TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A SUBMISSION UNDER 35 U.S.C. 371

ATTORNEY DOCKET NUMBER	
38767-0048US1	
U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)	

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US2016/031797	May 11, 2016	May 11, 2015

TITLE OF INVENTION
EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

APPLICANT(S) FOR DO/EO/US
Impossible Foods Inc.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a submission under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a submission under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☐ The US has been elected (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.
13. ☒ A preliminary amendment.
14. ☒ An Application Data Sheet under 37 C.F.R. 1.76.
15. ☐ A substitute specification.
16. ☐ A power of attorney and/or change of address letter.
17. ☒ A computer-readable form of the sequence listing in accordance with PCT Rule 13*ter*.2 and 37 C.F.R. 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☐ Other items or information:

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO. PCT/US2016/031797	ATTORNEY DOCKET NUMBER 38767-0048US1

The following fees have been submitted	CALCULATIONS	PTO USE ONLY
21. ☒ Basic national fee (37 CFR 1.492(a)).....$280	$280.00	
22. ☒ Examination fee (37 CFR 1.492(c)) If the written opinion prepared by ISA/US or the international preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4).....$0 All other situations.....$720	$720.00	
23. ☒ Search fee If the written opinion of the ISA/US or the international preliminary examination report prepared by IPEA/US indicates all claims satisfy provisions of PCT Article 33(1)-(4).....$0 Search fee (37 CFR 1.445(a)(2)) has been paid on the international application to the USPTO as an International Searching Authority.....$120 International Search Report prepared by an ISA other than the US and provided to the Office or previously communicated to the US by the IB.....$480 All other situations.....$600	$120.00	
Total of 21, 22 and 23 =	$1,120.00	

☐ Additional fee for specification and drawings filed in paper over 100 sheets (excluding sequence listing or computer program listing filed in an electronic medium). The fee is $400 for each additional 50 sheets of paper or fraction thereof.

Total Sheets	Extra Sheets	Number of each additional 50 or fraction thereof (round up to a whole number)	RATE	
-100 =	/50=		X $400	$0.00

Surcharge of **$140** for furnishing the oath or declaration later than 30 months from the earliest claimed priority date (37 C.F.R. 1.492(h)).	$140.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	18 - 20 =	0	x $80	$0.00	
Independent Claims	2 - 3 =	0	x $420	$0.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $780	$0.00	
			TOTAL OF ABOVE CALCULATIONS =	$1,260.00	
☐ Applicant claims small entity status. See 37 C.F.R. 1.27. Fees above are reduced by ½.				$0.00	
			SUBTOTAL =	$1,260.00	
Processing fee of **$140** for furnishing the English Translation later than 30 months from the earliest claimed priority date (37 C.F.R. 1.492(i)) +				$0.00	
			TOTAL NATIONAL FEE =	$1,260.00	
Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28, 3.31). **$40.00** per property +				$0.00	
			TOTAL FEES ENCLOSED =	$1,260.00	
			Amount to be refunded:	$0.00	
			Amount to be charged:	$1,260.00	

a. ☐ A check in the amount of $0.00 to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. 06-1050 in the amount of $1,260.00 to cover the above fees.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-1050.

d. ☐ Fees are to be charged to a credit card. **WARNING**: Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 C.F.R. 1.495 has not been met, a petition to revive (37 C.F.R. 1.137(a) or (b) must be filed and granted to restore the International Application to pending status.

SEND ALL CORRESPONDENCE TO:

PTO Customer No: 26191

/M. Angela Parsons; Reg No 44,282/
SIGNATURE

M. Angela Parsons, Ph.D.
NAME

44,282
REGISTRATION NUMBER

61591087.doc

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	38767-0048US1
	Application Number	
Title of Invention	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST	

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
	Smita		Shankar	

Residence Information (Select One) ● US Residency Non US Residency Active US Military Service

City	State/Province	Country of Residence
Millbrae	CA	US

Mailing Address of Inventor:

Address 1	120 S. El Camino Real, Apt. 115
Address 2	

City	State/Province	Postal Code	Country
Millbrae	CA	94030	US

Inventor 2 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Martin	Andrew	Hoyt	

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

City	State/Province	Country of Residence
San Francisco	CA	US

Mailing Address of Inventor:

Address 1	338 Garfield Street
Address 2	

City	State/Province	Postal Code	Country
San Francisco	CA	94132	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).

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	38767-0048US1
	Application Number	
Title of Invention	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST	

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

Customer Number	26191		
Email Address	apsi@fr.com	Add Email	Remove Email

Application Information:

Title of the Invention	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST		
Attorney Docket Number	38767-0048US1	**Small Entity Status Claimed**	☐
Application Type	Nonprovisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	11	**Suggested Figure for Publication (if any)**	

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	26191		

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	38767-0048US1
	Application Number	
Title of Invention	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST	

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)
	a 371 of international	PCTUS2016031797	2016-05-11
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCTUS2016031797	Claims benefit of provisional	62313491	2016-03-25
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCTUS2016031797	Claims benefit of provisional	62236506	2015-10-02
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCTUS2016031797	Claims benefit of provisional	62222388	2015-09-23
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCTUS2016031797	Claims benefit of provisional	62220366	2015-09-18
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCTUS2016031797	Claims benefit of provisional	62203052	2015-08-10
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCTUS2016031797	Claims benefit of provisional	62185921	2015-06-29
Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCTUS2016031797	Claims benefit of provisional	62183074	2015-06-22

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	38767-0048US1
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Title of Invention	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST	

Prior Application Status	Expired		Remove
Application Number	Continuity Type	Prior Application Number	Filing or 371(c) Date (YYYY-MM-DD)
PCTUS2016031797	Claims benefit of provisional	62159899	2015-05-11
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	38767-0048US1
	Application Number	
Title of Invention	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST	

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	38767-0048US1
	Application Number	
Title of Invention	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST	

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	Impossible Foods Inc.		
Mailing Address Information For Applicant:			
Address 1	525 Chesapeake Dr.		
Address 2			
City	Redwood City	**State/Province**	CA
Country	US	Postal Code	94063
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	38767-0048US1
	Application Number	
Title of Invention	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST	

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. ☐

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. 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	/M. Angela Parsons/			Date (YYYY-MM-DD)	2017-11-10
First Name	M. Angela	Last Name	Parsons, Ph.D.	Registration Number	44,282

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	38767-0048US1
	Application Number	
Title of Invention	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST	

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.

REMARKS

Applicants respectfully request entry of the amendments and remarks submitted herein. Claims 2-10, 12-14, 19, 20, 25-29 and 32-38 have been amended herein to remove the multiple dependencies. Claims 1-38 are currently pending.

In addition, Applicants have amended the specification to include a paragraph describing related applications and to claim the benefit of priority to such applications. Applicants submit that there is no new matter introduced by these amendments.

CONCLUSION

Applicants ask that claims 1-38 be examined. The enclosed filing fee takes into account the claims pending following entry of this Preliminary Amendment. Please apply any other charges or credits to Deposit Account 06-1050.

Respectfully submitted,

Date: /November 10, 2017/

/M. Angela Parsons/

M. Angela Parsons, Ph.D.
Reg. No. 44,282

Customer Number 26191
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945

61589476.doc

Amendments to the Claims:

Please amend claims 2-10, 12-14, 19, 20, 25-29 and 32-38 as follows. The claims and their status are shown.

1. (Original) A methylotrophic yeast cell comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises an exogenous nucleic acid encoding a transcriptional activator operably linked to at least one methanol-inducible promoter element.

2. (Currently Amended) The yeast cell of claim 1, wherein the methylotrophic yeast cell is of a yeast selected from the group consisting of *Candida*, *Hansenula*, *Pichia* and *Torulopsis*.

3. (Currently Amended) The yeast cell of claim 1 [[or 2]], wherein the methylotrophic yeast cell is of a *Pichia* yeast.

4. (Currently Amended) The yeast cell of claim 1 [[or 2]], wherein the cell is a *Pichia* cell.

5. (Currently Amended) The yeast cell of claim 1 ~~any of claims 1 to~~ [[4]], wherein the cell is a *Pichia pastoris* cell.

6. (Currently Amended) The yeast cell of claim 1 ~~any of claims 1 to 5~~, wherein the recombinant nucleic acid molecule is stably integrated into the genome of the methylotrophic yeast cell.

7. (Currently Amended) The yeast cell of claim 1 ~~any of claims 1 to 5~~, wherein the recombinant nucleic acid molecule is extrachromosomally expressed from a replication-competent plasmid.

8. (Currently Amended) The yeast cell of claim 1 ~~any of claims 1 to 7~~, wherein the exogenous nucleic acid encoding a transcriptional activator comprises a Mxr1 sequence from *Pichia pastoris*, a Adr1 sequence from *Hansenula polymorpha*, a Trm1 sequence from *Candida boidinii*, or a Trm2 sequence from *Candida boidinii*.

9. (Currently Amended) The yeast cell of claim 1 ~~any of claims 1 to 8~~, wherein the exogenous nucleic acid encoding a transcriptional activator comprises a Mxr1 sequence from *Pichia pastoris*.

10. (Currently Amended) The yeast cell of claim 1 ~~any of claims 1 to 9~~, wherein the transcriptional activator comprises a sequence shown in GenBank Accession No. ABD57365.

11. (Original) The yeast cell of claim 10, wherein the transcriptional activator is encoded by the nucleic acid sequence shown in GenBank Accession No. DQ395124.

12. (Currently Amended) The yeast cell of claim 1 ~~any of claims 1 to 11~~, wherein the at least one methanol-inducible promoter element is selected from the group consisting of an alcohol oxidase 1 (AOX1) promoter element from *Pichia pastoris*, an AOD1 promoter element from *Candida boidinii*, a MOX promoter element from *Hansenula polymorpha*, a MOD1 promoter element from *Pichia methanolica*, a DHAS promoter element from *Pichia pastoris*, a FLD1 promoter element from *Pichia pastoris*, and a PEX8 promoter element from *Pichia pastoris*.

13. (Currently Amended) The yeast cell of claim 1 ~~any of claims 1 to 11~~, wherein the at least one methanol-inducible promoter element is an alcohol oxidase 1 (AOX1) promoter element from *Pichia pastoris*.

14. (Currently Amended) The yeast cell of claim 1 ~~any of claims 1 to 13~~, further comprising a nucleic acid molecule comprising at least one heterologous nucleic acid encoding a polypeptide operably linked to at least one methanol-inducible promoter element.

15. (Original) The yeast cell of claim 14, wherein the heterologous nucleic acid encodes at least one polypeptide involved in the biosynthesis of an iron co-factor.

16. (Original) The yeast cell of claim 15, wherein the iron co-factor is heme.

17. (Original) The yeast cell of claim 16, wherein the at least one polypeptide involved in the biosynthesis of heme is selected from the group consisting of ALA synthase, ALA dehydratase, porphobilinogen deaminase, UPG III synthase, UPG III decarboxylase, CPG oxidase, PPG oxidase, and ferrochelatase.

18. (Original) The yeast cell of claim 16, wherein the at least one polypeptide involved in the biosynthesis of heme comprises two or more polypeptides selected from the group consisting of ALA synthase, ALA dehydratase, porphobilinogen deaminase, UPG III synthase, UPG III decarboxylase, CPG oxidase, PPG oxidase, and ferrochelatase.

19. (Currently Amended) The yeast cell of claim 1 ~~any of claims 1 to 18~~, wherein the yeast cell lacks heterologous sequences for selection.

20. (Currently Amended) A method for expressing a heterologous polypeptide in a cell, the method comprising:

providing the yeast cell of claim 1 ~~any of the preceding claims~~;

introducing a recombinant nucleic acid molecule into a methylotrophic yeast cell, the recombinant nucleic acid molecule comprising at least one heterologous nucleic acid encoding a polypeptide operably linked to at least one *Pichia pastoris* alcohol oxidase 1 (AOX1) promoter element; and

culturing the cell under conditions suitable for expression of the recombinant nucleic acid molecule,

thereby expressing the heterologous polypeptide.

21. (Original) The method of claim 20, wherein the heterologous polypeptide is a member of the globin family PF00042.

22. (Original) The method of claim 20, wherein the heterologous polypeptide is a plant hemoglobin.

23. (Original) The method of claim 22, wherein the plant hemoglobin is leghemoglobin.

24. (Original) The method of claim 23, wherein the leghemoglobin has a sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:6.

25. (Currently Amended) The method of claim 20 ~~any of claims 20 to 24~~, wherein the introducing step comprises a technique selected from the group consisting of transduction, electroporation, biolistic particle delivery, and chemical transformation.

26. (Currently Amended) The method of claim 20 ~~any of claims 20 to 25~~, wherein the conditions under which the cells are cultured comprises the addition of iron or a pharmaceutically or metabolically acceptable salt thereof.

27. (Currently Amended) The method of claim 20 ~~any of claims 20 to 26~~, wherein the culturing step comprises culturing the cell in the present of methanol.

28. (Currently Amended) The method of claim 20 ~~any of claims 20 to 26~~, wherein the culturing step comprises culturing the cell in the absence of methanol.

29. (Currently Amended) The method of claim 20 ~~any of claims 20 to 28~~, wherein the culturing step comprises culturing the cell in the absence of selection.

30. (Original) A methylotrophic *Pichia* yeast cell comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises:

a nucleic acid encoding a Mxr1 transcriptional activator sequence from *P. pastoris*;

a nucleic acid encoding a member of the globin family (PF00042); and

a nucleic acid encoding at least one polypeptide involved in heme biosynthesis.

31. (Original) The yeast cell of claim 30, wherein the nucleic acid encoding the Mxr1 transcriptional activator sequence is operably linked to a methanol-inducible promoter element.

32. (Currently Amended) The yeast cell ~~method~~ of claim 30, wherein the member of the globin family is leghemoglobin.

33. (Currently Amended) The yeast cell of claim 30 ~~any of claims 30 to 32~~, wherein the nucleic acid encoding the member of the globin family is operably linked to a methanol-inducible promoter element.

34. (Currently Amended) The yeast cell of claim 30 ~~any of claims 30 to 32~~, wherein the nucleic acid encoding the member of the globin family is operably linked to a constitutive promoter element.

35. (Currently Amended) The yeast cell of claim 30 ~~any of claims 30 to 34~~, wherein the nucleic acid encoding at least one polypeptide involved in heme biosynthesis is operably linked to a methanol-inducible promoter element.

36. (Currently Amended) The yeast cell of claim 30 ~~any of claims 30 to 34~~, wherein the nucleic acid encoding at least one polypeptide involved in heme biosynthesis is operably linked to a constitutive promoter element.

37. (Currently Amended) The yeast cell of claim 31 ~~any of claims 30, 31, 33, or 35~~, wherein the methanol-inducible promoter element is an alcohol oxidase I (AOX1) promoter element from *P. pastoris*.

38. (Currently Amended) The yeast cell of claim 34 ~~any of claims 30, 34 or 36~~, wherein the constitutive promoter element is a transcriptional elongation factor EF-1α gene (TEF1) promoter element from *P. pastoris*.

Amendments to the Specification:

Please add the following paragraph after the Title on page 1:

-- CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a National Stage Application under 35 U.S.C. § 371 and claims the benefit of International Application No. PCT/US2016/031797 filed May 11, 2016, which claims priority to U.S. Provisional Application No. 62/313,491 filed March 25, 2016, U.S. Provisional Application No. 62/236,506 filed October 2, 2015, U.S. Provisional Application No. 62/222,388 filed September 23, 2015, U.S. Provisional Application No. 62/220,366 filed September 18, 2015, U.S. Provisional Application No. 62/203,052 filed August 10, 2015, U.S. Provisional Application No. 62/185,921 filed June 29, 2015, U.S. Provisional Application No. 62/183,074 filed June 22, 2015, and U.S. Provisional Application No. 62/159,899 filed May 11, 2015. The disclosures of the foregoing applications are hereby incorporated by reference in their entirety. --

Attorney's Docket No.: 38767-0048US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Smita Shankar	Art Unit	:	Unknown
Serial No.	:	Unassigned	Examiner	:	Unknown
Filed	:	Herewith			
Title	:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST			

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

PRELIMINARY AMENDMENT

Prior to examination, please amend the application as indicated on the following pages.

Electronic Patent Application Fee Transmittal

Application Number:	
Filing Date:	
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Filer:	M. Angela Parsons
Attorney Docket Number:	38767-0048US1

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:				
NATIONAL STAGE FEE	1631	1	280	280
Natl Stage Search Fee - U.S. was the ISA	1641	1	120	120
NATIONAL STAGE EXAM - ALL OTHER CASES	1633	1	720	720
Pages:				
Claims:				
Miscellaneous-Filing:				
OATH/DECL > 30 MOS FROM 371 COMMENCEMENT	1617	1	140	140
Petition:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
		Total in USD ($)		**1260**

Electronic Acknowledgement Receipt

EFS ID:	30916645
Application Number:	15573300
International Application Number:	PCT/US2016/031797
Confirmation Number:	4807
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	M. Angela Parsons/Jennifer MacKenzie
Filer Authorized By:	M. Angela Parsons
Attorney Docket Number:	38767-0048US1
Receipt Date:	10-NOV-2017
Filing Date:	
Time Stamp:	14:59:04
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	$1260
RAM confirmation Number	111317INTEFSW00012049061050
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal of New Application	Trans.pdf	143792 6cd1426ce39713ba4c54507aa65aeb97a274360a	no	2
Warnings:					
Information:					
2	Application Data Sheet	ADS.pdf	1823463 738211bfa462eec9851aae9bf58bfd73752971fb	no	9
Warnings:					
Information:					
3		Pamd.pdf	108893 e9f0180d8e6f3db31b993de2e93259285f4fda91	yes	9

Multipart Description/PDF files in .zip description

Document Description	Start	End
Applicant Arguments/Remarks Made in an Amendment	9	9
Claims	3	8
Specification	2	2
Preliminary Amendment	1	1

Warnings:					
Information:					
4	Sequence Listing (Text File)	SEQUENCELISTING.TXT	44936	no	-
Warnings:					
Information:					

5	Fee Worksheet (SB06)	fee-info.pdf	37011 f26790cecc40822c58653f739c98ae403c41cf84	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	2158095	

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.

DocCode – SEQ.TXT

SCORE Placeholder Sheet for IFW Content

Application Number: 15573300 Document Date: 11/10/2017

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.

- Sequence Listing

At the time of document entry (noted above):

- USPTO employees may access SCORE content via eDAN using the Supplemental Content tab, or via the SCORE web page.
- External customers may access SCORE content via PAIR using the Supplemental Content tab.

Form Revision Date: August 26, 2013

PATENT COOPERATION TREATY PCT/US2016/031797

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92*bis*.1 and Administrative Instructions, Section 422)

To:

PARSONS, M. Angela
Fish & Richardson P.C.
P.O. Box 1022
Minneapolis, Minnesota 55440-1022
ÉTATS-UNIS D'AMÉRIQUE

Date of mailing *(day/month/year)*
20 July 2016 (20.07.2016)

Applicant's or agent's file reference
387670048WO1

IMPORTANT NOTIFICATION

International application No.
PCT/US2016/031797

International filing date *(day/month/year)*
11 May 2016 (11.05.2016)

1. The following indications appeared on record concerning:

☒ the applicant ☒ the inventor ☐ the agent ☐ the common representative

Name and Address
SHANKAR, Smita
HOYT, Martin Andrew

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

Name and Address
See Box 3

State of Nationality | State of Residence

Telephone No.

Facsimile No.

E-mail address
☐ Notifications by e-mail authorized

3. Further observations, if necessary:
The persons identified in Box 1 are now inventors only

4. A copy of this notification has been sent to:

☒ the receiving Office
☐ the International Searching Authority
☐ the Authority(ies) specified for supplementary search
☐ the International Preliminary Examining Authority
☐ the designated Offices concerned
☐ the elected Offices concerned
☐ other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. +41 22 338 70 90

Authorized officer
Cruz Juan
e-mail pt01.pct@wipo.int
Telephone No. +41 22 338 74 01

Form PCT/IB/306 (January 2009) 1/H3ME9GGMFPAK20

387670048WO1

PCT REQUEST

Original (for **SUBMISSION**)

0	**For receiving Office use only**	
0-1	International Application No.	PCT/US16/31797
0-2	International Filing Date	11-05-2016 11 MAY 2016
0-3	Name of receiving Office and "PCT International Application"	PCT INTERNATIONAL APPLICATION RO/US

0-4	**Form PCT/RO/101 PCT Request**	
0-4-1	Prepared Using	**PCT-SAFE [EFS-Web mode] Version 3.51.073.249 MT/FOP 20160401/0.20.5.24**
0-5	**Petition**	
	The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty	
0-6	**Receiving Office (specified by the applicant)**	**United States Patent and Trademark Office (USPTO) (RO/US)**
0-7	**Applicant's or agent's file reference**	**387670048WO1**
I	**Title of Invention**	**EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST**
II	**Applicant**	
II-1	This person is	**Applicant only**
II-2	Applicant for	**All designated States**
II-4	Name	**IMPOSSIBLE FOODS INC.**
II-5	Address	**525 Chesapeake Dr. Redwood City, California 94063 United States of America**
II-6	State of nationality	**US**
II-7	State of residence	**US**
III-1	**Applicant and/or inventor**	
III-1-1	This person is	**Applicant and inventor**
III-1-2	Applicant for	**All designated States**
III-1-4	Name (LAST, First)	**SHANKAR, Smita**
III-1-5	Address	**120 S. El Camino Real, Apt. 115 Millbrae, California 94030 United States of America**
III-1-6	State of nationality	**IN**
III-1-7	State of residence	**US**

387670048WO1

PCT REQUEST

Original (for **SUBMISSION**)

III-2	**Applicant and/or inventor**	
III-2-1	This person is	Applicant and inventor
III-2-2	Applicant for	All designated States
III-2-4	Name (LAST, First)	HOYT, Martin Andrew
III-2-5	Address	338 Garfield Street San Francisco, California 94132 United States of America
III-2-6	State of nationality	US
III-2-7	State of residence	US
IV-1	**Agent or common representative; or address for correspondence**	
	The person identified below is hereby/ has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:	Agent
IV-1-1	Name (LAST, First)	PARSONS, M. Angela
IV-1-2	Address	Fish & Richardson P.C. P.O. Box 1022 Minneapolis, Minnesota 55440-1022 United States of America
IV-1-3	Telephone No.	612-335-5070
IV-1-4	Facsimile No.	877-769-7945
IV-1-5	e-mail	apsi@fr.com
IV-1-5(a)	E-mail authorization The receiving Office, the International Searching Authority, the International Bureau and the International Preliminary Examining Authority are authorized to use this e-mail address, if the Office or Authority so wishes, to send notifications issued in respect of this international application:	exclusively in electronic form (no paper notifications will be sent)
IV-1-6	Agent's registration No.	44,282
IV-2	**Additional agent(s)**	additional agent(s) with same address as first named agent
IV-2-1	Name(s)	LAVOIE, Teresa A.(42,782)
V	**DESIGNATIONS**	
V-1	**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.**	
VI-1	**Priority claim of earlier national application**	
VI-1-1	Filing date	11 May 2015 (11.05.2015)
VI-1-2	Number	62/159,899
VI-1-3	Country or Member of WTO	US

PCT REQUEST

Original (for **SUBMISSION**)

VI-2	**Priority claim of earlier national application**	
VI-2-1	Filing date	22 June 2015 (22.06.2015)
VI-2-2	Number	62/183,074
VI-2-3	Country or Member of WTO	US
VI-3	**Priority claim of earlier national application**	
VI-3-1	Filing date	29 June 2015 (29.06.2015)
VI-3-2	Number	62/185,921
VI-3-3	Country or Member of WTO	US
VI-4	**Priority claim of earlier national application**	
VI-4-1	Filing date	10 August 2015 (10.08.2015)
VI-4-2	Number	62/203,052
VI-4-3	Country or Member of WTO	US
VI-5	**Priority claim of earlier national application**	
VI-5-1	Filing date	18 September 2015 (18.09.2015)
VI-5-2	Number	62/220,366
VI-5-3	Country or Member of WTO	US
VI-6	**Priority claim of earlier national application**	
VI-6-1	Filing date	23 September 2015 (23.09.2015)
VI-6-2	Number	62/222,388
VI-6-3	Country or Member of WTO	US
VI-7	**Priority claim of earlier national application**	
VI-7-1	Filing date	02 October 2015 (02.10.2015)
VI-7-2	Number	62/236,506
VI-7-3	Country or Member of WTO	US
VI-8	**Priority claim of earlier national application**	
VI-8-1	Filing date	25 March 2016 (25.03.2016)
VI-8-2	Number	62/313,491
VI-8-3	Country or Member of WTO	US
VI-9	**Priority document request**	
	The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s):	VI-1 VI-2 VI-3 VI-4 VI-5 VI-6 VI-7 VI-8

387670048WO1

PCT REQUEST

Original (for **SUBMISSION**)

VI-10	**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.		
VII-1	**International Searching Authority Chosen**	**United States Patent and Trademark Office (USPTO) (ISA/US)**	
VIII	**Declarations**	Number of declarations	
VIII-1	Declaration as to the identity of the inventor	–	
VIII-2	Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent	–	
VIII-3	Declaration as to the applicant's entitlement, as at the international filing date, to claim the priority of the earlier application	–	
VIII-4	Declaration of inventorship (only for the purposes of the designation of the United States of America)	–	
VIII-5	Declaration as to non-prejudicial disclosures or exceptions to lack of novelty	–	
IX	**Check list**	Number of sheets	Electronic file(s) attached
IX-1	Request (including declaration sheets)	5	✓
IX-2	Description	46	–
IX-3	Claims	5	–
IX-4	Abstract	1	✓
IX-5	Drawings	11	–
IX-7	TOTAL	68	
	Accompanying Items	Paper document(s) attached	Electronic file(s) attached
IX-8	Fee calculation sheet	✓	–
IX-20	**Figure of the drawings which should accompany the abstract**	None	
IX-21	**Language of filing of the international application**	English	
X-1	**Signature of applicant, agent or common representative**	/M. Angela Parsons; Reg No 44,282/	
X-1-1	Name (LAST, First)	PARSONS, M. Angela	
X-1-3	Capacity (if such capacity is not obvious from reading the request)		

387670048WO1

PCT REQUEST

Original (for **SUBMISSION**)

FOR RECEIVING OFFICE USE ONLY

10-1	**Date of actual receipt of the purported international application**	11-05-2016 11 MAY 2016
10-2	**Drawings:**	
10-2-1	Received	
10-2-2	Not received	
10-3	**Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application**	
10-4	**Date of timely receipt of the required corrections under PCT Article 11(2)**	
10-5	**International Searching Authority**	ISA/US
10-6	**Transmittal of search copy delayed until search fee is paid**	

FOR INTERNATIONAL BUREAU USE ONLY

11-1	**Date of receipt of the record copy by the International Bureau**	

PCT Recordation of Search History

Case/PCT Application Number: PCT/US16/31797

CLIN Number/Technical Field of PCT Application: 0007 Biotechnology

Date(s) During Which the Search was Conducted: 24 to 25 August 2016

Date of Completion of Recordation of Search History Form: 25 August 2016

Research Analyst Initials: CJF

Search Approval Official (SAO) Initials: DGF

Field of Search/Classification Information:

IC: C07K 14/39; C12N 1/19, 15/00, 15/29, 15/81; C12P 21/00; C12R 1/00, 1/84 (2016.01)

CPC: C07K 14/39; C12N 15/00, 15/81, 15/815; C12P 21/00; C12R 1/00, 1/84

Databases Searched (Patent and Non-Patent Literature (NPL), Including Sub-Databases and Files Searched) and Search Terms Used:

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); EBSCO Discovery; PubMed; Google; Google Scholar; Google Patents;

KEYWORDS: methylotrophic yeast, Pichia, pastoris, Mxr1, leghemoglobin, heme

Database Search String Recordation, Including Dates of Searches:

Patent Database Search Strategy/Results:

PatSeer

ID	Recent searches done (last 1000)	Count	Date
L33	(TACD: (Pichia OR pastoris) AND heme) AND (TACD: (Pichia OR pastoris) AND heme AND ((IC: C07K14/39 OR C12N1/19 OR C12N15/00 OR C12N15/29 OR C12N15/81 OR C12P21/00 OR C12R1/00 OR C12R1/84) OR (CPC: C07K14/39 OR C12N15/00 OR C12N15/81 OR C12N15/815 OR C12P21/00 OR C12R1/00 OR C12R1/84)))	3133	25-Aug-16
L32	TACD: (Pichia OR pastoris) AND heme AND ((IC: C07K14/39 OR C12N1/19 OR C12N15/00 OR C12N15/29 OR C12N15/81 OR C12P21/00 OR C12R1/00 OR C12R1/84) OR (CPC: C07K14/39 OR C12N15/00 OR C12N15/81 OR C12N15/815 OR C12P21/00 OR C12R1/00 OR C12R1/84))	3133	25-Aug-16
L31	TACD: (Pichia OR pastoris) AND heme	9404	25-Aug-16
L30	TAC: (Pichia OR pastoris) AND heme	84	25-Aug-16
L29	TA: (Pichia OR pastoris) AND heme	0	25-Aug-16
L28	TACD: (Pichia OR pastoris) AND Mxr1 AND ((IC: C07K14/39 OR C12N1/19 OR C12N15/00 OR C12N15/29 OR C12N15/81 OR C12P21/00 OR C12R1/00 OR C12R1/84) OR (CPC: C07K14/39 OR C12N15/00 OR C12N15/81 OR C12N15/815 OR C12P21/00 OR C12R1/00 OR C12R1/84))	33	25-Aug-16
L27	TACD: (Pichia OR pastoris) AND Mxr1	49	25-Aug-16
L26	TAC: (Pichia OR pastoris) AND Mxr1	12	25-Aug-16
L25	TA: (Pichia OR pastoris) AND Mxr1	0	25-Aug-16
L24	TACD: (Pichia OR pastoris) AND Mxr1 AND ((ALA W4 synthase*) OR (ALA W4 dehydratase*) OR (porphobilinogen W4 deaminase*) OR (UPG W4 III W4 synthase) OR (UPG W4 III W4 decarboxylase) OR (CPG W4 oxidase*) OR (PPG W4 oxidase) OR ferrochelatase*)	0	25-Aug-16
L23	TAC: (Pichia OR pastoris) AND Mxr1 AND ((ALA W4 synthase*) OR (ALA W4 dehydratase*) OR (porphobilinogen W4 deaminase*) OR (UPG W4 III W4 synthase) OR (UPG W4 III W4 decarboxylase) OR (CPG W4 oxidase*) OR (PPG W4 oxidase) OR ferrochelatase*)	0	25-Aug-16
L22	TA: (Pichia OR pastoris) AND Mxr1 AND ((ALA W4 synthase*) OR (ALA W4 dehydratase*) OR (porphobilinogen W4 deaminase*) OR (UPG W4 III W4 synthase) OR (UPG W4 III W4 decarboxylase) OR (CPG W4 oxidase*) OR (PPG W4 oxidase) OR ferrochelatase*)	0	25-Aug-16
L21	(TACD: leghemoglobin* AND ((ALA W4 synthase*) OR (ALA W4 dehydratase*) OR (porphobilinogen W4 deaminase*) OR (UPG W4 III W4 synthase) OR (UPG W4 III W4 decarboxylase) OR (CPG W4 oxidase*) OR (PPG W4 oxidase) OR ferrochelatase*)) AND ((IC: C07K14/39 OR C12N1/19 OR C12N15/00 OR C12N15/29 OR C12N15/81 OR C12P21/00 OR C12R1/00 OR C12R1/84) OR (CPC: C07K14/39 OR C12N15/00 OR C12N15/81 OR C12N15/815 OR C12P21/00 OR C12R1/00 OR C12R1/84))	11	25-Aug-16
L20	TACD: methylotrophic AND Pichia AND leghemoglobin*	4	24-Aug-16
L19	TACD: methylotrophic AND Pichia AND Mxr1	20	24-Aug-16
L18	TACD: methylotrophic AND Pichia AND Mxr1 W10 transcr* W10 activ* AND pastoris	0	24-Aug-16
L17	TAC: methylotrophic AND Pichia AND Mxr1 W10 transcr* W10 activ* AND pastoris	0	24-Aug-16
L16	TA: methylotrophic AND Pichia AND Mxr1 W10 transcr* W10 activ* AND pastoris	0	24-Aug-16
L15	TACD: leghemoglobin* AND ((ALA W4 synthase*) OR (ALA W4 dehydratase*) OR (porphobilinogen W4 deaminase*) OR (UPG W4 III W4	43	24-Aug-16

	synthase) OR (UPG W4 III W4 decarboxylase) OR (CPG W4 oxidase*) OR (PPG W4 oxidase) OR ferrochelatase*)		
L14	TAC: leghemoglobin* AND ((ALA W4 synthase*) OR (ALA W4 dehydratase*) OR (porphobilinogen W4 deaminase*) OR (UPG W4 III W4 synthase) OR (UPG W4 III W4 decarboxylase) OR (CPG W4 oxidase*) OR (PPG W4 oxidase) OR ferrochelatase*)	3	24-Aug-16
L13	TA: leghemoglobin* AND ((ALA W4 synthase*) OR (ALA W4 dehydratase*) OR (porphobilinogen W4 deaminase*) OR (UPG W4 III W4 synthase) OR (UPG W4 III W4 decarboxylase) OR (CPG W4 oxidase*) OR (PPG W4 oxidase) OR ferrochelatase*)	0	24-Aug-16
L12	TACD: Mxr1 AND ((ALA W4 synthase*) OR (ALA W4 dehydratase*) OR (porphobilinogen W4 deaminase*) OR (UPG W4 III W4 synthase) OR (UPG W4 III W4 decarboxylase) OR (CPG W4 oxidase*) OR (PPG W4 oxidase) OR ferrochelatase*)	1	24-Aug-16
L11	TAC: Mxr1 AND ((ALA W4 synthase*) OR (ALA W4 dehydratase*) OR (porphobilinogen W4 deaminase*) OR (UPG W4 III W4 synthase) OR (UPG W4 III W4 decarboxylase) OR (CPG W4 oxidase*) OR (PPG W4 oxidase) OR ferrochelatase*)	0	24-Aug-16
L10	TA: Mxr1 AND ((ALA W4 synthase*) OR (ALA W4 dehydratase*) OR (porphobilinogen W4 deaminase*) OR (UPG W4 III W4 synthase) OR (UPG W4 III W4 decarboxylase) OR (CPG W4 oxidase*) OR (PPG W4 oxidase) OR ferrochelatase*)	0	24-Aug-16
L9	TACD: Mxr1 AND leghemoglobin* AND ((ALA W4 synthase*) OR (ALA W4 dehydratase*) OR (porphobilinogen W4 deaminase*) OR (UPG W4 III W4 synthase) OR (UPG W4 III W4 decarboxylase) OR (CPG W4 oxidase*) OR (PPG W4 oxidase) OR ferrochelatase*)	0	24-Aug-16
L8	TAC: Mxr1 AND leghemoglobin* AND ((ALA W4 synthase*) OR (ALA W4 dehydratase*) OR (porphobilinogen W4 deaminase*) OR (UPG W4 III W4 synthase) OR (UPG W4 III W4 decarboxylase) OR (CPG W4 oxidase*) OR (PPG W4 oxidase) OR ferrochelatase*)	0	24-Aug-16
L7	TA: Mxr1 AND leghemoglobin* AND ((ALA W4 synthase*) OR (ALA W4 dehydratase*) OR (porphobilinogen W4 deaminase*) OR (UPG W4 III W4 synthase) OR (UPG W4 III W4 decarboxylase) OR (CPG W4 oxidase*) OR (PPG W4 oxidase) OR ferrochelatase*)	0	24-Aug-16
L6	TACD: Mxr1 W10 transcr* W10 activ* AND pastoris AND leghemoglobin* AND ((ALA W4 synthase*) OR (ALA W4 dehydratase*) OR (porphobilinogen W4 deaminase*) OR (UPG W4 III W4 synthase) OR (UPG W4 III W4 decarboxylase) OR (CPG W4 oxidase*) OR (PPG W4 oxidase) OR ferrochelatase*)	0	24-Aug-16
L5	TAC: Mxr1 W10 transcr* W10 activ* AND pastoris AND leghemoglobin* AND ((ALA W4 synthase*) OR (ALA W4 dehydratase*) OR (porphobilinogen W4 deaminase*) OR (UPG W4 III W4 synthase) OR (UPG W4 III W4 decarboxylase) OR (CPG W4 oxidase*) OR (PPG W4 oxidase) OR ferrochelatase*)	0	24-Aug-16
L4	TA: Mxr1 W10 transcr* W10 activ* AND pastoris AND leghemoglobin* AND ((ALA W4 synthase*) OR (ALA W4 dehydratase*) OR (porphobilinogen W4 deaminase*) OR (UPG W4 III W4 synthase) OR (UPG W4 III W4 decarboxylase) OR (CPG W4 oxidase*) OR (PPG W4 oxidase) OR ferrochelatase*)	0	24-Aug-16
L3	TACD: methylotrophic AND Pichia AND Mxr1 W10 transcr* W10 activ* AND pastoris AND leghemoglobin* AND ((ALA W4 synthase*) OR (ALA W4 dehydratase*) OR (porphobilinogen W4 deaminase*) OR (UPG W4 III W4 synthase) OR (UPG W4 III W4 decarboxylase) OR (CPG W4 oxidase*) OR (PPG W4 oxidase) OR ferrochelatase*)	0	24-Aug-16
L2	TAC: methylotrophic AND Pichia AND Mxr1 W10 transcr* W10 activ* AND	0	24-Aug-16

	pastoris AND leghemoglobin* AND ((ALA W4 synthase*) OR (ALA W4 dehydratase*) OR (porphobilinogen W4 deaminase*) OR (UPG W4 III W4 synthase) OR (UPG W4 III W4 decarboxylase) OR (CPG W4 oxidase*) OR (PPG W4 oxidase) OR ferrochelatase*)		
L1	TA: methylotrophic AND Pichia AND Mxr1 W10 transcr* W10 activ* AND pastoris AND leghemoglobin* AND ((ALA W4 synthase*) OR (ALA W4 dehydratase*) OR (porphobilinogen W4 deaminase*) OR (UPG W4 III W4 synthase) OR (UPG W4 III W4 decarboxylase) OR (CPG W4 oxidase*) OR (PPG W4 oxidase) OR ferrochelatase*)	0	24-Aug-16

Google Patents: 24 August 2016 to 25 August 2016

Search String	Results
methylotrophic yeast cell exogenous nucleic acid encode transcriptional activator methanol-inducible promoter element	2600
methylotrophic Pichia nucleic acid encoding Mxr1 transcriptional activator sequence from P pastoris	5
methylotrophic Pichia nucleic acid encoding Mxr1 transcriptional activator sequence from P pastoris PF00042	0
methylotrophic Pichia nucleic acid encoding Mxr1 transcriptional activator sequence from P pastoris leghemoglobin	0
methylotrophic Pichia nucleic acid encoding Mxr1 transcriptional activator sequence from P pastoris heme biosynthesis	0
methylotrophic Pichia nucleic acid encoding leghemoglobin	0
methylotrophic Pichia nucleic acid encoding polypeptide involved in heme biosynthesis	1660
methylotrophic Pichia nucleic acid encoding polypeptide ALA synthase	2370
methylotrophic Pichia nucleic acid encoding polypeptide "ALA synthase"	0
methylotrophic Pichia nucleic acid encoding polypeptide "ALA dehydratase"	0
methylotrophic Pichia nucleic acid encoding polypeptide "porphobilinogen"	9
methylotrophic Pichia nucleic acid encoding polypeptide "UPG III synthase"	0
methylotrophic Pichia nucleic acid encoding polypeptide "UPG III decarboxylase"	0
methylotrophic Pichia nucleic acid encoding polypeptide "CPG oxidase"	0
methylotrophic Pichia nucleic acid encoding polypeptide "PPG oxidase"	0
methylotrophic Pichia nucleic acid encoding polypeptide "ferrochelatase"	4
leghemoglobin linked to constitutive promoter	284
leghemoglobin linked to constitutive promoter pastoris.	55

Non-Patent Literature (NPL) Search Strategy/Results:

Google/Google Scholar: 25 August 2016

Search String	Results
methylotrophic yeast cell exogenous nucleic acid encode transcriptional activator methanol-inducible promoter element	28
methylotrophic Pichia nucleic acid encoding Mxr1 transcriptional activator sequence from P pastoris	23
Pichia nucleic acid encoding leghemoglobin	
Pichia nucleic acid encoding polypeptide involved in heme biosynthesis	691

PubMed: 25 August 2016

Search String	Results
methylotrophic yeast cell exogenous nucleic acid encode transcriptional activator methanol-inducible promoter element	0
methylotrophic Pichia nucleic acid encoding Mxr1 transcriptional activator sequence from P pastoris	0
Pichia nucleic acid encoding leghemoglobin	0
Pichia nucleic acid encoding polypeptide involved in heme biosynthesis	1

EBSCO Discovery: 25 August 2016

Search String	Results
methylotrophic yeast cell exogenous nucleic acid encode transcriptional activator methanol-inducible promoter element	51
methylotrophic Pichia nucleic acid encoding Mxr1 transcriptional activator sequence from P pastoris	18
Pichia nucleic acid encoding leghemoglobin	118
Pichia nucleic acid encoding polypeptide involved in heme biosynthesis	1248

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[Continued on next page]

(54) Title: EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

(57) Abstract: Methods and materials for genetically engineering methylotrophic yeast are provided.

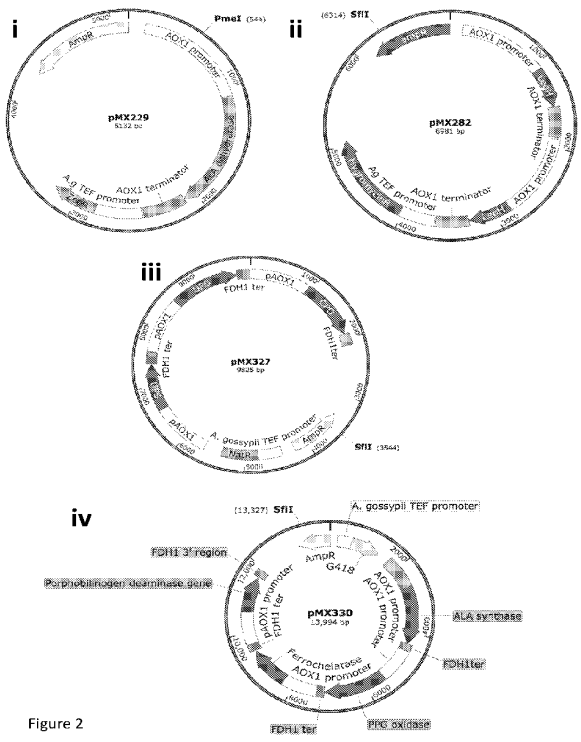


Figure 2

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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.

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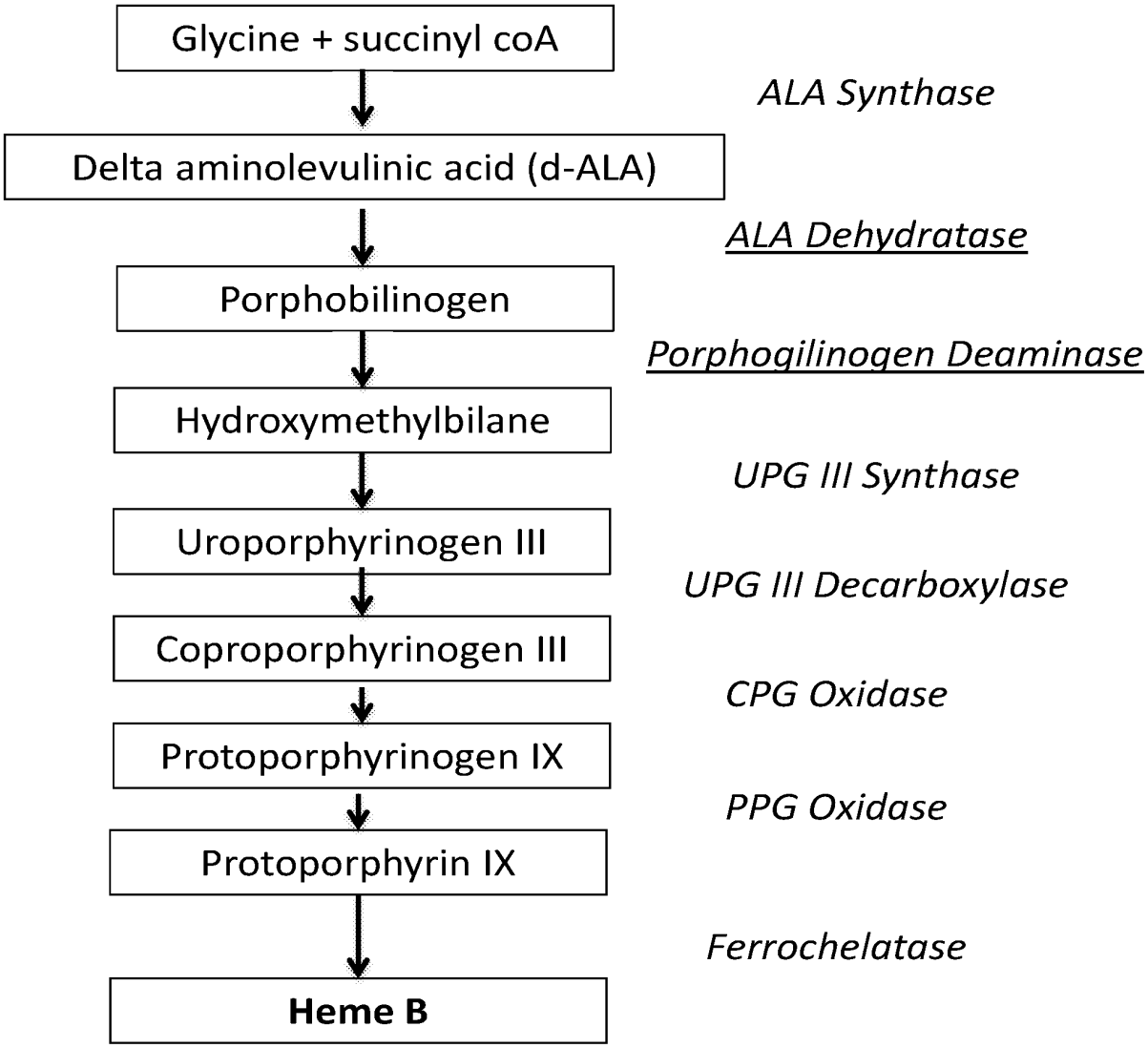

Glycine + succinyl coA
ALA Synthase
Delta aminolevulinic acid (d-ALA)
ALA Dehydratase
Porphobilinogen
Porphogilinogen Deaminase
Hydroxymethylbilane
UPG III Synthase
Uroporphyrinogen III
UPG III Decarboxylase
Coproporphyrinogen III
CPG Oxidase
Protoporphyrinogen IX
PPG Oxidase
Protoporphyrin IX
Ferrochelatase
Heme B

Figure 1

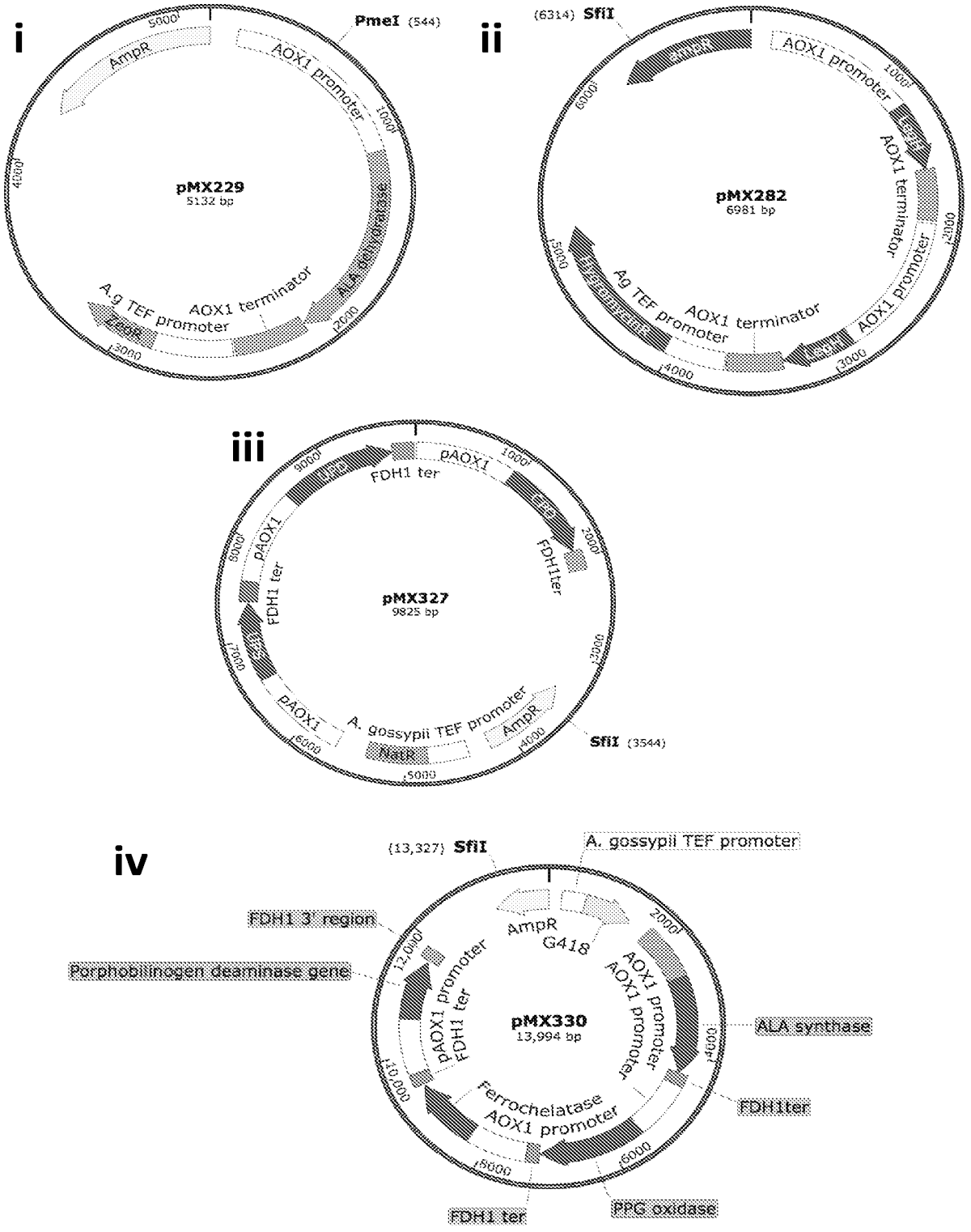

i
PmeI (544)
AmpR
AOX1 promoter
pMX229
5132 bp
ALA synthase
AOX1 terminator
A.g TEF promoter
NatR
ii
(6314) SfiI
AmpR
AOX1 promoter
pMX282
6981 bp
AOX1 terminator
A.g TEF promoter
NatR
AOX1 promoter
iii
pAOX1
FDH1 ter
AmpR
pMX327
9825 bp
FDH1ter
pAOX1
FDH1 ter
G418
pAOX1
A. gossypii TEF promoter
AmpR
NatR
SfiI (3544)
iv
(13,327) SfiI
A. gossypii TEF promoter
FDH1 3' region
AmpR
G418
Porphobilinogen deaminase gene
pAOX1 promoter
FDH1 ter
pMX330
13,994 bp
AOX1 promoter
ALA synthase
Ferrochelatase
AOX1 promoter
FDH1ter
FDH1 ter
PPG oxidase

Figure 2

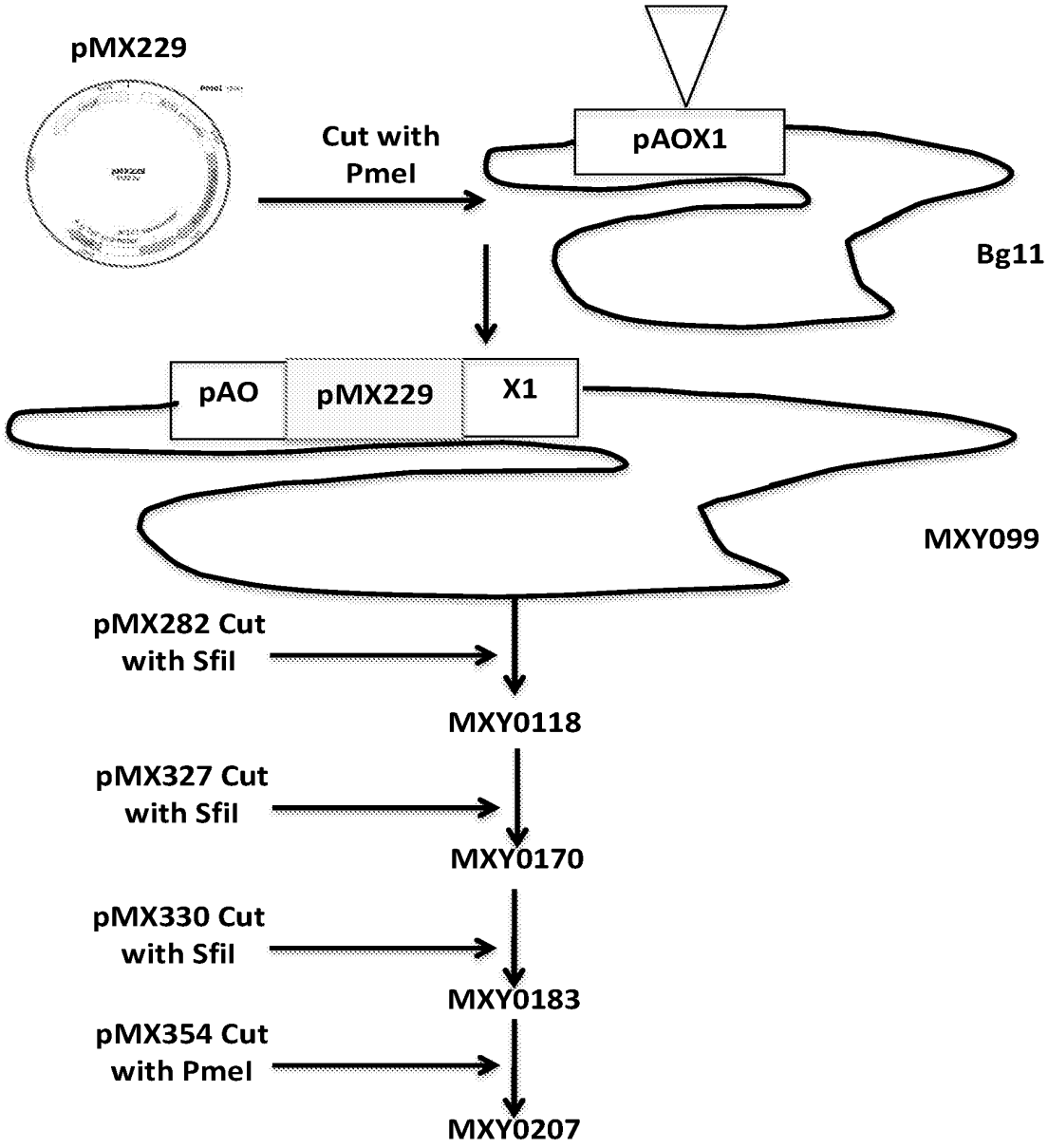

pMX229
Cut with
PmeI
pAOX1
Bg11
pAO
pMX229
X1
MXY099
pMX282 Cut
with SfiI
MXY0118
pMX327 Cut
with SfiI
MXY0170
pMX330 Cut
with SfiI
MXY0183
pMX354 Cut
with PmeI
MXY0207

Figure 3

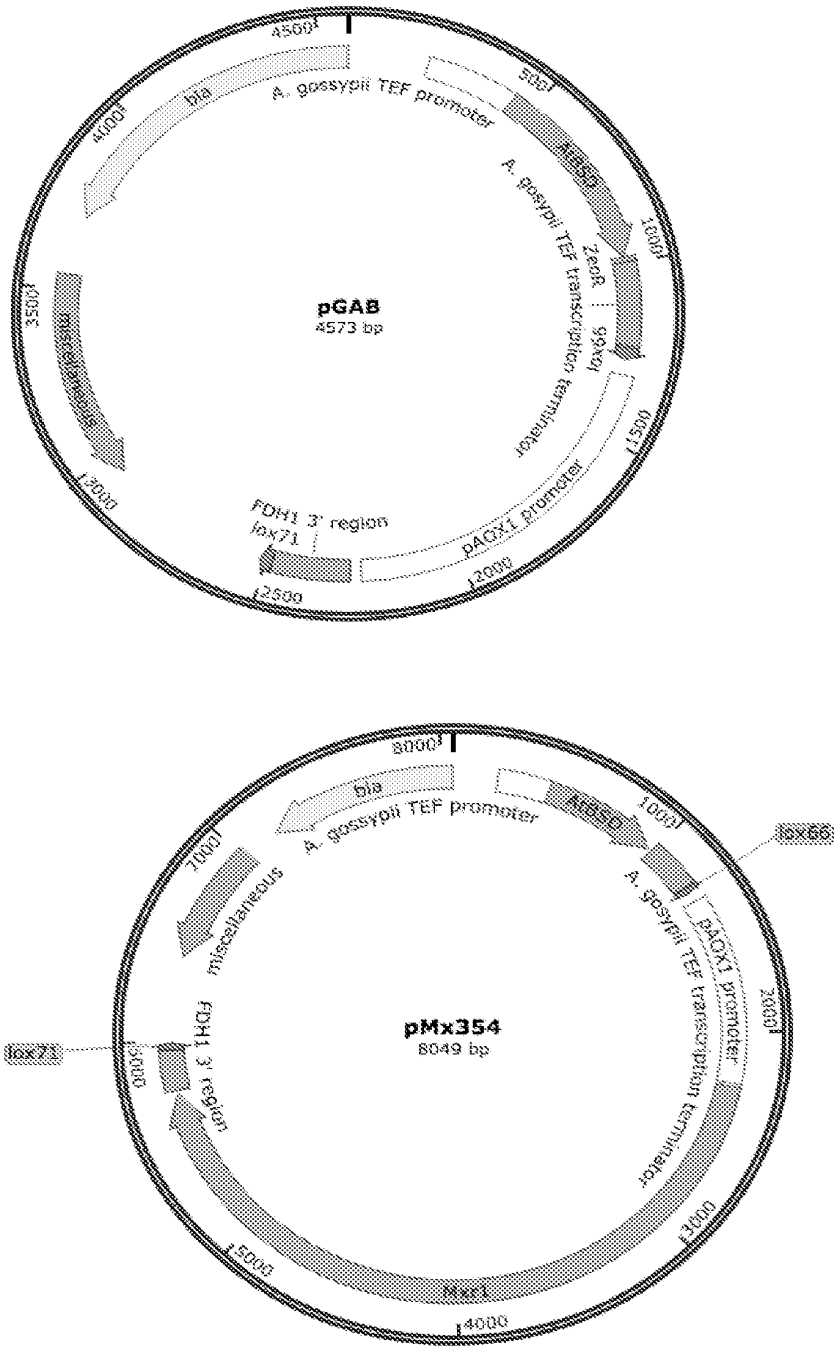

pGAB
4573 bp
bla
A. gossypii TEF promoter
AUR1
A. gossypii TEF transcription terminator
ZeoR
lox66
pAOX1 promoter
FDH1 3' region
lox71
pMx354
8049 bp
bla
A. gossypii TEF promoter
AUR1
miscellaneous
A. gossypii TEF transcription terminator
pAOX1 promoter
lox66
lox71
FDH1 3' region
Mxr1

Figure 4

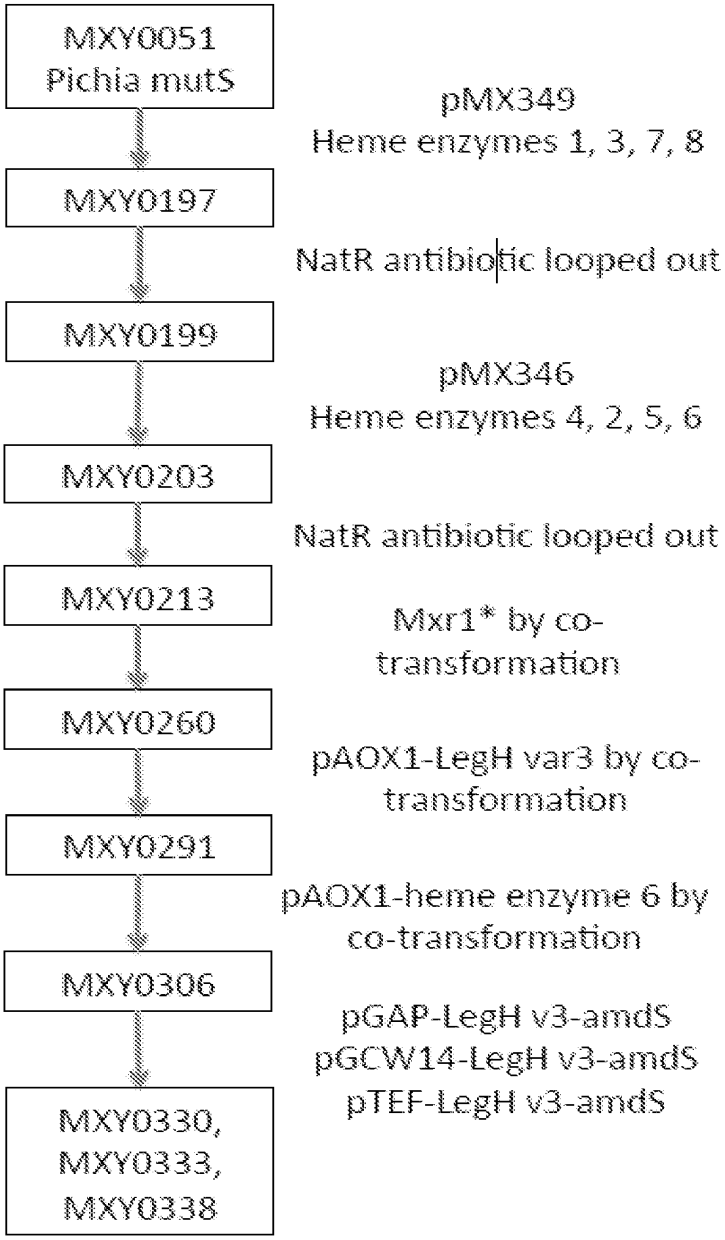

MXY0051
Pichia mutS
pMX349
Heme enzymes 1, 3, 7, 8
MXY0197
NatR antibiotic looped out
MXY0199
pMX346
Heme enzymes 4, 2, 5, 6
MXY0203
NatR antibiotic looped out
MXY0213
Mxr1* by co-transformation
MXY0260
pAOX1-LegH var3 by co-transformation
MXY0291
pAOX1-heme enzyme 6 by co-transformation
MXY0306
pGAP-LegH v3-amdS
pGCW14-LegH v3-amdS
pTEF-LegH v3-amdS
MXY0330,
MXY0333,
MXY0338

Figure 5

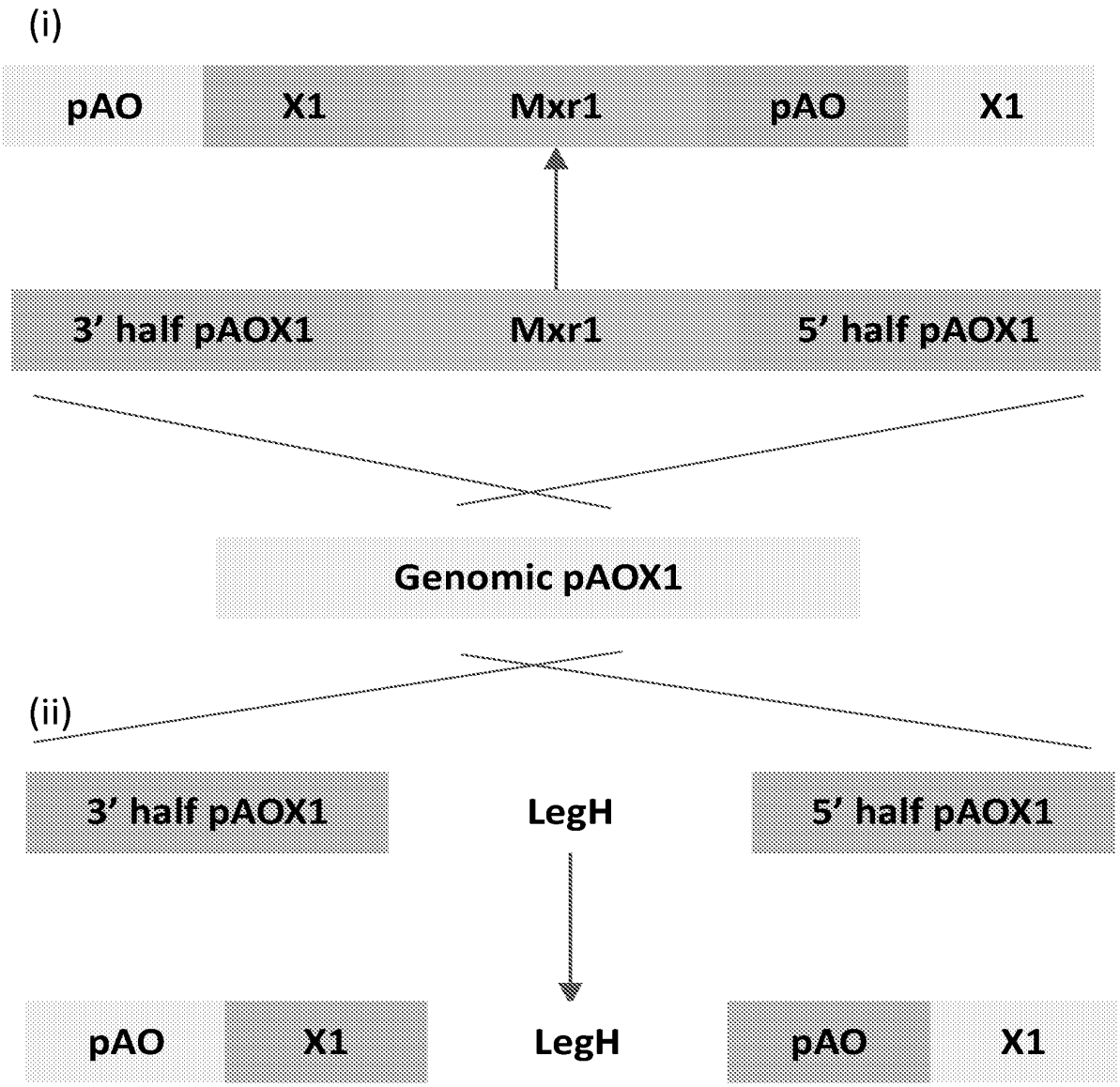

(i)
pAO
X1
Mxr1
pAO
X1
3' half pAOX1
Mxr1
5' half pAOX1
Genomic pAOX1
(ii)
3' half pAOX1
LegH
5' half pAOX1
pAO
X1
LegH
pAO
X1

Figure 6

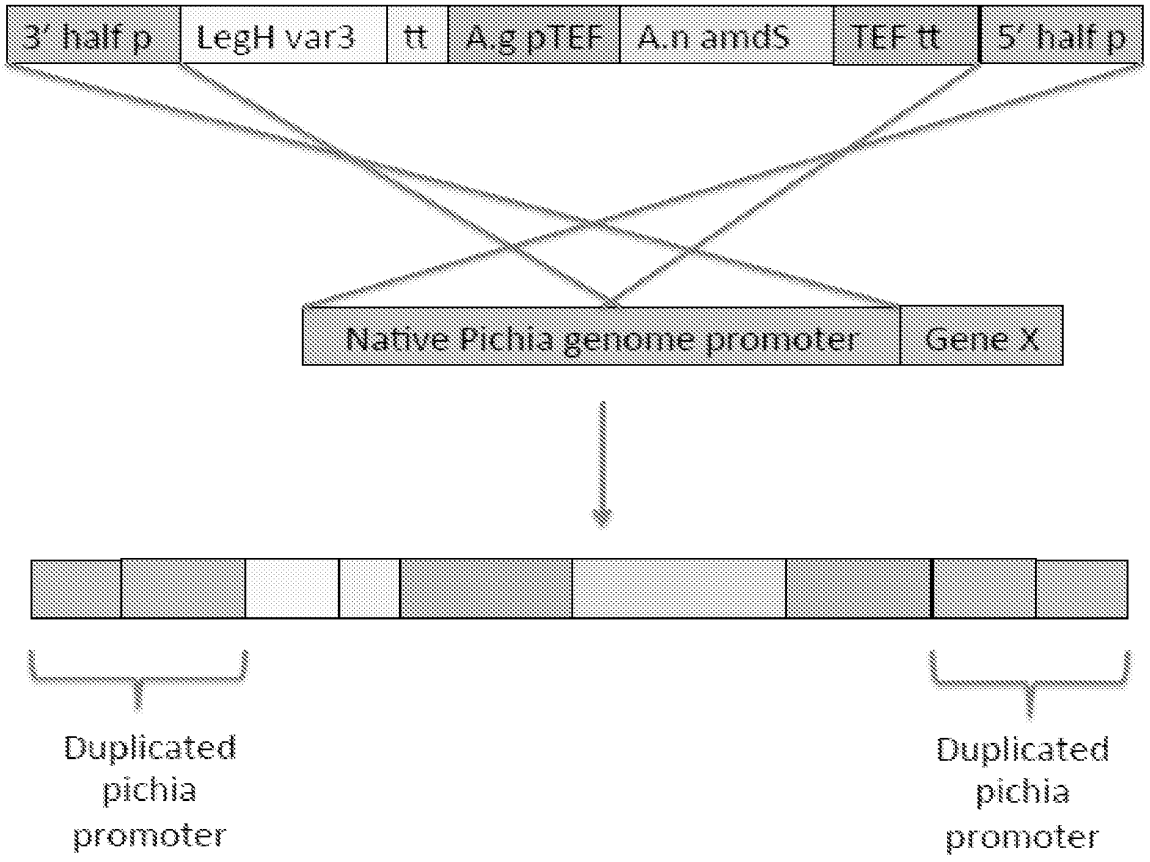

3' half p
LegH var3
tt
A.g pTEF
A.n amdS
TEF tt
5' half p
Native Pichia genome promoter
Gene X
Duplicated pichia promoter
Duplicated pichia promoter
Native Pichia promoter = pGAP, pTEF or pGCW14

Figure 7

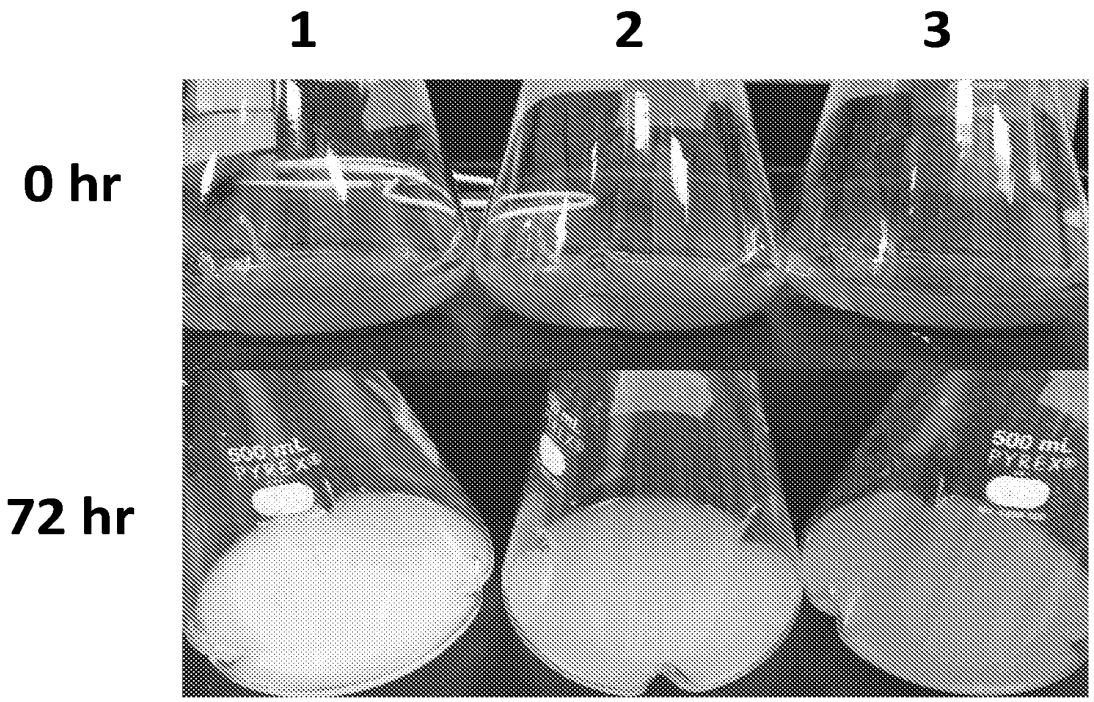
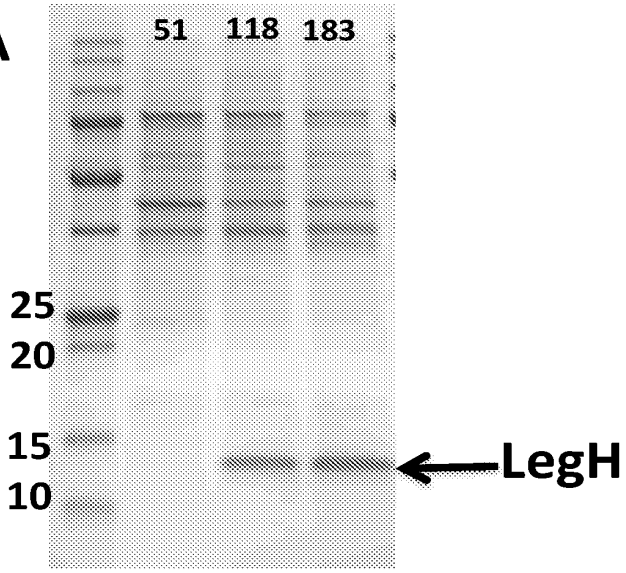


Figure 8

A

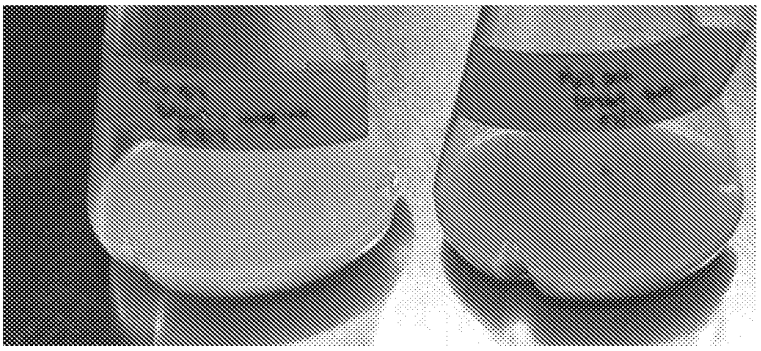


B

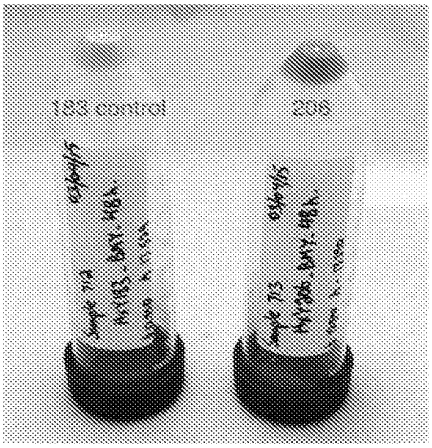
Strain	Relative improvement in yield (vs. MXY0118)	Heme-bound fraction of LegH
MXY0118	1	Sub-optimal
MXY0183	1.6	100%
MXY0207	3	100%

Figure 9

A



B



C

Strain	Relative yield of heme loaded LegH
183- BMGY	N/A
183- BMY	1
206- BMGY	3
206- BMY	5

Figure 10

MXY Strain	Yield on glycerol co-feed	Yield on dextrose co-feed
183	1X	1X
207	4.4X	4.4X
291	5.8X	5.8X
306	5.8X	5.8X
330	6.9X	6.9X
333	5.8X	5.8X
338	6.9X	6.9X

Figure 11

785221

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DocCode – APPENDIX

SCORE Placeholder Sheet for IFW Content

Application Number: 62203052 Document Date: 08/10/2015

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- Appendix

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Form Revision Date: August 26, 2013

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. _____

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Smita Martin Andrew	Shankar Hoyt	Millbrae, CA San Francisco, CA

Additional inventors are being named on the _____ *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max):

EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

☒ The address corresponding to Customer Number: 26191

OR

☐ Firm or Individual Name

Address

City	State	Zip
Country	Telephone	Email

ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Application Data Sheet. See 37 CFR 1.76

☐ CD(s), Number of CDs _____

☒ Drawing(s) *Number of Sheets* 10

☒ Other (specify) Appendix I & II

☒ Specification (e.g. description of the invention) *Number of Pages* 40

Fees Due: Filing Fee of $260 ($130 for small entity) ($65 for micro entity). If the specification and drawings exceed 100 sheets of paper, an application size fee is also due, which is $400 ($200 for small entity) ($100 for micro entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

METHOD OF PAYMENT OF THE FILING FEE AND APPLICATION SIZE FEE FOR THIS PROVISIONAL APPLICATION FOR PATENT

☐ Applicant claims small entity status. See 37 CFR 1.27.

☐ Applicant certifies micro entity status. See 37 CFR 1.29.
Applicant must attach form PTO/SB/15A or B or equivalent.

$260

TOTAL FEE AMOUNT ($)

☐ A check or money order made payable to the *Director of the United States Patent and Trademark Office* is enclosed to cover the filing fee and application size fee (if applicable).

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No

☐ Yes, the invention was made by an agency of the U.S. Government. The U.S. Government agency name is: _____

☐ Yes, the invention was made under a contract with an agency of the U.S. Government. The name of the U.S. Government agency and Government contract number are: _____

SIGNATURE /M. Angela Parsons/ Date /August 10, 2015/

TYPED or PRINTED NAME M. Angela Parsons, Ph.D. REGISTRATION NO. 44,282 *(if appropriate)*

TELEPHONE (612) 335-5070 Docket Number: 38767-0048P04

61198180.doc

Electronic Acknowledgement Receipt	
EFS ID:	23161776
Application Number:	62203052
International Application Number:	
Confirmation Number:	1010
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	M. Angela Parsons/Stacey Hill
Filer Authorized By:	M. Angela Parsons
Attorney Docket Number:	
Receipt Date:	10-AUG-2015
Filing Date:	
Time Stamp:	14:50:21
Application Type:	Provisional

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$260
RAM confirmation Number	1184
Deposit Account	061050
Authorized User	FISH & RICHARDSON P C
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

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	1561076 354f845bb8765c134cd92f95c14994396554f019	no	7
Warnings:					
Information:					
2		Application.pdf	234064 71c2414b058414eb9345a91e700c22be66f648d5	yes	40

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Specification	1	36
	Claims	37	39
	Abstract	40	40

Warnings:					
Information:					
3	Drawings-other than black and white line drawings	Figures.pdf	1835015 574816e724c3cf16261f962bf2fb1e79e4258a97	no	10
Warnings:					
Information:					
4	Appendix to the Specification	AppendixI.pdf	55361 f3fbc356ed1cc7ac140cd08007d92c4e2e6949fc	no	4
Warnings:					
Information:					
5	Appendix to the Specification	AppendixII.pdf	944785 e80203888e1ebf37d929c79e15cd43ef1b10d462	no	9
Warnings:					
Information:					
6	Power to Make Copies and/or Inspect	Transmittal.pdf	109359 464e4c418cf8d6393cec3e5dd53fa5c6ab711da0	no	1

Warnings:					
Information:					
7	Fee Worksheet (SB06)	fee-info.pdf	29913 01bd7d9945dfd2ad25798dcc48cb6c08d65d7acc	no	2
Warnings:					
Information:					
		Total Files Size (in bytes):	4769573		

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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.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	38767-0048P04
	Application Number	
Title of Invention	Expression Constructs and Methods of Genetically Engineering Methylotrophic Yeast	

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
	Smita		Shankar	

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

City	Millbrae	State/Province	CA	Country of Residence i	US

Mailing Address of Inventor:

Address 1	34 Broadway, Apt. 9		
Address 2			
City	Millbrae	State/Province	CA
Postal Code	94030	Country i	

Inventor 2 Remove

Legal Name

Prefix	Given Name	Middle Name	Family Name	Suffix
	Martin	Andrew	Hoyt	

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

City	San Francisco	State/Province	CA	Country of Residence i	US

Mailing Address of Inventor:

Address 1	925 Irving Street, Apt 106		
Address 2			
City	San Francisco	State/Province	CA
Postal Code	94122	Country i	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).

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	38767-0048P04
	Application Number	
Title of Invention	Expression Constructs and Methods of Genetically Engineering Methylotrophic Yeast	

☐ **An Address is being provided for the correspondence Information of this application.**			
Customer Number	26191		
Email Address	apsi@fr.com	Add Email	Remove Email

Application Information:

Title of the Invention	Expression Constructs and Methods of Genetically Engineering Methylotrophic Yeast		
Attorney Docket Number	38767-0048P04	**Small Entity Status Claimed**	☐
Application Type	Provisional		
Subject Matter	Utility		
Total Number of Drawing Sheets (if any)	10	**Suggested Figure for Publication (if any)**	

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 i

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	26191		

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	38767-0048P04
	Application Number	
Title of Invention	Expression Constructs and Methods of Genetically Engineering Methylotrophic Yeast	

Domestic Benefit/National Stage Information:

This section allows for the applicant to either claim benefit under 35 U.S.C. 119(e), 120, 121, or 365(c) or indicate National Stage entry from a PCT application. Providing this 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 blank.

Prior Application Status			Remove
Application Number	Continuity Type	Prior Application Number	Filing 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(d). 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(h)(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	38767-0048P04
	Application Number	
Title of Invention	Expression Constructs and Methods of Genetically Engineering Methylotrophic Yeast	

Authorization to Permit Access:

☐ Authorization to Permit Access to the Instant Application by the Participating Offices

If 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 World Intellectual Property Office (WIPO), and any other intellectual property offices in which a foreign application claiming priority to the instant patent application is filed access to the instant patent application. See 37 CFR 1.14(c) and (h). This box should not be checked if the applicant does not wish the EPO, JPO, KIPO, WIPO, or other intellectual property office in which a foreign application claiming priority to the instant patent application is filed to have access to the instant patent application.

In accordance with 37 CFR 1.14(h)(3), access will be provided to a copy of the instant patent application with respect to: 1) the instant patent application-as-filed; 2) any foreign application to which the instant patent application claims priority under 35 U.S.C. 119(a)-(d) if a copy of the foreign application that satisfies the certified copy requirement of 37 CFR 1.55 has been filed in the instant patent application; and 3) any U.S. application-as-filed from which benefit is sought in the instant patent application.

In accordance with 37 CFR 1.14(c), access may be provided to information concerning the date of filing this Authorization.

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	Impossible Foods Inc.

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	38767-0048P04
	Application Number	
Title of Invention	Expression Constructs and Methods of Genetically Engineering Methylotrophic Yeast	

Mailing Address Information:			
Address 1	525 Chesapeake Drive		
Address 2			
City	Redwood City	**State/Province**	CA
Country i	US	Postal Code	94063
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:

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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. ☐

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. Add

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Application Data Sheet 37 CFR 1.76	Attorney Docket Number	38767-0048P04
	Application Number	
Title of Invention	Expression Constructs and Methods of Genetically Engineering Methylotrophic Yeast	

Signature:

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NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications

Signature	/M. Angela Parsons/			Date (YYYY-MM-DD)	2015-08-10
First Name	M. Angela	Last Name	Parsons	Registration Number	44282

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

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.**

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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.

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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.

EFS Web 2.2.11

F&R Ref No.: 38767-0048P04

EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

TECHNICAL FIELD

This disclosure generally relates to DNA constructs and methods of using such DNA constructs to genetically engineer methylotrophic yeast.

BACKGROUND

Methylotrophic yeast such as *Pichia pastoris* are commonly used for expression of recombinant proteins. Constructs that can be used to efficiently express one or more polypeptides in a methylotrophic yeast are provided herein.

SUMMARY

This disclosure describes the use of *P. pastoris* strains overexpressing Mxr1 from the AOX1 promoter to increase expression of transgenes that also are expressed from the AOX1 promoter, which significantly improves the recombinant production of one or more proteins. In addition, expression of Mxr1 from the AOX1 promoter creates a positive feedback loop that allows for expression of other transgenes from the AOX1 promoter in the absence of methanol, the normally obligate inducer, when repressing carbon sources are depleted. Expression of Mxr1 in the strain resulted in an increase in the amount of protein produced.

In one aspect, a methylotrophic yeast cell is provided that includes a recombinant nucleic acid molecule. The recombinant nucleic acid molecule typically includes an exogenous nucleic acid encoding a transcriptional activator operably linked to at least one methanol-inducible promoter element. Representative methylotrophic yeast can be of the genus *Candida*, *Hansenula*, *Pichia* or Toruplosis. A representative methylotrophic yeast is *Pichia pastoris*.

In some embodiments, the recombinant nucleic acid molecule is stably integrated into the genome of the methylotrophic yeast cell. In some embodiments, the recombinant nucleic acid molecule is extrachromosomally expressed from a replication-competent plasmid.

In some embodiments, the exogenous nucleic acid encoding a transcriptional activator comprises a Mxr1 sequence from *Pichia pastoris*, a Adr1 sequence from *Hansenula polymorpha*,

a Trm1 sequence from *Candida boidinii*, and a Trm2 sequence from *Candida boidinii*. A representative nucleic acid encoding a transcriptional activator is shown in DQ395124. A representative transcriptional activator has an amino acid sequence shown in ABD57365.

In some embodiments, the at least one methanol-inducible promoter element is an alcohol oxidase 1 (AOX1) promoter element from *Pichia pastoris*, an AOD1 promoter element from *Candida boidinii*, a MOX promoter element from *Hansenula polymorpha*, a MOD1 promoter element from *Pichia mehanolica*, a DHAS promoter element from *Pichia pastoris*, or a PEX8 promoter element from *Pichia pastoris*.

In some embodiments, the methylotrophic yeast cell further includes a nucleic acid molecule that includes at least one heterologous nucleic acid encoding a polypeptide operably linked to at least one methanol-inducible promoter element. In some embodiments, the heterologous nucleic acid encodes a polypeptide involved in the biosynthesis of an iron co-factor (e.g., ALA synthase, ALA dehydratase, porphogilinogen deaminase, UPG III synthase, UPG III decarboxylase, CPG oxidase, PPG oxidase, and ferrochelatase).

In another aspect, a method for expressing a heterologous polypeptide in a cell is provided. Such a method typically includes providing a methylotrophic yeast cell as described herein; introducing a recombinant nucleic acid molecule into methylotrophic yeast cell, the recombinant nucleic acid molecule comprising at least one heterologous nucleic acid encoding a polypeptide operably linked to at least one *Pichia pastoris* alcohol oxidase 1 (AOX1) promoter element; and culturing the cell under conditions suitable for expression of the recombinant nucleic acid molecule, thereby expressing the heterologous polypeptide.

In some embodiments, the conditions under which the cells are cultured includes the addition of iron or a pharmaceutically or metabolically acceptable salt thereof. In some embodiments, the introducing step includes a technique such as transduction, electroporation, biolistic particle delivery, or chemical transformation. In some embodiments, the culturing step includes culturing the cell in the present of methanol.

In another aspect, a recombinant organism is provided that includes a transcriptional activator operably linked to the promoter it activates. In some embodiments, a recombinant organism is provided that expresses a polypeptide operably linked to the promoter. In yet another aspect, a method of expressing a polypeptide from an inducible promoter without addition of an inducer is provided as described herein.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the methods and compositions of matter belong. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the methods and compositions of matter, suitable methods and materials are described below. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

DESCRIPTION OF DRAWINGS

Figure 1 is a schematic depicting the steps involved in the heme biosynthesis pathway.

Figure 2 is a flow chart showing the strain lineage of the production strain, MXY0183.

Figure 3 are schematics of the plasmids used in the construction of production strain, MXY0183.

Figure 4 is a schematic showing the generation of the production strain, MXY0183, from the parent, Bg11 strain.

Figure 5 are schematics showing plasmids pGAB and pMx354.

Figure 6 is a photograph showing the phenotypic changes associated with strain, MXY0183. Shake flasks at the start of induction (0 hr) and 72 hr post-induction are shown. 1, MXY0051; 2, MXY0118; 3, MXY0183.

Figure 7 shows the production of LegH from the modified *P. pastoris* strains. Panel A) is a SDS gel showing lysates from *P. pastoris* strains grown in shake flasks: 51, MXY0051; 118, MXY0118; 183, MXY0183. Panel B) is a table comparing LegH production from strain MXY0183 and from strain MXY0118.

Figure 8 is a gel from colony PCR showing the presence of LegH and the heme biosynthetic enzymes in MXY0183 after > 200 generations of growth on non-selective media: 1, MXY0183 fresh strain; 2, Bg11 host strain; 3, MXY0183 passaged through non-selective media; 4, MXY0183 grown in a 1000 L fermenter for > 120 hours in non-selective induction media.

Figure 9 show data from the experiments described herein. Panel A) is a photograph of shake flask cultures of strains MXY0183 and MXY0206 after 48 hr of growth in repressing

carbon source. Panel B) is a photograph of cell pellets from shake flask cultures of strains MXY0183 and MXY0206 after 48 hr of growth in BMY media. Panel C) is a graph showing the relative yield of heme-loaded LegH in the absence of induction agent.

Figure 10 is a graph showing the relative yield of LegH and heme loading from select strains (MXY0121; MXY0118; MXY0183; MXY0207) grown in 2 L fermenter tanks.

DETAILED DESCRIPTION

Nucleic acid constructs are provided herein that allow for genetically engineering a cell to increase the recombinant expression of a polypeptide. In some embodiments, nucleic acid constructs are provided herein that allow for genetically engineering a cell to increase the recombinant expression of a polypeptide from an inducible promoter in the absence of the inducing molecule. Without being bound by any particular mechanism, the methods described herein create a positive feedback loop where the low level native expression of a transcriptional activator induces a promoter that is operably linked to a transcriptional activator. This leads to an increased expression of the transcriptional activator as well as one or more target polypeptides that are operably linked to the same inducible promoter.

Nucleic acid constructs are provided herein that allow for genetically engineering a methylotrophic yeast cell. While the methods are exemplified herein using a *Pichia* species (i.e., *P. pastoris*), other species of the *Pichia* genus can be used or species from any of the *Candida*, *Hansenula*, *Pichia* and *Toruplosis* genera.

Genetically engineering a methylotrophic yeast cell typically includes introducing a recombinant nucleic acid molecule into the cell. As described herein, a recombinant nucleic acid molecule as described herein typically includes an exogenous nucleic acid that encodes a transcriptional activator operably linked to at least one methanol-inducible promoter element.

Recombinant nucleic acid molecules used in the methods described herein are typically DNA, but RNA molecules can be used under the appropriate circumstances. As used herein, "exogenous" refers to any nucleic acid sequence that is introduced into the genome of a cell from an external source, where the external source can be the same or a different organism or a nucleic acid generated synthetically. For example, an exogenous nucleic acid can be a nucleic acid from one microorganism (e.g., one genus or species of methylotrophic yeast) that is introduced into a different genus or species of methylotrophic yeast, however, an exogenous nucleic acid also can

be a nucleic acid from a methyltrophic yeast that is introduced recombinantly into a methylotrophic yeast as an additional copy despite the presence of a corresponding native nucleic acid sequence. For example, *P. pastoris* contains an endogenous nucleic acid encoding a Mxr1 transcriptional activator; an additional *P. pastoris* Mxr1 nucleic acid (e.g., introduced recombinantly into P. pastoris) or modifying the endogenous *P. pastoris* Mxr1 nucleic acid is considered exogenous.

Transcriptional activators, and nucleic acids encoding transcriptional activators (e.g., exogenous nucleic acids encoding transcriptional activators), are known in the art. For example, a transcriptional activator from *Pichia pastoris* is the Mxr1 sequence, but suitable transcriptional activators also can be found in *Hansenula polymorpha* (the Adr1 sequence; see, for example, GenBank Accession No. AEOI02000005, bases 858873 to 862352, for the nucleic acid sequence and GenBank Accession No. ESX01253 for the amino acid sequence) and *Candida boidinii* (the Trm1 sequence; see, for example, GenBank Accession No. AB365355 for the nucleic acid sequence and GenBank Accession No. BAF99700 for the amino acid sequence; the Trm2 sequence; see, for example, GenBank Accession No. AB548760 for the nucleic acid sequence and GenBank Accession No. BAJ07608 for the amino acid sequence). A representative *P. pastoris* Mxr1 nucleic acid sequence can be found, for example, in GenBank Accession No. DQ395124, while a representative *P. pastoris* Mxr1 polypeptide sequence can be found, for example, in GenBank Accession No. ABD57365.

Transcriptional activators such as Mxr1 may be normally expressed at low levels. Therefore, it is desirable to place the transcriptional activator under control of a promoter that is constitutively expressed or inducible. It would be appreciated by a skilled artisan that a promoter is operably linked to the exogenous nucleic acid encoding the transcriptional activator. As used herein, "operably linked" means that a promoter or other expression element(s) are positioned relative to a nucleic acid in such a way as to direct or regulate expression of the nucleic acid (e.g., in-frame).

For genetically engineering methylotrophic yeast, a methanol-inducible promoter, or a promoter element therefrom, is useful. Methanol inducible promoters are known in the art. For example, a commonly used methanol-inducible promoter from *P. pastoris* is the promoter, or a portion thereof, from the alcohol oxidase 1 (AOX1) gene, which is strongly transcribed in response to methanol. Other methanol-inducible promoters, or promoter elements therefrom,

however, can be used, including, without limitation, the alcohol oxidase (AOD1) promoter from *Candida boidinii* (see, for example, GenBank Accession No. YSAAOD1A) or a promoter element therefrom, the alcohol oxidase (MOX) promoter from *Hansenula polymorpha* (see, for example, GenBank Accession No. X02425) or a promoter element therefrom, the MOD1 or MOD2 promoter from *Pichia mehanolica* (see, for example, Raymond et al., 1998, Yeast, 14:11-23; and Nakagawa et al., 1999, Yeast, 15:1223-30) or a promoter element therefrom, the DHAS promoter from *Pichia pastoris* (see, for example, GenBank Accession No. FJ752551) or a promoter element therefrom, or the PEX8 promoter from *Pichia pastoris* (see, for example, Kranthi et al., 2010, Yeast, 27:705-11) or a promoter element therefrom. All of these promoters are known to be induced by methanol.

A skilled artisan would understand that the recombinant nucleic acid molecule described herein can be stably integrated into the genome of the methylotrophic yeast cell, or can be extrachromosomally expressed from a replication-competent plasmid. Methods of achieving both are well known and routinely used in the art.

A strain that includes a recombinant nucleic acid molecule as described herein can be used to regulate (e.g., overexpress) a second recombinant nucleic acid in a cell (i.e., in a methylotrophic yeast cell). A second recombinant nucleic acid can include, for example, one or more heterologous polypeptides encoding one or more polypeptides of interest. Similar to the exogenous nucleic acid encoding the transcriptional activator, the heterologous nucleic acid encoding a polypeptide of interest can be operably linked to a methanol-inducible promoter element. Also similar to the exogenous nucleic acid encoding the transcriptional activator, a heterologous nucleic acid refers to any nucleic acid sequence that is not native to the genome or in the genome of an organism (e.g., a heterologous nucleic acid can be a nucleic acid from one microorganism (e.g., one genus or species of methylotrophic yeast) that is introduced into a different genus or species of methylotrophic yeast).

Simply by way of example, heterologous nucleic acids encoding the one or more polypeptides of interest can be the nucleic acids involved in the biosynthesis of a heme-co-factor. Exemplified herein are nucleic acids encoding the 8 different enzymes involved in heme biosynthesis as determined and annotated from the sequence of the *Pichia pastoris* genome. For example, heterologous nucleic acids encoding ALA synthase, ALA dehydratase, porphogilinogen

deaminase, UPG III synthase, UPG III decarboxylase, CPG oxidase, PPG oxidase, and ferrochelatase can be expressed in the methylotrophic yeast strains described herein.

In addition to the enzymes involved in iron-co-factor biosynthesis, it would be understood that a nucleic acid encoding a plant hemoglobin can be present. In the present case, a nucleic acid encoding soybean leghemoglobin (LegH) is present. LegH is a protein that binds to the iron co-factor, heme, which results in a characteristic absorption at 415 nm and a distinct red color. The protein is naturally found in root nodules of soybean, and the nucleic acid sequence used herein was codon optimized for expression in *P. pastoris*. See, for example, WO 2014/110539 and WO 2014/110532.

Alternatively, a heterologous nucleic acid encoding a polypeptide of interest can be, for example and without limitation, a dehydrin, a phytase, a protease, a catalase, a lipase, a peroxidase, an amylase, a transglutaminase, an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, a ligase, or an antibody against any such polypeptides. In other embodiments, a heterologous nucleic acid can encode one or more enzymes involved in the pathway for production of small molecules, such as ethanol, lactic acid, butanol, adipic acid, or succinic acid.

Similar to the recombinant nucleic acid molecule described herein, the second recombinant nucleic acid molecule can be stably integrated into the genome of the methylotrophic yeast cell, or can be extrachromosomally expressed from a replication-competent plasmid. It would be appreciated by a skilled artisan that a heterologous nucleic acid encoding a polypeptide of interest operably linked to a methanol-inducible promoter element can be separate from the recombinant nucleic acid molecule described herein, or can be contiguous with the exogenous nucleic acid encoding a transcriptional activator operably linked to a methanol-inducible promoter element contained within the recombinant nucleic acid molecule described herein. It also would be appreciated by a skilled artisan that, if the second nucleic acid molecule is contiguous with the recombinant nucleic acid molecule described herein, that a single methanol-inducible promoter, or promoter element therefrom, can be used to drive transcription of both or all of the genes (e.g., the exogenous nucleic acid encoding the transcriptional activator as well as the one or more heterologous nucleic acids encoding the polypeptide(s) of interest).

Methods of introducing nucleic acids into methylotrophic yeast cells are known in the art, and include, without limitation, transduction, electroporation, biolistic particle delivery, and chemical transformation.

In addition, methods of culturing methylotrophic yeast cells are known in the art. See, for example, Pichia Protocols, *Methods In Molecular Biology*, 389, Cregg, Ed., 2007, 2nd Ed., Humana Press, Inc. Under some circumstances, it may be desirable to introduce or add methanol to the culture media, although, as demonstrated herein, methanol is not required to obtain efficient expression at high levels of one or more polypeptides of interest. Under some circumstances (e.g., when one or more nucleic acids encoding enzyme(s) involved in an iron-co-factor biosynthesis are expressed), it may be desirable to supplement the culture media with iron or a pharmaceutically or metabolically acceptable (or GRAS) salt thereof.

Pichia strains are able to grow on methanol as the sole carbon source. Methanol utilization is initiated by the conversion of methanol to formaldehyde by the action of alcohol oxidase. The methylotrophic yeast, *Pichia pastoris*, contains two genes for alcohol oxidases, AOX1 and AOX2. Strains with reduced alcohol oxidase activity ("methanol utilization slow" or MutS strains) often produce more of a recombinant protein expressed from the AOX1 promoter than strains that do not have reduced alcohol oxidase activity. Strains mutated in both AOX genes and completely lacking alcohol oxidase activity cannot metabolize methanol, but can still be induced for expression from the AOX1 promoter by methanol. These strains retain the ability to use other carbon sources for growth, but still express heterologous proteins from the AOX1 promoter upon the addition of methanol. Because these strains do not metabolize methanol ("methanol utilization minus" or Mut- strains), much less methanol is required for induction of protein expression, and strains carrying these mutations avoid issues related to methanol feeding in large-scale fermentations. See, for example, Chiruvolu et al., 1997, Enzyme Microb. Technol., 21:277-83. It was determined herein that expression of LegH from the AOX1 promoter in Mut- strains greatly improved the LegH yield. Thus, a methylotrophic yeast having a mutation in both the AOX1 gene and the AOX2 gene can be used in the methods described herein.

The protein of interest, or a complex that includes one or more proteins of interest (e.g., heme-bound LegH, a dehydrin, a phytase, a protease a catalase, a lipase, a peroxidase, an amylase, a transglutaminase, an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, a ligase, or an antibody) can be purified from the yeast cells. Methods of purifying polypeptides are known in the art. As used herein, a "purified" polypeptide is a polypeptide that has been separated or purified from cellular components that naturally accompany it. Typically, the polypeptide is considered "purified" when it is at least 70% (e.g., at least 75%, 80%, 85%, 90%,

95%, or 99%) by dry weight, free from the polypeptides and naturally occurring molecules with which it is naturally associated. Since a polypeptide that is chemically synthesized is, by nature, separated from the components that naturally accompany it, a synthetic polypeptide is "purified."

As used herein, nucleic acids can include DNA and RNA, and includes nucleic acids that contain one or more nucleotide analogs or backbone modifications. A nucleic acid can be single stranded or double stranded, which usually depends upon its intended use. Also provided are nucleic acids and polypeptides that differ from a given sequence. Nucleic acids and polypeptides can have at least 50% sequence identity (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity) to a given nucleic acid or polypeptide sequence.

In calculating percent sequence identity, two sequences are aligned and the number of identical matches of nucleotides or amino acid residues between the two sequences is determined. The number of identical matches is divided by the length of the aligned region (i.e., the number of aligned nucleotides or amino acid residues) and multiplied by 100 to arrive at a percent sequence identity value. It will be appreciated that the length of the aligned region can be a portion of one or both sequences up to the full-length size of the shortest sequence. It also will be appreciated that a single sequence can align with more than one other sequence and hence, can have different percent sequence identity values over each aligned region.

The alignment of two or more sequences to determine percent sequence identity can be performed using the computer program ClustalW and default parameters, which allows alignments of nucleic acid or polypeptide sequences to be carried out across their entire length (global alignment). Chenna et al., 2003, Nucleic Acids Res., 31(13):3497-500. ClustalW calculates the best match between a query and one or more subject sequences, and aligns them so that identities, similarities and differences can be determined. Gaps of one or more residues can be inserted into a query sequence, a subject sequence, or both, to maximize sequence alignments. For fast pairwise alignment of nucleic acid sequences, the default parameters can be used (i.e., word size: 2; window size: 4; scoring method: percentage; number of top diagonals: 4; and gap penalty: 5); for an alignment of multiple nucleic acid sequences, the following parameters can be used: gap opening penalty: 10.0; gap extension penalty: 5.0; and weight transitions: yes. For fast pairwise alignment of polypeptide sequences, the following parameters can be used: word size: 1; window size: 5; scoring method: percentage; number of top diagonals: 5; and gap penalty: 3. For

multiple alignment of polypeptide sequences, the following parameters can be used: weight matrix: blosum; gap opening penalty: 10.0; gap extension penalty: 0.05; hydrophilic gaps: on; hydrophilic residues: Gly, Pro, Ser, Asn, Asp, Gln, Glu, Arg, and Lys; and residue-specific gap penalties: on. ClustalW can be run, for example, at the Baylor College of Medicine Search Launcher website or at the European Bioinformatics Institute website on the World Wide Web.

Changes can be introduced into a nucleic acid molecule, thereby leading to changes in the amino acid sequence of the encoded polypeptide. For example, changes can be introduced into nucleic acid coding sequences using mutagenesis (e.g., site-directed mutagenesis, PCR-mediated mutagenesis) or by chemically synthesizing a nucleic acid molecule having such changes. Such nucleic acid changes can lead to conservative and/or non-conservative amino acid substitutions at one or more amino acid residues. A "conservative amino acid substitution" is one in which one amino acid residue is replaced with a different amino acid residue having a similar side chain (see, for example, Dayhoff et al. (1978, in Atlas of Protein Sequence and Structure, 5(Suppl. 3):345-352), which provides frequency tables for amino acid substitutions), and a non-conservative substitution is one in which an amino acid residue is replaced with an amino acid residue that does not have a similar side chain.

As used herein, an "isolated" nucleic acid molecule is a nucleic acid molecule that is free of sequences that naturally flank one or both ends of the nucleic acid in the genome of the organism from which the isolated nucleic acid molecule is derived (e.g., a cDNA or genomic DNA fragment produced by PCR or restriction endonuclease digestion). Such an isolated nucleic acid molecule is generally introduced into a vector (e.g., a cloning vector, or an expression vector) for convenience of manipulation or to generate a fusion nucleic acid molecule, discussed in more detail below. In addition, an isolated nucleic acid molecule can include an engineered nucleic acid molecule such as a recombinant or a synthetic nucleic acid molecule.

Nucleic acids can be isolated using techniques routine in the art. For example, nucleic acids can be isolated using any method including, without limitation, recombinant nucleic acid technology, and/or the polymerase chain reaction (PCR). General PCR techniques are described, for example in PCR Primer: A Laboratory Manual, Dieffenbach & Dveksler, Eds., Cold Spring Harbor Laboratory Press, 1995. Recombinant nucleic acid techniques include, for example, restriction enzyme digestion and ligation, which can be used to isolate a nucleic acid. Isolated

nucleic acids also can be chemically synthesized, either as a single nucleic acid molecule or as a series of oligonucleotides.

Polypeptides can be purified from natural sources (e.g., a biological sample) by known methods such as DEAE ion exchange, gel filtration, and hydroxyapatite chromatography. A polypeptide also can be purified, for example, by expressing a nucleic acid in an expression vector. In addition, a purified polypeptide can be obtained by chemical synthesis. The extent of purity of a polypeptide can be measured using any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

A construct or vector containing a nucleic acid (e.g., a nucleic acid that encodes a polypeptide) also is provided. Constructs or vectors, including expression constructs or vectors, are commercially available or can be produced by recombinant DNA techniques routine in the art. A construct or vector containing a nucleic acid can have expression elements operably linked to such a nucleic acid, and further can include sequences such as those encoding a selectable marker (e.g., an antibiotic resistance gene). A construct or vector containing a nucleic acid can encode a chimeric or fusion polypeptide (i.e., a polypeptide operatively linked to a heterologous polypeptide, which can be at either the N-terminus or C-terminus of the polypeptide). Representative heterologous polypeptides are those that can be used in purification of the encoded polypeptide (e.g., 6xHis tag, glutathione S-transferase (GST))

Expression elements include nucleic acid sequences that direct and regulate expression of nucleic acid coding sequences. One example of an expression element is a promoter sequence. Expression elements also can include introns, enhancer sequences, response elements, or inducible elements that modulate expression of a nucleic acid. Expression elements can be of bacterial, yeast, insect, mammalian, or viral origin, and vectors can contain a combination of elements from different origins.

Vectors as described herein can be introduced into a host cell. As used herein, "host cell" refers to the particular cell into which the nucleic acid is introduced and also includes the progeny of such a cell that carry the vector. A host cell can be any prokaryotic or eukaryotic cell. For example, nucleic acids can be expressed in bacterial cells such as *E. coli*, or in insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art. Many methods for introducing nucleic acids into host cells, both *in vivo* and *in vitro*, are well known to those skilled in the art and include, without

limitation, electroporation, calcium phosphate precipitation, polyethylene glycol (PEG) transformation, heat shock, lipofection, microinjection, and viral-mediated nucleic acid transfer.

Nucleic acids can be detected using any number of amplification techniques (see, e.g., PCR Primer: A Laboratory Manual, 1995, Dieffenbach & Dveksler, Eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; and U.S. Patent Nos. 4,683,195; 4,683,202; 4,800,159; and 4,965,188) with an appropriate pair of oligonucleotides (e.g., primers). A number of modifications to the original PCR have been developed and can be used to detect a nucleic acid.

Nucleic acids also can be detected using hybridization. Hybridization between nucleic acids is discussed in detail in Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Sections 7.37-7.57, 9.47-9.57, 11.7-11.8, and 11.45-11.57). Sambrook et al. discloses suitable Southern blot conditions for oligonucleotide probes less than about 100 nucleotides (Sections 11.45-11.46). The Tm between a sequence that is less than 100 nucleotides in length and a second sequence can be calculated using the formula provided in Section 11.46. Sambrook et al. additionally discloses Southern blot conditions for oligonucleotide probes greater than about 100 nucleotides (see Sections 9.47-9.54). The Tm between a sequence greater than 100 nucleotides in length and a second sequence can be calculated using the formula provided in Sections 9.50-9.51 of Sambrook et al.

The conditions under which membranes containing nucleic acids are prehybridized and hybridized, as well as the conditions under which membranes containing nucleic acids are washed to remove excess and non-specifically bound probe, can play a significant role in the stringency of the hybridization. Such hybridizations and washes can be performed, where appropriate, under moderate or high stringency conditions. For example, washing conditions can be made more stringent by decreasing the salt concentration in the wash solutions and/or by increasing the temperature at which the washes are performed. Simply by way of example, high stringency conditions typically include a wash of the membranes in 0.2X SSC at 65°C.

In addition, interpreting the amount of hybridization can be affected, for example, by the specific activity of the labeled oligonucleotide probe, by the number of probe-binding sites on the template nucleic acid to which the probe has hybridized, and by the amount of exposure of an autoradiograph or other detection medium. It will be readily appreciated by those of ordinary

skill in the art that although any number of hybridization and washing conditions can be used to examine hybridization of a probe nucleic acid molecule to immobilized target nucleic acids, it is more important to examine hybridization of a probe to target nucleic acids under identical hybridization, washing, and exposure conditions. Preferably, the target nucleic acids are on the same membrane.

A nucleic acid molecule is deemed to hybridize to a nucleic acid but not to another nucleic acid if hybridization to a nucleic acid is at least 5-fold (e.g., at least 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 50-fold, or 100-fold) greater than hybridization to another nucleic acid. The amount of hybridization can be quantitated directly on a membrane or from an autoradiograph using, for example, a PhosphorImager or a Densitometer (Molecular Dynamics, Sunnyvale, CA).

Polypeptides can be detected using antibodies. Techniques for detecting polypeptides using antibodies include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence. An antibody can be polyclonal or monoclonal. An antibody having specific binding affinity for a polypeptide can be generated using methods well known in the art. The antibody can be attached to a solid support such as a microtiter plate using methods known in the art. In the presence of a polypeptide, an antibody-polypeptide complex is formed.

Detection (e.g., of an amplification product, a hybridization complex, or a polypeptide) is usually accomplished using detectable labels. The term "label" is intended to encompass the use of direct labels as well as indirect labels. Detectable labels include enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials.

Methods are described herein that can be used to generate a strain that lacks sequences for selection (i.e., that lacks a selectable marker). These methods include using a circular plasmid DNA vector and a linear DNA sequence; the circular plasmid DNA vector contains a selection marker and an origin of DNA replication (also known as an autonomously replicating sequence (ARS)), and the linear DNA sequence contains sequences for integration into the *Pichia* genome by homologous recombination. The linear DNA molecule additionally can include nucleic acid sequences encoding one or more proteins of interest such as, without limitation, heme-bound LegH, a dehydrin, a phytase, a protease a catalase, a lipase, a peroxidase, an amylase, a transglutaminase, an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, a ligase,

one or more enzymes involved in the pathway for production of small molecules, such as ethanol, lactic acid, butanol, adipic acid or succinic acid, or an antibody against any such proteins.

Pichia cells can be transformed with both DNA molecules and the transformants selected by the presence of the selectable marker on the circular plasmid. Transformants then can be screened for integration of the linear DNA molecule into the genome using, for example, PCR. Once transformants with the correct integration of the marker-free linear DNA molecule are identified, the cells can be grown in the absence of selection for the circular plasmid. Because the marker-bearing plasmid is not stably maintained in the absence of selection, the plasmid is lost, often very quickly, after selection is relaxed. The resulting strain carries the integrated linear DNA in the absence of heterologous sequences for selection.

This approach was used to construct MXY235, a marker-free version of strain MXY183, and MXY281, a marker-free version of strain MXY207. The expression of LegH protein in both marker-free strains was similar to the analogous strains that contained selection marker sequences. Therefore, this technique can be used to construct *Pichia* strains that lack a selection marker (e.g., a heterologous selection marker) with no significant impact on recombinant protein yield.

In accordance with the present invention, there may be employed conventional molecular biology, microbiology, biochemical, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. The invention will be further described in the following examples, which do not limit the scope of the methods and compositions of matter described in the claims.

EXAMPLES

Part A—Materials and Methods

Example 1—Polymerase Chain Reaction

Genes of interest were amplified from genomic DNA or plasmid DNA templates using Phusion Hi-fidelity DNA polymerase (New England Biolabs). Briefly, 0.6 μM each of forward and reverse primers are incubated with 10-50 ng of template DNA and 400 μM of nucleotide mix in the presence of 1-2 U of Phusion DNA polymerase. The reaction conditions were as follows:

1 cycle	Initial Denaturation	98°C	1min
25 cycles	Denaturation	98°C	10 sec
	Annealing		20 sec
	Extension	72°C	30 sec per kb
1 cycle	Final Extension	72°C	5min
1 cycle	Hold	4°C	Forever

Example 2—Plasmid Construction by Ligation

50-100 ng of restriction enzyme digested plasmid and 3X molar excess of PCR amplified inserts were incubated in the presence of T4 DNA ligase (New England Biolabs). Ligation was carried out at 16°C for greater than 2 hr. 2 µl of ligation reaction was transformed into DH10B electrocompetent *E. coli* cells

Example 3—Transformation into *E. coli* ElectroMax DH10B T1 Phage-Resistant Competent Cells

1.5-2 µl of ligation mixture was transformed into 20 µl of ElectroMax DH10B T1 Phage-Resistant Competent Cells (Invitrogen, Cat # 12033-015) by electroporation using MicroPulser (BioRad) set at 1.7 kV using a 1 mm gap cuvette (BioRad, Cat # 165-2089); after a pulse 1 ml SOC was added to cells and cells were incubated at 37°C for 1 h with shaking at 200 rpm. 10 µl of recovery mixture was plated on LB agar plates containing ampicillin at a concentration of 100 µg/ml. Plates were incubated overnight at 37°C.

Example 4—Linearization of Plasmid DNA for Transformation into *P. pastoris*

Plasmid DNA was digested with either PmeI restriction endonuclease (New England BioLabs, Cat # R0560L) in 1x CutSmart Buffer for 1-4 hours at 37°C or SfiI restriction endonuclease in 1x CutSmart Buffer for 1-4 hours at 50°C (New England BioLabs, Cat # R0123L). Linearized plasmid was gel purified from a 0.8% agarose gel using Zymoclean Gel DNA Recovery Kit (Zymo Research, Cat # D4002). DNA was eluted in 20 µl H_2O.

Example 5—Preparation of *P. pastoris* Transformation-Competent Cells

Selected strains of *P. pastoris* were grown to mid-exponential growth phase (~2 OD) in 25 ml YPD medium. Cells were collected by centrifugation at 930 x g for 15 minutes. The cell

pellet was resuspended in 2 ml of a solution of 80% YPD and 200 mM HEPES, pH 6.8. 75 µl of 1 M DTT was added. The resuspended cell pellet was mixed at 100 rpm at 30°C for 25 minutes. A 40 ml volume of ice cold, sterile water was added to the suspension, and the cells were collected by centrifugation at 1125 x g for 15 minutes and placed on ice. The cell pellet was resuspended in 40 ml ice cold water and collected as before for two additional wash steps. The cell pellet was then resuspended in 20 ml of ice cold 1 M sorbitol and collected by centrifugation as before. The final cell pellet was suspended in 0.3 ml ice cold, sterile sorbitol, aliquoted and frozen at -80°C.

Example 6—Transformation into *P. pastoris*

30-100 ng of linearized plasmid DNA was transformed into 30 µl of electrocompetent *P. pastoris* cells using a 1 mm gap GenePulser cuvette (BioRad) with a GenePulser (BioRad) set at 1.15 kV. 1 ml of YPD/1M sorbitol was added and mixed at a 1:1 ratio to the cells. The cells were allowed to recover for 3 h at 30°C with shaking at 100 rpm. 100 µl of the recovery mixture was plated on a YPD plate containing the appropriate antibiotic, and the rest of the cells were plated on a YPD plate with the appropriate antibiotic. Plates were incubated at 30°C for 48 hours. Primary transformation plates were streaked onto YPD plates with appropriate antibiotic, and plates were incubated for 48 h at 30°C. Individual clones were patched onto YPD plates with antibiotics and the patches were used to do colony PCR or gDNA prep to confirm integration into the chromosome and to grow the strains in shake flasks for further analysis.

Example 7—Growing Cultures in Shake Flasks for Production of LegH

A strain from a fresh patch was inoculated into growth media BMGY (BMY supplemented with 0.75% glycerol) and grown overnight at 30°C with shaking at 200 rpm. The next day, expression of LegH was induced with methanol by diluting the ON culture with BMMY media (BMY+ 1% methanol) supplemented with 0.1 mM Ammonium Fe(III) citrate. The culture was grown to an OD600 of 0.5-0.7. Antifoam was added to a final concentration of 0.01%. The cultures were grown for 72 hours total; cultures were supplemented with methanol every 24 hours by adding 1/10 of shake flask volume of 10x BMMY media (BMY+10% methanol). Cells were harvested after 72 h of induced growth by centrifugation.

Example 8—Shake Flask Medium

BMY media was prepared by dissolving 10 g of yeast extract and 20 g soytone in 790 ml water. The mixture was sterilized by autoclaving, and cooled to room temperature. 100 ml 1 M Potassium Phosphate buffer (pH 6.0) and 100 ml 10X Yeast Nitrogen Base without amino acids (13.4 g of YNB powder per 100 mL; Sigma-Aldrich) was filter sterilized (0.2 μm pore size PES) and added to the media. All components were FCC food grade or equivalent. No pH adjustment is required.

BMY Media Components

Component	Amount, per 1L
Yeast Extract	10 g
Soy peptone (BD)	20 g
Yeast Nitrogen Base without amino acids, 10X solution	100 mL (results in 13.4 g/L in BMY)
1M Potassium Phosphate Buffer, pH 6.0	100 mL

The following components were dissolved in water, and autoclaved to sterilize.

Low-Osmolarity Medium for Shake Flask

Component	Amount, g/L
Ammonium Sulfate	15.7
Potassium Phosphate Monobasic	9.4
Calcium Sulfate Dihydrate	0.43
Magnesium Sulfate Heptahydrate	11.7
Sodium Citrate Dihydrate	1.13

Example 9—Fermentation Medium and Feeds

The components indicated below were dissolved and the volume adjusted with water. The components were FCC food grade or equivalent. The medium was sterilized by autoclaving, by steaming in place, or with an equivalent.

Low-Osmolarity Medium with 95 g/L Glycerol for Fermentation

Component	Amount, g/L
Ammonium Sulfate	15.7
Potassium Phosphate Monobasic	9.4

Calcium Sulfate Dihydrate	0.43
Magnesium Sulfate Heptahydrate	11.7
Sodium Citrate Dihydrate	1.13
Glycerol, USP grade 99.7%	95

After sterilization, the medium was allowed to cool down to room temperature, and the following was added:

Component	Amount, mL/L
Trace Metals PTM1 Solution	2
Vitamin Solution	4
Sigma 204 antifoam or equivalent	1

Trace metals PTM1 solution is available as a powdered mix from Sunrise Science (Cat No. 4052-A-B-1L). Pouch A and pouch B were mixed in 950 mL water, and 5 mL sulfuric acid was added. Some precipitation is expected upon mixing; the mixture was filter sterilized (0.2 μm pore size PES) and stored at 4°C in the dark.

Vitamin solution recipe

Component	Amount, g/L
biotin	0.2
calcium pantothenate	1
folic acid	0.2
inositol	1
niacin	0.2
p-aminobenzoic acid	0.2
Pyridoxine hydrochloride	1
riboflavin	0.5
thiamine hydrochloride	1
B12	0.1

Alternatively, trace metals PTM1 can be made as follows:

Component	Amount
Cupric sulfate – 5H2O	6.0 g
Sodium iodide	0.08 g
Manganese sulfate – H2O	3.0 g
Sodium molybdate – 2H2O	0.2 g
Boric acid	0.02 g
Cobalt chloride	0.5 g

Zinc chloride	0.5 g
Ferrous sulfate – 7H2O	65.0 g
Biotin	0.2 g
Sulfuric acid	5.0 ml
Water	To a final volume of 1 L

The components are mixed together, filter sterilized and stored at room temperature.

The glycerol feed mix was prepared by mixing 17.5 g of AmberFerm 4000 into 320 mL water and stirring to dissolve. The water-Amberferm mixture was added to 850 g of glycerol and mixed well by vigorous stirring. The feed mix was sterilized by autoclaving.

Glycerol feed solution

Component	Amount, g/L
USP grade glycerol	850
Water	320
Sensient AmberFerm4000 soy hydrolysate	17.5

The methanol feed was made using 99-100% methanol supplemented with 12 mL/L of PTM1 solution.

Part B—Production of MXY0183

Example 10—Cloning Each Enzyme of Heme Biosynthesis Pathway into pGAN or pGAZ Integration Vector

pGAN (with the nat selection marker) and pGAZ (with the zeocin selection marker) were purchased from Biogrammatics, Inc (Carlsbad, CA). Each gene was placed under control of the AOX1 promoter, and the FDH terminator was placed immediately after the stop codon of each gene. The genes in the heme biosynthesis pathway were PCR amplified from WT *P. pastoris* strain or subcloned from previous constructs.

The heme biosynthetic pathway, including the enzymes involved in the biosynthesis of heme, are shown in Figure 1. The intermediates produced during the biosynthesis of heme are shown in the boxes, and the enzyme catalyzing each step is shown to the right. The rate limiting enzymatic steps, as shown in *S. cerevisiae*, are shown in red.

ALA synthase, ALA dehydratase, UPGIII synthase, UPGIII decarboxylase, CPG oxidase and PPG oxidase genes were PCR amplified with primers containing sites for recognition by the restriction endonuclease, BsaI. Oligonucleotides were synthesized by ElimBiopharm.

MxO0187	ALAsynth_F	GAGGGTCTCGGATGGAGTTTGTCGCCCGTC
MxO0188	ALAsynth_R	GAGGGTCTCGATTACAATCTGACTCCTGATGAGG
MxO0189	ALAdehyd_F	GAGGGTCTCGGATGGTGCATAAGGCTGAATACTTG
MxO0190	ALAdehyd_R	GAGGGTCTCGATTATTCAGATAACCACTCCAGG
MxO0191	UroporSynth_F	GAGGGTCTCGGATGCCAAAAGCCATTCTTCTGAAG
MxO0192	UroporSynth_R	GAGGGTCTCGATTAGTGCACTTTTTGTATAGAC
MxO0193	UroporDecarb_F	GAGGGTCTCGGATGAGTAGATTTCCAGAACTGAAG
MxO0194	UroporDecarb_R	GAGGGTCTCGATTATTGAGATCCAATGCG
MxO0195	CoproOx_F	GAGGGTCTCGGATGGCCATCGACTCTGATATC
MxO0196	CoproOx_R	GAGGGTCTCGATTATACCCATTCAATAGGAT
MxO0197	ProtoporOx_F	GAGGGTCTCGGATGCTGAAAAGTCTTGCACCAAA
MxO0198	ProtoporOx_R	GAGGGTCTCGATTAAATGCCACTGAGGGTAGC

Phusion High-Fidelity DNA Polymerase (New England BioLabs, Cat # M0530L) was used to amplify genes from genomic DNA. PCR products were obtained and purified using DNA Clean&Concentrator-5 (Cat # D4004) and DNA was eluted in 25 µl of H_2O. Vector DNA, pGAZ and pGAN, and PCR products were digested with BsaI (New England BioLabs, Cat # R0535S) in 50 µl reaction volume at 37°C.

Linearized vectors and digested PCR products were purified from 0.8% agarose gel using Zymoclean Gel DNA Recovery Kit (Zymo Research Cat # D4002). DNA was eluted in 20 µl H_2O. Ligation reactions were set up in 10 µl at 16°C overnight using T4 DNA Ligase (New England BioLabs, Cat # M0202S).

PBD and ferrochelatase genes were subcloned from previously constructed plasmids: pJAZ_PBD was digested with BstBI(Bsp119I) (ThermoScientific, FD0124)and NotI (ThermoScientific, FD0596) in 1x Fast Digest buffer for 5 min at 37°C. pJAZ_Ferroch was digested with MfeI (MunI, ThermoScientific, FD0753) and NotI (ThermoScientific, FD0596) in 1x Fast Digest buffer for 5 min at 37°C.

Digested products were purified from 0.8% agarose gel using Zymoclean Gel DNA Recovery Kit (Zymo Research Cat # D4002). DNA was eluted in 20 µl H_2O.

Ligation reactions were set up in 10 µl reaction at 16°C overnight using T4 DNA Ligase (New England BioLabs, Cat # M0202S).

1.5 µl of ligation mixture was transformed into 20 µl of ElectroMax DH10B T1 Phage-Resistant Competent Cells (Invitrogen, Cat # 12033-015) by electroporation using MicroPulser (BioRad) set at 1.7 kV; cells were incubated at 37°C in 1 ml SOC for 1 h with shaking at 200 rpm. 10 µl of recovery mixture was plated on LB agar plates containing ampicillin at concentration 100 µg/ml. Plates were incubated ON at 37°C. Colonies were screened by colony PCR for the presence of the insert. The sequences of the genes were confirmed.

Designation	Construct	Gene
pMx0308	pGAN-ALAsynth	ALAsynthase
pMx0309	pGAN-ALAD	ALAD
pMx0310	pGAN-UPGIIIsyn	Uroporphyrinogene synthase
pMx0311	pGAN-UPGIIIdecarb	Uroporphyrinogene decarboxylase
pMx0312	pGAN-CPGoxi	CPG oxidase
pMx0313	pGAN-PPGoxi	Protoporphyrin oxidase
pMx0314	pGAZ-ALAsyn	ALA synthase
pMx0315	pGAZ-ALAD	ALAD
pMx0316	pGAZ-UPGIIIsyn	Uroporphyrinogene synthase
pMx0317	pGAZ-UPGIIIdecarboxilase	UPGIIIdecarboxilase
pMx0318	pGAZ-PPGoxidase	PPGoxidase
pMx0319	pGAZ-CPG oxidase	CPG oxidase
pMx0320	pGAN-PBD	PBD
pMx0321	pGAZ-PGC	PBD
pMx0322	pGAZ-Fc	Ferrochelatase
pMx0323	pGAN-Fc	Ferrochelatase

Example 11—Assembling Heme Biosynthesis Genes on Plasmids for Integration into *P. pastoris* mutS Genome

The whole cassette "promoter-gene-terminator" was PCR amplified with primers containing sites for restriction endonucleases to assemble plasmids for integration into the *Pichia* genome.

Assembling Paox1_UPS_FDHterm- Paox1_UPD_FDHterm-Paox1_CPO_FDH term. on pGAN plasmid (pMx327)

pGAN-CPGoxidase (pMx312) was used as a vector to clone the UPS and UPD cassettes. UPG III synthase cassette was PCR amplified from pMx310 with primers to AOX1 promoter / FDH1 terminator containing NheI and SphI recognition sites for restriction endonucleases correspondingly:

MxO0399	NheI-pAOX1-F	CAA TCG CTA GCA TCC AAC ATC CAA AGA CGA AAG G
MxO0401	SphI-FDH1-R	GGA TAG CAT GCA CCT TAT CAA GAT AGC TAG AAA TAG AAA TGG

UPG III decarboxylase cassette was PCR amplified from pMx311 with primers to AOX1 promoter / FDH1 terminator containing SphI and AgeI recognition sites for restriction endonucleases correspondingly:

MxO0402	SphI-pAOX1I-F	CAA TAG CAT GCA ACA TCC AAA GAC GAA AGG TTG AAT G
MxO0404	AgeI-FDH1-R	CAT GGT ACC GGT ACC TTA TCA AGA TAG CTA GAA ATA GAA ATGG

Phusion High-Fidelity DNA Polymerase (New England BioLabs, Cat # M0530L) was used to amplify DNA from plasmids.

Obtained PCR products were purified using DNA Clean&Concentrator-5 (Zymo Research, Cat # D4004) and DNA was eluted in 25 µl of H_2O.

pGAN-CPGoxidase (pMx312) designated as a vector was digested in 1x CutSmart Buffer with NheI-HF (New England BioLabs, Cat # R3131S) and AgeI-HF (New England BioLabs, Cat # R3552S) over night at 37°C.

UPG III synthase cassette PCR product was digested in 1x CutSmart Buffer with NheI-HF (New England BioLabs, Cat # R3131S) and SphI-HF (New England BioLabs, Cat # R3182S) over night at 37°C.

UPG III decarboxylase cassette PCR product was digested in 1x CutSmart Buffer with SphI-HF (New England BioLabs, Cat # R3182S) and AgeI-HF (New England BioLabs, Cat # R3552S) over night at 37°C.

Digested vector and PCR products were gel purified from 0.8% agarose using Zymoclean Gel DNA Recovery Kit (Zymo Research, Cat # D4002). DNA was eluted in 20 µl H_2O.

Three way ligation between UPG III synthase cassette digested with NheI-SphI, UPG III decarboxylase cassette digested with SphI-AgeI and a vector digested with NheI-AgeI was set up in 10 µl at 16°C overnight using T4 DNA Ligase (New England BioLabs, Cat # M0202S).

1.5 µl of ligation mixture was transformed into 20 µl of ElectroMax DH10B T1 Phage-Resistant Competent Cells (Invitrogen, Cat # 12033-015) by electroporation using MicroPulser (BioRad) set at 1.7 kV; cells were incubated at 37°C in 1 ml SOC for 1 h with shaking at 200 rpm. 10 µl of recovery mixture was plated on LB agar plates containing ampicillin at a concentration of 100 µg/ml. Plates were incubated overnight at 37°C. Colonies were screened by colony PCR for the presence of the insert. The sequences of the junctions between vector and inserts were confirmed.

Assembling Paox1_ALAsynthase_FDH1term.- Paox1_PPGoxidase_FDH1term- Paox1_Fc_ FDH1term.- Paox1_PBD_ FDH1term cassette (pMx330)

a. PCR amplification of gene-cassettes:

ALAsynthase cassette was PCR amplified from pMx310 with primers to AOX1 promoter / FDH1 terminator containing NheI and XhoI recognition sites for restriction endonucleases correspondingly:

MxO0399	NheI-pAOX1-F	CAA TCG CTA GCA TCC AAC ATC CAA AGA CGA AAG G
MxO0400	XhoI-FDH1-R	GAT ATT GCT CGA GAC CTT ATC AAG ATA GCT AGA AAT AGA AAT G

PPGoxidase cassette was PCR amplified from pMx313 with primers to AOX1 promoter / FDH1 terminator containing XhoI and AflII recognition sites for restriction endonucleases correspondingly:

MxO0403	XhoI-pAOX1-F	CAA TCT CGA GAA CAT CCA AAG ACG AAA GGT TG
MxO0437	AflII-FDH1-R	CAA CCA TTT CTA TTT CTA GCT ATC TTG ATA AGG TCT TAA GTC CA

Ferrochelatase cassette was PCR amplified from pMx323 with primers to AOX1 promoter / FDH1 terminator containing AflII and AgeI recognition sites for restriction endonucleases correspondingly:

MxO0404	AgeI-FDH1-R	CAT GGT ACC GGT ACC TTA TCA AGA TAG CTA GAA ATA GAA ATG G

MxO0436	AflII-pAOX1-F	TTA CTT AAG TCC AAC ATC CAA AGA CGA AAG GTT G

G418 marker was PCR amplified from pJAG plasmid purchased from Biogrammatics using the following primers:

MxO0438	Mlu-G418-F	TCA CAG ACG CGT TGA ATT GTC C
MxO0439	BbvCI-G418-R	TTG CTC CTC AGC TTA GAA GAA CTC GTC CAA CAT CAA GTG

Phusion High-Fidelity DNA Polymerase (New England BioLabs, Cat # M0530L) was used to amplify DNA from plasmids. The PCR products were obtained and purified using DNA Clean&Concentrator-5 (Zymo Research, Cat # D4004) and DNA was eluted in 25 µl of H_2O.

b. Preparation of vectors

pGAZ-PBD (pMx321) designated as a vector was digested in 1x CutSmart Buffer with NheI-HF (New England BioLabs, Cat # R3131S) and XhoI (New England BioLabs, Cat # R0146S) overnight at 37°C.

pGAZ-ALAsyn.-PBD (pMx328) was digested in 1x NEBuffer3.1 with MluI (New England BioLabs, Cat # R0198S) and BbvCI (New England BioLabs, Cat # R0601S) overnight at 37°C.

pGAG-ALAsyn-PBD (pMx332) was digested in 1x CutSmart Buffer with XhoI (New England BioLabs, Cat # R0146S) and AgeI-HF (New England BioLabs, Cat # R3552S) overnight at 37°C.

c. Making intermediate constructs and assembling a final cassette

Digested vector and PCR products were gel purified from 0.8% agarose using Zymoclean Gel DNA Recovery Kit (Zymo Research, Cat # D4002). DNA was eluted in 20 µl of H_2O.

pGAZ-PBD (pMx321) vector, digested with NheI-XhoI restriction endonucleases, was ligated with ALAsynthase cassette PCR product digested with the same enzymes in 10 µl reaction at 16°C overnight using T4 DNA Ligase (New England BioLabs, Cat # M0202S) to yield a pGAZ-ALAsyn.-PBD plasmid (pMx328).

pGAG-ALAsyn-PBD (pMx332) digested with XhoI and AgeI–HF restriction endonucleases was ligated with PPGoxidase cassette and Ferrochelatase cassette PCR products digested with XhoI, AflII and AflII, AgeI-HF restriction endonucleases correspondingly in a three

way ligation reaction using T4 DNA Ligase (New England BioLabs, Cat # M0202S) to yield a pGAG-ALAsynthase_PPGoxidase_Fc_PBD (pMx330).

1.5 µl of ligation mixture was transformed into 20 µl of ElectroMax DH10B T1 Phage-Resistant Competent Cells (Invitrogen, Cat # 12033-015) by electroporation using MicroPulser (BioRad) set at 1.7 kV; cells were incubated at 37°C in 1 ml SOC for 1 h with shaking at 200 rpm. 10 µl of recovery mixture was plated on LB agar plates containing ampicillin at a concentration of 100 µg/ml. Plates were incubated overnight at 37°C. Colonies were screened by colony PCR for the presence of the insert. The sequences of junctions between the vector and the inserts were confirmed.

Designation	Construct	Gene
pMx0327	pGAN-UPGsyn_UPGdecarb_CPGoxy	UPGsyn_UPGdecarb_CPGoxy
pMx0328	pGAZ-ALAsyn-PGC	ALAsyn-PBD
pMx0330	pGAG-ALAsyn_PBD_PPG_Fc	ALAsyn_PBD_PPG_Fc
pMx0332	pGAG-ALAsyn_PBD	G418 marker

Example 12—Integration of Linearized Plasmids with Gene Cassettes into *P. pastoris* Bg11 Genome

The strains and the plasmids that were produced and used to generate the production strain, MXY0183, are shown in Figure 2 and Figure 3, respectively. The steps taken to make the modifications that led to production strain, MXY0183, are depicted in Figure 4.

The first enzyme to be introduced into the *P. pastoris* Bg11 genome was ALAD. A plasmid containing pAOX1-driven ALAD (pMX229, Figure 3i and Figure 4) was linearized using PmeI restriction enzyme (New England BioLab). Linearized plasmid was purified from 0.8% agarose gel as described and transformed into *P. pastoris* using homologous recombination at the native AOX1 locus, generating strain MXY099 (Figure 2 and Figure 4).

A plasmid containing two copies of the soybean LegH gene (sequence optimized for *P. pastoris*) under the control of the pAOX1 promoter designated pMX282 (Figure 3ii and Figure 4) was linearized using SfiI restriction enzyme. Linearized plasmid was purified from a 0.8% agarose gel as described and transformed into the *P. pastoris* strain containing ALAD, generating the strain MXY0118 (Figure 2 and Figure 3). qPCR was used and determined that strain MXY0118 contained 30 copies of the LegH gene, likely due to concatamerization of the plasmid, pMX282, at the time of recombination.

Plasmid pMX327 (Figure 3iii and Figure 4) containing genes encoding Uroporphyrinogen III synthase (UPS), Uroporphyrinogen III decarboxylase (UPD) and Coproporphyrinogen III oxidase (CPO) (the enzymes catalyzing steps 4, 5 and 6, respectively) under control of the AOX1 promoter was linearized with the SfiI restriction endonuclease and introduced into MXY0118, yielding strain MXY0170 (Figure 2 and Figure 4).

Genes encoding ALA synthase (ALAS), Protoporphyrin III oxidase (PPO), Ferrochelatase (FC) and Porphobilinogen deaminase (PBD) (the enzymes catalyzing steps 1, 7, 8 and 3, respectively) from the *P. pastoris* genome were assembled on plasmid pMX330 (Figure 2iv and Figure 3). pMX330 was linearized with the SfiI restriction endonuclease and transformed into MXY0170, leading to the generation of strain MXY0183 (Figure 2 and Figure 4).

The genotype of MXY0183 was confirmed using PCR and qPCR.

Part C—Production of MXY0207

Example 13—Construction of pGAB Expression Vector

The pGAB expression vector (Figure 5A) was constructed by replacing the open reading frame of the Zeocin resistance gene in the pGAZ vector (BioGrammatics, Inc., Carlsbad, CA) with the open reading frame from the Blasticidin S deaminase (BSD) gene from *Aspergillus terreus*, which allows for selection of transformants carrying the plasmid with the antibiotic Blasticidin S.

The BSD open reading frame was amplified from a commercially synthesized DNA molecule using oligonucleotide primers MxO0476 and MxO0477 using a high fidelity polymerase chain reaction as described herein.

ID	Description	Sequence
MxO0477	BSD_Reverse	TTA GTC TTG CTC CTC AGC TTA GCC
MxO0476	BSD_Forward	TCA CAG ACG CGT TGA ATT GTC C

The BSD PCR product was purified by gel electrophoresis on a 1% agarose gel in 1x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) and visualized using SYBR Safe DNA gel stain (Life Technologies, Carlsbad, CA). The desired DNA fragment was excised from the agarose gel and the DNA was recovered using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA).

The purified BSD PCR product and pGAZ vector were digested with 10 units each of the MluI and BbvCI restriction endonucleases (New England Biolabs, Ipswich, MA) for 1 hour at 37°C in 1x NEBuffer 3.1 (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 μg/ml BSA, pH 7.9 @ 25°C). Digested DNA products were recovered by gel electrophoresis as described above.

The purified, MluI and BbvCI digested BSD product and pGAZ vector were incubated with 400 units of T4 DNA ligase (New England Biolabs) in 1x T4 DNA ligase reaction buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT, pH 7.5 @ 25°C) in a 20 μl reaction, at 16°C for 2 hours in a 20 μl reaction. Electrocompetent E. coli DH10B cells were transformed with 2 μl of the ligation reaction and antibiotic resistant transformants were selected on LSB agar plates supplemented with 100 μg/μl ampicillin.

Example 14—Construction of Mxr1 Expression Vector

The Mxr1 expression vector, pMx354, was constructed by introducing the Mxr1 open reading frame into the pGAB vector (Figure 5B). The Mxr1 open reading frame was inserted into pGAB with the translation start immediately downstream of the methanol-inducible alcohol oxidase 1 (AOX1) promoter from *Pichia pastoris* and the translation stop signal immediately followed by the transcription terminator sequence from the *P. pastoris* FDH1 gene.

The open reading frame encoding the Mxr1 protein was amplified from genomic DNA isolated from *Pichia pastoris* strain Bg11 MutS obtained from BioGrammatics, Inc. (Carlsbad, CA). The Mxr1 open reading frame was amplified from *P. pastoris* genomic DNA with primers MxO0495 (TTT TGC GGC CGC ATG AGC AAT CTA CCC CCA ACT TTT G) and MxO0496 (AAA AGC GGC CGC CTA GAC ACC ACC ATC TAG TCG GTT), which appended flanking NotI restriction endonuclease recognition sites. Amplification was accomplished using the polymerase chain reaction as described herein.

The amplified Mxr1 PCR product and the pGAB vector were digested with 10 units of NotI restriction endonuclease (New England Biolabs) for 1 hour at 37°C in 1x NEBuffer 3.1 (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 μg/ml BSA, pH 7.9 @ 25°C). Following digestion, the NotI-digested pMx352 vector was treated with 5 units Antarctic phosphatase (New England Biolabs) for 15 minutes at 37°C in 1x Antarctic phosphatase buffer (50 mM Bis-Tris-Propane-HCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, pH 6 @ 25°C).

The NotI-digested amplified Mxr1 fragment and pMx352 vector were separated by electrophoresis on a 1% agarose gel in 1x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) and visualized using SYBR Safe DNA gel stain (Life Technologies, Carlsbad, CA). The desired DNA fragments were excised from the agarose gel and the DNA was recovered using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA).

The NotI-digested fragment containing Mxr1 open reading frame was introduced into pGAB at a NotI site immediately downstream of the AOX1 promoter by ligation. A mixture containing 137 ng of NotI-digested DNA encoding the Mxr1 open reading frame and 60 ng of NotI-digested, phosphatase-treated pMx352 was incubate with 400 units of T4 DNA ligase (New England Biolabs) in 1x T4 DNA ligase reaction buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT, pH 7.5 @ 25°C) in a 20 µl reaction, at 16°C, for 2 hours in a 20 µl reaction. Electrocompetent E. coli DH10B cells were transformed with 2 µl of the ligation reaction and antibiotic resistant transformants were selected on LSB agar plates supplemented with 100 µg/µl ampicillin. Plates were incubated overnight at 37°C. Colonies were screened for the presence of the insert by PCR using primers MxO0495 and MxO0496. The sequence of the final vector was confirmed by DNA sequencing.

Example 15—Construction of *P. pastoris* Strain MxY207

The pMx354 Mxr1 expression vector (Figure 5B) was introduced into the MXY0183 strain (Figure 1 and Figure 3) by DNA transformation.

The pMx354 vector (1.5 µg) was linearized at a unique PmeI site in the AOX1 promoter sequences by digestion with 20 units of the PmeI restriction endonuclaease (New England Biolabs) for 1 hour at 37°C in 1x NEBuffer 4 (50mM Potassium Acetate, 20mM Tris-acetate, 10mM Magnesium Acetate, 1mM DTT, pH 7.9@25°C).

The PmeI-digested pMX354 vector was purified by gel electrophoresis recovered using the Zymoclean Gel DNA Recovery Kit as described above.

Example 16—Protocol for Lab-Scale High Oxygen Transfer Fermentation

Seed Shake Flask Protocol

In a aseptic biosafety hood, low-osmo and BMY were mixed in a 9:1 low-osmo:BMY ratio. Glycerol, at a concentration of 12.5 g/L, was added to the medium. USP food grade

glycerol/glycerin (99.7% purity in a 50% v/v (63% w/w) glycerol/water solution) was used and autoclaved to sterilize. Sigma 204 or an equivalent antifoam was added to the medium at a concentration of 0.25 mL/L. Glycerol seed vials were retrieved, sprayed outside with 70% IPA or ethanol and thawed inside a biosafety hood at room temperature for about 5 min. Baffled shake flasks were inoculated with glycerol seed vials; 1 mL of inoculum vial were used for every 1 L of shake flask medium. Cultures were grown at 30°C for 24 hours with shaking (200 RPM with a 1" throw). A ratio of between 1:10 and 1:5 of actual medium volume : nominal shake flask volume was used. 2.8 L nominal volume flask with 250 to 500 mL of medium were routinely use with success. The OD at 600 nm was measured after 24 hours of growth; if the OD was 15 or higher, the culture was used to inoculate a fermenter. If the OD was less than 15, the culture was grown for 1-2 more hours before the OD was determined again. If an OD of 15 was not reached after 15 to 30 hours, the seed flask was considered to have failed.

Fermentation Protocol

The fermentation medium and feeds were prepared as described herein. The initial volume should be about 40% of the maximum fermenter volume, e.g., 4 L, if the maximum working volume of the fermenter is 10 L. This is because the process will approach the maximum working volume by the end of the fermentation. The fermenter is inoculated with shake flask seed at 10% inoculum-fermenter ratio, e.g. if 4 L of initial media are present in the fermenter, the fermenter is inoculated with about 0.4 L of shake flask seed. The total volume in the fermenter at this point is referred to T0 volume, e.g. 4.4 L in this representative example. Process controls include the following: 30°C temperature; dissolved oxygen controlled by agitation-aeration cascade to maintain a 20% saturation set point; and pH controlled via addition of 28% NH_4OH, the set point will depend on the phase of the process.

Batch phase (from inoculation to depletion of glycerol, signaled by DO spike): Depending on the responsiveness of the PID control for dissolved oxygen, a strong DO spike or a fast drop in agitation-aeration rates or a combination of both may be observed when the cells deplete the glycerol present in the medium. Fed-batch phase is initiated when this occurs. The duration of the batch phase is approximately 20 hours, but up to 24 hours is considered acceptable. The pH set point is 5.0. The wet cell weight at the end of the batch phase will be approximately 220 g/L.

Fed-batch phase: glycerol feed is initiated to achieve 12-14 g/L/hr of neat glycerol based on T0 volume. The federate was maintained until approximately 350 g/L wet cell weight was reached, which should take about 7-10 hours. The pH set point is 5.0.

Transition phase: A sample is taken before beginning the transition phase. Methanol feed was initiated to achieve 1 g/L/hr of neat methanol, based on the T0 volume, until 1-2 g/L methanol concentration was reached in the fermentation broth. The methanol feed rate was adjusted during the remainder of fermentation so as to maintain a methanol concentration of 0.25-1 g/L in broth. Glycerol federate was reduced from 12-14 g/L/hr to 8-9 g/L/hr of neat glycerol, based on T0 volume, linearly over the course of 2 hours. Stepwise reduction in feed rate every 20 min would be acceptable as well. The pH set point was changed to 3.5, and the fermentation was allowed to naturally adjust to the new set point (i.e., with no addition of acid).

Production phase (from end of glycerol feed ramp-down to end of fermentation): The pH set point was 3.5. A methanol concentration of 0.25-1 g/L was maintained in the fermentation broth. The feed rate of the glycerol was maintained at 8-9 g/L/hr of neat glycerol, based on the T0 volume. Samples were taken approximately every 12 hours. Samples were spun at 4000 to 7000 RCF at 4°C, and the supernatant decanted. The supernatant was saved in a separate tube. Pellets and 3 samples of 5 mL of supernatants at each time point were frozen at -80°C. If a 15-20% DO during production is unable to be maintained, even at maximum aeration and agitation rates for the vessel, the glycerol feed rate can be lowered up to 5 g/L/hr of neat glycerol, based on the T0 volume. Fermentation ended 60 hours after inoculation. At 1000 L scale, the harvest process consisted of shutting down feeds and aeration, chilling the broth to 8°C, and concentrating the paste using a sharples or disk stack centrifuge. Harvesting usually takes about 5-10 hours and does not incur a detectable loss of quality of the product. For lab scale, it is sufficient to collect, in addition to the 3x5 mL samples, an additional 50 mL sample at the end. Wet cell weight was >450 g/L, and spun pellets looked pink, as opposed to spun pellets from pre-induction samples, which looked more white. The color change of the broth from white to a more pronounced pink started following about 6-12 hours of induction.

Part D—Preliminary Results

Example 17—Preliminary Characterization of Strain MXY0183

Expression of the genes associated with strain MXY0183 resulted in phenotypic changes to the strain. Figure 6 shows photographs of shake flasks at the start of induction (0 hr) and 72 hr post-induction. The flasks designated #1 contain the host strain, MXY0051. The flasks designated #2 and #3 contain one of the intermediate strains (i.e., MXY0118, containing 30 copies of the LegH gene and the ALA dehydratase from the heme biosynthetic pathway) and the production strain (i.e., MXY0183, containing 30 copies of the LegH gene and the 8 enzymes from the heme biosynthetic pathway), respectively. The characteristic red color in flask #3 after 72 hours demonstrates the production of heme-bound LegH.

After growing in shake flasks, the *P. pastoric* strains indicated above, MXY0051, MXY0118, and MXY0183, were lysed and the proteins run on a SDS gel (Figure 7A). The arrow shows the position of the LegH protein. A comparison of LegH production in strain MXY0183 and in strain MXY0118 is shown in Figure 7B, which demonstrates the efficiency of heme loading of the LegH protein by the MXY0183 strain.

Experiments were then performed to determine the stability of the 9 transgenes in the MXY0183 strain (the gene encoding LegH and the genes encoding the 8 enzymes involved in heme biosynthesis). Colony PCR was performed on MXY0183 passaged through non-selective media (Figure 8, lane 3) and after more than 200 generations of growth on non-selective media (Figure 8, lanes 4) to amplify a portion of each of the 9 transgenes. Compared to the controls (Figure 8, lane 1, MXY0183 fresh strain; lane 2, Bg11 host strain), the 9 transgenes are stably maintained, even after prolonged passage in the absence of selective pressure. Lysates from the same cultures assayed in Figure 8 were run on a SDS gel to evaluate the translational patterns. Densitometry demonstrated that the LegH protein constitutes greater than 30% of the total soluble protein in the lysate.

Example 18—Preliminary Characterization of Strain MXY0207

Experiments were then performed to determine the benefits of overexpressing the transcriptional activator, Mxr1, in the presence of the genes encoding the 8 enzymes involved in heme biosynthesis. Strain MXY0183, which contains 30 copies of the LegH sequence and the genes encoding the 8 enzymes involved in heme biosynthesis, and strain MXY0206, which

contains 30 copies of the LegH sequence, the genes encoding the 8 enzymes involved in heme biosynthesis, and the Mxr1 transcriptional activator, were grown in shake flask cultures in the presence of glycerol, which is a repressing carbon source for these strains. Photographs of the shake flask cultures after 48 hr are shown in Figure 9A, and photographs of the pellets from cells grown on BMY media for 48 hours with no additional source of carbon are shown in Figure 9B; these experiments demonstrated that significant expression of transgenes (e.g. heme enzymes) under the control of the AOX1 promoter occurs in the absence of an inducing carbon source when a repressing carbon source is consumed in the growth medium of a strain in which Mxr1 is also expressed from the AOX1 promoter. The relative yield of heme-loaded LegH, when shake flask cultures were grown in the absence of induction agent, is shown in Figure 9C. These experiments demonstrated that significant production of a recombinant, heme-loaded protein is accomplished from AOX1 promoter-driven transgenes in the absence of methanol induction in Pichia strains in which Mxr1 expression is also driven by the AOX1 promoter.

Select strains were grown in 2 L fermenter tanks, and the relative yield of LegH and heme-loaded LegH was determined (Figure 10). While the production strain, MXY0183, produced a significant amount of LegH and heme-loaded it very effectively, the MXY0207 strain produced even more LegH and was able to produce enough heme to heme-load the LegH protein very effectively. It is noted that the strain referred to in Figure 10 as MXY0121 contains 4 copies of the LegH sequence and the ALA dehydratase sequence.

Example 19—Construction of Strain MxY228

The high affinity iron transport system in *Pichia pastoris* is composed of Fet3, a ferroxidase, and the iron permease, Ftr1. Expression of Fet3 is negatively regulated by Fep1, an iron-responsive GATA-type transcription factor. The disruption of the *Pichia* FEP1 gene leads to constitutively high levels of Fet3 mRNA expression irrespective of iron levels. See, for example, Miele et al., 2007, Arch. Biochem. Biophys., 465:172-9.

The addition of ferrous iron to protoporphyrin IX constitutes the final step in heme biosynthesis and is catalyzed by the enzyme ferrochelatase. Accordingly, heme synthesis requires maintenance of adequate intracellular iron levels. Accordingly, it was predicted that disruption of the *Pichia pastoris* FEP1 gene in strains overexpressing the heme pathway (e.g., MxY183, MxY207, and their derivatives) would de-regulate iron transport, allowing intracellular iron

concentration to increase and drive heme synthesis to completion. It also was predicted that this would reduce the accumulation of heme intermediates, and improve the formation of fully heme-loaded LegH.

Using the methods described herein, a FEP1 knockout mutant was created in the MxY207 strain background to generate strain MxY228. This FEP1 deletion mutant reduced the accumulation of heme biosynthetic intermediates and also increased the yield of LegH protein in shake flask cultures of this strain.

Part E—A Co-Transformation Method for Marker-Free Strain Construction

Example 20—Construction of the pMX382 Vector

A plasmid containing two copies of the soybean LegH gene (sequence-optimized for *P. pastoris*) under the control of the pAOX1 promoter designated pMX303 was used as a source of template for PCR amplification. The pMX303 vector was digested with PmeI restriction endonuclease, and the digested vector fragments were separated using agarose gel electrophoresis. A 1765 base pair DNA fragment was isolated and purified using DNA Clean&Concentrator-5 (Zymo Research, Cat # D4004) and the DNA was eluted in 12 µl of H_2O. The purified PmeI-digested DNA fragment was used as a template for a subsequent round of PCR amplification using primers MxO0617 and MxO0618 shown below.

MxO0617	AAACGCTGTCTTGGAACCTAATATGAC
MxO0618	AAACTGTCAGTTTTGGGCCATTTG

The resulting PCR product was composed of, in the 5' to 3' direction, the 3' end of the AOX1 promoter, followed by the LegH open reading frame, the AOX1 terminator, a short linker sequence, and the 5' end of the AOX1 promoter. The PCR product was obtained and purified by agarose gel electrophoresis as described herein. The purified PCR product was cloned into the pCR™-Blunt II-TOPO® vector using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, Cat # K2800-20) to create the pMX382 vector.

Example 21—Co-Transformation to Introduce the LegH Expression Cassette into *Pichia*

A linear LgH-expression cassette lacking heterologous sequences for selection was amplified from pMX382 using primers MxO0617 and MxO0618. PCR products were obtained

and purified as above, and DNA was eluted in H_2O. This linear LegH expression cassette was simultaneously introduced into *Pichia* strain MXY213 with the pIL75 plasmid by transformation. The pIL75 vector carries a panARS autonomous replication sequence (Liachko & Dunham, 2014, FEMS Yeast Res., 14:364-7), which allows for maintenance of the plasmid vector without integration into the genome of the transformed cells, and a kanMX marker for selection of transformants with the antibiotic G418. Transformed cells were selected on media supplemented with G418 for the presence of kanMX marker on the pIL75 plasmid. *Pichia* transformants were screened by colony PCR for transformants that took up both the pIL75 plasmid and had correctly integrated the LegH expression cassette using primers MxO0206 and MxO0565. Transformants with LegH expression cassette integrated into the genome were screened for LegH gene copy number using qPCR.

Example 22—Curing Transformants of Plasmid Vectors Bearing Selection Markers

In clones where the soybean LegH expression cassette was shown to be correctly integrated by colony PCR and in high copy number by qPCR, the pIL75 plasmid required for selection on G418 was eliminated by relaxing selection for the antibiotic. Transformants were streaked out for single colonies on media lacking G418 antibiotic. Because the panARS plasmid is not stably maintained in the absence of selection, the pIL75 was rapidly lost from the transformed cells under this condition. The resulting *Pichia* strain, MXY235, contains sequences for LegH expression in copy number similar to MXY207, but lacks heterologous sequences for selection.

Example 23—Construction of the pMX402 Vector

A plasmid containing the Mxr1 transcription regulator gene under the control of the pAOX1 promoter, designated pMX354, was used as a template for PCR amplification. The 3' end of the AOX1 promoter, the LegH open reading frame, and the AOX1 terminator were amplified from pMX354 using primers MxO0617 and MxO0647 shown below. The AOX1 terminator, linker and the 5' end of the AOX1 promoter were amplified from pMX382 using primers MxO0618 and MxO0646.

MxO0646	ACTAGATGGTGGTGTCTAGTCAAGAGGATGTCAGAATGCCATTTG
MxO0647	TCTGACATCCTCTTGACTAGACACCACCATCTAGTCGGTTTTCTAG

PCR products were obtained and purified using DNA Clean&Concentrator-5 and DNA was eluted in 12 μl of H_2O. The purified PCR products were then combined and used as a template for a subsequent round of PCR amplification using primers MxO0617 and MxO0618. The resulting PCR product was composed of the 3' end of the AOX1 promoter, followed by the Mxr1 open reading frame, the AOX1 terminator, a short linker sequence, and the 5' end of the AOX1 promoter. The PCR product was obtained and purified as described herein. The purified PCR product was cloned into the pCR™-Blunt II-TOPO® vector using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, Cat # K2800-20) to create the pMX402 vector.

Example 24—Co-Transformation to Construct Strain MXY281

A linear Mxr1 expression cassette lacking heterologous sequences for selection was amplified from pMX402 using primers MxO0617 and MxO0618. The PCR product was obtained and purified as above, and DNA was eluted in H_2O. The linear Mxr1 expression cassette was simultaneously introduced into *Pichia* strain MXY235 with the pIL75 plasmid by the co-transformation protocol described herein. The resulting transformants were screened by colony PCR for the presence of the Mxr1 gene under direction of the AOX1 promoter, and the positive clones were subsequently cured of the selection plasmid as described herein to generate strain MXY281.

Example 25—Appendices

Relevant sequences are shown in Appendix I. Representative materials and methods are shown in Appendix II.

It is to be understood that, while the methods and compositions of matter have been described herein in conjunction with a number of different aspects, the foregoing description of the various aspects is intended to illustrate and not limit the scope of the methods and compositions of matter. Other aspects, advantages, and modifications are within the scope of the following claims.

Disclosed are methods and compositions that can be used for, can be used in conjunction with, can be used in preparation for, or are products of the disclosed methods and compositions.

These and other materials are disclosed herein, and it is understood that combinations, subsets, interactions, groups, etc. of these methods and compositions are disclosed. That is, while specific reference to each various individual and collective combinations and permutations of these compositions and methods may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a particular composition of matter or a particular method is disclosed and discussed and a number of compositions or methods are discussed, each and every combination and permutation of the compositions and the methods are specifically contemplated unless specifically indicated to the contrary. Likewise, any subset or combination of these is also specifically contemplated and disclosed.

WHAT IS CLAIMED IS:

1. A methylotrophic yeast cell comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises an exogenous nucleic acid encoding a transcriptional activator operably linked to at least one methanol-inducible promoter element.

2. The methylotrophic yeast cell of claim 1, wherein the methylotrophic yeast cell is of a yeast selected from the group consisting of *Candida*, *Hansenula*, *Pichia* and Toruplosis.

3. The methylotrophic yeast cell of claim 1, wherein the cell is a *Pichia pastoris* cell.

4. The methylotrophic yeast cell of claim 1, wherein the recombinant nucleic acid molecule is stably integrated into the genome of the methylotrophic yeast cell.

5. The methylotrophic yeast cell of claim 1, wherein the recombinant nucleic acid molecule is extrachromosomally expressed from a replication-competent plasmid.

6. The methylotrophic yeast cell of claim 1, wherein the exogenous nucleic acid encoding a transcriptional activator comprises a Mxr1 sequence from *Pichia pastoris*, a Adr1 sequence from *Hansenula polymorpha*, a Trm1 sequence from *Candida boidinii*, and a Trm2 sequence from *Candida boidinii*.

7. The methylotrophic yeast cell of claim 1, wherein the nucleic acid encoding the transcriptional activator comprises a sequence shown in DQ395124.

8. The methylotrophic yeast cell of claim 1, wherein the transcriptional activator comprises a sequence shown in ABD57365.

9. The methylotrophic yeast cell of claim 1, wherein the at least one methanol-inducible promoter element is selected from the group consisting of an alcohol oxidase 1 (AOX1)

promoter element from *Pichia pastoris*, an AOD1 promoter element from *Candida boidinii*, a MOX promoter element from *Hansenula polymorpha*, a MOD1 promoter element from *Pichia mehanolica*, a DHAS promoter element from *Pichia pastoris*, and a PEX8 promoter element from *Pichia pastoris*.

10. The methylotrophic yeast cell of claim 1, further comprising a nucleic acid molecule comprising at least one heterologous nucleic acid encoding a polypeptide operably linked to at least one methanol-inducible promoter element.

11. The methylotrophic yeast cell of claim 10, wherein the heterologous nucleic acid encodes a polypeptide involved in the biosynthesis of an iron co-factor.

12. The methylotrophic yeast cell of claim 1, wherein the yeast cell lacks heterologous sequences for selection.

13. A method for expressing a heterologous polypeptide in a cell, the method comprising:

providing the methylotrophic yeast cell of any of the preceding claims;

introducing a recombinant nucleic acid molecule into methylotrophic yeast cell, the recombinant nucleic acid molecule comprising at least one heterologous nucleic acid encoding a polypeptide operably linked to at least one *Pichia pastoris* alcohol oxidase 1 (AOX1) promoter element; and

culturing the cell under conditions suitable for expression of the recombinant nucleic acid molecule,

thereby expressing the heterologous polypeptide.

14. The method of claim 13, wherein the conditions under which the cells are cultured comprises the addition of iron or a pharmaceutically or metabolically acceptable salt thereof.

15. The method of claim 13, wherein the introducing step comprises a technique selected from the group consisting of transduction, electroporation, biolistic particle delivery, and chemical transformation.

16. The method of claim 13, wherein the culturing step comprises culturing the cell in the present of methanol.

ABSTRACT OF THE DISCLOSURE

Methods and materials for genetically engineering methylotrophic yeast are provided.

5 61198170.doc

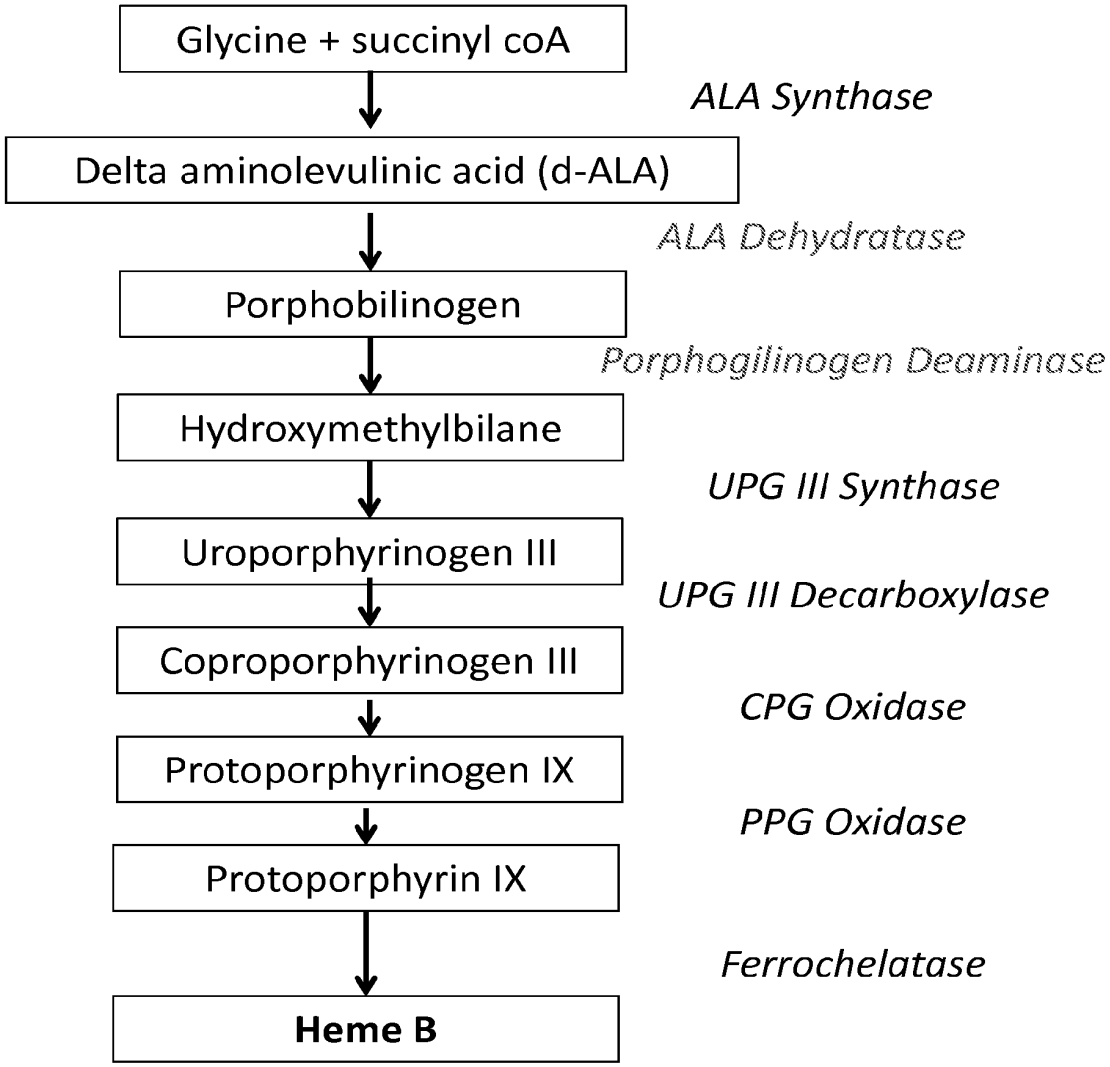

Glycine + succinyl coA
ALA Synthase
Delta aminolevulinic acid (d-ALA)
ALA Dehydratase
Porphobilinogen
Porphogilinogen Deaminase
Hydroxymethylbilane
UPG III Synthase
Uroporphyrinogen III
UPG III Decarboxylase
Coproporphyrinogen III
CPG Oxidase
Protoporphyrinogen IX
PPG Oxidase
Protoporphyrin IX
Ferrochelatase
Heme B

Figure 1

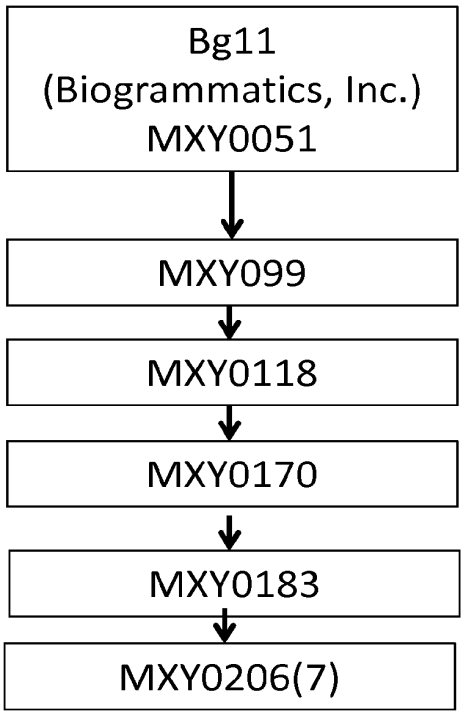

Bg11
(Biogrammatics, Inc.)
MXY0051
MXY099
MXY0118
MXY0170
MXY0183
MXY0206(7)

Figure 2

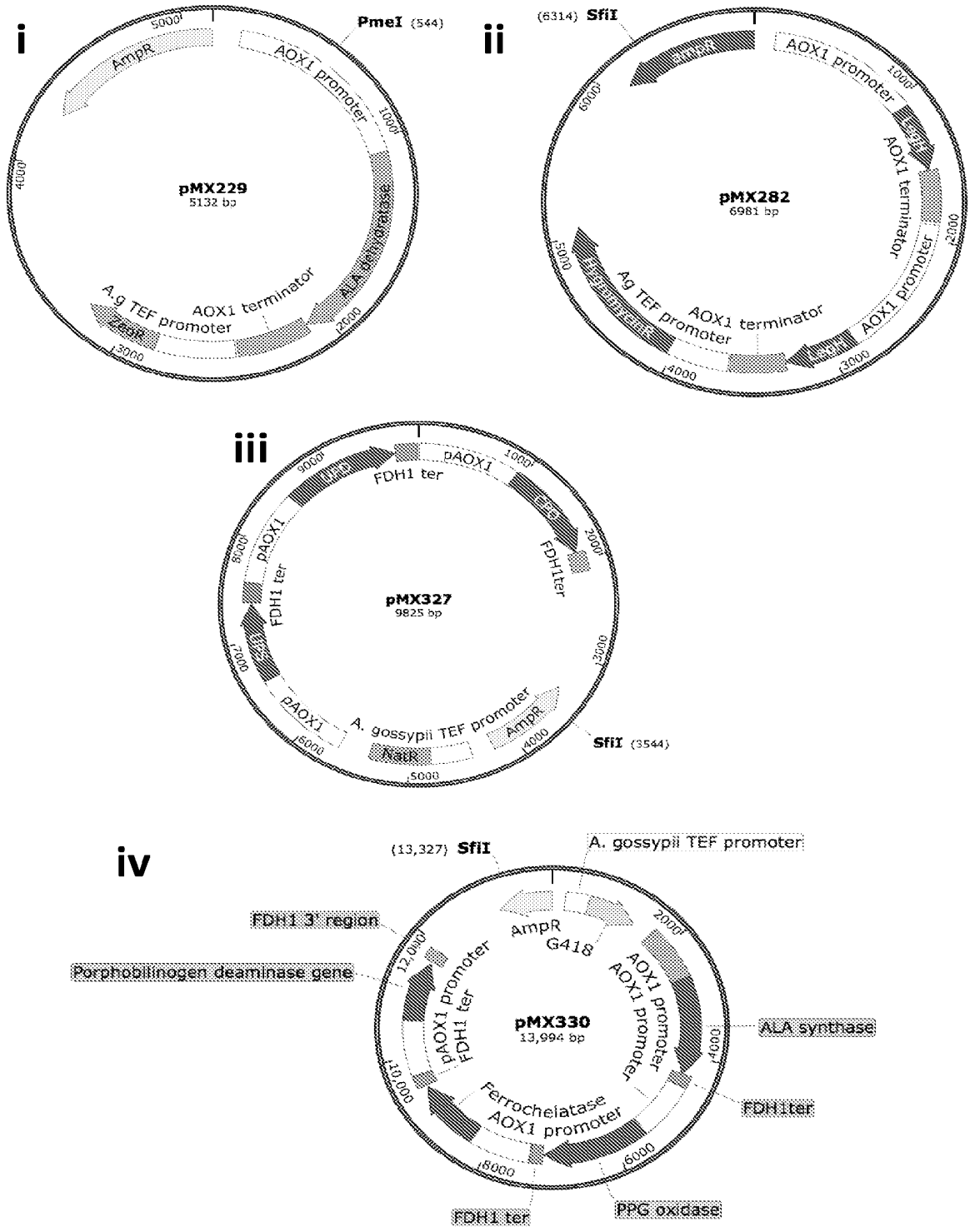

i
PmeI (544)
AmpR
AOX1 promoter
pMX229
5132 bp
ALA dehydratase
AOX1 terminator
A.g TEF promoter
ZeoR
ii
(6314) SfiI
AOX1 promoter
pMX282
6981 bp
AOX1 terminator
Ag TEF promoter
AOX1 terminator
AOX1 promoter
iii
pAOX1
FDH1 ter
pMX327
9825 bp
FDH1ter
pAOX1
FDH1 ter
pAOX1
A. gossypii TEF promoter
AmpR
NatR
SfiI (3544)
iv
(13,327) SfiI
A. gossypii TEF promoter
FDH1 3' region
AmpR
G418
Porphobilinogen deaminase gene
pAOX1 promoter
FDH1 ter
pMX330
13,994 bp
AOX1 promoter
ALA synthase
Ferrochelatase
AOX1 promoter
FDH1ter
FDH1 ter
PPG oxidase

Figure 3

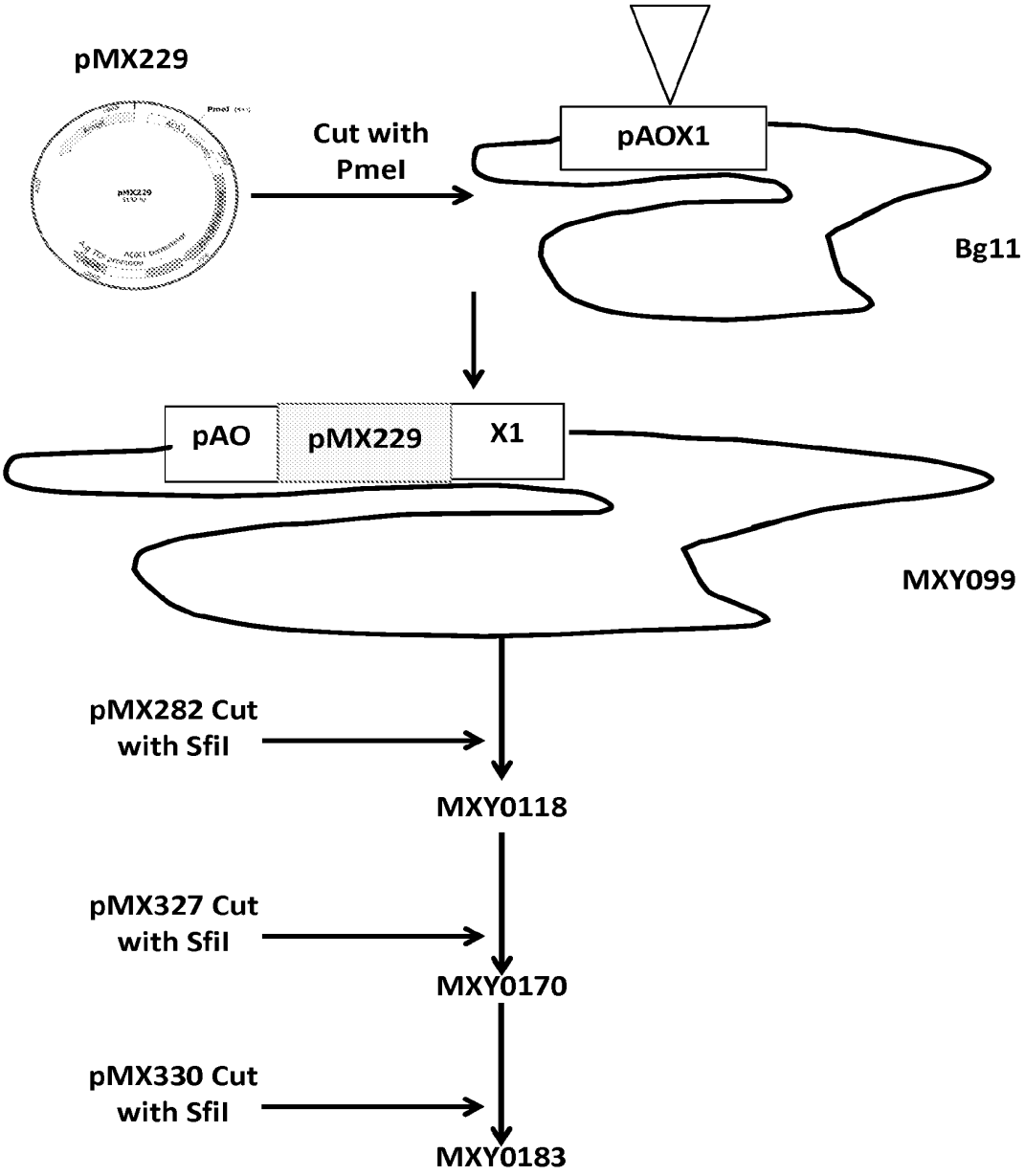

pMX229
Cut with
PmeI
pAOX1
Bg11
pAO
pMX229
X1
MXY099
pMX282 Cut
with SfiI
MXY0118
pMX327 Cut
with SfiI
MXY0170
pMX330 Cut
with SfiI
MXY0183

Figure 4

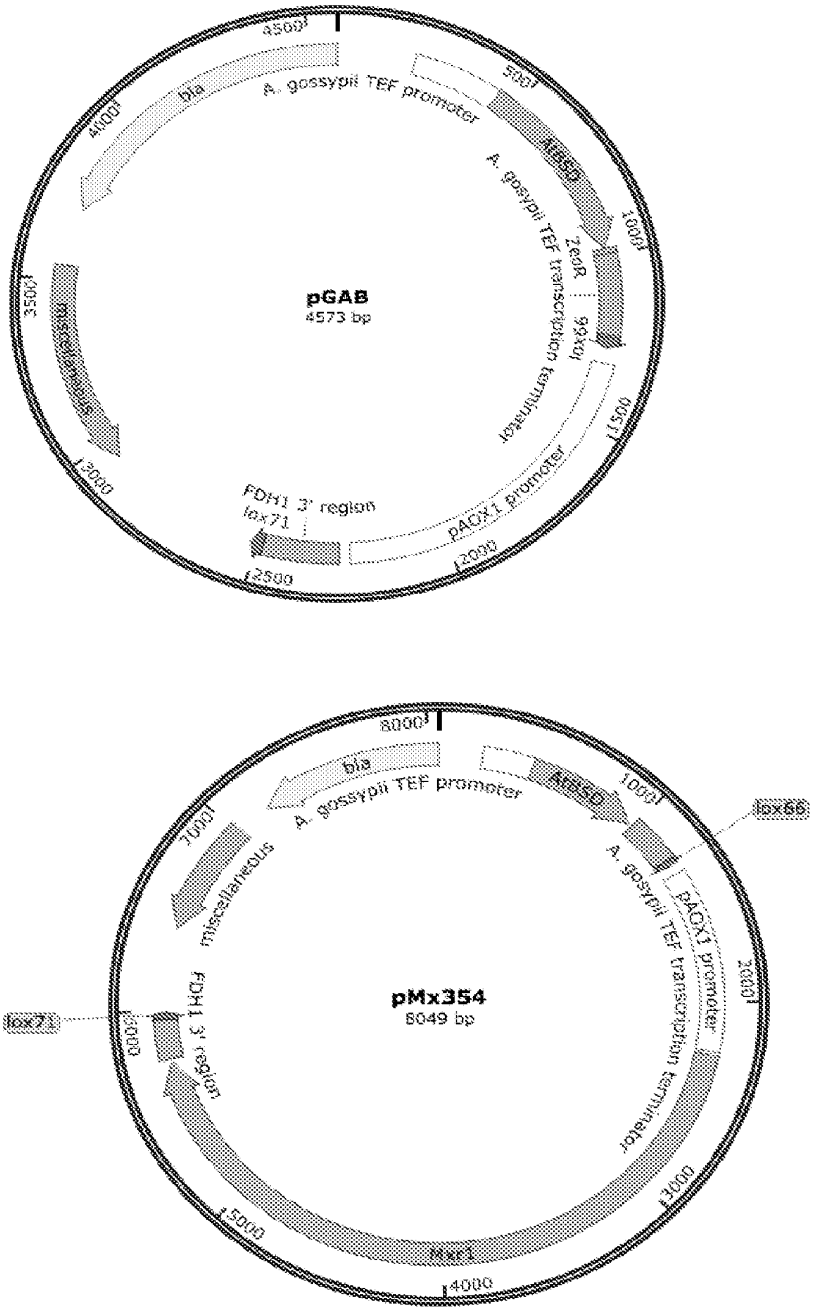

pGAB
4573 bp
bla
A. gossypii TEF promoter
AbleO
A. gossypii TEF transcription terminator
loxP
miscellaneous
FDH1 3' region
lox71
pAOX1 promoter
pMx354
8049 bp
lox66
Mxr1

Figure 5

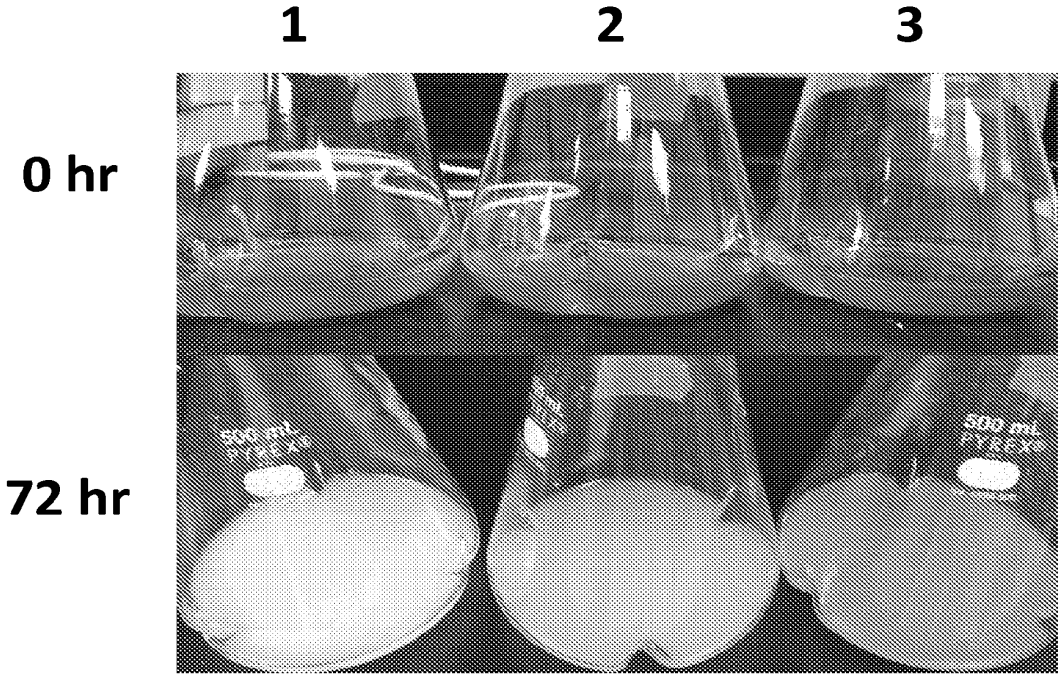
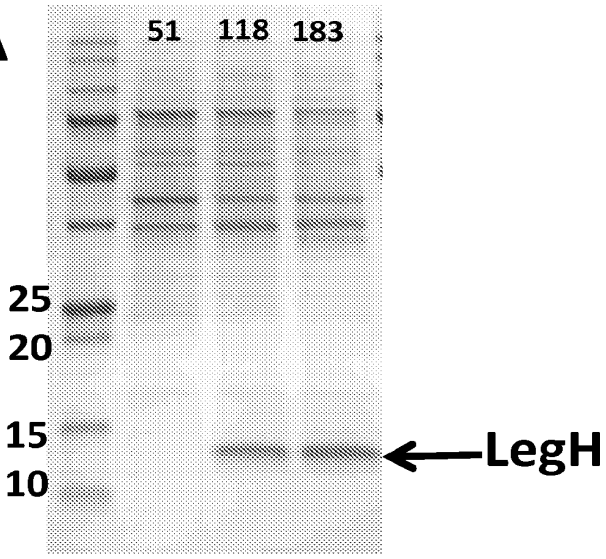


Figure 6

A



B

Strain	Amount of LegH relative to intermediate strain	Heme loading
MXY0118	1	Sub-optimal
MXY0183	2	100%

Figure 7

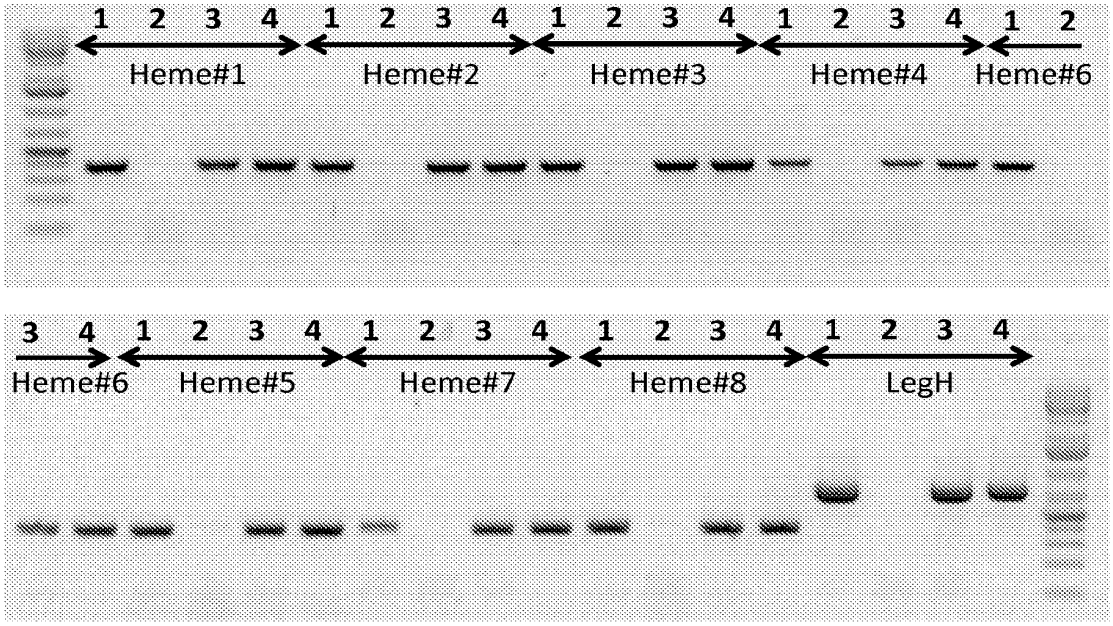
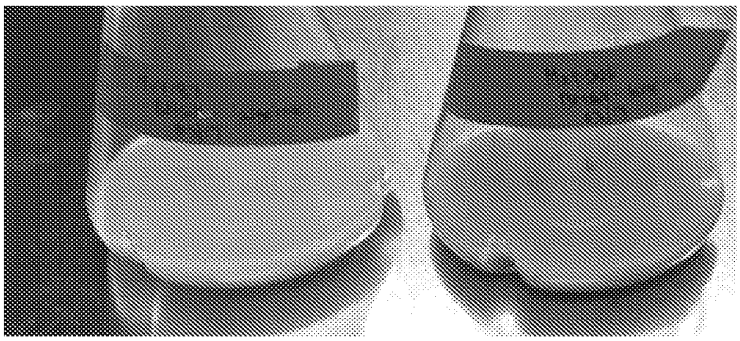
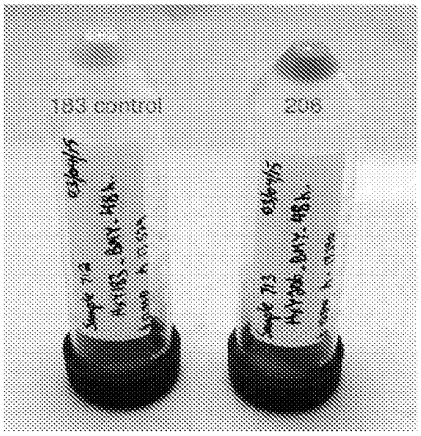

1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2
Heme#1
Heme#2
Heme#3
Heme#4
Heme#6
3 4 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
Heme#6
Heme#5
Heme#7
Heme#8
LegH

Figure 8

A



B



C

Strain	Relative yield of heme loaded LegH
183- BMGY	N/A
183- BMY	1
206- BMGY	3
206- BMY	5

Figure 9

Strain	Relative yield of LegH	Heme loading
MXY0121	1	100%
MXY0118	1.5	Sub-optimal
MXY0183	2.6	100%
MXY0207	3.2	100%

Figure 10

APPENDIX I

1. Heme biosynthesis enzyme 1- ALA Synthase

ATGGAGTTTGTCGCCCGTCAGTCCATGAATGCCTGTCCCTTTGTCAGGTCAACTTCTACCCACCA
TTTGAAGAAGTTGGCAGCAAACAGTTCTCTAGCTGCTACTGCTAGTCATTGTCCCGTGGTTGGCC
CTGCTCTCCAACAGCAGAGATACTACTCTCAACCTTCCAAGCCAGCCCAAGCCCAAACCTCCGAC
ATTGCTACTGGGATCAAGAAGGATGTTTCTCCGATCCGTATGGACTCTAATGAAACCGCCTTTGA
TTACAATGGAATGTATGAGTCTGATCTTGCGAATAAACGTAAAGATAACTCGTATCGTTATTTCA
ATAACATCAACCGTCTAGCCAAGGAGTTTCCCAAGGCACATCGCCAGACCGAAGATGACAAGGTG
ACCGTCTGGTGCTCTAACGACTACTTAGGAATGGGTAGGCATCCTGAGATTATCAAAACCATGAA
GGCTACCATGGACAAGTACGGTTCCGGAGCAGGAGGAACTAGGAACATTGCAGGTCATAACCACG
CCGCTATCAATTTGGAAAGCGAGTTGGCTTGCTTGAACAAGAAGGAAGCGGCTCTGGTGTTTTCA
TCATGTTTCATAGCTAACGATGCAATCATCTCGTTGTTGGGACAAAAAATCAAAAATTTGGTCAT
TTTCTCTGACCAGTCGAATCATGCTTCCATGATATTGGGTGTGCGTAACTCCAAAGCGAAGAAGC
ACATCTTCAAGCACAACAATTTGAAGGATCTGGAGTCGCAGTTAGCTCAGTACCCCAAGTCGACT
CCTAAACTGATCGCCTTCGAGTCAGTTTACTCTATGTGTGGATCTGTGGCTCCCATTGAGAAGAT
TTGCGATTTGGCTAAAAGGTACGGTGCCCTCACCTTCTTGGATGAAGTTCATGCTGTTGGAATGT
ATGGTCCTCATGGACAGGGTGTAGCTGAGCATTTGGACTTTGATCTGCATTTACAGTCTGGAATC
GCCAGTCCTAGCGTGGTGGACAAACGCACCATATTGGATCGTGTCGACATGATTACTGGTACTTG
CGGAAAGTCATTTGGTACTGTTGGAGGTTACGTTGCTGGTAGTGCCAACCTAATTGATTGGTTAA
GATCCTATGCGCCAGGTTTCATTTTCACTACCACACTTCCTCCTGCTATCATGGCTGGTACAGCC
ACTTCTGTTCGTATTGTTAGGGCCGACATTGAGGCCCGTATCAAGCAACAGCTTAATACTCGCTA
CGTCAAAGACTCATTTGAAAACCTTGGTATTCCAGTCATTCCAAACCCAAGTCACATTGTTCCTG
TTCTAGTTGGAAATGCTGCAGATGCCAAGAAGGCATCCGATATGTTAATGAACAAACACCGTATT
TATGTTCAAGCTATTAACTACCCTACTGTGCCTGTCGGTGAAGAACGACTAAGGATTACTCCTAC
TCCAGGTCATGGAAAGGAGATTTGTGACCAGCTGATCAGCGCTGTCGACGATGTTTTTACTGAGC
TTAATTTACCAAGAATCAACAAATGGCAGTCCCAAGGTGGTCATTGCGGTGTTGGTGATGCTAAT
TACGTACCAGAACCCAATCTGTGGACTCAGGACCAGCTCAGCTTGACAAACCAAGACTTGCACTC
CAATGTGCACAACCCAGTGATTGAGCAGATCGAAACCTCATCAGGAGTCAGATTGTAG

2. Heme biosynthesis enzyme 2- ALA dehydratase

ATGGTGCATAAGGCTGAATACTTGGACGACCACCCAACTCAGATTTCCAGCATTCTTTCAGGAGG
TTACAACCACCCATTACTTCGTGAATGGCAACATGAACGTCAACTCAACAAAAACATGTTCATCT
TTCCCCTGTTTGTCACAGATCGACCAGACGAAGAAGAACTTATTCCTAGTCTACCTAATATCAAG
AGGTTTGGCGTTAACAAGTTGATTCCTTATGTAGGAGGTTTGGTTTCCAAAGGATTGAGGGCGGT
GATCCTATTTGGTGTTCCTCTGAAGCCCGGTGTGAAAGATGAAGAAGGAACGGCCGCTGATGATC
CAGAGGGACCTGTTATCCAAGCCATCAAACACTTGAGAAAGAACTTTCCTGACCTGTATATCATC
ACCGATGTCTGTCTATGTGAGTACACCAGCCATGGACATTGTGGAATACTATATGAGGATGGCAC
TATCAACAGAGAGCTCTCAGTCCGTCGTATTGCTGCTGTAGCTGTCAAATATGCTCAAGCTGGAG
CCAACTCTGTGGCTCCTTCTGATATGACTGACGGCAGAATAAGAGATATTAAAGAAGGCTTACTA
AGTGCAGGACTGGCACATAAAACGTTTGTTATGTCCTACGCTGCAAAATTCTCTGGTAATTTGTA
TGGCCCTTTCAGAGATGCTGCAGGTTCCTGTCCATCTCAAGGGGACAGAAAATGTTACCAGCTTC
CTTCTGGAGGAAAAGGGTTGGCCCATCGTGCTCTGATTCGTGATATGAATGAAGGCACTGATGGA
ATTATTGTCAAACCATCTACATTCTATTTGGACATTGTCGCTGATGCTTATCAGCTTTGTAAAGA
CTATCCTATCTGCTGTTACCAGGTTTCTGGAGAGTACGCCATGCTACATGCAGCGGCAGAGAAGA
ATATTGTTGATCTGAAATCAATCGCTTTTGAAGCTCATCAAGGATTCTTGCGGGCTGGAGCTCGT
TTAATCATTAGTTACTTTACCCCTGAATTCCTGGAGTGGTTATCTGAATGA

3. Heme biosynthesis enzyme 3- Porphobilinogen deaminase

ATGAACCAAATCGAACAGAGCGGACCCATTGATTGCAGTTCCTTGAAATTGGGGTCCCGAAAGTC
CGCTCTGGCTATAATCCAGGCAGAAATCGTCCGCCAATTGATATTGAAAGAATACCCTGAATTGG
AGACGAAGTTGGTCAGTGTGTCCACCCTGGGGGACCAAGTCCAGAATAAAGCACTTTTCACGTTT

GGAGGAAAATCTTTGTGGACCAAAGAACTTGAGATGTTGTTGTTGGAGAGTGTGGGAGGATTTGA
CCAAATAGACATGATTGTACACTCGTTGAAAGACATGCCAACTCATTTACCAGACGAATTTGAGC
TGGGTTGCATTATTGAAAGAGAAGACCCTAGAGACGCTTTGGTCGTGCAAGATGGTTTATCTTAC
AAGTCATTGGCCGACCTTCCAGAGGGAGCTGTGGTCGGTACGTCTTCGGTTAGAAGATCGGCTCA
ACTACTGAAGAATTTCCCTCATCTGAAATTCAAATCTGTTAGAGGAAACCTTCAGACCAGACTAA
GAAAATTAGATGATCCAGATTCCGAGTACTGCTGTCTCCTCCTTGCAGCAGCCGGTTTAATCAGG
ACAGGCTTACAACACAGAATTTCAATGTATTTGAACGACGATGTGATGTACCACTCCGTCGGACA
AGGAGCATTAGGAGTAGAGATCAGAAAAGGTGACCAATTCATGAAAAATATCTGTGAAAAGATTG
GGCATAGAACCACCACCCTTCGTTGTCTTGCAGAGAGAGCACTGCTGAGATATCTAGAGGGAGGC
TGCTCGGTGCCAATTGGGGTCTCCACTATTTATAGCGAGGATACGAAGGAACTTACCATGAACTC
CCTAGTCGTCAGTTGTAACGGTCGTGACTCGGTAACAGAATCAATGACTGAAGTCGTGACTACTG
AAGAGCAAGCTGAAGATTTCGGTGAAAGGCTGGCCCAGAAGCTCATAGATCAAGGTGCGAAACGC
ATTCTTGACGAGATCAACTTCAACAAGATCAAAGAGATTAAGGAAGAGGGTTTACATTAA

4. Heme biosynthesis enzyme 4- Uroporphorinogen III synthase
ATGCCAAAAGCCATTCTTCTGAAGAATAAAACTACACCGAAGGATCCTTATCTGGAGAACTTCGT
AAGTAGTGGCTACTCGACCGATTTCGTACCACTTTTAGATCATATTCACATGGAGAAATCTGAGA
TCATCGCATTTCTCAAGACTGACTACTTTTTGCATAAAACTTTGGCGTTTATTATTACGTCCCAA
AGAGCTGTAGAAATGCTGAATGAGTGTATGCAAATACTGAGACGTACTGATCCTGAAATTACACA
AATCATCTATAGTAAACCTGTCTATACAGTTGGCCCTGCCACCTACAGAATACTTGCGGATGCTG
GCTTCGTGGATCTACGAGGCGGAGATAAGGCAGGAAACGGATCCATTCTAGCCCAGATAATTTTG
AATGATGACATTTACACTGGAATTGAAGATTCTGACAAGCATATAACGTTTTTCACGGGAGAAAC
AAGGAGAGACATAATTCCCAAATGTTTACTCTCTAACAACTTTCAACTTTACGAAAAGATTGTCT
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TCTTCAACACACCAAAGACCAATTTAATATTGCATCTATCGGGCCAACCACAGAAAAATACCTTC
TAAGCAAAAACCTGAAACCAAAAGTTGTGGCACCTAAGCCAGAGCCTATCTCTTTACTATTGTCT
ATACAAAAAGTGCACTAA

5. Heme biosynthesis enzyme 5- Uroporphorinogen III decarboxylase
ATGAGTAGATTTCCAGAACTGAAGAATGACCTTATTTTAAGGGCAGCTCGTGGTGAAAAAGTTGA
ACGTCCCCCAATATGGATTATGAGACAGGCCGGAAGATATCTTCCGGAGTACCATGAGGTCAAAG
GAGGTAGGGACTTCTTTGAAACTTGCAGGGATGCTGAGATTGCTTCTGAAATTACTATCCAGCCG
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AAAGCTATCTCATTGACCAGAAAAAAACTCAATGGACGAGTGCCTTTGCTTGGATTTTGTGGTGC
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AGTGGATCTACAAGTTTACCAAGGAATCTCATCAATTACTCCAACAGATCACTGACGTTGCAGTT
GAATTCTTAGCTCAGCAAGTTGTTGCAGGTGCCCAAATGTTACAAGTTTTTGAATCTTGGGGCGG
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TCTTGGTACGCCTTGGAGCAATTGTGCGACAGTGGTTATGATGTTGTCTCGTTGGATTGGTTATT
CCGTCCAAGTGATGCTGTCCAGATTGCTAACGGAAGAATCGCATTGCAAGGTAATCTTGACCCTG
GAACCATGTACGGCTCCAAAGAAACCATTTCCAAGAAAGTGGACAAAATGATCAAGGGTTTTGGT
GGAGGAAAGCAAAACTACATAATTAATTTTGGACACGGCACTCATCCATTCATGGATCCAGAACA
GATCAGATGGTTCTTACAAGAATGTCATCGCATTGGATCTCAATAG

6. Heme biosynthesis enzyme 6- Coproporphyrinogen III oxidase
ATGGCCATCGACTCTGATATCAATCTAAGCTCTCCCAATGATTCCATCCGTCAAAGGATGTTCGA
GCTTATCCAGCGGAAGCAACTCGAAATTGTCGCTGCATTGGAGGCAATTGAAGGAAACGATACCA
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AGAGGAAGATATGTCGAATTCAACTTAGTATTGGATAGAGGTACTCAATTTGGTTTGAGAACGCC
TGGATCTCGTGTTGAAAGTATTTTGATGTCGTTGCCAAGAACAGCTGGTTGGGTCTATGATCATC
ATCCAGAGCCTGGCTCCAGAGAAGAGGAGTTATTGCAGGTACTACAAAATCCTATTGAATGGGTA
TGA

7. Heme biosynthesis enzyme 7- Protoporphorinogen oxidase

ATGCTGAAAAGTCTTGCACCAAATTCCTCAATTGCCGTTTTAGGTTCAGGGATATCTGGATTGAC
TTTCAGCTTTTTTTTGAATCGGTTGCGTCCCGATGTTAAGATCCATATCTTTGAAAAATCCAAGC
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AAATCACCTGAATATTGATCTAGATGAGTTTGCATCTTTGCCAGACTTCAAGATGGAAATCACCA
AGATCCCCAACTGCATTCCCCAATATGAAGTTGGGTATCTTGATCTCAAGAGAAAGGTTCAGAAT
GCAGCCTCCAAAGAGTTCAACGACCAAATAAGTTTTGGAGGCATGGCATTTGGTGATGGTGTGGG
GATCCCTGACTGTGTCCAGAATGCATTCAAAGATTCGGCTACCCTCAGTGGCATTTAA

8. Heme biosynthesis enzyme 8- Ferrochelatase

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CATGAATATGGGTGGTCCCTCCACTGTCAAGGAAACCTATGACTTTTTATTTCGTCTTTTCTCGG
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GGAATTACTAAGGCCATTGCCTTTTCTCAATATCCGCAATTTAGTTATTCAACCACCGGATCATC
GATTAACGAACTTTACAGGCAATCGAAAATTTTGGACCCTGATCAATCTATTAAATGGACAGTTA
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CCAAAGCCTTGGCTGGGTGCTCAAACTGAAAAAATTACCAAGCAGCTAGCATCCAGTGATGTTCC
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TTGAACTGATTCAAGAACTACCTAATCCTTCAAAAGTAAAGCGAGTTGAATCGTTGAACGGAGAC
CAAACTTTCATTGACTCCTTGGCAGAACTAGTGAAGAGTCACATTGATTCGAAGGTTGTATTTTC
CAACCAGTTGCCATTGGATTCCATGCTGGGAGTAGTGTCAGATAATTCCCTCACAGATCCAAAAG
AGTTTTTCAGAGCCCATTGA

9. LegH

ATGGGTGCTTTCACCGAGAAGCAGGAAGCACTTGTTTCCTCTTCGTTCGAAGCTTTTAAGGCTAA
CATCCCTCAATACTCTGTTGTGTTTTACACGTCCATTCTAGAAAAAGCTCCTGCTGCCAAGGACC
TCTTCTCTTTTCTGTCCAACGGTGTAGATCCATCCAATCCCAAATTAACAGGTCACGCTGAGAAA
TTGTTCGGTTTAGTCAGAGATAGCGCTGGACAATTGAAAGCAAATGGTACTGTGGTTGCTGATGC
TGCCTTGGGCAGCATCCATGCACAGAAGGCAATTACAGACCCACAATTTGTTGTTGTGAAGGAAG
CTCTGCTTAAAACTATAAAGGAAGCCGTCGGAGACAAATGGAGTGACGAGTTGTCATCAGCTTGG
GAGGTAGCTTATGATGAGTTGGCCGCAGCAATCAAAAAGGCATTCTAA

APPENDIX II

1(a) Identification of the microorganism

The production strain *Pichia pastoris* MXY0183 is a genetically engineered derivative of the parent *Pichia pastoris* strain NRRL-Y11430 (ATCC 76273), which is deposited in the collection at the Northern Regional Research Laboratories (NRRL).

Since MXY0183 is derived from NRRL-Y11430, its taxonomy is identical to that of strain NRRL-Y11430. The morphological and growth characteristics for the notified strain MXY0183 are the same as that of parent strain NRRL-Y11430, as shown in Figure 1. Strain MXY0183 shows the same round to ovoid shape and reproduces by a division process known as budding all which are identical to the NRRL-Y11430 when grown on Yeast extract/ Peptone Dextrose Agar (YPD).

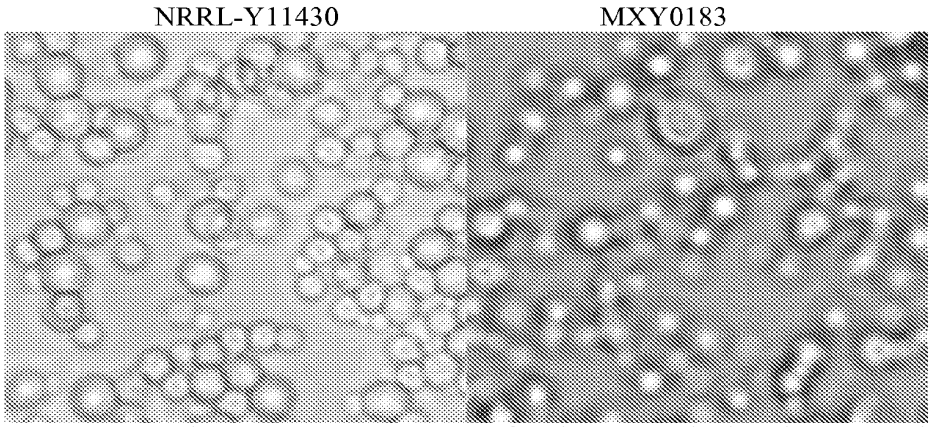


Figure 1: NRRL-Y11430 and MXY0183 grown on YPD grown for 2 days at 30C. The picture is at 40X magnification.

The sequence of an intermediate strain MXY0118 (assembled) was also evaluated for positive identification to *P. pastoris* and was shown to have 99.2% similarity to the *P. pastoris* genome sequence NCBI (AB018182.2).

Collectively, the information above shows that the MXY0183 toxonomy is that of *P. pastoris* in that:

- the strain is derived from *P. pastoris* strain NRRL-Y11430 (ATCC 76273),
- the morphological and growth characteristics is that of NRRL-Y11430 (ATCC 76273), and
- BLAST alignment using the intermediate strain MXY0118 18S rDNA sequence showed 99.2% sequence identity to a *P. pastoris* strain NCBI (AB018182.2).

1(b) Synonyms and common and superseded names

P. pastoris is the most commonly used name, although the organism is also referred to as *Komagataella pastoris*. In 2009, multi gene sequence analysis established that *P. pastoris* is actually *Komagataella phaffii*.

1(c) Strain History

The first *P. pastoris* strains were isolated from an oak tree and a chestnut tree and were deposited in the collection at the Northern Regional Research Laboratories (NRRL). Strain MXY0183 was isolated by the Applicant in 2013. The strain pedigree leading to the production strain MXY0183 is outlined in Figure 2.

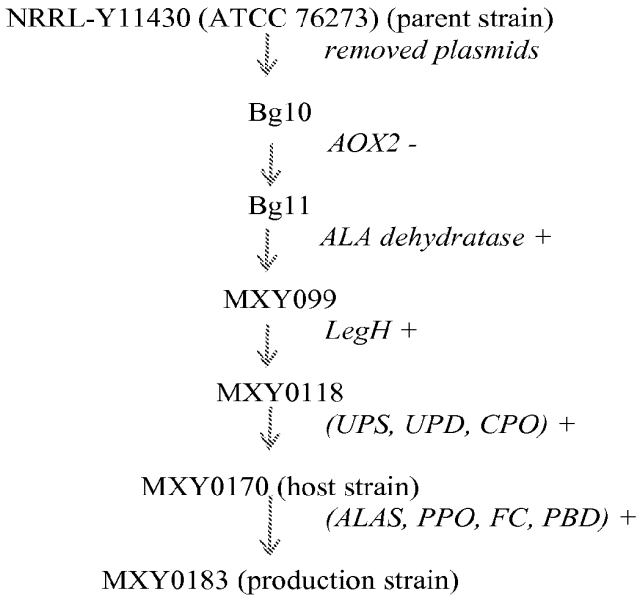


Figure 2. Strain lineage of production strain MXY0183.

1(d) Description of any modification to the micro-organism including:

1(di) The purpose of the modifications

Strain MXY0183 was developed to produce heme cofactor-bound soybean Leghemoglobin protein, referred to in this document as LegH (Glycine max LGB2, UniprotKB / Swiss-Prot # P02236).

1(dii) The methods and steps taken to make the modification

1) NRRL-Y-11430 strain

The native *P. pastoris* plasmids were removed using PCR primers unique to the plasmids to screen multiple single-colony isolates for the presence of the plasmids. One isolate without the plasmids was selected to become the wild-type (wt) strain, Bg10.

2) Bg10 strain

Alcohol oxidase 2 (Aox2) was deleted from Bg10 using homologous recombination to generate a strain that grows slower on the induction media and carbon source, methanol. The strain was named mutS (Methanol Utilization slow). The antibiotic resistance marker and background vector sequences used during homologous recombination were subsequently removed to generate a clean, antibiotic marker-free, Bg11 mutS strain.

3) Bg11 strain

Native *P. pastoris* ALAD was amplified from the genome and overexpressed under the control of the strong native *P. pastoris* promoter, pAOX1. A plasmid (Figure 3) containing pAOX1 driven ALAD, pMX229, was linearized using restriction enzyme digestion and transformed into *P. pastoris* using homologous recombination at the native AOX1 locus, generating strain MXY099.

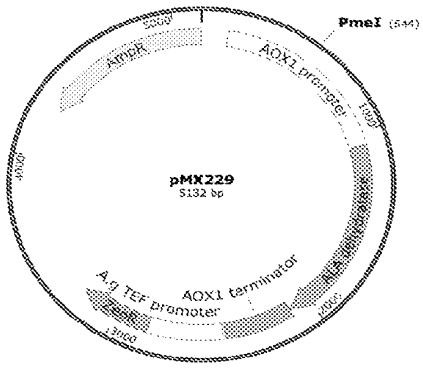


Figure 3: pMX229 plasmid

4) MXY099 strain

Two copies of soybean *LegH* (sequence optimized for *P. pastoris*) were expressed under the control of pAOX1 promoter in the vector pMX282 (Figure 4). The vector was linearized using restriction enzyme digestion (SfiI) and transformed into the *P. pastoris* strain containing ALAD, generating the strain MXY0118.

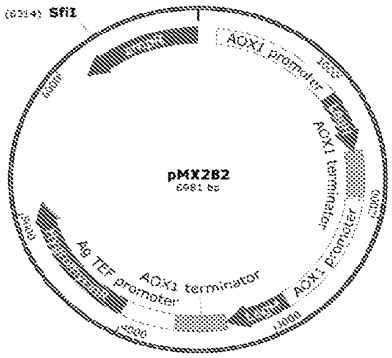


Figure 4: pMX282 plasmid

Next, enzymes needed to help catalyze the heme biosynthesis pathway to increase the heme bound to LegH were introduced into the strain (i.e., steps 5 and 6).

5) MXY0118 strain

Genes encoding the enzymes Uroporphyrinogen III synthase-UPS, Uroporphyrinogen III decarboxylase-UPD and Coproporphyrinogen III oxidase-CPO were amplified from the *P.*

pastoris genome and cloned into one plasmid pMX327, with each under the control of pAOX1 (Figure 5). The vector was linearized using restriction enzyme digestion (SfiI) and transformed into the *P. pastoris* strain, generating strain MXY0170.

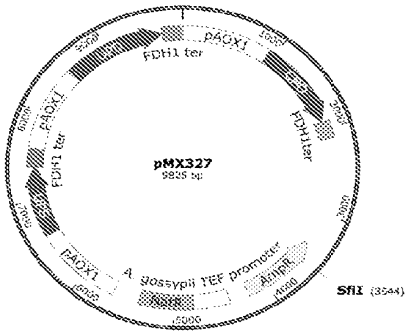


Figure 5: pMX327 plasmid

6) MXY0170 strain

Genes encoding the enzymes ALA synthase- ALAS, Protoporphyrin III oxidase-PPO, Ferrochelatase-FC and Porphobilinogen deaminase- PBD from the *P. pastoris* genome were cloned into one plasmid pMX330, with each under the control of pAOX1 (Figure 6). The vector was linearized using restriction enzyme digestion (SfiI) and transformed into the *P. pastoris* strain, generating strain MXY0183.

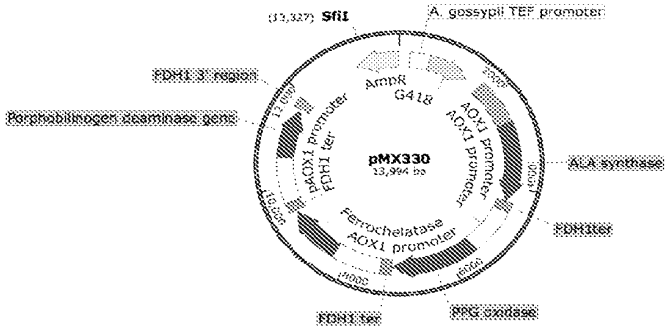


Figure 6: pMX330 plasmid

1(diii) Phenotypic and genotypic changes resulting from modification

Relative to parental strain NRRL-Y11430, strain MXY0183 exhibits the following genotypic changes:

- LegH gene (Figure 7)

Relative to parental strain NRRL-Y11430, strain MXY0183 exhibits the following phenotypic changes:

- Produces LegH (Table 1), higher titer with the additional heme enzymes.

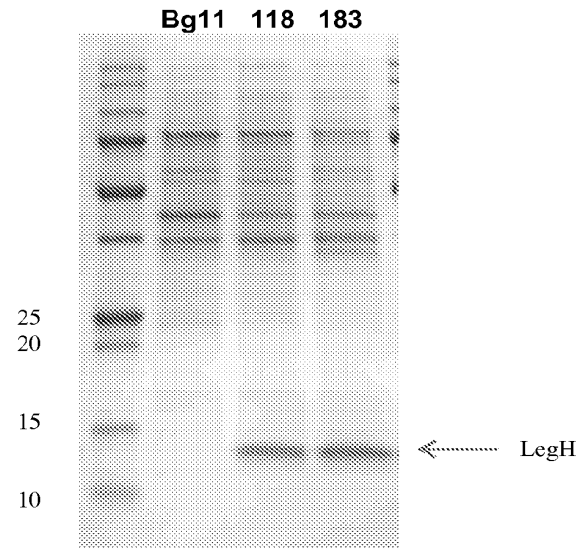


Figure 7. SDS gel showing LegH production from *P. pastoris* strains grown in shake flasks: Bg11 (unmodified host), MXY0118 and MXY0183.

Binding of heme cofactor to LegH is measured using the characteristic Soret peak absorbance (A415), and the ratio of A415/A280 is used to measure the fraction of protein bound to heme. It has been empirically determined that a A415/A280 ratio of greater than 2.5 indicates complete heme-bound LegH. Table 1 shows the improvement in heme binding between intermediate strain MXY0118 and production strain MXY0183, upon introducing the additional modifications discussed in Section 1d(ii) above.

Strain	LegH titer (gl/l)	Heme loading (A415/A280)
MXY0118	1.3	1.4
MXY0183	2	2.6

Table 1: LegH from MXY0118 and MXY0183.

1(div) The stability of the changes referred to herein

In order to ensure that the strain has not lost its ability to produce LegH after fermentation, a sample was taken and analyzed by SDS-gel analysis. Figure 8 shows that the LegH is still present.

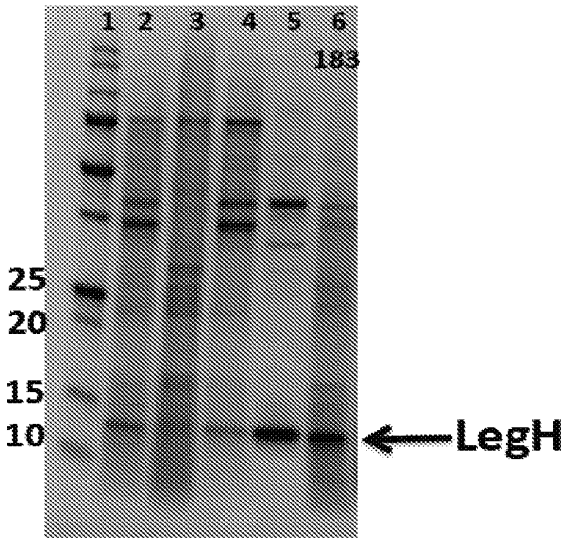


Figure 8. SDS gel showing lysate from MXY0183 grown in 1000 L fermentor for > 120 hrs (lane 6). Note: Lanes 1-5 represent process of purification of LegH from a different strain MXY0121: 1-Lysate, 2- Insoluble cell debris pellet, 3- Load on IEX column, 4- Elution from IEX column (NOTE: These lanes are unrelated to MXY0183- the samples happened to be run on the same gel).

1(dv) The nature, source and function of the inserted genetic material

The nature, source and function of the introduced genetic material in MXY0183 strain are shown in Table 2.

Base position	Size (bp)	Genetic element	Description/function	Origin
pMX229				
133-1071	939	pAOX1 promoter	Promoter of alcohol oxidase	*P. pastoris*
1072-2094	1023	ALA dehydratase	Heme enzyme- step 2	*P. pastoris*
2098-2447	350	AOX1 terminator	Transcription terminator	*P. pastoris*
2459-2837	379	pTEF promoter	Drives expression of antibiotic resistance gene	*Ashbya gossypii*
2838-3212	375	Sh bleomycin	Resistance to Zeocin	*Streptoalloteichus hindustanus*
4272-5132	861	Beta lactamase	Resistance to ampicillin	*E. coli*
pMX282				
133-1071	939	pAOX1 promoter	Promoter of alcohol oxidase	*P. pastoris*
1072-1527	456	LGB2	Leghemoglobin production	Glycine max
1528-1877	350	AOX1 terminator	Transcription terminator	*P. pastoris*
1898-2836	939	pAOX1 promoter	Promoter of alcohol oxidase	*P. pastoris*
2837-3292	456	LGB2	Leghemoglobin production	Glycine max
3293-3642	350	AOX1 terminator	Transcription terminator	*P. pastoris*
3654-4032	379	pTEF promoter	Drives expression of antibiotic resistance gene	*Ashbya gossypii*
4033-5061	1029	Hygromycin	Resistance to hygromycin	*E. coli*
pMX327				
1-939	939	pAOX1	Promoter of alcohol oxidase	*P. pastoris*
941-1915	975	Coproporphyrinogen oxidase	Heme enzyme – step 6	*P. pastoris*

1919-2122	204 bp	FDH1 terminator	Transcription terminator	*P. pastoris*
3551-4211	861	Beta lactamase	Resistance to ampicillin	*E. coli*
4419-4797	379	pTEF promoter	Drives expression of antibiotic resistance gene	*Ashbya gossypii*
4798-5367	570	NatR	Nourseothricin resistance	*Streptomyces noursei*
5650-6582	933	pAOX1	Promoter of alcohol oxidase	*P. pastoris*
6584-7378	795	Uroporphyrinogen III synthase	Heme enzyme- step 4	*P. pastoris*
7382-7585	204 bp	FDH1 terminator	Transcription terminator	*P. pastoris*
7592-8525	934	pAOX1	Promoter of alcohol oxidase	*P. pastoris*
8526-9608	1083	Uroporphyrinogen III decarboxylase	Heme enzyme- step 5	*P. pastoris*
9612-9815	204 bp	FDH1 terminator	Transcription terminator	*P. pastoris*
pMX330				
208-586	379	pTEF promoter	Drives expression of antibiotic resistance gene	*Ashbya gossypii*
587-1396	810	Geniticin (G418) resistance	Resistance to geniticin (G418)	Bacterial transposon Tn5
1701-2632	933	pAOX1 promoter	Promoter of alcohol oxidase	*P. pastoris*
2635-4317	1683	ALA synthase	Heme enzyme- step 1	*P. pastoris*
4318-4521	204 bp	FDH1 terminator	Transcription terminator	*P. pastoris*
4528-5460	933	pAOX1 promoter	Promoter of alcohol oxidase	*P. pastoris*
5462-7141	1680	Protoporhyrin III oxidase	Heme enzyme- step 7	*P. pastoris*
7145-7348	204 bp	FDH1 terminator	Transcription terminator	*P. pastoris*
7355-8288	934	pAOX1 promoter	Promoter of alcohol oxidase	*P. pastoris*
8305-9429	1125	Ferrochelatase	Heme enzyme- step 8	*P. pastoris*
9464-9667	204 bp	FDH1 terminator	Transcription terminator	*P. pastoris*
9684-10617	934	pAOX1 promoter	Promoter of alcohol oxidase	*P. pastoris*
10633-11667	1035	Porphobilinogen deaminase	Heme enzyme- step 3	*P. pastoris*
11702-11905	204 bp	FDH1 terminator	Transcription terminator	*P. pastoris*
13134-13994	861	Beta lactamase	Resistance to ampicillin	*E. coli*

Table 2. Nature, source and function of introduced genetic material in strain MXY0183 from each plasmid transformed into the strain i.e. pMX229, pMX282, pMX327 and pMX330.

1(e) a description of the methods that can be used to distinguish and detect the microorganism

When grown in the presence of methanol, which is an inducer of pAOX1 promoter, MXY0183 produces recombinant heme-bound LegH. As a result, the strain turns red in color within 24 hr post-induction both on agar plates containing methanol and in liquid cultures.

1(f) biological and ecological characteristics of the micro-organism

1(fi) The conditions required for, and conditions that limit survival, growth and replication

Optimum growth conditions for Strain MXY0183 include a target pH of 3.0 to 6.0 and temperatures of 28-35°C.

Ingredients required for growth and replication in submerged liquid cultures under aerophilic conditions are summarized in Table 3.

Salts	Glycerol (batch phase)
	Ammonium sulfate

	Potassium phosphate monobasic
	Calcium sulfate dihydrate
	Potassium sulfate
	Magnessium sulfate heptahydrate
	Sodium citrate dihydrate
	Soytone (Teknova)
Trace metals	Cupric sulfate-5H2O
	Sodium iodide
	Manganese sulfate monohydrate
	Sodium molybdatc dihyratc
	Boric acid
	Cobalt chloride hexahydrate
	Zinc chloride
	Ferrous sulfate heptahydrate
	Sulfuric acid
Vitamins	biotin
	calcium pantothenate
	folic acid
	Myoinositol
	nicotinic acid
	p-aminobenzoic acid
	Pyridoxine hydrochloride
	riboflavin
	thiamine hydrochloride
	vitamin B12 (cabalamin)
Feed	56% w/w Glycerol feed
	25% w/w Methanol feed
	30% w/w NH4OH for pH control
	204 antifoam

Table 3: Typical Fermentation Media

1(g) Description of the mode of action in relation to the intended use.

The intended use of strain MXY0183 is to produce commercial quantities of LegH in a contained facility with a capacity greater than 1000 L. In order to produce the protein, strain MXY0183 must be alive and growing aerobically for a period of 6 days.

2. Information in respect of the introduction of the micro-organism

2(a) The intended and potential uses

P. pastoris strain MXY0183 is intended for use solely as an intermediate in the production of a LegH enzyme in a contained fermentation facility greater than 1000 L in size.

2(b) The history of use

P. pastoris has been used as a production organism safely for several years. Enzyme products produced from *P. pastoris* have been used as food and feed additives. The parental strain NRRL Y-11430 was also the progenitor strain for GS115 (Cregg, 1985), a common *Pichia* strain provided in commercial kits by Invitrogen Corporation, and widely used as the parental

strain of many biotechnology products, including FDA approved proteins such as Kalibitor® (ecallantide, for the treatment of acute attacks of hereditary angioedema).

3. All other information

Production strain MXY0183 contains the following antibiotic resistance genes that were introduced for the purpose of strain construction only.

- Zeocin^R
- Hygromycin^R
- Nourseothricin^R
- Geniticin(G418)^R
- Ampicillin^R

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EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

TECHNICAL FIELD

This disclosure generally relates to DNA constructs and methods of using such DNA constructs to genetically engineer methylotrophic yeast.

BACKGROUND

Methylotrophic yeast such as *Pichia pastoris* are commonly used for expression of recombinant proteins. Constructs that can be used to efficiently express one or more polypeptides in a methylotrophic yeast are provided herein.

SUMMARY

This disclosure describes the use of *P. pastoris* strains that overexpress the transcriptional activator, Mxr1, from the AOX1 promoter to increase expression of transgenes that also are expressed from the AOX1 promoter, which significantly improves the recombinant production of one or more proteins. In addition, expression of Mxr1 from the AOX1 promoter creates a positive feedback loop that allows for expression of other transgenes from the AOX1 promoter in the absence of methanol, the normally obligate inducer, when repressing carbon sources are depleted. Expression of Mxr1 results in a significant increase in the amount of protein produced.

In one aspect, a methylotrophic yeast cell is provided that includes a recombinant nucleic acid molecule. The recombinant nucleic acid molecule typically includes an exogenous nucleic acid encoding a transcriptional activator operably linked to at least one methanol-inducible promoter element. Representative methylotrophic yeast can be of the genus *Candida*, *Hansenula*, *Pichia* or *Toruplosis*. A representative methylotrophic yeast is *Pichia pastoris*.

In some embodiments, the recombinant nucleic acid molecule is stably integrated into the genome of the methylotrophic yeast cell. In some embodiments, the recombinant nucleic acid molecule is extrachromosomally expressed from a replication-competent plasmid.

In some embodiments, the exogenous nucleic acid encoding a transcriptional activator comprises a Mxr1 sequence from *Pichia pastoris*, a Adr1 sequence from *Hansenula polymorpha*,

a Trm1 sequence from *Candida boidinii*, and a Trm2 sequence from *Candida boidinii*. A representative nucleic acid encoding a transcriptional activator is shown in DQ395124. A representative transcriptional activator has an amino acid sequence shown in ABD57365.

In some embodiments, the at least one methanol-inducible promoter element is an alcohol oxidase 1 (AOX1) promoter element from *Pichia pastoris*, an AOD1 promoter element from *Candida boidinii*, a MOX promoter element from *Hansenula polymorpha*, a MOD1 promoter element from *Pichia methanolica*, a DHAS promoter element from *Pichia pastoris*, a FLD1 promoter element from *Pichia pastoris*, or a PEX8 promoter element from *Pichia pastoris*.

In some embodiments, the methylotrophic yeast cell further includes a nucleic acid molecule that includes at least one heterologous nucleic acid encoding a polypeptide operably linked to at least one methanol-inducible promoter element. In some embodiments, the at least one heterologous nucleic acid encodes one or more polypeptides involved in the biosynthesis of an iron co-factor such as heme (e.g., ALA synthase, ALA dehydratase, porphogilinogen deaminase, UPG III synthase, UPG III decarboxylase, CPG oxidase, PPG oxidase, and/or ferrochelatase). In some embodiments, one or more of the polypeptides involved in the biosynthesis of the iron co-factor are linked to at least one methanol-inducible promoter element.

In another aspect, a method for expressing a heterologous polypeptide in a cell is provided. Such a method typically includes providing a methylotrophic yeast cell as described herein; introducing a recombinant nucleic acid molecule into methylotrophic yeast cell, the recombinant nucleic acid molecule comprising at least one heterologous nucleic acid encoding a polypeptide operably linked to at least one *Pichia pastoris* alcohol oxidase 1 (AOX1) promoter element; and culturing the cell under conditions suitable for expression of the recombinant nucleic acid molecule, thereby expressing the heterologous polypeptide.

In some embodiments, the conditions under which the cells are cultured includes the addition of iron or a pharmaceutically or metabolically acceptable salt thereof. In some embodiments, the introducing step includes a technique such as transduction, electroporation, biolistic particle delivery, or chemical transformation. In some embodiments, the culturing step includes culturing the cell in the present of methanol.

In another aspect, a recombinant organism is provided that includes a transcriptional activator operably linked to the promoter it activates. In some embodiments, a recombinant organism is provided that expresses a polypeptide operably linked to the promoter. In yet another

aspect, a method of expressing a polypeptide from an inducible promoter without addition of an inducer is provided as described herein.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the methods and compositions of matter belong. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the methods and compositions of matter, suitable methods and materials are described below. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

DESCRIPTION OF DRAWINGS

Figure 1 is a schematic depicting the steps involved in the heme biosynthesis pathway.

Figure 2 are schematics of plasmids used in the construction of production strain MXY0183.

Figure 3 is a schematic showing the generation of the production strains, MXY0183 and MXY0207, from the parent strain, Bg11.

Figure 4 are schematics showing plasmids pGAB and pMx354.

Figure 5 is a schematic showing the generation of the antibiotic selection free production strains, MXY0291 and MXY0338, from the parent strain, Bg11.

Figure 6 is a schematic of the linear pieces of DNA containing Mxr1 and LegH var 3 that were introduced by co-transformation to make the production strain MXY0291.

Figure 7 is a schematic showing the linear construct expressing LegH under control of native *Pichia* non-pAOX1 constitutive promoters.

Figure 8 is a photograph showing the phenotypic changes associated with strain, MXY0183. Shake flasks at the start of induction (0 hr) and 72 hr post-induction are shown. 1, MXY0051; 2, MXY0118; 3, MXY0183.

Figure 9 shows the production of LegH from the modified *P. pastoris* strains. Panel A is a SDS gel showing lysates from *P. pastoris* strains grown in shake flasks: 51, MXY0051; 118,

MXY0118; 183, MXY0183. Panel B is a table comparing LegH production from strains MXY0118, MXY0183, and MXY0207.

Figure 10 shows data from experiments with strain MXY0206. Panel A is a photograph of shake flask cultures of strains MXY0183 (left) and MXY0206 (right) after 48 hr of growth in repressing carbon source. Panel B is a photograph of cell pellets from shake flask cultures of strains MXY0183 (left) and MXY0206 (right) after 48 hr of growth in BMY media. Panel C is a graph showing the relative yield of heme-loaded LegH (in the absence of any induction agent).

Figure 11 is a summary table showing relative yields of strains described herein when grown in the presence of methanol with glycerol or methanol with glucose in 2 L fermenter tanks.

DETAILED DESCRIPTION

Nucleic acid constructs are provided herein that allow for genetically engineering a cell to increase the recombinant expression of a polypeptide. In some embodiments, nucleic acid constructs are provided herein that allow for genetically engineering a cell to increase the recombinant expression of a polypeptide from an inducible promoter in the absence of the inducing molecule. Without being bound by any particular mechanism, the methods described herein create a positive feedback loop where the low level native expression of a transcriptional activator induces a promoter that is operably linked to a transcriptional activator. This leads to an increased expression of the transcriptional activator as well as one or more target polypeptides that are operably linked to the same inducible promoter.

Nucleic acid constructs are provided herein that allow for genetically engineering a methylotrophic yeast cell. While the methods are exemplified herein using a *Pichia* species (i.e., *P. pastoris*), other species of the *Pichia* genus can be used or species from any of the *Candida*, *Hansenula*, *Pichia* and *Torulopsis* genera.

Genetically engineering a methylotrophic yeast cell typically includes introducing a recombinant nucleic acid molecule into the cell. As described herein, a recombinant nucleic acid molecule typically includes an exogenous nucleic acid that encodes a transcriptional activator operably linked to at least one inducible promoter element.

Recombinant nucleic acid molecules used in the methods described herein are typically DNA, but RNA molecules can be used under the appropriate circumstances. As used herein, "exogenous" refers to any nucleic acid sequence that is introduced into the genome of a cell from

an external source, where the external source can be the same or a different organism or a nucleic acid generated synthetically. For example, an exogenous nucleic acid can be a nucleic acid from one microorganism (e.g., one genus or species of methylotrophic yeast) that is introduced into a different genus or species of methylotrophic yeast, however, an exogenous nucleic acid also can be a nucleic acid from a methylotrophic yeast that is introduced recombinantly into a methylotrophic yeast as an additional copy despite the presence of a corresponding native nucleic acid sequence. For example, *P. pastoris* contains an endogenous nucleic acid encoding a Mxr1 transcriptional activator; an additional *P. pastoris* Mxr1 nucleic acid (e.g., introduced recombinantly into *P. pastoris*) or modifying the endogenous *P. pastoris* Mxr1 nucleic acid is considered exogenous.

Transcriptional activators, and nucleic acids encoding transcriptional activators (e.g., exogenous nucleic acids encoding transcriptional activators), are known in the art. For example, a transcriptional activator from *Pichia pastoris* is the Mxr1 sequence, but suitable transcriptional activators also can be found in *Hansenula polymorpha* (the Adr1 sequence; see, for example, GenBank Accession No. AEOI02000005, bases 858873 to 862352, for the nucleic acid sequence and GenBank Accession No. ESX01253 for the amino acid sequence) and *Candida boidinii* (the Trm1 sequence; see, for example, GenBank Accession No. AB365355 for the nucleic acid sequence and GenBank Accession No. BAF99700 for the amino acid sequence; the Trm2 sequence; see, for example, GenBank Accession No. AB548760 for the nucleic acid sequence and GenBank Accession No. BAJ07608 for the amino acid sequence). A representative *P. pastoris* Mxr1 nucleic acid sequence can be found, for example, in GenBank Accession No. DQ395124, while a representative *P. pastoris* Mxr1 polypeptide sequence can be found, for example, in GenBank Accession No. ABD57365.

Transcriptional activators such as Mxr1 may be normally expressed at low levels. Therefore, it is desirable to place the exogenous nucleic acid (i.e., the transcriptional activator) under control of a promoter that is inducible. As used herein, "operably linked" means that a promoter or other expression element(s) are positioned relative to a nucleic acid coding sequence in such a way as to direct or regulate expression of the nucleic acid (e.g., in-frame).

There are a number of inducible promoters that can be used when genetically engineering methylotrophic yeast. For example, a methanol-inducible promoter, or a promoter element therefrom, can be used. Methanol inducible promoters are known in the art. For example, a

commonly used methanol-inducible promoter from *P. pastoris* is the promoter, or a portion thereof, from the alcohol oxidase 1 (AOX1) gene, which is strongly transcribed in response to methanol. Other methanol-inducible promoters, or promoter elements therefrom, however, can be used, including, without limitation, the alcohol oxidase (AOD1) promoter from *Candida boidinii* (see, for example, GenBank Accession No. YSAAOD1A), the alcohol oxidase (MOX) promoter from *Hansenula polymorpha* (see, for example, GenBank Accession No. X02425), the MOD1 or MOD2 promoter from *Pichia methanolica* (see, for example, Raymond et al., 1998, Yeast, 14:11-23; and Nakagawa et al., 1999, Yeast, 15:1223-30), the DHAS promoter from *P. pastoris* (see, for example, GenBank Accession No. FJ752551) or a promoter element therefrom, the formaldehyde dehydrogenase (FLD1) promoter from *Pichia pastoris* (see, for example, GenBank Accession No. AF066054), or the PEX8 promoter from *P. pastoris* (see, for example, Kranthi et al., 2010, Yeast, 27:705-11). In some embodiments, the transcriptional activator is a Mit1 sequence from *Pichia pastoris* (see, for example, GenBank Accession No. CAY70887). All of these promoters are known to be induced by methanol.

A skilled artisan would understand that the recombinant nucleic acid molecule described herein can be stably integrated into the genome of the methylotrophic yeast cell, or can be extrachromosomally expressed from a replication-competent plasmid. Methods of achieving both are well known and routinely used in the art.

As demonstrated herein, the methanol-regulated transcriptional activators in *Pichia* can bind to the AOX1 promoter and act cooperatively with Mxr1 to activate transcription from the AOX1 promoter. In some embodiments, two methanol-regulated transcriptional activators (e.g., Mxr1 and Mit1) can be operably linked to a methanol inducible promoter element.

A strain that includes a recombinant nucleic acid molecule as described herein can be used to regulate (e.g., overexpress) a second recombinant nucleic acid molecule in the methylotrophic yeast cell. A second recombinant nucleic acid molecule can include, for example, one or more heterologous nucleic acids encoding one or more polypeptides of interest. Similar to the exogenous nucleic acid encoding the transcriptional activator, a heterologous nucleic acid refers to any nucleic acid sequence that is not native to the genome or in the genome of an organism (e.g., a heterologous nucleic acid can be a nucleic acid from one microorganism (e.g., one genus or species of methylotrophic yeast) that is introduced into a different genus or species of methylotrophic yeast).

Simply by way of example, heterologous nucleic acids encoding the one or more polypeptides of interest can be the nucleic acids involved in the biosynthesis of a heme-co-factor. Exemplified herein are nucleic acids encoding the 8 different enzymes involved in heme biosynthesis as determined and annotated from the sequence of the *Pichia pastoris* genome. For example, heterologous nucleic acids encoding ALA synthase, ALA dehydratase, porphobilinogen deaminase, UPG III synthase, UPG III decarboxylase, CPG oxidase, PPG oxidase, and ferrochelatase can be expressed in the methylotrophic yeast strains described herein. For genetically engineering methylotrophic yeast to contain more than one heterologous nucleic acids (e.g., transgenes), a combination of methanol-inducible and constitutive promoters, or elements therefrom, can be combined to further increase the expression of such nucleic acids.

Previous studies in *Saccharomyces cerevisiae* identified ALA dehydratase and porphobilinogen deaminase as rate limiting enzymes in heme biosynthesis (see, for example, Hoffman et al., 2003, Biochem. Biophys. Res. Commun., 310(4):1247-53). However, heterologous expression of individual heme enzymes in *P. pastoris* from the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter failed to overcome limitations associated with the expression of a recombinant protein containing a heme co-factor (see Krainer et al., 2015, Microb. Cell Fact., 13;14:4). As described herein, highly efficient expression of a recombinant heme containing protein in *P. pastoris* was achieved by co-expressing the entire heme biosynthetic pathway from methanol-inducible promoters, although it would be appreciated that one or more of the genes involved in the heme biosynthetic pathway could be expressed from one or more constitutive promoters.

In addition to the enzymes involved in iron-co-factor biosynthesis, it would be understood that a nucleic acid encoding a member of the globin family of proteins (PF00042 in the Pfam database) including plant hemoglobins can be present. In the Examples herein, a nucleic acid encoding soybean leghemoglobin (LegH) is present. LegH is a protein that binds to the iron co-factor, heme, which results in a characteristic absorption at 415 nm and a distinct red color. The LegH protein (also known as LGB2) is naturally found in root nodules of soybean (see, for example, UniprotKB Accession No. P02236), and the nucleic acid sequence used herein was codon optimized for expression in *P. pastoris*. See, for example, WO 2014/110539 and WO 2014/110532.

Alternatively, a heterologous nucleic acid encoding a polypeptide of interest can be, for example and without limitation, a dehydrin, a phytase, a protease, a catalase, a lipase, a peroxidase, an amylase, a transglutaminase, an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, a ligase, or an antibody against any such polypeptides. In other embodiments, a heterologous nucleic acid can encode one or more enzymes involved in the pathway for production of small molecules, such as ethanol, lactic acid, butanol, adipic acid, or succinic acid.

Similar to the exogenous nucleic acid encoding the transcriptional activator, the heterologous nucleic acid encoding a polypeptide of interest can be operably linked to an inducible promoter element (e.g., a methanol-inducible promoter element), or the heterologous nucleic acid encoding a polypeptide of interest can be operably linked to a constitutive promoter or constitutive promoter element. Inducible promoters and elements therefrom are discussed above. Constitutive promoters and constitutive promoter elements are known in the art. For example, a commonly used constitutive promoter from *P. pastoris* is the promoter, or a portion thereof, from the transcriptional elongation factor EF-1α gene (TEF1), which is strongly transcribed in a constitutive manner. Other constitutive promoters, or promoter elements therefrom, however, can be used, including, without limitation, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter from *P. pastoris* (see, for example, GenBank Accession No. U62648.1), the promoter from the potential glycosyl phosphatidyl inositol (GPI)-anchored protein, GCW14p (PAS_chr1-4_0586), from *P. pastoris* (see, for example, GenBank Accession No. XM_002490678), or the promoter from the 3-phosphoglycerate kinase gene (PGK1) from *P. pastoris* (see, for example, GenBank Accession No. AY288296).

Similar to the recombinant nucleic acid molecule described herein, the second recombinant nucleic acid molecule can be stably integrated into the genome of the methylotrophic yeast cell, or can be extrachromosomally expressed from a replication-competent plasmid.

It would be understood by the skilled artisan that a combination of inducible (e.g., methanol-inducible) and constitutive promoters (or promoter elements therefrom) can be combined to further increase the expression of any of the nucleic acids operably linked thereto.

It would be appreciated by a skilled artisan that a heterologous nucleic acid encoding a polypeptide of interest operably linked to a promoter element can be separate from the recombinant nucleic acid molecule described herein, or can be contiguous with the exogenous nucleic acid encoding a transcriptional activator operably linked to a promoter element contained

within the recombinant nucleic acid molecule described herein. It also would be appreciated by a skilled artisan that, if the second nucleic acid molecule is contiguous with the recombinant nucleic acid molecule described herein, that a single promoter, or promoter element therefrom, can be used to drive transcription of both or all of the genes (e.g., the exogenous nucleic acid encoding the transcriptional activator as well as the one or more heterologous nucleic acids encoding the polypeptide(s) of interest).

Methods of introducing nucleic acids into methylotrophic yeast cells are known in the art, and include, without limitation, transduction, electroporation, biolistic particle delivery, and chemical transformation.

In addition, methods of culturing methylotrophic yeast cells are known in the art. See, for example, Pichia Protocols, *Methods In Molecular Biology*, 389, Cregg, Ed., 2007, 2nd Ed., Humana Press, Inc. Under some circumstances, it may be desirable to introduce or add methanol to the culture media, although, as demonstrated herein, methanol is not required to obtain efficient expression at high levels of one or more polypeptides of interest. Under some circumstances (e.g., when one or more nucleic acids encoding enzyme(s) involved in an iron-co-factor biosynthesis are expressed), it may be desirable to supplement the culture media with iron or a pharmaceutically or metabolically acceptable (or GRAS) salt thereof.

Pichia strains are able to grow on methanol as the sole carbon source. Methanol utilization is initiated by the conversion of methanol to formaldehyde by the action of alcohol oxidase. The methylotrophic yeast, *Pichia pastoris*, contains two genes for alcohol oxidases, AOX1 and AOX2. Strains with reduced alcohol oxidase activity ("methanol utilization slow" or MutS strains) often produce more of a recombinant protein expressed from the AOX1 promoter than strains that do not have reduced alcohol oxidase activity. Strains mutated in both AOX genes and completely lacking alcohol oxidase activity cannot metabolize methanol, but can still be induced for expression from the AOX1 promoter by methanol. These strains retain the ability to use other carbon sources for growth, but still express heterologous proteins from the AOX1 promoter upon the addition of methanol. Because these strains do not metabolize methanol ("methanol utilization minus" or Mut- strains), much less methanol is required for induction of protein expression, and strains carrying these mutations avoid issues related to methanol feeding in large-scale fermentations. See, for example, Chiruvolu et al., 1997, Enzyme Microb. Technol., 21:277-83. It was determined herein that expression of LegH from the AOX1 promoter in Mut-

strains greatly improved the LegH yield. Thus, a methylotrophic yeast having a mutation in both the AOX1 gene and the AOX2 gene can be used in the methods described herein.

The protein of interest, or a complex that includes one or more proteins of interest (e.g., heme-bound LegH, a dehydrin, a phytase, a protease a catalase, a lipase, a peroxidase, an amylase, a transglutaminase, an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, a ligase, or an antibody) can be purified from the yeast cells. Methods of purifying polypeptides are known in the art. As used herein, a "purified" polypeptide is a polypeptide that has been separated or purified from cellular components that naturally accompany it. Typically, the polypeptide is considered "purified" when it is at least 70% (e.g., at least 75%, 80%, 85%, 90%, 95%, or 99%) by dry weight, free from the polypeptides and naturally occurring molecules with which it is naturally associated. Since a polypeptide that is chemically synthesized is, by nature, separated from the components that naturally accompany it, a synthetic polypeptide is "purified."

As used herein, nucleic acids can include DNA and RNA, and includes nucleic acids that contain one or more nucleotide analogs or backbone modifications. A nucleic acid can be single stranded or double stranded, which usually depends upon its intended use. Also provided are nucleic acids and polypeptides that differ from a given sequence. Nucleic acids and polypeptides can have at least 50% sequence identity (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity) to a given nucleic acid or polypeptide sequence.

In calculating percent sequence identity, two sequences are aligned and the number of identical matches of nucleotides or amino acid residues between the two sequences is determined. The number of identical matches is divided by the length of the aligned region (i.e., the number of aligned nucleotides or amino acid residues) and multiplied by 100 to arrive at a percent sequence identity value. It will be appreciated that the length of the aligned region can be a portion of one or both sequences up to the full-length size of the shortest sequence. It also will be appreciated that a single sequence can align with more than one other sequence and hence, can have different percent sequence identity values over each aligned region.

The alignment of two or more sequences to determine percent sequence identity can be performed using the computer program ClustalW and default parameters, which allows alignments of nucleic acid or polypeptide sequences to be carried out across their entire length (global alignment). Chenna et al., 2003, Nucleic Acids Res., 31(13):3497-500. ClustalW

calculates the best match between a query and one or more subject sequences, and aligns them so that identities, similarities and differences can be determined. Gaps of one or more residues can be inserted into a query sequence, a subject sequence, or both, to maximize sequence alignments. For fast pairwise alignment of nucleic acid sequences, the default parameters can be used (i.e., word size: 2; window size: 4; scoring method: percentage; number of top diagonals: 4; and gap penalty: 5); for an alignment of multiple nucleic acid sequences, the following parameters can be used: gap opening penalty: 10.0; gap extension penalty: 5.0; and weight transitions: yes. For fast pairwise alignment of polypeptide sequences, the following parameters can be used: word size: 1; window size: 5; scoring method: percentage; number of top diagonals: 5; and gap penalty: 3. For multiple alignment of polypeptide sequences, the following parameters can be used: weight matrix: blosum; gap opening penalty: 10.0; gap extension penalty: 0.05; hydrophilic gaps: on; hydrophilic residues: Gly, Pro, Ser, Asn, Asp, Gln, Glu, Arg, and Lys; and residue-specific gap penalties: on. ClustalW can be run, for example, at the Baylor College of Medicine Search Launcher website or at the European Bioinformatics Institute website on the World Wide Web.

Changes can be introduced into a nucleic acid molecule, thereby leading to changes in the amino acid sequence of the encoded polypeptide. For example, changes can be introduced into nucleic acid coding sequences using mutagenesis (e.g., site-directed mutagenesis, PCR-mediated mutagenesis) or by chemically synthesizing a nucleic acid molecule having such changes. Such nucleic acid changes can lead to conservative and/or non-conservative amino acid substitutions at one or more amino acid residues. A "conservative amino acid substitution" is one in which one amino acid residue is replaced with a different amino acid residue having a similar side chain (see, for example, Dayhoff et al. (1978, in Atlas of Protein Sequence and Structure, 5(Suppl. 3):345-352), which provides frequency tables for amino acid substitutions), and a non-conservative substitution is one in which an amino acid residue is replaced with an amino acid residue that does not have a similar side chain. Nucleic acid and/or polypeptide sequences may be modified as described herein to improve one or more properties including, without limitation, increased expression (e.g., transcription and/or translation), tighter regulation, deregulation, loss of catabolite repression, modified specificity, secretion, thermostability, solvent stability, oxidative stability, protease resistance, catalytic activity, and/or color.

As used herein, an "isolated" nucleic acid molecule is a nucleic acid molecule that is free of sequences that naturally flank one or both ends of the nucleic acid in the genome of the

organism from which the isolated nucleic acid molecule is derived (e.g., a cDNA or genomic DNA fragment produced by PCR or restriction endonuclease digestion). Such an isolated nucleic acid molecule is generally introduced into a vector (e.g., a cloning vector, or an expression vector) for convenience of manipulation or to generate a fusion nucleic acid molecule, discussed in more detail below. In addition, an isolated nucleic acid molecule can include an engineered nucleic acid molecule such as a recombinant or a synthetic nucleic acid molecule.

Nucleic acids can be isolated using techniques routine in the art. For example, nucleic acids can be isolated using any method including, without limitation, recombinant nucleic acid technology, and/or the polymerase chain reaction (PCR). General PCR techniques are described, for example in PCR Primer: A Laboratory Manual, Dieffenbach & Dveksler, Eds., Cold Spring Harbor Laboratory Press, 1995. Recombinant nucleic acid techniques include, for example, restriction enzyme digestion and ligation, which can be used to isolate a nucleic acid. Isolated nucleic acids also can be chemically synthesized, either as a single nucleic acid molecule or as a series of oligonucleotides.

Polypeptides can be purified from natural sources (e.g., a biological sample) by known methods such as DEAE ion exchange, gel filtration, and hydroxyapatite chromatography. A polypeptide also can be purified, for example, by expressing a nucleic acid in an expression vector. In addition, a purified polypeptide can be obtained by chemical synthesis. The extent of purity of a polypeptide can be measured using any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

A construct or vector containing a nucleic acid (e.g., a nucleic acid that encodes a polypeptide) also is provided. Constructs or vectors, including expression constructs or vectors, are commercially available or can be produced by recombinant DNA techniques routine in the art. A construct or vector containing a nucleic acid can have expression elements operably linked to such a nucleic acid, and further can include sequences such as those encoding a selectable marker (e.g., an antibiotic resistance gene). A construct or vector containing a nucleic acid can encode a chimeric or fusion polypeptide (i.e., a polypeptide operatively linked to a heterologous polypeptide, which can be at either the N-terminus or C-terminus of the polypeptide). Representative heterologous polypeptides are those that can be used in purification of the encoded polypeptide (e.g., 6xHis tag, glutathione S-transferase (GST))

Expression elements include nucleic acid sequences that direct and regulate expression of nucleic acid coding sequences. One example of an expression element is a promoter sequence. Expression elements also can include introns, enhancer sequences, response elements, or inducible elements that modulate expression of a nucleic acid. Expression elements can be of bacterial, yeast, insect, mammalian, or viral origin, and vectors can contain a combination of elements from different origins.

Vectors as described herein can be introduced into a host cell. As used herein, "host cell" refers to the particular cell into which the nucleic acid is introduced and also includes the progeny of such a cell that carry the vector. A host cell can be any prokaryotic or eukaryotic cell. For example, nucleic acids can be expressed in bacterial cells such as *E. coli*, or in insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art. Many methods for introducing nucleic acids into host cells, both *in vivo* and *in vitro*, are well known to those skilled in the art and include, without limitation, electroporation, calcium phosphate precipitation, polyethylene glycol (PEG) transformation, heat shock, lipofection, microinjection, and viral-mediated nucleic acid transfer.

Nucleic acids can be detected using any number of amplification techniques (see, e.g., PCR Primer: A Laboratory Manual, 1995, Dieffenbach & Dveksler, Eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; and U.S. Patent Nos. 4,683,195; 4,683,202; 4,800,159; and 4,965,188) with an appropriate pair of oligonucleotides (e.g., primers). A number of modifications to the original PCR have been developed and can be used to detect a nucleic acid.

Nucleic acids also can be detected using hybridization. Hybridization between nucleic acids is discussed in detail in Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Sections 7.37-7.57, 9.47-9.57, 11.7-11.8, and 11.45-11.57). Sambrook et al. discloses suitable Southern blot conditions for oligonucleotide probes less than about 100 nucleotides (Sections 11.45-11.46). The Tm between a sequence that is less than 100 nucleotides in length and a second sequence can be calculated using the formula provided in Section 11.46. Sambrook et al. additionally discloses Southern blot conditions for oligonucleotide probes greater than about 100 nucleotides (see Sections 9.47-9.54). The Tm between a sequence greater than 100 nucleotides in length and a

second sequence can be calculated using the formula provided in Sections 9.50-9.51 of Sambrook et al.

The conditions under which membranes containing nucleic acids are prehybridized and hybridized, as well as the conditions under which membranes containing nucleic acids are washed to remove excess and non-specifically bound probe, can play a significant role in the stringency of the hybridization. Such hybridizations and washes can be performed, where appropriate, under moderate or high stringency conditions. For example, washing conditions can be made more stringent by decreasing the salt concentration in the wash solutions and/or by increasing the temperature at which the washes are performed. Simply by way of example, high stringency conditions typically include a wash of the membranes in 0.2X SSC at 65°C.

In addition, interpreting the amount of hybridization can be affected, for example, by the specific activity of the labeled oligonucleotide probe, by the number of probe-binding sites on the template nucleic acid to which the probe has hybridized, and by the amount of exposure of an autoradiograph or other detection medium. It will be readily appreciated by those of ordinary skill in the art that although any number of hybridization and washing conditions can be used to examine hybridization of a probe nucleic acid molecule to immobilized target nucleic acids, it is more important to examine hybridization of a probe to target nucleic acids under identical hybridization, washing, and exposure conditions. Preferably, the target nucleic acids are on the same membrane.

A nucleic acid molecule is deemed to hybridize to a nucleic acid but not to another nucleic acid if hybridization to a nucleic acid is at least 5-fold (e.g., at least 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 50-fold, or 100-fold) greater than hybridization to another nucleic acid. The amount of hybridization can be quantitated directly on a membrane or from an autoradiograph using, for example, a PhosphorImager or a Densitometer (Molecular Dynamics, Sunnyvale, CA).

Polypeptides can be detected using antibodies. Techniques for detecting polypeptides using antibodies include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence. An antibody can be polyclonal or monoclonal. An antibody having specific binding affinity for a polypeptide can be generated using methods well known in the art. The antibody can be attached to a solid support such as a microtiter plate

using methods known in the art. In the presence of a polypeptide, an antibody-polypeptide complex is formed.

Detection (e.g., of an amplification product, a hybridization complex, or a polypeptide) is usually accomplished using detectable labels. The term "label" is intended to encompass the use of direct labels as well as indirect labels. Detectable labels include enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials.

Methods are described herein that can be used to generate a strain that lacks sequences for selection (i.e., that lacks a selectable marker). These methods include using a circular plasmid DNA vector and a linear DNA sequence; the circular plasmid DNA vector contains a selection marker and an origin of DNA replication (also known as an autonomously replicating sequence (ARS)), and the linear DNA sequence contains sequences for integration into the *Pichia* genome by homologous recombination. The linear DNA molecule additionally can include nucleic acid sequences encoding one or more proteins of interest such as, without limitation, heme-bound LegH, a dehydrin, a phytase, a protease a catalase, a lipase, a peroxidase, an amylase, a transglutaminase, an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, a ligase, one or more enzymes involved in the pathway for production of small molecules, such as ethanol, lactic acid, butanol, adipic acid or succinic acid, or an antibody against any such proteins.

Pichia cells can be transformed with both DNA molecules and the transformants selected by the presence of the selectable marker on the circular plasmid. Transformants then can be screened for integration of the linear DNA molecule into the genome using, for example, PCR. Once transformants with the correct integration of the marker-free linear DNA molecule are identified, the cells can be grown in the absence of selection for the circular plasmid. Because the marker-bearing plasmid is not stably maintained in the absence of selection, the plasmid is lost, often very quickly, after selection is relaxed. The resulting strain carries the integrated linear DNA in the absence of heterologous sequences for selection. Therefore, this approach can be used to construct *Pichia* strains that lack a selectable marker (e.g., a heterologous selection marker) with little to no impact on recombinant protein yield.

In accordance with the present invention, there may be employed conventional molecular biology, microbiology, biochemical, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. The invention will be further described in

the following examples, which do not limit the scope of the methods and compositions of matter described in the claims.

EXAMPLES

PART A. Materials and Methods

Example 1—Polymerase Chain Reaction

Genes of interest were amplified from genomic DNA or plasmid DNA templates using Phusion Hi-fidelity DNA polymerase (New England Biolabs). Briefly, 0.6 μM each of forward and reverse primers are incubated with 10-50 ng of template DNA and 400 μM of nucleotide mix in the presence of 1-2 U of Phusion DNA polymerase. The reaction conditions were as follows:

1 cycle	Initial Denaturation	98°C	1min
25 cycles	Denaturation	98°C	10 sec
	Annealing		20 sec
	Extension	72°C	30 sec per kb
1 cycle	Final Extension	72°C	5min
1 cycle	Hold	4°C	Forever

Example 2—Plasmid Construction by Ligation

50-100 ng of restriction enzyme digested plasmid and 3X molar excess of PCR amplified inserts were incubated in the presence of T4 DNA ligase (New England Biolabs). Ligation was carried out at 16°C for greater than 2 hr. 2 μl of ligation reaction was transformed into DH10B electrocompetent *E. coli* cells

Example 3—Transformation into *E. coli* ElectroMax DH10B T1 Phage-Resistant Competent Cells

1.5-2 μl of ligation mixture was transformed into 20 μl of ElectroMax DH10B T1 Phage-Resistant Competent Cells (Invitrogen, Cat # 12033-015) by electroporation using MicroPulser (BioRad) set at 1.7 kV using a 1 mm gap cuvette (BioRad, Cat # 165-2089); after a pulse 1 ml SOC was added to cells and cells were incubated at 37°C for 1 h with shaking at 200 rpm. 10 μl of recovery mixture was plated on LB agar plates containing ampicillin at a concentration of 100 μg/ml. Plates were incubated overnight at 37°C.

Example 4—Linearization of Plasmid DNA for Transformation into *P. pastoris*

Plasmid DNA was digested with either PmeI restriction endonuclease (New England BioLabs, Cat # R0560L) in 1x CutSmart Buffer for 1-4 hours at 37°C or SfiI restriction endonuclease in 1x CutSmart Buffer for 1-4 hours at 50°C (New England BioLabs, Cat # R0123L). Linearized plasmid was gel purified from a 0.8% agarose gel using Zymoclean Gel DNA Recovery Kit (Zymo Research, Cat # D4002). DNA was eluted in 20 µl H_2O.

Example 5—Preparation of *P. pastoris* Transformation-Competent Cells

Selected strains of *P. pastoris* were grown to mid-exponential growth phase (~2 OD) in 25 ml YPD medium. Cells were collected by centrifugation at 930 x g for 15 minutes. The cell pellet was resuspended in 2 ml of a solution of 80% YPD and 200 mM HEPES, pH 6.8. 75 µl of 1 M DTT was added. The resuspended cell pellet was mixed at 100 rpm at 30°C for 25 minutes. A 40 ml volume of ice cold, sterile water was added to the suspension, and the cells were collected by centrifugation at 1125 x g for 15 minutes and placed on ice. The cell pellet was resuspended in 40 ml ice cold water and collected as before for two additional wash steps. The cell pellet was then resuspended in 20 ml of ice cold 1 M sorbitol and collected by centrifugation as before. The final cell pellet was suspended in 0.3 ml ice cold, sterile sorbitol, aliquoted and frozen at -80°C.

Example 6—Transformation into *P. pastoris*

30-100 ng of linearized plasmid DNA was transformed into 30 µl of electrocompetent *P. pastoris* cells using a 1 mm gap GenePulser cuvette (BioRad) with a GenePulser (BioRad) set at 1.15 kV. 1 ml of YPD/1M sorbitol was added and mixed at a 1:1 ratio to the cells. The cells were allowed to recover for 3 h at 30°C with shaking at 100 rpm. 100 µl of the recovery mixture was plated on a YPD plate containing the appropriate antibiotic, and the rest of the cells were plated on a YPD plate with the appropriate antibiotic. Plates were incubated at 30°C for 48 hours. Primary transformation plates were streaked onto YPD plates with appropriate antibiotic, and plates were incubated for 48 h at 30°C. Individual clones were patched onto YPD plates with antibiotics and the patches were used to do colony PCR or gDNA prep to confirm integration into the chromosome and to grow the strains in shake flasks for further analysis.

Example 7—Growing Cultures in Shake Flasks for Production of LegH

A strain from a fresh patch was inoculated into growth media BMGY (BMY supplemented with 0.75% glycerol) and grown overnight at 30°C with shaking at 200 rpm. The next day, expression of LegH was induced with methanol by diluting the ON culture with BMMY media (BMY+ 1% methanol) supplemented with 0.1 mM Ammonium Fe(III) citrate. The culture was grown to an OD600 of 0.5-0.7. Antifoam was added to a final concentration of 0.01%. The cultures were grown for 72 hours total; cultures were supplemented with methanol every 24 hours by adding 1/10 of shake flask volume of 10x BMMY media (BMY+10% methanol). Cells were harvested after 72 h of induced growth by centrifugation.

Example 8—Shake Flask Medium

BMY media was prepared by dissolving 10 g of yeast extract and 20 g soytone in 790 ml water. The mixture was sterilized by autoclaving, and cooled to room temperature. 100 ml 1 M Potassium Phosphate buffer (pH 6.0) and 100 ml 10X Yeast Nitrogen Base without amino acids (13.4 g of YNB powder per 100 mL; Sigma-Aldrich) was filter sterilized (0.2 μm pore size PES) and added to the media. No pH adjustment is required.

BMY Media Components

Component	Amount, per 1L
Yeast Extract	10 g
Soy peptone (BD)	20 g
Yeast Nitrogen Base without amino acids, 10X solution	100 mL (results in 13.4 g/L in BMY)
1M Potassium Phosphate Buffer, pH 6.0	100 mL

The following components were dissolved in water, and autoclaved to sterilize.

Low-Osmolarity Medium for Shake Flask

Component	Amount, g/L
Ammonium Sulfate	15.7
Potassium Phosphate Monobasic	9.4
Calcium Sulfate Dihydrate	0.43
Magnesium Sulfate Heptahydrate	11.7
Sodium Citrate Dihydrate	1.13

Example 9—Fermentation Medium and Feeds

The components indicated below were dissolved and the volume adjusted with water. The components were FCC food grade or equivalent. The medium was sterilized by autoclaving, by steaming in place, or with an equivalent.

Low-Osmolarity Medium with 95 g/L Glycerol for Fermentation

Component	Amount, g/L
Ammonium Sulfate	15.7
Potassium Phosphate Monobasic	9.4
Calcium Sulfate Dihydrate	0.43
Magnesium Sulfate Heptahydrate	11.7
Sodium Citrate Dihydrate	1.13
Glycerol, USP grade 99.7%	95

After sterilization, the medium was allowed to cool down to room temperature, and the following was added:

Component	Amount, mL/L
Trace Metals PTM1 Solution	2
Vitamin Solution	4
Sigma 204 antifoam or equivalent	1

Trace metals PTM1 solution is available as a powdered mix from Sunrise Science (Cat No. 4052-A-B-1L). Pouch A and pouch B were mixed in 950 mL water, and 5 mL sulfuric acid was added. Some precipitation is expected upon mixing; the mixture was filter sterilized (0.2 μm pore size PES) and stored at 4°C in the dark.

Vitamin solution recipe

Component	Amount, g/L
biotin	0.2
calcium pantothenate	1
folic acid	0.2
inositol	1
niacin	0.2
p-aminobenzoic acid	0.2
Pyridoxine hydrochloride	1
riboflavin	0.5
thiamine hydrochloride	1
B12	0.1

Alternatively, trace metals PTM1 can be made as follows:

Component	Amount
Cupric sulfate – 5H2O	6.0 g
Sodium iodide	0.08 g
Manganese sulfate – H2O	3.0 g
Sodium molybdate – 2H2O	0.2 g
Boric acid	0.02 g
Cobalt chloride	0.5 g
Zinc chloride	0.5 g
Ferrous sulfate – 7H2O	65.0 g
Biotin	0.2 g
Sulfuric acid	5.0 ml
Water	To a final volume of 1 L

The components are mixed together, filter sterilized and stored at room temperature. The glycerol feed mix was prepared by mixing 17.5 g of AmberFerm 4000 into 320 mL water and stirring to dissolve. The water-Amberferm mixture was added to 850 g of glycerol and mixed well by vigorous stirring. The feed mix was sterilized by autoclaving.

Glycerol feed solution

Component	Amount, g/L
USP grade glycerol	850
Water	320
Sensient AmberFerm4000 soy hydrolysate	17.5

The methanol feed was made using 99-100% methanol supplemented with 12 mL/L of PTM1 solution.

Example 10—Protocol for Lab-Scale High Oxygen Transfer Fermentation

Seed Shake Flask Protocol

In a aseptic biosafety hood, low-osmolarity medium and BMY were mixed in a 9:1 low-osmo:BMY ratio. Glycerol, at a concentration of 12.5 g/L, was added to the medium. USP food grade glycerol/glycerin (99.7% purity in a 50% v/v (63% w/w) glycerol/water solution) was used and autoclaved to sterilize. Sigma 204 or an equivalent antifoam was added to the medium at a concentration of 0.25 mL/L. Glycerol seed vials were retrieved, sprayed outside with 70% IPA or ethanol and thawed inside a biosafety hood at room temperature for about 5 min. Baffled shake flasks were inoculated with glycerol seed vials; 1 mL of inoculum vial were used for every 1 L of shake flask medium. Cultures were grown at 30ºC for 24 hours with shaking (200 RPM with a 1" throw). A ratio of between 1:10 and 1:5 of actual medium volume : nominal shake flask volume was used. 2.8 L nominal volume flask with 250 to 500 mL of medium were routinely use with success. The OD at 600 nm was measured after 24 hours of growth; if the OD was 15 or higher, the culture was used to inoculate a fermenter. If the OD was less than 15, the culture was grown for 1-2 more hours before the OD was determined again. If an OD of 15 was not reached after 15 to 30 hours, the seed flask was considered to have failed.

Fermentation Protocol

The fermentation medium and feeds were prepared as described herein. The initial volume should be about 40% of the maximum fermenter volume, e.g., 4 L, if the maximum working volume of the fermenter is 10 L. This is because the process will approach the maximum working volume by the end of the fermentation. The fermenter is inoculated with shake flask seed at 10% inoculum-fermenter ratio, e.g. if 4 L of initial media are present in the fermenter, the fermenter is inoculated with about 0.4 L of shake flask seed. The total volume in the fermenter at this point is referred to T0 volume, e.g. 4.4 L in this representative example. Process controls include the following: 30°C temperature; dissolved oxygen controlled by agitation-aeration cascade to maintain a 20% saturation set point; and pH controlled via addition of 28% NH_4OH, the set point will depend on the phase of the process.

Batch phase (from inoculation to depletion of glycerol, signaled by DO spike): Depending on the responsiveness of the PID control for dissolved oxygen, a strong DO spike or a fast drop in agitation-aeration rates or a combination of both may be observed when the cells deplete the glycerol present in the medium. Fed-batch phase is initiated when this occurs. The duration of the batch phase is approximately 20 hours, but up to 24 hours is considered acceptable. The pH set point is 5.0. The wet cell weight at the end of the batch phase will be approximately 220 g/L.

Fed-batch phase: glycerol feed is initiated to achieve 12-14 g/L/hr of neat glycerol based on T0 volume. The federate was maintained until approximately 350 g/L wet cell weight was reached, which should take about 7-10 hours. The pH set point is 5.0.

Transition phase: A sample is taken before beginning the transition phase. Methanol feed was initiated to achieve 1 g/L/hr of neat methanol, based on the T0 volume, until 1-2 g/L methanol concentration was reached in the fermentation broth. The methanol feed rate was adjusted during the remainder of fermentation so as to maintain a methanol concentration of 0.25-1 g/L in broth. Glycerol federate was reduced from 12-14 g/L/hr to 8-9 g/L/hr of neat glycerol, based on T0 volume, linearly over the course of 2 hours. Stepwise reduction in feed rate every 20 min would be acceptable as well. The pH set point was changed to 3.5, and the fermentation was allowed to naturally adjust to the new set point (i.e., with no addition of acid).

Production phase (from end of glycerol feed ramp-down to end of fermentation): The pH set point was 3.5. A methanol concentration of 0.25-1 g/L was maintained in the fermentation

broth. The feed rate of the glycerol was maintained at 8-9 g/L/hr of neat glycerol, based on the T0 volume. Samples were taken approximately every 12 hours. Samples were spun at 4000 to 7000 RCF at 4°C, and the supernatant decanted. The supernatant was saved in a separate tube. Pellets and 3 samples of 5 mL of supernatants at each time point were frozen at -80°C. If a 15-20% DO during production is unable to be maintained, even at maximum aeration and agitation rates for the vessel, the glycerol feed rate can be lowered up to 5 g/L/hr of neat glycerol, based on the T0 volume. Fermentation ended 60 hours after inoculation. At 1000 L scale, the harvest process consisted of shutting down feeds and aeration, chilling the broth to 8°C, and concentrating the paste using a sharples or disk stack centrifuge. Harvesting usually takes about 5-10 hours and does not incur a detectable loss of quality of the product. For lab scale, it is sufficient to collect, in addition to the 3x5 mL samples, an additional 50 mL sample at the end. Wet cell weight was >450 g/L, and spun pellets looked pink, as opposed to spun pellets from pre-induction samples, which looked more white. The color change of the broth from white to a more pronounced pink started following about 6-12 hours of induction.

PART B. Construction of Production Strains

Production Strain MXY0183

Example 11—Cloning Each Enzyme of Heme Biosynthesis Pathway into pGAN or pGAZ Integration Vector

pGAN (with the nat selection marker) and pGAZ (with the zeocin selection marker) were purchased from Biogrammatics, Inc (Carlsbad, CA). Each gene was placed under control of the AOX1 promoter, and the FDH terminator was placed immediately after the stop codon of each gene. The genes in the heme biosynthesis pathway were PCR amplified from wild type *P. pastoris* strain or subcloned from previous constructs.

The heme biosynthetic pathway, including the enzymes involved in the biosynthesis of heme, are shown in Figure 1. The intermediates produced during the biosynthesis of heme are shown in the boxes, and the enzyme catalyzing each step is shown to the right. The rate limiting enzymatic steps, as shown in *S. cerevisiae*, are shown with underlining.

ALA synthase, ALA dehydratase, UPGIII synthase, UPGIII decarboxylase, CPG oxidase and PPG oxidase genes were PCR amplified with primers containing sites for recognition by the restriction endonuclease, BsaI. Oligonucleotides were synthesized by ElimBiopharm.

Primer Designation	Gene	Sequence	SEQ ID NO:
MxO0187	ALAsynth_F	GAGGGTCTCGGATGGAGTTTGTCGCCCGTC	19
MxO0188	ALAsynth_R	GAGGGTCTCGATTACAATCTGACTCCTGATGAGG	20
MxO0189	ALAdehyd_F	GAGGGTCTCGGATGGTGCATAAGGCTGAATACTTG	21
MxO0190	ALAdehyd_R	GAGGGTCTCGATTATTCAGATAACCACTCCAGG	22
MxO0191	UroporSynth_F	GAGGGTCTCGGATGCCAAAAGCCATTCTTCTGAAG	23
MxO0192	UroporSynth_R	GAGGGTCTCGATTAGTGCACTTTTTGTATAGAC	24
MxO0193	UroporDecarb_F	GAGGGTCTCGGATGAGTAGATTTCCAGAACTGAAG	25
MxO0194	UroporDecarb_R	GAGGGTCTCGATTATTGAGATCCAATGCG	26
MxO0195	CoproOx_F	GAGGGTCTCGGATGGCCATCGACTCTGATATC	27
MxO0196	CoproOx_R	GAGGGTCTCGATTATACCCATTCAATAGGAT	28
MxO0197	ProtoporOx_F	GAGGGTCTCGGATGCTGAAAAGTCTTGCACCAAA	29
MxO0198	ProtoporOx_R	GAGGGTCTCGATTAAATGCCACTGAGGGTAGC	30

Phusion High-Fidelity DNA Polymerase (New England BioLabs, Cat # M0530L) was used to amplify genes from genomic DNA. PCR products were obtained and purified using DNA Clean&Concentrator-5 (Cat # D4004) and DNA was eluted in 25 µl of H_2O. Vector DNA, pGAZ and pGAN, and PCR products were digested with BsaI (New England BioLabs, Cat # R0535S) in 50 µl reaction volume at 37°C.

Linearized vectors and digested PCR products were purified from 0.8% agarose gel using Zymoclean Gel DNA Recovery Kit (Zymo Research Cat # D4002). DNA was eluted in 20 µl H_2O. Ligation reactions were set up in 10 µl at 16°C overnight using T4 DNA Ligase (New England BioLabs, Cat # M0202S).

PBD and ferrochelatase genes were subcloned from previously constructed plasmids: pJAZ_PBD was digested with BstBI(Bsp119I) (ThermoScientific, FD0124)and NotI (ThermoScientific, FD0596) in 1x Fast Digest buffer for 5 min at 37°C. pJAZ_Ferroch was digested with MfeI (MunI, ThermoScientific, FD0753) and NotI (ThermoScientific, FD0596) in 1x Fast Digest buffer for 5 min at 37°C.

Digested products were purified from 0.8% agarose gel using Zymoclean Gel DNA Recovery Kit (Zymo Research Cat # D4002). DNA was eluted in 20 µl H_2O.

Ligation reactions were set up in 10 µl reaction at 16°C overnight using T4 DNA Ligase (New England BioLabs, Cat # M0202S).

1.5 µl of ligation mixture was transformed into 20 µl of ElectroMax DH10B T1 Phage-Resistant Competent Cells (Invitrogen, Cat # 12033-015) by electroporation using MicroPulser (BioRad) set at 1.7 kV; cells were incubated at 37°C in 1 ml SOC for 1 h with shaking at 200 rpm. 10 µl of recovery mixture was plated on LB agar plates containing ampicillin at concentration 100 µg/ml. Plates were incubated overnight at 37°C. Colonies were screened by colony PCR for the presence of the insert. The sequences of the genes were confirmed.

Designation	Construct	Gene
pMx0308	pGAN-ALAsynth	ALA synthase
pMx0309	pGAN-ALAD	ALAD
pMx0310	pGAN-UPGIIIsyn	Uroporphyrinogene synthase
pMx0311	pGAN-UPGIIIdecarb	Uroporphyrinogene decarboxylase
pMx0312	pGAN-CPGoxi	CPG oxidase
pMx0313	pGAN-PPGoxi	Protoporphyrin oxidase
pMx0314	pGAZ-ALAsyn	ALA synthase
pMx0315	pGAZ-ALAD	ALAD
pMx0316	pGAZ-UPGIIIsyn	Uroporphyrinogene synthase
pMx0317	pGAZ-UPGIIIdecarboxilase	UPGIII decarboxilase
pMx0318	pGAZ-PPGoxidase	PPG oxidase
pMx0319	pGAZ-CPG oxidase	CPG oxidase
pMx0320	pGAN-PBD	PBD
pMx0321	pGAZ-PGC	PBD
pMx0322	pGAZ-Fc	Ferrochelatase
pMx0323	pGAN-Fc	Ferrochelatase

Example 12 —Assembling Heme Biosynthesis Genes on Plasmids for Integration into *P. pastoris* mutS Genome

The whole cassette "promoter-gene-terminator" was PCR amplified with primers containing sites for restriction endonucleases to assemble plasmids for integration into the *Pichia* genome.

Assembling Paox1_UPS_FDHterm- Paox1_UPD_FDHterm-Paox1_CPO_FDH term. on pGAN plasmid (pMx327)

pGAN-CPGoxidase (pMx312) was used as a vector to clone the UPS and UPD cassettes. UPG III synthase cassette was PCR amplified from pMx310 with primers to AOX1 promoter / FDH1 terminator containing NheI and SphI recognition sites for restriction endonucleases correspondingly:

Primer Designation	Recognition Sites	Sequence	SEQ ID NO:
MxO0399	NheI-pAOX1-F	CAA TCG CTA GCA TCC AAC ATC CAA AGA CGA AAG G	31
MxO0401	SphI-FDH1-R	GGA TAG CAT GCA CCT TAT CAA GAT AGC TAG AAA TAG AAA TGG	32

UPG III decarboxylase cassette was PCR amplified from pMx311 with primers to AOX1 promoter / FDH1 terminator containing SphI and AgeI recognition sites for restriction endonucleases correspondingly:

Primer Designation	Recognition Sites	Sequence	SEQ ID NO:
MxO0402	SphI-pAOX1I-F	CAA TAG CAT GCA ACA TCC AAA GAC GAA AGG TTG AAT G	33
MxO0404	AgeI-FDH1-R	CAT GGT ACC GGT ACC TTA TCA AGA TAG CTA GAA ATA GAA ATGG	34

Phusion High-Fidelity DNA Polymerase (New England BioLabs, Cat # M0530L) was used to amplify DNA from plasmids.

Obtained PCR products were purified using DNA Clean&Concentrator-5 (Zymo Research, Cat # D4004) and DNA was eluted in 25 µl of H_2O.

pGAN-CPGoxidase (pMx312) designated as a vector was digested in 1x CutSmart Buffer with NheI-HF (New England BioLabs, Cat # R3131S) and AgeI-HF (New England BioLabs, Cat # R3552S) over night at 37°C.

UPG III synthase cassette PCR product was digested in 1x CutSmart Buffer with NheI-HF (New England BioLabs, Cat # R3131S) and SphI-HF (New England BioLabs, Cat # R3182S) over night at 37°C.

UPG III decarboxylase cassette PCR product was digested in 1x CutSmart Buffer with SphI-HF (New England BioLabs, Cat # R3182S) and AgeI-HF (New England BioLabs, Cat # R3552S) over night at 37°C.

Digested vector and PCR products were gel purified from 0.8% agarose using Zymoclean Gel DNA Recovery Kit (Zymo Research, Cat # D4002). DNA was eluted in 20 µl H_2O.

Three way ligation between UPG III synthase cassette digested with NheI-SphI, UPG III decarboxylase cassette digested with SphI-AgeI and a vector digested with NheI-AgeI was set up in 10 µl at 16°C overnight using T4 DNA Ligase (New England BioLabs, Cat # M0202S).

1.5 µl of ligation mixture was transformed into 20 µl of ElectroMax DH10B T1 Phage-Resistant Competent Cells (Invitrogen, Cat # 12033-015) by electroporation using MicroPulser (BioRad) set at 1.7 kV; cells were incubated at 37°C in 1 ml SOC for 1 h with shaking at 200 rpm. 10 µl of recovery mixture was plated on LB agar plates containing ampicillin at a concentration of 100 µg/ml. Plates were incubated overnight at 37°C. Colonies were screened by colony PCR for the presence of the insert. The sequences of the junctions between vector and inserts were confirmed.

Assembling Paox1_ALAsynthase_FDH1term.- Paox1_PPGoxidase_FDH1term- Paox1_Fc_ FDH1term.- Paox1_PBD_ FDH1term cassette (pMx330)

a. PCR amplification of gene-cassettes:

ALAsynthase cassette was PCR amplified from pMx310 with primers to AOX1 promoter / FDH1 terminator containing NheI and XhoI recognition sites for restriction endonucleases correspondingly:

Primer Designation	Recognition Sites	Sequence	SEQ ID NO:
MxO0399	NheI-pAOX1-F	CAA TCG CTA GCA TCC AAC ATC CAA AGA CGA AAG G	35
MxO0400	XhoI-FDH1-R	GAT ATT GCT CGA GAC CTT ATC AAG ATA GCT AGA AAT AGA AAT G	36

PPGoxidase cassette was PCR amplified from pMx313 with primers to AOX1 promoter / FDH1 terminator containing XhoI and AflII recognition sites for restriction endonucleases correspondingly:

Primer Designation	Recognition Sites	Sequence	SEQ ID NO:
MxO0403	XhoI-pAOX1-F	CAA TCT CGA GAA CAT CCA AAG ACG AAA GGT TG	37
MxO0437	AflII-FDH1-R	CAA CCA TTT CTA TTT CTA GCT ATC TTG ATA AGG TCT TAA GTC CA	38

Ferrochelatase cassette was PCR amplified from pMx323 with primers to AOX1 promoter / FDH1 terminator containing AflII and AgeI recognition sites for restriction endonucleases correspondingly:

Primer Designation	Recognition Sites	Sequence	SEQ ID NO:
MxO0404	AgeI-FDH1-R	CAT GGT ACC GGT ACC TTA TCA AGA TAG CTA GAA ATA GAA ATG G	39
MxO0436	AflII-pAOX1-F	TTA CTT AAG TCC AAC ATC CAA AGA CGA AAG GTT G	40

G418 marker was PCR amplified from pJAG plasmid purchased from Biogrammatics using the following primers:

Primer Designation	Recognition Sites	Sequence	SEQ ID NO:
MxO0438	Mlu-G418-F	TCA CAG ACG CGT TGA ATT GTC C	41
MxO0439	BbvCI-G418-R	TTG CTC CTC AGC TTA GAA GAA CTC GTC CAA CAT CAA GTG	42

Phusion High-Fidelity DNA Polymerase (New England BioLabs, Cat # M0530L) was used to amplify DNA from plasmids. The PCR products were obtained and purified using DNA Clean&Concentrator-5 (Zymo Research, Cat # D4004) and DNA was eluted in 25 µl of H_2O.

b. Preparation of vectors

pGAZ-PBD (pMx321) designated as a vector was digested in 1x CutSmart Buffer with NheI-HF (New England BioLabs, Cat # R3131S) and XhoI (New England BioLabs, Cat # R0146S) overnight at 37°C.

pGAZ-ALAsyn.-PBD (pMx328) was digested in 1x NEBuffer3.1 with MluI (New England BioLabs, Cat # R0198S) and BbvCI (New England BioLabs, Cat # R0601S) overnight at 37°C.

pGAG-ALAsyn-PBD (pMx332) was digested in 1x CutSmart Buffer with XhoI (New England BioLabs, Cat # R0146S) and AgeI-HF (New England BioLabs, Cat # R3552S) overnight at 37°C.

c. Making intermediate constructs and assembling a final cassette

Digested vector and PCR products were gel purified from 0.8% agarose using Zymoclean Gel DNA Recovery Kit (Zymo Research, Cat # D4002). DNA was eluted in 20 µl of H_2O.

pGAZ-PBD (pMx321) vector, digested with NheI-XhoI restriction endonucleases, was ligated with ALAsynthase cassette PCR product digested with the same enzymes in 10 µl reaction at 16°C overnight using T4 DNA Ligase (New England BioLabs, Cat # M0202S) to yield a pGAZ-ALAsyn.-PBD plasmid (pMx328).

pGAG-ALAsyn-PBD (pMx332) digested with XhoI and AgeI–HF restriction endonucleases was ligated with PPGoxidase cassette and Ferrochelatase cassette PCR products digested with XhoI, AflII and AflII, AgeI-HF restriction endonucleases correspondingly in a three way ligation reaction using T4 DNA Ligase (New England BioLabs, Cat # M0202S) to yield a pGAG-ALAsynthase_PPGoxidase_Fc_PBD (pMx330).

1.5 µl of ligation mixture was transformed into 20 µl of ElectroMax DH10B T1 Phage-Resistant Competent Cells (Invitrogen, Cat # 12033-015) by electroporation using MicroPulser (BioRad) set at 1.7 kV; cells were incubated at 37°C in 1 ml SOC for 1 h with shaking at 200 rpm. 10 µl of recovery mixture was plated on LB agar plates containing ampicillin at a concentration of 100 µg/ml. Plates were incubated overnight at 37°C. Colonies were screened by colony PCR for the presence of the insert. The sequences of junctions between the vector and the inserts were confirmed.

Designation	Construct	Gene
pMx0327	pGAN-UPGsyn_UPGdecarb_CPGoxy	UPGsyn_UPGdecarb_CPGoxy
pMx0328	pGAZ-ALAsyn-PGC	ALAsyn-PBD
pMx0330	pGAG-ALAsyn_PBD_PPG_Fc	ALAsyn_PBD_PPG_Fc
pMx0332	pGAG-ALAsyn_PBD	G418 marker

Example 13—Integration of Linearized Plasmids with Gene Cassettes into *P. pastoris* Bg11 Genome

The plasmids that were used to generate the production strain, MXY0183, are shown in Figure 2. The steps taken to make the modifications that led to production strain, MXY0183, are depicted in Figure 3.

The first enzyme to be introduced into the *P. pastoris* Bg11 genome was ALAD. A plasmid containing pAOX1-driven ALAD (pMX229, Figure 2i and Figure 3) was linearized using PmeI restriction enzyme (New England BioLab). Linearized plasmid was purified from 0.8% agarose gel as described and transformed into *P. pastoris* using homologous recombination at the native AOX1 locus, generating strain MXY099 (Figure 3).

A plasmid containing two copies of the soybean LegH gene (sequence optimized for *P. pastoris*; SEQ ID NO:3) under the control of the pAOX1 promoter designated pMX282 (Figure 2ii and Figure 3) was linearized using SfiI restriction enzyme. Linearized plasmid was purified from a 0.8% agarose gel as described and transformed into the *P. pastoris* strain containing ALAD, generating the strain MXY0118 (Figure 3). qPCR was used and determined that strain MXY0118 contained several copies of the LegH gene, likely due to concatamerization of the plasmid, pMX282, at the time of recombination.

Plasmid pMX327 (Figure 2iii and Figure 3) containing genes encoding Uroporphyrinogen III synthase (UPS), Uroporphyrinogen III decarboxylase (UPD) and Coproporphyrinogen III oxidase (CPO) (the enzymes catalyzing steps 4, 5 and 6, respectively) under control of the AOX1 promoter was linearized with the SfiI restriction endonuclease and introduced into MXY0118, yielding strain MXY0170 (Figure 3).

Genes encoding ALA synthase (ALAS), Protoporphyrin III oxidase (PPO), Ferrochelatase (FC) and Porphobilinogen deaminase (PBD) (the enzymes catalyzing steps 1, 7, 8 and 3, respectively) from the *P. pastoris* genome were assembled on plasmid pMX330 (Figure 2iv and Figure 3). pMX330 was linearized with the SfiI restriction endonuclease and transformed into MXY0170, leading to the generation of strain MXY0183 (Figure 3). The genotype of MXY0183 was confirmed using PCR and qPCR.

Production Strain MXY0207

Example 14—Construction of pGAB Expression Vector

The pGAB expression vector (Figure 4A) was constructed by replacing the open reading frame of the Zeocin resistance gene in the pGAZ vector (BioGrammatics, Inc., Carlsbad, CA) with the open reading frame from the Blasticidin S deaminase (BSD) gene from *Aspergillus terreus*, which allows for selection of transformants carrying the plasmid with the antibiotic Blasticidin S.

The BSD open reading frame was amplified from a commercially synthesized DNA molecule using oligonucleotide primers MxO0476 and MxO0477 using a high fidelity polymerase chain reaction as described herein.

Primer Designation	Description	Sequence	SEQ ID NO:
MxO0477	BSD_Reverse	TTA GTC TTG CTC CTC AGC TTA GCC	43
MxO0476	BSD_Forward	TCA CAG ACG CGT TGA ATT GTC C	44

The BSD PCR product was purified by gel electrophoresis on a 1% agarose gel in 1x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) and visualized using SYBR Safe DNA gel stain (Life Technologies, Carlsbad, CA). The desired DNA fragment was excised from the agarose gel and the DNA was recovered using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA).

The purified BSD PCR product and pGAZ vector were digested with 10 units each of the MluI and BbvCI restriction endonucleases (New England Biolabs, Ipswich, MA) for 1 hour at 37°C in 1x NEBuffer 3.1 (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 µg/ml BSA, pH 7.9 @ 25°C). Digested DNA products were recovered by gel electrophoresis as described above.

The purified, MluI and BbvCI digested BSD product and pGAZ vector were incubated with 400 units of T4 DNA ligase (New England Biolabs) in 1x T4 DNA ligase reaction buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT, pH 7.5 @ 25°C) in a 20 µl reaction, at 16°C for 2 hours in a 20 µl reaction. Electrocompetent E. coli DH10B cells were transformed with 2 µl of the ligation reaction and antibiotic resistant transformants were selected on LSB agar plates supplemented with 100 µg/µl ampicillin.

Example 15—Construction of Mxr1 Expression Vector

The Mxr1 expression vector, pMx354, was constructed by introducing the Mxr1 open reading frame into the pGAB vector (Figure 4B). The Mxr1 open reading frame was inserted into pGAB with the translation start immediately downstream of the methanol-inducible alcohol oxidase 1 (AOX1) promoter from *Pichia pastoris* and the translation stop signal immediately followed by the transcription terminator sequence from the *P. pastoris* FDH1 gene.

The open reading frame encoding the Mxr1 protein was amplified from genomic DNA isolated from *Pichia pastoris* strain Bg11 MutS obtained from BioGrammatics, Inc. (Carlsbad, CA). The Mxr1 open reading frame was amplified from *P. pastoris* genomic DNA with primers MxO0495 (TTT TGC GGC CGC ATG AGC AAT CTA CCC CCA ACT TTT G (SEQ ID NO:45)) and MxO0496 (AAA AGC GGC CGC CTA GAC ACC ACC ATC TAG TCG GTT (SEQ ID NO:46)), which appended flanking NotI restriction endonuclease recognition sites. Amplification was accomplished using the polymerase chain reaction as described herein.

The amplified Mxr1 PCR product and the pGAB vector were digested with 10 units of NotI restriction endonuclease (New England Biolabs) for 1 hour at 37°C in 1x NEBuffer 3.1 (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 µg/ml BSA, pH 7.9 @ 25°C). Following digestion, the NotI-digested pMx352 vector was treated with 5 units Antarctic phosphatase (New England Biolabs) for 15 minutes at 37°C in 1x Antarctic phosphatase buffer (50 mM Bis-Tris-Propane-HCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, pH 6 @ 25°C).

The NotI-digested amplified Mxr1 fragment and pMx352 vector were separated by electrophoresis on a 1% agarose gel in 1x TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) and visualized using SYBR Safe DNA gel stain (Life Technologies, Carlsbad, CA). The desired DNA fragments were excised from the agarose gel and the DNA was recovered using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA).

The NotI-digested fragment containing Mxr1 open reading frame was introduced into pGAB at a NotI site immediately downstream of the AOX1 promoter by ligation. A mixture containing 137 ng of NotI-digested DNA encoding the Mxr1 open reading frame and 60 ng of NotI-digested, phosphatase-treated pMx352 was incubate with 400 units of T4 DNA ligase (New England Biolabs) in 1x T4 DNA ligase reaction buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, 10 mM DTT, pH 7.5 @ 25°C) in a 20 µl reaction, at 16°C, for 2 hours in a 20 µl reaction. Electrocompetent E. coli DH10B cells were transformed with 2 µl of the ligation reaction and

antibiotic resistant transformants were selected on LSB agar plates supplemented with 100 µg/µl ampicillin. Plates were incubated overnight at 37°C. Colonies were screened for the presence of the insert by PCR using primers MxO0495 and MxO0496. The sequence of the final vector was confirmed by DNA sequencing.

During cloning, 6 additional amino acids were introduced at the N-terminus of Mxr1. The Mxr1 open reading frame is shown under the section "Nucleic acid sequences", with residual amino acids from the cloning shown with underlining. *Pichia* production strains containing the Mxr1 sequence having the additional 6-amino acids at the N-terminus and *Pichia* strains containing the wild type Mxr1 (i.e., without the additional 6 amino acids at the N-terminus) were indistinguishable in fermentation tanks.

Example 16—Construction of Native Mxr1 Expression Vector

A plasmid containing the Mxr1 transcription regulator gene under the control of the pAOX1 promoter, designated pMX354, was used as a template for PCR amplification. The 3' end of the AOX1 promoter, the LegH open reading frame, and the AOX1 terminator were amplified from pMX354 using primers MxO0617 and MxO0647 shown below. The AOX1 terminator, linker and the 5' end of the AOX1 promoter were amplified from pMX382 using primers MxO0618 and MxO0646.

Primer Designation	Sequence	SEQ ID NO:
MxO0646	ACTAGATGGTGGTGTCTAGTCAAGAGGATGTCAGAATGCCATTTG	47
MxO0647	TCTGACATCCTCTTGACTAGACACCACCATCTAGTCGGTTTTCTAG	48

PCR products were obtained and purified using DNA Clean&Concentrator-5 and DNA was eluted in 12 µl of H_2O. The purified PCR products were then combined and used as a template for a subsequent round of PCR amplification using primers MxO0617 and MxO0618. The resulting PCR product was composed of the 3' end of the AOX1 promoter, followed by the Mxr1 open reading frame, the AOX1 terminator, a short linker sequence, and the 5' end of the AOX1 promoter. The PCR product was obtained and purified as described herein. The purified PCR product was cloned into the pCR™-Blunt II-TOPO® vector using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, Cat # K2800-20) to create the pMX402 vector.

Example 17—Construction of *P. pastoris* Strains MXY0206 and MXY0207

The pMx354 Mxr1 expression vector (Figure 4B) was introduced into the MXY0183 strain by DNA transformation (Figure 3).

The pMx354 vector (1.5 μg) was linearized at a unique PmeI site in the AOX1 promoter sequences by digestion with 20 units of the PmeI restriction endonuclease (New England Biolabs) for 1 hour at 37°C in 1x NEBuffer 4 (50mM Potassium Acetate, 20mM Tris-acetate, 10mM Magnesium Acetate, 1mM DTT, pH 7.9@25°C).

The PmeI-digested pMX354 vector was purified by gel electrophoresis recovered using the Zymoclean Gel DNA Recovery Kit as described above. The linearized pMX354 vector was introduced into strain MXY0183 by transformation and selection on blasticidin-containing medium. Two independent clones were obtained from the transformation, and they were designated MXY0206 and MXY0207. The presence of an additional copy of Mxr1 under the control of the AOX1 promoter in these strains was confirmed by PCR.

Production Strain MXY0291

Example 18—Construction of Strains MXY0213 and MXY0260

Figure 5 shows the steps taken to construct antibiotic marker free strain MXY0213 that contains 7 enzymes of the heme biosynthetic pathway. A linear piece of DNA containing variant Mxr1 (6 extra amino acids at N terminus) under pAOX1, with homology to the pAOX1 promoter on each end, was introduced using co-transformation (Figure 5 and Figure 6i). This linear Mxr1 expression cassette was simultaneously introduced into *Pichia* strain MXY213 with the pIL75 plasmid by transformation. The pIL75 vector carries a panARS autonomous replication sequence (Liachko & Dunham, 2014, FEMS Yeast Res., 14:364-7), which allows for maintenance of the plasmid vector without integration into the genome of the transformed cells, and a kanMX marker for selection of transformants with the antibiotic G418. Transformed cells were selected on media supplemented with G418 for the presence of kanMX marker on the pIL75 plasmid. *Pichia* transformants were screened by colony PCR for transformants that took up both the pIL75 plasmid and had correctly integrated the Mxr1 expression cassette.

Example 19—Co-Transformation to Introduce the LegH Expression Cassette into *Pichia*

A plasmid containing a different Pichia pastoris-codon optimized variant of soybean LegH gene (variant 3; SEQ ID NO:5) under the control of the pAOX1 promoter designated pMX399 was used as a source of template for PCR amplification of the gene. The backbone from TOPO cloning plasmid pMX401was PCR amplified. The insert and vector were assembled using Gibson assembly (NEB Gibson assembly kit) to generate plasmid pMX422. This plasmid was used as a template for a subsequent round of PCR amplification using primers MxO0617 and MxO0618 shown below.

Primer Designation	Sequence	SEQ ID NO:
MxO0617	AAACGCTGTCTTGGAACCTAATATGAC	49
MxO0618	AAACTGTCAGTTTTGGGCCATTTG	50

The resulting PCR product was composed of, in the 5' to 3' direction, the 3' end of the AOX1 promoter, followed by the LegH var 3 open reading frame, the AOX1 terminator, a short linker sequence, and the 5' end of the AOX1 promoter (Figure 6ii). The PCR product was obtained and purified by agarose gel electrophoresis as described herein.

Transformants with LegH expression cassette integrated into the genome were screened by PCR and characterized for LegH gene copy number using qPCR.

Example 20—Curing Transformants of Plasmid Vectors Bearing Selection Markers

In clones where the soybean LegH expression cassette was shown to be correctly integrated by colony PCR and in high copy number by qPCR, the pIL75 plasmid required for selection on G418 was eliminated by relaxing selection for the antibiotic. Transformants were streaked out for single colonies on media lacking G418 antibiotic. Because the panARS plasmid is not stably maintained in the absence of selection, the pIL75 was rapidly lost from the transformed cells under this condition. The resulting *Pichia* strain, MXY0291, contains sequences for LegH expression in copy number similar to MXY0207, but lacks heterologous sequences for selection.

Production Strains MXY0330, MXY0333, and MXY0338

Example 21—Construction of Strain MXY0306

Genotype PCR of strain MXY0291 revealed that a portion of the CPGoxidase coding sequence had been deleted during construction of this strain. The full-length CPGoxidase coding region was restored by replacement of the truncated copy. Briefly, a linear DNA fragment containing the pAOX1 promoter and full-length CPGoxidase coding region was generated by PCR amplification from plasmid pMX312 using primers MxO0866 and MxO0867 shown below.

Primer Designation	Sequence	SEQ ID NO:
MxO0866	ACGCTGTCTTGGAACCTAATATGAC	51
MxO0867	TACCCATTCAATAGGATTTTGTAGTACCTGC	52

The linear pAOX1-CPGoxidase DNA fragment was introduced into strain MXY0291 by co-transformation with the pIL75 plasmid. Transformants were selected on media containing G418 and then screened for the presence of the full-length CPGoxidase coding region by PCR. An isolate containing the full-length CPGoxidase was identified and subsequently cured of the plasmid vector required for selection on G418 as described above. This strain was designated MXY0306 (see Figure 5).

Example 22—Linear Constructs for Hybrid Promoter Strains

LegH variant 3 was expressed under the direction of each of the three native *Pichia pastoris* constitutive promoters indicated herein. The linear constructs are shown in Figure 7, and contained the 3' half of the promoter, followed by LegH var3, followed by the FDH1 transcription terminator. This was immediately followed by the antibiotic resistance cassette containing the pTEF promoter from *Ashbya gossypii*, the acetamidase gene (amdS) from *Aspergillus nidulans* and the TEF terminator from *Ashbya gossypii*. Finally, the construct contained the 5' half of the promoter. This linear cassette was amplified using the oligonucleotide primers listed in the Table below to generate constructs that contain several hundred base pairs on the 5' and 3' ends that are homologous to the respective promoter in the native *Pichia* genome.

Primers used to amplify the linear constructs

Primer designation	Sequence	SEQ ID NO:
MXO0718	GAGCTTCTTCTACGGCCCCC	53

MXO0723	TCCAGCAGAGTAAAATTTCCTAGGGAC	54
MXO0724	CTCTTTTAGGTTTTAAGTTGTGGGAACAGTAACA	55
MXO0729	GTGGGTGCTTCTTTGCGTGG	56
MXO0730	AGAATTGCCATCAAGAGACTCAGGACT	57
MXO0735	GATAGAGAGAAATCGCAAACTTTGAGAGGAAG	58

Competent MXY0306 cells were transformed with each of the linear cassettes and transformants containing the amdS selection cassette were selected based on their ability to grow on agar plates containing acetamide as the sole nitrogen source. These strains were purified, isolated and the presence of LegH under control of the constitutive promoter was verified by PCR (Figure 5).

Example 23—Nucleic Acid Sequences

Mxr1 nucleic acid sequence (the underlined nucleotides encode 6 amino acid at N-term introduced during cloning) (SEQ ID NO:1)

ATGCGAGACCGCGGCCGCATGAGCAATCTACCCCCAACTTTTGGTTCCACTAGACAATCTCCAGA
AGACCAATCACCTCCCGTGCCCAAGGAGCTGTCATTCAATGGGACCACACCCTCAGGAAAGCTAC
GCTTATTTGTCTGTCAGACATGTACTCGAGCATTTGCTCGTCAGGAACACTTGAAACGACACGAA
AGGTCTCACACCAAGGAGAAACCTTTCAGCTGCGGCATTTGTTCTCGTAAATTCAGCCGTCGAGA
TCTGTTATTGAGACATGCCCAAAAACTGCACAGCAACTGCTCTGATGCGGCCATAACAAGACTAA
GGCGCAAGGCAACTCGTCGGTCTTCTAATGCCGCGGGTTCCATATCTGGTTCTACTCCGGTGACA
ACGCCAAATACTATGGGTACGCCCGAAGATGGCGAGAAACGAAAAGTTCAGAAACTGGCCGGCCG
CCGGGACTCAAATGAACAGAAACTGCAACTGCAACAACAACATCTACAGCAACAACCACAGTTGC
AATACCAACAATCTCTTAAGCAGCATGAAAATCAAGTCCAGCAGCCTGATCAAGATCCATTGATA
TCCCCGAGAATGCAATTATTCAATGATTCCAACCATCACGTAAACAATTTGTTTGATCTTGGACT
AAGAAGAGCTTCCTTCTCCGCCGTTAGTGGAAATAATTATGCCCATTATGTGAATAATTTTCAAC
AAGATGCCTCTTCTACCAATCCAAATCAAGATTCAAATAATGCCGAATTTGAGAATATTGAATTT
TCTACCCCACAAATGATGCCCGTTGAAGATGCTGAAACTTGGATGAACAACATGGGTCCAATTCC
GAACTTCTCTCTCGATGTGAACAGGAACATTGGTGATAGCTTTACAGATATACAACACAAGAATT
CAGAGCCTATTATATCCGAACCGCCCAAGGACACCGCTCCAAACGACAAGAAGTTGAATGGCTAC
TCTTTTTACGAAGCCCCCATCAAGCCATTAGAATCCCTATTTTCTGTCAGGAATACAAAGAGAAA
CAAGTATAAAACAAATGACGACTCTCCAGACACCGTGGATAATAACTCCGCACCGGCTGCTAATA
CCATTCAAGAACTTGAGTCTTCTTTGAATGCATCCAAGAATTTTTGCTTGCCAACTGGTTATTCC
TTCTATGGTAATTTGGACCAACAGACTTTCTCTAACACGTTATCATGCACTTCTTCTAATGCCAC
AATTTCGCCCATTCTACTCGATAACTCCATTAATAATAACTCCACTAGTGACGTGAGACCAGAAT
TTAGAACACAAAGTGTCACCTCTGAAATGAGTCAAGCCCCTCCCCCTCCTCAAAAAAACAACTCG
AAATATTCCACCGAAGTTCTTTTTACCAGCAACATGCGGTCGTTTATTCACTACGCTCTTTCCAA
GTATCCTTTTATTGGTGTGCCCACTCCAACTCTTCCGGAGAACGAAAGACTAAATGAATATGCTG
ATTCATTCACCAACCGTTTCTTAAATCATTATCCTTTCATACATGTCACGATTCTCAAAGAATAC
TCCCTTTTCAAGGCAATTTTAGATGAGAATGAGTCGACTAAGAACTGGGAAAATAATCAGTTTTA
CTTAGAGAACCAACGAATATCAATTGTTTGTCTTCCTCTTTTGGTGGCTACGATAGGTGCAGTAC
TATCAAACAACAAAAAGGATGCTTCGAATTTATACGAAGCTTCAAGGCGTTGTATTCATGTTTAC

TTAGATTCCAGGAAAAAGATACCCACTTCCTTGTCCGCAAATAACAATGACTCTCCACTTTGGCT
AATTCAATCCCTGACGTTATCTGTTATGTATGGGTTATTTGCGGACAATGACATTAGTTTGAATG
TCGTGATCAGACAAGTTAACGCACTTAATTCTCTGGTCAAGACTTCGGGCCTGAATAGGACCTCA
ATTATAGATCTTTTCAACATCAACAAACCTTTGGATAATGAACTCTGGAATCAATTCGTGAAAAT
AGAGTCCACCGTAAGGACAATCCACACGATTTTTCAAATCAGTTCCAACTTAAGCGCCTTGTACA
ATATTATTCCATCGTTGAAAATTGATGACCTAATGATTACTCTACCAGTTCCCACAACACTTTGG
CAAGCTGATTCTTTTGTGAAATTCAAAAGTCTAAGTTACGGAAATCAGATCCCTTTTCAATATAC
AAGAGTACTACAGAATTTGATTGATTACAATCAGCCATTGAGCGATGGAAAATTTTTGTATGAAA
ACCATGTAAGTGAGTTTGGACTCATATGCCTACAGAATGGTCTACACCAATACAGCTATTTCCAA
AAATTGACTGCTGTCAATAACAGAGAAGATGCGCTATTCACAAAGGTTGTTAATTCACTTCACAG
TTGGGATAGGATGATTTCGAATTCTGATTTGTTTCCAAAGAAGATATATCAGCAGAGTTGCTTGA
TTTTGGACTCAAAGTTGCTTAATAATTTCCTGATTGTCAAGAGCTCATTGAAAGTTTCGACCGGA
GACGTTAGTTCTTTGAATAAGTTAAAAGAAAACGTGTGGCTTAAAAACTGGAATCAAGTGTGTGC
TATCTATTATAACAGCTTCATGAACATTCCTGCTCCCAGTATTCAAAAGAAGTACAATGACATAG
AGTTTGTGGATGACATGATTAATTTGAGTCTAATCATCATCAAGATTATGAAACTCATTTTCTAT
AACAATGTCAAAGACAATTATGAGGATGAAAATGACTTCAAATTGCAAGAGTTAAATTTAACATT
TGACAATTTTGATGAGAAAATATCCTTGAATTTGACAATATTATTCGATATATTTTTGATGATCT
ACAAGATAATTACCAATTACGAAAAGTTTATGAAGATCAAACACAAGTTTAATTACTACAATTCT
AATTCGAATATAAGCTTCTTGCATCATTTCGAACTCTCCTCGGTTATCAATAACACCCAAATGAA
CCAGAATGATTATATGAAAACAGATATTGATGAAAAGCTTGATCAGCTTTTCCACATCTATCAAA
CATTTTTCCGGCTGTATCTGGATTTAGAAAAGTTTATGAAGTTCAAATTCAACTATCATGACTTT
GAGACAGAGTTTTCAAGTCTCTCAATATCCAATATACTGAACACTCATGCTGCTTCTAACAATGA
CACAAATGCTGCTGATGCTATGAATGCCAAGGATGAAAAAATATCTCCCACAACTTTGAATAGCG
TATTACTTGCTGATGAAGGAAATGAAAATTCCGGTCGTAATAACGATTCAGACCGCCTGTTCATG
CTGAACGAGCTAATTAATTTTGAAGTAGGTTTGAAATTTCTCAAGATAGGTGAGTCATTTTTTGA
TTTCTTGTATGAGAATAACTACAAGTTCATCCACTTCAAAAACTTAAATGACGGAATGTTCCACA
TCAGGATATACCTAGAAAACCGACTAGATGGTGGTGTCTAG

Mxr1 protein sequence (the underlined 6 amino acid at N-term introduced during cloning) (SEQ ID NO:2)

MRDRGRMSNLPPTFGSTRQSPEDQSPPVPKELSFNGTTPSGKLRLFVCQTCTRAFARQEHLKRHE
RSHTKEKPFSCGICSRKFSRRDLLLRHAQKLHSNCSDAAITRLRRKATRRSSNAAGSISGSTPVT
TPNTMGTPEDGEKRKVQKLAGRRDSNEQKLQLQQQHLQQQPQLQYQQSLKQHENQVQQPDQDPLI
SPRMQLFNDSNHHVNNLFDLGLRRASFSAVSGNNYAHYVNNFQQDASSTNPNQDSNNAEFENIEF
STPQMMPVEDAETWMNNMGPIPNFSLDVNRNIGDSFTDIQHKNSEPIISEPPKDTAPNDKKLNGY
SFYEAPIKPLESLFSVRNTKRNKYKTNDDSPDTVDNNSAPAANTIQELESSLNASKNFCLPTGYS
FYGNLDQQTFSNTLSCTSSNATISPILLDNSINNNSTSDVRPEFRTQSVTSEMSQAPPPPQKNNS
KYSTEVLFTSNMRSFIHYALSKYPFIGVPTPTLPENERLNEYADSFTNRFLNHYPFIHVTILKEY
SLFKAILDENESTKNWENNQFYLENQRISIVCLPLLVATIGAVLSNNKKDASNLYEASRRCIHVY
LDSRKKIPTSLSANNNDSPLWLIQSLTLSVMYGLFADNDISLNVVIRQVNALNSLVKTSGLNRTS
IIDLFNINKPLDNELWNQFVKIESTVRTIHTIFQISSNLSALYNIIPSLKIDDLMITLPVPTTLW
QADSFVKFKSLSYGNQIPFQYTRVLQNLIDYNQPLSDGKFLYENHVSEFGLICLQNGLHQYSYFQ
KLTAVNNREDALFTKVVNSLHSWDRMISNSDLFPKKIYQQSCLILDSKLLNNFLIVKSSLKVSTG
DVSSLNKLKENVWLKNWNQVCAIYYNSFMNIPAPSIQKKYNDIEFVDDMINLSLIIIKIMKLIFY
NNVKDNYEDENDFKLQELNLTFDNFDEKISLNLTILFDIFLMIYKIITNYEKFMKIKHKFNYYNS
NSNISFLHHFELSSVINNTQMNQNDYMKTDIDEKLDQLFHIYQTFFRLYLDLEKFMKFKFNYHDF

ETEFSSLSISNILNTHAASNNDTNAADAMNAKDEKISPTTLNSVLLADEGNENSGRNNDSDRLFM
LNELINFEVGLKFLKIGESFFDFLYENNYKFIHFKNLNDGMFHIRIYLENRLDGGV*

Pichia pastoris-Codon-optimized LegH nucleic acid sequence (SEQ ID NO:3)

ATGGGTGCTTTCACCGAGAAGCAGGAAGCACTTGTTTCCTCTTCGTTCGAAGCTTTTAAGGCTAA
CATCCCTCAATACTCTGTTGTGTTTTACACGTCCATTCTAGAAAAAGCTCCTGCTGCCAAGGACC
TCTTCTCTTTTCTGTCCAACGGTGTAGATCCATCCAATCCCAAATTAACAGGTCACGCTGAGAAA
TTGTTCGGTTTAGTCAGAGATAGCGCTGGACAATTGAAAGCAAATGGTACTGTGGTTGCTGATGC
TGCCTTGGGCAGCATCCATGCACAGAAGGCAATTACAGACCCACAATTTGTTGTTGTGAAGGAAG
CTCTGCTTAAAACTATAAAGGAAGCCGTCGGAGACAAATGGAGTGACGAGTTGTCATCAGCTTGG
GAGGTAGCTTATGATGAGTTGGCCGCAGCAATCAAAAAGGCATTCTAA

Pichia pastoris-Codon-optimized LegH amino acid sequence (SEQ ID NO:4)

MGAFTEKQEALVSSSFEAFKANIPQYSVVFYTSILEKAPAAKDLFSFLSNGVDPSNPKLTGHAEK
LFGLVRDSAGQLKANGTVVADAALGSIHAQKAITDPQFVVVKEALLKTIKEAVGDKWSDELSSAW
EVAYDELAAA
IKKAF

Pichia pastoris-Codon-optimized LegH variant 3 nucleic acid sequence (SEQ ID NO:5)

ATGGGTGCATTTACAGAAAAACAAGAGGCTTTAGTATCCTCATCTTTTGAAGCTTTCAAAGCCAA
TATTCCTCAATACTCCGTTGTTTTCTATACGTCCATTTTGGAAAAGGCTCCAGCAGCTAAGGACC
TTTTCTCTTTCTTGTCGAACGGCGTGGATCCCTCAAATCCTAAGCTGACTGGTCACGCCGAGAAG
CTTTTTGGTTTGGTCAGAGACAGCGCCGGACAGCTGAAAGCTAACGGTACAGTTGTGGCAGATGC
TGCCTTGGGATCTATACATGCACAAAAGGCTATCACCGACCCACAGTTTGTGGTTGTAAAAGAGG
CTCTACTCAAAACTATCAAGGAAGCAGTTGGTGACAAATGGAGCGATGAATTGTCCAGTGCATGG
GAGGTCGCTTACGATGAGTTAGCTGCTGCAATCAAAAAGGCTTTCTAA

Pichia pastoris-Codon-optimized LegH variant 3 amino acid sequence (SEQ ID NO:6)

MGAFTEKQEALVSSSFEAFKANIPQYSVVFYTSILEKAPAAKDLFSFLSNGVDPSNPKLTGHAEK
LFGLVRDSAGQLKANGTVVADAALGSIHAQKAITDPQFVVVKEALLKTIKEAVGDKWSDELSSAW
EVAYDELAAAIKKAF

Pichia pastoris pAOX1 promoter (SEQ ID NO:7)

GATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTTGCCATCCGACATCCACAGGTCCAT
TCTCACACATAAGTGCCAAACGCAACAGGAGGGGATACACTAGCAGCAGACCGTTGCAAACGCAG
GACCTCCACTCCTCTTCTCCTCAACACCCACTTTTGCCATCGAAAAACCAGCCCAGTTATTGGGC
TTGATTGGAGCTCGCTCATTCCAATTCCTTCTATTAGGCTACTAACACCATGACTTTATTAGCCT
GTCTATCCTGGCCCCCCTGGCGAGGTTCATGTTTGTTTATTTCCGAATGCAACAAGCTCCGCATT
ACACCCGAACATCACTCCAGATGAGGGCTTTCTGAGTGTGGGGTCAAATAGTTTCATGTTCCCCA
AATGGCCCAAAACTGACAGTTTAAACGCTGTCTTGGAACCTAATATGACAAAAGCGTGATCTCAT
CCAAGATGAACTAAGTTTGGTTCGTTGAAATGCTAACGGCCAGTTGGTCAAAAAGAAACTTCCAA
AAGTCGGCATACCGTTTGTCTTGTTTGGTATTGATTGACGAATGCTCAAAAATAATCTCATTAAT
GCTTAGCGCAGTCTCTCTATCGCTTCTGAACCCCGGTGCACCTGTGCCGAAACGCAAATGGGGAA
ACACCCGCTTTTTTGGATGATTATGCATTGTCTCCACATTGTATGCTTCCAAGATTCTGGTGGGAA
TACTGCTGATAGCCTAACGTTCATGATCAAAATTTAACTGTTCTAACCCCTACTTGACAGCAATA
TATAAACAGAAGGAAGCTGCCCTGTCTTAAACCTTTTTTTTTATCATCATTATTAGCTTACTTTC

ATAATTGCGACTGGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTAACGACAACTTGAGAA
GATCAAAAAACAACTAATTATTCGAAACG

Pichia pastoris pGAP promoter (SEQ ID NO:8)

CGACTATTATCGATCAATGAAATCCATCAAGATTGAAATCTTAAAATTGCCCCTTTCACTTGACA
GGATCCTTTTTTGTAGAAATGTCTTGGTGTCCTCGTCCAATCAGGTAGCCATCTCTGAAATATCT
GGCTCCGTTGCAACTCCGAACGACCTGCTGGCAACGTAAAATTCTCCGGGGTAAAACTTAAATGT
GGAGTAATGGAACCAGAAACGTCTCTTCCCTTCTCTCTCCTTCCACCGCCCGTTACCGTCCCTAG
GAAATTTTACTCTGCTGGAGAGCTTCTTCTACGGCCCCCTTGCAGCAATGCTCTTCCCAGCATTA
CGTTGCGGGTAAAACGGAGGTCGTGTACCCGACCTAGCAGCCCAGGGATGGAAAAGTCCCGGCCG
TCGCTGGCAATAATAGCGGGCGGACGCATGTCATGAGATTATTGGAAACCACCAGAATCGAATAT
AAAAGGCGAACACCTTTCCCAATTTTGGTTTCTCCTGACCCAAAGACTTTAAATTTAATTTATTT
GTCCCTATTTCAATCAATTGAACAACTATCAAAACACG

Pichia pastoris pGCW14 promoter (SEQ ID NO:9)

CAGGTGAACCCACCTAACTATTTTTAACTGGGATCCAGTGAGCTCGCTGGGTGAAAGCCAACCAT
CTTTTGTTTCGGGGAACCGTGCTCGCCCCGTAAAGTTAATTTTTTTTTCCCGCGCAGCTTTAATC
TTTCGGCAGAGAAGGCGTTTTCATCGTAGCGTGGGAACAGAATAATCAGTTCATGTGCTATACAG
GCACATGGCAGCAGTCACTATTTTGCTTTTTAACCTTAAAGTCGTTCATCAATCATTAACTGACC
AATCAGATTTTTTGCATTTGCCACTTATCTAAAAATACTTTTGTATCTCGCAGATACGTTCAGTG
GTTTCCAGGACAACACCCAAAAAAAGGTATCAATGCCACTAGGCAGTCGGTTTTATTTTTGGTCA
CCCACGCAAAGAAGCACCCACCTCTTTTAGGTTTTAAGTTGTGGGAACAGTAACACCGCCTAGAG
CTTCAGGAAAAACCAGTACCTGTGACCGCAATTCACCATGATGCAGAATGTTAATTTAAACGAGT
GCCAAATCAAGATTTCAACAGACAAATCAATCGATCCATAGTTACCCATTCCAGCCTTTTCGTCG
TCGAGCCTGCTTCATTCCTGCCTCAGGTGCATAACTTTGCATGAAAAGTCCAGATTAGGGCAGAT
TTTGAGTTTAAAATAGGAAATATAAACAAATATACCGCGAAAAAGGTTTGTTTATAGCTTTTCGC
CTGGTGCCGTACGGTATAAATACATACTCTCCTCCCCCCCCTGGTTCTCTTTTTCTTTTGTTACT
TACATTTTACCGTTCCGTCACTCGCTTCACTCAACAACAAAA

Pichia pastoris pTEF1 promoter (SEQ ID NO:10)

ATAACTGTCGCCTCTTTTATCTGCCGCACTGCATGAGGTGTCCCCTTAGTGGGAAAGAGTACTGA
GCCAACCCTGGAGGACAGCAAGGGAAAAATACCTACAACTTGCTTCATAATGGTCGTAAAAACAA
TCCTTGTCGGATATAAGTGTTGTAGACTGTCCCTTATCCTCTGCGATGTTCTTCCTCTCAAAGTT
TGCGATTTCTCTCTATCAGAATTGCCATCAAGAGACTCAGGACTAATTTCGCAGTCCCACACGCA
CTCGTACATGATTGGCTGAAATTTCCCTAAAGAATTTcTTTTTCACGAAAATTTTTTTTTTACAC
AAGATTTTCAGCAGATATAAAATGGAGAGCAGGACCTCCGCTGTGACTCTTCTTTTTTTTCTTTT
ATTCTCACTACATACATTTTAGTTATTCGCCAAC

Heme biosynthesis enzyme 1- ALA Synthase (SEQ ID NO:11)

ATGGAGTTTGTCGCCCGTCAGTCCATGAATGCCTGTCCCTTTGTCAGGTCAACTTCTACCCACCA
TTTGAAGAAGTTGGCAGCAAACAGTTCTCTAGCTGCTACTGCTAGTCATTGTCCCGTGGTTGGCC
CTGCTCTCCAACAGCAGAGATACTACTCTCAACCTTCCAAGCCAGCCCAAGCCCAAACCTCCGAC
ATTGCTACTGGGATCAAGAAGGATGTTTCTCCGATCCGTATGGACTCTAATGAAACCGCCTTTGA
TTACAATGGAATGTATGAGTCTGATCTTGCGAATAAACGTAAAGATAACTCGTATCGTTATTTCA
ATAACATCAACCGTCTAGCCAAGGAGTTTCCCAAGGCACATCGCCAGACCGAAGATGACAAGGTG
ACCGTCTGGTGCTCTAACGACTACTTAGGAATGGGTAGGCATCCTGAGATTATCAAAACCATGAA

GGCTACCATGGACAAGTACGGTTCCGGAGCAGGAGGAACTAGGAACATTGCAGGTCATAACCACG
CCGCTATCAATTTGGAAAGCGAGTTGGCTTGCTTGAACAAGAAGGAAGCGGCTCTGGTGTTTTCA
TCATGTTTCATAGCTAACGATGCAATCATCTCGTTGTTGGGACAAAAAATCAAAAATTTGGTCAT
TTTCTCTGACCAGTCGAATCATGCTTCCATGATATTGGGTGTGCGTAACTCCAAAGCGAAGAAGC
ACATCTTCAAGCACAACAATTTGAAGGATCTGGAGTCGCAGTTAGCTCAGTACCCCAAGTCGACT
CCTAAACTGATCGCCTTCGAGTCAGTTTACTCTATGTGTGGATCTGTGGCTCCCATTGAGAAGAT
TTGCGATTTGGCTAAAAGGTACGGTGCCCTCACCTTCTTGGATGAAGTTCATGCTGTTGGAATGT
ATGGTCCTCATGGACAGGGTGTAGCTGAGCATTTGGACTTTGATCTGCATTTACAGTCTGGAATC
GCCAGTCCTAGCGTGGTGGACAAACGCACCATATTGGATCGTGTCGACATGATTACTGGTACTTG
CGGAAAGTCATTTGGTACTGTTGGAGGTTACGTTGCTGGTAGTGCCAACCTAATTGATTGGTTAA
GATCCTATGCGCCAGGTTTCATTTTCACTACCACACTTCCTCCTGCTATCATGGCTGGTACAGCC
ACTTCTGTTCGTATTGTTAGGGCCGACATTGAGGCCCGTATCAAGCAACAGCTTAATACTCGCTA
CGTCAAAGACTCATTTGAAAACCTTGGTATTCCAGTCATTCCAAACCCAAGTCACATTGTTCCTG
TTCTAGTTGGAAATGCTGCAGATGCCAAGAAGGCATCCGATATGTTAATGAACAAACACCGTATT
TATGTTCAAGCTATTAACTACCCTACTGTGCCTGTCGGTGAAGAACGACTAAGGATTACTCCTAC
TCCAGGTCATGGAAAGGAGATTTGTGACCAGCTGATCAGCGCTGTCGACGATGTTTTTACTGAGC
TTAATTTACCAAGAATCAACAAATGGCAGTCCCAAGGTGGTCATTGCGGTGTTGGTGATGCTAAT
TACGTACCAGAACCCAATCTGTGGACTCAGGACCAGCTCAGCTTGACAAACCAAGACTTGCACTC
CAATGTGCACAACCCAGTGATTGAGCAGATCGAAACCTCATCAGGAGTCAGATTGTAG

Heme biosynthesis enzyme 2- ALA dehydratase (SEQ ID NO:12)

ATGGTGCATAAGGCTGAATACTTGGACGACCACCCAACTCAGATTTCCAGCATTCTTTCAGGAGG
TTACAACCACCCATTACTTCGTGAATGGCAACATGAACGTCAACTCAACAAAAACATGTTCATCT
TTCCCCTGTTTGTCACAGATCGACCAGACGAAGAAGAACTTATTCCTAGTCTACCTAATATCAAG
AGGTTTGGCGTTAACAAGTTGATTCCTTATGTAGGAGGTTTGGTTTCCAAAGGATTGAGGGCGGT
GATCCTATTTGGTGTTCCTCTGAAGCCCGGTGTGAAAGATGAAGAAGGAACGGCCGCTGATGATC
CAGAGGGACCTGTTATCCAAGCCATCAAACACTTGAGAAAGAACTTTCCTGACCTGTATATCATC
ACCGATGTCTGTCTATGTGAGTACACCAGCCATGGACATTGTGGAATACTATATGAGGATGGCAC
TATCAACAGAGAGCTCTCAGTCCGTCGTATTGCTGCTGTAGCTGTCAAATATGCTCAAGCTGGAG
CCAACTCTGTGGCTCCTTCTGATATGACTGACGGCAGAATAAGAGATATTAAAGAAGGCTTACTA
AGTGCAGGACTGGCACATAAAACGTTTGTTATGTCCTACGCTGCAAAATTCTCTGGTAATTTGTA
TGGCCCTTTCAGAGATGCTGCAGGTTCCTGTCCATCTCAAGGGGACAGAAAATGTTACCAGCTTC
CTTCTGGAGGAAAAGGGTTGGCCCATCGTGCTCTGATTCGTGATATGAATGAAGGCACTGATGGA
ATTATTGTCAAACCATCTACATTCTATTTGGACATTGTCGCTGATGCTTATCAGCTTTGTAAAGA
CTATCCTATCTGCTGTTACCAGGTTTCTGGAGAGTACGCCATGCTACATGCAGCGGCAGAGAAGA
ATATTGTTGATCTGAAATCAATCGCTTTTGAAGCTCATCAAGGATTCTTGCGGGCTGGAGCTCGT
TTAATCATTAGTTACTTTACCCCTGAATTCCTGGAGTGGTTATCTGAATGA

Heme biosynthesis enzyme 3- Porphobilinogen deaminase (SEQ ID NO:13)

ATGAACCAAATCGAACAGAGCGGACCCATTGATTGCAGTTCCTTGAAATTGGGGTCCCGAAAGTC
CGCTCTGGCTATAATCCAGGCAGAAATCGTCCGCCAATTGATATTGAAAGAATACCCTGAATTGG
AGACGAAGTTGGTCAGTGTGTCCACCCTGGGGGACCAAGTCCAGAATAAAGCACTTTTCACGTTT
GGAGGAAAATCTTTGTGGACCAAAGAACTTGAGATGTTGTTGTTGGAGAGTGTGGGAGGATTTGA
CCAAATAGACATGATTGTACACTCGTTGAAAGACATGCCAACTCATTTACCAGACGAATTTGAGC
TGGGTTGCATTATTGAAAGAGAAGACCCTAGAGACGCTTTGGTCGTGCAAGATGGTTTATCTTAC
AAGTCATTGGCCGACCTTCCAGAGGGAGCTGTGGTCGGTACGTCTTCGGTTAGAAGATCGGCTCA
ACTACTGAAGAATTTCCCTCATCTGAAATTCAAATCTGTTAGAGGAAACCTTCAGACCAGACTAA

GAAAATTAGATGATCCAGATTCCGAGTACTGCTGTCTCCTCCTTGCAGCAGCCGGTTTAATCAGG
ACAGGCTTACAACACAGAATTTCAATGTATTTGAACGACGATGTGATGTACCACTCCGTCGGACA
AGGAGCATTAGGAGTAGAGATCAGAAAAGGTGACCAATTCATGAAAAATATCTGTGAAAAGATTG
GGCATAGAACCACCACCCTTCGTTGTCTTGCAGAGAGAGCACTGCTGAGATATCTAGAGGGAGGC
TGCTCGGTGCCAATTGGGGTCTCCACTATTTATAGCGAGGATACGAAGGAACTTACCATGAACTC
CCTAGTCGTCAGTTGTAACGGTCGTGACTCGGTAACAGAATCAATGACTGAAGTCGTGACTACTG
AAGAGCAAGCTGAAGATTTCGGTGAAAGGCTGGCCCAGAAGCTCATAGATCAAGGTGCGAAACGC
ATTCTTGACGAGATCAACTTCAACAAGATCAAAGAGATTAAGGAAGAGGGTTTACATTAA

Heme biosynthesis enzyme 4- Uroporphyrinogen III synthase (SEQ ID NO:14)
ATGCCAAAAGCCATTCTTCTGAAGAATAAAACTACACCGAAGGATCCTTATCTGGAGAACTTCGT
AAGTAGTGGCTACTCGACCGATTTCGTACCACTTTTAGATCATATTCACATGGAGAAATCTGAGA
TCATCGCATTTCTCAAGACTGACTACTTTTTGCATAAAACTTTGGCGTTTATTATTACGTCCCAA
AGAGCTGTAGAAATGCTGAATGAGTGTATGCAAATACTGAGACGTACTGATCCTGAAATTACACA
AATCATCTATAGTAAACCTGTCTATACAGTTGGCCCTGCCACCTACAGAATACTTGCGGATGCTG
GCTTCGTGGATCTACGAGGCGGAGATAAGGCAGGAAACGGATCCATTCTAGCCCAGATAATTTTG
AATGATGACATTTACACTGGAATTGAAGATTCTGACAAGCATATAACGTTTTTCACGGGAGAAAC
AAGGAGAGACATAATTCCCAAATGTTTACTCTCTAACAACTTTCAACTTTACGAAAAGATTGTCT
ACAAGACTCTTCCTAGGGATGATATCGTGACTAGATTCAAGTCTGCCGTTGACAGCATCGACCAA
TCGCAAAGAAGTTCCAGTTGGGTGGTCTTCTTTTCGCCTCAAGGAACAGAGGACATTGTAACGTA
TCTTCAACACACCAAAGACCAATTTAATATTGCATCTATCGGGCCAACCACAGAAAAATACCTTC
TAAGCAAAAACCTGAAACCAAAAGTTGTGGCACCTAAGCCAGAGCCTATCTCTTTACTATTGTCT
ATACAAAAAGTGCACTAA

Heme biosynthesis enzyme 5- Uroporphyrinogen III decarboxylase (SEQ ID NO:15)
ATGAGTAGATTTCCAGAACTGAAGAATGACCTTATTTTAAGGGCAGCTCGTGGTGAAAAAGTTGA
ACGTCCCCCAATATGGATTATGAGACAGGCCGGAAGATATCTTCCGGAGTACCATGAGGTCAAAG
GAGGTAGGGACTTCTTTGAAACTTGCAGGGATGCTGAGATTGCTTCTGAAATTACTATCCAGCCG
ATTACGCATTTTGACGGTCTGATCGATGCAGCTATTATCTTCAGTGATATCTTGGTGATTCCTCA
AGCTATGGGCATGGAAGTTAAGATGGTGGACAAAGTTGGCCCACAGTTCCCCAATCCGCTAAGAA
AACCGTCTGACTTGGATCATTTGAAAAAAGACGTTGACGTTTTGAAGGAACTCGATTGGGCCTTC
AAAGCTATCTCATTGACCAGAAAAAAACTCAATGGACGAGTGCCTTTGCTTGGATTTTGTGGTGC
TCCTTGGACTCTACTGGTTTATATGACTGAAGGAGGCGGTACCAAGATGTTTCGATTTGCAAAAG
AGTGGATCTACAAGTTTACCAAGGAATCTCATCAATTACTCCAACAGATCACTGACGTTGCAGTT
GAATTCTTAGCTCAGCAAGTTGTTGCAGGTGCCCAAATGTTACAAGTTTTTGAATCTTGGGGCGG
TGAATTGGGGCCTGATGAATTCGATGAGTTTTCTTTGCCTTATTTGAGACAGATTTCCTCTAAAC
TTCCCCTGAGGTTGAAGGAACTTGGAATCACAGAGAATGTTCCCATAACTGTCTTTGCTAAAGGC
TCTTGGTACGCCTTGGAGCAATTGTGCGACAGTGGTTATGATGTTGTCTCGTTGGATTGGTTATT
CCGTCCAAGTGATGCTGTCCAGATTGCTAACGGAAGAATCGCATTGCAAGGTAATCTTGACCCTG
GAACCATGTACGGCTCCAAAGAAACCATTTCCAAGAAAGTGGACAAAATGATCAAGGGTTTTGGT
GGAGGAAAGCAAAACTACATAATTAATTTTGGACACGGCACTCATCCATTCATGGATCCAGAACA
GATCAGATGGTTCTTACAAGAATGTCATCGCATTGGATCTCAATAG

Heme biosynthesis enzyme 6- Coproporphyrinogen III oxidase (SEQ ID NO:16)
ATGGCCATCGACTCTGATATCAATCTAAGCTCTCCCAATGATTCCATCCGTCAAAGGATGTTCGA
GCTTATCCAGCGGAAGCAACTCGAAATTGTCGCTGCATTGGAGGCAATTGAAGGAAACGATACCA
AATTTCGTTCTGATTCTTGGGAAAGAGGAGCCGAAGGTGGAGGAGGAAGATCTATGCTTATTCAA

GATGGAAGAGTGTTTGAAAAGGCTGGTGTAAATATTTCCAAGGTTCATGGCGTATTGCCTCCTCA
AGCTGTGAGCCAGATGAGAAATGACCACTCCAAGCTAGATCTGCCTGCGGGAACCTCTCTGAAGT
TCTTTGCCTGTGGGCTTTCGTTGGTCATTCATCCCCATAATCCCCATGCTCCAACTACCCATCTG
AATTATCGCTACTTCGAAACTTGGGATGAAACTGGAAAGCCTCACACCTGGTGGTTTGGGGGCGG
TGCTGATTTAACGCCTTCGTACCTGTATCCCGAGGATGCCAAGCAATTCCATCAAGCCCATAAGG
ATGCCCTGGACAAACACGATGTTAGCTTGTACCCGAGATTCAAAAAGTGGTGTGATGAATACTTT
CTGATCAAACATCGAAATGAAACTAGAGGTATTGGGGGTATTTTCTTTGATGATTTTGACGAGTT
TGATGCTGAGAGGTCCCTGAAGTTGGTTGAAGATTGTTTCAATGCTTTCTTGGAATCTTATCCCG
CTATCACTCGAAAAAGGATGGACACCCCTTCAACTGATGCTGAGAAGAACTGGCAACAAATTAGA
AGAGGAAGATATGTCGAATTCAACTTAGTATTGGATAGAGGTACTCAATTTGGTTTGAGAACGCC
TGGATCTCGTGTTGAAAGTATTTTGATGTCGTTGCCAAGAACAGCTGGTTGGGTCTATGATCATC
ATCCAGAGCCTGGCTCCAGAGAAGAGGAGTTATTGCAGGTACTACAAAATCCTATTGAATGGGTA
TGA

Heme biosynthesis enzyme 7- Protoporphyrinogen oxidase (SEQ ID NO:17)

ATGCTGAAAAGTCTTGCACCAAATTCCTCAATTGCCGTTTTAGGTTCAGGGATATCTGGATTGAC
TTTCAGCTTTTTTTTGAATCGGTTGCGTCCCGATGTTAAGATCCATATCTTTGAAAAATCCAAGC
AGGTTGGAGGATGGATCAGATCAGAAGAGCATGAAACTTTTCATTTTGAAAAGGGACCCAGAACT
TTGAGAGGCACAAATACGGGTACCTTGATGTTGTTGGATCTTCTTACCAAGATAGGAGCAAATGA
CAAGGTCCTGGGACTGCACAAAGATTCTCTTGCTAATAAAAAGTATCTGTTGTCCCCGTTCTCAG
ATGTTCACGGAAACAACGCAAAGCTTCTTCAAGTGCCACAGGATTTCAGCTCTTTTGTAAAGTTC
ATGTTTGACCCGTTGTCTAAGGATCTCATTCTCGGTCTTTTGAAAGAACCATGGCAACCAAAATT
AAAGTATTCAGATGAGTCGGTTGACCATTTTTTCAACAGAAGATTTGCTACCAAACTATCAGAGA
ATATCGTCAGCGCAATTGTGCATGGAATCTATGCGGGCGACGTGAAGAAGTTAAGTGTGAAAGCC
ATCTTCCCTAGGCTCCCTGAGATGGAACAGGAAAGTGGCTCTATTATAAGGTATATGATCGCCCA
ATACAGGACAAAAAAGAACGTCAAACAAAAAGTTGACCCTTTTTTGGCAGATTATGAAAAATTGA
TCGGTACATCTTTGAGTTTCAAAAATATTTCTTTGTTTCTGAAAAACTTTCCCATGCTGAGTTTT
CAGGGTGGACTACAGAAACTTCCCATCTCATTGAAGAACCATTTATCACAGATTGAAAACATCAA
GTTTCATTTTGACAGCAAAATCAAAAACATTGCTTTGGAGAGCGGTAAGGTGGCATTGACTGACC
ATGATCAGGTTTATCTTGTTGACCATGTGAGATCTACCATTAATACCAACGAATTGGCCAAAATC
ATTTCACCCGTTGTTCCAAGTTCTACTAAGAAAAAATCCGTTTTCAAATCCAAAGCGAATGGCCC
AGGGCTGGTCAAATGTTTGAGCTGGCTACACTATACAAATATACTAATGTGCAACATTTATATAC
CTAAGCACGTCTCAAAATCTATCACCGGATTTGGATACTTGGTTCCTCGATCAATGTCTTCTCAG
GCATCCAAACTTCTCGGTGTCATATTTGACTCAGACATCGAGACTGCAATGACTCCTAATTTTAC
AGAGGCCAACATTACGGCGATAAACAGTAACTCTGCATCTCCCAAGCAACTCCAAAAGTTTTCTG
ACCAATTCGTCAATAATGATCTCCCTAAATACACCAAGTTGACGCTAATGCTTGGAGGTCATTAT
CTCAAGTCGGAGGCAGACATGCCCGGTTCCGCAGAGAGTAAACATGCTGTCAAGGCGATTCTGTC
AAATCACCTGAATATTGATCTAGATGAGTTTGCATCTTTGCCAGACTTCAAGATGGAAATCACCA
AGATCCCCAACTGCATTCCCCAATATGAAGTTGGGTATCTTGATCTCAAGAGAAAGGTTCAGAAT
GCAGCCTCCAAAGAGTTCAACGACCAAATAAGTTTTGGAGGCATGGCATTTGGTGATGGTGTGGG
GATCCCTGACTGTGTCCAGAATGCATTCAAAGATTCGGCTACCCTCAGTGGCATTTAA

Heme biosynthesis enzyme 8- Ferrochelatase (SEQ ID NO:18)

ATGCTTAACCGTCGTTTCCAATCTACCGTGTCCTCGAGTCTGAACAAGGGCACTGGAATAGTGTT
CATGAATATGGGTGGTCCCTCCACTGTCAAGGAAACCTATGACTTTTTATTTCGTCTTTTCTCGG
ACGGAGATTTAATCCCGTTTGGCAGATTTCAGAACATCCTGGCCCGCTTCATTGCAAGTAGAAGA
ACACCCAAAATTGAATCCTACTACAAAGCTATCGGAGGTGGGTCTCCTATCCGAAAGTGGTCTGA

ATACCAGAGTTCTAAACTATGTGAAAAATTAGACATTATCAGTCCACAATCGGCTCCTCATAAGC
CTTATGTTGCCTTCAGATACGCTAATCCTCTCACTGAAGATACTTTACAAAAGATGAAAAATGAT
GGAATTACTAAGGCCATTGCCTTTTCTCAATATCCGCAATTTAGTTATTCAACCACCGGATCATC
GATTAACGAACTTTACAGGCAATCGAAAATTTTGGACCCTGATCAATCTATTAAATGGACAGTTA
TAGATCGCTGGCCTGACCACCCAGCCTTAGTTAAAACTTTCGCAGCTCATATCAAAGATACTCTA
AACAGATTCAAAACTGAAAATGGACTGACTGACACAAAAGACGTCGTCCTCCAATTCAGTGCTCA
TTCTTTACCAATGGATATTGTCAATAAAGGAGATTCGTATCCTGCAGAAGTCGCAGCGAGTGTCT
TTGCCATTATGAAAGAACTTAACTTCTCAAATCCTTATAAATTAACCTGGCAATCACAGGTTGGC
CCAAAGCCTTGGCTGGGTGCTCAAACTGAAAAAATTACCAAGCAGCTAGCATCCAGTGATGTTCC
TGGAGTCGTTTTGGTTCCTATTGCCTTTACCTCTGATCATATTGAAACTCTCCATGAACTGGATA
TTGAACTGATTCAAGAACTACCTAATCCTTCAAAAGTAAAGCGAGTTGAATCGTTGAACGGAGAC
CAAACTTTCATTGACTCCTTGGCAGAACTAGTGAAGAGTCACATTGATTCGAAGGTTGTATTTTC
CAACCAGTTGCCATTGGATTCCATGCTGGGAGTAGTGTCAGATAATTCCCTCACAGATCCAAAAG
AGTTTTTCAGAGCCCATTGA

Part C. Results and Discussion

Example 24— Characterization of Strain MXY0183

Optimum growth conditions for Strain MXY0183 include a target pH of 3.0 to 6.0 and temperatures of 28-35°C. In order to produce the LegH protein, strain MXY0183 must be alive and growing aerobically for a period of 6 days.

Expression of the genes associated with strain MXY0183 resulted in phenotypic changes to the strain. Figure 8 shows photographs of shake flasks at the start of induction (0 hr) and 72 hr post-induction. The flasks designated #1 contain the host strain, MXY0051. The flasks designated #2 and #3 contain one of the intermediate strains (i.e., MXY0118, containing >10 copies of the LegH gene and the ALA dehydratase from the heme biosynthetic pathway) and the production strain (i.e., MXY0183, containing > 10 copies of the LegH gene and the 8 enzymes from the heme biosynthetic pathway), respectively. The characteristic red color in flask #3 after 72 hours demonstrates the production of heme-bound LegH.

After growing in shake flasks, the *P. pastoris* strains indicated above, MXY0051, MXY0118, and MXY0183, were lysed and the proteins run on a SDS gel (Figure 9A). The arrow shows the position of the LegH protein. A comparison of LegH production in strain MXY0183 and in strain MXY0118 is shown in Figure 9B, which demonstrates the efficiency of heme loading of the LegH protein by the MXY0183 strain.

Example 25— Characterization of Strain MXY0207

Experiments were then performed to determine the benefits of overexpressing the transcriptional activator, Mxr1, in the presence of the genes encoding the 8 enzymes involved in heme biosynthesis. Strain MXY0183, which contains >10 copies of the LegH sequence and the genes encoding the 8 enzymes involved in heme biosynthesis, and sister strains MXY0206 and MXY0207, which contain >10 copies of the LegH sequence, the genes encoding the 8 enzymes involved in heme biosynthesis, and the Mxr1 transcriptional activator, were grown in shake flask cultures in the presence of glycerol, which is a repressing carbon source for these strains. Photographs of the shake flask cultures after 48 hr are shown in Figure 10A, and photographs of the pellets from cells grown on BMY media for 48 hours with no additional source of carbon are shown in Figure 10B; these experiments demonstrated that significant expression of transgenes (e.g. heme enzymes) under the control of the AOX1 promoter occurs in the absence of an inducing carbon source when a repressing carbon source is consumed in the growth medium of a strain in which Mxr1 is also expressed from the AOX1 promoter. The relative yield of heme-loaded LegH, when shake flask cultures were grown in the absence of induction agent, is shown in Figure 10C. These experiments demonstrated that significant production of a recombinant, heme-loaded protein is accomplished from AOX1 promoter-driven transgenes in the absence of methanol induction in Pichia strains in which Mxr1 expression is also driven by the AOX1 promoter.

Select strains were grown in 2 L fermenter tanks, and the relative yield of LegH and heme-loaded LegH was determined (Figure 11). Compared to strain MXY0183, the MXY0207 strain produced even more LegH and was able to produce enough heme to heme-load the LegH protein very effectively.

Example 26—Characterization of Strain MXY0291

As described above in Examples 18-20, strain MXY0291 was constructed to recapitulate the LegH production ability of MXY0207, while being free of antibiotic resistance genes. It was determined that strain MXY0291 contained ~ 16 copies of LegH var3, Mxr1 and 7 of the 8 heme biosynthetic enzymes. When grown in 2 L fermenter tanks, this strain showed improved LegH yield compared to MXY0207. This improvement was seen both in induction media containing methanol / glycerol and methanol / dextrose (D-glucose) (Figure 11).

Example 27—Characterization of Hybrid Promoter Strains

Additional copies of soybean leghemoglobin (LegH) were expressed under three different constitutive promoters, pGAP, pGCW14 and pTEF1, in a strain that already contains several copies of LegH, all heme biosynthetic enzymes, and the transcriptional factor Mxr1 under control of the promoter, pAOX1 (referred to above as MXY0291). When induced by methanol in the presence of dextrose (i.e., D-glucose), the constitutive promoters and pAOX1 drive expression of LegH while only the pAOX1 promoter drives expression of the heme enzymes. This leads to further improvement in LegH yield compared to previous strain MXY0291 (Figure 11).

It is to be understood that, while the methods and compositions of matter have been described herein in conjunction with a number of different aspects, the foregoing description of the various aspects is intended to illustrate and not limit the scope of the methods and compositions of matter. Other aspects, advantages, and modifications are within the scope of the following claims.

Disclosed are methods and compositions that can be used for, can be used in conjunction with, can be used in preparation for, or are products of the disclosed methods and compositions. These and other materials are disclosed herein, and it is understood that combinations, subsets, interactions, groups, etc. of these methods and compositions are disclosed. That is, while specific reference to each various individual and collective combinations and permutations of these compositions and methods may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a particular composition of matter or a particular method is disclosed and discussed and a number of compositions or methods are discussed, each and every combination and permutation of the compositions and the methods are specifically contemplated unless specifically indicated to the contrary. Likewise, any subset or combination of these is also specifically contemplated and disclosed.

WHAT IS CLAIMED IS:

1. A methylotrophic yeast cell comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises an exogenous nucleic acid encoding a transcriptional activator operably linked to at least one methanol-inducible promoter element.

2. The yeast cell of claim 1, wherein the methylotrophic yeast cell is of a yeast selected from the group consisting of *Candida*, *Hansenula*, *Pichia* and *Torulopsis*.

3. The yeast cell of claim 1 or 2, wherein the methylotrophic yeast cell is of a *Pichia* yeast.

4. The yeast cell of claim 1 or 2, wherein the cell is a *Pichia* cell.

5. The yeast cell of any of claims 1 to 4, wherein the cell is a *Pichia pastoris* cell.

6. The yeast cell of any of claims 1 to 5, wherein the recombinant nucleic acid molecule is stably integrated into the genome of the methylotrophic yeast cell.

7. The yeast cell of any of claims 1 to 5, wherein the recombinant nucleic acid molecule is extrachromosomally expressed from a replication-competent plasmid.

8. The yeast cell of any of claims 1 to 7, wherein the exogenous nucleic acid encoding a transcriptional activator comprises a Mxr1 sequence from *Pichia pastoris*, a Adr1 sequence from *Hansenula polymorpha*, a Trm1 sequence from *Candida boidinii*, or a Trm2 sequence from *Candida boidinii*.

9. The yeast cell of any of claims 1 to 8, wherein the exogenous nucleic acid encoding a transcriptional activator comprises a Mxr1 sequence from *Pichia pastoris*.

10. The yeast cell of any of claims 1 to 9, wherein the transcriptional activator comprises a sequence shown in GenBank Accession No. ABD57365.

11. The yeast cell of claim 10, wherein the transcriptional activator is encoded by the nucleic acid sequence shown in GenBank Accession No. DQ395124.

12. The yeast cell of any of claims 1 to 11, wherein the at least one methanol-inducible promoter element is selected from the group consisting of an alcohol oxidase 1 (AOX1) promoter element from *Pichia pastoris*, an AOD1 promoter element from *Candida boidinii*, a MOX promoter element from *Hansenula polymorpha*, a MOD1 promoter element from *Pichia methanolica*, a DHAS promoter element from *Pichia pastoris*, a FLD1 promoter element from *Pichia pastoris*, and a PEX8 promoter element from *Pichia pastoris*.

13. The yeast cell of any of claims 1 to 11, wherein the at least one methanol-inducible promoter element is an alcohol oxidase 1 (AOX1) promoter element from *Pichia pastoris*.

14. The yeast cell of any of claims 1 to 13, further comprising a nucleic acid molecule comprising at least one heterologous nucleic acid encoding a polypeptide operably linked to at least one methanol-inducible promoter element.

15. The yeast cell of claim 14, wherein the heterologous nucleic acid encodes at least one polypeptide involved in the biosynthesis of an iron co-factor.

16. The yeast cell of claim 15, wherein the iron co-factor is heme.

17. The yeast cell of claim 16, wherein the at least one polypeptide involved in the biosynthesis of heme is selected from the group consisting of ALA synthase, ALA dehydratase, porphobilinogen deaminase, UPG III synthase, UPG III decarboxylase, CPG oxidase, PPG oxidase, and ferrochelatase.

18. The yeast cell of claim 16, wherein the at least one polypeptide involved in the biosynthesis of heme comprises two or more polypeptides selected from the group consisting of ALA synthase, ALA dehydratase, porphobilinogen deaminase, UPG III synthase, UPG III decarboxylase, CPG oxidase, PPG oxidase, and ferrochelatase.

19. The yeast cell of any of claims 1 to 18, wherein the yeast cell lacks heterologous sequences for selection.

20. A method for expressing a heterologous polypeptide in a cell, the method comprising:

providing the yeast cell of any of the preceding claims;

introducing a recombinant nucleic acid molecule into a methylotrophic yeast cell, the recombinant nucleic acid molecule comprising at least one heterologous nucleic acid encoding a polypeptide operably linked to at least one *Pichia pastoris* alcohol oxidase 1 (AOX1) promoter element; and

culturing the cell under conditions suitable for expression of the recombinant nucleic acid molecule,

thereby expressing the heterologous polypeptide.

21. The method of claim 20, wherein the heterologous polypeptide is a member of the globin family PF00042.

22. The method of claim 20, wherein the heterologous polypeptide is a plant hemoglobin.

23. The method of claim 22, wherein the plant hemoglobin is leghemoglobin.

24. The method of claim 23, wherein the leghemoglobin has a sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:6.

25. The method of any of claims 20 to 24, wherein the introducing step comprises a technique selected from the group consisting of transduction, electroporation, biolistic particle delivery, and chemical transformation.

26. The method of any of claims 20 to 25, wherein the conditions under which the cells are cultured comprises the addition of iron or a pharmaceutically or metabolically acceptable salt thereof.

27. The method of any of claims 20 to 26, wherein the culturing step comprises culturing the cell in the present of methanol.

28. The method of any of claims 20 to 26, wherein the culturing step comprises culturing the cell in the absence of methanol.

29. The method of any of claims 20 to 28, wherein the culturing step comprises culturing the cell in the absence of selection.

30. A methylotrophic *Pichia* yeast cell comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises:

a nucleic acid encoding a Mxr1 transcriptional activator sequence from *P. pastoris*;

a nucleic acid encoding a member of the globin family (PF00042); and

a nucleic acid encoding at least one polypeptide involved in heme biosynthesis.

31. The yeast cell of claim 30, wherein the nucleic acid encoding the Mxr1 transcriptional activator sequence is operably linked to a methanol-inducible promoter element.

32. The method of claim 30, wherein the member of the globin family is leghemoglobin.

33. The yeast cell of any of claims 30 to 32, wherein the nucleic acid encoding the member of the globin family is operably linked to a methanol-inducible promoter element.

34. The yeast cell of any of claims 30 to 32, wherein the nucleic acid encoding the member of the globin family is operably linked to a constitutive promoter element.

35. The yeast cell of any of claims 30 to 34, wherein the nucleic acid encoding at least one polypeptide involved in heme biosynthesis is operably linked to a methanol-inducible promoter element.

36. The yeast cell of any of claims 30 to 34, wherein the nucleic acid encoding at least one polypeptide involved in heme biosynthesis is operably linked to a constitutive promoter element.

37. The yeast cell of any of claims 30, 31, 33, or 35, wherein the methanol-inducible promoter element is an alcohol oxidase I (AOX1) promoter element from *P. pastoris*.

38. The yeast cell of any of claims 30, 34 or 36, wherein the constitutive promoter element is a transcriptional elongation factor EF-1α gene (TEF1) promoter element from *P. pastoris*.

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Sequence Listing was accepted.
If you need help call the Patent Electronic Business Center at (866)
217-9197 (toll free).
Reviewer: Anjum, Durreshwar
Timestamp: [year=2017; month=11; day=21; hr=14; min=1; sec=35; ms=367; ]
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Application No: 15573300 Version No: 1.0

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Glu Arg Ser His Thr Lys Glu Lys Pro Phe Ser Cys Gly Ile Cys Ser
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Arg Lys Phe Ser Arg Arg Asp Leu Leu Leu Arg His Ala Gln Lys Leu
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His Ser Asn Cys Ser Asp Ala Ala Ile Thr Arg Leu Arg Arg Lys Ala
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Thr Arg Arg Ser Ser Asn Ala Ala Gly Ser Ile Ser Gly Ser Thr Pro
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Lys Val Gln Lys Leu Ala Gly Arg Arg Asp Ser Asn Glu Gln Lys Leu
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Gln Ser Leu Lys Gln His Glu Asn Gln Val Gln Gln Pro Asp Gln Asp
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Glu Asn Glu Ser Thr Lys Asn Trp Glu Asn Asn Gln Phe Tyr Leu Glu
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Phe Leu Ile Val Lys Ser Ser Leu Lys Val Ser Thr Gly Asp Val Ser
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Ser Leu Asn Lys Leu Lys Glu Asn Val Trp Leu Lys Asn Trp Asn Gln
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Val Cys Ala Ile Tyr Tyr Asn Ser Phe Met Asn Ile Pro Ala Pro Ser
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Lys Leu Phe Gly Leu Val Arg Asp Ser Ala Gly Gln Leu Lys Ala Asn
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Gly Thr Val Val Ala Asp Ala Ala Leu Gly Ser Ile His Ala Gln Lys
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Ala Ile Thr Asp Pro Gln Phe Val Val Val Lys Glu Ala Leu Leu Lys
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Thr Ile Lys Glu Ala Val Gly Asp Lys Trp Ser Asp Glu Leu Ser Ser
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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 NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/573,300	11/10/2017		1260	38767-0048US1	38	2

CONFIRMATION NO. 4807

26191
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

FILING RECEIPT

OC000000096963148

Date Mailed: 01/29/2018

Receipt is acknowledged of this non-provisional 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 APPLICANT, 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 Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections 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**

Inventor(s)

Smita Shankar, Millbrae, CA;
Martin Andrew Hoyt, San Francisco, CA;

Applicant(s)

Impossible Foods Inc., Redwood City, CA;

Power of Attorney: The patent practitioners associated with Customer Number 26191

Domestic Priority data as claimed by applicant

This application is a 371 of PCT/US2016/031797 05/11/2016
which claims benefit of 62/313,491 03/25/2016
and claims benefit of 62/236,506 10/02/2015 *
and claims benefit of 62/222,388 09/23/2015 *
and claims benefit of 62/220,366 09/18/2015 *
and claims benefit of 62/203,052 08/10/2015 *
and claims benefit of 62/185,921 06/29/2015 *
and claims benefit of 62/183,074 06/22/2015 *
and claims benefit of 62/159,899 05/11/2015 *
(*)Data provided by applicant is not consistent with PTO records.

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.

If Required, Foreign Filing License Granted: 01/25/2018
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/573,300**
Projected Publication Date: 05/10/2018
Non-Publication Request: No
Early Publication Request: No
Title

EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

Preliminary Class

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).

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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technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

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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

U.S. APPLICATION NUMBER NO.	FIRST NAMED INVENTOR	ATTY. DOCKET NO.
15/573,300	Smita Shankar	38767-0048US1

26191
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

INTERNATIONAL APPLICATION NO.	
PCT/US2016/031797	
I.A. FILING DATE	PRIORITY DATE
05/11/2016	05/11/2015

CONFIRMATION NO. 4807
371 FORMALITIES LETTER



Date Mailed: 01/29/2018

Notification of Insufficiency Under 37 CFR 1.492 and/or 1.495 (DO/EO/US)

The following items have been submitted by the applicant or the International Bureau to the United States Patent and Trademark Office as a Designated / Elected Office (37 CFR 1.495).

- Priority Document
- Copy of the International Application filed on 11/10/2017
- Copy of the International Search Report filed on 11/10/2017
- Preliminary Amendments filed on 11/10/2017
- Sequence Listing txt file (EFS or physical medium) filed on 11/21/2017
- Sequence Listing (PDF or paper) filed on 11/21/2017
- U.S. Basic National Fees filed on 11/10/2017
- Authorize Access to Search Results filed on 11/10/2017
- Priority Documents filed on 11/10/2017
- Authorization to Permit Access filed on 11/10/2017
- Application Data Sheet (37 CFR 1.76) filed on 11/10/2017

The following items **MUST** be furnished within the period set forth below:

- Additional claim fees of $**1800** as a undiscounted entity, including any required multiple dependent claim fee, are required. Applicant must submit the additional claim fees or cancel the additional claims for which fees are due.

SUMMARY OF FEES DUE:

Total additional fees required for this application is $**1800** for an Undiscounted Entity:

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: **Smita Shankar** and **Martin Andrew Hoyt**

Total additional claim fee(s) for this application is $**1800**

- $**1800** for **18** total claims over 20.

FORM PCT/DO/EO/923 (371 Formalities Notice)

ALL OF THE ITEMS SET FORTH ABOVE MUST BE SUBMITTED WITHIN TWO (2) MONTHS FROM THE DATE OF THIS NOTICE. FAILURE TO PROPERLY RESPOND WILL RESULT IN ABANDONMENT.

The time period set above may be extended by filing a petition and fee for extension of time under the provisions of 37 CFR 1.136(a).

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)

Registered users of EFS-Web may alternatively submit their reply to this notice via EFS-Web.
https://sportal.uspto.gov/authenticate/AuthenticateUserLocalEPF.html

For more information about EFS-Web please call the USPTO Electronic Business Center at **1-866-217-9197** or visit our website at http://www.uspto.gov/ebc.

If you are not using EFS-Web to submit your reply, you must include a copy of this notice.

JUELETHIA A PALMER

Telephone: (571) 272-9050

FORM PCT/DO/EO/923 (371 Formalities Notice)

PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 15/573,300

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	300
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	520
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	760
TOTAL CLAIMS (37 CFR 1.16(i))	38 minus 20 =	* 18			OR	x 100 =	1800
INDEPENDENT CLAIMS (37 CFR 1.16(h))	2 minus 3 =	*				x 460 =	0.00
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).						0.00
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	3380

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($)	ADDITIONAL FEE($)		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.

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

Substitute for Form PTO-1360
(For use with Form PTO/SB/06)

Application Number	Filing Date
15573300	

Applicant(s) Smita Shankar

* May be used for additional claims or amendments

CLAIMS	AS FILED		AFTER FIRST AMENDMENT		AFTER SECOND AMENDMENT		*		*		*	
	Indep	Depend	Indep	Depend	Indep	Depend	Indep	Depend	Indep	Depend	Indep	Depend
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Total Depend	0	↵	36	↵	0	↵						
Total Claims	0		38		0							

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Attorney's Docket No.: 38767-0048US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Smita Shankar	Art Unit	:	Unknown
Serial No.	:	15/573,300	Examiner	:	Unknown
Filed	:	November 10, 2017	Conf. No.	:	4807
Title	:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST			

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REQUEST FOR CORRECTED OFFICIAL FILING RECEIPT

Applicants note that the Official Filing Receipt mailed January 29, 2018 in the above-referenced application indicates that the Domestic Priority data provided by Applicants are not consistent with PTO records for seven of the eight provisional applications to which the present application claims the benefit of priority. Applicants have checked the filing documents as well as PAIR and cannot identify any inconsistency in any of the priority data. By way of confirmation, Applicants have attached the first page from the Official Filing Receipt for each of the provisional applications to which the present applications claims the benefit of priority.

Applicants request issuance of a Corrected Official Filing Receipt in the above-referenced application in which the priority claim is not indicated as being inconsistent with PTO records. In the alternative, Applicants request that the Office correct any inconsistencies in *their* records.

No fees are believed due. If this is incorrect, please apply any charges, or credits, to Deposit Account No. 06-1050.

Respectfully submitted,

Date: /March 12, 2018/

/M. Angela Parsons/
M. Angela Parsons, Ph.D.
Reg. No. 44,282

Customer Number 26191
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945
61648007.doc

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
62/159,899	05/11/2015		260	38767-0048P01		

CONFIRMATION NO. 4140

FILING RECEIPT

26191
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022



Date Mailed: 05/22/2015

Receipt is acknowledged of this provisional patent application. It will not be examined for patentability and will become abandoned not later than twelve months after its filing date. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, 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 Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections 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**

Inventor(s)

Smita Shankar, Millbrae, CA;
Martin Andrew Hoyt, San Francisco, CA;

Applicant(s)

Impossible Foods Inc., Redwood City, CA;

Power of Attorney:
M. Parsons--44282

If Required, Foreign Filing License Granted: 05/21/2015

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 62/159,899**

Projected Publication Date: None, application is not eligible for pre-grant publication

Non-Publication Request: No

Early Publication Request: No

Title

Expression Constructs and Methods of Genetically Engineering Methylotrophic Yeast

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

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
62/183,074	06/22/2015		260	38767-0048P02		

CONFIRMATION NO. 6145

FILING RECEIPT

26191
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

OC000000078089517

Date Mailed: 07/06/2015

Receipt is acknowledged of this provisional patent application. It will not be examined for patentability and will become abandoned not later than twelve months after its filing date. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, 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 Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections 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**

Inventor(s)

Smita Shankar, Millbrae, CA;
Martin Andrew Hoyt, San Francisco, CA;

Applicant(s)

Impossible Foods Inc., Redwood City, CA;

Power of Attorney:
M. Parsons--44282

If Required, Foreign Filing License Granted: 07/02/2015

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 62/183,074**

Projected Publication Date: None, application is not eligible for pre-grant publication

Non-Publication Request: No

Early Publication Request: No

Title

Expression Constructs and Methods of Genetically Engineering Methylotrophic Yeast

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

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
62/185,921	06/29/2015		260	38767-0048P03		

CONFIRMATION NO. 9336

26191
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

FILING RECEIPT



Date Mailed: 07/10/2015

Receipt is acknowledged of this provisional patent application. It will not be examined for patentability and will become abandoned not later than twelve months after its filing date. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, 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 Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections 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**

Inventor(s)

Smita Shankar, Millbrae, CA;
Martin Andrew Hoyt, San Francisco, CA;

Applicant(s)

Impossible Foods Inc., Redwood City, CA;

Power of Attorney:
M. Parsons--44282

If Required, Foreign Filing License Granted: 07/09/2015

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 62/185,921**

Projected Publication Date: None, application is not eligible for pre-grant publication

Non-Publication Request: No

Early Publication Request: No

Title

Expression Constructs and Methods of Genetically Engineering Methylotrophic Yeast

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
62/203,052	08/10/2015		260	38767-0048P04		

CONFIRMATION NO. 1010

26191
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

FILING RECEIPT



Date Mailed: 08/25/2015

Receipt is acknowledged of this provisional patent application. It will not be examined for patentability and will become abandoned not later than twelve months after its filing date. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, 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 Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections 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**

Inventor(s)

Smita Shankar, Millbrae, CA;
Martin Andrew Hoyt, San Francisco, CA;

Applicant(s)

Impossible Foods Inc., Redwood City, CA

Power of Attorney:
M. Parsons--44282

If Required, Foreign Filing License Granted: 08/24/2015

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 62/203,052**

Projected Publication Date: None, application is not eligible for pre-grant publication

Non-Publication Request: No

Early Publication Request: No

Title

Expression Constructs and Methods of Genetically Engineering Methylotrophic Yeast

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

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
62/220,366	09/18/2015		260	38767-0048P05		

CONFIRMATION NO. 6058

FILING RECEIPT

26191
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

OC000000077829824

Date Mailed: 10/05/2015

Receipt is acknowledged of this provisional patent application. It will not be examined for patentability and will become abandoned not later than twelve months after its filing date. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, 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 Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections 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**

Inventor(s)

Smita Shankar, Millbrae, CA;
Martin Andrew Hoyt, San Francisco, CA;

Applicant(s)

Impossible Foods Inc., Redwood City, CA;

Power of Attorney:
M. Parsons--44282

If Required, Foreign Filing License Granted: 10/02/2015

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 62/220,366**

Projected Publication Date: None, application is not eligible for pre-grant publication

Non-Publication Request: No

Early Publication Request: No

Title

EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

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

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
62/222,388	09/23/2015		260	38767-0048P06		

CONFIRMATION NO. 2991

FILING RECEIPT

26191
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022



Date Mailed: 10/07/2015

Receipt is acknowledged of this provisional patent application. It will not be examined for patentability and will become abandoned not later than twelve months after its filing date. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, 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 Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections 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**

Inventor(s)

Smita Shankar, Millbrae, CA;
Martin Andrew Hoyt, San Francisco, CA;

Applicant(s)

Impossible Foods Inc., Redwood City, CA

Power of Attorney:
M. Parsons--44282

If Required, Foreign Filing License Granted: 10/06/2015

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 62/222,388**

Projected Publication Date: None, application is not eligible for pre-grant publication

Non-Publication Request: No

Early Publication Request: No

Title

EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

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

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
62/236,506	10/02/2015		260	38767-0048P07		

CONFIRMATION NO. 5696

FILING RECEIPT

26191
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

OC000000078131292

Date Mailed: 10/20/2015

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Inventor(s)

Smita Shankar, Millbrae, CA;
Martin Hoyt, San Francisco, CA;

Applicant(s)

Impossible Foods Inc., Redwood City, CA

Power of Attorney:
M. Parsons--44282

If Required, Foreign Filing License Granted: 10/19/2015

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 62/236,506**

Projected Publication Date: None, application is not eligible for pre-grant publication

Non-Publication Request: No

Early Publication Request: No

Title

EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
62/313,491	03/25/2016		260	38767-0048P08		

CONFIRMATION NO. 6991

FILING RECEIPT

26191
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

OC000000082103806

Date Mailed: 04/12/2016

Receipt is acknowledged of this provisional patent application. It will not be examined for patentability and will become abandoned not later than twelve months after its filing date. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, 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 Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections 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**

Inventor(s)

Smita Shankar, Millbrae, CA;
Martin Andrew Hoyt, San Francisco, CA;

Applicant(s)

Impossible Foods Inc., Redwood City, CA;

Power of Attorney:
M. Parsons--44282

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.

If Required, Foreign Filing License Granted: 04/11/2016

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 62/313,491**

Projected Publication Date: None, application is not eligible for pre-grant publication

Non-Publication Request: No

Early Publication Request: No

Electronic Acknowledgement Receipt

EFS ID:	32027704
Application Number:	15573300
International Application Number:	
Confirmation Number:	4807
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	M. Angela Parsons/Judith Witzel
Filer Authorized By:	M. Angela Parsons
Attorney Docket Number:	38767-0048US1
Receipt Date:	12-MAR-2018
Filing Date:	10-NOV-2017
Time Stamp:	17:10:06
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	Request for Corrected Filing Receipt	ReqCorrectFR.pdf	65844 86ded8dec124d954b82bc6b214c4ccc1d488fc25	no	1

Warnings:

Information:					
2	Miscellaneous Incoming Letter	0048P0xfilingreceipts.pdf	128717 0bf0d1e3c923e5f57d32adc53372f9672f18783d	no	8
Warnings:					
Information:					
			Total Files Size (in bytes):	194561	

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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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/573,300	11/10/2017	1656	1260	38767-0048US1	38	2

CONFIRMATION NO. 4807

CORRECTED FILING RECEIPT

26191
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

OC000000098012474

Date Mailed: 03/15/2018

Receipt is acknowledged of this non-provisional 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 APPLICANT, 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 Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections 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**

Inventor(s)

Smita Shankar, Millbrae, CA;
Martin Andrew Hoyt, San Francisco, CA;

Applicant(s)

Impossible Foods Inc., Redwood City, CA;

Power of Attorney: The patent practitioners associated with Customer Number 26191

Domestic Priority data as claimed by applicant

This application is a 371 of PCT/US2016/031797 05/11/2016
which claims benefit of 62/313,491 03/25/2016
and claims benefit of 62/236,506 10/02/2015
and claims benefit of 62/222,388 09/23/2015
and claims benefit of 62/220,366 09/18/2015
and claims benefit of 62/203,052 08/10/2015
and claims benefit of 62/185,921 06/29/2015
and claims benefit of 62/183,074 06/22/2015
and claims benefit of 62/159,899 05/11/2015

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.

If Required, Foreign Filing License Granted: 01/25/2018
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/573,300**
Projected Publication Date: 05/10/2018
Non-Publication Request: No
Early Publication Request: No
Title

EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

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

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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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Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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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.

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/573,300	11/10/2017	1656	1260	38767-0048US1	38	2

CONFIRMATION NO. 4807

CORRECTED FILING RECEIPT

26191
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

OC000000098072633

Date Mailed: 03/16/2018

Receipt is acknowledged of this non-provisional 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 APPLICANT, 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 Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections 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**

Inventor(s)

Smita Shankar, Millbrae, CA;
Martin Andrew Hoyt, San Francisco, CA;

Applicant(s)

Impossible Foods Inc., Redwood City, CA;

Power of Attorney: The patent practitioners associated with Customer Number 26191

Domestic Priority data as claimed by applicant

This application is a 371 of PCT/US2016/031797 05/11/2016
which claims benefit of 62/313,491 03/25/2016
and claims benefit of 62/236,506 10/02/2015
and claims benefit of 62/222,388 09/23/2015
and claims benefit of 62/220,366 09/18/2015
and claims benefit of 62/203,052 08/10/2015
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and claims benefit of 62/159,899 05/11/2015

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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: 01/25/2018
The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/573,300**
Projected Publication Date: 05/10/2018
Non-Publication Request: No
Early Publication Request: No
Title

EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

Preliminary Class

435

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

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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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Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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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

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SelectUSA

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technology, manufacture products, deliver services, and grow your business, visit http://www.SelectUSA.gov or call +1-202-482-6800.

Attorney Docket No.: 38767-0048US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Smita Shankar	Art Unit	:	Unknown
Serial No.	:	15/573,300	Examiner	:	Unknown
Filed	:	November 10, 2017	Conf. No.	:	4807

Title : EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

MAIL STOP MISSING PARTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REPLY TO NOTICE OF INSUFFICIENT FEES

In response to the Notice of Insufficiency under 37 CFR § 1.492 and/or 1.495 mailed January 29, 2018, Applicant, as a Large Entity, submits herewith payment of the additional claim fees in the amount of $1,800.

Under 37 CFR § 1.136, please extend the period of time for responding to the January 29, 2018 Notice for one month to and including April 29, 2018.

The fees in the amount of $2,000 ($1,800 for claim fees and $200 for extension of time fees) are being paid with this reply via the Electronic Filing System (EFS). Please apply any other charges, or credits, to Deposit Account No. 06-1050, referencing the above-indicated Attorney Docket Number.

Respectfully submitted,

Date: /April 9, 2018/

/M. Angela Parsons/
M. Angela Parsons, Ph.D.
Reg. No. 44,282

Customer Number 26191
Fish & Richardson P.C.
Telephone: (612) 335-5070
Facsimile: (877) 769-7945

61649914.doc

Electronic Patent Application Fee Transmittal

Application Number:	15573300
Filing Date:	10-Nov-2017
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Filer:	M. Angela Parsons/Judith Witzel
Attorney Docket Number:	38767-0048US1

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:				
CLAIMS IN EXCESS OF 20	1615	18	100	1800
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension-of-Time:				
Extension - 1 month with $0 paid	1251	1	200	200
Miscellaneous:				
		Total in USD ($)		**2000**

Electronic Acknowledgement Receipt

EFS ID:	32279484
Application Number:	15573300
International Application Number:	
Confirmation Number:	4807
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	M. Angela Parsons/Judith Witzel
Filer Authorized By:	M. Angela Parsons
Attorney Docket Number:	38767-0048US1
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Attorney Docket No.: 38767-0048US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Smita Shankar	Art Unit	:	1656
Serial No.	:	15/573,300	Examiner	:	Unknown
Filed	:	November 10, 2017	Conf. No.	:	4807
Title	:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST			

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INFORMATION DISCLOSURE STATEMENT

Please consider the references listed on the enclosed PTO-1449 Form. Foreign patent documents and non-patent literature are enclosed; U.S. patents and patent application publications will be provided upon request. Copy of Communications from foreign Patent Offices in counterpart applications also are enclosed.

This statement is being filed before the receipt of a first Office Action on the merits. Therefore, no fees are believed due. If this is incorrect, please apply any charges, or credits, to Deposit Account No. 06-1050, referencing the above-indicated Attorney Docket Number.

Please contact the undersigned if there are any questions regarding this statement.

Respectfully submitted,

Date: /April 13, 2018/

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Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Impossible Foods Inc.	
		Filing Date November 10, 2017	Group Art Unit 1656

U.S. Patent Documents

Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
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	3.	4,800,159	01/24/1989	Mullis et al.			
	4.	4,886,753	12/01/1989	Marcker			
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	15.	2017/0349906	12/07/2017	Shankar et al.			

Foreign Patent Documents or Published Foreign Patent Applications

Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	16.	EP 2058398	05/13/2009	EP				
	17.	WO 1998/012913	04/02/1998	WIPO				
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	22.	WO 2013/010042	01/17/2013	WIPO				
	23.	WO 2014/008729	01/16/2014	WIPO				

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		Filing Date November 10, 2017	Group Art Unit 1656

Foreign Patent Documents or Published Foreign Patent Applications								
Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes	Translation No
	24.	WO 2014/110532	07/17/2014	WIPO				
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	Applicant Impossible Foods Inc.	
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	Applicant Impossible Foods Inc.	
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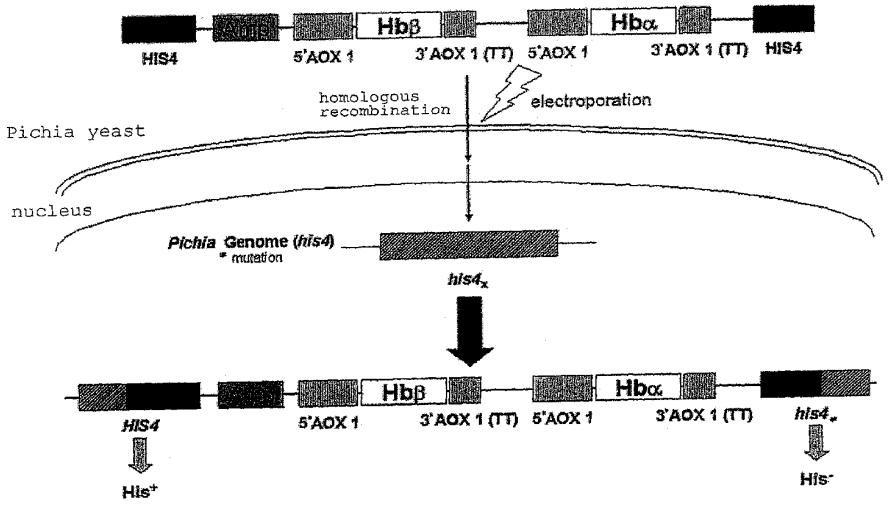
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(54) Production of recombinant human hemoglobin using pichia yeast

(57) The present invention provides a production method of a functional recombinant human hemoglobin, which comprises transforming a yeast of the genus Pichia with an expression vector containing a DNA encoding a human hemoglobin α-chain (Hbα), which is under regulation of a promoter functional in a yeast of the genus Pichia, and an expression vector containing a DNA encoding a human hemoglobin β-chain (Hbβ), which is under regulation of a promoter functional in a yeast of the genus Pichia, culturing the yeast in a medium, and recovering the human hemoglobin from the obtained culture. The present invention also provides an expression vector containing AOX1 promoter, DNA encoding Hbβ, terminator functional in a yeast of the genus Pichia, AOX1 promoter, DNA encoding Hbα and terminator functional in the yeast of the genus Pichia in this order from the upstream side in transcriptional direction.

FIG. 3



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Description

TECHNICAL FIELD OF THE INVENTION

[0001] The present invention relates to production of a recombinant human hemoglobin and an expression vector therefor. More particularly, the present invention relates to a production method of a human hemoglobin using a yeast of the genus Pichia, as well as an expression vector comprising in tandem an expression cassette containing a DNA encoding a human hemoglobin β-chain and an expression cassette containing a DNA encoding a human hemoglobin α-chain.

BACKGROUND OF THE INVENTION

[0002] Hemoglobin is a protein contained in red blood cells at a high concentration, and has a function to carry various gas molecules including oxygen. Structurally, it is a heterotetramer consisting of two α-chains and two β-chains.

[0003] As a preparation utilizing hemoglobin, a red blood cell substitute has been developed. There have so far been designed an intramolecular crosslinking type which is a hemoglobin modification form, a polymerization type, a polymer bond type, and hemoglobin endoplasmic reticulum in which hemoglobin is encapsulated in liposome. Particularly, there is a practical prospect of hemoglobin endoplasmic reticulum as a single administration oxygen transfusion for critical care, based on the property data of its shape, hemoglobin purity and the like, and the biological data of safety, in vivo retention, in vivo kinetics, oxygen-carrying ability and the like.

[0004] On the other hand, a preparation using a human blood-derived hemoglobin is associated with the risk of contamination with unknown virus and the like, and its use for human body is problematic in terms of safety. However, there is a possibility of provision of a safe hemoglobin preparation by a transformed microorganism using the gene recombination technique.

[0005] Until now, recombinant hemoglobin has been produced mainly using a system with Escherichia coli as a host cell. However, this method has problems in that (1) unless an enzyme (methionine aminopeptidase and the like) is coexpressed with hemoglobin and the like, addition of redundant first methionine to the N-terminal occurs, leading to a primary sequence different from that of a naturally occurring type, (2) aminolaevulinic acid needs to be added to a culture medium for the purpose of heme synthesis and the like.

[0006] Two cases have been reported so far as regards the production of recombinant hemoglobin using Saccharomyces cerevisiae (see Wagenbach, M. et al., Bio/Technol., 9: 57-61 (1991) and Coghlan, D. et al., Eur. J. Biochem., 207: 931-936 (1992)). In this system, since the first methionine is cleaved by an enzyme S. cerevisiae itself has, the primary sequence completely matches with that of a naturally occurring type, and addition of aminolaevulinic acid for heme synthesis is not necessary. However, this method is not entirely sufficient due to the problem of yield and the like, and therefore, an industrial production of recombinant human hemoglobin has not been practiced yet.

[0007] One of known industrial yeasts other than the genus Saccharomyces is a yeast of the genus Pichia. This yeast can grow with methanol as a sole carbon source, and when grown in methanol, an enzyme necessary for the treatment of methanol and its metabolism intermediate is disinhibited and expressed. Production of heterologous protein utilizing the methanol-utilizing pathway has been studied and applied to the production of albumin for blood preparations (see e.g., JP-A-6-22784). The expression amount thereof is known to be very high, and albumin of a 10 g order can be produced from 1 L of a medium.

[0008] However, there often exist cases where the expression efficiency of heterologous protein is low due to the combination (compatibility) of the object heterologous protein and a host cell. Particularly, in the case of a heterooligomer protein such as hemoglobin, since many conditions of balanced expression amounts of respective subunits, oligomer formation and the like need to be met, the desired high expression cannot be obtained easily. In fact, a yeast of the genus Pichia is used for industrial production of human protein in a very few cases.

[0009] An object of the present invention is to provide a means for producing a safe recombinant protein such as human hemoglobin or albumin, globulin and the like, which has sufficient function and activity similar to those of the naturally occurring type and is free of viral contamination and the like, easily in a large amount.

[0010] The present inventors have conducted intensive studies in an attempt to solve the above-mentioned problems and succeeded in recovering various proteins or peptides such as a human hemoglobin and the like, particularly a heterooligomer protein and the like, in a high yield by transforming Pichia pastoris, which is one of the yeasts of the genus Pichia, with an expression vector containing a DNA encoding plural proteins or peptide chains, for example, an expression vector containing a DNA encoding α-chain and β-chain of human hemoglobin, and cultivating the obtained transformant in a medium, which resulted in the completion of the present invention.

SUMMARY OF THE INVENTION

[0011] Accordingly, the present invention provides

(1) a production method of a functional recombinant human hemoglobin, which comprises transforming a yeast of the genus Pichia with an expression vector containing a DNA encoding a human hemoglobin α-chain, which is under regulation of a promoter functional in a yeast of the genus Pichia, and an expression vector containing a DNA encoding a human hemoglobin β-chain, which is under regulation of a promoter functional in a yeast of the genus Pichia, culturing the yeast in a medium, and recovering the human hemoglobin from the obtained culture;
(2) the method of the above-mentioned (1), wherein the DNA encoding the human hemoglobin α-chain and the DNA encoding the human hemoglobin β-chain are on the same vector;
(3) the method of the above-mentioned (2), wherein the DNA encoding the human hemoglobin β-chain is at the upstream side in the transcriptional direction;
(4) the method of any of the above-mentioned (1) - (3), wherein the DNA encoding the human hemoglobin α-chain and the DNA encoding the human hemoglobin β-chain are under regulation of different promoters;
(5) the method of any of the above-mentioned (1) - (4), wherein the DNA encoding the human hemoglobin α-chain and the DNA encoding the human hemoglobin β-chain are under regulation of an alcoholoxidase 1 promoter;
(6) the method of the above-mentioned (5), wherein the transformed yeast of the genus Pichia is cultivated in a liquid medium requiring methanol as a single carbon source;
(7) the method of the above-mentioned (6), wherein the culture is a feeding culture;
(8) the method of any of the above-mentioned (1) - (7), wherein the yeast of the genus Pichia is Pichia pastoris; and
(9) an expression vector comprising an alcoholoxidase 1 promoter, a DNA encoding the human hemoglobin β-chain, a terminator functional in a yeast of the genus Pichia, an alcoholoxidase 1 promoter, a DNA encoding a human hemoglobin α-chain and a terminator functional in a yeast of the genus Pichia in this order from the upstream side in the transcriptional direction.
The present invention also provides
(10) a method of producing an expression vector comprising a DNA encoding plural proteins, which is under control of a promoter functional in a yeast of the genus Pichia, wherein the vector and a DNA encoding one of the plural proteins are ligated without a dephosphorylation treatment of the vector;
(11) the method of the above-mentioned (10), wherein (i) an expression vector containing a DNA encoding one or more proteins, which is under control of a promoter functional in a yeast of the genus Pichia, wherein the expression vector is digested with a restriction enzyme capable of cleavage thereof other than the inside of the promoter and the DNA and the both ends produced thereby are not dephosphorylated is provided, (ii) a DNA encoding other protein, which is under control of a promoter functional in a yeast of the genus Pichia, wherein the both ends are complementary to the end of the expression vector of the above-mentioned (i) is provided, and (iii) the expression vector of the above-mentioned (i) and the DNA of the above-mentioned (ii) are ligated;
(12) a production method of a recombinant protein, which comprises culturing, in a medium, a yeast of the genus Pichia transformed with an expression vector obtained by the method of the above-mentioned (10) or (11), and recovering the plural proteins from the obtained culture;
(13) the method of the above-mentioned (12), wherein the plural proteins are polymerized in the culture to form a functional protein complex; and the like.

[0012] Since the human hemoglobin provided by the method of the present invention is produced using a yeast of the genus Pichia as a host by the gene recombination technique, it is free of a risk of contamination with an unknown virus and the like, which is a problem specific to blood-derived preparations, and can be used safely for human body and the like. Using a yeast of the genus Pichia, moreover, a human hemoglobin can be produced in a large amount, which has similar function and properties as the naturally occurring type. Accordingly, administration thereof to a human body etc. causes less influence such as side effects and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013]

Fig. 1 is an explanatory drawing of the principle of connection between hemoglobin α-chain expression cassette and β-chain expression cassette.
Fig. 2 is an explanatory drawing of the outline of the procedures for preparing hemoglobin in the Examples.
Fig. 3 is an explanatory drawing of the recombination method of hemoglobin with Pichia chromosome in the Examples.

DETAILED DESCRIPTION OF THE INVENTION

[0014] As a DNA encoding the human hemoglobin α-chain to be used in the present invention (hereinafter sometimes to be abbreviated as "Hbα"), a DNA containing a base sequence encoding an amino acid sequence the same or substantially the same as the amino acid sequence shown in SEQ ID NO: 2, and as a DNA encoding a human hemoglobin β-chain (hereinafter sometimes to be abbreviated as "Hbβ"), a DNA containing a base sequence encoding an amino acid sequence the same or substantially the same as the amino acid sequence shown in SEQ ID NO: 4 can be mentioned.

[0015] As the amino acid sequence the same or substantially the same as the amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4), an amino acid sequence having a homology of not less than about 80%, preferably not less than about 90%, more preferably not less than about 95%, particularly preferably not less than about 97% with the amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4) and the like can be mentioned. As used herein, by the "homology" is meant the proportion (%) of the same amino acid and similar amino acid residues relative to the total overlapping amino acid residues in the optimal alignment when two amino acid sequences are aligned using a mathematical algorithm known in the art (preferably, the algorithm is capable of considering introduction of a gap into one or both of the sequences for the optimal alignment). The "similar amino acid" means an amino acid similar in the physicochemical properties. For example, amino acids classified in the same group such as aromatic amino acids (Phe, Trp, Tyr), aliphatic amino acids (Ala, Leu, Ile, Val), polar amino acids (Gln, Asn), basic amino acids (Lys, Arg, His), acidic amino acids (Glu, Asp), amino acids (Ser, Thr) having a hydroxyl group, amino acids (Gly, Ala, Ser, Thr, Met) with small side chain and the like can be mentioned. It is predicted that substitution with such similar amino acids will not alter protein phenotypes (namely, preservative amino acid substitution). Specific examples of preservative amino acid substitution are well known in the art and are described in various literatures (see e.g., Bowie et al., Science, 247: 1306-1310 (1990)).

[0016] The homology of the amino acid sequence in the present specification can be calculated using homology calculation algorithm NCBI BLAST (National Center for Biotechnology Information Basic Local Alignment Search Tool) under the following conditions (expectancy=10; allowing gap; matrix=BLOSUM62; filtering=OFF). Other algorithms for determining the homology of the amino acid sequence include, for example, the algorithm described in Karlin et al., Proc. Natl. Acad. Sci. USA, 90: 5873-5877 (1993) [this algorithm is incorporated in the NBLAST and XBLAST program (version 2.0) (Altschul et al., Nucleic Acids Res., 25: 3389-3402 (1997))], the algorithm described in Needleman et al., J. Mol. Biol., 48: 444-453 (1970) [this algorithm is incorporated in the GAP program in the GCG software package], the algorithm described in Myers and Miller, CABIOS, 4: 11-17 (1988) [this algorithm is incorporated in the ALIGN program (version 2.0) which is a part of the CGC sequence alignment software package], the algorithm described in Pearson et al., Proc. Natl. Acad. Sci. USA, 85: 2444-2448 (1988) [this algorithm is incorporated in the FASTA program in the GCG software package] and the like, and they can also be used preferably.

[0017] More preferably, an amino acid sequence substantially same as the amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4) has homology of not less than about 80%, preferably not less than about 90%, more preferably not less than about 95%, particularly preferably not less than about 97%, to the amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4).

[0018] A protein containing an amino acid sequence substantially same as the amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4) means a protein containing an amino acid sequence substantially same as the aforementioned amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4) and having a substantially equivalent activity to that of the protein containing the amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4).

[0019] The substantially equivalent activity includes, for example, the physiological activity of human hemoglobin, for example, an activity to carry a gas molecule (e.g., oxygen and the like), and the like. The "substantially equivalent" means that the activities are qualitatively same. Therefore, the activity of oxygen-carrying ability and the like is preferably equivalent (e.g., about 0.5- to about 2-fold). However, quantitative elements such as the level of the activity, molecular weight of the protein and the like may be different.

[0020] The activity of oxygen-carrying ability and the like (e.g., autoxidation rate constant, oxygen affinity, cooperativity and the like) can be measured according to, but not limited to, a method known per se, for example, the method described in JP-A-2004-500871 and the like.

[0021] In addition, Hbα (or Hbβ) to be used in the present invention includes, for example, a protein containing (1) an amino acid sequence wherein one or more (preferably about 1 - 30, more preferably about 1 - 10, particularly preferably 1 - several (2, 3, 4 or 5)) amino acids are deleted from the amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4), (2) an amino acid sequence wherein one or more (preferably about 1 - 30, more preferably about 1 - 10, particularly preferably 1 - several (2, 3, 4 or 5)) amino acids are added to the amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4), (3) an amino acid sequence wherein one or more (preferably about 1 - 30, more preferably about 1 - 10, particularly preferably 1 - several (2, 3, 4 or 5)) amino acids are inserted in the amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4), (4) an amino acid sequence wherein one or more (preferably about 1 - 30, more preferably about 1 - 10, particularly preferably 1 - several (2, 3, 4 or 5)) amino acids are substituted by other amino acids in the

amino acid sequence shown in SEQ ID NO: 2 (or SEQ ID NO: 4), or (5) a combination of these, and the like.

[0022] When the amino acid sequence is inserted, deleted or substituted as mentioned above, the site of the insertion, deletion or substitution is not particularly limited as long as the activity of protein is maintained.

[0023] Human hemoglobin in the present invention is used to mean not only adult hemoglobin (HbA1) but encompass HbA2 wherein α-chain and δ-chain are bonded, fetal hemoglobin (HbF) wherein α-chain and γ-chain are bonded, and the like.

[0024] For the production of HbA2 and HbF, therefore, a DNA encoding δ-chain and γ-chain instead of β-chain is used. The amino acid sequence and base sequence of δ-chain are registered, for example, in GenBank accession Nos. NP_000510 and NM_000519. On the other hand, the amino acid sequence and base sequence of γ-chain are registered, for example, in GenBank accession Nos. NP_000550 and NM_000559.

[0025] Examples of the DNA encoding Hbα (or Hbβ) include human genomic DNA, cDNA derived from Hbα (and/or Hbβ)-producing cells (e.g., red blood cell and the like) of adult, synthetic DNA and the like. The genomic DNA and cDNA encoding Hbα (or Hbβ) can also be directly amplified by Polymerase Chain Reaction (hereinafter to be abbreviated as "PCR method") and Reverse Transcriptase-PCR (hereinafter to be abbreviated as "RT-PCR method"), using a genomic DNA fraction and total RNA or an mRNA fraction prepared from a producing-cell or tissue (e.g., blood and the like) thereof as respective templates. Alternatively, the genomic DNA and cDNA encoding Hbα (or Hbβ) can be respectively cloned by colony or plaque hybridization method, PCR method and the like, from a genomic DNA library and cDNA library prepared by inserting genomic DNA and total RNA or an mRNA fraction prepared from the above-mentioned cell or tissue into suitable vectors. The vector to be used for the library may be any such as bacteriophage, plasmid, cosmid, phagemid and the like.

[0026] Examples of the DNA encoding Hbα (or Hbβ) include DNA containing the base sequence shown in SEQ ID NO: 1 (or SEQ ID NO: 3), DNA having a base sequence hybridizing with the base sequence shown in SEQ ID NO: 1 (or SEQ ID NO: 3) under stringent conditions, and encoding a protein having substantially equivalent activity (e.g., oxygen-carrying ability and the like) to that of the aforementioned Hbα (or Hbβ) and the like.

[0027] As the DNA capable of hybridizing to the base sequence shown in SEQ ID NO: 1 (or SEQ ID NO: 3) under stringent conditions, for example, DNA containing a base sequence having homology of not less than about 70%, preferably not less than about 80%, more preferably not less than about 90%, particularly preferably not less than about 95%, to the base sequence shown in SEQ ID NO: 1 (or SEQ ID NO: 3) and the like are used.

[0028] The homology of the base sequence in the present specification can be calculated using a homology calculation algorithm NCBI BLAST (National Center for Biotechnology Information Basic Local Alignment Search Tool) under the following conditions (expectancy=10; allowing gap; filtering=ON; match score=1; mismatch score=-3). Preferable examples of other algorithms usable for determining homology of the base sequence include the above-mentioned homology calculation algorithms for amino acid sequence.

[0029] Hybridization can be performed according to a method known per se or a method according to the method, for example, the method described in Molecular Cloning, ver. 2 (J. Sambrook et al., Cold Spring Harbor Lab. Press, 1989) and the like. When a commercially available library is used, moreover, hybridization can be performed according to the method described in the attached instruction manual. Preferably, hybridization can be performed under stringent conditions.

[0030] The high stringent conditions include, for example, a hybridization reaction at 45°C in 6×SSC (sodium chloride/sodium citrate), and washing one or more times at 65°C in 0.2×SSC/0.1% SDS and the like.

[0031] Those of ordinary skill in the art can easily adjust to the desired stringency by appropriately changing the salt concentration of hybridization solution, temperature of hybridization reaction, probe concentration, length of probe, number of mismatches, hybridization reaction time, salt concentration of washing solution, temperature of washing and the like.

[0032] A DNA encoding Hbα (or Hbβ) can be cloned by amplifying by PCR method using a synthetic DNA primer having a part of the base sequence encoding Hbα (or Hbβ), or hybridizing DNA incorporated into a suitable expression vector to with a labeled DNA fragment or synthetic DNA encoding a part or full region of Hbα (or Hbβ).

[0033] A DNA encoding Hbα (or Hbβ) can be obtained from human genomic DNA or RNA (cDNA) as mentioned above. It is also possible to construct a DNA encoding the full-length Hbα (or Hbβ) by chemically synthesizing a DNA chain or connecting a synthesized, partly overlapping oligo DNA short chain by PCR method. The advantage of constructing a full-length DNA by a combination of chemical synthesis and PCR method is that the codon to be used can be designed over the full length of the gene in accordance with the host to be introduced into the gene. Plural codons encoding the same amino acid are not uniformly used and the frequency of use varies depending on the species. In general, a codon contained in a gene highly expressing in a certain species contains a large amount of codon frequently used in the species. On the other hand, in many of the genes with a low expression amount, the presence of a codon used less frequently often becomes a bottleneck in the high expression. There are many reports on the examples wherein, in the expression of a heterologous gene, the expression amount of the heterologous protein was increased by substituting the gene sequence with a codon highly frequently used in the host organism, and therefore, such alteration

of the codon to be used is expected to be effective for increasing the expression amount of a heterologous gene.

[0034] For the above-mentioned reasons, a DNA encoding Hbα (or Hbβ) can be altered to a codon more suitable for a yeast of the genus Pichia into which the DNA is to be introduced (that is, a codon highly frequently used in the host organism). The frequency of use of the codon in each yeast of the genus Pichia is defined by the ratio of use of each codon to all the genes present on the genomic sequence of the host, for example, by the number of use per 1000 codons. In addition, the frequency of use of codon can also be approximately calculated from plural representative gene sequences in the case of a biological entity for which the complete genomic sequences have not been identified. As for the data of frequent use of codon in yeast of the genus Pichia to be genetically modified, for example, the genetic code usage frequency database (http://www.kazusa.or.jp/codon/index.html) disclosed in the homepage of Kazusa DNA Research Institute can be used. Alternatively, a document indicating the frequency of use of codon in each yeast of the genus Pichia may be referred to, or the data of frequency of use of codon in the yeast of the genus Pichia to be used may be constructed by oneself. By referring to the obtained data and the gene sequence to be introduced, the codon used in the gene sequence, which shows a low frequency of use in the host yeast, can be substituted to a codon encoding the same amino acid and showing a high frequency of use.

[0035] Another advantage of constructing a DNA encoding the full-length Hbα (or Hbβ) by chemically synthesizing a DNA chain, or connecting a synthesized, partly overlapping oligo DNA short chain by PCR method is easy construction of an expression vector thereafter. For example, in the below-mentioned Examples, an expression cassette of Hbβ and an expression cassette of Hbα are connected in tandem, utilizing the complementarity of sticky end produced by digestion with restriction enzymes BamH I and Bgl II (see Fig. 1. Utilizing the complementarity of sticky end, multicopy expression cassette can be repeatedly integrated in tandem into a vector). Therefore, the DNA encoding Hbα and the DNA encoding Hbβ are preferably free of a BamH I recognition site. For example, Hbβ cDNA registered as GenBank accession No. NM_000518 in the NCBI database has a BamH I recognition site (GGATCC) at the 347th - 352nd positions. In the base sequence shown in SEQ ID NO: 3, the base sequence of the corresponding part (base Nos. 297 - 302) is TGACCC, where a mutation has been introduced to not contain a BamH I recognition site. In the below-mentioned Examples, moreover, an EcoR I recognition sequence is added to both ends of a DNA encoding Hbα and a DNA encoding Hbβ to construct an expression cassette by inserting a DNA encoding Hbα and a DNA encoding Hbβ between a promoter and a terminator functional in a yeast of the genus Pichia (see Fig. 2). Therefore, a DNA encoding Hbα and a DNA encoding Hbβ is preferably free of a EcoR I recognition site. For example, Hbβ cDNA registered as GenBank accession No. NM_000518 in the NCBI database has an EcoR I recognition site (GAATTC) at the 414th to 419th positions. In the base sequence shown in SEQ ID NO: 3, the base sequence of the corresponding part (base Nos. 370 - 375) is GAGTTT, where a mutation has been introduced to not contain an EcoR I recognition site. When a restriction enzyme recognition site in a protein-coding sequence is to be altered, it is desirable to substitute a base, thereby to not change the amino acid sequence of Hbα (or Hbβ) utilizing degeneration of the codon.

[0036] A DNA cloned as mentioned above can be used as it is depending on the object or used after digestion with a restriction enzyme or adding a linker. The DNA may have ATG as a translation initiation codon at the 5' end side thereof and TAA, TGA or TAG as a translation stop codon at the 3' end side thereof. These translation initiation codon and translation stop codon can be added using a suitable synthetic DNA adapter.

[0037] An expression vector containing a DNA encoding Hbα (or Hbβ) can be produced, for example, by ligating a DNA fragment encoding Hbα (or Hbβ) cloned as mentioned above to a downstream of a promoter in a suitable expression vector using a restriction enzyme and a DNA ligase.

[0038] The vector to be used is not particularly limited as long as it can be maintained stably by autonomous replication in a fungus body of yeast of the genus Pichia or integration into a yeast genome. Examples of the autonomously-replicable vector include YEp vector, YRp vector, YCp vector and the like. In addition, examples of the vector to be integrated into a yeast genome include YIp vector and YRp vector.

[0039] Examples of the promoter functional in the yeast of the genus Pichia include promoters derived from a yeast, such as PHO5 promoter, PGK promoter, GAP promoter, ADH promoter derived from S. cerevisiae and the like, alcohol oxidase (AOX) 1 promoter, AOX2 promoter, dihydroxyacetone synthase promoter, P40 promoter, ADH promoter, folic acid dehydrogenase promoter derived from P. pastoris and the like. In addition, the above-mentioned promoter derived from a yeast may be a mutant promoter modified to further improve the gene expression efficiency, for example, mutant AOX2 (mAOX2) promoter [Ohi et al., Mol. Gen. Genet., 243, 489-499 (1994); JP-A-4-299984] and the like. Preferably, the promoter is a promoter of an enzyme gene necessary for treating methanol or a metabolic intermediate thereof, in order to use a methanol-metabolizing system in the yeast of the genus Pichia, such as AOX1 promoter, mAOX2 promoter and the like, more preferably AOX1 promoter.

[0040] The expression vector containing the DNA encoding Hbα (or Hbβ) of the present invention preferably further contains transcription terminator sequence (terminator) functional in a yeast of the genus Pichia (e.g., AOX1 terminator etc.), enhancer sequence, selection marker gene usable for selecting yeast (auxotrophic gene, for example, HIS4, LEU2, ARG4 and URA3 gene derived from P. pastoris or S. cerevisiae, and the like, or antibiotic resistance gene, for example, resistance gene to cycloheximide, G-418, chloramphenicol, bleomycin, hygromycin etc., and the like) and the like, and

when desired, may contain replicable unit functional in yeast. For preparation of the vector in a large amount, moreover, the vector more preferably contains a replicable unit functional in Escherichia coli and a selection marker gene usable for selecting Escherichia coli (e.g., resistance gene to ampicillin and tetracycline etc.).

[0041] When the expression vector is of a type incorporated into a yeast genome, the vector preferably further contains a sequence homologous to a yeast genome necessary for homologous recombination. As such homology sequence, the aforementioned auxotrophic gene sequence can be mentioned. Accordingly, in one preferable embodiment, the expression vector of the present invention is one wherein an expression cassette of the above-mentioned Hbα (or Hbβ) is inserted in an auxotrophic gene (in the present specification, the "expression cassette" means a unit enabling gene expression, whose minimal unit is a protein-coding sequence configured under regulation of a promoter, with preference given to a unit comprising promoter-protein-coding region-terminator) (see Fig. 3).

[0042] An expression cassette of Hbα and an expression cassette of Hbβ may be carried on individual vectors or the same vector. In the former case, two vectors desirably contain different selection marker genes. However, in a preferable embodiment of the present invention, the expression cassette of Hbα and the expression cassette of Hbβ are configured on the same vector. In this case, the expression cassette of Hbα and the expression cassette of Hbβ may be configured in any order. For example, the expression cassettes may be configured head-to-head or tail-to-tail on an opposite chain. Preferably, they are configured in the same transcriptional direction, more preferably, the expression cassettes are configured in the order of the expression cassette of Hbβ and the expression cassette of Hbα, from the upstream side in the transcriptional direction. When the DNA encoding Hbα and the DNA encoding Hbβ are transcribed in the same direction, the transcription may be performed monocistronically from the same promoter or dicistronically from separate promoters. In the case of monocistronic transcription, the expression vector does not contain a terminator at the 3' side of the coding sequence in the upstream. In addition, an internal ribosome entry site (IRES) is preferably contained at the 5' side of the coding sequence in the downstream. However, in a preferable embodiment of the present invention, the DNA encoding Hbα and the DNA encoding Hbβ are transcribed dicistronically from separate promoters.

[0043] In a particularly preferable embodiment of the present invention, an expression vector containing AOX1 promoter, DNA encoding Hbβ, terminator functional in a yeast of the genus Pichia (preferably AOX1 terminator), AOX1 promoter, DNA encoding Hbα and terminator functional in the yeast of the genus Pichia (preferably AOX1 terminator) in this order from the upstream side in transcriptional direction is used.

[0044] For construction of an expression vector comprising the above-mentioned expression cassette of Hbβ and the expression cassette of Hbα ligated in tandem, generally, a site immediately upstream (when expression cassette of Hbα is contained) of the promoter of the expression vector containing one of the expression cassettes or a site immediately downstream (when expression cassette of Hbβ is contained) of the terminator thereof is cleaved with a suitable restriction enzyme (e.g., in Fig. 2, Hbβ-pAO815 is cleaved with BamH I) to linearize the vector, and ligated using the other expression cassette cleaved by treating with a restriction enzyme providing the same sticky end (or blunt end) (e.g., in Fig. 2, expression cassette of Hbα is cleaved out from Hbα-pAO815 by double digestion with BamH I and Bgl II) and a DNA ligase. At this time, in general, a dephosphorylation treatment of the end (e.g., calf intestine alkaline phosphatase (CIAP) treatment) is performed to prevent self-ligation on the vector side. In some cases, the total ligation efficiency can be enhanced when the dephosphorylation treatment is not applied.

[0045] The expression vector obtained as mentioned above can be introduced into the fungus body of the target yeast of the genus Pichia using, for example, a known transformation technique such as competent cell method, protoplast method, calcium phosphate coprecipitation method, polyethylene glycol method, lithium method, electroporation method, microinjection method, liposome fusion method, particle gun method and the like.

[0046] While the yeast of the genus Pichia to be used in the present invention is not particularly limited, for example, P. pastoris, Pichia acaciae, Pichia angusta, Pichia anomala, Pichia capsulata, Pichia ciferrii, Pichia etchellsii, Pichia fabianii, Pichia farinosa, Pichia guilliermondii, Pichia inositovora, Pichia jadinii, Pichia methanolica, Pichia norvegensis, Pichia ofunaensis, Pichia pinus and the like can be used. Preferred is P. pastoris, particularly, auxotrophic mutant P. pastoris strain (e.g., P. pastoris GTS115 strain ($HIS4^-$) [NNRL Y-15851], P. pastoris GS190 strain ($ARG4^-$) [NNRLY-18014), P. pastoris PPF1 ($HIS4^-$, $URA4^-$) [NNRL Y-18017] and the like).

[0047] By cultivating the transformed yeast of the genus Pichia by a method generally used in the art, a functional human hemoglobin can be produced. The medium to be used needs to contain at least a carbon source and an inorganic or organic nitrogen source necessary for the growth of the host cell. Examples of the carbon source include methanol, glycerol, glucose, sucrose, dextran, soluble starch and the like. In addition, examples of the inorganic or organic nitrogen source include ammonium salts, nitrate salts, amino acid, corn steep liquor, peptone, casein, meat extract, yeast extract, soybean cake, potato extract and the like. When desired, moreover, other nutrients, for example, inorganic salts such as calcium chloride, sodium dihydrogenphosphate, magnesium chloride and the like, vitamins such as biotin and the like, antibiotic and the like can be added.

[0048] Examples of the medium to be used include conventional natural medium (e.g., YPD medium, YPM medium, YPG medium etc.) and synthetic medium. As the pH and culture temperature of the medium, those suitable for the growth of yeast and production of human hemoglobin are employed. For example, pH of about 5 - about 8, culture

temperature of about 20°C - about 30°C are preferable. In addition, aeration and agitation are performed as necessary. The culture is generally performed for about 48 - about 120 hr.

[0049] For example, when a promoter whose expression is induced by methanol, such as AOX1 promoter, mAOX2 promoter and the like, is used as a promoter functional in the fungus body of a yeast of the genus Pichia, a method of liquid aeration-agitation culture using natural medium controlled to pH about 6.0, which contains glycerol as a carbon source for the growth of fungus body and methanol as an Hbα and Hbβ expression inducer is most preferable. When the expression of Hbα and Hbβ is not preferable for the growth of fungus body, a method including first increasing the amount of fungus body with a carbon source other than methanol, and inducing the expression of Hbα and Hbβ by addition of methanol is more preferable. In a culture in a jarfermenter, moreover, a high density culture method is suitable for the production of human hemoglobin. The culture may be performed by any of batch culture, feeding culture and continuous culture, with preference given to feeding culture method. That is, for a certain period, a method including culturing the host fungus body in a medium (initial medium) containing a carbon energy source suitable for the growth (e.g., glucose etc.) and/or a nutrient source, and confining human hemoglobin in the system until completion of the culture while additionally supplying a substrate controlling the expression of Hbα and Hbβ (that is, methanol) to the medium from a certain point in time according to the situation can be used (see e.g., JP-A-3-83595).

[0050] Human hemoglobin produced in the culture can be collected in the yeast fungus body by applying the culture after completion of cultivation to centrifugation and/or filtration, and subsequently isolated and purified from the fungus body according to a method known per se. As such method, a method utilizing the solubility such as salting out, solvent precipitation and the like; a method mainly utilizing difference in the molecular weight such as dialysis, ultrafiltration, gel filtration method, SDS-polyacrylamide gel electrophoresis and the like; a method utilizing difference in the electric charge such as ion exchange chromatography and the like; a method utilizing specific affinity such as affinity chromatography and the like; a method utilizing difference in hydrophobicity such as reversed-phase high performance liquid chromatography and the like; a method utilizing difference in the isoelectric point such as isoelectric focusing and the like; and the like can be used. These methods can be appropriately combined.

[0051] Examples of a method for confirming the isolated and purified human hemoglobin include known Western blotting method, activity measurement method and the like. In addition, the structure of the purified hemoglobin can be clarified by amino acid analysis, N-terminal amino acid sequence, primary structure analysis and the like. Human hemoglobin obtained in this way has the same primary structure (that is, removal by cleavage of first methionine) and higher-order structure as those of the naturally occurring type, and shows the physiological activity of the same quality as the naturally occurring type.

[0052] The recombinant human hemoglobin produced in the present invention can be administered to the living body, for example, as a red blood cell substitute. For administration as a red blood cell substitute, hemoglobin can be administered by encapsulating in liposome or dispersing in emulsion. The methods of preparation making and method of administration to human are described in, for example, JP-A-2004-307404, JP-A-2006-104069, JP-A-2002-161047, JP-A-2001-348341, JP-A-08-003063, JP-A-08-003062, JP-A-07-017874, JP-A-06-321802, JP-A-06-072892 and the like.

SEQUENCE LISTING FREE TEXT

[0053]

SEQ ID NO: 5
primer.
SEQ ID NO: 6
primer.
SEQ ID NO: 7
primer.
SEQ ID NO: 8
primer.
SEQ ID NO: 9
primer.
SEQ ID NO: 10
primer.
SEQ ID NO: 11
primer.
SEQ ID NO: 12
primer.
SEQ ID NO: 13
Hbα cDNA having EcoR I site at 5' and 3'-ends.

SEQ ID NO: 14
Hbβα cDNA having EcoR I site at 5' and 3'-ends.
SEQ ID NO: 15
primer.
SEQ ID NO: 16
primer.
SEQ ID NO: 17
primer.
SEQ ID NO: 18
primer.
SEQ ID NO: 19
primer.

[0054] The present invention is explained in more detail in the following by referring to Examples, which are mere exemplifications and do not limit the scope of the present invention.

EXAMPLES

(1) amplification of hemoglobin gene

[0055] Using plasmid pBEX transfected with human hemoglobin gene (hereinafter referred to as "pBEX-Hb". Available from Tadayuki Uno (Graduate School and School of Pharmaceutical Sciences, Osaka University), a coinventor of the present application) as a template, and Hbα-sense primer of SEQ ID NO: 5 and Hbα-antisense primer of SEQ ID NO: 6 as synthetic primers of the DNA sequence of hemoglobin α-chain, and Hbβ-sense primer of SEQ ID NO: 7 and Hbβ-antisense primer of SEQ ID NO: 8 as synthetic primers of the DNA sequence of hemoglobin β-chain, respectively, mutation of EcoR I restriction enzyme site, which would become a obstruction of subsequent manipulation, was performed (QuikChange XL Site-Directed Mutagenesis Kit, Stratagene). As for mutation reaction conditions, DNA was treated for 30 sec at 95°C, after which a 12-cycle reaction of denaturation (95°C, 30 sec), annealing (55°C, 1 min) and extension (68°C, 10 min) was performed. After the reaction, the template plasmid was digested by Dpn I, and each of obtained pBEX-Hb(αΔ) and pBEX-Hb(βΔ) were transfected into XL-10-Gold ultracompetent cells to perform transformation. The transformants, which were transfected with the objective plasmids pBEX-Hb(αΔ) or pBEX-Hb(βΔ), were screened in ampicillin-added medium, and the plasmids were purified from the obtained transformants (QIAprep Spin Miniprep Kit, manufactured by QIAGEN). The plasmids were digested with EcoR I (manufactured by Takara Shuzo Co., Ltd.), and the mutation was confirmed.

[0056] Using the obtained pBEX-Hb(αΔ) and pBEX-Hb(βΔ) as templates, and Hbα-sense primer of SEQ ID NO: 9 and Hbα-antisense primer of SEQ ID NO: 10 as synthetic primers of the DNA sequence of hemoglobin α-chain, and Hbβ-sense primer of SEQ ID NO: 11 and Hbβ-antisense primer of SEQ ID NO: 12 as synthetic primers of the DNA sequence of hemoglobin β-chain, PCR was performed using a polymerase (KOD-plus-, manufactured by Toyobo Co., Ltd.). As for PCR reaction conditions, DNA was treated for 2 min at 94°C, after which a 30-cycle reaction of denaturation (94°C, 15 sec), annealing (65°C, 30 sec) and extension (68°C, 1 min) was performed. By this PCR, DNA fragments, wherein EcoR I restriction enzyme recognition sites were added to 5' end and 3' end of the DNA sequences encoding hemoglobin α-chain and β-chain, respectively, were obtained. The DNA fragments amplified by PCR were purified by phenol extraction, ethanol precipitation, then digested with restriction enzyme EcoR I (manufactured by Takara Shuzo Co., Ltd.). After the restriction enzyme treatment, DNA fragments were subjected to 2% agarose electrophoresis, and bands corresponding to each of DNA fragments (DNA fragment encoding hemoglobin α-chain;Hbα, SEQ ID NO: 13 and DNA fragment encoding hemoglobin β-chain; Hbβ, SEQ ID NO: 14) were cut out, and subjected to gel extraction (QIAquick Gel Extraction Kit, manufactured by QIAGEN).

(2) ligation of hemoglobin gene

[0057] E. coli JM109 (manufactured by Takara Shuzo Co., Ltd.) was transfected with pA0815 (manufactured by Invitrogen), a shuttle vector for Escherichia coli and yeast, to perform transformation. The transformant, which was transfected with pA0815, was screened in ampicillin-added medium, and the plasmid was purified from the obtained transformant (QIAprep Spin Miniprep Kit, manufactured by QIAGEN). The plasmid was digested with EcoR I (manufactured by Takara Shuzo Co., Ltd.), double-digested with EcoR I and Sal I (manufactured by Takara Shuzo Co., Ltd.) and double-digested with BamH I and Bgl II (manufactured by Takara Shuzo Co., Ltd.), by which restriction enzyme map was constructed, and the plasmid was confirmed to be an objective plasmid vector.

[0058] Escherichia coli, wherein the transfection of pA0815 was confirmed, was further cultured, and the plasmid was

purified from grown bacterial bodies (QIAGEN plasmid Maxi Kit, manufactured by QIAGEN). Purified pA0815 was digested with EcoR I (manufactured by Takara Shuzo Co., Ltd.), purified by phenol extraction and ethanol precipitation, after which dephosphorylation treatment was performed (Alkaline Phosphatase E. coli C75, manufactured by Takara Shuzo Co., Ltd.).

[0059] Each of Hbα and Hbβ was ligated to dephosphorylation-treated pA0815 (DNA Ligation kit Ver. 1, manufactured by Takara Shuzo Co., Ltd.) to give Hbα-pAO815 and Hbβ-pAO815, respectively. Each of the obtained Hbα-pAO815 and Hbβ-pAO815 was transfected into E. coli JM109 (manufactured by Takara Shuzo Co., Ltd.) to perform transformation. The transformants, which were transfected with either the objective plasmids Hbα-pA0815 or Hbβ-pAO815, were screened in ampicillin-added medium, and the plasmids were purified from the obtained transformants (QIAprep Spin Miniprep Kit, QIAGEN manufactured by). The plasmids were digested with EcoR I (manufactured by Takara Shuzo Co., Ltd.), and double-digested with BamH I and Bgl II (manufactured by Takara Shuzo Co., Ltd.), by which restriction enzyme map was constructed, and the plasmids were confirmed to be Hbα-pAO815 and Hbβ-pAO815.

[0060] Furthermore, full sequences of hemoglobin α-chain and β-chain gene were confirmed by ABI Prism 310 Genetic Analyzer (Applied Biosystems) using 5' AOX1 sequencing primer of SEQ ID NO: 15 (manufactured by Invitrogen), Hbα-sequence primer 1 of SEQ ID NO: 16 and Hbα-sequence primer 2 of SEQ ID NO: 17 for α-chain, and 5' AOX1 sequencing primer of SEQ ID NO: 15 (manufactured by Invitrogen), Hbβ-sequence primer 1 of SEQ ID NO: 18 and Hbβ-sequence primer 2 of SEQ ID NO: 19 for β-chain.

[0061] Escherichia coli, wherein the transfection of Hbα-pAO815 or Hbβ-pAO815 were confirmed, was further cultured, and the plasmids were purified from grown bacterial bodies (QIAGEN plasmid Maxi Kit, manufactured by QIAGEN). Purified Hbα-pAO815 was digested with BamH I and Bgl II (manufactured by Takara Shuzo Co., Ltd.), after which a band corresponding to Hbα expression cassette was cut out, and gel extraction was performed (QIAquick Gel Extraction Kit, manufactured by QIAGEN).

[0062] On the other hand, purified Hbβ-pAO815 was digested with BamH I (manufactured by Takara Shuzo Co., Ltd.) without performing dephosphorylation treatment, and purified by phenol extraction and ethanol precipitation.

[0063] Hbα expression cassette was ligated to Hbβ-pAO815 (DNA Ligation kit Ver. 1, manufactured by Takara Shuzo Co., Ltd.), which was enzyme-digested by BamH I, to give Hbβ-Hbα-pAO815. Obtained Hbβ-Hbα-pAO815 was transfected into E. coli DH5α (manufactured by Toyobo Co., Ltd.) to perform transformation. The transformant, which was transfected with the objective plasmid Hbβ-Hbα-pAO815, was screened in ampicillin-added medium, and the plasmid was purified from the obtained transformant (QIAprep Spin Miniprep Kit, manufactured by QIAGEN). The plasmid was digested with EcoR I (manufactured by Takara Shuzo Co., Ltd.), and double-digested with BamH I and Bgl II (manufactured by Takara Shuzo Co., Ltd.), by which restriction enzyme map was constructed, and the plasmid was confirmed to be Hbβ-Hbα-pAO815. Fig. 2 shows the procedure for construction of Hbβ-Hbα-pAO815.

(3) expression of hemoglobin

[0064] Hbβ-Hbα-pAO815 was digested with restriction enzyme Sal I, purified by phenol extraction and ethanol precipitation, and subsequently transformed into HIS4 gene locus of Pichia yeast (GS115 strain) by homologous recombination using an electroporation apparatus (Gene Pulser II Electroporation System, manufactured by BIO-RAD) (see Fig. 3). The obtained transformant was cultured in BMMY liquid medium, and stocked in glycerol after confirmation of expression of hemoglobin.

(4) purification of hemoglobin

[0065] The transformed Pichia yeast was cultured in BMGY liquid medium for 48 hr, and subsequently in BMMY medium for 96 hr as adding 1% methanol every 12 hr. The yeast was precipitated by centrifugation (6,000 g x 10 min), resuspended in disrupt buffer, and glass beads (425 - 600 μm) was added, after which the suspension was stirred intensively to disrupt the yeast.

[0066] The disrupted fungus body was separated by centrifugation at 10,000 g for 30 min, and the supernatant was collected. This was dialyzed against 10 mM Tris-HCl buffer (pH 7.5), and passed through Q-Sepharose™ Fast Flow column (manufactured by Amersham Biosciences), furthermore, bonded to the Q-Sepharose™ Fast Flow column by pH adjustment to 8.5, and hemoglobin was eluted by concentration gradient of 0 to 300 mM NaCl. This was replaced with 200 mM sodium acetate buffer (pH 5.5), bonded to Blue Sepharose CL-6B column (manufactured by Amersham Biosciences), and hemoglobin was eluted by concentration gradient of 0 to 3 M NaCl. Subsequently, this eluate was dialyzed against 0.5 M ammonium sulfate/100 mM sodium phosphate buffer (pH 7.0), bonded to HiTrap Butyl FF column (manufactured by Amersham Biosciences), and hemoglobin was eluted with 70% ethanol. Finally, CO was blown into the eluate and the eluate was preserved at 4°C.

(5) confirmation of hemoglobin

[0067] The final sample was subjected to SDS polyacrylamide gel electrophoresis, and a single band of predicted molecular weight, about 15,000, was detected. Also, N-terminal amino acid analysis was performed, and it showed that the band completely corresponded with the amino acid sequence of human blood-derived hemoglobin. Furthermore, molecular weights of α-chain and β-chain were estimated by MALDI TOF-MS, 15,122 and 15,866, respectively, corresponding with calculated weights.

[0068] The production method of the present invention is free of a risk of contamination with virus and the like. Therefore, it is extremely useful in that it can conveniently provide human hemoglobin in large amounts, which can be safely administered to the living body for medical purposes.

[0069] While the present invention has been described with emphasis on preferred embodiments, it is obvious to those skilled in the art that the preferred embodiments can be modified. The present invention intends that the present invention can be embodied by methods other than those described in detail in the present specification. Accordingly, the present invention encompasses all modifications encompassed in the gist and scope of the appended "CLAIMS."

[0070] In addition, the contents disclosed in any publication cited herein, including patents and patent applications, are hereby incorporated in their entireties by reference, to the extent that they have been disclosed herein.

SEQUENCE LISTING

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Claims

1. A production method of a functional recombinant human hemoglobin, which comprises transforming a yeast of the genus Pichia with an expression vector containing a DNA encoding a human hemoglobin α-chain, which is under regulation of a promoter functional in a yeast of the genus Pichia, and an expression vector containing a DNA encoding a human hemoglobin β-chain, which is under regulation of a promoter functional in a yeast of the genus Pichia, culturing the yeast in a medium, and recovering the human hemoglobin from the obtained culture.

2. The method of claim 1, wherein the DNA encoding the human hemoglobin α-chain and the DNA encoding the human hemoglobin β-chain are on the same vector.

3. The method of claim 2, wherein the DNA encoding the human hemoglobin β-chain is at the upstream side in the transcriptional direction.

4. The method of any of claims 1 to 3, wherein the DNA encoding the human hemoglobin α-chain and the DNA encoding the human hemoglobin β-chain are under regulation of different promoters.

5. The method of any of claims 1 to 4, wherein the DNA encoding the human hemoglobin α-chain and the DNA encoding the human hemoglobin β-chain are under regulation of an alcoholoxidase 1 promoter.

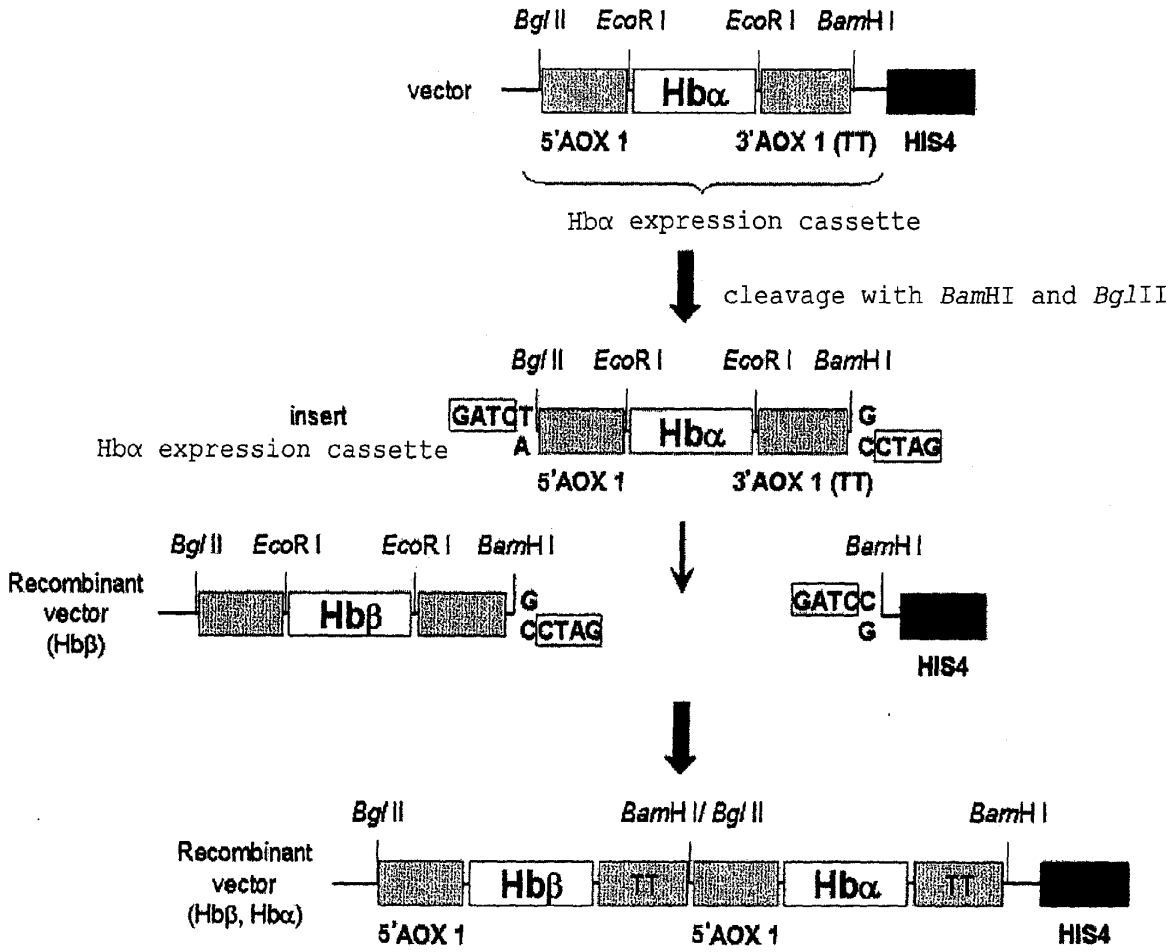
6. The method of claim 5, wherein the transformed yeast of the genus Pichia is cultivated in a liquid medium requiring methanol as a single carbon source.

7. The method of claim 6, wherein the culture is a feeding culture.

8. The method of any of claims 1 to 7, wherein the yeast of the genus Pichia is Pichia pastoris.

9. An expression vector comprising an alcoholoxidase 1 promoter, a DNA encoding the human hemoglobin β-chain, a terminator functional in a yeast of the genus Pichia, an alcoholoxidase 1 promoter, a DNA encoding a human hemoglobin α-chain and a terminator functional in a yeast of the genus Pichia in this order from the upstream side in the transcriptional direction.

FIG. 1



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FIG. 2

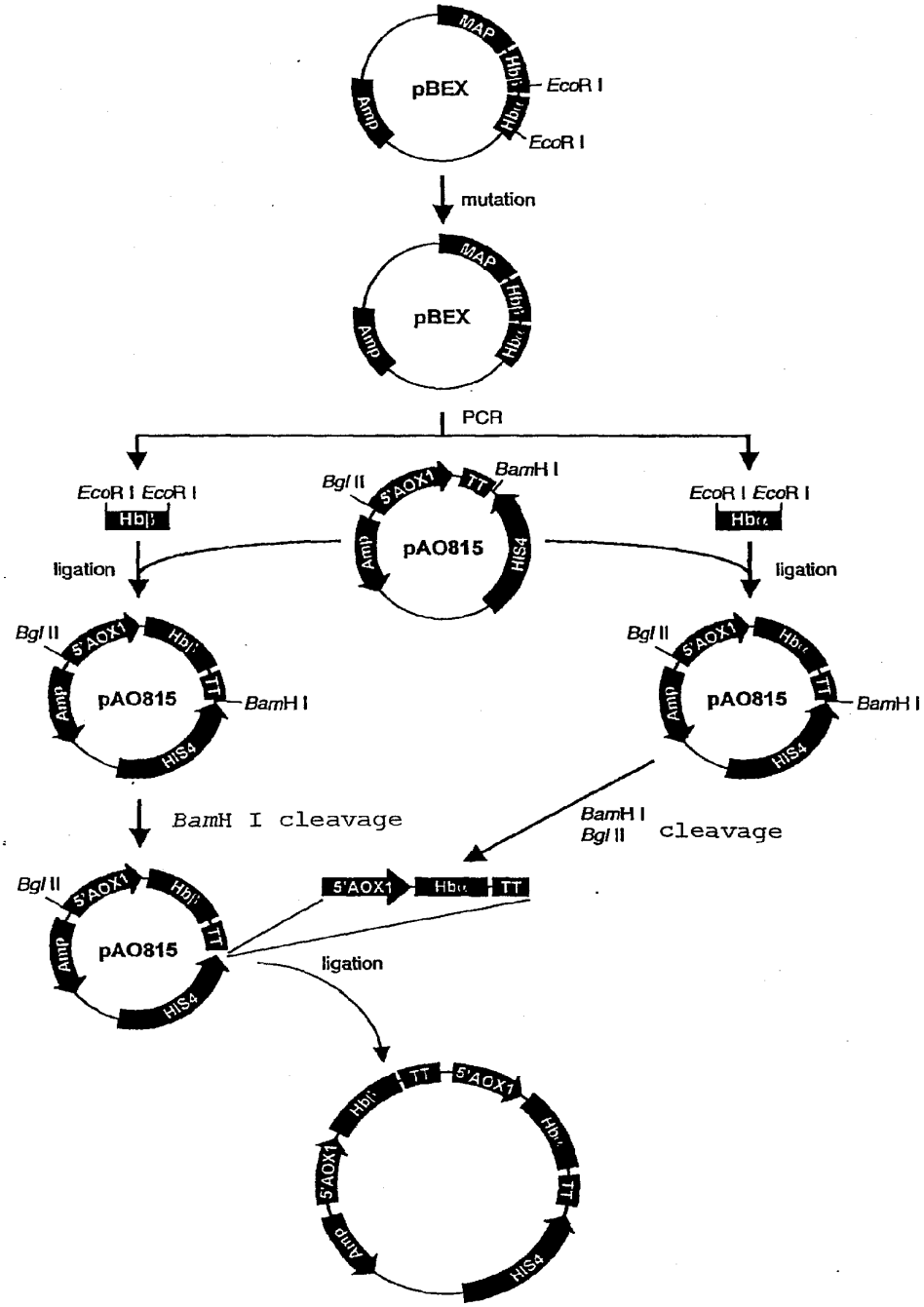
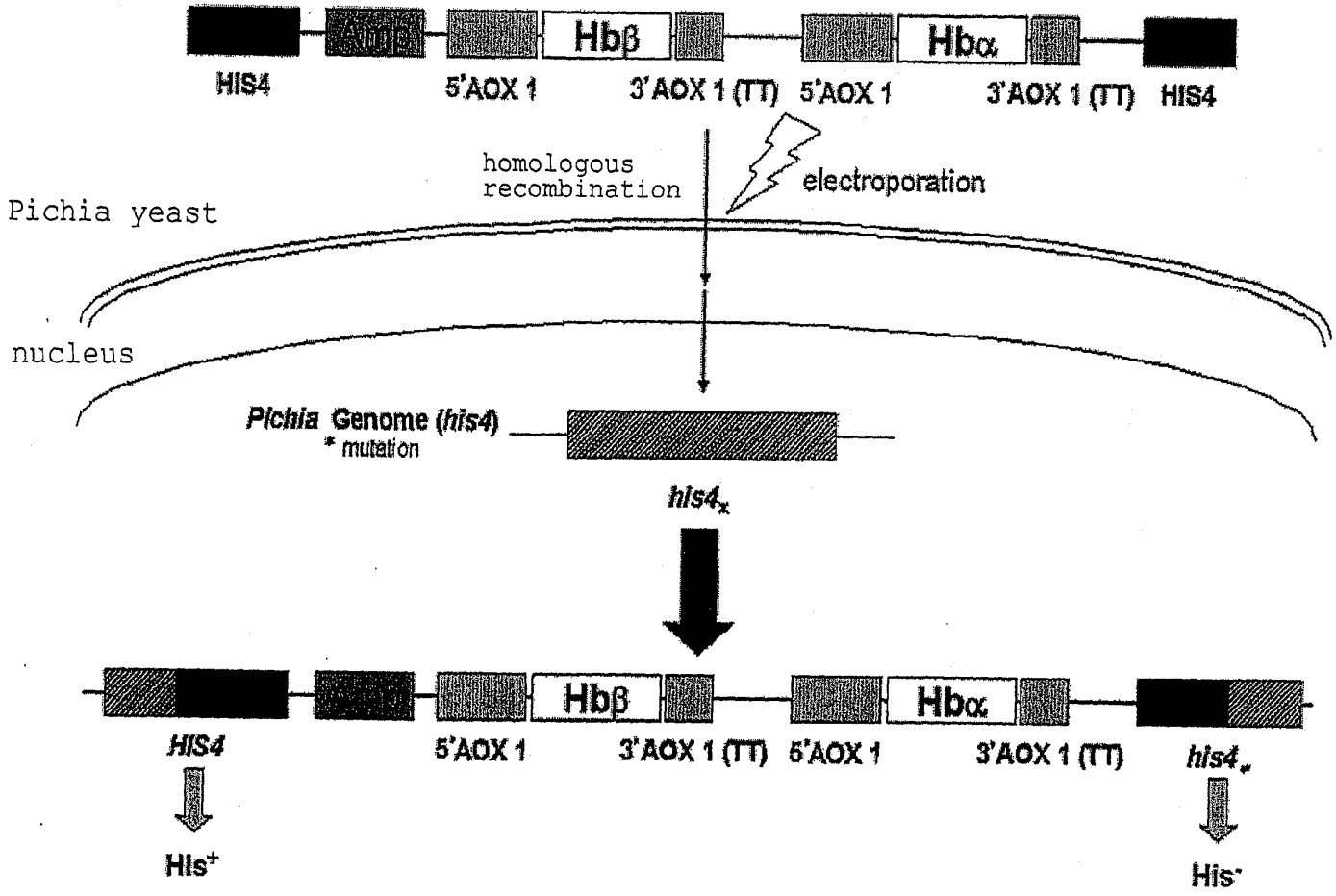

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FIG. 3



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European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 07 12 0389

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	GILBERT S C ET AL: "Increase in copy number of an integrated vector during continuous culture of Hansenula polymorpha expressing functional human haemoglobin" YEAST, vol. 10, no. 12, 1994, pages 1569-1580, XP002474658 ISSN: 0749-503X	1-4,6,7	INV. C12N15/12 C07K14/805
Y	* abstract * * figure 1 * * page 1579, right-hand column, last paragraph - page 1580, left-hand column, paragraph 3 *	5,8,9	
X	WO 90/13645 A (SOMATOGEN INC [US]) 15 November 1990 (1990-11-15) * abstract * * page 38, last paragraph - page 39, paragraph 1 *	1	
Y	MACAULEY-PATRICK SUE ET AL: "Heterologous protein production using the Pichia pastoris expression system" YEAST, vol. 22, no. 4, March 2005 (2005-03), pages 249-270, XP002474659 ISSN: 0749-503X * abstract * * page 251, paragraph 4 *	5,8,9	TECHNICAL FIELDS SEARCHED (IPC) C07K

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	2 April 2008	Loubradou, Gabriel

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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O : non-written disclosure
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E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 07 12 0389

DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	CEREGHINO JOAN LIN ET AL: "Heterologous protein expression in the methylotrophic yeast Pichia pastoris" FEMS MICROBIOLOGY REVIEWS, ELSEVIER, AMSTERDAM, NL, vol. 24, no. 1, January 2000 (2000-01), pages 45-66, XP002195512 ISSN: 0168-6445 * abstract * * page 48, left-hand column, paragraph 1; table 3 *	5,8,9	
Y	GELLISSEN G: "Heterologous protein production in methylotrophic yeasts" APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER VERLAG, BERLIN, DE, vol. 54, no. 6, December 2000 (2000-12), pages 741-750, XP002333889 ISSN: 0175-7598 * abstract; figure 1; tables 2,3 *	5,8,9	
A	RASCHKE W C ET AL: "Inducible expression of a heterologous protein in Hansenula polymorpha using the alcohol oxidase 1 promoter of Pichia pastoris" GENE: AN INTERNATIONAL JOURNAL ON GENES AND GENOMES, ELSEVIER, AMSTERDAM, NL, vol. 177, no. 1, 24 October 1996 (1996-10-24), pages 163-167, XP004043391 ISSN: 0378-1119 * the whole document *		TECHNICAL FIELDS SEARCHED (IPC)

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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	2 April 2008	Loubradou, Gabriel

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T : theory or principle underlying the invention
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& : member of the same patent family, corresponding document

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European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 07 12 0389

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
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A	GASSER B ET AL: "Engineering of Pichia pastoris for improved production of antibody fragments" BIOTECHNOLOGY AND BIOENGINEERING, WILEY & SONS, HOBOKEN, NJ, US, vol. 94, no. 2, 5 June 2006 (2006-06-05), pages 353-361, XP003018989 ISSN: 0006-3592 * the whole document *		
A	US 6 172 039 B1 (DE ANGELO JOSEPH [US] ET AL) 9 January 2001 (2001-01-09) * the whole document *		TECHNICAL FIELDS SEARCHED (IPC)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	2 April 2008	Loubradou, Gabriel

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 07 12 0389

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02-04-2008

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

REFERENCES CITED IN THE DESCRIPTION

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(57) Abstract

The present invention relates to genetic-engineering of plants for enhanced oxygen assimilation and utilization. More particularly, this invention relates to producing transgenic plants engineered to express globin proteins such as, for example, hemoglobin, myoglobin, and hemoproteins. The engineered plants of the invention achieve quicker germination, are faster growing or maturing crops, produce higher crop yields, and/or contain higher levels of desired plant metabolites, particularly alkaloids. The invention also relates to mutant *Vitreoscilla* hemoglobin proteins, polynucleotides encoding the same, and host cells containing such polynucleotides.

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EXPRESSION OF GLOBIN PROTEINS IN PLANTS

1. INTRODUCTION

The present invention relates to genetic-engineering of plants for enhanced oxygen assimilation and utilization. More particularly, this invention relates to producing transgenic plants engineered to express oxygen-binding proteins, particularly globins such as, for example, hemoglobin, myoglobin, and hemoproteins. The engineered plants may be used to achieve quicker germination, faster growing or maturing crops, higher crop yields and/or plants with higher levels of desired plant metabolites, particularly alkaloids.

2. BACKGROUND OF THE INVENTION

Genetic engineering of plants through transgenic approaches has rapidly become a diverse and promising field. Transformation techniques are applied both to study plant development and physiology, and in attempts to generate plants with advantageous agricultural properties.

For example, much effort has focused on improving crop production by transforming plants with herbicide or pesticide resistance genes. When grown under field conditions, the resulting transgenic plants can tolerate and survive applications of herbicides or pesticides which would destroy non-transformed plants. Alternatively, plants themselves have been genetically engineered to express products which are shown to act as natural insecticides such as *Bacillus thuringensis* toxin proteins (*see* U.S. Patent No. 5,380,831, issued Jan. 10, 1995).

Genetic engineering has also been attempted to generate plants with altered compositions of primary metabolites such as starch, sugar, and oils. For example, a bacterial levan sucrase gene was transformed into plants to increase levels of fructans in the resulting transformant. In another approach, the activity of certain enzymes involved in the metabolic synthesis of oils has been decreased using antisense technology in order to design vegetable oils exhibiting the desired properties. (*Reviewed in* Knauf, 1995, Curr. Opin. Biotech. 6:165-70).

In addition to attempts to alter primary metabolic compositions of plants (*i.e.* carbohydrates, oils, amino acids and proteins), it would also be desirable to alter secondary metabolite compositions. Secondary metabolites are those specialized plant products required by cells in small amounts (*e.g.* hormones), or

are highly specialized biomolecules (*e.g.* nucleotides, pigments, toxins, antibiotics, and alkaloids). Generally, secondary metabolites can be defined as compounds that have no recognized role in the maintenance of fundamental life processes of the cell, but which may perform other advantageous functions for the organism as a whole. (*See* for example, Bell, 1981, "The Physiological Role(s) of Secondary (Natural) Products" in The Biochemistry of Plants, v. 7, p. 1, Academic Press, Inc.)

The production of useful secondary metabolites from plants and plant cell cultures is an important aspect of plant technology. About 25% of the prescription drugs used in the industrialized world contain ingredients extracted from higher plants (Tyler, V.E., 1988, Planta Med. 54:95-100). The most prominent group of plant substances used in medicine are the alkaloids (Nickell, L.G., 1980, "Products," Plant tissue culture as a source of biochemicals, Staba, E.J., Ed., Boca Raton, CRC Press Inc., 235-269). Alkaloids are secondary metabolites which protect plants against phytophagocytosis by higher organisms or invasion by pathogens (Robins, R.J. *et al.*, 1991, Planta Med. 57:27-35). Plant alkaloids are generally derived from simple amino acids that interact with acetate and terpenoid units and undergo aromatic hydroxylations (Robins, *supra*). Examples of plant alkaloids are nicotine, scopolamine, hyoscyamine, ajmalicine, serpentine, piperine, leucenol, mimosine, ricinine, pelletierine, cocaine, hygrine, lupinine and anagyrine.

Several examples of introducing a new metabolic pathway or redirecting previously existing ones to increase production of secondary metabolites have been described (Bailey, *supra*; Berlin, J. *et al.*, 1994, Stud. Plant Sci. 4:57-81; Lilius, G., Holmberg, N. and Bülow, L., 1996, Bio/Technology 14:177-180). However, apparently all of these genetic engineering approaches entail either the up-regulation of a limiting enzyme in the pathway (for example through transformation and expression of the cloned gene), or the down-regulation of competing enzymatic pathways (for example through anti-sense technology). (*See* for review, Robins et al., 1991, Planta. Med. 57:27-35.)

Plant cell suspension cultures have often been used for industrial production of plant secondary metabolites. During *in vitro* culture, a critical parameter influencing secondary metabolite formation, especially in scale-up cultures, is the

level of dissolved oxygen. For instance, it has been demonstrated that a high dissolved oxygen concentration promotes the production of ajmalicine in *Catharantus roseus* batch cultures (Schaltmann, J.E. *et al.*, 1995, Biotech. Bioeng. 45:435-439). Similarly, increased alkaloid levels with increasing aeration have been observed in *Berberis wilsonae* (Breuling, M. *et al.*, 1985, Plant Cell Rep. 4:220-223). From these results it has been suggested that oxygen is a limiting substrate in plant tissue culture secondary metabolism, as well as for primary metabolism, especially for industrial scale-up processes. However, this need for increased oxygen has always been thought to be a need for extracellular oxygen during fermentation batch processes.

Aerobic metabolism has been successfully enhanced in fermentation microorganisms and cultured mammalian cells by engineering the host to express the *Vitreoscilla* hemoglobin gene (VHb) (Khosla, C., Bailey, J.E., 1988, Nature 331:633-635; Khosla, C., Bailey, J.E., 1988, Mol. Gen. Genet. 214:158-161; DeModena, *supra*; Chen, W. *et al.*, 1994, Biotechnol. Prog. 10:308-313; Pendse, G.J. and Bailey, J.E., 1994, Biotechnol. Bioeng. 44:1367-1370). This metabolic engineering strategy has been shown effective, for example, in increasing total cell protein synthesis by oxygen-limited *Escherichia coli* (Khosla, C. *et al.*, 1990, Bio/Technology 8:849-853 and U.S. Patent No. 5,049,493, issued Sept. 17, 1991), improving lysine yield and titer in cultivations of *Corynebacterium glutamicum* (Sander, F. *et al.*, 1994, Proc. 6th Eur. Congress Biotechnol., Alberghina, L., Frontali, L. and Sensi, P. (eds.) Elsevier Science B.V., Amsterdam 607-610), and in increasing actinorhodin and cephalosporin C production in *Streptomyces coelicolor* and *Acremonium chrysogenum*, respectively (Magnolo, S.K. *et al.*, 1991, Bio/Technology 9:473-476; DeModena, J.A. *et al.*, 1993, Bio/Technology 11:926-929).

Hemoglobin and globin-like proteins exist ubiquitously in mammals, and are less frequently found in plants. The few plant hemoglobin-like molecules described, which include lupin and soybean leghemoglobin, are largely thought to be associated with nitrogen-fixation activities of these plants, although some researchers claim that hemoglobin-like proteins occur in the roots of all plants. However, in no case have these proteins been overexpressed in plants. Although several major plant metabolic pathways are known to be oxygen-dependent,

including chlorophyll and heme biosynthesis, fatty acid desaturation and cysteine, glycine and serine biosynthesis, oxygen has not been thought to be a limiting factor in metabolism in intact plants. Rather, the observed effects of dissolved oxygen on metabolism in plant tissue cultures has been attributed to mass transport problems during large-scale, or even batch culture, fermentations (*see* Schlatmann et al., 1991, *supra*.). Thus, until the present invention, the advantages of altering useable levels of intracellular oxygen in intact plants have not been recognized.

3. SUMMARY OF THE INVENTION

The present invention is directed to the generation of plants with improved agronomic or medicinal characteristics by metabolically engineering an increased intracellular oxygen level or increased utilization of oxygen. Such engineered plants display enhanced production of valuable secondary metabolites, in particular those metabolites which are synthesized by oxygen-dependent pathways, as well as other unexpected and valuable properties such as more rapid germination, higher levels of chlorophyll, increased overall growth metabolism, and increased rate of accumulation of biomass.

The invention encompasses both genetically engineered plants and methods of producing the same. Therefore, in one of its embodiments, the invention encompasses a method of metabolically engineering plants by increasing intracellular levels of oxygen or intracellular utilization of oxygen. The invention includes a method of producing a plant with an improved agronomic or medicinal characteristic by transforming the plant with polynucleotides encoding an oxygen-binding protein. Particularly preferred oxygen-binding proteins are globins, which reversibly bind oxygen through a heme group. In an exemplary embodiment, the oxygen-binding protein is *Vitreoscilla* hemoglobin. Preferably, the polynucleotides encoding the oxygen-binding protein are operably linked to a strong, constitutively expressed plant promoter, for example, the CaMV 35S promoter. Another aspect of the invention is a plant or plant cell which has been genetically engineered by the methods of the invention. The invention encompasses plants and plant cells which are both dicotyledons and monocotyledons.

Still another aspect of the invention is mutant *Vitreoscilla* hemoglobin proteins, polynucleotides encoding the same, and host cells containing such mutant

proteins and polynucleotides. The host cells can be bacteria, plant, animal, fungi or algae. The invention also encompasses a method of generating polynucleotides encoding mutant *Vitreoscilla* hemoglobin proteins. The method entails amplifying a polynucleotide that encodes *Vitreoscilla* hemoglobin protein using a low fidelity polymerase chain reaction, transforming the amplified polynucleotides into bacteria, and selecting bacteria that contain a polynucleotide sequence encoding the mutant *Vitreoscilla* hemoglobin protein. In a preferred aspect of this method, bacterial colonies containing mutant *Vitreoscilla* hemoglobin proteins are selected by virtue of their color phenotype. Polynucleotides encoding mutant *Vitreoscilla* hemoglobin proteins that are isolated by this method, as well as the mutant proteins themselves, and host cells containing such polynucleotides, are also within the scope of the present invention.

4. BRIEF DESCRIPTION OF THE FIGURES

Figure 1: FIG. 1A. The last steps in the *Nicotiana tabaccum* biosynthesis of the secondary metabolites nicotine and anabasine. The reaction catalyzed by nicotine synthase, the oxygen-dependent enzyme catalyzing the conversion of nicotinic acid and N-methyl-Δ'-pyrrolinium salt into nicotine, is labelled in bold. FIG. 1B. Oxygen-dependent steps in chlorophyll and heme biosynthesis in plants. The last oxidation of chlorophyll a to chlorophyll b has only been observed *in vitro* (Scheer, H. (ed.), 1991, Chlorophylls CRC Press, Boca Raton, FL, USA 421-425).

Figure 2. Construction of the Ti-expression vector containing the *Vitreoscilla* VHb gene using PCR. The gene was placed under control of the CaMV 35S promoter and transcription termination was achieved by the NOS region. The approximate positions of the PCR primers are indicated by solid arrows. Right and left border of the T-DNA are indicated by RB and LB, respectively. NOS-prom, NPT II and NOS are abbreviations for the nopaline synthase promoter, the neomycine phosphotransferase II gene, and the nopaline synthase 3' transcription termination region, respectively.

Figure 3. Dried weight measurements at different time intervals are shown as mean values. Bars correspond to the standard error. The data from the first two time points (15 and 20 days) correspond to 12 plants but weighed in triplets,

and the data from the last two time points are from 8 plants individually weighed. This experiment was duplicated and the data points were subjected to statistical analysis using one-way analysis of variance (ANOVA).

Figure 4. Nicotine and anabasine production in the transgenic lines, He3 and He4, and a wild-type line. The nicotine concentrations are given μg/g fresh weight and the anabasine concentrations as μg/g freeze-dried plant. The nicotine and anabasine concentrations were measured in eight plants and six plants, respectively. All measurements were performed in duplicate, with the standard deviations being less than 1% and 25%, respectively.

Figure 5. The chlorophyll contents in the two transgenic tobacco lines and a wild-type control measured as μg chlorophyll/ml x mg fresh weight. The bars represent the mean values of 18 plants. The standard errors of the mean were less than 10%, 8% and 5% for wild-type tobacco, He3 and He4, respectively.

Figure 6. Schematic map of the pUC derivative plasmid pJFK2 used for transformation of rice.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to metabolic genetic engineering by effectively increasing the intracellular level of available oxygen in plants. The invention is based, in part, on the discovery that transforming plants so as to express oxygen-binding proteins, and in particular heme-containing globins, improves the properties of the transformed plants in unexpected ways. The resulting plants produce higher levels of secondary metabolites whose production is oxygen sensitive. Surprisingly, however, these genetically engineered plants also exhibit better growth characteristics, more rapid germination, and improved vegetative yield. Without intending to be limited to a particular mechanism, it is believed that these surprising results are due to either an increased level of intracellular oxygen, or an increased efficiency in intracellular oxygen utilization.

Accordingly, the targets for engineering are genes encoding proteins involved in binding oxygen, particularly those which bind oxygen reversibly such as the globins. The target genes include, but are not limited to, those encoding *Vitreoscilla* hemoglobin (VHb), horse heart myoglobin, E. coli hemoprotein, B.

subtilis hemoprotein, yeast flavohemoglobin, soybean leghemoglobin, lupin leghemoglobin, and sperm whale myoglobin. In fact, an engineered increased expression of any endogenous plant hemoglobin is also within the scope of the invention.

Particularly suitable for use in the present invention are those oxygen-binding proteins which have relatively high k_{off} rates such as VHb (k_{off} 5600 s^{-1}; Orii and Webster, 1986, J. Biol. Chem. 261:3544-3547) or relatively low oxygen affinity such as horse heart myoglobin (K_D 0.79 μM; Wittenberg et al., 1985, in Nitrogen fixation research progress, H.J. Evand et al. Eds. Martinus Nijhoff Publishers , Dordrecht, p. 354). Therefore, particularly preferred oxygen binding proteins may be those proteins with a k_{off} rate for oxygen of greater than 10 s^{-1}, or a K_D less than 0.5 μM, although it will be understood that oxygen-binding proteins with rate constants outside of these parameters will also be useful. Other examples of preferred oxygen-binding proteins are globins such as hemoglobin, myoglobin, and leghemoglobins. The properties of many oxygen-binding proteins, including globins, are disclosed in the literature. Additionally, techniques for determining the oxygen-binding properties of a protein such as a globin are well known to one of skill in the art and may be performed without undue experimentation.

A particularly advantageous oxygen-binding protein for use in the instant invention, as described herein by way of working example, is *Vitreoscilla* hemoglobin ("VHb"). The complete sequence of the VHb gene is described in U.S. Patent No. 5,049,493, *supra*. Mutants of VHb which bind oxygen are also within the scope of the present invention. As described more fully below by way of a working example, genes encoding functional mutants of VHb are generated through PCR mutagenesis, and then tested in a simple phenotypic screen for their ability to bind oxygen. Using these techniques, several VHb mutants have been obtained for use in the present invention.

Also encompassed within the scope of the invention are any nucleotide sequence which hybridizes to the complement of the nucleotide sequence of the VHb gene under highly stringent conditions, *e.g.*, hybridization to filter-bound DNA in 0.5 M $NaHPO_4$, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing

Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encodes a functionally equivalent gene product; and any nucleotide sequence that hybridizes to the complement of the nucleotide sequence of the VHb gene under less stringent conditions, such as moderately stringent conditions, *e.g.*, washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel et al., 1989, *supra*), yet which still encodes a functionally equivalent Vhb gene product. Functional equivalents of VHb include naturally occurring hemoglobin genes present in other species, and mutant VHb whether naturally occurring or engineered which retain at least some of the functional activity of VHb (*i.e.*, reversible oxygen binding). Species from which genes encoding functional equivalents of VHb include but are not limited to bacterial species, and in particular those bacterial species found in soil, dung, and in other oxygen-accessible, but poorly aerated, environments.

Furthermore, using the teachings of the present invention one of skill in the art can also isolate genes encoding other bacterial proteins which reversibly bind oxygen through a heme group without relying upon sequence homology with known proteins. For example, one may isolate and culture a bacterial organism, prepare genomic DNA from the cultured bacteria, and then construct an expression library with the DNA in *E. coli* using well known techniques. The expression library is plated to obtain isolated colonies. Those *E. coli* colonies carrying plasmids which expresses an oxygen-binding heme protein may be identified phenotypically by their red color. The expression library DNA clones encoding oxygen-binding proteins may be further characterized and subcloned into plant expression vectors. The expression of such oxygen-binding heme proteins in plants is accordingly encompassed by the invention.

The invention also encompasses degenerate variants of nucleotide sequences which encode the amino acid sequence of the VHb protein, VHb mutants, and functional equivalents of VHb encoded by nucleotide sequences which hybridize to the complement of the nucleotide sequence of the VHb gene. For example, the nucleotide sequence may be altered so as to optimize amino acid codon usage for expression in the chosen host cell.

The levels of oxygen-binding proteins can be altered or their expression can be enhanced or otherwise modified (*e.g.*, ectopic expression or tissue specific expression) to engineer a plant with desirable properties. The engineering is

accomplished by transforming plants with nucleic acid constructs described herein. The transformed plants or their progenies are screened for plants that express the desired protein or exhibit the desired altered expression of the oxygen-binding protein, altered expression of the corresponding mRNA, altered secondary metabolite levels, increased growth rate, enhanced vegetative yield and/or increased germination rate.

Engineered plants exhibiting the desired physiological and/or agronomic changes can be used in plant breeding or directly in agricultural production. These plants having one altered protein also may be crossed with other altered plants engineered with alterations in other pathways (*e.g.*, cross an oxygen-binding expressing plant to a plant which has been otherwise genetically engineered) to produce lines with even further enhanced physiological and/or agronomic properties compared to the parents.

The invention is illustrated by working examples of plants engineered for expression of *Vitreoscilla* hemoglobin (VHb). In all instances, engineered plants that exhibit expression also show better growth characteristics, increased rate of seed germination, improved vegetative yield and/or enhanced levels of secondary metabolites over control, wild-type plants.

The invention is based, in part, on the surprising discovery that transformation of plants with oxygen-binding proteins provides several advantages. Plants were transformed with expression plasmids encoding an oxygen-binding protein in an attempt to develop plants with increased drought tolerance. As a model system, *Nicotiana tabaccum* (tobacco) was first chosen to be transformed with expression constructs encoding the oxygen-binding protein *Vitreoscilla* hemoglobin (VHb). However, since there is an oxygen-dependent step in the nicotine biosynthesis pathway, the production of nicotine was examined.

The VHb gene was introduced into *Nicotiana tabaccum* (tobacco) via *Agrobacterium* mediated gene transfer. Transcription and translation of the VHb gene and message were demonstrated by reverse transcriptase PCR and Western immunoblot analysis, respectively. Transgenic tobacco plants expressing VHb exhibited on average 34% greater nicotine content than wild-type controls. They also contained an altered distribution of nicotine relative to the less desirable alkaloid anabasine. Additional effects of VHb expression on the physiology of the

transgenic tobacco plants were also noted; these plants contained on average 10-20% more chlorophyll than controls. Moreover, the plants germinated more quickly and exhibited a more rapid rate of dry weight increase. Thus, engineering of plants to express additional oxygen-binding proteins not only produced increased alkaloid levels and altered distributions of alkaloids, but also enhanced plant growth.

In yet another illustration of the advantages of the present invention, plants from the genus *Datura* were transformed with the same expression construct used to transform the tobacco plants. *Datura* is the major commercial source for production of the secondary metabolites scopolamine and hyoscyamine, which are used as sedatives and as starting material for semisynthetic derivatives of these metabolites. Surprisingly, scopolamine and hyoscyamine levels in the transgenic plants were increased as much as 5 to 6 fold over levels found in non-transformed plants.

In still another illustration of the invention, plants from the genus *Oryzae* were transformed by the ballistic method with a polynucleotide construct that directs the expression of the VHb hemoglobin gene. Thus, the present invention is generally applicable for altering the oxygen utilization in all plant types in order to obtain plant varieties with advantageous medicinal and agronomic qualities. For example, plants which germinate more quickly and accumulate biomass more rapidly will find particular application in agricultural environments where arable land and/or the length of growing season are limiting factors to production. Even a small increase in biomass production and/or rate of growth can prove significant. Additionally, the increased levels of economically important secondary metabolites may enable production of metabolites from previously economically unfeasible plant sources.

Further, the invention encompasses a method of generating polynucleotides encoding mutant *Vitreoscilla* hemoglobin proteins. The method entails amplifying a polynucleotide that encodes *Vitreoscilla* hemoglobin protein using a low fidelity polymerase chain reaction, transforming the amplified polynucleotides into bacteria, and selecting bacteria that contain a polynucleotide sequence encoding the mutant *Vitreoscilla* hemoglobin protein. In a preferred aspect of this method, bacterial colonies containing mutant *Vitreoscilla* hemoglobin proteins are selected

by virtue of their color phenotype; a red or pink color indicates that the bacteria express an oxygen-binding heme protein. Polynucleotides encoding mutant *Vitreoscilla* hemoglobin proteins that are isolated by this method, as well as the mutant proteins themselves, and host cells containing such polynucleotides, are within the scope of the present invention. The host cells can be bacteria, plant, animal, fungi or algae. Such mutant *Vitreoscilla* hemoglobin proteins are useful in any number of applications for which wild-type *Vitreoscilla* hemoglobin proteins are advantageous, including but not limited to increasing cell growth and increasing cell production.

5.1. PRODUCTION OF TRANSGENIC PLANTS

According to the present invention, a desirable plant or plant cell may be obtained by transforming a plant cell with the nucleic acid encoding an oxygen-binding protein, particularly a globin, and in one embodiment VHb, as described herein. In some instances, it may be desirable to engineer a plant with several different gene constructs. Such engineering may be accomplished by transforming a plant or plant cell with all of the desired gene constructs simultaneously. Alternatively, the engineering may be carried out sequentially. That is, transforming with one gene construct, obtaining the desired transformant after selection and screening, transforming the transformant with a second gene construct, and so on.

In an embodiment of the present invention, *Agrobacterium* is employed to introduce the gene constructs into plants. Such transformations preferably use binary *Agrobacterium* T-DNA vectors (Bevan, 1984, Nuc. Acid Res. 12:8711-8721), and the co-cultivation procedure (Horsch et al., 1985, Science 227:1229-1231). Generally, the *Agrobacterium* transformation system is used to engineer dicotyledonous plants (Bevan et al., 1982, Ann. Rev. Genet. 16:357-384; Rogers et al., 1986, Methods Enzymol. 118:627-641) The *Agrobacterium* transformation system may also be used to transform, as well as transfer, DNA to monocotyledonous plants and plant cells (*see* Hernalsteen et al., 1984, EMBO J. 3:3039-3041; Hooykass-Van Slogteren et al., 1984, Nature 311:763-764; Grimsley et al., 1987, Nature 325:1677-179; Boulton et al., 1989, Plant Mol. Biol. 12:31-40; Gould et al., 1991, Plant Physiol. 95:426-434).

In other embodiments, various alternative methods for introducing recombinant nucleic acid constructs into plants and plant cells may also be utilized. These other methods are particularly useful where the target is a monocotyledonous plant. Alternative gene transfer and transformation methods include, but are not limited to, protoplast transformation through calcium-, polyethylene glycol (PEG)- or electroporation-mediated uptake of naked DNA (*see* Paszkowski et al., 1984, EMBO J 3:2717-2722, Potrykus et al., 1985, Mol. Gen. Genet. 199:169-177; Fromm et al., 1985, Proc. Natl. Acad. Sci. USA 82:5824-5828; Shimamoto, 1989, Nature 338:274-276) and electroporation of plant tissues (D'Halluin et al., 1992, Plant Cell 4:1495-1505). Additional methods for plant cell transformation include microinjection, silicon carbide mediated DNA uptake (Kaeppler et al., 1990, Plant Cell Reporter 9:415-418), and microprojectile bombardment (*see* Klein et al., 1988, Proc. Natl. Acad. Sci. USA 85:4305-4309; Gordon-Kamm et al., 1990, Plant Cell 2:603-618).

According to the present invention, a wide variety of plants may be engineered for the desired physiological and agronomic characteristics described herein using nucleic acid constructs encoding oxygen-binding proteins and the various transformation methods mentioned above. Target plants for engineering include, but are not limited to, those of tobacco, *Datura*, maize, wheat, rice, soybean, tomato, carrots, peanut, potato, sugar beets, sunflower, yam, *Arabidopsis*, rape seed, petunia and other ornamental flowers, turfgrass, barley, alfalfa, potato, cassava, sweet potato, lettuce, sugar cane, fruit trees (*e.g.* cherry, apple, peach, pear, plum, orange, lemon), nut trees (*e.g.* walnut, almond), banana, peanut, strawberry, cauliflower, artichoke, cucumber, melon, grape, pepper, cabbage, endive, leek, lettuce, spinach, arrowroot, radish, bean and pea.

According to the present invention, desired plants may be obtained by engineering the gene constructs described herein into a variety of plant cell types, including but not limited to, protoplasts, tissue culture cells, tissue and organ explants, pollen, embryos as well as whole plants. In an embodiment of the present invention, the engineered plant material is selected or screened for transformants (*i.e.*, those that have incorporated or integrated the introduced gene construct(s)) following the approaches and methods described below. An isolated transformant may then be regenerated into a plant. Alternatively, the engineered

plant material may be regenerated into a plant, or plantlet, before subjecting the derived plant, or plantlet, to selection or screening for the marker gene traits. Procedures for regenerating plants from plant cells, tissues or organs, either before or after selecting or screening for marker gene(s), are well known to those skilled in the art.

A transformed plant cell, callus, tissue or plant may be identified and isolated by selecting or screening the engineered plant material for traits encoded by the marker genes present on the transforming DNA. For instance, selection may be performed by growing the engineered plant material on media containing inhibitory amounts of the antibiotic or herbicide to which the transforming marker gene construct confers resistance. Further, transformed plants and plant cells may also be identified by screening for the activities of any visible marker genes (*e.g.*, the ß-glucuronidase, luciferase, B or C1 genes) that may be present on the recombinant nucleic acid constructs of the present invention. Such selection and screening methodologies are well known to those skilled in the art.

Physical and biochemical methods may also be used to identify a plant or plant cell transformant containing the gene constructs of the present invention. These methods include but are not limited to: 1) Southern analysis or PCR amplification for detecting and determining the structure of the recombinant DNA insert; 2) northern blot, S-1 RNase protection, primer-extension or reverse transcriptase-PCR amplification for detecting and examining RNA transcripts of the gene constructs; 3) enzymatic assays for detecting enzyme or ribozyme activity, where such gene products are encoded by the gene construct; 4) protein gel electrophoresis, western blot techniques, immunoprecipitation, or enzyme-linked immunoassays, where the gene construct products are proteins; 5) biochemical measurements of compounds produced as a consequence of the expression of the introduced gene constructs. Additional techniques, such as *in situ* hybridization, enzyme staining, and immunostaining, may also be used to detect the presence or expression of the recombinant construct in specific plant organs and tissues. The methods for doing all these assays are well known to those skilled in the art.

5.1.1. PLANTS TRANSFORMED TO EXPRESS OXYGEN-BINDING PROTEINS

In accordance with the present invention, a plant that expresses a recombinant oxygen-binding protein gene may be engineered by transforming a plant cell with a gene construct comprising a plant promoter operably associated with a sequence encoding oxygen-binding protein. (Operably associated is used herein to mean that transcription controlled by the "associated" promoter would produce a functional messenger RNA, whose translation would produce the enzyme.) The plant promoter may be constitutive or inducible. Useful constitutive promoters include, but are not limited to, the CaMV 35S promoter, the T-DNA mannopine synthetase promoter, and their various derivatives. Useful inducible promoters include but are not limited to the promoters of ribulose bisphosphate carboxylase (RUBISCO) genes, chlorophyll a/b binding protein (CAB) genes, heat shock genes, the defense responsive gene (*e.g.*, phenylalanine ammonia lyase genes), wound induced genes (*e.g.*, hydroxyproline rich cell wall protein genes), chemically-inducible genes (*e.g.*, nitrate reductase genes, gluconase genes, chitinase genes, PR-1 genes *etc.*), dark-inducible genes (*e.g.*, asparagine synthetase gene (Coruzzi and Tsai, U.S. Patent 5,256,558, October 26, 1993, Gene Encoding Plant Asparagine Synthetase) to name just a few.

In yet another embodiment of the present invention, it may be advantageous to transform a plant with a gene construct operably linking a modified or artificial promoter to a sequence encoding oxygen-binding protein. Typically, such promoters, constructed by recombining structural elements of different promoters, have unique expression patterns and/or levels not found in natural promoters. *See, e.g.* Salina et al., 1992, Plant Cell 4:1485-1493, for examples of artificial promoters constructed from combining cis-regulatory elements with a promoter core.

In a preferred embodiment of the present invention, the associated promoter is a strong and/or embryo-specific plant promoter such that the oxygen-binding protein is overexpressed in the transgenic plant. Additionally, particular promoters may be used to direct tissue specific expression of the oxygen-binding protein. For example, root-specific expression of an oxygen-binding protein may be engineered.

Further, recombinant expression of an oxygen-binding protein may be directed to specific cellular compartments. One of skill in the art may direct expression of the oxygen-binding protein intracellularly, to the chloroplasts, or to the mitochondria, to give just a few examples.

In yet another preferred embodiment of the present invention, the overexpression of oxygen-binding protein in plants may be engineered by increasing the copy number of the oxygen-binding protein gene. One approach to producing such transgenic plants is to transform with nucleic acid constructs that contain multiple copies of a plant expression construct directing the expression of an oxygen-binding protein gene (*i.e.*, operatively linked to a plant promoter). Another approach is to repeatedly transform successive generations of a plant line with one or more copies of the plant expression construct. Yet another approach is to place an oxygen-binding protein expression construct in a nucleic acid construct containing an amplification-selectable marker (ASM) gene such as the glutamine synthetase or dihydrofolate reductase gene. Cells transformed with such constructs are subjected to culturing regimes that select cell lines with increased copies of the oxygen-binding protein gene. *See* Donn et al., 1984, J. Mol. Appl. Genet. 2:549-562, for a selection protocol used to isolate of a plant cell line containing amplified copies of the GS gene. Because the desired gene is closely linked to the ASM, cell lines that amplified the ASM gene are also likely to have amplified the desired oxygen-binding protein gene. Cell lines with amplified copies of the oxygen-binding protein gene can then be regenerated into transgenic plants.

5.1.2. SCREENING OF TRANSFORMED PLANTS FOR THOSE HAVING DESIRED ALTERED TRAITS

It will be recognized by those skilled in the art that in order to obtain transgenic plants having the desired engineered traits, screening of transformed plants (i.e., those having an gene construct of the invention) having those traits may be required. For example, where the plants have been engineered for overexpression of an oxygen-binding protein gene, transformed plants are examined for those expressing the oxygen-binding protein gene at the desired level and in the desired tissues and developmental stages. The plants exhibiting the

desired physiological changes, *e.g.*, oxygen-binding protein expression, may then be subsequently screened for those plants that have the desired phenotypic changes at the plant level (e.g., increased chlorophyll, heme, amino acid, or alkaloid biosynthesis, increased fatty acid desaturation, or increased levels of oxmoprotectants such as glycine betaine which requires oxygen for its biosynthesis). The same principle applies to obtaining transgenic plants having tissue-specific expression of a heterologous gene by the use of a tissue specific promoter driven expression construct.

Alternatively, the transformed plants may be directly screened for those exhibiting the desired structural changes. In one embodiment, such screening may be for the color of the transformed plants. In other embodiments, the screening of the transformed plants may be for improved agronomic characteristics (e.g., faster growth, greater vegetative or reproductive yields, or improved protein contents, etc.), as compared to unengineered progenitor plants, when cultivated under various growth conditions (e.g., soils or media containing different amount of nutrients, water content, and/or salt content).

According to the present invention, plants engineered with oxygen-binding protein expression constructs, particularly *Vitreoscilla* hemoglobin, may exhibit improved vigorous growth characteristics, increased levels of chlorophyll, and of secondary plant metabolites, particularly alkaloids.

Engineered plants and plant lines possessing such improved agronomic characteristics may be identified by examining any of following parameters: 1) the rate of growth, measured in terms of rate of increase in fresh or dry weight, or rate of increase in height; 2) vegetative yield of the mature plant, in terms of fresh or dry weight; 3) the rate of germination; 4) the level of chlorophyll content; 5) the level of a secondary metabolite of interest, such as nicotine, scopolamine, hyoscyamine; and 6) the color of the plant. The procedures and methods for examining these parameters are well known to those skilled in the art.

According to the present invention, a desired plant is one that exhibits improvement over the control plant (*i.e.*, progenitor plant) in one or more of the aforementioned parameters. In an embodiment, a desired plant is one that shows at least 5% increase over the control plant in at least one parameter. In a preferred embodiment, a desired plant is one that shows at least 20% increase over the

control plant in at least one parameter. Most preferred is a plant that shows at least 50% increase in at least one parameter.

6. EXAMPLE: EXPRESSION OF VITREOSCILLA HEMOGLOBIN IN TOBACCO

An extensively investigated plant alkaloid is nicotine, the principal alkaloid found in tobacco (*Nicotiana tabaccum*). The biosynthetic pathways for the tobacco alkaloids have become accepted but regulation of this pathway is not yet fully understood, and all of its enzymes are not characterized (Bush, L.P., 1981, Recent Adv. Tob. Sci. 7:75-106). However, the activity of the final enzyme in nicotine biosynthesis, nicotine synthase, which catalyzes the formation of (s)-nicotine from nicotinic acid and N-methyl-Δ'-pyrrolinium chloride, has proven to be oxygen-dependent (Figure 1) (Friesen, J.B. and Leete, E., 1990, Tetrahedron Lett. 31:6295-6298), and the competing pathway from nicotinic acid and N-methyl-Δ'-pyrrolinium chloride to anabasine is not, so far as is known, stimulated by oxygen. Thus, this system was chosen as a first experiment to investigate the effects of increasing oxygen availability by providing oxygen-binding proteins to the plant. In this example, tobacco was transformed with a VHb expression vector via *Agrobacterium* mediated gene transfer. Subsequent assays were performed to determine: (i) if the VHb gene was correctly transcribed and translated in tobacco, (ii) the response of nicotine and anabasine accumulation to VHb expression, and, based on other features observed for the VHb-expressing transgenic plants, (iii) chlorophyll content and growth rate of VHb containing plants.

6.1. Experimental protocol

Recombinant DNA techniques and bacterial strains

Plasmid procedures were performed according to the methods described in Sambrook, J. *et al.*, 1990, Molecular cloning. A laboratory manual, Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, and PCR was performed using standard protocols (Saiki, *supra*). *Escherichia coli* strain TGI (F *'traD36lacI9Δ[lacZ]M15proA+B+/supE[hsdM-mcrB]5[r_K-m_K-McrB-]thi[lac-proAB][lac-prof*) was cultivated and transformed, using standard techniques (Sambrook, J., *supra*). Restriction enzymes were used according to the suppliers'

recommendations (GIBCO BRL, Labdesign). *Agrobacterium tumefaciens* strain LBA 4404 (Walden, R. *et al.*, 1990, MMCB 1:175-194) was cultivated in LB medium (tryptone 10 g/l, yeast extract 5g/l, NaCl 10 g/l) and transformed with the aid of *E.coli* HB 101, harboring conjugative plasmid pRK 2013 (Figurski, D.H. and Helinski, D.R., 1979, Proc. Natl. Acad. Sci. U.S.A. 76:1648-1652). Extraction of genomic tobacco DNA for PCR analysis was performed as described by Edwards, K. *et al.*, 1991, Nucleic Acids Res. 19:1349).

DNA-constructions

PCR was used to amplify the VHb gene from plasmid pINT1 (Khosla, C. and Bailey J.E., 1989, J. Mol. Biol. 210:79-90). The 5' primer H1 (with sequence 5'- GCA AAC AGG ATC CCG CGT CTA GAG G 3') was complementary to a region upstream of the transcription initiation codon containing several restriction enzyme sites, the 3' primer H2 (with sequence 5'- GCA GAT TTG TAC GCT CAA GCG GTT GAA TGA GCT CCC AAA G - 3') was complementary to the coding region of the VHb gene upstream of the stop codon; primers were synthesized at the Biomolecular Unit, Lund University. The stop codon was also altered, using PCR mediated mutagenesis, from TAA to TGA, which is a more frequently used stop codon in tobacco. The amplified PCR fragment was inserted into pUC19 which was subsequently transformed into *E. coli*. Bacteria expressing the VHb gene exhibit a red tint when pelleted. One of the best expressing *E. coli* clones, pUH4, was chosen for further experiments. To verify that the cloned PCR fragment of the VHb gene expressed a functional protein, difference spectrum analysis was used (Webster, D.A. and Liu, C.Y., 1974, J. Biol. Chem. 249:4257-4260). Analysis of the CO spectrum of recombinant *E. coli* harboring pUH4 revealed a pronounced peak at 419 nm which is characteristic for the VHb activity (Lilius, *supra*). The VHb gene fragment was cleaved out with *XbaI* and *SacI* and ligated into plasmid pBin121:2 (Jefferson, R.A., 1987, Plant. Mol. Biol. Rep. 5:387-405) giving pBH4 (Figure 2).

Plant material

Nicotiana tabaccum SR. plants were exposed to 16 h light at 25°C and were either kept in soil or in sterile jars on MS-media (Murashige, T. and Skoog, F., 1962, Physiol. Plant 15:473-497). The tobacco plants were transformed by *Agrobacterium*-mediated gene transfer as described by Rogers, S.G. and Fraley,

R.T., 1986, Methods Enzymol. 118:627-684. Stable heterozygous transformants of the F_1 generation were used in all plant experiments.

Transcription analysis using RT-PCR

Polyadenylated mRNA was isolated from 100 mg leaf material using oligo(dT)-cellulose as in the Pharmacia Quick Prep™ manual. The VHb gene transcript was amplified by *rTth DNA polymerase* and the primers according to the instructions given by Perkin Elmer.

Western immunoblot analysis

Protein extracts of two transgenic tobacco lines and a wild-type control were prepared as follows: One gram leaf and stem material was homogenized in 0.1 M sodium phosphate, 5 mM DTT and 5 mM EDTA buffer at pH 7.5. The homogenates were centrifuged in 1.5 mL test tubes at 12,000 rpm for 15 min; the protein-containing supernatants were decanted and used for further studies. The proteins were separated on 15 % SDS-PAGE gels and blotted onto Immobilon P nylon membranes supplied by Millipore. The electrophoreses and blotting were performed as described by Laemmli (Laemmli, U.K., 1970, Nature 277:680-685) and Sambrook, *supra*, respectively. The VHb monomers were detected by VHb antiserum generated in rabbit, and visualized by peroxidase conjugated swine anti-rabbit immunoglobulins (Dako).

Alkaloid analysis

Transgenic tobacco and wild-type plants were analyzed for their nicotine and anabasine levels. The plants were grown for 30 days on MS medium, then harvested and homogenized in approximately 40 mL 0.1 M Tris-HCl buffer pH 9.0, centrifuged and stored at -70°C. The nicotine and anabasine concentrations were determined using capillary column gas chromatography as described by Curvall *et al.*, 1982, J. Chrom. 232:283-293.

Chlorophyll assay

Leaf material from three-week old seedlings was homogenized in 1.5 ml tubes and the chlorophyll was extracted with ice-cold 80% acetone. The debris was removed and the supernatants were collected. The absorbance of the chlorophyll extracts were monitored at 645 and 633 nm. The absorbencies were normalized to the amount of plant material used in each extraction. The amounts

of chlorophyll-A and B were calculated as described by Arnon, D.I., 1949, Plant Physiol. 24:1-15.

6.2. Results

Preparation of Transgenic Tobacco

A vector was constructed for expression of the VHb gene in plants (Figure 2). Genomic DNA from several tobacco transformants was purified and analyzed by PCR to determine if the VHb gene was inserted into the genome. Approximately 90% of the analyzed plants were positive. PolyA+ mRNA was isolated from plant leaves of two PCR-positive transgenic plant lines, He3 and He4, and a wild-type control line. Two polynucleotide primers, H1 and H2 (see Experimental protocol), were used in a reverse transcriptase PCR reaction to amplify and detect the mRNA transcript of the VHb gene. Both transgenic tobacco lines exhibited a full-length VHb mRNA transcript which corresponded well to the theoretical size of 460 base pairs.

Expression of VHb protein in tobacco could easily be detected by Western immunoblot analysis on leaf and stem protein extracts. A 15.7 kDa protein that cross-reacted with a VHb antiserum was present in both transgenic tobacco lines but absent in the wild-type control. The level of VHb expression using the 35S CaMV promoter of pBi121:2 is approximately 0.1 % of total protein. However, the presence of several cytochromes in tobacco precluded the use of the CO binding assay for VHb activity (Saiki, *et al.*, 1988, Science 239:487-491).

Improved overall growth in transgenic tobacco

Transgenic tobacco seeds of two transgenic lines, He3 and He4, from the F1 generation and a wild-type control were germinated in soil and their development was followed visually and by harvesting plants at regular intervals. Germination was markedly accelerated in plants transformed with VHb-expression constructs, particularly in the transgenic line He4, over that in non-transformed plants. Analysis showed that the germination time for the transformed plants was approximately 3-4 days compared to 6-8 days for wild-type tobacco. Additionally, the growth rates and final yields were higher in the transgenic plants (Figure 3). For instance, the dried weights were enhanced by 80 to 100% after 35 days of

growth. Furthermore, the period between germination and flowering of the transgenic plants was reduced by 3-5 days compared to wild-type.

Alkaloid analysis of the VHb-expressing transgenic tobacco Extracts of two VHb-expressing transgenic tobacco plant lines, He3 and He4, and a wild-type control were analyzed for nicotine and anabasine content by gas chromatography (Figure 4). Compared with the wild-type, the transgenic plaints (He3) were holding on average 34% higher ratio of nicotine corresponding to 108 μg/g dry weight. At the same time, the anabasine levels decreased substantially in the VHb-expressing transgenic plants. The highest yielding individual transgenic tobacco plant had 130 μg/g dry weight of nicotine which is very similar to the highest nicotine content in tobacco given in the literature (Sheen, S.J., 1988, J. Food Sci. 53:1572-1573), 140 μg/g dry weight.

Chlorophyll content of VHb-expressing transgenic tobacco

Visual observation of VHb-expressing tobacco transgenes indicated that many of these plants appeared more green than wild-type control plants. As depicted in Figure 1B, several steps in chlorophyll and heme biosynthesis are oxygen dependent. Therefore, chlorophyll and growth rate assays were conducted on several Fl generation plants obtained from wild-type tobacco and from the VHb-expressing clones designated He3 and He4. Assays of chlorophyll content showed small but significant increases in chlorophyll content particularly in the He4 plants (Figure 5). These plants also grew about 20-40% faster than the wild-type as determined by height. Differences in growth rate between transgenic and wild-type plants were not observed when these plants were grown in an atmosphere with reduced oxygen.

6.3. Discussion

Engineering VHb expression in tobacco plants influenced nicotine (and anabasine) production, but indications of more global effects of VHb expression began with the observation that young transgenic plants exhibit a darker green shade of color than do wild-type plants. Subsequent measurements of chlorophyll levels revealed 10-20% increased levels in the He3 plants which also corresponded 20-40% increased growth rates as determined by plant heights. Cloning of VHb in tobacco affected the He3 and He4 plants somewhat differently. Enhanced levels of

nicotine and chlorophyll were associated with He3 and He4 plants, respectively. Such differences among clones are common, particularly when using integrating vectors.

One hypothesis to explain this dramatic shift in nicotine and anabasine production in VHb-expressing transgenic tobacco plants, is that the presence of VHb increases the oxygen availability, which in turn significantly affects distribution of secondary metabolite production in the whole tobacco plant. Alternatively, these shifts in secondary metabolite production in VHb-expressing transgenes may result from other metabolic effects of VHb such as an increase in the ATP synthesis rate and/or membrane energy. Availability of more energy in the cell might lead to the improvement of many metabolic functions including increased nicotine production.

7. EXAMPLE: EXPRESSION OF VITREOSCILLA HEMOGLOBIN IN DATURA

Hyoscyamine and scopolamine are tropane alkaloids produced by plants; hyoscyamine is the levorotatory component of racemic atropine. Atropine, hyoscyamine, and scopolamine affect the parasympathetic nervous system and exhibit a wide range of pharmaceutical activity. In medicine they are used to relieve Parkinson-ism, dilate pupils, increase the heart rate, counteract toxic agents, and reduce secretions such as sweat. Scopolamine produces fewer undesirable side effects than hyoscyamine and is the preferred substance for treatment of motion sickness and production of derivative drugs for gastric disorders. Therefore, there has been a long-standing interest in raising the scopolamine level of producing plants. *Datura innoxia* a medicinal plant grown in many tropical and sub-tropical countries is one of the principal sources of tropane alkaloids. The biosynthesis of these alkaloids involves several oxygen-requiring steps.

7.1. Experimental Protocol

Recombinant DNA techniques, Transcription analysis and Western immunoblot analysis were performed as above for tobacco. The same transformation vector which was used for tobacco was also used for *Datura*.

Plant material

Plants of *Datura innoxia* Mill were exposed to 16 h light at 24°C and were kept in sterile jars on basal medium (BM) containing Murashige and Skoog's macro salts, and Nitsch and Nitsch micro salts and vitamins (Sangwan *et al.*, 1991, Plant Cell Rep. 10:90-93). The plants were transformed by *Agrobacterium*-mediated gene transfer using the leaf disc method as described by Sangwan *et al.* (1991). Transformants of the F_0 generation were used in this experiment.

Alkaloid analysis

Transgenic *Datura* and wild-type plants were analyzed for their scopolamine and hyoscyamine levels. The plants were grown in soil until they were budding, then the leaves were harvested and dried. The scopolamine and hyoscyamine levels were determined using HPLC as described by Gontier *et al.*, 1994, Agro-Food-Ind. Hi-Tech 5, 26-28.

7.2. Results

Several transgenic plants were positive for the VHb gene as determined by PCR of genomic DNA. PolyA$^+$ mRNA was isolated from plant leaves of six PCR-positive transgenic plants and two wild-type plants. All six transgenes exhibited a full-length VHb transcript. Preliminary results showed that the *Datura* plants transformed with the VHb expression construct also exhibited increased growth rates as compared to the wild-type plants.

Extracts from the six transgenic and two wild-type *Datura* plants were analyzed for scopolamine and hyoscyamine content by HPLC. Results are presented in the following table.

TABLE I

Transgenic plant No.	Production of Scopolamine mg/100 g D.W.	Production of Hyoscyamine mg/100 g D.W.
1	115	47
2	148	35
3	246	20
4	86	28
5	118	17
6	70	10
Control A	26	7.1
Control B	55	8.0

7.3. Discussion

Similarly to the results in tobacco, engineering *Datura* plants to express a VHb gene also resulted in transformed plants which both grew faster and contained altered levels of desired secondary metabolites. These results show that the expression of oxygen-binding proteins in whole plants, particularly proteins which reversibly bind oxygen via a heme group such as VHb, is generally applicable to obtain improved transformed plants.

8. EXAMPLE: PHENOTYPIC SCREEN FOR VITREOSCILLA HEMOGLOBIN MUTANTS

In order to create a library of randomly mutated VHbs, we used an earlier construct, pINT1 which carries the VHb gene as a template (Khosla and Bailey, 1989, *supra.*). The VHb sequence used as a template encoded the wild-type VHb protein with an additional 8 amino acid extension - MTMITPSF - at the amino-terminal end of the protein. However, all numbering conventions given herein correspond to the wild-type amino acid sequence positions as described in U.S. Patent No. 5,049,493 *supra.*. The VHb gene was amplified by low fidelity PCR with an estimated error rate of 2-4% (Leung *et al.*, 1989, Technique, 1, 11-15) using primers H1 and H2.

8.1. Experimental Protocol

PCR

Low fidelity PCR was performed with 1 mM dCTP, dGTP and dTTP, 0.2 mM dATP, 0.45 mM of each primer, 0.1 ng template, 1% 1 M mercaptoethanol, 10% DMSO, 0.5 mM $MnCl_2$ and 10 U Taq DNA polymerase. A temperature profile was employed of 2 min. at 95°C, hold at 70°C while the Taq DNA polymerase was added, 30 cycles of 15 sec. at 92°C, 30 sec. at 50°C, 1 min. at 72°C and an additional 5 cycles of 15 sec. at 92°C, 5 min. at 72°C. The amplified PCR fragments were inserted into pUC19 which was subsequently transformed into *E coli.* Sequencing of the mutated genes was performed on an automatic sequencer with the terminator chemistry.

Screening the library for colonies carrying mutated VHb

The plasmid library was transformed into *E. coli.* TG1 and selected on LB agar (Sambrook *et al.*) by visually assessing the color of the colonies. Expression of wild-type VHb results in a red colored colony. The red color phenotype is indicative of a VHb protein capable of binding oxygenated heme. Approximately 10,000 colonies were identified ranging in color from white to deep red.

Western immunoblot analysis

Overnight cultures were pelleted, resuspended in a sodium phosphate buffer (50 mM, pH 7.0) and sonicated to obtain protein extracts. The proteins were separated on 15% SDS-PAGE gels and blotted onto Immobilon P nylon membranes supplied by Millipore. The electrophoresis and blotting were performed as described by Laemmli (1970) and Sambrook *et al.* (1989), respectively. The VHb mutants were detected by VHb antiserum generated in rabbit, and visualized by peroxidase conjugated swine anti-rabbit immunoglobulins (Dako).

8.2. Results

Randomly picked colonies, which exhibited the correct size VHb (15.7 kDa) according to Western blot, were sequenced. The following altered VHb amino acid sequences were identified.

TABLE II.

ALTERED VHB PROTEINS OBTAINED THROUGH PCR MUTAGENESIS

ISOLATE	AMINO ACID ALTERATION
A	$Ile^{129}Thr$
B	$His^{36}Arg$
C	$Lys^{79}Asn$
D	$Phe^{33}Tyr$, $Gln^{53}Arg$
E	$Lys^{124}Glu$, $Phe^{133}Leu$, $Ile^{134}Thr$
F	$Gly^{21}Asp$, $Val^{136}Glu$, $Ala^{138}Thr$
G	$Leu^{51}Ser$, $Val^{83}Ala$, $Tyr^{126}His$
H	$Lys^{11}Glu$, $Lys^{107}Glu$, $Lys^{124}Stop$

8.3. Discussion

The PCR mutagenesis technique allowed the phenotypic selection of VHb mutants which retain at least some functional activity of the VHb, as evidenced by their ability to bind oxygenated heme. Although these mutants were generated in a VHb protein with an 8 amino acid amino-terminal extension, these mutants will be similarly effective in a VHb protein with the wild-type amino-terminus. Further, this approach will enable the easy selection and generation of any altered hemoglobin molecule through mutagenesis of the coding sequence, followed by selection for clones which encode heme containing proteins which retain functional activity.

9. EXAMPLE: TRANSFORMATION OF RICE WITH A VITREOSCILLA HEMOGLOBIN EXPRESSION CONSTRUCT

This example illustrates the transformation of rice using particle bombardment with a polynucleotide that directs the expression of Vitreoscilla hemoglobin in plant cells.

9.1 Experimental Protocol

Plasmid constructions

pJFK2 was constructed as a derivative of pUC that contains the *vhb* gene under the regulation of the CaMV 35S promoter and a nos polyadenylation

sequence. Additionally, the stop codon of the *vhb* gene was altered using PCR mediated mutagenesis from TAA to TGA (a more frequently used stop codon in plants). For the selection of transgenic rice tissue, the *aph*IV gene from *E.coli* which confers resistance to Hygromycin was included in the transformation construct. This marker gene was under the control of the CaMV 35S promoter and the CaMV polyadenylation sequence. A map of pJFK is shown in Figure 6.

The pJFK2 plasmid was digested with I-SceI, and the larger fragment containing *vhb* gene and hygromycin resistance gene (and lacking the ampicillin resistance gene) was isolated for use in posterior bombardment of plant embryos.

Rice transformation

Immature *Oryza sativa* embryos of the Japonica variety Taipei 309 were aseptically isolated 10-14 days after pollination from green-house-plants and plated scutulum site up on solid MS medium (Murashige and Skoog, 1962) containing 3% sucrose, 2 mg l^{-1} 2,4-D and 50 mg l^{-1} cefotaxime (MS1). After 4-6 days (28°C, darkness) embryos were transferred to solid MS medium containing 10% sucrose, 2 mg l^{-1} 2,4-D and 50 mg l^{-1} cefotaxime (MS2) and subjected within 1 hour to microprojectile bombardment with a particle inflow gun. The DNA fragment containing *vhb* and *aph* IV genes (5μg) was precipitated on 1-3 μm gold particles (Aldrich) as described by Vain et al., 1993, Plant Cell Rep. 12:84-88. Gold particles (400 μg per bombardment) were accelerated to the target with a particle inflow gun (Finer et al., 1992, Plant Cell Rep. 11:323-328) at a pressure of 6 bar. Embryos were placed 16 cm below the syringe filter. Twenty-four hours post-bombardment embryos were subjected to selection on a solid selection media (MS1 medium containing 20 mg l^{-1} hygromycin B) and incubated at 28°C in the dark.

After 1 week embryos were transferred to a liquid selection media, R2 medium (Ohira et al., 1973, Plant Cell Physiol. 14:113-121) supplemented with: 3% sucrose, 1 mg l^{-1} thiamine, 1 mg l^{-1} 2,4-D, 50 mg l^{-1} cefotaxime and 20 mg l^{-1} hygromycin B. The embryos were incubated with shaking at 28°C in the dark and subcultured weekly. Developing calli were isolated 3 to 6 weeks later, and transferred to a callus increasing media (R2 medium supplemented with: 6% sucrose, MS vitamins, 100 mg l^{-1} inositol, 2 mg l^{-1} 2,4-D, 50 mg l^{-1} cefotaxime and 20 mg l^{-1} hygromycin B). The calli were incubated in this media at 28°C in the dark and subcultured weekly.

Resistant calli were transferred to solid R2 regeneration medium supplemented with 2% sucrose, 3% sorbitol, 20 mg l^{-1} Hygromycin B, 1 mg l^{-1} of zeatin, 0.5 mg l^{-1} IAA, MS vitamins and 0.65% agarose. The callus tissue was maintained at 28°C with 12 h of light in order to enhance shoot formation. The calli were then subcultured every 3 weeks until shoots had reached a length of 2-3 cm. They were transferred to half-strength MS rooting medium without hormones, supplemented with 1.5% sucrose and 0.3% gelride®. After 2-4 weeks of cultivation, plantlets were transferred directly to the greenhouse and planted in soil. Plantlets were grown in 7 liter aquaculture pots with fertilizer enriched earth, 3 plants per pot, (day: 12 h, 28°C, 80% humidity; night: 12h, 21°C, 60% humidity) until they flowered and set seeds.

Analysis of transgenic rice plants

To check for presence of the transgene, complexity of insertion(s) and number of copies present, Southern blot analysis was performed as described previously (Burkhardt et al., 1997). A PCR-amplified, DIG-labeled (Boehringer), 300-bp fragment of the coding region of *vhb* was used as a probe.

Transgene expression is confirmed using western immunoblots as described above and in the literature (Wünn et al., 1996, Bio/Technology 14:171-176).

9.2 Results

Transformed plants were analyzed by Southern blot and were found to carry the transgene. Western blot is performed and demonstrates that the transformed plants express the *vhb* transgene. Seeds from the transgenic R0 plants are collected and R1 plants are grown. Growth of the R1 generation plants, as compared to control plants without the transgene, is more rapid.

Although the invention is described in detail with reference to specific embodiments thereof, it will be understood that variations which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings such modifications are intended to fall within the scope of the appended claims. Various publications are cited herein, each of the disclosures of which is incorporated by reference in its entirety.

WHAT IS CLAIMED IS:

1. A plant which has been genetically engineered by transformation with a polynucleotide encoding a globin protein.

2. The plant of claim 1, wherein the globin protein is selected from the group consisting of *Vitreoscilla* hemoglobin, horse heart myoglobin, E. coli hemoprotein, B. subtilis hemoprotein, yeast flavohemoglobin, soybean leghemoglobin, lupin leghemoglobin, and sperm whale myoglobin, or their functional equivalents.

3. The plant of claim 1 or 2, wherein the polynucleotide encoding the globin protein is operably linked to a plant promoter.

4. The plant of claim 3, wherein the plant promoter is a strong, constitutively expressed plant promoter.

5. The plant of claim 4, wherein said strong, constitutively expressed plant promoter is a CaMV 35S promoter.

6. The plant of claim 3, wherein the plant promoter is a tissue specific plant promoter.

7. The plant of any one of claims 1-6, wherein the polynucleotide is introduced into a plant cell via microprojectile bombardment.

8. The plant of any one of claims 1-6, wherein the polynucleotide is introduced into a plant cell on a Ti plasmid.

9. The plant of any one of claims 1-8, wherein the plant is a dicotyledon.

10. The plant of claim 9, wherein the plant is from the genus *Nicotiana*.

11. The plant of claim 10, wherein the plant is *Nicotiana tabaccum*.

12. The plant of claim 9, wherein the plant is from the genus *Datura*.

13. The plant of any one of claims 1-8, wherein the plant is a monocotyledon.

14. The plant of claim 13, wherein the plant is from the genus *Oryza*.

15. A method of producing a plant with an improved agronomic or medicinal characteristic comprising transforming the plant with a polynucleotide encoding a globin protein, wherein the transformed plant exhibits an increased rate of growth, an increase in vegetative yield of the mature plant, an increased rate of germination, and/or an increased chlorophyll content, relative to a non-transformed plant.

16. The method of claim 15, wherein the globin protein is selected from the group consisting of *Vitreoscilla* hemoglobin, horse heart myoglobin, E. coli hemoprotein, B. subtilis hemoprotein, yeast flavohemoglobin, soybean leghemoglobin, lupin leghemoglobin, and sperm whale myoglobin, or their functional equivalents.

17. The method of claim 15 or 16, wherein the polynucleotide encoding the globin protein is operably linked to a plant promoter.

18. The method of claim 17, wherein the plant promoter is a strong, constitutively expressed plant promoter.

19. The method of claim 18, wherein the strong, constitutively expressed plant promoter is a CaMV 35S promoter.

20. The method of claim 17, wherein the plant promoter is a tissue specific plant promoter.

21. The method of any one of claims 15-20, wherein the polynucleotide is introduced into a plant cell via microprojectile bombardment.

22. The method of any one of claims 15-20, wherein the polynucleotide is transformed into a plant cell on a Ti plasmid.

23. The method of any one of claims 15-22, wherein the plant is a dicotyledon.

24. The method of claim 23, wherein the plant is from the genus *Nicotiana*.

25. The method of claim 24, wherein the plant is *Nicotiana tabaccum*.

26. The method of claim 25, wherein the plant further exhibits an elevated level of nicotine.

27. The method of claim 23, wherein the plant is from the genus *Datura*.

28. The method of claim 27, wherein the plant further exhibits an elevated level of scopolamine and/or hyoscyamine.

29. The method of claim 17-22, wherein the plant is a monocotyledon.

30. The plant of claim 29, wherein the plant is from the genus *Oryza*.

31. A plant produced by the method of claim 17.

32. A method of altering the distribution of secondary metabolites in a plant, comprising transforming a target plant or target plant cell with a polynucleotide encoding a globin protein operably linked to a plant promoter.

33. The method of claim 32, wherein the plant is from the genus *Nicotiana* and the distribution of nicotine is altered relative to anabasine.

34. The method of claim 32, wherein the plant is from the genus *Datura* and the distribution of scopolamine and/or hyoscyamine is altered.

35. A mutant *Vitreoscilla* hemoglobin protein, wherein the mutant *Vitreoscilla* hemoglobin protein differs from the wild type *Vitreoscilla* hemoglobin protein by having an amino acid substitution selected from the group consisting of: $Ile^{129}Thr$; $His^{36}Arg$; $Lys^{79}Asn$; $Phe^{33}Tyr$, $Gln^{53}Arg$; $Lys^{124}Glu$, $Phe^{133}Leu$, $Ile^{134}Thr$; $Gly^{21}Asp$, $Val^{136}Glu$, $Ala^{138}Thr$; $Leu^{51}Ser$, $Val^{83}Ala$, $Tyr^{126}His$; and $Lys^{11}Glu$, $Lys^{107}Glu$, $Lys^{124}Stop$.

36. The mutant *Vitreoscilla* hemoglobin protein of Claim 35 which further comprises an amino-terminal amino acid extension having the sequence MetThrMetIleThrProSerPhe.

37. A polynucleotide that encodes the mutant *Vitreoscilla* hemoglobin protein of Claim 35 or 36.

38. A host cell that contains the polynucleotide of Claim 37.

39. The host cell of Claim 38, wherein the host cell is selected from the group consisting of a plant cell, a bacterial cell, an animal cell, a fungal cell and an algae cell.

40. A method of generating a polynucleotide encoding mutant *Vitreoscilla* hemoglobin protein comprising:

amplifying a polynucleotide that encodes *Vitreoscilla* hemoglobin protein using a low fidelity polymerase chain reaction;

transforming the amplified polynucleotides into bacteria; and

selecting bacteria that contain a polynucleotide sequence encoding a mutant *Vitreoscilla* hemoglobin protein.

41. The method of Claim 40, wherein said selecting step comprises assaying the color of the bacterial cells.

42. A polynucleotide that encodes a mutant *Vitreoscilla* hemoglobin protein isolated by the method of Claim 40 or Claim 41.

43. A mutant *Vitreoscilla* hemoglobin protein encoded by the polynucleotide of Claim 42.

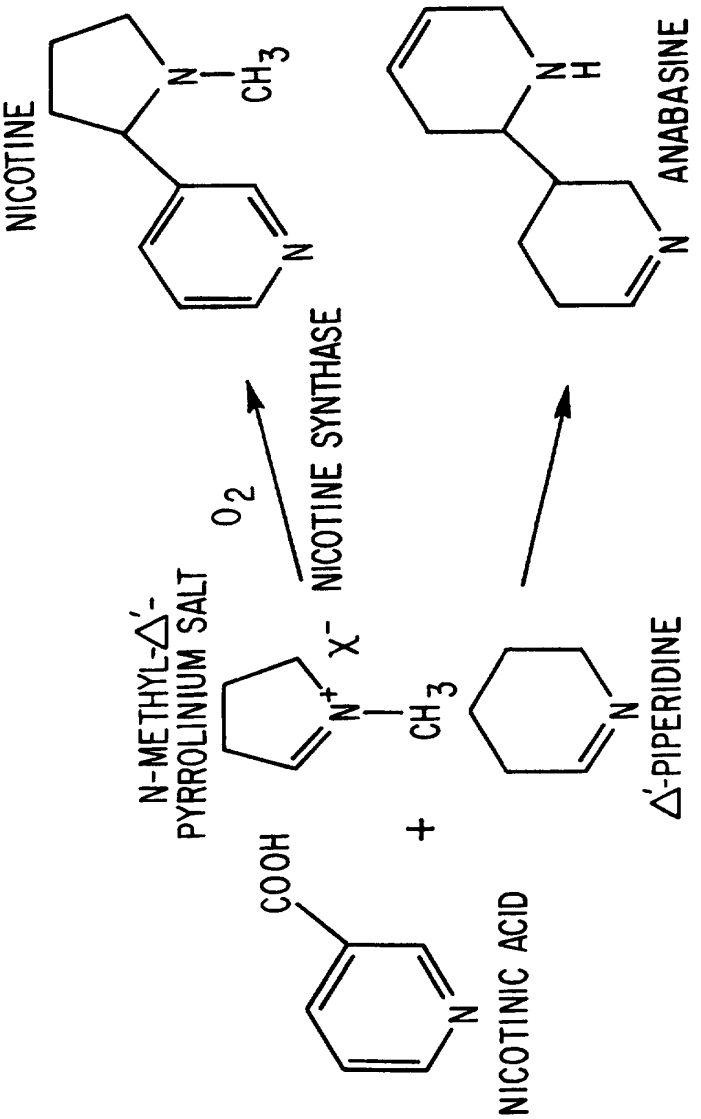

NICOTINE
ANABASINE
N-METHYL-Δ'-PYRROLINIUM SALT
O_2
NICOTINE SYNTHASE
X^-
Δ'-PIPERIDINE
NICOTINIC ACID
COOH

FIG. 1A

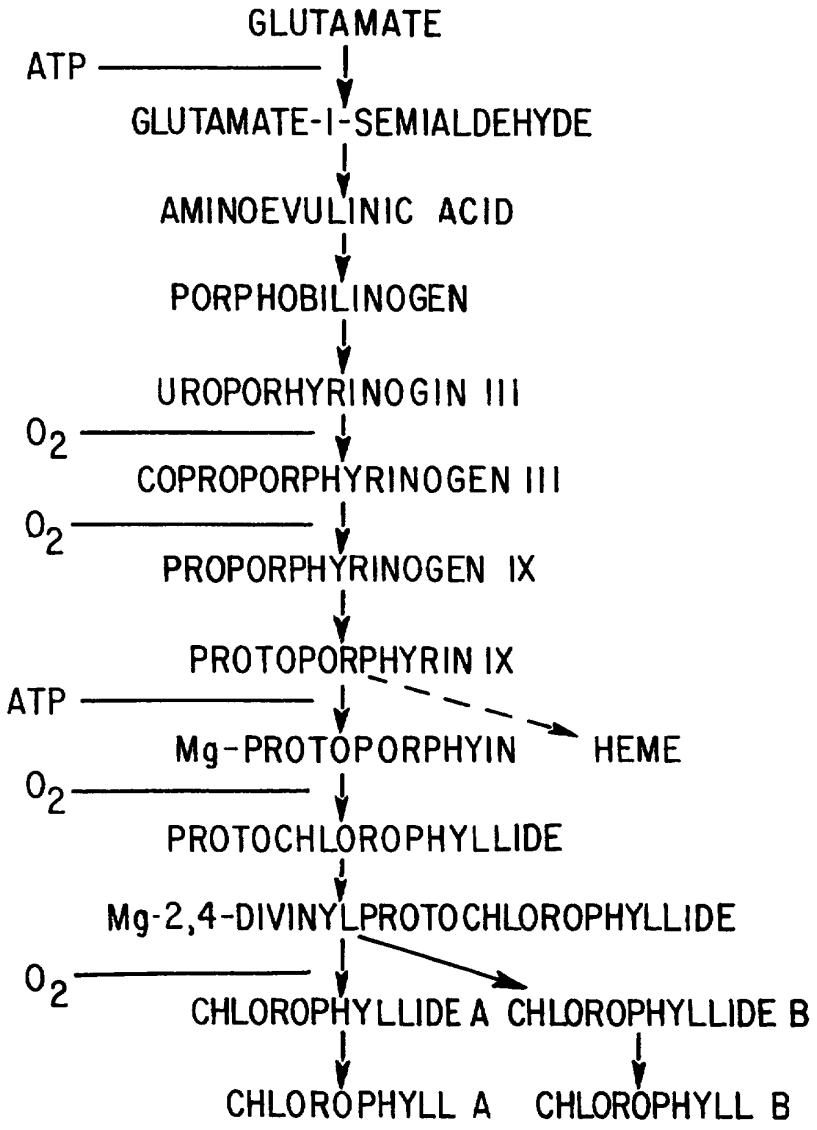

GLUTAMATE
ATP
GLUTAMATE-1-SEMIALDEHYDE
AMINOEVULINIC ACID
PORPHOBILINOGEN
UROPORHYRINOGIN III
O_2
COPROPORPHYRINOGEN III
O_2
PROPORPHYRINOGEN IX
PROTOPORPHYRIN IX
ATP
Mg-PROTOPORPHYIN
HEME
O_2
PROTOCHLOROPHYLLIDE
Mg-2,4-DIVINYLPROTOCHLOROPHYLLIDE
O_2
CHLOROPHYLLIDE A
CHLOROPHYLLIDE B
CHLOROPHYLL A
CHLOROPHYLL B

FIG. 1B

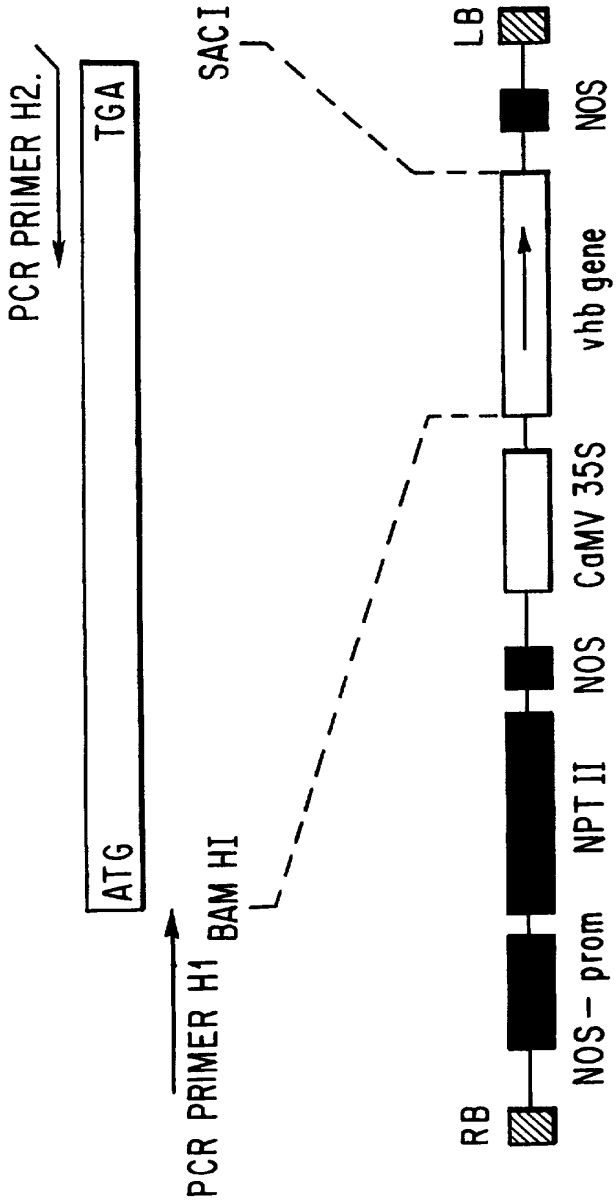

PCR PRIMER H2.
TGA
ATG
SACI
BAM HI
PCR PRIMER H1
LB
NOS
vhb gene
CaMV 35S
NOS
NPT II
NOS- prom
RB

FIG. 2

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U.S. Patent No. 10,689,656

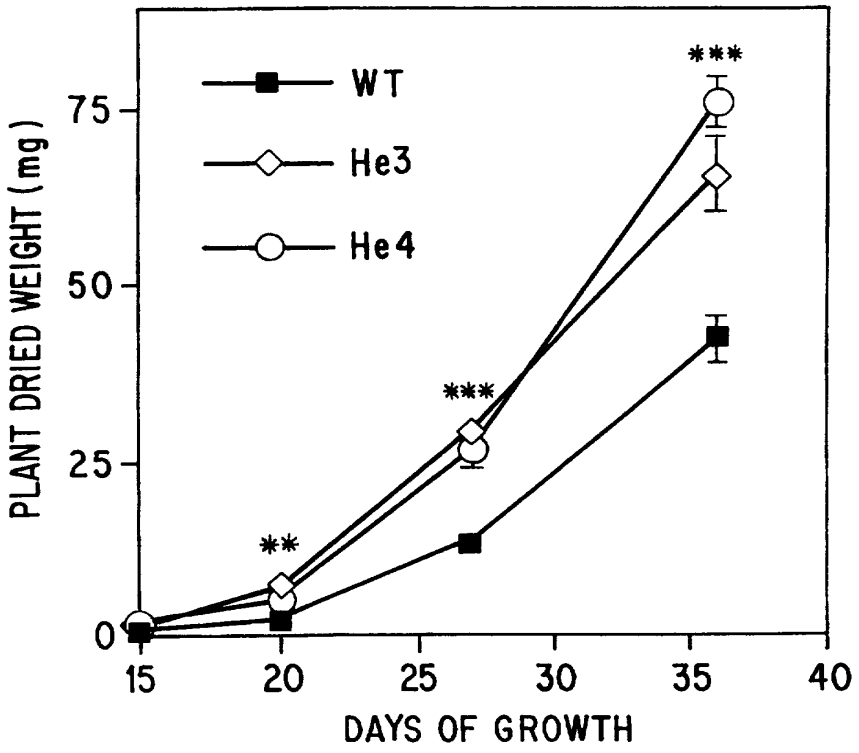


FIG. 3

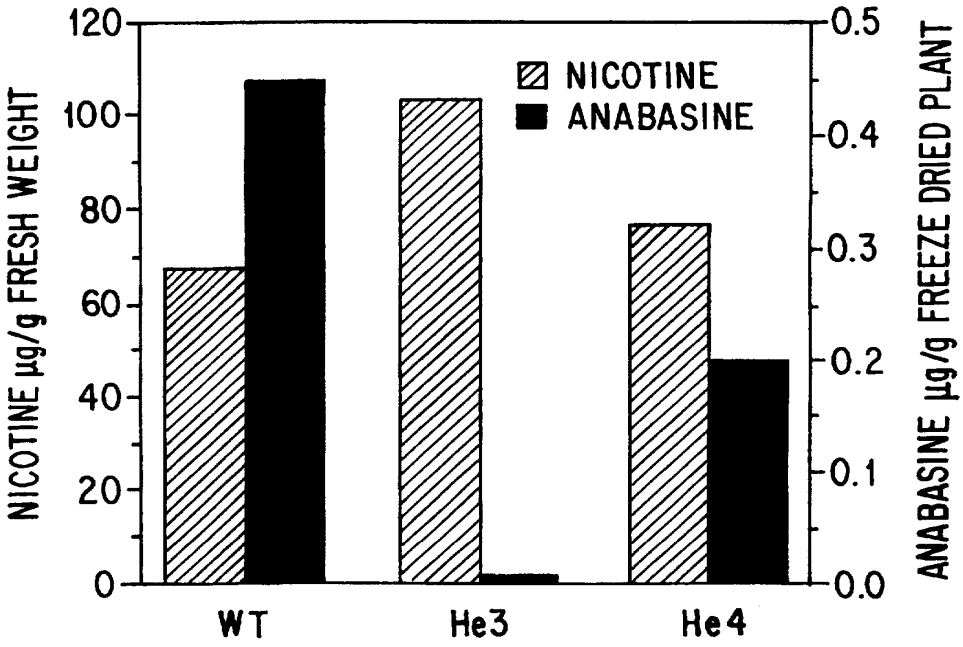


FIG. 4

Motif Exhibit 1040, Page 295 of 1537

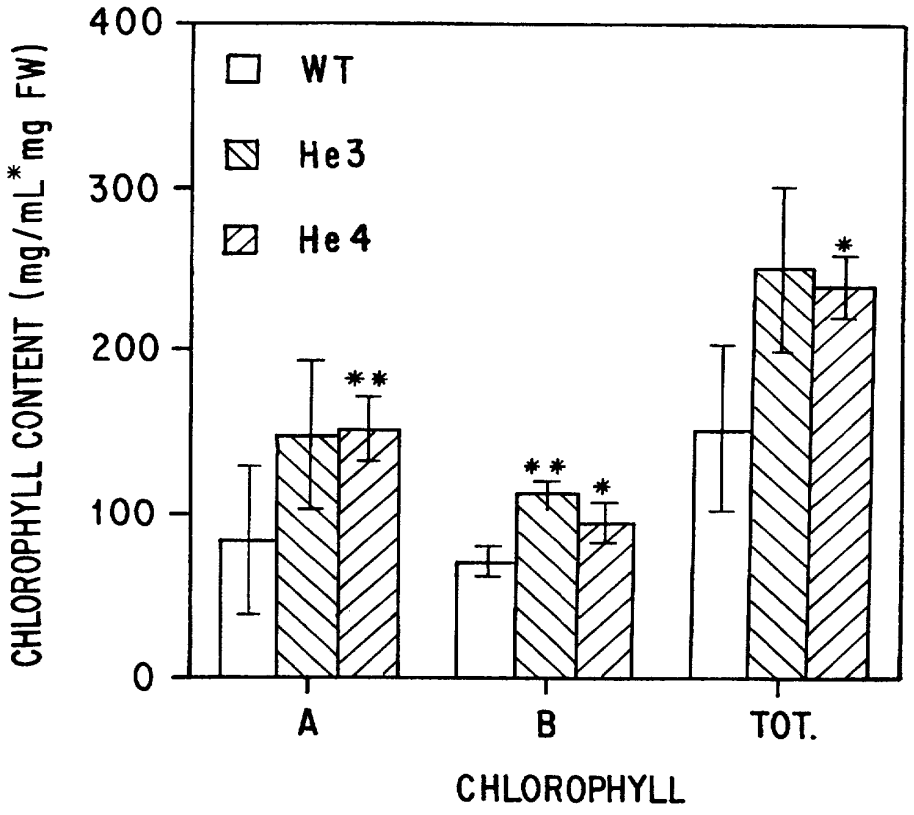

400
300
200
100
0
CHLOROPHYLL CONTENT (mg/mL*mg FW)
WT
He3
He4
**
**
*
*
A
B
TOT.
CHLOROPHYLL

FIG. 5

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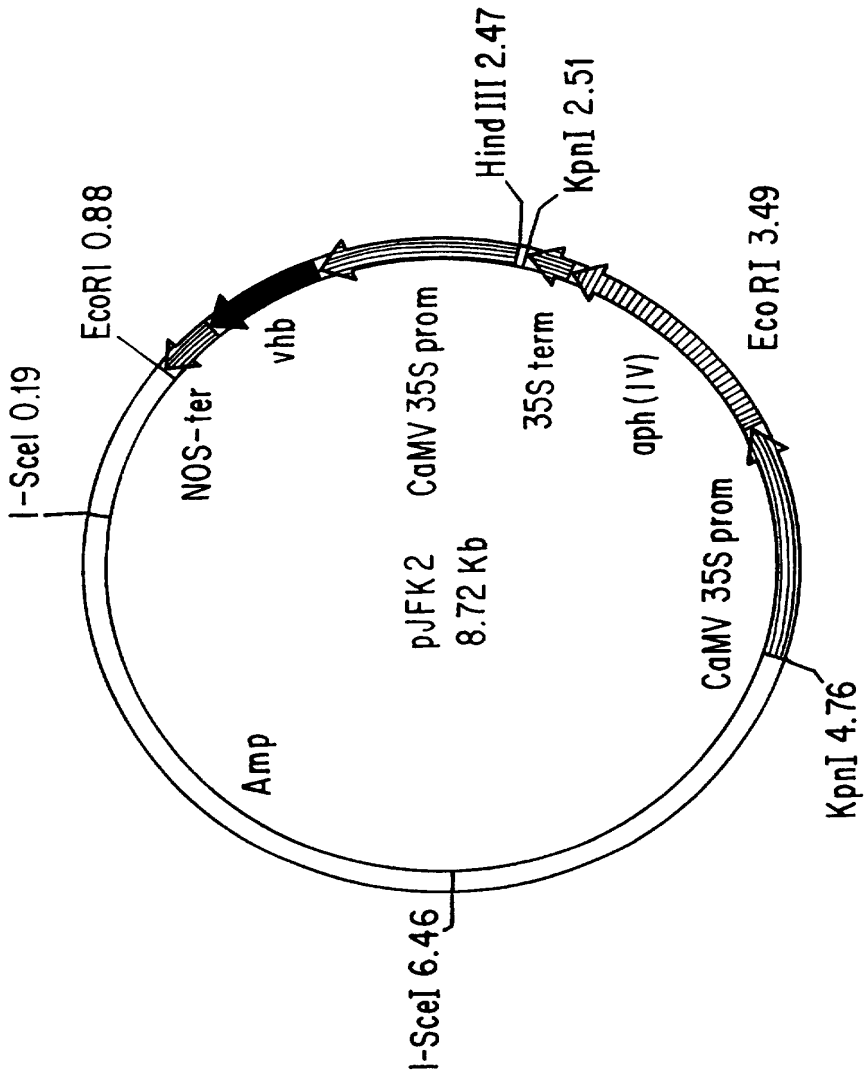


FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17246

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01H 5/00; C12N 5/14, 15/31, 15/82
US CL :435/172.3, 320.1, 419; 536/23.7; 800/205, DIG40, DIG43, DIG57
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)

U.S. : 435/172.3, 320.1, 419; 536/23.7; 800/205, DIG40, DIG43, DIG57

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 practicable, search terms used)

APS, STN, BIOSIS, EMBASE, CA, WPIDS
oygen binding, hemoglobin, plant, transgenic, transformation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DE ZOETEN et al. The Expression, Localization, and Effect of a Human Interferon in Plants. Virology. 1989, Vol. 172, pages 213-222, see entire article.	1-6, 1-12, 14-20, 24-28, 31-34
Y	KHOSLA et al. Heterologous Expression of a Bacterial Haemoglobin Improves the Growth Properties of Recombinant Escherichia coli. Nature. 18 February 1988, Vol. 331, pages 633-635, see entire article.	1-6, 10-12, 14-20, 24-28, 31-34
Y	BOGUSZ et al. Nonlegume Hemoglobin Genes Retain Organ-Specific Expression in Heterologous Transgenic Plants. The Plant Cell. July 1990, Vol. 2, pages 633-641, see abstract.	1-6, 10-12, 14-20, 24-28, 31-34

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"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
"A"	document defining the general state of the art which is not considered to be of particular relevance	"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
"E"	earlier document published on or after the international filing date	"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
"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)	"&"	document member of the same patent family
"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		

Date of the actual completion of the international search 28 OCTOBER 1997	Date of mailing of the international search report 12 DEC 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer AMY NELSON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17246

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 7, 8, 9, 13, 21-23, 29-30
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-6, 10-12, 14-20, 24-28, 31-34

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17246

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claim(s)1-6, 10-12, 14-20, 24-28, 31-34, drawn to method of producing transgenic plants comprising a globin gene, and transgenic plants thereby obtained.
Group II, claim(s) 35-36 and 43, drawn to mutant hemoglobin protein.
Group III, claim(s) 37-42, drawn to method of making mutant hemoglobin gene and mutant hemoglobin gene thereby obtained.

The inventions listed as Groups I, II and III do not relate to a single 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:

Group I is drawn toward wild type globin genes and Group III is drawn toward mutant globin genes and hence they lack a special technical feature.

Both Group III encompasses many different mutant genes and hence the mutant genes of Group III are not linked by a special technical feature to the proteins of Group II.

DNA and proteins have completely different structures, compositions and functions, and mutant DNA molecules and proteins differ structurally and functionally from wild type DNA and proteins. Hence, the inventions of Group I, II and III are not so linked as to form a single inventive concept under PCT Rule 13.1.

(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

(19) Weltorganisation für geistiges Eigentum
Internationales Büro

(43) Internationales Veröffentlichungsdatum
15. Juli 2004 (15.07.2004)

PCT

(10) Internationale Veröffentlichungsnummer
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(51) Internationale Patentklassifikation[7]: A01H 1/00

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23. Dezember 2003 (23.12.2003)

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(81) Bestimmungsstaaten *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Bestimmungsstaaten *(regional)*: ARIPO Patent (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Veröffentlicht:
— *ohne internationalen Recherchenbericht und erneut zu veröffentlichen nach Erhalt des Berichts*

Zur Erklärung der Zweibuchstaben-Codes und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

WO 2004/057946 A2

(54) Title: METHOD FOR ALTERING THE CONTENT OF RESERVE SUBSTANCES IN PLANTS

(54) Bezeichnung: VERFAHREN ZUR VERÄNDERUNG DES GEHALTS VON SPEICHERSTOFFEN IN PFLANZEN

(57) Abstract: The invention relates to a method for altering the content of reserve substances in plants during which leghemoglobin and/or hemoglobin expressing transformed plants are used. The invention also relates to corresponding plants and to the use thereof.

(57) Zusammenfassung: Die Erfindung betrifft ein Verfahren zur Veränderung des Gehalts von Speicherstoffen in Pflanzen, wobei Leghemoglobin und/oder Hemoglobin exprimierende transformierte Pflanzen eingesetzt werden, entsprechende Pflanzen sowie deren Verwendung.

Verfahren zur Veränderung des Gehalts von Speicherstoffen in Pflanzen

Die Erfindung betrifft ein Verfahren zur Veränderung des Gehalts von Speicherstoffen in Pflanzen durch Einsatz von Leghemoglobin und/oder Hemoglobin exprimierender transformierter Pflanzen, entsprechende Pflanzen sowie deren Verwendung.

Speicherstoffe in Pflanzen dienen als Reservestoffe und werden von pflanzlichen Geweben gebildet und intrazellulär abgelagert. Als Speicherstoffe dienen z. B. Polysaccharide (Kohlenhydrate wie Lichenin, Stärke, Glykogen, Polyfructosane), Proteine, Fette, auch Polyphosphate und Polyhydroxyalkanoate. Die Speicherstoffe können bei Bedarf wieder in den Stoff- und Energiewechsel eingeschleust werden, beispielsweise bei Nahrungsmangel, der Keimung von Samen, dem Wachstum und sonstigen energieverbrauchenden Vorgängen.

Speicherstoffe sind wertvolle Rohstoffe für die Ernährung (Getreide, Nüsse, Früchte, Obst, Gewürze etc.) und bilden die Basis vieler menschlicher Nahrungsmittel und können auch als Lieferanten für technische Fette und Öle dienen. Andere Speicherstoffe werden wegen ihrer pharmakologisch wirksamen Inhaltsstoffe arzneilich genutzt. (Quelle: CD Römpp Chemie Lexikon – Version 1.0, Stuttgart/New York: Georg Thieme Verlag 1995).

Auch werden in zunehmendem Maße die Speicherstoffe als nachwachsende und ökologisch verträgliche Rohstoffe für die Industrie angewendet, wie dies z. B. durch die Verwendung von Stärke zur Herstellung von Verpackungsmaterialien oder z. B. von Pflanzenölen als Kraftstoffe, wie Biodiesel oder Schmierstoffe möglich ist.