1.Change of Correspondence Address and/or Indication Of Fee Address (37 CFR 1.33 & 1.363)

| Current Correspondence Address: | Current Indicated Fee Address : |
|---|---|
| 122515<br>Silvia Salvadori, P.C.<br>Silvia Salvadori<br>485 Central Park West Unit 5C<br><br>New York NY 10025<br>UNITED STATES<br>2128971938<br>eofficeaction@appcoll.com | |
| ☐ Change of correspondence address requested, system generated AIA/122-EFS form attached | ☐ Fee Address indication requested, system generated SB/47-EFS form attached |

## 2.Entity Status

**Change in Entity Status**

○ Applicant certifying micro entity status; system generated Micro Entity certification form attached. See 37 CFR 1.29.
Note: Absent a valid certification of micro entity status, issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment. If this box is checked, you will be prompted to choose a micro entity status on the gross income basis (37 CFR 1.29(a)) or the institution of higher education basis (37 CFR 1.29(d)), and make the applicable certification online.

○ Applicant asserting small entity status. See 37 CFR 1.27.
Note: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

◉ Applicant changing to regular undiscounted fee status.
Note: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

Doc Code: IFEE PTOL/85B-EFS

Document Description: Issue Fee Payment (PTO-85B)

**3.The Following Fee(s) Are Submitted:**

| | |
|---|---|
| ☒ Issue Fee | ☐ I authorize USPTO to apply my previously paid issue fee to the current fees due |
| ☐ Publication Fee | ☐ The Director is hereby authorized to apply my previously paid issue fee to the current fee due and to charge deficient fees to Deposit Account Number _____ |
| | ☒ If **in addition to** the payment of the issue fee amount submitted with this form, there are any discrepancies in any amount(s) due, the Director is authorized to charge any deficiency, or credit any overpayment, to Deposit Account Number 506423 . The **issue fee must be submitted** with this form. **If payment of the issue fee does not accompany this form, checking this box and providing a deposit account number will NOT be effective to satisfy full payment of the fee(s) due.** |

**4.Firm and/or Attorney Names To Be Printed**

**NOTE: If no name is listed, no name will be printed**
For printing on the patent front page, list to be displayed as entered

1. SILVIA SALVADORI, P.C.
2. SILVIA SALVADORI
3.

**5.Assignee Name(s) and Residence Data To Be Printed**

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

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|---|---|---|---|---|
| Radici Chimica S.p.A. | Bergamo | | ITALY | corporation |

**6.Signature**

I certify, in accordance with 37 CFR 1.4(d)(4) that I am an attorney or agent registered to practice before the Patent and Trademark Office who has filed and has been granted power of attorney in this application. I also certify that this Fee(s) Transmittal form is being transmitted to the USPTO via EFS-WEB on the date indicated below.

| | | | |
|---|---|---|---|
| **Signature** | /Silvia Salvadori/ | **Date** | 09-04-2023 |
| **Name** | Silvia Salvadori | **Registration Number** | 48265 |

WEB IFEE 1.0

# Electronic Patent Application Fee Transmittal

| | |
|---|---|
| **Application Number:** | 16630567 |
| **Filing Date:** | 13-Jan-2020 |
| **Title of Invention:** | BIOLOGICAL METHODS FOR PREPARING TERPENES |
| **First Named Inventor/Applicant Name:** | Kimberly Ann Aeling |
| **Filer:** | Silvia Salvadori |
| **Attorney Docket Number:** | SS118-1026US |

Filed as Large Entity

**Filing Fees for U.S. National Stage under 35 USC 371**

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Basic Filing:** | | | | |
| UTILITY APPL ISSUE FEE | 1501 | 1 | 1200 | 1200 |
| PUBL. FEE- EARLY, VOLUNTARY, OR NORMAL | 1504 | 1 | 0 | 0 |
| **Pages:** | | | | |
| **Claims:** | | | | |
| **Miscellaneous-Filing:** | | | | |
| **Petition:** | | | | |
| **Patent-Appeals-and-Interference:** | | | | |

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Post-Allowance-and-Post-Issuance:** | | | | |
| **Extension-of-Time:** | | | | |
| **Miscellaneous:** | | | | |
| | | **Total in USD ($)** | | **1200** |

| Electronic Acknowledgement Receipt | |
|---|---|
| **EFS ID:** | 48534142 |
| **Application Number:** | 16630567 |
| **International Application Number:** | |
| **Confirmation Number:** | 5356 |
| **Title of Invention:** | BIOLOGICAL METHODS FOR PREPARING TERPENES |
| **First Named Inventor/Applicant Name:** | Kimberly Ann Aeling |
| **Customer Number:** | 122515 |
| **Filer:** | Silvia Salvadori |
| **Filer Authorized By:** | |
| **Attorney Docket Number:** | SS118-1026US |
| **Receipt Date:** | 04-SEP-2023 |
| **Filing Date:** | 13-JAN-2020 |
| **Time Stamp:** | 10:12:23 |
| **Application Type:** | U.S. National Stage under 35 USC 371 |

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| Submitted with Payment | yes |
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| Authorized User | Silvia Salvadori |
| The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:<br>37 CFR 1.21 (Miscellaneous fees and charges) | |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Issue Fee Payment (PTO-85B) | Web85b.pdf | 60648<br>bc2463b3a53760f70457c5ac13d4d4fba2143516 | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 2 | Fee Worksheet (SB06) | fee-info.pdf | 40218<br>4112205a7768c3dcd249ddfe82f7e82308b302c1 | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| | | **Total Files Size (in bytes):** | 100866 | | |

**This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.**

**New Applications Under 35 U.S.C. 111**
**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**
**National Stage of an International Application under 35 U.S.C. 371**
**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**
**New International Application Filed with the USPTO as a Receiving Office**
**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

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United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NO. | ISSUE DATE | PATENT NO. | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 16/630,567 | 10/10/2023 | 11781148 | SS118-1026US | 5356 |

122515 7590 09/20/2023

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# ISSUE NOTIFICATION

The projected patent number and issue date are specified above. The patent will issue electronically. The electronically issued patent is the official patent grant pursuant to 35 U.S.C. § 153. The patent may be accessed on or after the issue date through Patent Center at https://patentcenter.uspto.gov/. The patent will be available in both the public and the private sides of Patent Center. Further assistance in electronically accessing the patent, or about Patent Center, is available by calling the Patent Electronic Business Center at 1-888-217-9197.

The USPTO is implementing electronic patent issuance with a transition period, during which period the USPTO will mail a ceremonial paper copy of the electronic patent grant to the correspondence address of record. Additional copies of the patent (i.e., certified and presentation copies) may be ordered for a fee from the USPTO's Certified Copy Center at https://certifiedcopycenter.uspto.gov/index.html. The Certified Copy Center may be reached at (800)972-6382.

**Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)**
(application filed on or after May 29, 2000)

The Patent Term Adjustment is 245 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Center (https://patentcenter.uspto.gov).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Patents Stakeholder Experience (OPSE), Stakeholder Support Division (SSD) at (571)-272-4200.

INVENTOR(s) (Please see PATENT CENTER site https://patentcenter.uspto.gov for additional inventors):

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| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 16/630,567 | 01/13/2020 | Kimberly Ann Aeling | SS118-1026US | 5356 |

122515 7590 10/10/2023

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| EXAMINER | |
|---|---|
| NOAKES, SUZANNE MARIE | |

| ART UNIT | PAPER NUMBER |
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| 1656 | |

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|---|---|
| 10/10/2023 | ELECTRONIC |

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

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| APPLICATION NO. | ISSUE DATE | PATENT NO. |
|---|---|---|
| 16/630,567 | 10-Oct-2023 | 11781148 |

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# EGRANT NOTIFICATION

Your electronic patent grant (eGrant) is now available, which can be accessed via Patent Center at https://patentcenter.uspto.gov

The electronic patent grant is the official patent grant under 35 U.S.C. 153. For more information, please visit https://www.uspto.gov/electronicgrants