| | | | | | |
|---|---|---|---|---|---|
| 51 | Non Patent Literature | UniprotKB_P02236.pdf | 327004<br>ab00618946067fa01a116daf620036595c465473 | no | 9 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 52 | Non Patent Literature | Wu_1993.pdf | 738633<br>0b98dbb7a130135c219aca9469298d519f816374 | no | 1 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 53 | Non Patent Literature | Xie_Yang_2013.pdf | 9459123<br>992060b721155591cc1153780780d779df2addda | no | 9 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 54 | Non Patent Literature | Zhang_2013.pdf | 10261001<br>7d01444ac2d66d08111e76e9468061aec39b0285 | no | 8 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| | | | **Total Files Size (in bytes):** | 250816860 | |

**This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.**

**New Applications Under 35 U.S.C. 111**
**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**
**National Stage of an International Application under 35 U.S.C. 371**
**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**
**New International Application Filed with the USPTO as a Receiving Office**
**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
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 ACCEPTANCE LETTER**

*OC000000099235613*

Date Mailed: 05/03/2018

## NOTICE OF ACCEPTANCE OF APPLICATION UNDER 35 U.S.C 371 AND 37 CFR 1.495

The applicant is hereby advised that the United States Patent and Trademark Office, in its capacity as a Designated / Elected Office (37 CFR 1.495), has ACCEPTED the above identified international application for national patentability examination in the United States Patent and Trademark Office.

The United States Application Number assigned to the application is shown above. A Filing Receipt will be issued for the present application in due course. **THE DATE APPEARING ON THE FILING RECEIPT AS THE "FILING DATE or 371(c) DATE" IS THE DATE ON WHICH THE LAST OF THE 35 U.S.C. 371 (c)(1) and (c)(2) REQUIREMENTS HAS BEEN RECEIVED IN THE OFFICE. THIS DATE IS SHOWN BELOW.** The filing date of the above identified application is the international filing date of the international application (Article 11(3) and 35 U.S.C. 363)

11/10/2017
DATE OF RECEIPT OF 35 U.S.C.
371(c)(1) and (c)(2) REQUIREMENTS

The following items have been received:

- Copy of the International Application filed on 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

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**



FORM PCT/DO/EO/903 (371 Acceptance Notice)

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)

JUELETHIA A PALMER

Telephone: (571) 272-9050



FORM PCT/DO/EO/903 (371 Acceptance Notice)

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NUMBER | FILING or 371(c) DATE | GRP ART UNIT | FIL FEE REC'D | ATTY.DOCKET.NO | TOT CLAIMS | IND CLAIMS |
|---|---|---|---|---|---|---|
| 15/573,300 | 11/10/2017 | 1656 | 3060 | 38767-0048US1 | 38 | 2 |

**CONFIRMATION NO. 4807**

**UPDATED FILING RECEIPT**

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

OC000000099233812

Date Mailed: 05/03/2018

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

Since the rights granted by a U.S. patent extend only throughout the territory of the United States and have no effect in a foreign country, an inventor who wishes patent protection in another country must apply for a patent in a specific country or in regional patent offices. Applicants may wish to consider the filing of an international application under the Patent Cooperation Treaty (PCT). An international (PCT) application generally has the same effect as a regular national patent application in each PCT-member country. The PCT process **simplifies** the filing of patent applications on the same invention in member countries, but **does not result** in a grant of "an international patent" and does not eliminate the need of applicants to file additional documents and fees in countries where patent protection is desired.

Almost every country has its own patent law, and a person desiring a patent in a particular country must make an application for patent in that country in accordance with its particular laws. Since the laws of many countries differ in various respects from the patent law of the United States, applicants are advised to seek guidance from specific foreign countries to ensure that patent rights are not lost prematurely.

Applicants also are advised that in the case of inventions made in the United States, the Director of the USPTO must issue a license before applicants can apply for a patent in a foreign country. The filing of a U.S. patent application serves as a request for a foreign filing license. The application's filing receipt contains further information and guidance as to the status of applicant's license for foreign filing.

Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

For information on preventing theft of your intellectual property (patents, trademarks and copyrights), you may wish to consult the U.S. Government website, http://www.stopfakes.gov. Part of a Department of Commerce initiative,

this website includes self-help "toolkits" giving innovators guidance on how to protect intellectual property in specific countries such as China, Korea and Mexico. For questions regarding patent enforcement issues, applicants may call the U.S. Government hotline at 1-866-999-HALT (1-866-999-4258).

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## Title 35, United States Code, Section 184

## Title 37, Code of Federal Regulations, 5.11 & 5.15

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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.

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## *SelectUSA*

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The U.S. offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to promote and facilitate business investment. SelectUSA provides information assistance to the international investor community; serves as an ombudsman for existing and potential investors; advocates on behalf of U.S. cities, states, and regions competing for global investment; and counsels U.S. economic development organizations on investment attraction best practices. To learn more about why the United States is the best country in the world to develop

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

## 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.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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 | FIRST NAMED APPLICANT | ATTY. DOCKET NO./TITLE |
|---|---|---|---|
| 15/573,300 | 11/10/2017 | Smita Shankar | 38767-0048US1 |

**CONFIRMATION NO. 4807**

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

**PUBLICATION NOTICE**

*OC000000099436524*

**Title:**EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

**Publication No.**US-2018-0127764-A1
**Publication Date:**05/10/2018

## NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Public Records Division. The Public Records Division can be reached by telephone at (571) 272-3150 or (800) 972-6382, by facsimile at (571) 273-3250, by mail addressed to the United States Patent and Trademark Office, Public Records Division, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently https://portal.uspto.gov/pair/PublicPair. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

---

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

Attorney Docket No.: 38767-0048US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | | | | | |
|---|---|---|---|---|---|
| First Named Inventor | : | Smita Shankar | Art Unit | : | 1656 |
| Application 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 AMENDMENT**
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the references listed on the enclosed PTO-1449 Form. Non-patent literature is enclosed; U.S. patents and patent application publications will be provided upon request.

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: /June 19, 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

61702850.doc

Sheet 1 of 1

| Substitute Disclosure Form / U.S. Department of Commerce Patent and Trademark Office | Attorney Docket No. | Application No. |
|---|---|---|
| **Information Disclosure Statement by Applicant** (Use several sheets if necessary) (37 CFR §1.98(b)) | 38767-0048US1 | 15/573,300 |
| | 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 |
| | 1. | 9,938,327 | 04/10/2018 | 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 |
| | -- | -- | -- | -- | | | | |

| Other Documents (include Author, Title, Date, and Place of Publication) | | |
|---|---|---|
| Examiner Initial | Desig. ID | Document |
| | 2. | GenBank Accession No. AAA33980, "leghemoglobin [Glycine max]," April 1993, 1 page |

| Examiner Signature | Date Considered |
|---|---|
| | |

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 32934253 |
| **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/Shannon Stadnick |
| **Filer Authorized By:** | M. Angela Parsons |
| **Attorney Docket Number:** | 38767-0048US1 |
| **Receipt Date:** | 19-JUN-2018 |
| **Filing Date:** | 10-NOV-2017 |
| **Time Stamp:** | 11:41:58 |
| **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 | Information Disclosure Statement (IDS) Form (SB08) | Transmittal.pdf | 71206<br>ae5b1624527d36d8a8cee11fcee5c8ef70a2d63e | no | 1 |

**Warnings:**

| | | | | | |
|---|---|---|---|---|---|
| **Information:** | | | | | |
| This is not an USPTO supplied IDS fillable form | | | | | |
| 2 | Information Disclosure Statement (IDS) Form (SB08) | 1449.pdf | 122599<br>642a739d544c6c4b85a1abab8c85db389bb37886 | no | 1 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| This is not an USPTO supplied IDS fillable form | | | | | |
| 3 | Non Patent Literature | GENBANK_AAA33980_1993.pdf | 34646<br>4dc7d4d9178ad15c8836f895535ed374e6edbe71 | no | 1 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| | | **Total Files Size (in bytes):** | 228451 | | |

**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.**

Attorney Docket No.: 38767-0048US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | | | | | |
|---|---|---|---|---|---|
| First Named Inventor | : | Smita Shankar | Art Unit | : | 1656 |
| Application 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 AMENDMENT**
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Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the references listed on the enclosed PTO-1449 Form. Non-patent literature is 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: /July 6, 2018/

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

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Facsimile: (877) 769-7945

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Sheet 1 of 1

| Substitute Disclosure Form / U.S. Department of Commerce Patent and Trademark Office | Attorney Docket No. | Application No. |
|---|---|---|
| **Information Disclosure Statement by Applicant** (Use several sheets if necessary) (37 CFR §1.98(b)) | 38767-0048US1 | 15/573,300 |
| | 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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**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 |
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| | -- | -- | -- | -- | | | | |

**Other Documents (include Author, Title, Date, and Place of Publication)**

| Examiner Initial | Desig. ID | Document |
|---|---|---|
| | 1. | [No Author Listed] Impossible Foods Inc. "GRAS Notification for Soybean Leghemoglobin Protein Derived from Pichia Pastoris." GRAS notice 000737, Retrieved from internet <URL:https://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm, 1063 pages |

| Examiner Signature | Date Considered |
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EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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# Electronic Acknowledgement Receipt

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|---|---|
| **EFS ID:** | 33104519 |
| **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/Shannon Stadnick |
| **Filer Authorized By:** | M. Angela Parsons |
| **Attorney Docket Number:** | 38767-0048US1 |
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| **Application Type:** | U.S. National Stage under 35 USC 371 |

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| 1 | Information Disclosure Statement (IDS) Form (SB08) | Transmittal.pdf | 71017<br>8226f314d920cec683df2bdc9bb7edc4638459b7 | no | 1 |

**Warnings:**

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| This is not an USPTO supplied IDS fillable form | | | | | |
| 2 | Information Disclosure Statement (IDS) Form (SB08) | 1449.pdf | 123078<br>7322a202f50aeadd034ff992bee1f685c2a0463f | no | 1 |
| **Warnings:** | | | | | |
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| This is not an USPTO supplied IDS fillable form | | | | | |
| 3 | Non Patent Literature | GRAS_Notification_Soybean_Leghemoglobin.pdf | 16322875<br>0be2cfd827f92640e30e9c0c934f712b5526b01e | no | 1063 |
| **Warnings:** | | | | | |
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**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.**

Attorney Docket No.: 38767-0048US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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|---|---|---|---|---|---|
| First Named Inventor | : | Smita Shankar | Art Unit | : | 1656 |
| Application 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 AMENDMENT**
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the documents listed on the disclosure form submitted with this statement. Copies of the documents are being submitted with this statement to the extent required under 37 CFR § 1.98(a)(2). A copy of a Communication from a foreign Patent Office in a counterpart application also is being submitted.

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 applicable under 37 CFR § 1.17(p), or credits, to Deposit Account 06-1050, referencing the above-indicated Attorney Docket Number.

Please contact the undersigned if there are any questions regarding this statement.

Respectfully submitted,

Date: /November 16, 2018/

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

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Facsimile: (877) 769-7945

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Sheet 1 of 1

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| | | Applicant: Impossible Foods Inc. | |
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| U.S. Patent Documents | | | | | | | |
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| Examiner Initial | Desig. ID | Document Number | Publication Date | Patentee | Class | Subclass | Filing Date If Appropriate |

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| | 1. | WO 2015/153666 | 10/8/2015 | WIPO | | | | |

| Other Documents (include Author, Title, Date, and Place of Publication) | | |
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| Examiner Initial | Desig. ID | Document |
| | 2. | Krainer et al., "Optimizing cofactor availability for the production of recombinant heme peroxidase in Pichia pastoris", MICROBIAL CELL FACTORIES, vol. 14, no. 1, 13 January 2015 |
| | 3. | Liu et al., "Balanced globin protein expression and heme biosynthesis improve production of human hemoglobin in Saccharomyces cerevisiae", METABOLIC ENGINEERING, vol. 21, pages 9-16, 2014 |
| | 4. | Fraser et al., "Safety Evaluation of Soy Leghemoglobin Protein Preparation Derived From Pichia pastoris , Intended for Use as a Flavor Catalyst in Plant-Based Meat", INTERNATIONAL JOURNAL OF TOXICOLOGY, vol. 37, no. 3, 11 April 2018, pages 241-262 |
| | 5. | Proulx et al., "Iron Bioavailability of Hemoglobin from Soy Root Nodules Using a Caco-2 Cell Culture Model", J Agric Food Chem, Vol 54, Pages 1518-1522, 2006 |

| Examiner Signature | Date Considered |
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EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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(54) Title: GROUND MEAT REPLICAS

(57) Abstract: This document relates to ground meat replicas, and more particularly to plant-based products that mimic ground meat, including the fibrousness, heterogeneity in texture, beefy flavor, and red-to-brown color transition during cooking of ground meat. For example, this document provides meat replicas that include proteins that are selected based upon the temperature at which they gel and/or denature to replicate the behavior and qualities of meat during cooking, i.e., the firming, syneresis (water release), chew texture, or mouthfeel.

# GROUND MEAT REPLICAS

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Application Serial No. 61/973,181, filed on March 31, 2014, and to U.S. Application Serial No. 62/058,230, filed on October 1, 2014, the disclosure of which are incorporated by reference in their entirety.

## TECHNICAL FIELD

This disclosure relates to meat replicas, such as ground meat replicas, and more particularly to plant-based products that mimic the texture, appearance, and sensory aspects of ground meat, including the texture, appearance, and sensory aspects of cooking and eating ground meat, such as the fibrousness, heterogeneity in texture, beefy flavor, and red-to-brown color transition during cooking of ground meat. This disclosure also relates to compositions and methods for altering the flavor of a food product or a food replica, such as a cheese or meat replica.

## BACKGROUND

Meat substitute compositions typically are extruded soy/grain mixtures that fail to replicate the experience of cooking and eating meat. Common limitations of plant-based meat substitute products include a texture and mouth-feel more homogenous than that of equivalent meat products. Furthermore, as these products must largely be sold pre-cooked, with artificial flavors and aromas pre-incorporated, they fail to replicate the aromas, flavors, and other key features, such as texture and mouth-feel, associated with cooking or cooked meat, and they also may have added off flavors. As a result, these products mainly appeal to a limited consumer base that is already committed to vegetarianism, but have failed to appeal to the larger consumer segment accustomed to eating meat. It would be useful to have improved plant-based meat substitutes, which better replicate the fibrousness, texture, aromas and flavors of meat during and/or after cooking.

## SUMMARY

This document is based on methods and materials for making plant-based products that can mimic ground meat, including the fibrousness, heterogeneity in texture, beefy or other meat flavor, and red-to-brown color transition during cooking of ground meat, without off flavors. For example, this document provides meat replicas that include proteins that are selected based upon the temperature at which they gel and/or denature to replicate the behavior and qualities of meat during cooking, i.e., the firming, syneresis (water release), chew texture, or mouthfeel. For example, the temperature of denaturing and gelling of the proteins selected to be in the meat replica can be similar to that of proteins typically found in meat (e.g., actin and myosin). Further the plant-based products provided herein can include flavoring agents (e.g., flavorings, flavoring precursors, and/or flavoring compounds) that can provide meaty flavors, such that a plant-based meat replica has a more natural flavor and does not have off flavors. This document therefore also provides methods for making plant-based products containing such flavoring agents.

In one aspect, this document features a meat replica composition that includes about 5% to about 88% (e.g., about 40% to about 88%, about 45% to about 60%, or about 15% to about 55%) by weight of a meat dough; about 0% to about 40% (e.g., about 1% to about 30%, about 5% to about 25%, or about 15% to about 25%) by weight of a carbohydrate-based gel; about 5% to about 35% (e.g., about 10% to about 15%, about 12% to about 18%, or about 20% to about 25%) by weight of a fat; about 0.00001% to about 10% (e.g., about 3% to about 7%, about 0.001% to about 2%, or about 0.00001% to about 2%) by weight of a flavoring agent; about 0% to about 15% (e.g., about 2% to about 15% or about 2% to about 10%) by weight of a binding agent; and about 0.01% to about 4% (e.g., about 0.05% to about 1%, or about 0.5% to about 2%) by weight of a heme-containing protein and/or an iron salt. The meat dough can include a flavoring agent. The fat can include a flavoring agent. The meat dough can be about 45% to about 60% by weight of the composition. The carbohydrate-based gel can be about 10% to about 25% by weight of the composition. The fat can be about 10% to about 15% by weight of the composition. The flavoring agent can be about 3% to about 7% or about

0.001% to about 2% by weight of the composition. The flavoring agent can include one or more flavor precursors, a flavoring, or a flavoring compound. The flavoring agent can be a combination of a flavoring and one or more flavor precursors. The binding agent can be about 2% to about 10% by weight of the composition. The binding agent can include one or more proteins that have been chemically or enzymatically modified to improve their textural and/or flavor properties, or to modify their denaturation and gelling temperatures. The heme-containing protein can be about 0.01% to about 2% by weight of the composition. The composition can include the heme-containing protein and the iron salt. The meat dough can include an isolated plant protein, an edible fibrous component, an optional flavoring agent, and an optional fat. The binding agent can be a conglycinin protein.

In another aspect, this document features a meat replica composition that includes about 5% to about 80% (e.g., about 20% to about 30%) by weight of a meat dough; about 5% to about 35% (e.g., about 15% to about 25%) by weight of a fat; about 15% to about 40% (e.g., about 15% to about 25%) by weight of an edible fibrous component; about 0.1% to about 18% (e.g., about 7% to about 18%) by weight of a carbohydrate-based gel; about 0% to about 10% (e.g., about 0% to about 10%) by weight of a flavoring agent; about 0.5% to about 15% (e.g., about 5% to about 15%) by weight of a binding agent; and about 0.1% to about 8% (e.g., about 2% to about 8%) by weight of a heme-containing protein and/or an iron salt.

In another aspect, this document features a method of making a ground meat replica. The method can include (a) heating a dough to a temperature ranging from 150°F to 250°F, the dough comprising an isolated plant protein, an optional edible fibrous component, one or more optional flavoring agents, and an optional fat; (b) combining the dough, after heating, with a fat, the fat optionally containing a flavoring agent and/or an isolated plant protein; and (c) combining the dough from step (b) with a carbohydrate-based gel, an optional edible fibrous component, an optional binding agent, a highly conjugated heterocyclic ring complexed to an iron ion and/or an iron salt, and one or more optional flavoring agents to make the ground meat replica. The method can further include breaking the dough from step (b) into pieces before combining with the

carbohydrate-based gel, the optional edible fibrous component, the optional binding agent, the highly conjugated heterocyclic ring complexed to an iron ion and/or the iron salt, and one or more optional flavoring agents.

In another aspect, this document features a method of flavoring a meat dough. The method can include (a) combining a first highly conjugated heterocyclic ring complexed to an iron ion and/or a first iron salt with one or more flavor precursors and an optional fat; (b) heating the mixture to form one or more flavor compounds; and (c) making a dough comprising an isolated plant protein, an optional edible fibrous component, and the mixture from step (b). The method can further include (d) combining the dough, after heating, with a fat, the fat optionally containing a flavoring agent and/or an isolated plant protein; and (e) combining the dough of step (d) with a carbohydrate-based gel, an optional binding agent, a second highly conjugated heterocyclic ring complexed to an iron ion and/or a second iron salt, and one or more optional flavoring agents to make a ground meat replica. The method can further include breaking the dough from step (d) into pieces before combining with the carbohydrate-based gel, the optional binding agent, the second highly conjugated heterocyclic ring complexed to an iron ion and/or the second iron salt, and one or more optional flavoring agents.

In another aspect, this document features a method of flavoring a meat dough, where the method includes (a) making a dough comprising an isolated plant protein, an optional edible fibrous component, one or more optional flavoring agents, and an optional fat; (b) making a flavored fat by combining a fat with a highly conjugated heterocyclic ring complexed to an iron ion and/or a first iron salt, and one or more flavor precursors and heating the mixture; and (c) combining the dough, after heating, with the flavored fat. The method can further include combining the dough of step (c) with a carbohydrate-based gel, an optional binding agent, a second highly conjugated heterocyclic ring complexed to an iron ion and/or a second iron salt, and one or more optional flavoring agents to make a ground meat replica. The method can further include breaking the dough of step (c) before combining with the carbohydrate-based gel, the optional binding agent, the second highly conjugated heterocyclic ring complexed to an iron ion and/or the second iron salt, and one or more optional flavoring agents.

This document also features a method of making a ground meat replica, where the method includes (a) combining an iron salt with one or more flavor precursors and an optional fat; (b) heating the mixture to form one or more flavor compounds; (c) making a dough comprising an isolated plant protein, an optional edible fibrous component, and the mixture from step (b); (d) combining the dough, after heating, with a fat, the fat optionally containing a flavoring agent and/or an isolated plant protein; and (e) combining the dough of step (d) with a carbohydrate-based gel, an optional binding agent, an iron salt, an optional highly conjugated heterocyclic ring complexed to an iron ion, and one or more optional flavoring agents to make the ground meat replica. The method can further include breaking the dough from step (d) into pieces before combining with the carbohydrate-based gel, the optional binding agent, the iron salt, the optional highly conjugated heterocyclic ring complexed to an iron ion, and one or more optional flavoring agents. In some embodiments, a highly conjugated heterocyclic ring complexed to an iron ion can be combined with the iron salt, the one or more flavor precursors, and the fat before heating the mixture.

In yet another aspect, this document features a method of making a ground meat replica. The method can include (a) making a dough comprising an isolated plant protein, an optional edible fibrous component, one or more optional flavoring agents, and an optional fat; (b) making a flavored fat by combining a fat with an iron salt and one or more flavor precursors and heating the mixture; (c) combining the dough, after heating, with the flavored fat; and (d) combining the dough of step (c) with a carbohydrate-based gel, an optional binding agent, an iron salt, an optional highly conjugated heterocyclic ring complexed to an iron ion, and one or more optional flavoring agents to make the ground meat replica. The method can further include breaking the dough from step (c) before combining with the carbohydrate-based gel, the optional binding agent, the iron salt, the optional highly conjugated heterocyclic ring complexed to an iron ion, and one or more optional flavoring agents. In some embodiments, a highly conjugated heterocyclic ring complexed to an iron ion can be combined with the fat, the iron salt, and the one or more flavor precursors before heating the mixture.

In any of the methods or compositions described herein, the iron salt can be iron gluconate, iron chloride, iron oxalate, iron nitrate, iron citrate, iron ascorbate, ferrous sulfate, ferric pyrophosphate, or any other aqueous soluble salt.

In any of the methods or compositions described herein, the heme-containing protein can be a non-animal heme-containing protein, such as a plant-derived heme-containing protein (e.g., leghemoglobin). Further, in some embodiments, the heme-containing protein can be isolated or isolated and purified.

In any of the methods or compositions described herein, wherein the one or more flavor precursors can be a sugar, a sugar alcohol, a sugar acid, a sugar derivative, an oil, a free fatty acid, an amino acid or derivative thereof, a nucleoside, a nucleotide, a vitamin, an acid, a peptide, a phospholipid, a protein hydrolysate, a yeast extract, or a mixture thereof. For example, the flavor precursor can be selected from the group consisting of glucose, fructose, ribose, arabinose, glucose-6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, inositol, maltose, sucrose, maltodextrin, glycogen, nucleotide-bound sugars, molasses, a phospholipid, a lecithin, inosine, inosine monophosphate (IMP), guanosine monophosphate (GMP), pyrazine, adenosine monophosphate (AMP), lactic acid, succinic acid, glycolic acid, thiamine, creatine, pyrophosphate, vegetable oil, algal oil, sunflower oil, corn oil, soybean oil, palm fruit oil, palm kernel oil, safflower oil, flaxseed oil, rice bran oil, cottonseed oil, olive oil, sunflower oil, canola oil, flaxseed oil, coconut oil, mango oil, a free fatty acid, cysteine, methionine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan, valine, arginine, histidine, alanine, asparagine, aspartate, glutamate, glutamine, glycine, proline, serine, tyrosine, glutathione, an amino acid derivative, urea, pantothenic acid, ornithine, niacin, glycerol, citrulline, taurine, biotin, borage oil, fungal oil, blackcurrant oil, betaine, beta carotene, B-vitamins, N-Acetyl L-cysteine, iron glutamate and a peptone, or mixtures thereof.

In any of the methods or compositions described herein, the isolated plant protein in the dough can include wheat gluten, a dehydrin protein, an albumin, a globulin, or a zein, or mixtures thereof.

In any of the methods or compositions described herein, the optional edible fibrous component can include plant fibers from carrot, bamboo, pea, broccoli, potato,

sweet potato, corn, whole grains, alfalfa, kale, celery, celery root, parsley, cabbage, zucchini, green beans, kidney beans, black beans, red beans, white beans, beets, cauliflower, nuts, apple skins, oats, wheat, or psyllium, or a mixture thereof.

In any of the methods or compositions described herein, the edible fibrous component can include an extruded mixture of isolated plant proteins. The extruded mixture can contain wheat gluten and soy protein isolate, and optionally can further contain a flavoring agent (e.g., a flavoring such as yeast extract, a protein hydrolysate, or an oil; a flavor compound; or a flavor precursor). In some embodiments, the edible fibrous component can be a solution-spun protein fiber (e.g., a solution-spun protein fiber containing a prolamin such as corn zein, pea prolamin, kafirin, secalin, hordein, avenin, or a mixture thereof).

In any of the methods or compositions described herein, the fat can be a non-animal fat, an animal fat, or a mixture of non-animal and animal fat. The fat can be an algal oil, a fungal oil, corn oil, olive oil, soy oil, peanut oil, walnut oil, almond oil, sesame oil, cottonseed oil, rapeseed oil, canola oil, safflower oil, sunflower oil, flax seed oil, palm oil, palm kernel oil, coconut oil, babassu oil, shea butter, mango butter, cocoa butter, wheat germ oil, borage oil, black currant oil, sea-buckhorn oil, macadamia oil, saw palmetto oil, conjugated linoleic oil, arachidonic acid enriched oil, docosahexaenoic acid (DHA) enriched oil, eicosapentaenoic acid (EPA) enriched oil, palm stearic acid, sea-buckhorn berry oil, macadamia oil, saw palmetto oil, or rice bran oil; or margarine or other hydrogenated fats. In some embodiments, for example, the fat is algal oil. The fat can contain the flavoring agent and/or the isolated plant protein (e.g., a conglycinin protein).

In any of the methods or compositions described herein, the dough can include the flavoring agent. In any of the methods or compositions, the non-animal fat in the dough can include a flavoring agent. The flavoring agent can be selected from the group consisting of a vegetable extract, a fruit extract, an acid, an antioxidant, a carotenoid, a lactone, and combinations thereof. The antioxidant can be epigallocatechin gallate. The carotenoid can be lutein, β-carotene, zeaxanthin, trans-β-apo-8'-carotenal, lycopene, or

canthaxanthin. The vegetable extract can be from a cucumber or tomato. The fruit extract can be from a melon or pineapple.

In any of the methods or compositions described herein, the carbohydrate based gel can have a melting temperature between about 45°C and about 85°C. The carbohydrate-based gel can include agar, pectin, carrageenan, konjac, alginate, chemically-modified agarose, or mixtures thereof.

In any of the methods or compositions described herein, the ground meat replica can contain the binding agent. The binding agent can be an isolated plant protein (e.g., a RuBisCO, an albumin, a gluten, a conglycinin, or mixtures thereof). The denaturation temperature of the binding agent can be between about 40°C and about 80°C. The binding agent can be a carbohydrate based gel that becomes firm upon cooking to 140°F to 190°F. The carbohydrate based gel can contain methylcellulose, hydroxypropylmethyl cellulose, guar gum, locust bean gum, xanthan gum, or a mixture thereof. The binding agent can be egg albumin or collagen.

In any of the methods or compositions described herein, the highly conjugated heterocyclic ring complexed to an iron ion can be a heme moiety, or a porphyrin, porphyrinogen, corrin, corrinoid, chlorin, bacteriochlorophyll, corphin, chlorophyllin, bacteriochlorin, or isobacteriochlorin moiety complexed to an iron ion. The heme moiety can be a heme-containing protein (e.g., a non-symbiotic hemoglobin, a Hell's gate globin I, a flavohemoprotein, a leghemoglobin, a heme-dependent peroxidase, a cytochrome c peroxidase, or a mammalian myoglobin). In some embodiments, the heme-containing protein can be a leghemoglobin. The leghemoglobin can be from soybean, pea, or cowpea.

In another aspect, this document features a method of increasing the meat flavor or masking off flavors from plant material in a food product. The method can include adding, to the food product, one or more lactones at a concentration of $10^{-3}$ to $10^{-11}$ of the food product, wherein the lactones are selected from the group consisting of tetrahydro-6-methyl-2H-pyran-2-one, delta-octalactone, 5-ethyldihydro-2(3H)-furanone, butyrolactone, dihydro-5-pentyl-2(3H)-furanone, dihydro-3-methylene-2,5-furandione, l-pentoyl lactone, tetrahydro-2H-pyran-2-one, 6-heptyltetrahydro-2H-pyran-2-one, γ-

octalactone, 5-hydroxymethyldihydrofuran-2-one, 5-ethyl-2(5H)-furanone, 5-acetyldihydro-2(3H)-furanone, trans-3-methyl-4-octanolide 2(5H)-furanone, 3-(1,1-dimethylethyl)-2,5-urandione, 3,4-dihydroxy-5-methyl-dihydrofuran-2-one, 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, δ-tetradecalactone, and dihydro-4-hydroxy-2(3H)-furanone. In some embodiments, the lactones can be 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, butyrolactone, γ-octalactone, and δ-tetradecalactone. The food product can be a meat replica. The meat replica can be free of animal products.

This document also features a method of increasing the meat flavor or masking off flavors from plant material in a food product, where the method includes adding, to the food product, one or more carotenoids at a concentration of between 0.00001% and 0.1% of the food product, wherein the carotenoids are selected from the group consisting of β-carotene, zeaxanthin, lutein, trans-β-apo-8'-carotenal, lycopene, canthaxanthin, and combinations thereof. The food product can be a meat replica. The meat replica can be free of animal products.

In another embodiment, this document features a method of increasing the meat flavor of a meat replica. The method can include adding, to the meat replica, a vegetable juice, a vegetable puree, a vegetable extract, a fruit juice, a fruit puree, or a fruit extract to the meat replica at a concentration from 0.0001% to 10% of the meat replica. The vegetable juice, vegetable puree, vegetable extract, a fruit juice, a fruit puree, or a fruit extract can be a *Cucumis* juice, puree, or extract (e.g., a juice, puree, or extract from a cucumber or a melon). The method vegetable juice, vegetable puree, vegetable extract, fruit juice, fruit puree, or fruit extract can be cooked or otherwise treated to denature proteins before adding to the meat replica. The meat replica can be free of animal products.

In another aspect, this document features a food product or food replica product containing a heme-containing protein and one or more lactones at a concentration of $10^{-3}$ to $10^{-11}$ of the food product, wherein the one or more lactones are selected from the group consisting of tetrahydro-6-methyl-2H-pyran-2-one, delta-octalactone, 5-ethyldihydro-2(3H)-furanone, butyrolactone, dihydro-5-pentyl-2(3H)-furanone, dihydro-3-methylene-2,5-furandione, l-pentoyl lactone, tetrahydro-2H-pyran-2-one, 6-heptyltetrahydro-2H-

pyran-2-one, γ-octalactone, 5-hydroxymethyldihydrofuran-2-one, 5-ethyl-2(5H)-furanone, 5-acetyldihydro-2(3H)-furanone, trans-3-methyl-4-octanolide 2(5H)-furanone, 3-(1,1-dimethylethyl)-2,5-urandione, 3,4-dihydroxy-5-methyl-dihydrofuran-2-one, 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, δ-tetradecalactone, and dihydro-4-hydroxy-2(3H)-furanone. For example, the one or more lactones can be 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, butyrolactone, γ-octalactone, and δ-tetradecalactone. The food product or food replica product can be a meat replica. The meat replica can be free of animal products.

This document also features a food product or food replica product containing a heme-containing protein and one or more carotenoids at a concentration of between 0.00001% and 0.1% of the food product, wherein the one or more carotenoids are selected from the group consisting of β-carotene, zeaxanthin, lutein, trans-β-apo-8'-carotenal, lycopene, canthaxanthin, and combinations thereof. The food product or food replica product can be a meat replica. The meat replica can be free of animal products.

In another aspect, this document features a food product or food replica product containing (a) a heme-containing protein, and (b) a vegetable juice, a vegetable puree, a vegetable extract, a fruit juice, a fruit puree, or a fruit extract at a concentration from 0.0001% to 10% of the food product. The vegetable juice, vegetable puree, vegetable extract, a fruit juice, a fruit puree, or a fruit extract can be a *Cucumis* juice, puree, or extract. The *Cucumis* juice, puree, or extract can be from a cucumber or a melon. The vegetable juice, vegetable puree, vegetable extract, fruit juice, fruit puree, or fruit extract can have been cooked or otherwise treated to denature proteins before being added to the food replica product. For example, the vegetable juice, vegetable puree, vegetable extract, fruit juice, fruit puree, or fruit extract can have been heated to a temperature of about 60°C to about 100°C before being added to the food replica product. The food product can be free of animal products.

In another aspect, this document features a food replica product containing one or more lactones at a concentration of $10^{-3}$ to $10^{-11}$ of the food product, wherein the one or more lactones are selected from the group consisting of tetrahydro-6-methyl-2H-pyran-2-one, delta-octalactone, 5-ethyldihydro-2(3H)-furanone, butyrolactone, dihydro-5-pentyl-

2(3H)-furanone, dihydro-3-methylene-2,5-furandione, l-pentoyl lactone, tetrahydro-2H-pyran-2-one, 6-heptyltetrahydro-2H-pyran-2-one, γ-octalactone, 5-hydroxymethyldihydrofuran-2-one, 5-ethyl-2(5H)-furanone, 5-acetyldihydro-2(3H)-furanone, trans-3-methyl-4-octanolide 2(5H)-furanone, 3-(1,1-dimethylethyl)-2,5-urandione, 3,4-dihydroxy-5-methyl-dihydrofuran-2-one, 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, δ-tetradecalactone, and dihydro-4-hydroxy-2(3H)-furanone. The one or more lactones can be 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, butyrolactone, γ-octalactone, and δ-tetradecalactone.

In still another aspect, this document features a food replica product containing one or more carotenoids at a concentration of between 0.00001% and 0.1% of the food product, wherein the one or more carotenoids are selected from the group consisting of β-carotene, zeaxanthin, lutein, trans-β-apo-8'-carotenal, lycopene, canthaxanthin, and combinations thereof.

This document also features a food replica product containing a vegetable juice, a vegetable puree, a vegetable extract, a fruit juice, a fruit puree, or a fruit extract at a concentration from 0.0001% to 10% of the food product. The vegetable juice, vegetable puree, vegetable extract, fruit juice, fruit puree, or fruit extract can be a *Cucumis* juice, puree, or extract (e.g., a *Cucumis* juice, puree, or extract from a cucumber or a melon). The vegetable juice, vegetable puree, vegetable extract, fruit juice, fruit puree, or fruit extract can have been cooked or otherwise treated to denature proteins before being added to the food replica product. For example, the vegetable juice, vegetable puree, vegetable extract, fruit juice, fruit puree, or fruit extract can have been heated to a temperature of about 60°C to about 100°C before being added to the food replica product.

In some embodiments, the food replica products provided herein can be free of animal products, wheat gluten, soy protein, and/or tofu.

Any of the food replica products provided herein can contain one or more of a meat dough, a carbohydrate-based gel, a non-animal fat, and a binding agent.

Any of the food replica products provided herein can be a meat replica. Further materials and methods for making meat replicas can be found in, for example, U.S.

Publication No. 2014/0193547, and PCT publications WO 2014/110532 and WO 2014/110539, each of which is incorporated herein by reference in its entirety.

Any of the food replica products provided herein can be a cheese replica. The cheese replica can contain a nut milk, a cross-linking enzyme, or a cheese culture. Further materials and methods for making cheese replicas can be found in, for example, U.S. Publication No. 2014/0127358, and PCT publication WO 2014/110540, both of which are incorporated herein by reference in their entirety.

In yet another aspect, this document features a ground meat replica containing (a) a dough that contains an isolated plant protein, an optional edible fibrous component, one or more optional flavoring agents, and an optional fat; (b) a fat, the fat optionally containing a flavoring agent and/or an isolated plant protein; and (c) a carbohydrate-based gel, a binding agent, a highly conjugated heterocyclic ring complexed to an iron ion and/or an iron salt, an optional edible fibrous component, and one or more optional flavoring agents. The binding agent can be an isolated plant protein (e.g., a RuBisCO, an albumin, a gluten, a conglycinin, or mixtures thereof). The denaturation temperature of the binding agent can be between about 40°C and about 80°C.

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 this invention pertains. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims. The word "comprising" in the claims may be replaced by "consisting essentially of" or with "consisting of," according to standard practice in patent law.

## DETAILED DESCRIPTION

In general, this document provides methods and materials for producing meat replicas, including ground meat replicas (e.g., ground beef, ground chicken, ground turkey, ground lamb, or ground pork), as well as replicas of cuts of meat and fish. Broadly, the document provides methods for making ground meat replicas that include preparing a meat replica dough (referred to herein as "meat dough") that includes an optional edible fibrous component, combining the meat dough with a fat (typically a non-animal-based fat, although it is to be noted that an animal-based fat could be used) that can optionally include a flavoring agent and/or an isolated plant protein, adding a carbohydrate-based gel, an optional edible fibrous component, a binding agent, a highly conjugated heterocyclic ring complexed to an iron ion and/or an iron salt, and one or more flavoring agents to make the replica. After combining the meat dough with the fat, the mixture can be broken into smaller pieces before adding further ingredients.

The meat dough can incorporate an edible fibrous component to help achieve a textural heterogeneity and fibrousness in the meat replica that resembles the heterogeneity and texture of ground meat (e.g., ground beef). Incorporating flavoring agents into multiple components of the meat replica (e.g., two or more of the meat dough, the edible fibrous component, the non-animal-based fat, or the assembled replica), helps mimic the sensory properties of ground meat. In some embodiments, flavoring agents are incorporated into three components of the meat replica. In some embodiments, flavoring agents are incorporated into four components of the meat replica.

As described herein, the flavoring agents can be flavor precursors, flavor compounds produced from reacting flavor precursors with iron, or flavorings such as extracts (e.g., a malt extract, a yeast extract, a vegetable or fruit extract, such as a cucumber extract or a melon extract, or a peptone) or protein hydrolysates such as vegetable protein hydrolysates, soy protein hydrolysates, yeast protein hydrolysates, algal protein hydrolysates, or meat protein hydrolysates or flavor compounds, natural or synthetic. Flavor precursors can react, e.g., with the iron in a highly conjugated heterocyclic ring complexed to an iron ion or an iron salt, with each other, or with flavorings, upon heating. Accordingly, in the meat replicas described herein,

combinations of pre-cooked, *i.e.*, reacted, flavor components, uncooked flavor precursors that can react (e.g., with the iron salt and/or highly conjugated heterocyclic ring complexed to an iron ion or with each other) during cooking of the replicas, or flavorings or flavor compounds that introduce a flavor without requiring a reaction, can be incorporated into the meat replica to reproduce the sensory experience of cooking and eating cooked ground meat. The flavor and/or aroma profile of the ground meat product can be modulated by the type and concentration of the flavor precursors, the pH of the reaction, the length of cooking, the type and amount of iron complex (e.g., a heme-cofactor such as a heme-containing protein, or heme bound to non-peptidic polymer or macromolecule), the temperature of the reaction, and the amount of water activity in the product, among other factors.

A highly conjugated heterocyclic ring complexed to an iron ion is referred to herein as an iron complex. Such iron complexes include heme moieties or other highly conjugated heterocylic rings complexed to an iron ion. "Heme" refers to a prosthetic group bound to iron ($Fe^{2+}$ or $Fe^{3+}$) in the center of a porphyrin ring. Thus, an iron complex can be a heme moiety, or a porphyrin, porphyrinogen, corrin, corrinoid, chlorin, bacteriochorophyll, corphin, chlorophyllin, bacteriochlorin, or isobacteriochlorin moiety complexed to an iron ion. The heme moiety can be a heme cofactor such as a heme-containing protein; a heme moiety bound to a non-peptidic polymer or other macromolecule such as a liposome, a polyethylene glycol, a carbohydrate, a polysaccharide, or a cyclodextrin.

In some embodiments, the iron complex is a heme-containing protein that is isolated and purified. As used herein, the term "isolated and purified" with respect to a protein or a protein fraction indicates that the protein or protein fraction has been separated from other components of the source material (e.g., other animal, plant, fungal, algal, or bacterial proteins), such that the protein or protein fraction is at least 50% (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99%) free, by dry weight, of the other components of the source material.

As used herein, an "enriched" protein or protein fraction composition is at least 2-fold (e.g., at least 3-fold, 4-fold, 5-fold, 10-fold, 20-fold, 50-fold, or 100-fold) enriched in that protein or protein fraction relative to the source material.

The term "heme containing protein" can be used interchangeably with "heme containing polypeptide" or "heme protein" or "heme polypeptide" and includes any polypeptide that can covalently or noncovalently bind a heme moiety. In some embodiments, the heme-containing polypeptide is a globin and can include a globin fold, which comprises a series of seven to nine alpha helices. Globin type proteins can be of any class (e.g., class I, class II, or class III), and in some embodiments, can transport or store oxygen. For example, a heme-containing protein can be a non-symbiotic type of hemoglobin or a leghemoglobin. A heme-containing polypeptide can be a monomer, i.e., a single polypeptide chain, or can be a dimer, a trimer, tetramer, and/or higher order oligomer. The life-time of the oxygenated $Fe^{2+}$ state of a heme-containing protein can be similar to that of myoglobin or can exceed it by 10%, 20%, 30%, 50%, 100% or more under conditions in which the heme-protein-containing consumable is manufactured, stored, handled or prepared for consumption. The life-time of the unoxygenated $Fe^{2+}$ state of a heme-containing protein can be similar to that of myoglobin or can exceed it by 10%, 20%, 30%, 50%, 100% or more under conditions in which the heme-protein-containing consumable is manufactured, stored, handled or prepared for consumption

Non-limiting examples of heme-containing polypeptides can include an androglobin, a cytoglobin, a globin E, a globin X, a globin Y, a hemoglobin, a myoglobin, an erythrocruorin, a beta hemoglobin, an alpha hemoglobin, a protoglobin, a cyanoglobin, a cytoglobin, a histoglobin, a neuroglobins, a chlorocruorin, a truncated hemoglobin (e.g., HbN or HbO), a truncated 2/2 globin, a hemoglobin 3 (e.g., Glb3), a cytochrome, or a peroxidase.

Heme-containing proteins that can be used in the ground meat replicas described herein can be from mammals (e.g., farms animals such as cows, goats, sheep, pigs, ox, or rabbits), birds, plants, algae, fungi (e.g., yeast or filamentous fungi), ciliates, or bacteria. For example, a heme-containing protein can be from a mammal such as a farm animal (e.g., a cow, goat, sheep, pig, fish, ox, or rabbit) or a bird such as a turkey or chicken.

Heme-containing proteins can be from a plant such as *Nicotiana tabacum* or *Nicotiana sylvestris* (tobacco); *Zea mays* (corn), *Arabidopsis thaliana*, a legume such as *Glycine max* (soybean), *Cicer arietinum* (garbanzo or chick pea), *Pisum sativum* (pea) varieties such as garden peas or sugar snap peas, *Phaseolus vulgaris* varieties of common beans such as green beans, black beans, navy beans, northern beans, or pinto beans, *Vigna unguiculata* varieties (cow peas), *Vigna radiata* (mung beans), *Lupinus albus* (lupin), or *Medicago sativa* (alfalfa); *Brassica napus* (canola); *Triticum sps.* (wheat, including wheat berries, and spelt); *Gossypium hirsutum* (cotton); *Oryza sativa* (rice); *Zizania sps.* (wild rice); *Helianthus annuus* (sunflower); *Beta vulgaris* (sugarbeet); *Pennisetum glaucum* (pearl millet); *Chenopodium sp.* (quinoa); *Sesamum sp.* (sesame); *Linum usitatissimum* (flax); or *Hordeum vulgare* (barley). Heme-containing proteins can be isolated from fungi such as *Saccharomyces cerevisiae*, *Pichia pastoris*, *Magnaporthe oryzae*, *Fusarium graminearum*, *Aspergillus oryzae*, *Trichoderma reesei*, *Myceliopthera thermophile*, *Kluyveramyces lactis*, or *Fusarium oxysporum*. Heme-containing proteins can be isolated from bacteria such as *Escherichia coli*, *Bacillus subtilis, Bacillus licheniformis, Bacillus megaterium, Synechocistis sp., Aquifex aeolicus, Methylacidiphilum infernorum*, or thermophilic bacteria such as *Thermophilus spp.* The sequences and structure of numerous heme-containing proteins are known. *See for example*, Reedy, *et al.*, *Nucleic Acids Research*, 2008, Vol. 36, Database issue D307–D313 and the Heme Protein Database available on the world wide web at http://hemeprotein.info/heme.php.

In some embodiments, a non-symbiotic hemoglobin can be from any plant. In some embodiments, a non-symbiotic hemoglobin can be from a plant selected from the group consisting of soybean, sprouted soybean, alfalfa, golden flax, black bean, black eyed pea, northern bean, tobacco, pea, garbanzo, moong bean, cowpeas, pinto beans, pod peas, quinoa, sesame, sunflower, wheat berries, spelt, barley, wild rice, and rice.

In some embodiments, a leghemoglobin can be a soy, pea, or cowpea leghemoglobin.

In some embodiments, isolated plant proteins are used. As used herein, the term "isolated" with respect to a protein or a protein fraction (e.g., a 7S fraction) indicates that the protein or protein fraction has been separated from other components of the source

material (e.g., other animal, plant, fungal, algal, or bacterial proteins), such that the protein or protein fraction is at least 2% (e.g., at least 5%, 10%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99%) free, by dry weight, of the other components of the source material. Thus, in some embodiments, the iron complex can be a heme-containing protein (e.g., a plant heme-containing protein) that is isolated. Proteins can be separated on the basis of their molecular weight, for example, by size exclusion chromatography, ultrafiltration through membranes, or density centrifugation. In some embodiments, the proteins can be separated based on their surface charge, for example, by isoelectric precipitation, anion exchange chromatography, or cation exchange chromatography. Proteins also can be separated on the basis of their solubility, for example, by ammonium sulfate precipitation, isoelectric precipitation, surfactants, detergents or solvent extraction. Proteins also can be separated by their affinity to another molecule, using, for example, hydrophobic interaction chromatography, reactive dyes, or hydroxyapatite. Affinity chromatography also can include using antibodies having specific binding affinity for the heme-containing protein, nickel nitroloacetic acid (NTA) for His-tagged recombinant proteins, lectins to bind to sugar moieties on a glycoprotein, or other molecules which specifically binds the protein.

Example 2 describes a method for isolating RuBisCO from a plant (e.g., spinach or alfalfa). The extraction process can be improved further by adding reductants such as metabisulfite (about 2% w/v solution or more) to the initial extraction buffer and maintaining anaerobic conditions through the process and/or by adding 0.05-1% v/v cationic flocculants such as Superfloc 781G,Magnafloc LT 7989 (BASF), or Tramfloc 863A to the extraction buffer to the extraction buffer. The resuspended protein pellet from such methods, upon microfiltration at a pH of 7.0, would still perform, provide the same color, and have the same denaturation properties.

Example 4 describes a method for isolating conglycinin (also can be referred to as a 7S fraction) from a plant such as soybean. Other sources of 7S include seeds such as, without limitation, peas, chickpeas, mung beans, kidney beans, fava beans, cowpeas, pine nuts, rice, corn, and sesame. Soluble proteins can be extracted from defatted soybean

flour, and then the mixture acidified (e.g., to a pH of 4.5) to precipitate the proteins. Conglycinin can be resolubilized and concentrated, e.g., using ultrafiltration.

In some embodiments, the isolated protein is decolorized. For example, the RuBisCO concentrates can be decolorized (pH 7-9) by passing over columns packed with activated carbon. The colorants can bind to the column while RuBisCO can be isolated in the filtrate. Alternatively, RuBisCO concentrates can be decolorized by incubating the solution with a FPX66 (Dow Chemicals) resin packed in a column or batch mode. The slurry is incubated for 30 minutes and then the liquid is separated from the resin. The colorants can bind to the resin and RuBisCO can be collected in the column flow-through.

In some embodiments, the isolated protein can be purified and decolorized as described in Example 3. See also "Methods for Extracting and Purifying Native Proteins" filed on October 1, 2014, U.S. Ser. No. 62/058,211.

In some embodiments, a decolorized isolated plant protein can provide an increased shelf-life stability to the red color of the meat replica as compared to a corresponding meat replica including an isolated plant protein without decolorization. In some embodiments, the decolorized protein lead to an improved flavor profile of the meat replica as compared to that observed in a meat replica with the corresponding isolated plant protein without decolorization.

Heme-containing or other proteins also can be recombinantly produced using polypeptide expression techniques (e.g., heterologous expression techniques using bacterial cells, insect cells, fungal cells such as yeast, plant cells such as tobacco, soybean, or *Arabidopsis*, or mammalian cells). For example, leghemoglobin can be recombinantly produced in *E. coli* or *Pichia pastoris* as described in Example 1. In some cases, standard polypeptide synthesis techniques (e.g., liquid-phase polypeptide synthesis techniques or solid-phase polypeptide synthesis techniques) can be used to produce heme-containing proteins synthetically. In some cases, *in vitro* transcription-translation techniques can be used to produce heme-containing proteins.

In some embodiments, the meat replicas described herein are substantially or entirely composed of ingredients derived from non-animal sources, e.g., plant, fungal, or

microbial-based sources. In some embodiments, a meat replica may include one or more animal-based products. For example, a meat replica can be made from a combination of plant-based and animal-based sources.

*Making the Meat Replica*

A meat dough can be prepared by mixing an isolated plant protein and an optional edible fibrous component, an optional flavoring agent, and optional non-animal fat, and adding an aqueous component such as water or a broth to the mixture and kneading or otherwise mixing, manually or mechanically, to form a dough. The aqueous component can be heated before adding to the mixture of plant protein and fibrous component. Once the meat dough is formed, the meat dough is heated (e.g., steamed or boiled) to a temperature ranging from 150°F to 250°F (e.g., 160°F to 240°F, 170°F to 230°F, 180°F to 220°F, or 190°F to 212°F). For example, a meat dough can be steamed by placing in a rice cooker, steam cabinet, or tunnel steamer. A meat dough can be heated by applying dry heat, for example, by placing in a bread maker or oven, or by immersing in hot water or broth. Boiling in broth can improve the meat dough flavor because beneficial flavors and off-flavor masking agents can be absorbed into the dough. Texture properties may also be modulated by choice of the cooking method.

As used herein, the term "isolated plant protein" indicates that the plant protein (e.g., a heme-containing protein, wheat gluten, dehydrin protein, an albumin, a globulin, conglycinin, glycinin, or a zein, or mixtures thereof) or plant protein fraction (e.g., a 7S fraction) has been separated from other components of the source material (e.g., other animal, plant, fungal, algal, or bacterial proteins), such that the protein or protein fraction is at least 2% (e.g., at least 5%, 10%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99%) free, by dry weight, of the other components of the source material. For example, wheat gluten can be used alone or in combination with one or more other proteins (e.g., dehydrins). Dehydrins can be particularly useful for enhancing the juiciness and texture in the ground meat replicas. In some embodiments, the meat replica can be formulated to be gluten free, and, for

example, a blend of maize starch, tapioca flour, rice flour, and guar gum can be substituted for the wheat gluten in the meat dough.

The edible fibrous component can be a plant fiber, an extruded mixture of isolated plant proteins (e.g., wheat gluten or other isolated plant protein, such as glutelins, albumins, legumins, vicillins, convicillins, glycinins and protein isolates such as from any seed or bean, including soy, pea, lentil, etc.), or a solution-spun protein fiber. In some embodiments, the solution-spun protein fiber is a prolamin solution-spun protein fiber. The prolamin can be from any plant source (e.g., corn or pea) and can include zein, prolamin, kafirin, secalin, hordein, or avenin. The texture of the ground meat product (e.g., meat patty) depends on properties of the edible fibrous component such as fibrousness and tensile strength. As described herein, the extruded mixture of isolated plant proteins or solution spun protein fibers can be referred to as connective tissue replicas and the fibrousness and tensile strength of the connective tissue replicas can be controlled by co-variation of extrusion parameters such as temperature, throughput, and die size. For example, combinations of lower extrusion temperatures, medium/low throughputs and smaller dies favor production of highly fibrous tissues with low tensile strength, while higher extrusion temperatures, higher throughputs and larger dies favor production of low fibrousness tissue replicas with very high tensile strengths.

The fibrousness and tensile strength of connective tissue replicas also can be modulated by changing the composition of the extrusion mixture. For example, by increasing the ratio of isolated plant protein (e.g., soy protein such as conglycinin) to wheat gluten to 3:1 w/w, and simultaneously decreasing water content in the extrusion mixture to 50%, a connective tissue replica with thinner fibers and larger tensile strength can be made.

The texture of a meat dough also can be modified by adding cream of tartar to the preparation. For example, meat dough preparations containing cream of tartar may be more cohesive, with a form factor after grinding that is similar to ground beef, such that it is readily shaped. Cream of tartar can be added between 0.05% and 2.5% (e.g., 0.5%).

The appearance of the ground meat replica can be modulated by shredding the edible fibrous component into pieces of the desired size and shape. In some

embodiments, edible fibrous component can be shredded using commercial shredders, e.g., a Cuisineart chopper/grinder, UM 12 with a dull blade attachment, Comitrol shredder (Urschel Laboratories, Indiana) or a similar shredder. The size of the fibers can be adjusted to imitate the fibrous appearance of meat by the type of shredder, choice of blade, and screen type, and adjusting the time of shredding.

In other embodiments, the edible fibrous component can be separated into fibers by carding, using hand-held carders or carding machines, for example, Pat Green carder. By varying the size and spacing of pins on the carding drums, the size of the fibers can be adjusted to imitate the fibrous appearance of meat.

In other embodiments, the edible fibrous component can be separated into fibers by pushing it through rollers (for example, a KITCHENAID® pasta attachment), followed by gentle shredding using, for example, a dull blade on a UM 12 machine. By varying the number of rollers and the spacing between the rollers, the size of the fibers can be adjusted to imitate the fibrous appearance of meat.

The fibrousness, tensile strength, and appearance of the connective tissue replicas can be tailored to imitate specific ground meat products (e.g., ground beef or different cuts of beef that can be ground).

In some embodiments, the edible fibrous component includes soluble or insoluble plant fibers. For example, plant fibers from carrot, bamboo, pea, broccoli, potato, sweet potato, corn, whole grains, alfalfa, kale, celery, celery root, parsley, cabbage, zucchini, green beans, kidney beans, black beans, red beans, white beans, beets, cauliflower, nuts, apple skins, oats, wheat, or psyllium, or a mixture thereof, can be used as the edible fibrous component.

In some embodiments, the edible fibrous component can include compounds that prevent development of off-flavors during the extrusion process. High temperature and low moisture conditions to which the extrusion mixture is exposed during the extrusion process lead to formation of compounds associated with grainy, woody, nutty, rubbery and other off-flavors. Including certain classes of compounds such as antioxidants or carotenoids can help reduce the formation of off-flavor compounds. For example, the

extruded mixture can include canthaxanthin to prevent development of grainy off-flavors. Carotenoids can be about 0% to about 1% by weight of the edible fibrous component.

In some embodiments, meat doughs are formed using roughly equal proportions of isolated plant protein and edible fibrous component. It will be appreciated that the ratio can be varied as desired to tailor the properties of the end product.

In some embodiments, a broth such as a flavored broth can be used in the meat dough. For example, a meat dough can be formed using roughly equal proportions of isolated plant protein and a broth.

In some embodiments, a flavor broth includes flavor mixtures created by pre-reacting (cooking) flavor precursors before adding into the meat dough. Flavor precursor molecules or compositions can be added to a pre-reaction mixture in purified form and/or can be derived from ingredients in the uncooked meat dough that contain and/or are enriched with one or more of the particular flavor precursors or compositions, including, for example, coconut oil, cysteine, glucose, ribose, thiamine, algal oil, lactic acid, and or yeast extract. The resultant flavor and/or aroma profile can be modulated by the type and concentration of the flavor precursors, the pH of the reaction, the length of cooking, the temperature of cooking, the type and amount of iron complex (e.g., an iron containing protein, a heme cofactor such as a heme-containing protein, or ferrous chlorophyllin) or iron salt (iron gluconate), the temperature of the reaction, and the amount of water activity in the product, among other factors. The flavor broth can contain non-animal products (e.g., plant) or it can be a combination of animal and non-animal based precursors (e.g., lard). The flavor broth can bring flavors into the consumable food product that result in taste and smell of beef, bacon, pork, lamb, goat, turkey, duck, deer, yak, bison, chicken or desirable meat flavor.

In some embodiments, a flavored broth can be made by combining an iron complex (e.g., an isolated heme-containing protein) and/or an iron salt (e.g., iron gluconate, iron chloride, oxalate, nitrate, citrate, ascorbate, ferrous sulfate, ferric pyrophosphate, or any other aqueous soluble salt) with one or more flavor precursors and a fat (e.g., a non-animal-based fat), and heating the mixture to obtain a flavored broth containing one or more flavor compounds. Suitable flavor precursors include sugars,

sugar alcohols, sugar derivatives, free fatty acids, triglycerides, alpha-hydroxy acids, dicarboxylic acids, amino acids and derivatives thereof, nucleosides, nucleotides, vitamins, peptides, phospholipids, lecithin, pyrazine, creatine, pyrophosphate and organic molecules. For example, sugars, sugar alcohols, sugar acids, and sugar derivatives can include glucose, fructose, ribose, sucrose, arabinose, glucose-6-phosphate, fructose-6-phosphate, fructose 1,6-diphosphate, inositol, maltose, mannose, glycerol, molasses, maltodextrin, glycogen, galactose, lactose, ribitol, gluconic acid, glucuronic acid, amylose, amylopectin, or xylose. Free fatty acids can include caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, alpha linolenic acid, gamma linolenic acid, arachidic acid, arachidonic acid, behenic acid, eicosapentaenoic acid, petroselinic acid or erucic acid. Triglycerides can include fatty acid esters of caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid, alpha linolenic acid, gamma linolenic acid, arachidic acid, arachidonic acid, behenic acid, eicosapentaenoic acid, petroselinic acid or erucic acid. Amino acids and derivatives thereof can include cysteine, cystine, a cysteine sulfoxide, allicin, selenocysteine, methionine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan, 5-hydroxytryptophan, valine, arginine, histidine, alanine, asparagine, aspartate, glutamate, glutamine, glycine, proline, serine, tyrosine, ornithine, carnosine, citrulline, carnitine, ornithine, theanine, and taurine. Phospholipids can include a plurality of amphipathic molecules comprising fatty acids, glycerol and polar groups. The fatty acids are selected from the group consisting of oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, conjugated oleic acid, or esters of: oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, or conjugated oleic acid, or glycerol esters of oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid,

pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, or conjugated oleic acid, or triglyceride derivatives of oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, or conjugated oleic acid. In some embodiments, the polar groups are selected from the group consisting of choline, ethanolamine, serine, phosphate, glycerol-3-phosphate, inositol and inositol phosphates.

Nucleosides and nucleotides can include inosine, inosine monophosphate (IMP), guanosine, guanosine monophosphate (GMP), adenosine, or adenosine monophosphate (AMP). Vitamins can include thiamine, Vitamin B2, Vitamin B9, Vitamin C, 4-aminobenzoic acid, choline, niacin, Vitamin B8, Vitamin B12, biotin, Betaine, Vitamin A, beta carotene, Vitamin D, Vitamin B6, or Vitamin E. Acids such as acetic acid, caffeic acid, glycolic acid, aspartic acid, pantothenic acid, alpha hydroxy acids such as lactic acid or glycolic acid, tricarboxylic acids such as citric acid, or dicarboxylic acids such as succinic acid or tartaric acid. Peptides and protein hydrolysates can include glutathione, vegetable protein hydrolysates, soy protein hydrolysates, wheat protein hydrolysates, corn protein hydrolysates, yeast protein hydrolysates, algal protein hydrolysates, and meat protein hydrolysates. Extracts can include a malt extract, a yeast extract, or peptone.

For example, in some embodiments, a broth can be made by combining an iron complex (e.g., an isolated and purified heme-containing protein such as leghemoglobin) and/or an iron salt (e.g., iron gluconate, iron chloride, oxalate, nitrate, citrate, ascorbate, ferrous sulfate, ferric pyrophosphate, or any other aqueous soluble salt) with one or more flavor precursors (e.g., a precursor mix shown in Table 2 or Table 13) and a fat (e.g., a non-animal-based fat), and heating the mixture to obtain a flavored broth containing one or more flavor compounds. A non-animal fat can include plant derived oils, algal oils, or oils from bacteria or fungi. Suitable plant derived oils include coconut oil, mango oil, sunflower oil, cottonseed oil, safflower oil, rice bran oil, cocoa butter, palm kernel oil,

palm fruit oil, palm oil, soybean oil, rapeseed oil, canola oil, corn oil, sesame oil, walnut oil, almond oil, flaxseed, jojoba oil, castor, grapeseed oil, peanut oil, olive oil, borage oil, algal oil, fungal oil, black currant oil, babassu oil, shea butter, mango butter, wheat germ oil, blackcurrant oil, sea-buckhorn oil, macadamia oil, saw palmetto oil, conjugated linoleic oil, arachidonic acid enriched oil, docosahexaenoic acid (DHA) enriched oil, eicosapentaenoic acid (EPA) enriched oil, or margarine. The oils can be hydrogenated (e.g., a hydrogenated vegetable oil) or non-hydrogenated. Oil fractions such as stearin (e.g., palm stearin) or olein also can be used. For example, the non-animal fat can be coconut oil, or a combination of coconut oil and stearin. In some embodiments, the fat can contain non-animal (e.g., plant) products, or it can be a combination of animal and non-animal based precursors (e.g., lard), or exclusively animal-based fat.

In some embodiments, a flavored broth can be made by combining water, a non-animal based fat such as coconut oil, and a flavoring agent such as an acid (e.g., lactic acid), a carotenoid (e.g., lutein), or an antioxidant, and heating the mixture to make a broth.

After heating the meat dough as described above, a non-animal fat optionally containing a flavoring agent can be combined with the meat dough. Typically, the meat dough is allowed to cool (e.g., to room temperature) before combining the meat dough with the non-animal fat. The non-animal fat can be flavored by combining the non-animal fat with an iron complex or iron salt and one or more flavor precursors (described above) and heating the mixture to produce the flavor compounds. The heated mixture can be cooled so that the non-animal-based fat can solidify. One or more additional non-animal fats (e.g., algal oil), one or more masking agents (e.g., a lactone such as butyrolactone, delta-tridecalactone, gamma decalactone, delta-dodecalactone, γ-octalactone, dihydro-5-methyl 2(3H)-furanone, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, δ-tetradecalactone, or combinations thereof), or one or more flavoring compounds (e.g., acetoin, carotenoid, antioxidant, vegetable or fruit juice, puree, or extract) can be added before the mixture solidifies to improve the flavor of the non-animal fat. In some embodiments, a combination of 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, butyrolactone, γ-octalactone, and/or δ-tetradecalactone can be used as a

masking agent. Adding one or more lactones (e.g., at a concentration of $10^{-3}$ to $10^{-10}$) can result in a decrease in off flavors perceived as grain, eggy, bitterness, cardboard, livery, or mushroom and increase desired flavors such as creamy, buttery, caramelized, fatty, fresh, and fruity. For example, combinations of two, three, or four lactones can be used to mask properties such as bitterness. In addition, lactones also can be used at concentrations between $10^{-3}$ to $10^{-11}$ to provide desired flavors such as creamy, buttery, caramelized, fatty, fresh, fruity, tallow and meaty notes to the meat replica. Thus, lactones can be used as masking agents or as flavoring agents. Lactones can act as masking agents in other products, including, without limitation, dairy replicas such as milks, cheeses, and yogurts, or protein supplements such as protein bars and protein powders. Combinations of lactones can provide a unique flavor profile important in creating meat flavors (e.g., fatty tallow and sweet aromatics) in a food product such as a meat replica or providing a beef flavor to a non-beef food product. The meat replicas improve in overall liking and meatiness rating when lactones are added to the product. In some embodiments, for example, a combination of butyrolactone, delta-tetradecalactone, and 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone can be used to provide a meaty flavor. Lactones can be added to vegetable oil to make the fat taste more like animal fat and have an increase in perception of mouth coating. The lactones also can be added to increase the sweetness of the product without a change in the sugar content. It is to be noted that agents such as lactones and carotenoids can be used to flavor food replicas (e.g., plant-based food replicas), including meat or cheese replicas, and also can be used to alter the flavor of food products such as meats and cheeses (e.g., to increase meat or cheese flavors).

In some embodiments, carotenoids such as β-carotene, zeaxanthin, lutein, trans-β-apo-8'-carotenal, lycopene, and canthaxanthin can be used to control the creation of desirable flavors and prevent undesirable flavors from being created in food products such as plant based food products (e.g., meat replicas described herein). Carotenoids can be used to reduce off plant flavors in other food products, including dairy replicas. It was found that each type of carotenoid had different properties in creating desirable flavors and controlling off flavors. See, Examples 18 and 26. The carotenoids can increase

sweet and fatty notes that improve meat replicas when added between 0.00001% and 0.1%.

Carotenoids can be added to the meat replica by adding them into the flavor emulsion or the flavor broth. The carotenoids can be added before or after cooking. The carotenoids can be added between 0.00001% and 0.1%. When the carotenoids are added before cooking, they can act as a substrate in the reaction flavor mixtures creating the flavors before their addition into a meat replica. The carotenoids also change the pathway for other flavors being generated by acting as antioxidant. With the addition of carotenoids, the flavor emulsion can have improved flavor quality; there is a decrease in off oxidized notes (waxy, fishy, painty), decrease in other off notes (earthy, mushroom, grainy, beany), and an increase in sweet, fatty, meaty, and fresh flavors. Each carotenoid has different resulting flavor profiles. For example, adding lycopene to the flavor emulsion before cooking results in a bland flavor, whereas β-carotene is very flavorful with added fatty and meaty notes compared to the control. The flavor profile of adding the carotenoids before cooking has a large effect on the flavor profile. When adding carotenoids after cooking, there can still be beneficial effects especially in terms of decreasing off flavor generated with storage. Other flavor precursor molecules in the flavor emulsion or flavor broth have an impact on the effect of the carotenoids. The resultant flavor and/or aroma profile can be modulated by the type and concentration of the flavor precursors, the pH of the reaction, the length of cooking, the temperature of cooking, the type and amount of iron complex (e.g., a heme cofactor such as a heme-containing protein, or ferrous chlorophyllin) or iron salt (iron gluconate), the temperature of the reaction, and the amount of water activity in the product, among other factors, all of which change how the carotenoids change the flavor profile. Particular examples include how carotenoids can reduce or prevent the creation of flavor compounds generated in plant oils, particularly when there is metal in the oil source. Carotenoids, when added to flavor emulsions with fat and oils that have poly unsaturated fatty acids like linoleic, gamma linoleic, DHA, and EPA, can prevent off fishy, painty, and vegetable flavor notes and facilitate the generation of meatiness, and sweet notes.

Particularly carotenoids can reduce grainy, woody, earthy, mushroom, planty and oxidized notes. Carotenoids can be added to different parts of plant-based products to have different impact. Carotenoids can reduce or prevent the creation off flavor compounds generated in wheat flours including wheat gluten. For example, lutein can be added to raw meat dough and reduce overall flavor intensity, reduce grain, woody, and oxidized notes in the cooked meat dough and in the final product. These changes in flavor character is supported by reduction in particular flavor compounds as seen by SPME Gas chromatography–mass spectrometry (GC-MS) in some cases and in other cases there is no change in flavor compounds but an observed reduction in the grain character, suggesting that carotenoids act by changing chemical reactions that are taking place and by masking particular flavors. Additionally, carotenoid added to the meat dough resulted in the samples being described as more fatty and sweeter than the control without carotenoids. The main compounds that decreased with lutein included oxidized flavor compound like alcohols and aldehydes, including (Z)-2-nonenal, (E,E)-2,4-nonadienal, and 1-penten-3-ol; additionally, sulfur compounds were decreased with lutein, including methanethiol, 2-acetylthiazole, and dimethyl sulfide; many of these compounds were also described as grainy and oxidized notes by trained flavor scientist by Gas Chromatography-Olfactometry (GCO).

Antioxidants such as epigallocatechin gallate (EGCG) also can be used to reduce off flavors in food products such as plant-based products (e.g., a meat replica). Antioxidants like EGCG, which is found in (and can be purified from) green tea extracts, can be added from 0.0001% and 0.1%. Antioxidants including EGCG also can be added to meat dough and change the flavor profile of both the cooked meat dough and the consumer products created from the dough. The EGCG decreases the overall flavor of the dough and particular decreases off flavors like grainy, and oxidized flavors as described by trained flavor scientist and confirmed using GCMS.

Vegetables or fruits (juice, purees, or extracts) can be added to meat replicas to increase the perceived meat flavor (e.g., the meatiness) and likeability of the products, as well as increase the perceived fattiness and fat mouth coating. Additionally, they can cause tasters to have an increase in salivation when eating the products, leading to an

increase in perceived juiciness in meat replicas. The type of meat flavors that the vegetable or fruit enhances depends on the type and processing. Examples include added tallow fatty notes from cucumber and melons that are enhanced with cooking; added sweet aromatics, char meat, and savory notes from honeydew; added sweet aromatics, and freshness from pineapple and, added savory, browned meat flavor from tomato.

The vegetable or fruit can be added to meat replicates in the form of juices, purees, extracts created from pressing, juicing, stream distillate, pressure distillation, solvent assisted flavor extraction, or other methods. The vegetable or fruit can be uncooked or untreated, or can be cooked or otherwise treated (e.g., by pasteurization or by enzyme inactivation) to denature proteins (e.g., lipoxygenase). The flavor profiles – both meatiness and amount of off notes, including green or vegetable notes of the fruit or vegetable – can change depending on cooking or other treatment, and depending on the amount and process of cooking or other treatment. Many of the flavors in fruit and vegetable extracts, purees, and juices are created by enzymes. These enzymes can create desirable or undesirable flavors, and the desired flavor depends upon the application for the extracts and juices. Selection of the appropriate type of fruit or vegetable and treatment allows the creation of flavors appropriate for meat replicas. In addition, during processing it can be desirable to deactivate enzymes that can cause off flavors. A particular enzyme that can generate off flavors in the extracts when added to meat replicates is lipoxygenase, which is particularly active in the skin of fruits and vegetables. Disruption of the skin can increase lipoxygenase activity. Therefore, enzyme inactivation before cutting the skin of the fruit or vegetable can help to reduce off flavors. In the generation of fruit and vegetable extracts, purees, or juices, the enzymes can be deactivated by heating above 60°C, high pressure pasteurization, or enzyme inhibition. In some embodiments, for example, lipoxygenase can be inhibited by the addition of inhibitors such as epigallocatechin gallate (EGCG), or by addition of other redox active enzymes. In some embodiments, the whole fruit or vegetable can be cooked or treated before penetrating the skin or cooking can occur after cutting of the product. The cooking or other treatment can be rapid (minutes) or long (hours). When cooking is used, the temperature can be slightly elevated from room temperature to under pressure above

120°C. For example, the fruit or vegetable can be cooked at a temperature of 60-100°C (e.g., 70-80°C, 80-90°C, or 90-100°C). The process can include blending, straining, and or pressing. The seeds can be removed in some cases or the seeds can remain.

For example, cucumber puree added to a meat replica can provide additional fatty tallow flavor but can also bring green vegetable notes along. When the fruit is cooked first, there is a decrease in a few compounds including but not limited to 2-nonenal and 2,6-nonadienal that are responsible for the green, and strong cucumber notes. Additionally, there is an increase in buttery, fatty, and tallow flavors, which could come from an increase in the concentration of lactones as seen by SPME GC-MS. The cooking of tomatoes also enhances the meaty notes while decreasing the green and tomatoes flavors.

The fruits or vegetables flavor liquids can be added to different components of the products, for example added to the meat dough before cooking, added to the fat emulsion after or before cooking, added to a gelled matrix, added to the fully assembled product, or added to the unreacted flavor broth. The extract can be added from 0.0001% for extracts to up to 10% for purees and juices.

Acids such as lactic acid can be added to the meat dough to lower the pH and change the flavor reactions that occur with cooking and processing. Beef has a pH of around 5.5; to achieve meat dough at pH 5.5 additional acidity is needed. Lactic acid brings along a desirable fresh, sourness like that seen in beef.

In other embodiments, the non-animal fat can include an isolated plant protein. For example, an emulsion can be made by combining a plant derived oil, algal oil, or oil from bacteria or fungi and an optional flavor agent with an aqueous solution of an isolated plant protein (e.g., conglycinin from soy), then homogenizing the mixture using, for example, a high-speed homogenizer and heating it for a short period of time, for example, 5 min at 90°C. Physical properties of the emulsion, such as melting temperature, firmness, brittleness, color can be modulated by using different types of isolated proteins, changing the protein concentration, oil-to-water ratio, speed of homogenization, heating temperature and heating time. For example, emulsions with a high oil-to-water ratio and low protein concentration are more brittle and melt easier,

while emulsions with lower oil-to-water ratio and a higher protein concentration are softer, less brittle, and more sticky, and melt at higher temperatures.

In some embodiments, an emulsion can be made by combining a plant derived oil, algal oil, or oil from bacteria or fungi and an optional flavoring agent with an aqueous solution of isolated proteins (for example, soy conglycinin) having a pH >10 (for example, pH 12) with, for example, sodium hydroxide. Agitation, stirring or homogenization of this mixture leads to the formation of an emulsion. After the emulsion is formed, the pH can be adjusted to neutral or an acidic pH by adding, for example, hydrochloric or lactic acid. Physical properties of these emulsions can be controlled by changing protein type, protein concentration, pH level at the time of homogenization, speed of homogenization and oil-to-water ratio.

In other embodiments, an emulsion can be made by mixing a plant derived oil, algal oil, or oil from bacteria or fungi, an aqueous solution of salt and flavoring agents (e.g., flavor precursors), and emulsifiers. For example, mono/di-glycerides, lecithins, phospholipids, Tween surfactants, sodium stearoyl lactylate, or DATEM (diacetyl tartaric acid ester of monoglyceride) can be used as emulsifiers. Physical properties of these emulsions can be controlled by changing emulsifier type and concentration, speed of homogenization and oil-to-water ratio.

The solidified, optionally flavor-infused and/or protein containing fat can be combined with the meat dough, and the mixture of the meat dough and non-animal fat can be broken into smaller pieces, e.g., by chopping, grinding, cutting, mincing, shearing, or tearing. In some embodiments, shearing can be applied to the dough while heating, resulting in a dough that firms up and eventually breaks into pieces during the cooking process. Accordingly, a separate step for breaking into pieces would not be necessary.

A carbohydrate-based gel and an optional binding agent can be added to the dough-fat mixture. The carbohydrate-based gels also are useful for developing the texture of the meat replica and providing juiciness to the final product without making it soggy. Typically, carbohydrate-based gels that have a melting temperature between about 45°C and about 85°C are used. Non-limiting examples of suitable carbohydrate-

based gels include agar, pectin, carrageenan, konjac (also known as glucomannan), alginate, chemically modified agarose, or mixtures thereof.

The binding agent can be an isolated plant protein or a carbohydrate-based gel. Non-limiting examples of suitable plant proteins include RuBisCO, an albumin, a gluten, a glycinin, a conglycinin, a legumin, a globulin, a vicilin, a conalbumin, a gliadin, a glutelin, a glutenin, a hordein, a prolamin, a phaseolin, a proteinoplast, a secalin, a triticeae gluten, a zein, an oleosin, a caloleosin, a steroleosin, or mixtures thereof (e.g., albumin fractions). The plant proteins can be obtained from any source, including soy, peas or lentils. In some embodiments, useful binding agents can be obtained from a non-plant-based source. For example, egg albumin or collagen can be used as a binding agent in some embodiments.

When the binding agent is a protein, it is useful for the denaturation temperature of the protein to be less than the melting temperature of the carbohydrate-based gel. For example, the denaturation temperature of suitable protein-binding agents (e.g., RuBisCO, albumin, soybean conglycinin, or a gluten, or mixtures thereof) can be between about 40°C and about 80°C. This allows the carbohydrate based gel to melt after the protein binding agent denatures and binds the meat replica together, and provides better texture and form to the meat replica.

In some embodiments, the proteins used as binding agents may be chemically or enzymatically modified to improve their textural and/or flavor properties. For example, proteins may be partially proteolyzed using food-grade enzymes such as papain to result in better water-release profile during gelation and cooking. In some embodiments, the proteins used as binding agents may be chemically or enzymatically modified to modify the denaturation and gelling temperature of the proteins, for example, to achieve a specific gelling temperature (e.g., 52°C to mimic myosin or 68°C to mimic actin). In some instances, proteins such as proteases may be used to reduce bitterness that may be present in purified protein fractions.

In some embodiments, the binding agent is a carbohydrate-based gel. For example, a carbohydrate based gel that becomes firm upon cooking to 140°F to 190°F (e.g., 150°F to 180°F). Non-limiting examples of carbohydrate-based gels include

methylcellulose, modified starches such as hydroxypropylmethyl cellulose, guar gum, locust bean gum, xanthan gum, or mixtures thereof.

In addition, an iron-complex and/or an iron salt and a flavoring agent can be added to the meat replica. The iron-complex and/or iron salt can be the same or different than the iron-complex and/or iron salt used to flavor the meat dough, connective tissue replica, or non-animal-based fat. The flavoring agent can be a flavor precursor or mixture of flavor precursors (described above) such that upon cooking the meat replica, the iron-complex and/or iron salt and flavor precursor can react and produce flavor compounds. The flavoring agent also can be a flavoring such as yeast extract, hydrolyzed protein, or a flavor compound. Flavor compounds can include, for example, phenylacetic acid, (E,E)-2,4-nonadienal, aquaresin onion, oil soluble onion, p-cresol, acetonyl acetate, 4-hydroxy-2,5-dimethyl-3(2H)-furanone, (E,E)-2,4-octadienal, 2-methyl-1-butane thiol, 2-methyl-3-furyl tetrasulfide, ethyl 2-mercaptopropionate, 2-mercapto-3-butanol (mixture of isomers), n-decane-d22, oil soluble garlic, sulfurol, sulfuryl acetate, mercapto-3-butanol, spiromeat, 1-penten-3-one, 2-methyl-3-furanthiol, 2-methyl-3-tetrahydrofuranthiol, oleic acid, dipropyl trisulfide, difurfuryl disulfide, methylcyclopentenolone, 3-methylthio hexanal, butyric acid, butyrolactone, 5-methyl-2(3H)-furanone, furaneol, 1-(1H-pyrrol-2-yl)-ethanone, hexanoic acid, and combinations thereof. Additional flavor compounds may be purchased commercially from companies such as Sigma Aldrich (St. Louis, MO), Penta Manufacturing Co. (Fairfield, NJ), Advanced Biotech (Totowa, NJ), Firmenich (Meyrin, Switzerland), Givaudan (Vernier, Switzerland), International Flavors and Fragrances (New York, NY), and Wild Flavors (Erlanger, KY).

In some embodiments, seasonings agents such as edible salts (e.g., sodium or potassium chloride), garlic, or herbs (e.g., rosemary, thyme, basil, sage, or mint), emulsifiers (e.g., lecithin), additional fiber (e.g., zein or inulin), minerals (e.g., iodine, zinc, and/or calcium), meat shelf life extenders (e.g., carbon monoxide, nitrites, sodium metabisulfite, Bombal, vitamin E, rosemary extract, green tea extract, catechins and other antioxidants) can be incorporated into the meat replica.

Meat replicas described herein also can include a natural coloring agent such as turmeric or beet juice, or an artificial coloring agent such as an azo dye, triphenylmethane, xanthene, quinine, indigoid, titanium dioxide, red #3, red #40, blue #1, or yellow #5, or any combination of natural and/or artificial coloring agents.

Any of the replicas described herein can be shaped to the desired use, e.g., formed into patties, loaves, chubs, meatballs, or nuggets, and used in any type of food product that ground meat would be used, e.g., as taco filling, or in casseroles, sauces, toppings, soups, stews, meatballs, or meatloaves. In some embodiments, a meat replica can be formed, for example, into meatballs or nuggets, and then coated with breadcrumbs, rice, or a flour (e.g., oat flour or coconut flour) for ease of convenience.

*Meat Replica*

A meat replica described herein can include about 5% to about 88% (e.g., about 10% to about 40%, about 25% to about 35%, about 40% to about 88%, or 45% to about 60%) by weight of a meat replica dough; about 0% to about 40% (e.g., about 15% to about 25%) by weight of a carbohydrate-based gel; about 3% to about 35% by weight of a non-animal fat (e.g., about 10% to about 15%); about 0.00001% to about 10% by weight of a flavoring agent; about 0% to about 15% (e.g., about 2% to about 15% or about 2% to about 10%) by weight of a binding agent; and about 0.01% to about 4% (e.g., about 0.05% to about 1%, or about 0.2% to about 2%) by weight of an iron complex such as a heme-containing protein and/or an iron salt. The amount of flavoring agent can vary depending on the type of flavoring agent. In some embodiments, a flavoring agent can be about 0.5% to about 7% of the meat replica. For example, a flavoring agent such as a mixture of flavor precursors can be about 0.5% to about 7% of the meat replica (e.g., about 1% to about 3%; about 3% to about 6%; about 4% to about 7%). In some embodiments, a flavoring agent such as a flavoring compound can be about 0.00001% to about 2% of the meat replica.

As described herein one or more, two or more, three or more, or four or more of the components can include a flavoring agent. For example, the meat dough can include a flavoring agent (e.g., a flavoring compound produced by combining an iron complex or

iron salt with one or more flavor precursors and heating) or can include a flavoring such as yeast extract in the edible fibrous component. The non-animal fat also can include a flavoring agent (e.g., a flavoring compound produced by combining an iron complex or iron salt with one or more flavor precursors and heating). The replica also can include an iron complex or iron salt and one or more flavor precursors that can react upon cooking the replica, enhancing the sensory experience of cooking the replica. In addition, the replica can include a flavoring or flavoring compound.

In some embodiments, the components are produced at the desired particle sizes and then compressed together for 5 minutes to 24 hours (e.g., 10 minutes to 2 hours, 1 to 4 hours, 4 to 8 hours, 6 to 12 hours, or 12 to 24 hours) to allow the components to adhere into a meat replica. The meat replica may then be ground to replicate the attributes of a ground meat. The meat replica can be compressed into any desired form to replicate the shape and density of, for example, a steak, a tenderloin, a chop, or a fillet. The meat replica also may be further processed into a processed meat such as a sausage.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

## EXAMPLES

### EXAMPLE 1

**Isolation and Purification of Leghemoglobin**

A nucleic acid encoding *Glycine max* leghemoglobin C2 (Uniprot KB P02236) with an N-terminal His6 epitope tag and a TEV cleavage site was cloned into the pJexpress401 vector (DNA2.0), and transformed into *E. coli* BL21. Transformed cells were grown by fed-batch fermentation supplemented with kanamycin, 0.1 mM ferric chloride and 10 µg/ml 5-aminolevulinic acid. Expression was induced by 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and cells were grown at 30°C for 24 hr. Cells were concentrated by centrifugation and resuspended in 20 mM potassium phosphate pH 7.8, 100 mM NaCl. Cells were lysed by high-pressure homogenization and clarified by centrifugation and microfiltration. Leghemoglobin was purified from the soluble lysate using zinc-charged IMAC sepharose fast flow resin (GE Healthcare).

Bound leghemoglobin was eluted off the resin with 500 mM potassium phosphate monobasic, 100 mM NaCl. Purified leghemoglobin was neutralized and concentrated using ultrafiltration. Concentrated leghemoglobin was reduced with 20 mM Na dithionite. Na dithionite was removed by diafiltration. Leghemoglobin concentration was determined by soret peak absorbance and adjusted to 60-70 mg/ml. The final leghemoglobin product was frozen in liquid nitrogen, lyophilized, and stored at -20°C. Purity (partial abundance) of leghemoglobin was analyzed by SDS-PAGE and determined to be ~ 80%. Analysis of UV-VIS spectra (250-700nm) revealed spectral signature consistent with heme-loaded leghemoglobin.

*Glycine max* leghemoglobin C2 and eight *Pichia pastoris* heme biosynthesis genes (listed in Table 1) were cloned into the *Pichia pastoris* expression vector pJA (BioGrammatics Inc.; Carlsbad, CA) under the control of the *pAOX1* methanol inducible promoter. *Pichia pastoris* strain Bg11 (BioGrammatics, Inc.) was transformed with linearized plasmids, and stable integrants were selected by antibiotic resistance.

**TABLE 1**

| Gene | Species | Function | UniprotKB # |
|---|---|---|---|
| Leghemoglobin C2 | *Glycine max* | Leghemoglobin production | P02236 |
| ALA synthase | *Pichia pastoris* | Heme enzyme-step 1 | F2QS71 |
| ALA dehydratase | *Pichia pastoris* | Heme enzyme-step 2 | F2QZA1 |
| Porphobilinogen deaminase | *Pichia pastoris* | Heme enzyme-step 3 | F2QP90 |
| Uroporphyrinogen III synthase | *Pichia pastoris* | Heme enzyme-step 4 | F2QSR5 |
| Uroporphyrinogen III decarboxylase | *Pichia pastoris* | Heme enzyme-step 5 | F2QUW1 |
| Coproporphyrinogen oxidase | *Pichia pastoris* | Heme enzyme – step 6 | F2QUX3 |

| | | | |
|---|---|---|---|
| Protoporphyrinogen III oxidase | *Pichia pastoris* | Heme enzyme-step 7 | F2R0D |
| Ferrochelatase | *Pichia pastoris* | Heme enzyme-step 8 | F2QWX6 |
| Sh bleomycin | *Streptoalloteichus hindustanus* | Resistance to Zeocin | P17493 |
| Beta lactamase | *E. coli* | Resistance to ampicillin | Q9L5C7 |
| Hygromycin | *E. coli* | Resistance to hygromycin | P00557 |
| NatR | *Streptomyces noursei* | Nourseothricin resistance | O33583 |
| Neomycin resistance | Synthetic bacterial transposon Tn5 | Resistance to geniticin (G418) | n/a |

Transformed *Pichia* cells were grown by fed-batch fermentation and leghemoglobin expression was induced with methanol for 120 hours at 30°C. Cells were concentrated by centrifugation, resuspended in water, and lysed by high pressure homogenization. Solids were removed by treatment with Tramfloc 863A, centrifugation, and 0.2 μm microfiltration (Koch Membrane Systems). The soluble lysate was concentrated and diafiltered with water using 3 kDa ultrafiltration (Spectrum Laboratories). The formulated lysate was partially purified using HPA25L anion exchange resin (Mitsubishi) to a final purity of ~40%. The partially purified leghemoglobin solution was re-formulated by concentration and water diafiltration using 3 kDa ultrafiltration (Spectrum Laboratories) and further purified using Q Fast Flow anion exchange resin (GE Lifesciences). The final leghemoglobin product was concentrated using 3 kD ultrafiltration and frozen at -20°C. The final product was ~80% pure and contained 80 g/L leghemoglobin.

## EXAMPLE 2

### Isolation of RuBisCO

One kg of fresh spinach leaves was macerated in a Vita-prep 3 blender (Vitamix Corp, Cleveland, OH) at a ratio of 1:1 with potassium phosphate buffer (pH 7.4) containing 0.1M NaCl. The extraction was performed for 10 min at the highest setting (3HP motor). The temperature was maintained at less than 30°C. The pH was adjusted to 7.4 post-grinding using a 10 M NaOH solution. The homogenate was centrifuged at 3500 g for 5 minutes, simulating the conditions at scale (with a GEA Westfalia decanter GCE-345 at about a 1 gpm feed rate). The pellet was discarded. The liquid centrate (about 1.6 L) then was microfiltered using a 0.2 μm modified polyethersulfone (mPES) membrane in a hollow fiber format (KrosFlo K02E20U-05N from Spectrum Laboratories Inc. Rancho Dominguez, CA). The retentate (about 0.25 L) was diafiltered using about 1.5 L of the extraction buffer. The permeate from this filtration step (~3 L) was concentrated using a 10 kDa mPES membrane (MiniKros N02E010-05N from Spectrum Laboratories Inc. Rancho Dominguez, CA) to about 0.1 L. The protein concentrate had a pH of about 7.4. A concentrated acid solution such as 6M Hydrochloric Acid was slowly added to the concentrate to decrease the pH to 5. The mixture was stirred vigorously for 30 minutes using a magnetic stir plate or a homogenizer and then centrifuged at 3500 g for 5 minutes to obtain an off white pellet and a brown centrate. The centrate was discarded and the protein pellet was washed with deionized water. The pellet was resuspended in 0.05-0.1L DI water. The solution was mixed vigorously into a uniform slurry and the pH was slowly raised to 11 using a concentrated base solution such as 10M sodium hydroxide. The resulting solution was clear yellow. The pH was then reduced to 9 to maintain the clear mixture. The product was dried using a spray dryer, or frozen and dried using a freeze dryer. This material was analyzed using a Leco FP-528 Nitrogen Combustion Analyzer (Leco, St. Joseph, MI) by the AAOC method (AOAC, 2000). Protein was calculated as % nitrogen x 6.25 and was calculated to be 86% protein. The product obtained was slightly decolored and retained the low temperature denaturation property.

## EXAMPLE 3

### Isolation and decolorization of RuBisCO

One kg of fresh spinach leaves were macerated in a Vita-prep 3 bender (Vitamix Corp., Cleveland, OH) in a ratio of 1:1 (w/w) with potassium phosphate buffer (pH 7.4) containing 8% (w/v) PEG (Carbowax Sentry PEG 8000; Dow Chemicals, Midland, MI) and 0.1% (w/v) cationic flocculant (863A; Tramfloc, Inc., Houston, TX). The extraction was performed for 3 minutes at the highest setting (3HP motor) maintaining the temperature at less than 30°C at all times. The pH was adjusted to 7.4 post-grinding, using a 10 M NaOH solution. The homogenate was centrifuged at 3500 g for 5 minutes using a bench top centrifuge (Allegra X15R, SX4750 rotor; Beckman Coulter, Inc., Pasadena, CA). The pellet was discarded and the supernatant (about 1.6 L) was collected separately. Magnesium sulfate heptahydrate salt (K+S KALI GmbH, Kassel, Germany) was added to the supernatant to attain 1M concentration. The solution was mixed thoroughly and centrifuged at 5451g for 3 minutes using a bench top centrifuge (Allegra X15R, SX4750 rotor; Beckman Coulter, Inc.). Three layers formed in the centrifuge bottle, and the remaining green solids separated out as a pellet (about 0.1 L). The PEG layer (about 0.3 L) separated and formed the top layer, selectively fractionating the color compounds and odorous compounds. A clear product remaining in the middle layer was then microfiltered using a 0.2 μm modified polyethersulfone (mPES) membrane in a hollow fiber format (Spectrum Laboratories Inc.). The retentate (about 0.25 L) was diafiltered using about 0.75 L of 1M magnesium sulfate solution. The permeate from this filtration step (about 3 L) was concentrated using a 70 kDa mPES membrane (Spectrum Laboratories, Inc.) to about 0.1 L. This was further diafiltered with about 0.5L DI water in 5 steps. The protein concentrate had a pH of about 7 and conductivity less than 5 mS/cm. The resulting protein concentrate was clear pale yellow. The product was dried using a spray dryer, or frozen and dried using a freeze dryer. This material was analyzed using the standard 660 nm Pierce protein assay and SDS gel densitometry. The dry solids were analyzed using the IR moisture analyzer. The flocculant and PEG concentration in the final product were analyzed using titration methods. The protein concentration was about 91% (w/w), and the total solids about 95% (w/w). The PEG and flocculant

concentrations were analyzed at less than 0.2% (w/w). The product was over 90% pure with over 90% recovery through the process. The product obtained was decolored and retained the low temperature denaturation property.

## EXAMPLE 4

**Isolation of soluble soybean conglycinin**

The soluble conglycinin fraction of soybean proteins (the 7S fraction) was obtained using the following method: 1 kg of defatted soy flour (CHS HONEYSOY® PDI 90) was mixed with 10 L deionized water in a vessel fitted with an overhead mixer. After the clumps of flour were dispersed, the pH of the slurry was adjusted to 8 with 2N NaOH. The mixture was stirred for 1 hour at 4°C to extract all soluble proteins. The pH of the mixture then was adjusted to 5.8 using 2N $H_2SO_4$ and mixed for an additional 1 hour at 4°C. The mixture was then centrifuged to remove insoluble carbohydrate and protein (glycinins) at 10000 g for 10 minutes in a JLA 8.1 rotor (JHC centrifuge, Beckman Coulter Inc.). The soluble supernatant was further acidified to pH 4.5 using 2N $H_2SO_4$ and mixed for 1 hour at 4°C. The acidified mixture was then centrifuged at 10000g for 10 minutes to collect the precipitated proteins and the supernatant containing lipoxygenase, soybean lecithin and trypsin inhibitors was discarded. The conglycinin in the pH 4.5 precipitated protein fraction was resolubilized by resuspending the pellet in 4 volumes of water (approximately 2L) and adjusted to pH 8 using 2N NaOH. The mixture was stirred at 4°C for 1 hr. The pH of the mixture was once again dropped to 5.8 using 2N $H_2SO_4$ to minimize co-purification of contaminant proteins. The mixture was centrifuged at 15000g for 20 minutes to collect the soluble conglycinin in the supernatant. The conglycinin fraction was concentrated using ultrafiltration (70 kDa mPES ultrafiltration membrane, 2600sq. cm, Spectrum Laboratories Inc.). The resulting protein solution (approx. 0.5L at 10% protein concentration) comprises 55-65% pure conglycinin and gels at 65°C. The protein then was freeze-dried and stored at room temperature until used in making of the meat-replicas.

## EXAMPLE 5

**Preparation of dough broth for pre-flavoring meat dough**

A dough broth was created by mixing a 1X precursor mix 1 (see Table 2), 0.5% leghemoglobin (LegH, isolated and purified as described in Example 1), and 18% Refined, Bleached, and Deodorized (RBD) coconut oil (from Shay and company, Milwaukie, OR), and stirring as the solution was heated until boiling, then simmered at a low boil for 10 minutes. This solution is referred to as the "dough broth" and was used for creating the meat dough of Example 10. Incubating the coconut oil with the LegH and precursor mix generates savory or meaty flavors in the broth including caramelized, fatty, beefy, nutty, sulfur, metallic, buttery, sweet, savory, and umami.

**TABLE 2**
**Composition of magic mixes**

| Precursor | Precursor mix 1 (mM) | Precursor mix 2 (mM) |
|---|---|---|
| Alanine | 15.0 | 7.5 |
| Arginine | 0.6 | 0.3 |
| Asparagine | 0.8 | 0.4 |
| Aspartate | 0.8 | 0.4 |
| Cysteine | 9.0 | 9.0 |
| Glutamic acid | 50.0 | 50.0 |
| Glutamine | 0.7 | 0.3 |
| Glycine | 1.3 | 0.7 |
| Histidine | 0.6 | 0.3 |
| Isoleucine | 0.8 | 0.4 |
| Leucine | 2.0 | 1.0 |
| Lysine | 5.0 | 2.5 |
| Methionine | 1.0 | 0.5 |
| Phenylalanine | 0.6 | 0.3 |
| Proline | 0.9 | 0.4 |
| Threonine | 0.8 | 0.4 |
| Tryptophan | 1.5 | 0.8 |
| Tyrosine | 0.6 | 0.3 |
| Valine | 1.0 | 0.5 |
| Glucose | 5.6 | 2.8 |
| Ribose | 5.0 | 5.0 |
| Thiamine | 0.2 | 0.2 |
| IMP + GMP | 2.0 | 1.0 |
| Lactic acid | 9.0 | 4.5 |
| Creatine | 3.0 | 1.5 |
| L-Taurine | 40.0 | 20.0 |
| Glutathione | 2.0 | 1.0 |
| N-Acetyl L-cysteine | 10.0 | 5.0 |

**EXAMPLE 6**

**Preparation of flavor infused fat replica**

A flavored fat replica was created by mixing a solution of LegH (from Example 1) at 0.5%, 1x precursor mix 1 (Table 1), and 30% RBD coconut oil (Shay and company,

Milwaukie, OR) and stirring the mixture as it was heated until boiling, then simmered at a low boil for 10 minutes. The solution was cooled to allow the oil to solidify. Once the oil was solidified, it was separated from the aqueous layer and used in preparing the burger described in Example 11. Incubating the coconut oil with the LegH and precursor mix infuses flavor notes in the oil including savory, meaty, beef fat, slightly sweet and sulfur.

## EXAMPLE 7

### Preparation of "soft connective" tissue replica

A soft connective tissue replica was prepared using soy protein isolate (SUPRO® EX38 (Solae)), Vital wheat gluten (131100, Guisto's Specialty Foods, San Francisco, CA), and water. A Nano 16 extruder ((Leistritz Advanced Technologies Corp., Somerville, NJ) was used, with a custom-made cooling die (round, ID 6.5 mm, length 300 mm), a cooling water circulator, and a high pressure water pump (Optos, Eldex Laboratories Inc.).

Fifty (50) g of soy protein isolate and 50 g of wheat gluten powder were thoroughly mixed with manual mixing and tumbling for 5 min, and then loaded into the loading tube of the extruder's batch feeder. The dry mixture was fed into the extruder at the rate of 2.4 g/min. Water was fed by the pump into the second zone of the extruder's barrel at the rate of 3.6 ml/min. Screw speed of the extruder was maintained at 120 RPM. A temperature gradient was set along the extruder barrel as follows: feed zone - 25°C, zone 1 – 30°C, zone 2 – 60°C, zone 3 – 110°C, zone 4 – 110°C. The die plate was neither actively heated, nor cooled. The cooling die was cooled by the cooling water circulator maintaining the die at 24°C.

The soft connective replica produced by this method was off-white in color and highly fibrous/filamentous, with a neutral taste and flavor. Tensile strength of this material was low and comparable to that of tender beef roast.

## EXAMPLE 8

**Preparation of "tough fibrous connective" tissue replica**

To prepare a tough fibrous connective tissue replica, 50 g of soy protein isolate and 50 g of wheat gluten powder were thoroughly mixed by manual mixing and tumbling for 5 min, and loaded into the loading tube of the extruder's batch feeder. The dry mixture was fed into the extruder at the rate of 3.6 g/min. Water was fed by the pump into the second zone of the extruder's barrel at the rate of 5.4 ml/min. Screw speed of the extruder was maintained at 120 RPM. A temperature gradient was set along the extruder barrel as follows: feed zone - 25°C, zone 1 – 37°C, zone 2 – 61°C, zone 3 – 135°C, zone 4 – 135°C. The die plate was neither actively heated, nor cooled. The cooling die was cooled by the cooling water circulator maintaining the die at 26°C.

The tough fibrous connective replica produced by this method was light tan in color and was a fibrous/layered material having a neutral taste and flavor. Tensile strength of this material was high and comparable to that of cooked beef tendons.

## EXAMPLE 9

**Preparation of pre-flavored "soft connective" tissue**

To prepare a flavored soft connective tissue replica, 50 g of soy protein isolate, 50 g of wheat gluten powder, 1 g of yeast extract #9 (Flavor house Inc., X11020), and yeast extract #21 (Biospringer 1405/40 MGl) were thoroughly mixed by manual mixing and tumbling for 5 min, and loaded into the loading tube of the extruder's batch feeder and extruded as described in Example 7. The pre-flavored soft connective tissue had a savory taste, with an increase in the flavor complexity and decrease in off notes as compared to the soft connective tissue produced in Example 7.

## EXAMPLE 10

**Preparation of "meat dough"**

"Meat dough" for the ground beef-replica was prepared using the following ingredients:

a. Vital wheat gluten (#131100, Guisto's Specialty Foods, San Francisco, CA)

b. soft connective tissue replica (see Example 7, the pre-flavored soft connective tissue of Example 9 also could be used)

c. dough broth (see Example 5)

A 100g portion of meat dough was prepared as follows. First, 25 g of the soft connective tissue replica was hand shredded lengthwise into approximately 1-inch long pieces. The shredded soft connective replica was combined with 25 g dry wheat gluten in a mixing bowl and gently hand tossed to mix evenly. In a separate container, 50 mL of dough broth was brought to a boil and simmered on low for 10 minutes. The hot dough broth was added to the dry gluten-connective tissue replica mix and kneaded on a stand mixer (e.g., KITCHENAID® Professional 600 Series 6 Quart Bowl-Lift Stand Mixer model KP26M1XER with dough-mixing attachment, set at speed 2) for 30 seconds to form the meat dough.

Once kneaded, the meat dough was formed into a slab and transferred to another vessel for steaming. The meat dough was steamed (in an Aroma Rice cooker Model No. ARC-1030SB) until the internal temperature reached approximately 200°F and held at that temperature for additional 20 minutes. After steaming, the dough was transferred to a container on ice to allow it to cool down to room temperature. The steamed meat dough also can be stored at this point at 4°C for up to a week. Before forming the beef-patty replicas, the steamed meat dough was hand torn into smaller pieces, approximately 1 inch cubes. The mixture is now ready for use in the formation of a beef-patty replica (described in Examples 11 and 12).

## EXAMPLE 11

### Assembly and cooking of burger

A replica burger containing the ingredients in Table 3 was prepared. The 1% agar preparation was made by adding 1 g of agar powder (item 6410, Now Foods Bloomingdale, IL) to 99 ml of water in a glass beaker. The agar was fully solubilized by heating the mixture to 100°C while stirring, and then cooling in an ice bath for 20 – 30 min until a firm gel firmed. The gel was then transferred to a coffee grinder (Cuisinart®

Model # CUI DCG-20N) and ground for 20 seconds to break it into small pieces for mixing.

**TABLE 3**
**Composition of Burger**

| Ingredient | % |
|---|---|
| Meat dough (Example 10) | 54.1 |
| 1% agar preparation | 20.0 |
| Coconut oil with flavor system (Example 6) | 13.5 |
| 16x precursor mix 2 (Table 1) | 5.9 |
| RuBisCO preparation (dry) (Example 2) | 5.3 |
| LegH preparation (dry) (Example 1, *E. coli*) | 1.2 |
| Total | 100 |

The meat dough (Example 10) and flavored coconut oil (Example 6) were mixed by hand in a bowl. A typical batch size was 100 g to 2000 g. The mixture was then ground using a stand mixer fitted with a food grinder attachment (KITCHENAID® Professional 600 Series 6 Quart Bowl-Lift Stand Mixer model KP26M1XER and KITCHENAID® Food Grinder model FGA, St. Joseph, MI) on speed setting 1. The mixture was fed by a screw conveyor past a rotating knife installed in front of a fixed-hole plate. The ground tissue was collected in a bowl.

The following ingredients then were added in the ratios shown in Table 3: 1% agar preparation, RuBisCO (approximately 50% by weight RuBisCO), 16x precursor mix 2, and LegH (350-650 mg/g). Ingredients were added in the order listed here and the material was mixed gently after each addition. Thirty (30) g or 90 g portions of ground tissue were then formed by hand into round patty shapes. Typical dimensions for 30 g patties were 50 mm x 12 mm. Typical dimensions for 90 g patties were 70 mm x 18 mm. During the assembly, grinding, and forming, all materials were kept cold (4 – 15°C). Patties were refrigerated until cooked. Patties were cooked on a preheated (325 – 345°F) non-stick skillet and heated to an internal temperature of 160°F while flipping every 2 minutes. Typical cook times ranged from 12 to 15 minutes. Cooked patties had an appearance, texture, and flavor similar to ground beef as judged by a trained sensory

panel. In addition to cooking in a patty format, the unformed material also can be used in a variety of dishes such as taco filling, casseroles, sauces, toppings, soups, stews, or loaves.

## EXAMPLE 12

### Adding flavor molecules to the burger

A replica burger containing the ingredients in Table 4 was prepared.

**TABLE 4**
**Composition of burger**

| Ingredient | % |
|---|---|
| Unflavored meat dough | 54.1 |
| 1% agar preparation (see Example 11) | 20.0 |
| Coconut oil | 13.5 |
| 16x precursor mix 2 (Table 1) | 5.9 |
| RuBisCO preparation (dry) (Example 2) | 5.3 |
| LegH preparation (dry) (Example 1, *E. coli*) | 1.2 |
| Phenylacetic acid (CAS # 103-82-2) | 0.003% |
| Furaneol (CAS #3658-77-3) | 0.003% |
| 2-Mercapto-3-butanol (CAS # 37887-04-0) | 0.0015% |
| Garlic, oil soluble (Kalsec) | 0.0015% |
| Total | 100 |

The unflavored meat dough (wheat gluten, unflavored soft connective tissue, and water) and coconut oil were mixed by hand in a bowl and ground as described in Example 11. The following ingredients were then added at the ratios in Table 4: 1% agar preparation, RuBisCO (approximately 50% by weight), 16x precursor mix 2, and LegH (350-650 mg/g). Flavor compounds and garlic oil were diluted to 1x $10^{-2}$ then added at the concentration listed in Table 4. Ingredients were added in the order listed here and the material was mixed gently after each addition. 100 g portions of ground tissue were then formed by hand into round patty shapes. During the assembly, grinding, and forming, all materials were kept cold (4 – 15°C). Patties were cooked on a preheated (325 – 345°F) non-stick skillet and heated to an internal temperature of 160°F while flipping

every 2 minutes. The patties typically cooked in 12 to 15 minutes. Cooked patties had appearance, texture, and flavor similar to ground beef. These patties did not have as much depth in flavor as burgers created with pre-flavored dough and fat, however these burgers had additional flavor notes associated with beef as judged by a trained sensory panel.

## EXAMPLE 13

**Preparation of solution-spun zein fibers for connective tissue replica**

Solution-spun zein fibers were produced using zein powder (Prairie Gold Inc., Bloomington, IL), ethanol (190 proof Everclear by Luxco), sodium hydroxide (Fisher Scientific), glycerol, (Fisher Scientific), and water. Fifty (50) g of zein powder, ten (10) g of glycerol, thirty six (36) g of ethanol, and four (4) g of water were mixed in a glass jar for 5 min using a homogenizer. The pH of the solution was adjusted to 7.0 with a 1M solution of sodium hydroxide in ethanol. The solution was loaded into a 1 ml syringe with a 30-gauge needle. The syringe was mounted on a syringe pump (New Era Syringe Pumps, Inc.), which was installed vertically, needle pointing down, over a custom-made fiber spooler with a Delrin spool. The spooling rod was set to rotate at 3 RPM.

The syringe pump was set to 0.12 ml/h and activated. When a drop of solution formed at the end of the needle, it was picked up with a spatula and stretched into a fiber. The end of the fiber was touched to the spooling rod until it adhered. A heating fan, pointing to the spooling rod at the place of fiber attachment, was then switched on to facilitate fiber drying. Fiber was spooled until the syringe was empty, after which the syringe was reloaded and the above procedure repeated. After spooling, the fibers were pre-cured in a 110°C oven for 1 hour, and then finished by baking at 175°C for 5 minutes.

Zein fibers obtained by this process were semi-clear, light yellow colored fibers, 60-80 micrometer thick, as measured by light microscopy. They were very flexible in air and water, maintaining high tensile strength similar to animal connective tissue (10-15 MPa) even after water immersion for several hours.

## EXAMPLE 14

**Preparation of dough broth with iron gluconate for pre-flavoring meat dough**

A dough broth was created by mixing a 1X precursor mix 1 (see Table 2), 1 mM iron gluconate, and 18% refined, bleached, and deodorized (RBD) coconut oil (Shay and company), and stirring as the solution was heated until boiling, then simmered at a low boil for 30 minutes. This solution is referred to the "iron gluconate dough broth" and can be used instead of the "dough broth" that is used in the meat dough of Example 10. Incubating the coconut oil with the iron gluconate and precursor mix generates savory and or meaty flavors in the broth including pork, beefy, sulfur, metallic, sweet, savory, and umami in the broth.

## EXAMPLE 15

**Preparation of flavor infused fat replica with iron gluconate**

Flavored fat replica containing iron gluconate was created by mixing a solution of LegH at 0.25%, 1 mM iron gluconate, 1x precursor mix 1 (Table 1), and 30% RBD coconut oil (Shay and company) and stirring the mixture as it was heated until boiling, then simmered at a low boil for 10 minutes. The solution was cooled to 4°C to allow the oil to solidify. Once the oil was solidified, it was separated from the aqueous layer and used instead of the flavored fat replica in preparing the burger described in Example 13. Incubating the coconut oil with the LegH, iron gluconate, and precursor mix infuses flavor notes in the oil including savory, meaty, beef fat notes, sweet, metallic and sulfur notes.

## EXAMPLE 16

**Preparation of meat dough containing cream of tartar**

A meat dough was prepared as follows using the ingredients shown in Table 5.

**TABLE 5**
**Composition of Meat Dough**

| Ingredient | % |
|---|---|
| Gluten flour | 48.2 |
| Water | 35.0 |
| Coconut oil | 9.0 |
| 1M lactic acid solution | 6.0 |
| Hydrolyzed vegetable protein | 1.3 |
| Cream of tartar | 0.5 |
| Total | 100.0 |

First, water, coconut oil, 1M lactic acid solution, and hydrolyzed vegetable protein were mixed and heated to 60°C to make a broth. Heating was done to melt and help distribute the coconut oil. Gluten flour (vital wheat gluten #131100, Guisto's Specialty Foods, San Francisco, CA) and cream of tartar were mixed in a separate container. The warm broth was then added to the dry mixture and kneaded with a stand mixer (e.g., KITCHENAID® Professional 600 Series 6 Quart Bowl-Lift Stand Mixer model KP26M1XER with dough-mixing attachment, set at speed 2) for 30 seconds to form the meat dough. Once kneaded, the meat dough was formed into a slab and transferred to another vessel for steaming. The meat dough was steamed (e.g., in an Aroma Rice cooker Model No. ARC-1030SB) until the internal temperature reached approximately 88°C. After steaming, the dough was transferred to a container on ice to allow it to cool down to 4°C. Cream of tartar modified the texture of the dough in an advantageous way. When compared to the meat dough of Example 10, the meat dough of the present example was more cohesive, had a form factor after grinding that was more similar to ground beef, and had improved raw handling characteristics so that it was easier to shape and form patties.

**EXAMPLE 17**

**Preparation of meat dough containing lutein**

A meat dough was prepared as follows using the ingredients shown in Table 6.

**TABLE 6**
**Composition of Meat Dough**

| Ingredient | % |
|---|---|
| Water | 50 |
| gluten flour | 40 |
| coconut oil | 9.0 |
| lutein SAF preparation | 1 |
| Total | 100.0 |

First, water, coconut oil (Shay and company, Milwaukie, OR), and lutein (FloraGLO Lutein 20% SAF, DSM Nutritional Products, Overland Park, KS) were mixed and heated to greater than 25°C to make a broth. Heating was done to melt and help distribute the coconut oil and lutein. The warm broth was then added to gluten flour (vital wheat gluten PROLIGHT® LF, ADM, Chicago, IL) and kneaded with a stand mixer (e.g., KITCHENAID® Professional 600 Series 6 Quart Bowl-Lift Stand Mixer model KP26M1XER with dough-mixing attachment), set at speed "2" for 30 seconds to form the meat dough. Once kneaded, the meat dough was formed into a slab and transferred to another vessel for steaming as in Example 16 then transferred to a container on ice to allow it to cool down to 4°C. A control batch of meat dough was made as described above except no lutein was added. The meat dough containing lutein was described as having less grainy flavor and being closer to beef than the control meat dough.

**EXAMPLE 18**

**Decreasing grain and off flavors by the addition of carotenoids**

Meat dough was prepared as follows using the ingredients shown in Table 7.

**TABLE 7**
**Composition of Meat Dough**

| Ingredient | % |
|---|---|
| Gluten flour (ADM PROLITE® Low Flavor Vital Wheat Gluten (Montreal, Canada)) | 40.0 |
| Tap water | 50.0 |
| Coconut oil | 10.0 |
| Carotenoids (DSM) | 0.005 |
| Total | 100.0 |

First, melted coconut oil (50°C) was mixed with carotenoids, then this was mixed into the water. The broth was vigorously stirred, then quickly the wheat gluten flour was added to the broth and mixed well with a spoon. The formed raw meat dough was transferred to a metal ramekin or glass beaker for steaming as described in Example 16 and then transferred to a container on ice to allow it to cool down to 4°C.

The addition of carotenoids modified the flavor of the dough in an advantageous way; when compared to the meat dough with no carotenoids (Example 10), five trained flavor scientists described the meat dough with carotenoid as having less grain flavor, less oxidized notes, and overall less off flavors in four tastings. Table 8 presents the summarized sensory results from a panel of samples with different carotenoids evaluated by five trained flavor scientist on grain flavor. The trained flavor scientists rated the samples from 1-5 on the grain flavor, with 1 being the lowest in grain flavor and 5 being the highest. The reduction in off flavors in the meat dough with the lutein was supported by SPME Gas chromatography–mass spectrometry (GC-MS) data. Additionally, carotenoids (lycopene, beta-carotene, zeaxanthin, canthaxanthin, and lutein) added to the meat dough resulted in the samples being described as more fatty and sweeter.

With the addition of lutein in the meat dough, most flavor compounds decreased dependent on the concentration of lutein. See Table 9; lutein concentrations were at none,

0.0005%, and 0.005%. The main compounds that decreased with the carotenoids included oxidized flavor compound like alcohols and aldehydes, including (Z)-2-nonenal, (E,E)-2,4-nonadienal, and 1-penten-3-ol; additionally, sulfur compounds were decreased, including methanethiol, 2-acetylthiazole, and dimethyl sulfide; many of these compounds also were described as grainy and oxidized notes by trained flavor scientist by Gas Chromatography-Olfactometry (GCO).

**TABLE 8**

**Sensory emulation of meat dough with the addition of carotenoids for reduction in grain flavor**

| Meat dough with: | Grain ranking: 1-5 (1 = lowest) |
|---|---|
| Control coconut oil blank | 3.0 ± 0.8 |
| plus Lycopene | 2.8 ± 0.6 |
| plus beta-carotene | 1.5 ± 0.7 |
| plus Zeaxanthin | 1.6 ± 0.8 |
| plus Canthaxanthin | 1.6 ± 0.8 |
| plus lutein | 1.9 ± 0.8 |

**TABLE 9**

**Flavor compounds affect by the addition of carotenoids in wheat gluten flour upon cooking. Data collected by SPME GCMS.**

| Compounds | Peak Area | | |
|---|---|---|---|
| | Lutein Amount | | |
| | None | Low | High |
| 2,4-Decadienal, (E,E)- | | | |
| 2-Pyrrolidinone | | | |
| Dimethyl trisulfide | | | |
| 2-Acetylthiazole | | | |
| Carbon disulfide | | | |
| Methanethiol | | | |
| Pentanoic acid | | | |
| Acetic acid | | | |
| Butanoic acid | | | |
| Propanoic acid | | | |
| Ethanone, 1-(2-furanyl)- | | | |
| 5-Hepten-2-one, 6-methyl- | | | |
| 4-Heptenal, (Z)- | | | |
| 3-Furaldehyde | | | |
| 2-Nonenal, (Z)- | | | |
| Phenylacetaldehyde | | | |
| 2,4-Nonadienal, (E,E)- | | | |
| Acetaldehyde | | | |
| Butanal, 3-methyl- | | | |
| Propanal, 2-methyl- | | | |
| Acetone | | | |
| Furfural | | | |
| Butanal | | | |
| 3-Octen-2-one | | | |
| 3,5-Octadien-2-one, (E,E)- | | | |
| Phenol | | | |
| 1-Penten-3-ol | | | |
| Benzyl alcohol | | | |
| 2-Penten-1-ol, (Z)- | | | |
| 1-Butanol, 3-methyl- | | | |
| Butyrolactone | | | |
| 2(3H)-Furanone, 5-ethyldihydro- | | | |
| 2(5H)-Furanone, 5-ethyl- | | | |

## EXAMPLE 19

**Decreased grain and off flavor by the addition of antioxidants**

A meat dough was prepared as follows using the ingredients shown in Table 10.

**TABLE 10**
**Composition of Meat Dough**

| Ingredient | % |
|---|---|
| Gluten flour (ADM PROLITE® Low Flavor Vital Wheat Gluten) | 40.0 |
| Tap water | 50.0 |
| Coconut oil | 9.95 |
| EGCG (Swanson Superior Herbs) | 0.05 |
| Total | 100.0 |

EGCG (epigallocatechin gallate) was solubilized in water, then melted coconut oil (50°C) was mixed into the water. The broth was vigorously stirred, then quickly the wheat gluten flour was added to the broth and mixed well with a spoon. The formed raw meat dough was transferred to a metal ramekin or glass beaker for steaming as described in Example 16, and then transferred to a container on ice to allow it to cool down to 4°C.

The reduction in off flavors as described by trained flavor scientists in the meat dough with the addition of EGCG is supported by SPME GC-MS data. The GCMS data showed multiple flavor compounds that were no longer detectable or decreased by at least 2-fold, including compounds 2-pentyl-furan, 6-methyl-5-hepten-2-one, 1-penten-3-ol, 2-penten-1-ol, methyl-pyrazine, butanal, 5-ethyl-2(5H)-furanone, 5-ethyldihydro -2(3H)-furanone, 2-nonenal, phenylacetaldehyde, and 3,5-octadien-2-one that were described by GCO as being grainy and oxidized notes.

## EXAMPLE 20

**Preparation of meat dough with decreased grain flavor by washing the wheat gluten**

Meat dough was prepared as follows using the ingredients shown in Table 11.

**TABLE 11**
**Composition of Meat Dough**

| Ingredient | % |
|---|---|
| Gluten flour | 40.0 |
| Tap water | 50.0 |
| Coconut oil | 10 |
| Total | 100.0 |

Wheat gluten flour (ADM PROLITE® Low Flavor Vital Wheat Gluten) was slowly stirred into a solution that contained 10X washing solution (50 mM NaCl), then mixed well to prevent clump formation. The solution was set on ice for five minutes, during which the wheat gluten settled to the bottom. A second wash step was followed by removing the first wash solution and stirring into 10X fresh washing solution. The second solution was discarded and a final wash with tap water followed. The water wash solution was discarded, then the washed wheat gluten was measured to determine that the correct amount of water was incorporated. Water was added or pressed out so that the wheat gluten dough weight was equal to the amount of initial wheat gluten flour measured out and the theoretical amount of water. Melted coconut oil was added, and the dough was hand kneaded for 30 seconds to incorporate the oil. The formed raw meat dough was transferred to a metal ramekin or glass beaker for steaming as in Example 16 and then transferred to a container on ice to allow it to cool down to 4°C.

The washing step modified the flavor of the dough in an advantageous way; when compared to the non-washed meat dough, five trained flavor scientists described the washed meat dough as having less grain flavor, less oxidized notes, and overall less off flavors in four tastings. The reduction in off flavors in the washed meat dough is

supported by SPME GC-MS coupled with GCO that compared non-washed to washed meat dough. In the washed meat dough, flavor compounds decreased, including oxidized flavor compounds such as alcohols and aldehydes, and particular compounds including 1-(2-furanyl)-ethanone, methyl-pyrazine, pentanoic acid, 3-methyl-1-butanol, 2,3-butanedione, benzyl alcohol, (E,E) 3,5-octadien-2-one, (E)-2-nonenal, (E,E)-2,4-decadienal, and 1-octen-3-one, which were determined to be odor active compounds by GCO and the detection of these compounds was either decreased or was not detected in the washed meat dough.

## EXAMPLE 21

### Preparation of bloody agar

Bloody agar was prepared using the ingredients shown in Table 12.

**TABLE 12**
**Composition of Bloody Agar**

| Ingredient | % |
|---|---|
| Flavor broth | 41.5 |
| Leghemoglobin, 50 mg/ml liquid | 26.7 |
| 17x Liquid Magic Mix | 17.3 |
| RuBisCO, dry (Example 2) | 12.0 |
| 1 M lactic acid solution | 1.5 |
| Agar powder | 1 |
| Total | 100.0 |

Agar powder (Now Foods, Bloomingdale, IL) was dissolved in a mixture of lactic acid and flavor broth (made as in Example 5, except 10% coconut oil and Magic Mix 1 from Table 13 was used) by heating to 100°C in a stirred beaker. The solution was cooled to 65°C by immersion in an ice bath. 17x liquid magic mix (Magic Mix 2 from Table 13) and leghemoglobin, both at 4°C, then were added, causing the temperature of the mixture to decrease to 50°C. It is important that the mixture be cooled before adding

the leghemoglobin to prevent the leghemoglobin from denaturing. The dry RuBisCO then was added and the mixture was stirred vigorously by hand. It is important that the temperature be between 40°C and 60°C when the RuBisCO is added. If the temperature is too high, the RuBisCO can denature and will not function as a firming agent during cooking of the final product. If the temperature is too low, the agar will solidify and hinder generation of a homogenous mixture.

**TABLE 13**
**Composition of Magic Mixes**

| Precursor | Precursor mix 1 (mM) | Precursor mix 2 (mM) |
|---|---|---|
| Alanine | 15.0 | 7.5 |
| Arginine | 0.6 | 0.3 |
| Asparagine | 0.8 | 0.4 |
| Aspartate | 0.8 | 0.4 |
| Cysteine | 9.0 | 9.0 |
| Glutamic acid | 20 | 20 |
| Glutamine | 0.7 | 0.3 |
| Glycine | 1.3 | 0.7 |
| Histidine | 0.6 | 0.3 |
| Isoleucine | 0.8 | 0.4 |
| Leucine | 2.0 | 1.0 |
| Lysine | 5.0 | 2.5 |
| Methionine | 1.0 | 0.5 |
| Phenylalanine | 0.6 | 0.3 |
| Proline | 0.9 | 0.4 |
| Threonine | 0.8 | 0.4 |
| Tryptophan | 1.5 | 0.8 |
| Tyrosine | 0.6 | 0.3 |
| Valine | 1.0 | 0.5 |
| Glucose | 5.6 | 2.8 |
| Ribose | 5.0 | 5.0 |
| Thiamine | 0.2 | 0.2 |
| IMP + GMP | 2.5 | 1.3 |
| Lactic acid | 10.0 | 5.0 |
| Creatine | 3.0 | 1.5 |
| L-Taurine | 10.0 | 5.0 |

## EXAMPLE 22

**Preparation of bloody agar**

Bloody agar was prepared using the ingredients shown in Table 14.

**TABLE 14**
**Composition of Bloody Agar**

| Ingredient | % |
|---|---|
| Water | 81.1 |
| 1 M lactic acid solution | 9.2 |
| 17x Liquid Magic Mix (Table 12) | 4.4 |
| Leghemoglobin, 87 mg/ml liquid | 4.3 |
| Agar powder | 1.0 |
| Total | 100.0 |

Agar powder (Agar 100, TIC Gums, White Marsh, MD) was dissolved in a mixture of water and lactic acid by heating to at least 91°C in a stirred beaker. Heating was done to fully solubilize the agar. The solution then was cooled to 50 - 70°C and a premixture of the leghemoglobin (*Pichia* expressed, Example 1) and 17x Liquid Magic mix (Table 12) was added. If the temperature is too high, the leghemoglobin can denature and will not function as intended for flavor reaction chemistry. If the temperature is too low, the agar will solidify and hinder generation of a homogenous mixture. The mixture then was stirred and further cooled to 4 - 25°C. The finished product has a ketchup like appearance and texture.

## EXAMPLE 23

**Preparation of adipose replica emulsion with improved melting, adhesive and mouth feel properties**

To prepare one hundred (100) g of adipose replica, 1 g of dry precursor mix 1 (Table 12) was dissolved in 18.8 ml of water and the pH was adjusted to 6 with a concentrated NaOH solution. A frozen solution of leghemoglobin (5.5%) was added to

the precursor solution and placed on a stirring hot plate maintained at 160°C, with a 250 RPM rotation speed.

In a separate container, thirty five (35) g of coconut oil (Shay and company, Milwaukie, OR) and thirty five (35) g of palm stearin were melted together in a 60°C water bath. The melted oil mixture was slowly (about 12 ml/min) added to the solution of precursors and leghemoglobin, while increasing the stirring rate to 450 RPM.

The resulting thick emulsion was maintained at the same temperature and stirring rate for 23 min after the oil was added. The emulsion then was transferred to a 600 ml beaker and placed on ice and into the refrigerator for rapid cooling. When the emulsion reached 25°C, 0.35 g of algal vegetable oil and 0.35 g of acetoin were added to the emulsion and rapidly mixed in with a spatula. Lactones for improved flavor and masking off-flavors (as described in Example 31) were added in the following amounts: 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone was added to a final concentration of $2.5*10^{-5}$ %, butyrolactone was added to a final concentration of $2.5*10^{-8}$ %, and δ-tetradecalactone was added to a final concentration $5*10^{-9}$ %. The emulsion was homogenized for 2.5 min using a hand-held homogenizer at setting 6. The emulsion was incubated at 4°C until it was fully solid.

After solidification, the adipose replica emulsion was off-white to a slightly browned color, waxy solid at room temperature, with flavors that were characterized as savory, meaty, bloody, and chicken fat-like. When incorporated into the ground beef replica, the stickiness of the replica was increased as was the ability of the replica to be handled and shaped.

## EXAMPLE 24

**Preparation of adipose replica emulsion stabilized by soy conglycinin protein**

To prepare 100 g of adipose replica, 1.5 g of isolated soy conglycinin powder from Example 4 was dissolved in 28.5 ml of water and placed on a hot stir plate. In a separate container, 70 g of coconut oil (Shay and company) were melted in a 60°C water bath. The melted oil mixture was slowly (about 12 ml/min) added to the solution of purified protein under constant stirring. The resulting emulsion was heated up to 90°C

temperature and maintained at this temperature for 5 min. The emulsion then was transferred to a 600 ml beaker and placed on ice and into the refrigerator for rapid cooling. When the emulsion reached 25°C, 0.35 g of algal vegetable oil were added to the emulsion and rapidly mixed in with a spatula. Lactones for improved flavor and masking off-flavors (as described in Example 31) were added in the following amounts: 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone was added to a final concentration of 2.5*10-5 %, butyrolactone was added to a final concentration of 2.5*10-8 %, and δ-tetradecalactone was added to a final concentration 5*10-9 %. The emulsion was homogenized for 2.5 min using a hand-held homogenizer at setting 6 and incubated in the 4°C refrigerator until it was fully solid. After solidification, the adipose replica emulsion was white to slight off-white color, solid at room temperature, with bland, very neutral flavor and texture characterized as similar to rendered beef fat.

## EXAMPLE 25

**Preparation of adipose replica emulsion stabilized by soy conglycinin protein by a pH excursion method**

To prepare 100 g of adipose replica, 0.5 g of isolated soy glycinin protein powder from Example 4 was dissolved in 29.5 ml of water in a beaker. The pH of the protein solution was adjusted to 12 using a 2 M solution of sodium hydroxide. In a separate container, 70 g of coconut oil (Shay and company) were melted in a 60°C water bath. The melted oil mixture was slowly (about 12 ml/min) added to the solution of purified protein under constant stirring. 0.35 g of algal vegetable oil were added to the emulsion and rapidly mixed in with a spatula. The pH of the protein solution was adjusted to 12 using a 2 M solution of sodium hydroxide, and the emulsion was homogenized for 30 s (thirty seconds) using a hand-held homogenizer at setting 6 and incubated at 4°C until it was fully solid. After solidification, the adipose replica emulsion was white to slight off-white color, solid at room temperature, with bland, very neutral flavor and cottage cheese-like texture.

## EXAMPLE 26

**Adding carotenoids in flavor emulsion to increase meaty flavors**

Each carotenoid (canthaxanthin, β-carotene, lutein, or lycopene) (Shay and company, Milwaukie, OR) was individually dissolved in coconut oil or water at 10%, dependent on solubility. The carotenoids were added to the flavor emulsion before cooking by mixing a solution of LegH at 0.5%, 1x precursor mix 1 (Table 1), 30% RBD coconut oil, and the individual 10% carotenoid solutions (final carotenoid concentration in emulsion was 0.025%), and stirring the mixture as it was heated until boiling, then simmered at a low boil for 10 minutes. Additional algal vegetable oil at 0.7% was added for additional precursors for flavor creation as described in Example 32. Lactones for improved flavor and masking off-flavors (as described in Example 31) were added in the following amounts: 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone was added to a final concentration of 2.5*10-5 %, butyrolactone was added to a final concentration of 2.5*10-8 %, and δ-tetradecalactone was added to a final concentration 5*10-9 %. The emulsion was homogenized for 2.5 min using a hand-held homogenizer at setting 6 then incubated at 4°C until it was fully solid. The emulsions were then added to the taco meat prepared as described in Example 30. The samples then were compared with the different carotenoids to a control with no carotenoids added. The samples were evaluated by at least five trained flavor scientists. The results are summarized in Table 15.

**TABLE 15**

**Flavor descriptions from tacos tasted with flavor emulsion prepared with different carotenoids**

| Carotenoid | Summary - common | Summary - unique |
|---|---|---|
| canthaxanthin | more brown (2)[§], similar to control (2), lower green floral/veg (2), toasted grain (2), more fatty (2), odd/chemical/vitamin(2) | sweet, more beefy, less grain, sl* floral |
| β-carotene | more meaty/beefy (3), more fatty (2), less green/floral off, less chickeny | more savory, less savory, sl bitter, sl carrot, sl bland |
| lutein | similar to control (3), more brown (2) | lower green floral, floral, cardboard/grain, more butter, vegetable stock, pleasant, no off flavors |
| lycopene | fatty (2), grain (3) | lower green/floral/grain, more vegetable, less meaty, less off, savory, same as control |

*slightly

[§]Numbers in parentheses indicate the number of tasters with that response.

**EXAMPLE 27**

**Assembly and cooking of burger**

A replica burger containing the ingredients in Table 16 was prepared.

**TABLE 16**
**Composition of Burger**

| Ingredient | % |
|---|---|
| Meat dough (Example 16) | 26.9 |
| Bloody agar (Example 21) | 33.9 |
| Flavored emulsion (Example 21) | 20.0 |
| Soft connective tissue (Example 7) | 19.2 |
| Total | 100.0 |

Chilled meat dough was ground using a stand mixer fitted with a food grinder attachment (KITCHENAID® Professional 600 Series 6 Quart Bowl-Lift Stand Mixer model KP26M1XER and KITCHENAID® Food Grinder model FGA, St. Joseph, MI) on speed setting 1. In this equipment, material is fed by a screw conveyor past a rotating knife installed in front of a fixed-hole plate. Soft connective tissue was shredded using a Universal Machine (UM-12, Stephen Machinery GmbH, Schwarzenbeck , Germany) fitted with a blunt blade and run for 20 – 30 seconds at slow speed. Flavored emulsion was chilled to -20°C and then chopped with a mini chopper (Mini-Prep® Plus Processor model DLC-2L Cuisinart, Stamford, CT) in a single step process. Approximate 400g of emulsion was placed in the mini chopper and processed on the chop setting for 60 seconds to yield pieces of 1 – 3 mm in length.

Ground meat dough, shredded soft connective tissue, and flavored emulsion pieces were then mixed. During mixing, the mixture was kept at -5 to 4°C to prevent the fat from melting. The bloody agar was then added and mixed until it was thoroughly incorporated. The total batch size was 1 kg. 50 g or 150 g portions of ground tissue then were formed by hand into round patty shapes. Typical dimensions for 50 g patties were 55 mm x 15 mm. Typical dimensions for 150 g patties were 100 mm x 22 mm. Patties were refrigerated until cooked. Patties were cooked on a preheated (325 – 345°F) non-stick skillet and heated to an internal temperature of 160°F while flipping every 2 minutes. Cooked patties had an appearance, texture, and flavor similar to ground beef. In addition to cooking in patty format, the unformed material also can be used in a variety of dishes such as taco filling, casseroles, sauces, toppings, soups, stews, or loaves.

## EXAMPLE 28

**Assembly and cooking of burger**

A replica burger containing the ingredients in Table 17 was prepared.

**TABLE 17**
**Composition of Burger**

| Ingredient | % |
|---|---|
| Meat dough (Example 17) | 26.8 |
| Fat emulsion (Example 23) | 20 |
| Soft connective tissue (Example 7) | 19.2 |
| Bloody agar (Example 22) | 13.0 |
| Hydration liquids | 10.5 |
| Soy conglycinin, dry | 10.5 |
| Total | 100.0 |

Chilled meat dough was ground using a stand mixer fitted with a food grinder attachment (KITCHENAID® Professional 600 Series 6 Quart Bowl-Lift Stand Mixer model KP26M1XER and KITCHENAID® Food Grinder model FGA, St. Joseph, MI) on speed setting 1. Soft connective tissue was shredded using a Universal Machine (UM-12, Stephan Machinery GmbH, Schwarzenbeck , Germany) fitted with a blunt blade and run for 20 – 30 seconds at slow speed. Flavored emulsion was chilled to -20°C and then chopped with a SALADSHOOTER® National Presto Industries, Inc. Eau Claire, WI) to yield pieces of 1 – 3 mm in length. Ground meat dough, shredded soft connective tissue, flavored emulsion pieces, and dry soy conglycinin were then mixed. During mixing, the mixture was kept at -5 to 4°C to prevent the fat from melting. Hydration liquids (a 1:1 mixture of leghemoglobin and 17x Liquid Magic mix as described in Example 22) were then added and the mixture was held at 4°C for a minimum of 15 minutes to allow the dry soy conglycinin to hydrate. Finally bloody agar was added and mixed until it was thoroughly incorporated. The total batch size was 200 g. 50 g portions of ground tissue

then were formed by hand into round patty shapes. Typical dimensions for 50 g patties were 55 mm x 15 mm. Patties were refrigerated until cooked. Patties were cooked on a preheated (325 – 345°F) non-stick skillet and heated to an internal temperature of 170°F while flipping every minute. Cooked patties had an appearance, texture, and flavor similar to ground beef. In addition to cooking in patty format, the unformed material also can be used in a variety of dishes such as taco filling, casseroles, sauces, toppings, soups, stews, or loaves.

**EXAMPLE 29**

**Assembly and cooking of burger with 10% meat dough**

A replica burger containing the ingredients in Table 18 was prepared.

**TABLE 18**
**Composition of Burger**

| Ingredient | % |
|---|---|
| Meat dough (Example 17) | 10 |
| Fat emulsion (Example 23) | 20 |
| Soft connective tissue (Example 7) | 36 |
| Bloody agar (Example 22) | 13.0 |
| Hydration liquids | 10.5 |
| Soy conglycinin, dry | 10.5 |
| Total | 100.0 |

Chilled meat dough was ground using a stand mixer fitted with a food grinder attachment (KITCHENAID® Professional 600 Series 6 Quart Bowl-Lift Stand Mixer model KP26M1XER and KITCHENAID® Food Grinder model FGA, St. Joseph, MI) on speed setting 1. Soft connective tissue was shredded using a Universal Machine (UM-12, Stephan Machinery GmbH, Schwarzenbeck, Germany) fitted with a blunt blade and run for 20 – 30 seconds at slow speed. Flavored emulsion was chilled to -20°C and then chopped with a SALADSHOOTER® National Presto Industries, Inc. Eau Claire, WI) to yield pieces of 1 – 3 mm in length. Ground meat dough, shredded soft connective tissue, flavored emulsion pieces, and dry soy conglycinin were then mixed. During mixing, the

mixture was kept at -5 to 4°C to prevent the fat from melting. Hydration liquids (a 1:1 mixture of leghemoglobin and 17x Liquid Magic mix as described in Example 22) were then added and the mixture was held at 4°C for a minimum of 15 minutes to allow the dry soy conglycinin to hydrate. Finally, bloody agar was added and mixed until it was thoroughly incorporated. The total batch size was 200 g. Fifty (50) g portions of ground tissue then were formed by hand into round patty shapes. Typical dimensions for 50 g patties were 55 mm x 15 mm. Patties were refrigerated until cooked. Patties were cooked on a preheated (325–345°F) non-stick skillet and heated to an internal temperature of 170°F while flipping every minute. Cooked patties had an appearance, texture, and flavor similar to ground beef. In addition to cooking in patty format, the unformed material also can be used in a variety of dishes such as taco filling, casseroles, sauces, toppings, soups, stews, or loaves.

## EXAMPLE 30

**Assembly and cooking of "taco meat"**

A replica "taco meat" containing the ingredients in Table 19 was prepared.

**TABLE 19**
**Composition of Burger**

| Ingredient | % |
|---|---|
| Meat dough (Example 17) | 29.9 |
| Fat emulsion (Example 23) | 22.3 |
| Soft connective tissue (Example 7) | 21.5 |
| Bloody agar (Example 22) | 14.5 |
| Hydration liquids | 11.7 |
| Total | 100.0 |

Chilled meat dough was ground, soft connective tissue was shredded, and the flavored emulsion was chopped as described in Example 28. Ground meat dough, shredded soft connective tissue, and flavored emulsion pieces were then mixed. During mixing, the mixture was kept at -5 to 4°C to prevent the fat from melting. Hydration liquids (a 1:1 mixture of leghemoglobin and 17x Liquid Magic mix as described in

Example 22) and bloody agar was then added and mixed. The total batch size was 20 g. The mixed tissue was then cooked on a preheated (325 – 345°F) non-stick skillet to 160°F. Cooked tissue had an appearance, texture, and flavor similar to ground beef. The material resembled taco meat in appearance; without the 7S protein, the meat did not firm up and stick together as much.

## EXAMPLE 31

### Using lactones as masking agents

Lactones were diluted in either water or oil depending on solubility. The diluted lactones were then added to the flavored emulsion (Example 23) as indicated in Table 20 and homogenized. The final concentrations of the lactones are given in Table 20. The flavored emulsion was added for a final of 20% of the ground meat (e.g., taco meat) (all components of the meat replica without RuBisCO). The flavored emulsion was mixed with meat dough, connective tissue, magic mix, and heme as indicated in Example 17 but without the RuBisCO. The ground meat was then tested by five trained flavor scientists for overall taste, any reduction in off flavors, and overall improvement. The summarized results are indicated in Table 20. The addition of particular lactones and combinations of lactones resulted in a decrease in off flavors including grain, eggy, bitterness, livery, and mushroom. Unique combinations were required for particular masking properties like bitterness. The lactones also increased desired flavors of creamy, buttery, caramelized, fatty, fresh, and fruity.

**TABLE 20**

**Sensory data on addition of lactones as masking agents in meat replicas**

| Compound | Conc. (%) in Meat Tested | Compound | Conc. (%) in Meat Tested | Compound | Conc. (%) in Meat Tested | % of testers who preferred over control | Common Flavor Descriptors | Masks or prevents off flavors? |
|---|---|---|---|---|---|---|---|---|
| Butyrolactone | $2.5*10^{-9}$ | γ-Octalactone | $2.5*10^{-8}$ | - | - | 80% | Sour, savory | Bitterness |
| Delta-Tridecalactone | $2.5*10^{-5}$ | 5-Ethyl-4-hydroxy-2-methyl-3(2H)-furanone | $2.5*10^{-6}$ | - | - | 75% | Salty, savory, creamy/buttery | Bitterness |
| 2(3H)-Furanone, Dihydro-5-Methyl | $2.5*10^{-z8}$ | 5-Ethyl-4-hydroxy-2-methyl-3(2H)-furanone | $2.5*10^{-6}$ | - | - | 75% | Bland, fatty, salty | Bitterness, grain |
| 2(3H)-Furanone, Dihydro-5-Methyl | $2.5*10^{-8}$ | 5-Ethyl-4-hydroxy-2-methyl-3(2H)-furanone | $2.5*10^{-6}$ | Delta-Tridecalactone | $2.5*10^{-6}$ | 83% | Buttery, fatty, bright/bitter, savory | Eggy, grain |
| 2(3H)-Furanone, Dihydro-5-Methyl | $2.5*10^{-8}$ | 5-Ethyl-4-hydroxy-2-methyl-3(2H)-furanone | $2.5*10^{-6}$ | Delta-Tridecalactone | $2.5*10^{-6}$ | 83% | Sl sweet, fatty | Grain |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Butyrolactone | $2.5*10^{-8}$ | 5-Ethyl-4-hydroxy-2-methyl-3(2H)-furanone | $2.5*10^{-6}$ | *y-Octalactone* | $2.5*10^{-7}$ | 83% | Creamy, metallic | Eggy |
| Gamma Decalactone | $5 * 10^{-5}$ | | | | | 40% | Caramelized/aromatic sweet, salty | Grain |
| Delta-Tridecalactone | $5 * 10^{-5}$ | | | | | 60% | Caramelized/aromatic sweet | Mushroom |
| *Delta-dodecalactone* | $1 * 10^{-5}$ | | | | | 60% | Buttery, fatty/oily, sl beefy | Grain |
| *4-hydroxy-2,5-dimethyl-3(2H)-furanone* | $1*10^{-5}$ | | | | | 60% | Sl sweet/caramelized | Grain |
| *y-Octalactone* | $1 * 10^{-5}$ | | | | | 80% | Fatty, sweet/caramelized | Grain |
| Butyrolactone | $2.5 * 10^{-8}$ | | | | | 83% | Bright/sharp/bitter, sweet, fatty | Eggy |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| δ-Tetradecalactone | $5*10^{-9}$ | | | | | 83% | Brothy, fatty | Eggy, grain |
| 2(3H)-Furanone, Dihydro-5-Methyl | $5*10^{-9}$ | | | | | 100% | Mild savory, slight sweet | Eggy, grain |

## EXAMPLE 32

**Adding polyunsaturated fats for the creation of meaty fat flavor**

Algal vegetable oil (DSM life's omega 45 02412-0100) was added to the flavored emulsion (Example 23), and then homogenized, for a final concentration of 0.07% in the meat replica. The flavor emulsion was added to the replica as described in Example 27. The addition of algal vegetable oil resulted in a replica with an increase in tallow taste, fattiness, and overall meatiness, as described by trained flavor scientists.

The addition of algal oil increased the precursors including eicosapentaenoic acid and docosahexaenoic acid that are needed for the creation of fatty flavor molecules. As detected by SPME Gas chromatography–mass spectrometry (GC-MS), the addition of algae oil to the precursor mix and hemoglobin as compared to the control without algal oil created flavors including nonane, (E,E)-3,5-octadien-2-one, 1-hepten-3-ol, 1-penten-3-one, 2-propyl furan, n-caproic acid vinyl ester, 3-ethyl-2-methyl-1,3-hexadiene, 1-ethyl-5-methylcyclopentene, trans-2-(2-pentenyl)furan, 1-penten-3-ol, 4,7-dimethyl-undecane, 1-octanol, 3-ethyl-pyridine, 3-ethylcyclopentanone, (Z)-2-octen-1-ol, 2-n-heptylfuran, (Z)-2-decenal, hexanoic acid, (E,E)-2,4-nonadienal, 6-methyl-2-heptanone, (Z)-2-heptenal, (E,E)-2,4-heptadienal, 1-hexanol, (E,E)-2,4, decadienal, (E,Z)-2,6-nonadienal, and 1-octen-3-ol.

## EXAMPLE 33

**Creating *Cucumis* slurries for meat replicas**

To create a boiled cucumber slurry, an entire non-permeated crisp variety cucumber (with the skin not peeled off or otherwise disrupted) was used. A water bath was heated to 80-90°C, and the entire cucumber was placed into the water bath and cooked until the internal temperature of the cucumber was equilibrated with the temperature of the water bath, around 30 minutes for this example. The cucumber then was taken out of the bath and the skin of the cucumber was completely removed from the flesh. The flesh was blended with the seeds and then sieved to separate out any of the larger particulates. The blended flesh then was used as the slurry and added to the meat

replicas. This same method was used with other varieties in the *Cucumis* genus including honeydew melon and cantaloupe.

## EXAMPLE 34

**Creating *Cucumis* extracts using solvent assisted flavor extraction**

An extract was created using solvent assisted flavor extraction (SAFE), and water as the solvent. SAFE works by pulling the flavor compounds out of the material with pressure and a slightly elevated temperature.

An extract was created by removing all the skin of a crisp variety cucumber, and cutting the cucumber into pieces then blended with a magic bullet. The cucumber slurry was then poured into the sample inlet of the SAFE glassware that was under pressure using an Edwards 12 floor vacuum, and the temperature was set at 40°C with a water pump, and a warm water-bath for the sample round bottom flask. A small amount (2-4 mL of sample) of the cucumber slurry was put in the sample round bottom flask. The slurry immediately appeared to boil as it traveled into the sample round bottom flask. When the visual boiling stopped, more sample volume was added to the sample round bottom flask. This continued until the entire sample was let into the SAFE glassware setup. Once the entire sample was gone, the sample keep extracting as the water bath reach 40°C then extracted for additional 20 minutes. As the extract was taking place the collection round bottom flask was submerged in liquid nitrogen and cold finger inlet filled with liquid nitrogen. The extract was then collected from the collection round bottom flask.

## EXAMPLE 35

**Adding *Cucumis* liquid to meat replicas to increase meatiness and fattiness by adding to gelled matrix**

Bloody agar as outlined in Example 22 was made as described other than with the addition of *Cucumis* liquid replacing DI water. The *Cucumis* liquid was one of the following: (i) a commercially available water extract from a cantaloupe melon, added at 2% of the DI water, (ii) – (v) a slurry, cooked or not cooked, of either honeydew melon

(6.25%) or cucumber (3.2%) as described in Example 33, or (vi) solvent assisted flavor extract of cucumber (pressure distillate) as described in Example 34. The addition of these cucumber and melon extracts brings certain elements of a meaty flavor profile to enhance the overall preferences and meatiness of replicas. As demonstrated by SPME GC-MS, and confirmed as odor active compounds by trained flavor scientist using GCO, many of the compounds are aldehydes, lactones, many of which are seen in beef. Compounds that are similar between beef and *Cucumis* include, without limitation, nonanal, 2-decenal, 2-nonenal, 2-heptenal, 2,6-nonadienal, 2,4-decadienal, 2-undecenal, 2-octenal, 2-nonenal, dodecanal, 2,4-heptadienal, 2,6-nonadienal, 2,4-nonadienal, 2,4-octadienal, decanal, 5-(methylenecyclopropyl)-pentanal, 6-nonenal, 3,7-dimethyl 1,6-octadien-3-ol, 2-nonen-1-ol, 3-nonen-1-ol, 3,5-octadien-2-one, 2,3-butanedione, 2-methyl-cyclopentanone, 2-butanone, á-ionone, 6-octen-2-one, dihydro-5-pentyl -2(3H)-furanone, l-menthone, n-caproic acid vinyl ester, 4-methyloctanoic acid, and acetic acid ethenyl ester.

When the bloody agar with the extracts were added to meat replicas as described in Example 30, there was an increase in sweet aromatics, fattiness, and in some cases tallow and beefy flavors, see Table 21 for the full description from five trained flavor scientist of additional flavor notes that were not seen in the control and blind control. Additionally, it was observed that the addition of these melon and cucumber extracts also decreases the perception of off flavors including grainy, earthy, woody, and astringent.

Three of these extracts were tested in a formal descriptive panel with 8 trained panelists and compared to one control replica indicated as such, a blind control not indicated as a control, 80:20 beef, and three additional samples with the *Cucumis* liquid (cooked cucumber slurry tested at 0.37% of the final taco meat, cooked honeydew slurry tested at 0.73% of the final taco meat, and Cantaloupe extract (from TREATT) at 0.24% of the final taco meat). The results showed that all three samples were rated as higher in fattiness and sweet aromatics, with a decrease in off notes of earthy, grainy, astringent, and green. The cantaloupe extract had an additional off note from the control of a sweet melon flavor unlike what is tasted in beef. The other two samples the cooked honeydew

slurry and cucumber slurry made as described in Example 33, were both rated as higher in meatiness and fattiness than both controls, and had no additional off notes described.

**TABLE 21**
**Flavor descriptions from adding *Cucumis* liquid to meat replicas**

| Cucumis liquid | Flavor Description when added to tacos | |
|---|---|---|
| | Increase in desirable flavors | Decrease in undesirable flavors |
| Cooked honeydew slurry | Fatty, butter, fresh, sweet, mouthwatering, and fatty mouth coating | Green, grain, earthy |
| Cooked cucumber slurry | Beef tallow, more buttery, fatty, fresh, fruit, fatty mouth coating | Green, grain, earthy |
| Honeydew slurry | Savory, melon, slight butter, pork, sweet aromatic, fatty, mouthwatering, mouth coating | Green, grain, earthy |
| Cucumber slurry | Fruity, fatty, cucumber, vegetable stock and melon | Green, grain, earthy |
| Cantaloupe extract | Sweet, fruity, aromatic, slight butter, melon, candy | Green, grain, earthy |
| Cucumber SAFE | Less chicken, freshness, sweet, little cucumber | Green, grain, earthy |

## OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

**WHAT IS CLAIMED IS:**

1. A meat replica composition comprising:

(a) about 5% to about 88% by weight of a meat dough;

(b) about 0% to about 40% by weight of a carbohydrate-based gel;

(c) about 5% to about 35% by weight of a non-animal fat;

(d) about 0.00001% to about 10% by weight of a flavoring agent;

(e) about 0% to about 15% by weight of a binding agent; and

(f) about 0.01% to about 4% by weight of a heme-containing protein and/or an iron salt.

2. The composition of claim 1, wherein said meat dough comprises a flavoring agent.

3. The composition of claim 1 or 2, wherein said non-animal fat comprises a flavoring agent.

4. The composition of any one of claims 1-3, wherein said meat dough is about 45% to about 60% by weight of said composition.

5. The composition of any one of claims 1-4, wherein said carbohydrate-based gel is about 10% to about 25% by weight of said composition.

6. The composition of any one of claims 1-5, wherein said non-animal fat is about 10% to about 15% by weight of said composition.

7. The composition of any one of claims 1-6, wherein said flavoring agent is about 3% to about 7% by weight of said composition.

8. The composition of any one of claims 1-7, wherein said flavoring agent comprises one or more flavor precursors.

9. The composition of any one of claims 1-7, wherein said flavoring agent is about 0.001% to about 2% by weight of said composition.

10. The composition of claim 9, wherein said flavoring agent comprises a flavoring or a flavoring compound.

11. The composition of any one of claims 1-10, wherein said flavoring agent is a combination of a flavoring and one or more flavor precursors.

12. The composition of any one of claims 1-11, wherein said binding agent is about 2% to about 10% by weight of said composition.

13. The composition of any one of claims 1-12, wherein said binding agent comprises one or more proteins that have been chemically or enzymatically modified to improve their textural and/or flavor properties, or to modify the denaturation and gelling temperature of the one or more proteins.

14. The composition of any one of claims 1-13, wherein said heme-containing protein is about 0.01% to about 2% by weight of said composition.

15. The composition of any one of claims 1-14, wherein said composition comprises said heme-containing protein and said iron salt.

16. The composition of any one of claims 1-15, wherein said meat dough comprises an isolated plant protein, an edible fibrous component, an optional flavoring agent, and an optional non-animal fat.

17. The composition of claim 12, wherein said binding agent is a conglycinin protein.

18. A meat replica composition comprising:

(a) about 5% to about 80% by weight of a meat dough;

(b) about 5% to about 35% by weight of a non-animal fat;

(c) about 15% to about 40% by weight of an edible fibrous component;

(d) about 0.1% to about 18% by weight of a carbohydrate-based gel;

(e) about 0% to about 10% by weight of a flavoring agent;

(f) about 0.5% to about 15% by weight of a binding agent; and

(g) about 0.1% to about 8% by weight of a heme-containing protein and/or an iron salt.

19. The meat replica composition of claim 18, comprising:

(a) about 20% to about 30% by weight of a meat dough;

(b) about 15% to about 25% by weight of a non-animal fat;

(c) about 15% to about 25% by weight of an edible fibrous component;

(d) about 7% to about 18% by weight of a carbohydrate-based gel;

(e) about 0% to about 10% by weight of a flavoring agent;

(f) about 5% to about 15% by weight of a binding agent; and

(g) about 2% to about 8% by weight of a heme-containing protein and/or an iron salt.

20. A method of making a ground meat replica, said method comprising:

(a) heating a dough to a temperature ranging from 150°F to 250°F, said dough comprising an isolated plant protein, an optional edible fibrous component, one or more optional flavoring agents, and an optional non-animal fat;

(b) combining said dough, after heating, with a non-animal fat, said non-animal fat optionally containing a flavoring agent and/or an isolated plant protein; and

(c) combining the dough from step (b) with a carbohydrate-based gel, an optional edible fibrous component, an optional binding agent, a highly conjugated heterocyclic ring complexed to an iron ion and/or an iron salt, and one or more optional flavoring agents to make said ground meat replica.

21. The method of claim 20, said method further comprising breaking the dough from step (b) into pieces before combining with said carbohydrate-based gel, said optional edible fibrous component, said optional binding agent, said highly conjugated heterocyclic ring complexed to an iron ion and/or said iron salt, and one or more optional flavoring agents.

22. A method of flavoring a meat dough, said method comprising:

(a) combining a first highly conjugated heterocyclic ring complexed to an iron ion and/or a first iron salt with one or more flavor precursors and an optional non-animal fat;

(b) heating the mixture to form one or more flavor compounds; and

(c) making a dough comprising an isolated plant protein, an optional edible fibrous component, and the mixture from step (b).

23. The method of claim 22, further comprising:

(d) combining said dough, after heating, with a non-animal fat, said non-animal fat optionally containing a flavoring agent and/or an isolated plant protein; and

(e) combining the dough of step (d) with a carbohydrate-based gel, an optional binding agent, a second highly conjugated heterocyclic ring complexed to an iron ion and/or a second iron salt, and one or more optional flavoring agents to make a ground meat replica.

24. The method of claim 23, said method further comprising breaking the dough from step (d) into pieces before combining with said carbohydrate-based gel, said optional binding agent, said second highly conjugated heterocyclic ring complexed to an iron ion and/or said second iron salt, and one or more optional flavoring agents.

25. A method of flavoring a meat dough, said method comprising:

(a) making a dough comprising an isolated plant protein, an optional edible fibrous component, one or more optional flavoring agents, and an optional non-animal fat;

(b) making a flavored non-animal fat by combining a non-animal fat with a highly conjugated heterocyclic ring complexed to an iron ion and/or a first iron salt, and one or more flavor precursors and heating the mixture; and

(c) combining said dough, after heating, with said flavored non-animal fat.

26. The method of claim 25, further comprising combining the dough of step (c) with a carbohydrate-based gel, an optional binding agent, a second highly conjugated heterocyclic ring complexed to an iron ion and/or a second iron salt, and one or more optional flavoring agents to make a ground meat replica.

27. The method of claim 26, further comprising breaking the dough of step (c) before combining with said carbohydrate-based gel, said optional binding agent, said second highly conjugated heterocyclic ring complexed to an iron ion and/or said second iron salt, and one or more optional flavoring agents.

28. A method of making a ground meat replica, said method comprising:

(a) combining an iron salt with one or more flavor precursors and an optional non-animal fat;

(b) heating the mixture to form one or more flavor compounds;

(c) making a dough comprising an isolated plant protein, an optional edible fibrous component, and the mixture from step (b);

(d) combining said dough, after heating, with a non-animal fat, said non-animal fat optionally containing a flavoring agent and/or an isolated plant protein; and

(e) combining the dough of step (d) with a carbohydrate-based gel, an optional binding agent, an iron salt, an optional highly conjugated heterocyclic ring complexed to an iron ion, and one or more optional flavoring agents to make said ground meat replica.

29. The method of claim 28, further comprising breaking the dough from step (d) into pieces before combining with said carbohydrate-based gel, said optional binding agent, said iron salt, said optional highly conjugated heterocyclic ring complexed to an iron ion, and one or more optional flavoring agents.

30. A method of making a ground meat replica, said method comprising:

(a) making a dough comprising an isolated plant protein, an optional edible fibrous component, one or more optional flavoring agents, and an optional non-animal fat;

(b) making a flavored non-animal fat by combining a non-animal fat with an iron salt and one or more flavor precursors and heating the mixture;

(c) combining said dough, after heating, with said flavored non-animal fat; and

(d) combining the dough of step (c) with a carbohydrate-based gel, an optional binding agent, an iron salt, an optional highly conjugated heterocyclic ring complexed to an iron ion, and one or more optional flavoring agents to make said ground meat replica.

31. The method of claim 30, further comprising breaking the dough from step (c) before combining with said carbohydrate-based gel, said optional binding agent, said iron salt, said optional highly conjugated heterocyclic ring complexed to an iron ion, and one or more optional flavoring agents.

32. The method of any one of claims 20-31, wherein said iron salt is iron gluconate, iron chloride, iron oxalate, iron nitrate, iron citrate, iron ascorbate, ferrous sulfate, ferric pyrophosphate, or any other aqueous soluble salt.

33. The method of claim 28, wherein a highly conjugated heterocyclic ring complexed to an iron ion is combined with said iron salt, said one or more flavor precursors, and said non-animal fat before heating said mixture.

34. The method of claim 30, wherein a highly conjugated heterocyclic ring complexed to an iron ion is combined with said non-animal fat, said iron salt, and said one or more flavor precursors before heating said mixture.

35. The method of any one of claims 22-31, wherein said one or more flavor precursors is a sugar, a sugar alcohol, a sugar acid, a sugar derivative, an oil, a free fatty acid, an amino acid or derivative thereof, a nucleoside, a nucleotide, a vitamin, an acid, a peptide, a phospholipid, a protein hydrolysate, a yeast extract, or a mixture thereof.

36. The method of claim 35, wherein said flavor precursor is selected from the group consisting of glucose, fructose, ribose, arabinose, glucose-6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, inositol, maltose, sucrose, maltodextrin, glycogen, nucleotide-bound sugars, molasses, a phospholipid, a lecithin, inosine, inosine monophosphate (IMP), guanosine monophosphate (GMP), pyrazine, adenosine monophosphate (AMP), lactic acid, succinic acid, glycolic acid, thiamine, creatine, pyrophosphate, vegetable oil, algal oil, sunflower oil, corn oil, soybean oil, palm fruit oil,

palm kernel oil, safflower oil, flaxseed oil, rice bran oil, cottonseed oil, olive oil, sunflower oil, canola oil, flaxseed oil, coconut oil, mango oil, a free fatty acid, cysteine, methionine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan, valine, arginine, histidine, alanine, asparagine, aspartate, glutamate, glutamine, glycine, proline, serine, tyrosine, glutathione, an amino acid derivative, urea, pantothenic acid, ornithine, niacin, glycerol, citrulline, taurine, biotin, borage oil, fungal oil, blackcurrant oil, betaine, beta carotene, B-vitamins, N-Acetyl L-cysteine, iron glutamate and a peptone, or mixtures thereof.

37. The method of any one of claims 20-36, wherein said isolated plant protein in said dough comprises wheat gluten.

38. The method of any one of claims 20-36, wherein said isolated plant protein in said dough comprises wheat gluten, a dehydrin protein, an albumin, a globulin, or a zein, or mixtures thereof.

39. The method of any one of claims 20-38, wherein said ground meat replica comprises said optional edible fibrous component.

40. The method of claim 39, wherein said optional edible fibrous component comprises plant fibers from carrot, bamboo, pea, broccoli, potato, sweet potato, corn, whole grains, alfalfa, kale, celery, celery root, parsley, cabbage, zucchini, green beans, kidney beans, black beans, red beans, white beans, beets, cauliflower, nuts, apple skins, oats, wheat, or psyllium, or a mixture thereof.

41. The method of claim 39, wherein said optional edible fibrous component comprises an extruded mixture of isolated plant proteins.

42. The method of claim 41, wherein said extruded mixture comprises wheat gluten and soy protein isolate.

43. The method of claim 41 or claim 42, wherein said extruded mixture further comprises a flavoring agent.

44. The method of claim 43, wherein said flavoring agent is a flavoring, a flavor compound, or a flavor precursor.

45. The method of claim 44, wherein said flavoring is yeast extract, a protein hydrolysate, or an oil.

46. The method of claim 39, wherein said edible fibrous component is a solution-spun protein fiber.

47. The method of claim 46, wherein said solution-spun protein fiber comprises a prolamin.

48. The method of claim 47, wherein said prolamin is corn zein, pea prolamin, kafirin, secalin, hordein, avenin, or a mixture thereof.

49. The method of any one of claims 20-48, wherein said non-animal fat is an algal oil, a fungal oil, corn oil, olive oil, soy oil, peanut oil, walnut oil, almond oil, sesame oil, cottonseed oil, rapeseed oil, canola oil, safflower oil, sunflower oil, flax seed oil, palm oil, palm kernel oil, coconut oil, babassu oil, shea butter, mango butter, cocoa butter, wheat germ oil, borage oil, black currant oil, sea-buckhorn oil, macadamia oil, saw palmetto oil, conjugated linoleic oil, arachidonic acid enriched oil, docosahexaenoic acid (DHA) enriched oil, eicosapentaenoic acid (EPA) enriched oil, palm stearic acid, sea-buckhorn berry oil, macadamia oil, saw palmetto oil, or rice bran oil; or margarine or other hydrogenated fats.

50. The method of any one of claims 20-48, wherein said non-animal fat is algal oil.

51. The method of claim 49 or claim 50, wherein said non-animal fat comprises said optional flavoring agent.

52. The method of any one of claims 49-51, said non-animal fat comprising said optional isolated plant protein.

53. The method of claim 52, wherein said optional isolated plant protein is a conglycinin protein.

54. The method of any one of claims 20, 21, or 23-53, wherein said dough comprises said flavoring agent.

55. The method of any one of claims 20-52, wherein said non-animal fat in said dough comprises a flavoring agent.

56. The method of claim 54 or claim 55, wherein said flavoring agent is selected from the group consisting of a vegetable extract, a fruit extract, an acid, an antioxidant, a carotenoid, a lactone, and combinations thereof.

57. The method of claim 56, wherein said antioxidant is epigallocatechin gallate.

58. The method of claim 56, wherein said carotenoid is lutein, β-carotene, zeaxanthin, trans-β-apo-8'-carotenal, lycopene, or canthaxanthin.

59. The method of claim 56, wherein said vegetable extract is from a cucumber or tomato.

60. The method of claim 56, wherein said fruit extract is from a melon or pineapple.

61. The method of any one of claims 20-60, wherein said carbohydrate based gel has a melting temperature between about 45°C and about 85°C.

62. The method of claim 61, wherein said carbohydrate-based gel comprises agar, pectin, carrageenan, konjac, alginate, chemically-modified agarose, or mixtures thereof.

63. The method of any one of claims 20-62, wherein said ground meat replica comprises said optional binding agent.

64. The method of claim 63, wherein said optional binding agent is an isolated plant protein.

65. The method of claim 64, wherein the denaturation temperature of said binding agent is between about 40°C and about 80°C.

66. The method of claim 64, wherein said isolated plant protein is a RuBisCO, an albumin, a gluten, a conglycinin, or mixtures thereof.

67. The method of claim 63, wherein said binding agent is a carbohydrate based gel that becomes firm upon cooking to 140°F to 190°F.

68. The method of claim 67, wherein said carbohydrate based gel comprises methylcellulose, hydroxypropylmethyl cellulose, guar gum, locust bean gum, xanthan gum, or a mixture thereof.

69. The method of claim 63, wherein said binding agent is egg albumin or collagen.

70. The method of any one of claims 20-69, wherein said highly conjugated heterocyclic ring complexed to an iron ion is a heme moiety, or a porphyrin, porphyrinogen, corrin, corrinoid, chlorin, bacteriochorophyll, corphin, chlorophyllin, bacteriochlorin, or isobacteriochlorin moiety complexed to an iron ion.

71. The method of claim 70, wherein said heme moiety is a heme-containing protein.

72. The method of claim 71, wherein said heme-containing protein is a non-symbiotic hemoglobin, a Hell's gate globin I, a flavohemoprotein, a leghemoglobin, a heme-dependent peroxidase, a cytochrome c peroxidase, or a mammalian myoglobin.

73. The method of claim 71, wherein said heme-containing protein is a leghemoglobin.

74. The method of claim 73, wherein said leghemoglobin is from soybean, pea, or cowpea.

75. A method of increasing the meat flavor or masking off flavors from plant material in a food product, said method comprising adding, to said food product, one or more

lactones at a concentration of $10^{-3}$ to $10^{-11}$ of said food product, wherein said lactones are selected from the group consisting of tetrahydro-6-methyl-2H-pyran-2-one, delta-octalactone, 5-ethyldihydro-2(3H)-furanone, butyrolactone, dihydro-5-pentyl-2(3H)-furanone, dihydro-3-methylene-2,5-furandione, l-pentoyl lactone, tetrahydro-2H-pyran-2-one, 6-heptyltetrahydro-2H-pyran-2-one, γ-octalactone, 5-hydroxymethyldihydrofuran-2-one, 5-ethyl-2(5H)-furanone, 5-acetyldihydro-2(3H)-furanone, trans-3-methyl-4-octanolide 2(5H)-furanone, 3-(1,1-dimethylethyl)-2,5-urandione, 3,4-dihydroxy-5-methyl-dihydrofuran-2-one, 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, δ-tetradecalactone, and dihydro-4-hydroxy-2(3H)-furanone.

76. The method of claim 75, wherein said lactones are 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, butyrolactone, γ-octalactone, and δ-tetradecalactone.

77. A method of increasing the meat flavor or masking off flavors from plant material in a food product, said method comprising adding, to said food product, one or more carotenoids at a concentration of between 0.00001% and 0.1% of said food product, wherein said carotenoids are selected from the group consisting of β-carotene, zeaxanthin, lutein, trans-β-apo-8'-carotenal, lycopene, canthaxanthin, and combinations thereof.

78. The method of any one of claims 75-77, wherein said food product is a meat replica.

79. The method of claim 78, wherein said meat replica is free of animal products.

80. A method of increasing the meat flavor of a meat replica, said method comprising adding, to said meat replica, a vegetable juice, a vegetable puree, a vegetable extract, a fruit juice, a fruit puree, or a fruit extract to said meat replica at a concentration from 0.0001% to 10% of said meat replica.

81. The method of claim 80, wherein said vegetable juice, vegetable puree, vegetable extract, a fruit juice, a fruit puree, or a fruit extract is a *Cucumis* juice, puree, or extract.

82. The method of claim 81, wherein said *Cucumis* juice, puree, or extract is from a cucumber or a melon.

83. The method of any one of claims 80-82, wherein said vegetable juice, vegetable puree, vegetable extract, fruit juice, fruit puree, or fruit extract is cooked or otherwise treated to denature proteins before adding to said meat replica.

84. The method of any one of claims 80-83, wherein said meat replica is free of animal products.

85. A food product or food replica product comprising a heme-containing protein and one or more lactones at a concentration of $10^{-3}$ to $10^{-11}$ of said food product, wherein said one or more lactones are selected from the group consisting of tetrahydro-6-methyl-2H-pyran-2-one, delta-octalactone, 5-ethyldihydro-2(3H)-furanone, butyrolactone, dihydro-5-pentyl-2(3H)-furanone, dihydro-3-methylene-2,5-furandione, l-pentoyl lactone, tetrahydro-2H-pyran-2-one, 6-heptyltetrahydro-2H-pyran-2-one, γ-octalactone, 5-hydroxymethyldihydrofuran-2-one, 5-ethyl-2(5H)-furanone, 5-acetyldihydro-2(3H)-furanone, trans-3-methyl-4-octanolide 2(5H)-furanone, 3-(1,1-dimethylethyl)-2,5-urandione, 3,4-dihydroxy-5-methyl-dihydrofuran-2-one, 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, δ-tetradecalactone, and dihydro-4-hydroxy-2(3H)-furanone.

86. The food product or food replica product of claim 85, wherein said one or more lactones are 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, butyrolactone, γ-octalactone, and δ-tetradecalactone.

87. A food product or food replica product comprising a heme-containing protein and one or more carotenoids at a concentration of between 0.00001% and 0.1% of said food product, wherein said one or more carotenoids are selected from the group consisting of β-carotene, zeaxanthin, lutein, trans-β-apo-8'-carotenal, lycopene, canthaxanthin, and combinations thereof.

88. The food product or food replica product of any one of claims 85-87, wherein said food replica product is a meat replica.

89. The food product or food replica product of claim 88, wherein said meat replica is free of animal products.

90. A food product or food replica product comprising (a) a heme-containing protein, and (b) a vegetable juice, a vegetable puree, a vegetable extract, a fruit juice, a fruit puree, or a fruit extract at a concentration from 0.0001% to 10% of said food product.

91. The food product or food replica product of claim 90, wherein said vegetable juice, vegetable puree, vegetable extract, a fruit juice, a fruit puree, or a fruit extract is a *Cucumis* juice, puree, or extract.

92. The food product or food replica product of claim 91, wherein said *Cucumis* juice, puree, or extract is from a cucumber or a melon.

93. The food product or food replica product of any one of claims 90-92, wherein said vegetable juice, vegetable puree, vegetable extract, fruit juice, fruit puree, or fruit extract was cooked or otherwise treated to denature proteins before being added to said food replica product.

94. The food product or food replica product of claim 93, wherein said vegetable juice, vegetable puree, vegetable extract, fruit juice, fruit puree, or fruit extract was heated to a temperature of about 60°C to about 100°C before being added to said food replica product.

95. The food product or food replica product of any one of claims 90 to 94, wherein said food product is free of animal products.

96. The food product or food replica product of any one of claims 85 to 95, further comprising one or more of a meat dough, a carbohydrate-based gel, a non-animal fat, and a binding agent.

97. A food replica product comprising one or more lactones at a concentration of $10^{-3}$ to $10^{-11}$ of said food product, wherein said one or more lactones are selected from the

group consisting of tetrahydro-6-methyl-2H-pyran-2-one, delta-octalactone, 5-ethyldihydro-2(3H)-furanone, butyrolactone, dihydro-5-pentyl-2(3H)-furanone, dihydro-3-methylene-2,5-furandione, l-pentoyl lactone, tetrahydro-2H-pyran-2-one, 6-heptyltetrahydro-2H-pyran-2-one, γ-octalactone, 5-hydroxymethyldihydrofuran-2-one, 5-ethyl-2(5H)-furanone, 5-acetyldihydro-2(3H)-furanone, trans-3-methyl-4-octanolide 2(5H)-furanone, 3-(1,1-dimethylethyl)-2,5-urandione, 3,4-dihydroxy-5-methyl-dihydrofuran-2-one, 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, δ-tetradecalactone, and dihydro-4-hydroxy-2(3H)-furanone.

98. The food replica product of claim 97, wherein said one or more lactones are 5-ethyl-4-hydroxy-2-methyl-3(2H)-furanone, butyrolactone, γ-octalactone, and δ-tetradecalactone.

99. A food replica product comprising one or more carotenoids at a concentration of between 0.00001% and 0.1% of said food product, wherein said one or more carotenoids are selected from the group consisting of β-carotene, zeaxanthin, lutein, trans-β-apo-8'-carotenal, lycopene, canthaxanthin, and combinations thereof.

100. A food replica product comprising a vegetable juice, a vegetable puree, a vegetable extract, a fruit juice, a fruit puree, or a fruit extract at a concentration from 0.0001% to 10% of said food product.

101. The food replica product of claim 100, wherein said vegetable juice, vegetable puree, vegetable extract, a fruit juice, a fruit puree, or a fruit extract is a *Cucumis* juice, puree, or extract.

102. The food replica product of claim 101, wherein said *Cucumis* juice, puree, or extract is from a cucumber or a melon.

103. The food replica product of claim 100-102, wherein said vegetable juice, vegetable puree, vegetable extract, fruit juice, fruit puree, or fruit extract was cooked or otherwise treated to denature proteins before being added to said food replica product.

104. The food replica product of claim 103, wherein said vegetable juice, vegetable puree, vegetable extract, fruit juice, fruit puree, or fruit extract was heated to a temperature of about 60°C to about 100°C before being added to said food replica product.

105. The food replica product of any one of claims 97 to 104, wherein said food product is free of animal products.

106. The food replica product of any one of claims 97 to 105, further comprising one or more of a meat dough, a carbohydrate-based gel, a non-animal fat, and a binding agent.

107. The food replica product of any one of claims 97 to 105, wherein said food replica product is a meat replica.

108. The food replica product of any one of claims 97 to 105, wherein said food replica product is a cheese replica.

109. The food replica product of claim 108, wherein said cheese replica comprises a nut milk, a cross-linking enzyme, or a cheese culture.

110. A ground meat replica comprising:

(a) a dough comprising an isolated plant protein, an optional edible fibrous component, one or more optional flavoring agents, and an optional non-animal fat;

(b) a non-animal fat, said non-animal fat optionally containing a flavoring agent and/or an isolated plant protein; and

(c) a carbohydrate-based gel, a binding agent, a highly conjugated heterocyclic ring complexed to an iron ion and/or an iron salt, an optional edible fibrous component, and one or more optional flavoring agents.

111. The ground meat replica of claim 110, wherein said binding agent is an isolated plant protein.

112. The ground meat replica of claim 110, wherein said isolated plant protein is a RuBisCO, an albumin, a gluten, a conglycinin, or mixtures thereof.

113. The ground meat replica of claim 110, wherein the denaturation temperature of said binding agent is between about 40°C and about 80°C.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/023679

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A23J 3/22 (2015.01)
CPC - A23J 3/227 (2015.05)
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)
IPC(8) - A23J 3/14, 3/22 (2015.01)
CPC - A23J 3/14, 3/227 (2015.05) (keyword delimited)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 426/573, 574 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Orbit, Google Patents, Public AppFT and PatFT, Google Scholar
Search terms used: "meat replica" isolated plant protein dough cucumis juice cucumber food flavor meat vegetarian

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | US 2008/0254199 A1 (ORCUTT et al) 16 October 2008 (16.10.2008) entire document | 99, 100, 103, 104 |
| --- | --- | --- |
| Y | | 28-34, 77-83, 87-94, 101, 102, 113 |
| Y | WO 2013/010042 A1 (SAND HILL FOODS INC) 17 January 2013 (17.01.2013) entire document | 1-3, 18-27, 33-36, 85-94, 110-113 |
| Y | US 2006/0204644 A1 (CAVALLINI et al) 14 September 2006 (14.09.2006) entire document | 1-3, 18-36, 77-83, 110-113 |
| Y | US 2002/0034570 A1 (KRAMMER et al) 21 March 2002 (21.03.2002) entire document | 75, 76, 85, 86, 97, 98 |
| Y | US 2003/0212281 A1 (SINHA et al) 13 November 2003 (13.11.2003) entire document | 75, 76, 85, 86, 97, 98 |
| Y | WO 2013/010037 A1 (LYRICAL FOODS INC) 17 January 2013 (17.01.2013) entire document | 75, 76, 97, 98 |
| Y | US 2010/0310738 A1 (LUDWIG) 09 December 2010 (09.12.2010) entire document | 81, 82, 91, 92, 101, 102 |

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 26 May 2015 | 28 AUG 2015 |
| Name and mailing address of the ISA/US<br>Mail Stop PCT, Attn: ISA/US, Commissioner for Patents<br>P.O. Box 1450, Alexandria, Virginia 22313-1450<br>Facsimile No. 571-273-8300 | Authorized officer:<br>Blaine R. Copenheaver<br>PCT Helpdesk: 571-272-4300<br>PCT OSP: 571-272-7774 |

Form PCT/ISA/210 (second sheet) (July 2009)

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/023679

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search 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.: 4-17, 37-74, 84, 95, 96, 105-109
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

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 additional fees, this Authority did not invite payment of additional fees.

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.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)

# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 34331732 |
| **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/Kirsten Neuman |
| **Filer Authorized By:** | M. Angela Parsons |
| **Attorney Docket Number:** | 38767-0048US1 |
| **Receipt Date:** | 16-NOV-2018 |
| **Filing Date:** | 10-NOV-2017 |
| **Time Stamp:** | 12:55:22 |
| **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 | Information Disclosure Statement (IDS) Form (SB08) | Transmittal.pdf | 71599<br>aa461326b071536c8f8e8a61d83c920b23b0a397 | no | 1 |

**Warnings:**

| | | | | | |
|---|---|---|---|---|---|
| **Information:** | | | | | |
| This is not an USPTO supplied IDS fillable form | | | | | |
| 2 | Information Disclosure Statement (IDS) Form (SB08) | PTO_1449.pdf | 129305<br>ed766e7ce6b6829d2a8b99fd259d150d85420bcd | no | 1 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| This is not an USPTO supplied IDS fillable form | | | | | |
| 3 | Foreign Reference | WO2015153666.pdf | 4239746<br>dd533a2627e72a51277fad8900957465bdb8dcd6 | no | 95 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 4 | Non Patent Literature | Krainer_2015.pdf | 812849<br>f8856e181fb28566698835f8b87500245dd99b7e | no | 9 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 5 | Non Patent Literature | Liu_2014.pdf | 1340871<br>a378a0613196d7c57d864fd6b409cf5496f1f601 | no | 8 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 6 | Non Patent Literature | Fraser_2018.pdf | 1590629<br>f5dd39b1808c2a6bfd0a7d24d387bd3aa625f03d | no | 22 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 7 | Non Patent Literature | Proulx_2006.pdf | 3780409<br>14ad119e38b88f96df17a78c360e7f3ea1bb359d | no | 5 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| | | | **Total Files Size (in bytes):** | 11965408 | |

**This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.**

**New Applications Under 35 U.S.C. 111**

**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**

**National Stage of an International Application under 35 U.S.C. 371**

**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**

**New International Application Filed with the USPTO as a Receiving Office**

**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 15/573,300 | 11/10/2017 | Smita Shankar | 38767-0048US1 | 4807 |

26191 7590 04/08/2019
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

| EXAMINER | |
|---|---|
| LEITH, NANCY J | |

| ART UNIT | PAPER NUMBER |
|---|---|
| 1636 | |

| NOTIFICATION DATE | DELIVERY MODE |
|---|---|
| 04/08/2019 | ELECTRONIC |

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

PATDOCTC@fr.com

PTOL-90A (Rev. 04/07)

| *Office Action Summary* | **Application No.** 15/573,300 | **Applicant(s)** Shankar et al. | |
|---|---|---|---|
| | **Examiner** NANCY J. LEITH | **Art Unit** 1636 | **AIA (FITF) Status** Yes |

*-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --*

**Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 2 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

1)☑ Responsive to communication(s) filed on 16 November 2018.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims***

5) ☑ Claim(s) 1-38 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☐ Claim(s) _____ is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☑ Claim(s) 1-38 are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

**Application Papers**

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

**Priority under 35 U.S.C. § 119**

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

**Certified copies:**

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

1) ☑ Notice of References Cited (PTO-892)

2) ☐ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

## DETAILED ACTION

## REQUIREMENT FOR UNITY OF INVENTION

As provided in 37 CFR 1.475(a), a national stage application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept ("requirement of unity of invention"). Where a group of inventions is claimed in a national stage application, the requirement of unity of invention shall be fulfilled only when there is a technical relationship among those inventions involving one or more of the same or corresponding special technical features. The expression "special technical features" shall mean those technical features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art.

The determination whether a group of inventions is so linked as to form a single general inventive concept shall be made without regard to whether the inventions are claimed in separate claims or as alternatives within a single claim. See 37 CFR 1.475(e).

## WHEN CLAIMS ARE DIRECTED TO MULTIPLE CATEGORIES OF INVENTIONS

As provided in 37 CFR 1.475(b), a national stage application containing claims to different categories of invention will be considered to have unity of invention if the claims are drawn only to one of the following combinations of categories:

(1) A product and a process specially adapted for the manufacture of said product; or

(2) A product and process of use of said product; or

(3) A product, a process specially adapted for the manufacture of the said product, and a use of the said product; or

(4) A process and an apparatus or means specifically designed for carrying out the said process; or

(5) A product, a process specially adapted for the manufacture of the said product, and an apparatus or means specifically designed for carrying out the said process.

Otherwise, unity of invention might not be present. See 37 CFR 1.475(c).

Restriction is required under 35 U.S.C. 121 and 372.

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

In accordance with 37 CFR 1.499, applicant is required, in reply to this action, to elect a single invention to which the claims must be restricted.

Group I, claims 1-19 and 30-38, drawn to a methylotrophic yeast cell comprising a nucleic acid molecule encoding a transcriptional activator operably linked to a methanol-inducible promoter.

Group II, claims 20-29, drawn to a method for expressing a heterologous polypeptide in a yeast cell.

According to PCT Rule 13.2, unity of invention exists only when the shared same or corresponding technical feature is a contribution over the prior art. The inventions listed as Groups I-II do not relate to a single general inventive concept because they lack the same or corresponding technical feature. The technical feature of Group I is a methylotrophic yeast cell comprising a nucleic acid encoding a transcriptional activator operably linked to a methanol-inducible promoter, which is shown by Zhou *et al.* (U.S. Patent Application Publication No. 2015/0299716, published October 22, 2015, and filed January 9, 2015) to lack an inventive step.

Zhou discloses a methylotrophic yeast that comprises an exogenous nucleic acid encoding a transcriptional activator (Mit1) operably linked to a methanol-inducible promoter (abstract and paragraphs [0002] and [0017]).

Thus, Zhou discloses the features of the instantly claimed invention. Therefore, the technical feature of the methylotrophic yeast cell does not make a contribution over the prior art and does not constitute a special technical feature.

Accordingly, Groups I-II are not so linked by the same of a corresponding special technical feature as to form a single general inventive concept.

Applicant is advised that the reply to this requirement to be complete must include (i) an election of an invention to be examined even though the requirement may be traversed (37 C.F.R. § 1.143) and (ii) identification of the claims encompassing the elected invention.

The election of an invention may be made with or without traverse. To preserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the restriction requirement, the election shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure

to timely traverse the requirement will result in the loss of right to petition under 37 C.F.R. § 1.144. If claims are added after the election, applicant must indicate which of these claims are readable on the elected invention.

Should applicant traverse on the ground that the inventions have unity of invention (37 C.F.R. § 1.475(a)), applicant must provide reasons in support thereof. Applicant may submit evidence or identify such evidence now of record showing the inventions to be obvious variants or clearly admit on the record that this is the case.

Where such evidence or admission is provided by applicant, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other invention.

Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be corrected in compliance with 37 CFR 1.48(a) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. A request to correct inventorship under 37 CFR 1.48(a) must be accompanied by an application data sheet in accordance with 37 CFR 1.76 that identifies each inventor by his or her legal name and by the processing fee required under 37 CFR 1.17(i).

The examiner has required restriction between product or apparatus claims and process claims. Where applicant elects claims directed to the product/apparatus, and all product/apparatus claims are subsequently found allowable, withdrawn process claims that include all the limitations of the allowable product/apparatus claims should be considered for rejoinder. All claims directed to a nonelected process invention must include all the limitations of an allowable product/apparatus claim for that process invention to be rejoined.

In the event of rejoinder, the requirement for restriction between the product/apparatus claims and the rejoined process claims will be withdrawn, and the rejoined process claims will be fully examined for patentability in accordance with 37 CFR 1.104. Thus, to be allowable, the rejoined claims must meet all criteria for patentability including the requirements of 35 U.S.C. 101, 102, 103 and 112. Until all claims to the elected product/apparatus are found allowable, an otherwise proper restriction requirement between product/apparatus claims and process claims may be maintained. Withdrawn process claims that are not commensurate in scope with an allowable product/apparatus claim will not be rejoined. See MPEP § 821.04. Additionally, in order for rejoinder to occur, applicant is advised that the process claims should be amended during prosecution to require the limitations of the product/apparatus claims. **Failure to do so may result in no rejoinder.** Further, note that the prohibition against double patenting rejections of 35 U.S.C. 121 does not apply where the restriction requirement is withdrawn by the examiner before the patent issues. See MPEP § 804.01.

***Conclusion***

Any inquiry concerning this communication or earlier communications from the examiner should be directed to NANCY J LEITH whose telephone number is (313)446-4874. The examiner can normally be reached on Monday - Thursday 8:00 AM - 6:30 PM.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, HEATHER CALAMITA can be reached on (571) 272-2876. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

NANCY J. LEITH
Primary Examiner
Art Unit 1636

/NANCY J LEITH/
Primary Examiner, Art Unit 1636

| *Notice of References Cited* | Application/Control No. 15/573,300 | Applicant(s)/Patent Under Reexamination Shankar et al. | |
|---|---|---|---|
| | Examiner NANCY J. LEITH | Art Unit 1636 | Page 1 of 1 |

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| * | | Document Number Country Code-Number-Kind Code | Date MM-YYYY | Name | CPC Classification | US Classification |
|---|---|---|---|---|---|---|
| * | A | US-20150299716-A1 | 10-2015 | Zhou; Xiangshan | C07K14/39 | 435/69.1 |
| | B | | | | | |
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**FOREIGN PATENT DOCUMENTS**

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**NON-PATENT DOCUMENTS**

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| | V | |
| | W | |
| | X | |

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

| Index of Claims | Application/Control No. | Applicant(s)/Patent Under Reexamination |
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| | Examiner | Art Unit |
| | NANCY J. LEITH | 1636 |

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| = | Allowed | ÷ | Restricted | I | Interference | O | Objected |

**CLAIMS**

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

| CLAIM | | DATE | | | | | | | | |
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| Final | Original | 04/02/2019 | | | | | | | | |
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# BIB DATA SHEET

**CONFIRMATION NO. 4807**

| SERIAL NUMBER | FILING or 371(c) DATE<br>RULE | CLASS | GROUP ART UNIT | ATTORNEY DOCKET NO. |
|---|---|---|---|---|
| 15/573,300 | 11/10/2017 | 435 | 1636 | 38767-0048US1 |

**APPLICANTS**
Impossible Foods Inc., Redwood City, CA;

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

**** CONTINUING DATA ***************************
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 ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****
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| Foreign Priority claimed ❑ Yes ☑ No<br>35 USC 119(a-d) conditions met ❑ Yes ☑ No<br>Verified and Acknowledged /NANCY J LEITH/ Examiner's Signature | ❑ Met after Allowance<br>Initials | STATE OR COUNTRY | SHEETS DRAWINGS | TOTAL CLAIMS | INDEPENDENT CLAIMS |
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| | | CA | 11 | 38 | 2 |

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MINNEAPOLIS, MN 55440-1022
UNITED STATES

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

| FILING FEE RECEIVED | | |
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| 3060 | FEES: Authority has been given in Paper<br>No._____ to charge/credit DEPOSIT ACCOUNT<br>No._____ for following: | ❑ All Fees<br>❑ 1.16 Fees (Filing)<br>❑ 1.17 Fees (Processing Ext. of time)<br>❑ 1.18 Fees (Issue)<br>❑ Other _____<br>❑ Credit |

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| First Named Inventor | : | Smita Shankar | Art Unit | : | 1636 |
| Application No. | : | 15/573,300 | Examiner | : | Nancy J. Leith |
| Filed | : | November 10, 2017 | Conf. No. | : | 4807 |
| Title | : | EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST | | | |

**MAIL STOP AMENDMENT**
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the documents listed on the disclosure form submitted with this statement. Copies of the documents are being submitted with this statement to the extent required under 37 CFR § 1.98(a)(2).

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 applicable under 37 C.F.R. § 1.17(p), or credits, to Deposit Account No. 06-1050, referencing the above-referenced Attorney Docket Number.

Please contact the undersigned if there are any questions regarding this statement.

Respectfully submitted,

Date: /June 4, 2019/

/M. Angela Parsons/
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Customer Number 26191
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Telephone: (612) 335-5070
Facsimile: (877) 769-7945

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Sheet 1 of 1

| Substitute Disclosure Form (37 CFR §1.98(b)) | U.S. Department of Commerce Patent and Trademark Office | Attorney Docket No. | Application No. |
|---|---|---|---|
| **Information Disclosure Statement by Applicant** (Use several sheets if necessary) | | 38767-0048US1 | 15/573,300 |
| | | Applicant: Impossible Foods Inc. | |
| | | Filing Date: November 10, 2017 | Group Art Unit: 1636 |

| U.S. Patent Documents | | | | | | | |
|---|---|---|---|---|---|---|---|
| Examiner Initial | Desig. ID | Document Number | Publication Date | Patentee | Class | Subclass | Filing Date If Appropriate |
| | 1. | 2018/0371469 | 12/27/2018 | Shankar et al. | | | |

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|---|---|---|---|---|---|---|---|---|
| Examiner Initial | Desig. ID | Document Number | Publication Date | Country or Patent Office | Class | Subclass | Translation Yes | Translation No |
| | 2. | EP 2669375 | 12/4/2013 | EP | | | | |
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| Examiner Signature | Date Considered |
|---|---|
| EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. | |

Substitute Disclosure Form

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# (54) YEAST FOR TRANSFORMATION AND PROCESS FOR PRODUCING PROTEIN

(57) An object of the present invention it to improve the production efficiency of a target protein in a transformant using a yeast cell as a host. The present invention provides a yeast expressing an MPP1 homolog at high levels, in particular, a methanol-assimilating yeast, a transformant prepared by introducing a gene encoding a target protein into said yeast, and a method for producing a target protein using said transformant.

EP 2 669 375 A1

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## Description

### Technical Field

**[0001]** The present invention is intended to improve protein production using a yeast host. The present invention relates to a yeast in which a particular transcription factor gene is introduced and/or activated and a method for producing a protein using such yeast.

### Background Art

**[0002]** In recent years, hosts suitable for proteins of interest, such as animal cells (e.g., CHO), insects (e.g., silk worm), insect cells, animals (e.g., chickens and cows), and microorganisms (e.g., *E. coli* and yeast), have been used in order to produce proteins via genetic recombination. In particular, yeast cells can be cultured in large-scale, high-density culture systems in cost-effective media, and proteins can be produced at low cost. In addition, proteins can be secreted and expressed in a culture solution of yeast cells by using a signal peptide or the like, therefore, a process for purifying proteins is easy. Further, yeast cells are advantageous in that they are eukaryotic organisms and post-translational modifications such as glycosylation is possible.

**[0003]** When a yeast is used as a host, however, productivity varies significantly depending on target proteins, and products may occasionally be influenced by the type of carbon source utilized. Examples of such influence include saccharification caused by glucose and oxidation caused by methanol.

**[0004]** In order to overcome such problems, use of methanol-assimilating yeasts, such as yeasts of the genus *Pichia*, yeasts of the genus *Candida*, and yeasts of the genus *Torulopsis* as hosts has been examined, and production of the green fluorescent protein, human serum albumin, the hepatitis B surface antigen, the human epidermal growth factor, and hirudin has been reported (Non-Patent Document 1).

**[0005]** In order to produce a protein, a nucleotide sequence region (i.e., a promoter) is necessary for transcription of a gene encoding such protein in a site upstream of such gene. In addition to a RNA polymerase that reads the nucleotide sequence of the template DNA and synthesizes (transcribes) complementary RNA, a variety of transcription regulators are involved in promotion or suppression of transcription.

**[0006]** Methanol-assimilating yeasts begin to utilize methanol as a nutrient source, such utilization is mediated by an alcohol oxidase (AOX) or methanol oxidase (MOX), and the AOX or MOX gene is transcribed prior thereto. A nucleotide sequence region (i.e., a promoter) for transcription of the AOX or MOX gene is located in an upstream region thereof. For example, an MOX promoter is known to have two different transcription initiation points depending on the carbon source (Non-Patent Document 2). Non-Patent Document 2 also suggests that a plurality of factors are associated with the MOX promoter.

**[0007]** Since the AOX and MOX promoters for methanol-assimilating yeasts are potent promoters, such promoters are used for protein production. In order to achieve the high-level production of a target protein via a general means, many problems still need to be resolved.

### Prior Art Documents

#### Non-Patent Documents

**[0008]**

Non-Patent Document 1: Gellissen, G., Appl. Microbiol. Biotechnol., Dec. 2000; 54(6): 741-50
Non-Patent Document 2: V., Genu et al., Eur. J. Biochem., 270, 2467-2475, 2003

### Summary of the Invention

#### Problem to be Solved by the Invention

**[0009]** It is an object of the present invention to improve the production efficiency of a target protein using yeasts, and especially methanol assimilating yeasts, by enhancing or activating the transcription or translation levels through, for example, promoter modification, high-level expression of transcription factors, and modification of hosts. More particularly, the present invention is intended to identify a factor that activates a promoter.

Means for Solving the Problem

**[0010]** The present invention includes the following inventions.

(1) A yeast for transformation, wherein the capacity for target protein production is improved by high-level expression of an MPP1 homolog.
(2) The yeast for transformation according to (1), wherein the MPP1 homolog is any of the polypeptides (a), (b), and (c) below:

(a) a polypeptide consisting of the amino acid sequence as shown in SEQ ID NO: 38;
(b) a polypeptide consisting of an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 38 by substitution, deletion, insertion, and/or addition of one or a plurality of amino acids;
(c) a polypeptide consisting of an amino acid sequence having 85% or higher sequence identity with the amino acid sequence as shown in SEQ ID NO: 38;
or any of the polypeptides (d), (e), and (f) below:
(d) a polypeptide consisting of the amino acid sequence as shown in SEQ ID NO: 41;
(e) a polypeptide consisting of an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 41 by substitution, deletion, insertion, and/or addition of one or a plurality of amino acids; or
(f) a polypeptide consisting of an amino acid sequence having 85% or higher sequence identity with the amino acid sequence as shown in SEQ ID NO: 41.

(3) The yeast for transformation according to (1), wherein the MPP1 homolog is partially composed of any of the polypeptides (g), (h), and (i) below:

(g) a polypeptide consisting of the amino acid sequence as shown in SEQ ID NO: 49;
(h) a polypeptide consisting of an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 49 by substitution, deletion, insertion, and/or addition of one or a plurality of amino acids;
(i) a polypeptide consisting of an amino acid sequence having 85% or higher sequence identity with the amino acid sequence as shown in SEQ ID NO: 49;
or any of the polypeptides (j), (k), and (l) below:
(j) a polypeptide consisting of the amino acid sequence as shown in SEQ ID NO: 50;
(k) a polypeptide consisting of an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 50 by substitution, deletion, insertion, and/or addition of one or a plurality of amino acids; or
(l) a polypeptide consisting of an amino acid sequence having 85% or higher sequence identity with the amino acid sequence as shown in SEQ ID NO: 50.

(4) The yeast for transformation according to any of (1) to (3), wherein the transcription factors are expressed at high levels.
(5) The yeast for transformation according to (4), wherein the transcription factor acts additively or synergistically with the MPP1 homolog.
(6) The yeast for transformation according to (4) or (5), wherein the transcription factor is any of the polypeptides (m), (n), and (o) below:

(m) a polypeptide consisting of the amino acid sequence as shown in SEQ ID NO: 46;
(n) a polypeptide consisting of an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 46 by substitution, deletion, insertion, and/or addition of one or a plurality of amino acids; and
(o) a polypeptide consisting of an amino acid sequence having 85% or higher sequence identity with the amino acid sequence as shown in SEQ ID NO: 46.

(7) The yeast for transformation according to any of (1) to (6), wherein the gene consisting of any of the nucleotide sequences (p), (q), (r), and (s) is expressed at high levels:

(p) a nucleotide sequence encoding the MPP1 homolog;
(q) the nucleotide sequence as shown in SEQ ID NO: 10 or 11;
(r) a nucleotide sequence hybridizing under stringent conditions to a nucleotide sequence complementary to the nucleotide sequence as shown in SEQ ID NO: 10 or 11; and
(s) a nucleotide sequence having 85% or higher sequence identity with the nucleotide sequence as shown in SEQ ID NO: 10 or 11.

(8) The yeast for transformation according to (7), wherein the gene consisting of any of the nucleotide sequences (t), (u), (v), and (w) below is expressed at high levels:

(t) a nucleotide sequence encoding a transcription factor;
(u) a nucleotide sequence as shown in SEQ ID NO: 12;
(v) a nucleotide sequence hybridizing under stringent conditions to a nucleotide sequence complementary to the nucleotide sequence as shown in SEQ ID NO: 12; and
(w) a nucleotide sequence having 85% or higher sequence identity with the nucleotide sequence as shown in SEQ ID NO: 12.

(9) The yeast for transformation according to any of (1) to (8), wherein the expression level of the target protein is controlled by a promoter that is activated by the MPP1 homolog.
(10) The yeast for transformation according to (9), wherein the promoter is a methanol-inducible promoter.
(11) The yeast for transformation according to (10), wherein the methanol-inducible promoter is selected from the group consisting of the AMO promoter, the AOX1 promoter, the AOX2 promoter, the CAT promoter, the DAK promoter, the DAO promoter, the DHAS promoter, the FAO promoter, the FDH promoter, the FGH promoter, the FMD promoter, the GAP promoter, the MOX promoter, the MPP1 promoter, the Mut3 promoter, the Mxr1 promoter, the PER6 promoter, the PER10 promoter, the Pex3 promoter, the Pex5 promoter, the Pex8 promoter, the Pex10 promoter, the PHO1 promoter, the Prm1 promoter, the SNF1 promoter, the Swi1p promoter, the Trm1 promoter, the Trm2 promoter, the YNA1 promoter, and the YNA2 promoter.
(12) The yeast for transformation according to (10), wherein the methanol-inducible promoter is selected from the group consisting of the AOX promoter, the DHAS promoter, the FMD promoter, and the MOX promoter.
(13) The yeast for transformation according to any of (1) to (12), which is a transformant of the methanol-assimilating yeast.
(14) The yeast for transformation according to (13), wherein the methanol-assimilating yeast is selected from the group consisting of a yeast of the genus *Pichia*, a yeast of the genus *Candida*, and a yeast of the genus *Torulopsis*.
(15) The yeast for transformation according to (14), wherein the yeast of the genus *Pichia* is selected from the group consisting of *Pichia angusta*, *Pichia pastoris*, *Pichia methanolica*, and *Pichia minuta*.
(16) A method for producing a target protein, characterized by comprising using the yeast for transformation according to any of (1) to (15).
(17) The method for producing a target protein according to (16), wherein the yeast for transformation according to any of (1) to (15) is cultured.
(18) The method for producing a target protein according to (17), wherein culture is conducted using glucose, and/or glycerol, and/or methanol as a carbon source.
(19) The method for producing a target protein according to (18), wherein culture is conducted using glucose, or glycerol, or methanol as a carbon source.
(20) A target protein produced by the method according to any of (16) to (19).

**[0011]** This patent application claims priority from Japanese Patent Application No. 2011-015719 filed on January 27, 2011, and includes part or all of the contents as disclosed in the description thereof.

## Effects of the Invention

**[0012]** The present invention provides a yeast for transformation in which a promoter-activating factor are expressed at high levels. With the use of the yeast for transformation according to the present invention, a target protein can be secreted and produced efficiently.

## Brief Description of the Drawings

**[0013]**

Fig. 1 shows the secretion-expression levels of a fully humanized anti-TNF-α Fab antibody by various transformed yeasts prepared in Example 8 (1: a transformant of *Pichia angusta* expressing a fully humanized anti-TNF-α Fab antibody; 2: a transformant of *Pichia angusta* expressing PaMPP1 (pUCPgapPaMPP1TmG418-introduced) and fully humanized anti-TNF-α Fab antibody; 3: a transformant of *Pichia angusta* expressing PpMPP1 (pUCPgapPpMPP1TmG418-introduced) and fully humanized anti-TNF-α Fab antibody; 4: a transformant of *Pichia angusta* expressing Mut3 (pUCPgapMut3TmG418-introduced) and fully humanized anti-TNF-α Fab antibody; 5: a transformant of *Pichia angusta* co-expressing PaMPP1 and Mut3 (pUCPgapMut3PgapPaMPP1TmG418-intro-

duced) and fully humanized anti-TNF-α Fab antibody; 6: a transformant of *Pichia angusta* co-expressing PaMPP1, Mut3, and PpMPP1 (pUCPgapPpMPP1PgapMut3PgapPaMPP1TmG418-introduced) and fully humanized anti-TNF-α Fab antibody; 7: a transformant of *Pichia pastoris* expressing fully humanized anti-TNF-α Fab antibody; 8: a transformant of *Pichia pastoris* expressing PaMPP1 (pUCPgapPaMPP1TmG418-introduced) and fully humanized anti-TNF-α Fab antibody; 9: a transformant of *Pichia pastoris* expressing PpMPP1 (pUCPgapPpMPP1TmG418-introduced) and fully humanized anti-TNF-α Fab antibody; 10: a transformant of *Pichia pastoris* expressing Mut3 (pUCPgapMut3TmG418-introduced) and fully humanized anti-TNF-α Fab antibody; and 11: a transformant of *Pichia pastoris* co-expressing PaMPP1/Mut3 (pUCPgapMut3PgapPaMPP1TmG418-introduced) and fully humanized anti-TNF-α Fab antibody).

Embodiments for Carrying out the Invention

**[0014]** Hereafter, the present invention is described in detail with reference to the preferred embodiments.

1. Yeast for transformation

**[0015]** The yeast for transformation of the present invention exhibits improved capacity to produce a target protein through the high-level expression of a factor that activates a promoter for transcription and expression of a gene encoding a target protein in the yeast. In the present invention, the term "factor that activates a promoter" refers to "MPP1" or a functional homolog thereof (hereafter, MPP1 and a functional homolog thereof are collectively referred to as "MPP1 homologs").

**[0016]** The term "promoter" refers to a nucleotide sequence region located upstream of a gene encoding a protein. In addition to RNA polymerase, a variety of transcription regulators involved in promotion and suppression of transcription bind to or act on such region to read the nucleotide sequence of the template gene and then synthesize (transcribe) complementary RNA.

**[0017]** "MPP1" is a protein involved in the regulation of peroxisome protein levels. Specifically, MPP1 is PaMPP1 as shown in SEQ ID NO: 38 derived from *Pichia angusta* described in "Transcriptional downregulation of peroxisome numbers affects selective peroxisome degradation in Hansenula polymorpha" (J. Biol. Chem., 2003, 278: 40749-40756) or PpMPP1 as shown in SEQ ID NO: 41 derived from *Pichia pastoris* with the GenBank Accession Number XP_002493066.

**[0018]** A functional homolog of MPP1 is not particularly limited, provided that it is a polypeptide having functions as a factor that activates a promoter equivalent to those of PaMPP1 or PpMPP1 derived from a yeast of the genus *Pichia* described above. Functional homologs of MPP1 may be isolated from *Eumycetes*, including other yeasts or molds. Examples include polypeptides, such as CBF78897 TPA: Putative Zn(II)2Cys6 transcription factor (Eurofung) (*Aspergillus nidulans* FGSC A4), CBF82226 TPA: Putative Zn(II)2Cys6 transcription factor (Eurofung) (*Aspergillus nidulans* FGSC A4), EDK37118 hypothetical protein PGUG_01216 (*Pichia guilliermondii* ATCC 6260), EDK41734 hypothetical protein PGUG_05832 (*Pichia guilliermondii* ATCC 6260), EDP47334 C6 transcription factor, putative (*Aspergillus fumigatus* A1163), EDP53358 C6 transcription factor, putative (*Aspergillus fumigatus* A1163), EEH16200 conserved hypothetical protein (*Paracoccidioides brasiliensis* Pb03), EEH22621 conserved hypothetical protein (*Paracoccidioides brasiliensis* Pb03), EEH42856 conserved hypothetical protein (*Paracoccidioides brasiliensis* Pb18), EEH49391 C6 zinc finger domain-containing protein (*Paracoccidioides brasiliensis* Pb18), EEQ42087 conserved hypothetical protein (*Candida albicans* WO-1), EEQ88974 C6 zinc finger domain-containing protein (*Ajellomyces dermatitidis* ER-3), EER38331 C6 zinc finger protein (*Ajellomyces capsulatus* H143), EFQ30548 hypothetical protein GLRG_05692 (*Glomerella graminicola* M1.001), EFQ34763 hypothetical protein GLRG_09907 (*Glomerella graminicola* M1.001), EFQ35786 C6 zinc finger domain-containing protein (*Glomerella graminicola* M1.001), EFQ86342 hypothetical protein PTT_18442 (*Pyrenophora teres f. teres* 0-1), EFQ86946 hypothetical protein PTT_17743 (*Pyrenophora teres f teres* 0-1), EFR00222 C6 zinc finger domain-containing protein (*Arthroderma gypseum* CBS 118893), XP_001210705 conserved hypothetical protein (*Aspergillus terreus* NIH2624), XP_001210874 conserved hypothetical protein (*Aspergillus terreus* NIH2624), XP_001212782 conserved hypothetical protein (*Aspergillus terreus* NIH2624), XP_001215119 conserved hypothetical protein (*Aspergillus terreus* NIH2624), XP_001216311 predicted protein (*Aspergillus terreus* NIH2624), XP_001216865 predicted protein (*Aspergillus terreus* NIH2624), XP_001216868 conserved hypothetical protein (*Aspergillus terreus* NIH2624), XP_001221354 hypothetical protein CHGG_02133 (*Chaetomium globosum* CBS 148.51), XP_001224209 hypothetical protein CHGG_04995 (*Chaetomium globosum* CBS 148.51), XP_001246901 hypothetical protein CIMG_00672 (*Coccidioides immitis* RS), XP_001257329 hypothetical protein NFIA_047680 (*Neosartorya fischeri* NRRL 181), XP_001257817 sexual development transcription factor RosA (*Neosartorya fischeri* NRRL 181), XP_001259009 C6 zinc finger domain protein (*Neosartorya fischeri* NRRL 181), XP_001259056 hypothetical protein NFIA_005210 (*Neosartorya fischeri* NRRL 181), XP_001259127 hypothetical protein NFIA_005940 (*Neosartorya fischeri* NRRL 181), XP_001259586 C6 zinc finger domain protein (*Neosartorya fischeri* NRRL 181), XP_001262439 hypothetical protein NFIA_

029740 (*Neosartorya fischeri* NRRL 181), XP_001266583 hypothetical protein NFIA_101700 (*Neosartorya fischeri* NRRL 181), XP_001270410 conserved hypothetical protein (*Aspergillus clavatus* NRRL 1), XP_001270412 conserved hypothetical protein (*Aspergillus clavatus* NRRL 1), XP_001387003 predicted protein (*Scheffersomyces stipitis* CBS 6054), XP_001387109 predicted protein (*Scheffersomyces stipitis* CBS 6054), XP_001388653 hypothetical protein An01g02150 (*Aspergillus niger*), XP_001391257 hypothetical protein An07g01700 (*Aspergillus niger*), XP_001394851 hypothetical protein An11g08910 (*Aspergillus niger*), XP_001397318 hypothetical protein An16g00040 (*Aspergillus niger*), XP_001398002 hypothetical protein An16g07020 (*Aspergillus niger*), XP_001400256 hypothetical protein An02g11270 (*Aspergillus niger*), XP_001402162 hypothetical protein An04g08040 (*Aspergillus niger*), XP_001482069 hypothetical protein PGUG_05832 (*Meyerozyma guilliermondii* ATCC 6260), XP_001485545 hypothetical protein PGUG_01216 (*Meyerozyma guilliermondii* ATCC 6260), XP_001525560 conserved hypothetical protein (*Lodderomyces elongisporus* NRRL YB-4239), XP_001552259 hypothetical protein BC1G_08737 (*Botryotinia fuckeliana* B05.10), XP_001590624 hypothetical protein SS1G_08364 (*Sclerotinia sclerotiorum* 1980), XP_001791576 hypothetical protein SNOG_00909 (*Phaeosphaeria nodorum* SN15), XP_001798983 hypothetical protein SNOG_08674 (*Phaeosphaeria nodorum* SN15), XP_001800139 hypothetical protein SNOG_09853 (*Phaeosphaeria nodorum* SN15), XP_001800140 hypothetical protein SNOG_09854 (*Phaeosphaeria nodorum* SN15), XP_001820352 hypothetical protein (*Aspergillus oryzae* RIB40), XP_001820847 hypothetical protein (*Aspergillus oryzae* RIB40), XP_001822149 hypothetical protein (*Aspergillus oryzae* RIB40), XP_001825067 hypothetical protein (*Aspergillus oryzae* RIB40), XP_001827368 hypothetical protein (*Aspergillus oryzae* RIB40), XP_001933596 conserved hypothetical protein (*Pyrenophora tritici-repentis* Pt-1C-BFP), XP_001939276 C6 zinc finger domain containing protein (*Pyrenophora tritici-repentis* Pt-1C-BFP), XP_002145025 C6 transcription factor (ArcA), putative (*Penicillium marneffei* ATCC 18224), XP_002149655 conserved hypothetical protein (*Penicillium marneffei* ATCC 18224), XP_002149656 C6 transcription factor, putative (*Penicillium marneffei* ATCC 18224), XP_002152992 conserved hypothetical protein (*Penicillium marneffei* ATCC 18224), XP_002340358 C6 transcription factor (ArcA), putative (*Talaromyces stipitatus* ATCC 10500), XP_002374058 conserved hypothetical protein (*Aspergillus flavus* NRRL3357), XP_002375724 C6 transcription factor, putative (*Aspergillus flavus* NRRL3357), XP_002376570 conserved hypothetical protein (*Aspergillus flavus* NRRL3357), XP_002379231 C6 transcription factor (ArcA), putative (*Aspergillus flavus* NRRL3357), XP_002381760 C6 transcription factor, putative (*Aspergillus flavus* NRRL3357), XP_002417371 conserved hypothetical protein (*Candida dubliniensis* CD36), XP_002417723 zinc cluster transcription factor, putative (*Candida dubliniensis* CD36), XP_002481660 C6 transcription factor, putative (*Talaromyces stipitatus* ATCC 10500), XP_002484214 conserved hypothetical protein (*Talaromyces stipitatus* ATCC 10500), XP_002494969 ZYRO0B00242p (*Zygosaccharomyces rouxii*), XP_002498535 ZYRO0G125 (*Zygosaccharomyces rouxii*), XP_002541155 predicted protein (*Uncinocarpus reesii* 1704), XP_002549474 hypothetical protein CTRG_03771 (*Candida tropicalis* MYA-3404), XP_002549678 predicted protein (*Candida tropicalis* MYA-3404), XP_002557168 Pc12g02800 (*Penicillium chrysogenum* Wisconsin 54-1255), XP_002557176 Pc12g02890 (*Penicillium chrysogenum* Wisconsin 54-1255), XP_002558943 Pc13g05080 (*Penicillium chrysogenum* Wisconsin 54-1255), XP_002560015 Pc14g00200 (*Penicillium chrysogenum* Wisconsin 54-1255), XP_002563281 Pc20g07580 (*Penicillium chrysogenum* Wisconsin 54-1255), XP_002567816 Pc21g07760 (*Penicillium chrysogenum* Wisconsin 54-1255), XP_002628177 C6 zinc finger domain-containing protein (*Ajellomyces dermatitidis* SLH14081), XP_002795625 conserved hypothetical protein (*Paracoccidioides brasiliensis* Pb01), XP_002848121 C6 zinc finger domain-containing protein (*Arthroderma otae* CBS 113480), XP_003001329 C6 zinc finger domain-containing protein (*Verticillium albo-atrum* VaMs.102), XP_003001370 C6 zinc finger domain-containing protein (*Verticillium albo-atrum* VaMs.102), XP_003002986 conserved hypothetical protein (*Verticillium albo-atrum* VaMs.102), XP_003002987 C6 zinc finger domain-containing protein (*Verticillium albo-atrum* VaMs.102), XP_003009478 C6 zinc finger domain-containing protein (*Verticillium albo-atrum* VaMs.102), XP_003010314 conserved hypothetical protein (*Arthroderma benhamiae* CBS 112371), XP_003024130 conserved hypothetical protein (*Trichophyton verrucosum* HKI 0517), XP_003040390 hypothetical protein NECHADRAFT_44605 (*Nectria haematococca* mpVI 77-13-4), XP_003040613 hypothetical protein NECHADRAFT_79789 (*Nectria haematococca* mpVI 77-13-4), XP_003042194 hypothetical protein NECHADRAFT_42482 (*Nectria haematococca* mpVI 77-13-4), XP_003045088 hypothetical protein NECHADRAFT_43849 (*Nectria haematococca* mpVI 77-13-4), XP_003051420 hypothetical protein NECHADRAFT_80852 (*Nectria haematococca* mpVI 77-13-4), XP_003051651 hypothetical protein NECHADRAFT_92631 (*Nectria haematococca* mpVI 77-13-4), XP_003052094 hypothetical protein NECHADRAFT_78932 (*Nectria haematococca* mpVI 77-13-4), XP_003068062 Fungal Zn binuclear cluster domain containing protein (*Coccidioides posadasii* C735 delta SOWgp), XP_363960 hypothetical protein MGG_01887 (*Magnaporthe oryzae* 70-15), XP_370281 hypothetical protein MGG_06778 (*Magnaporthe oryzae* 70-15), XP_384237 hypothetical protein FG04061.1 (*Gibberella zeae* PH-1), XP_384367 hypothetical protein FG04191.1 (*Gibberella zeae* PH-1), XP_388176 hypothetical protein FG08000.1 (*Gibberella zeae* PH-1), XP_391514 hypothetical protein FG11338.1 (*Gibberella zeae* PH-1), XP_456995 DEHA2B00638p (*Debaryomyces hansenii* CBS767), XP_460402 DEHA2F00946p (*Debaryomyces hansenii* CBS767), XP_570940 hypothetical protein CNE02410 (*Cryptococcus neoformans var. neoformans* JEC21), XP_659433 hypothetical protein AN1829.2 (*Aspergillus nidulans* FGSC A4), XP_660561 hypothetical protein AN2957.2 (*Aspergillus nidulans* FGSC A4), XP_662863 hypothetical protein AN5259.2

(*Aspergillus nidulans* FGSC A4), XP_664794 hypothetical protein AN7190.2 (*Aspergillus nidulans* FGSC A4), XP_746554 C6 transcription factor (*Aspergillus fumigatus* Af293), XP_748614 C6 transcription factor (*Aspergillus fumigatus* Af293), XP_751365 C6 transcription factor (*Aspergillus fumigatus* Af293), XP_753541 C6 transcription factor (ArcA) (*Aspergillus fumigatus* Af293), and XP_775527 hypothetical protein CNBE2410 (*Cryptococcus neoformans var. neoformans* B-3501A).

**[0019]** The MPP1 homolog of the present invention is preferably PaMPP1, PpMPP1, CBF82226 TPA: Putative Zn(II) 2Cys6 transcription factor (Eurofung) (*Aspergillus nidulans* FGSC A4), EDK37118 hypothetical protein PGUG_01216 (*Pichia guilliermondii* ATCC 6260), EDP47334 C6 transcription factor, putative (*Aspergillus fumigatus* A1163), EEH22621 conserved hypothetical protein (*Paracoccidioides brasiliensis* Pb03), EEH49391 C6 zinc finger domain-containing protein (*Paracoccidioides brasiliensis* Pb18), EEQ42087 conserved hypothetical protein (*Candida albicans* WO-1), EFQ34763 hypothetical protein GLRG_09907 (*Glomerella graminicola* M1.001), EFQ86342 hypothetical protein PTT_16442 (*Pyrenophora teres f. teres* 0-1), EFQ86946 hypothetical protein PTT_17743 (*Pyrenophora teres f teres* 0-1), EFR00222 C6 zinc finger domain-containing protein (*Arthroderma gypseum* CBS 118893), XP_001216865 predicted protein (*Aspergillus terreus* NIH2624), XP_001246901 hypothetical protein CIMG_00672 (*Coccidioides immitis* RS), XP_001259586 C6 zinc finger domain protein (*Neosartorya fischeri* NRRL 181), XP_001262439 hypothetical protein NFIA_029740 (*Neosartorya fischeri* NRRL 181), XP_001387003 predicted protein (*Scheffersomyces stipitis* CBS 6054), XP_001485545 hypothetical protein PGUG_01216 (*Meyerozyma guilliermondii* ATCC 6260), XP_001525560 conserved hypothetical protein (*Lodderomyces elongisporus* NRRL YB-4239), XP_001552259 hypothetical protein BC1G_08737 (*Botryotinia fuckeliana* B05.10), XP_001798983 hypothetical protein SNOG_08674 (*Phaeosphaeria nodorum* SN15), XP_001933596 conserved hypothetical protein (*Pyrenophora tritici-repentis* Pt-1C-BFP), XP_001939276 C6 zinc finger domain containing protein (*Pyrenophora tritici-repentis* Pt-1C-BFP), XP_002152992 conserved hypothetical protein (*Penicillium marneffei* ATCC 18224), XP_002340358 C6 transcription factor (ArcA), putative (*Talaromyces stipitatus* ATCC 10500), XP_002379231 C6 transcription factor (ArcA), putative (*Aspergillus flavus* NRRL3357), XP_002417371 conserved hypothetical protein (*Candida dubliniensis* CD36), XP_002417723 zinc cluster transcription factor, putative (*Candida dubliniensis* CD36), XP_002481660 C6 transcription factor, putative (*Talaromyces stipitatus* ATCC 10500), XP_002494969 ZYRO0B00242p (*Zygosaccharomyces rouxii*), XP_002541155 predicted protein (*Uncinocarpus reesii* 1704), XP_002549474 hypothetical protein CTRG_03771 (*Candida tropicalis* MYA-3404), XP_002549678 predicted protein (*Candida tropicalis* MYA-3404), XP_002795625 conserved hypothetical protein (*Paracoccidioides brasiliensis* Pb01), XP_003002986 conserved hypothetical protein (*Verticillium albo-atrum* VaMs.102), XP_003009478 C6 zinc finger domain-containing protein (*Verticillium albo-atrum* VaMs.102), XP_003045088 hypothetical protein NECHADRAFT_43849 (*Nectria haematococca* mpVI 77-13-4), XP_003068062 Fungal Zn binuclear cluster domain containing protein (*Coccidioides posadasii* C735 delta SOWgp), XP_363960 hypothetical protein MGG_01887 (*Magnaporthe oryzae* 70-15), XP_384367 hypothetical protein FG04191.1 (*Gibberella zeae* PH-1), XP_456995 DEHA2B00638p (*Debaryomyces hansenii* CBS767), XP_746554 C6 transcription factor (*Aspergillus fumigatus* Af293), or XP-753541 C6 transcription factor (ArcA) (*Aspergillus fumigatus* Af293).

**[0020]** The MPP1 homolog of the present invention is more preferably PaMPP1, PpMPP1, XP_001246901 hypothetical protein CIMG_00672 (*Coccidioides immitis* RS), XP_001262439 hypothetical protein NFIA_029740 (*Neosartorya fischeri* NRRL 181), XP_001387003 predicted protein (*Scheffersomyces stipitis* CBS 6054), XP_001552259 hypothetical protein BC1G_08737 (*Botryotinia fuckeliana* B05.10), XP_002340358 C6 transcription factor (ArcA), putative (*Talaromyces stipitatus* ATCC 10500), XP_002549678 predicted protein (*Candida tropicalis* MYA-3404), XP_384367 hypothetical protein FG04191.1 (*Gibberella zeae* PH-1), or XP_456995 DEHA2B00638p (*Debaryomyces hansenii* CBS767).

**[0021]** The MPP1 homolog of the present invention may be a polypeptide having an amino acid sequence having at least 85% or higher, preferably 90% or higher, more preferably 95% or higher, further preferably 97% or higher, and most preferably 99% or higher sequence identity with the amino acid sequence of the MPP1 homolog, provided that it is capable of exerting functions substantially equivalent to those of the MPP1 homolog.

**[0022]** A promoter activated by an MPP1 homolog is not particularly limited, provided that it is activated by an MPP1 homolog and capable of realizing high-level expression of the target protein in a yeast. A promoter activated by an MPP1 homolog is preferably a methanol-inducible promoter.

**[0023]** The methanol-inducible promoter of the present invention is not particularly limited, provided that it has transcription activity when a carbon source is methanol. For example, it is preferably the ADR1 promoter, the ALD promoter, the Alg3 promoter, the AMO promoter, the AOX1 promoter, the AOX2 promoter, the Atg1 promoter, the Atg2 promoter, the Atg3 promoter, the Atg4 promoter, the Atg5 promoter, the Atg6 promoter, the Atg7 promoter, the Atg8 promoter, the Atg9 promoter, the Atg10 promoter, the Atg11 promoter, the Atg12 promoter, the Atg13 promoter, the Atg15 promoter, the Atg16 promoter, the Atg17 promoter, the Atg18 promoter, the Atg19 promoter, the Atg21 promoter, the Atg22 promoter, the Atg24 promoter, the Atg26 promoter, the Atg28 promoter, the CAT promoter, the CNX promoter, the CPY promoter, the DAK promoter, the DAO promoter, the DHAS promoter, the DIC1 promoter, the DNM1 promoter, the FAO promoter, the FDH promoter, the FGH promoter, the FIG4 promoter, the FMD promoter, the Fmp22 promoter, the GAP promoter, the Gasip promoter, the GCR1 promoter, the GDH promoter, the GSH promoter, the HARO7 promoter, the

HpPmp20 promoter, the HSA1 promoter, the HSV2 promoter, the KEX1 promoter, the KEX2 promoter, the MAL1 promoter, the MAS promoter, the MDV1 promoter, the MLN promoter, the Mnn9p promoter, the MOX promoter, the MPP1 promoter, the Mut3 promoter, the Mxr1 promoter, the Nil2p promoter, the OCH promoter, the PAH2 promoter, the PAK1 promoter, the PDD1 promoter, the PEP4 promoter, the PEP 12 promoter, the PepN promoter, the PER6 promoter, the PER10 promoter, the Pex1 promoter, the Pex3 promoter, the Pex4 promoter, the Pex5 promoter, the Pex6 promoter, the Pex7 promoter, the Pex8 promoter, the Pex10 promoter, the Pex11 promoter, the Pex12 promoter, the Pex13 promoter, the Pex17 promoter, the Pex19 promoter, the Pex20 promoter, the Pex22 promoter, the Pex23 promoter, the Pex24 promoter, the Pex25 promoter, the Pex26 promoter, the Pex32 promoter, the PHO1 promoter, the PIP2 promoter, the POX1 promoter, the PPI promoter, the PRAI promoter, the Prm1 promoter, the PYC promoter, the RHO promoter, the SLA2 promoter, the SNF1 promoter, the STL1 promoter, the Swilp promoter, the TOR promoter, the TPS1 promoter, the TPS3 promoter, the Trm1 promoter, the Trm2 promoter, the Vac8p promoter, the Vam3p promoter, the Vam7p promoter, the YAP promoter, the VPS1 promoter, the Yhflp promoter, the YNA1 promoter, the YNA2 promoter, the YNI1 promoter, the YNR1 promoter, the YNT1 promoter, the YPT1 promoter, or the Ypt7p promoter, and more preferably the AOX promoter, the DHAS promoter, the FMD promoter, or the MOX promoter.

**[0024]** The MPP1 homolog of the present invention is preferably PaMPP1, and PaMPP1 is not particularly limited, provided that it is a polypeptide having the function of a factor that activates the promoter mentioned above. In addition to a polypeptide consisting of the amino acid sequence as shown in SEQ ID NO: 38, it may be a polypeptide consisting of an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 38 by substitution, deletion, insertion, and/or addition of one or a plurality of amino acids or an amino acid sequence having 85% or higher, 90% or higher, 95% or higher, and more preferably 99% or higher sequence identity with the amino acid sequence as shown in SEQ ID NO: 38. The number of amino acids defined by the term "a plurality of amino acids" is not particularly limited, provided that functions of the MPP1 homolog are retained. The number of amino acids is preferably up to 102, more preferably up to 100, further preferably up to 90, and most preferably up to 80, 70, 60, 50, 40, 30, 20, 10, 5, 4, 3, or 2.

**[0025]** The MPP1 homolog of the present invention is preferably PpMPP1, and PpMPP1 is not particularly limited, provided that it is a polypeptide having the function of factor that activates the promoter mentioned above. In addition to a polypeptide consisting of the amino acid sequence as shown in SEQ ID NO: 41, it may be a polypeptide consisting of an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 41 by substitution, deletion, insertion, and/or addition of one or a plurality of amino acids or an amino acid sequence having 85% or higher, 90% or higher, 95% or higher, and more preferably 99% or higher sequence identity with the amino acid sequence as shown in SEQ ID NO: 41. The number of amino acids defined by the term "a plurality of amino acids" is not particularly limited, provided that the functions of the MPP1 homolog are retained. The number of amino acids is preferably up to 133, more preferably up to 130, further preferably up to 120, and most preferably up to 110, 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 4, 3, or 2.

**[0026]** According to the present invention, amino acid sequence identity can be determined by a method well known in the art using sequence analysis software and the like. An example is a homology search conducted with the use of genetic information processing software (GENETYX Ver. 10, network edition, Genetyx).

**[0027]** The MPP1 homolog of the present invention may be a polypeptide consisting of the amino acid sequence as shown in SEQ ID NO: 49. PaMPP1 as shown in SEQ ID NO: 49 is a polypeptide composed of amino acid residues 28 to 67, which may be capable of binding to a promoter. By combining the polypeptide as shown in SEQ ID NO: 49 with another polypeptide and allowing expression of the protein in a host at high levels, the effects of promoter activation can be attained. In addition to the amino acid sequence as shown in SEQ ID NO: 49, it may be a polypeptide consisting of an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 49 by substitution, deletion, insertion, and/or addition of one or a plurality of amino acids or an amino acid sequence having 85% or higher, 90% or higher, 95% or higher, and more preferably 99% or higher sequence identity with the amino acid sequence as shown in SEQ ID NO: 49. The number of amino acids defined by the term "a plurality of amino acids" is not particularly limited, provided that functions of the MPP1 homolog are retained. The number of amino acids is preferably up to 6, more preferably up to 5, further preferably up to 4, and most preferably up to 3 or 2.

**[0028]** The MPP1 homolog of the present invention may be a polypeptide consisting of the amino acid sequence as shown in SEQ ID NO: 50. PpMPP1 as shown in SEQ ID NO: 50 is a polypeptide composed of amino acid residues 58 to 98, which may be capable of binding to a promoter. By combining the polypeptide as shown in SEQ ID NO: 50 with another polypeptide and allowing expression of the protein in a host at high levels, the effects of promoter activation can be attained. In addition to the amino acid sequence as shown in SEQ ID NO: 50, it may be a polypeptide consisting of an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 50 by substitution, deletion, insertion, and/or addition of one or a plurality of amino acids or an amino acid sequence having 85% or higher, 90% or higher, 95% or higher, and more preferably 99% or higher sequence identity with the amino acid sequence as shown in SEQ ID NO: 50. The number of amino acids defined by the term "a plurality of amino acids" is not particularly limited, provided that the functions of the MPP1 homolog are retained. The number of amino acids is preferably up to 6, more preferably up to 5, further preferably up to 4, and most preferably up to 3 or 2.

**[0029]** In the present invention, the term "expression" refers to transcription or translation of a nucleotide sequence that causes production of a protein, which is a gene product. Such expression may be constant regardless of conditions such as external stimulus or growth conditions, or it may be influenced by such conditions. A promoter that activates expression is not particularly limited, provided that such promoter activates expression of a nucleotide sequence encoding a target protein.

**[0030]** The term "high-level expression" used in the present invention refers to an increased protein level in a host or culture solution or an increased mRNA level in the host, compared with a general level. For example, the protein or mRNA level may be measured using an antibody that recognizes a protein or by a technique such as RT-PCR, Northern hybridization, or hybridization using DNA arrays, and the determined level may be compared with that of a non-modified strain, such as a parent strain or wild-type strain. Thus, high-level expression can be confirmed.

**[0031]** The high-level expression of the MPP1 homolog can be realized by conventional techniques, such as modification of the MPP1 homolog gene of the chromosome and/or the vector (e.g., increase of the copy number, insertion of a promoter, or modification of a codon), introduction of the MPP1 homolog gene into a host, or production of a strain expressing MPP1 homolog gene at high levels via introduction of a mutation into the host, in addition to the induction of expression of a gene encoding the MPP1 homolog of the chromosome and/or the vector.

**[0032]** A strain expressing the MPP1 homolog gene at high levels via introduction of a mutation into the host can be obtained via conventional techniques, such as mutation of the host with the aid of a drug or ultraviolet rays, followed by selection of a strain expressing the MPP homolog at high levels.

**[0033]** The term "protein" used in the present invention is composed of two or more amino acids joined by peptide bonds, including short-chain proteins generally referred to as peptides or oligopeptides and long-chain proteins referred to as polypeptides.

**[0034]** The "target protein" of the present invention is not particularly limited, and it can be, for example, an enzyme derived from a microorganism or a protein produced by a multicellular organism, such as an animal or plant. Examples thereof include phytase, protein A, protein G, protein L, amylase, glucosidase, cellulase, lipase, protease, glutaminase, peptidase, nuclease, oxidase, lactase, xylanase, trypsin, pectinase, isomerase, and fluorescent protein. Preferable examples include human and/or animal therapeutic proteins.

**[0035]** Specific examples of human and/or animal therapeutic proteins include hepatitis B virus surface antigen, hirudin, antibody, human antibody, partial antibody, human partial antibody, serum albumin, human serum albumin, epidermal growth factor, human epidermal growth factor, insulin, growth hormone, erythropoietin, interferon, antihemophilic factor, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), thrombopoietin, IL-1, IL-6, tissue plasminogen activator (TPA), urokinase, leptin, and stem cell growth factor (SCF).

**[0036]** The term "antibody" refers to a heterotetrameric protein composed of two L- and H-polypeptide chains joined by disulfide bonds. An antibody is not particularly limited, provided that it is capable of binding to a particular antigen.

**[0037]** The term "partial antibody" refers to Fab antibody, $(Fab)_2$ antibody, scFv antibody, diabody antibody, or a derivative of any thereof. A partial antibody is not particularly limited, provided that it is capable of binding to a particular antigen. The term "Fab antibody" refers to a heteromeric protein comprising the L-chain and the Fd chain of the antibody joined by S-S bonds, or a heteromeric protein comprising the L-chain and the Fd chain of the antibody joined with each other without S-S bonds. A heteromeric protein is not particularly limited, provided that it is capable of binding to a particular antigen.

**[0038]** An amino acid constituting a protein may be a naturally occurring, non-natural, or modified amino acid. The amino acid sequence of a protein may be artificially modified or de novo designed.

**[0039]** In the present invention, the term "capacity for producing a target protein" refers to the capacity for producing a target protein in a host cell or the potential that can be achieved via introduction of a gene encoding such protein.

**[0040]** The term "yeast for transformation" used in the present invention refers to a yeast capable of producing a target protein at high levels via introduction of a gene encoding the target protein. Such yeast preferably expresses the MPP1 homolog at high levels and more preferably expresses PaMPP1, PpMPP1, a polypeptide comprising the amino acid sequence as shown in SEQ ID NO: 49, or a polypeptide comprising the amino acid sequence as shown in SEQ ID NO: 50 at high levels.

**[0041]** Examples of techniques of yeast transformation include known techniques such as electroporation, the lithium acetate method, and the spheroplast method, although the techniques are not limited thereto. For example, *Pichia angusta* is generally transformed via electroporation as described in "Highly-efficient electrotransformation of the yeast *Hansenula polymorpha*" (Curr. Genet., 25: 305.310.).

**[0042]** Expression of the "transcription factor" may be enhanced, in addition to that of the factor that activates the aforementioned promoter in the yeast for transformation of the present invention. The term "transcription factor" used in the present invention refers to a substance that activates the initiation of transcription or protein production by a promoter in a mechanism for protein production by a promoter.

**[0043]** The high-level expression of the transcription factor can be realized by conventional techniques, such as modification of the transcription factor gene of the chromosome and/or the vector (e.g., increase of the number of copies,

insertion of a promoter, or modification of a codon), introduction of a transcription factor gene into a host, or production of a strain expressing the transcription factor gene at high levels via introduction of a mutation into a host, in addition to induction of the expression of a gene encoding the transcription factor of the chromosome and/or vector (i.e., the transcription factor gene).

**[0044]** A strain expressing the transcription factor gene at high levels via introduction of a mutation into the host can be obtained via conventional techniques, such as mutation of the host with the aid of a drug or ultraviolet rays, followed by selection of a strain expressing the transcription factor gene at high levels.

**[0045]** A preferable transcription factor acts additively or synergistically with the MPP1 homolog, although a transcription factor is not limited thereto. An example of a transcription factor is Mut3 derived from *Pichia angusta*. Mut3 is a polypeptide with the GenBank Accession Number AY046886, the sequence of which is as shown in SEQ ID NO: 46.

**[0046]** The transcription factor of the present invention may be a homolog of Mut3 isolated from another yeast cell. An example thereof is Trm1 of *Candida boidinii*.

**[0047]** Mut3 of the present invention is preferably a polypeptide consisting of the amino acid sequence as shown in SEQ ID NO: 46. Alternatively, it may be a polypeptide consisting of an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 46 by substitution, deletion, insertion, and/or addition of one or a plurality of amino acids or an amino acid sequence having 85% or higher, 90% or higher, 95% or higher, and more preferably 99% or higher sequence identity with the amino acid sequence as shown in SEQ ID NO: 46. The number of amino acids defined by the term "a plurality of amino acids" is not particularly limited, provided that functions of the transcription factor are retained. The number of amino acids is preferably up to 139, more preferably up to 130, further preferably up to 120, and most preferably up to 110, 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 4, 3, or 2.

**[0048]** The MPP1 homolog gene or transcription factor gene of the present invention may be DNA encoding an MPP1 homolog or transcription factor. Preferable DNA encoding an MPP1 homolog is as shown in SEQ ID NO: 10 or 11 in the Sequence Listing, and preferable DNA encoding a transcription factor is as shown in SEQ ID NO: 12 in the Sequence Listing. Alternatively, DNA encoding an MPP1 homolog or transcription factor may encompass a nucleotide sequence encoding a polypeptide hybridizing under stringent conditions to a nucleotide sequence complementary to the nucleotide sequence as shown in SEQ ID NO: 10, 11, or 12 in the Sequence Listing and have functions as an MPP1 homolog or transcription factor. Further, DNA may encompass a nucleotide sequence having 85% or higher, preferably 90% or higher, more preferably 95% or higher, and further preferably 99% or higher sequence identity with the nucleotide sequence as shown in SEQ ID NO: 10, 11, or 12 and may encode a polypeptide having functions as an MPP1 homolog or transcription factor.

**[0049]** The "DNA hybridizing under stringent conditions" can be obtained by, for example, conducting hybridization in the presence of 0.7 to 1.0 M NaCl at 65°C using a filter on which colony- or plaque-derived DNA is immobilized and washing the filter with a 2x SSC solution (a 1x SSC solution comprises 150 mM sodium chloride and 15 mM sodium citrate) at 65°C. Such DNA can be obtained by washing the filter, preferably with a 0.5x SSC solution at 65°C, more preferably with a 0.2x SSC solution at 65°C, and further preferably with a 0.1x SSC solution at 65°C.

**[0050]** According to the present invention, nucleotide sequence identity can be determined by a method well known in the art using sequence analysis software or the like. An example is a homology search conducted with the use of genetic information processing software (GENETYX Ver. 10, network edition, Genetyx).

**[0051]** In the present invention, yeasts are not particularly limited, although methanol-assimilating yeasts are preferred. Examples of methanol-assimilating yeasts include those of the genus *Pichia*, the genus *Candida*, and the genus *Torulopsis*. Among yeasts of the genus *Pichia*, *Pichia angusta*, *Pichia pastoris*, *Pichia methanolica*, and *Pichia minuta* are particularly preferable, although the yeasts are not limited thereto.

**[0052]** *Pichia angusta* is also referred to as *Hansenula polymorpha*. *Pichia minuta* is also referred to as *Ogataea minuta*.

**[0053]** In general, "methanol-assimilating yeasts" can be cultured with the use of methanol as a sole carbon source. However, the ability to utilize methanol may be lost due to artificial modification or mutation.

**[0054]** Examples of preferable *Pichia angusta* strains include, but are not particularly limited to, NCYC495 (ATCC14754), 8V (ATCC34438), and DL-1 (ATCC26012). Such strains can be obtained from the American Type Culture Collection or other institutions. Strains derived from such strains can also be used, and examples of leucine auxotrophs include NCYC495-derived BY4329, 8V-derived BY5242, and DL-1-derived BY5243. These strains can be distributed by the National BioResource Project. In the present invention, strains derived from the strains mentioned above can also be used.

**[0055]** Examples of preferable *Pichia pastoris* strains include, but are not particularly limited to, Y-11430 and X-33. Such strains can be obtained from the Northern Regional Research Laboratory or other institutions. Strains derived from such strains can also be used. In the present invention, strains derived from the strains mentioned above can also be used.

2. Method for protein production

**[0056]** An example of a method for producing a protein using the yeast for transformation of the present invention is

a method comprising culturing the yeast for transformation and allowing a protein to accumulate in the yeast or a culture supernatant. While a method involving the use of methanol-assimilating yeast is described as a preferable example, methods are not limited thereto.

**[0057]** Yeasts for transformation can be generally cultured with the use of any medium containing a nutrient source that can be utilized by methanol-assimilating yeasts. For example, a common medium obtained by adequately mixing carbon sources, such as sugars (e.g., glucose, sucrose, and maltose), organic acids (e.g., lactic acid, acetic acid, citric acid, and propionic acid), alcohols (e.g., methanol, ethanol, and glycerol), carbohydrates (e.g., paraffin), fats and oils (e.g., soybean oil and rapeseed oil), or mixtures of any thereof, nitrogen sources, such as ammonium sulfate, ammonium phosphate, urea, yeast extract, meat extract, peptone, and corn steep liquor, and other nutrient sources, such as inorganic salts and vitamins, can be used, and culture can be carried out in a batch or continuous system.

**[0058]** When culturing the yeast for transformation, a single type of carbon source may be used, or a mixture of two or more types of carbon sources may be used. Culture may be initiated in the presence of a carbon source, or a carbon source may be added during culture.

**[0059]** The yeast for transformation may be subjected to culture alone or in combinations of two or more. The yeast for transformation may be subjected to culture in the presence of other microorganisms.

**[0060]** In the present invention, culture can be carried out under general conditions. For example, culture can be carried out at a pH of 2.5 to 10.0 between 10°C and 48°C in an aerobic environment for 10 hours to 10 days.

**[0061]** Proteins produced with the use of the yeast for transformation of the present invention may be present in a culture supernatant, or proteins may be accumulated in the yeast. Alternatively, proteins may be isolated therefrom via any technique. Proteins can be isolated from a culture supernatant or yeast by performing known protein purification techniques in adequate combination. For example, the transformants are first cultured in an adequate medium, and a culture solution is subjected to centrifugation or filtration to remove yeasts from the culture supernatant. The obtained culture supernatant is then subjected to techniques such as salting-out (e.g., ammonium sulfate precipitation or sodium phosphate precipitation), solvent precipitation (e.g., protein fractional precipitation with acetone or ethanol), dialysis, gel filtration chromatography, ion-exchange chromatography, hydrophobic chromatography, affinity chromatography, reverse phase chromatography, or ultrafiltration. Any such techniques may be carried out alone or in combination, and the proteins of the present invention are isolated from the culture supernatant.

**[0062]** The isolated proteins can be used without processing. Alternatively, such proteins may be subjected to pharmacological modification such as PEGylation, modification for the purpose of impartation of enzyme or isotope functions, or other types of modification before use. Also, proteins may be used in various forms of formulations.

**[0063]** When the high-level expression of the MPP1 homolog or transcription factor is to be attained via introduction of the MPP1 homolog gene or the transcription factor gene into a host, it is preferable that an expression vector containing such genes be constructed and the resulting vector then be introduced into a host.

**[0064]** The term "expression vector" used in the present invention refers to a nucleic acid molecule having a function of expressing a target gene in a transformed host cell. The expression vector has, for example, an expression cassette, a homologous recombination region, a selection marker gene, such as an auxotrophic complementary gene or drug tolerant gene, and an autonomously replicating sequence. The vector after transformation may be integrated into the chromosome, or it may be present in the form of an autonomously replicating vector. Examples of autonomously replicating vectors include the YEp vector, the YRp vector, and the YCp vector. In the case of the genus *Pichia*, examples of vectors include, but are not particularly limited to, the pPICHOLI, pHIP, pHRP, and pHARS vectors.

**[0065]** The "expression cassette" is composed of the MPP1 homolog gene and/or the transcription factor gene as promoter activating factors and a promoter to express such genes. The expression cassette may comprise a terminator gene. Accordingly, the expression cassettes include a cassette comprising the MPP1 homolog gene as a promoter activating factor (i.e., the MPP1 homolog gene expression cassette), a cassette comprising the transcription factor gene (i.e., the transcription factor gene expression cassette), and a cassette comprising both the MPP1 homolog gene and the transcription factor gene. The expression cassette can be constructed in a plasmid such as pUC19, or it can be prepared via PCR.

**[0066]** A promoter used for MPP1 homolog gene expression is not particularly limited, and a promoter that can induce gene expression with a selected carbon source may adequately be used.

**[0067]** When a carbon source is methanol, examples of promoters for MPP1 homolog gene expression include, but are not particularly limited to, the MOX promoter, the FMD promoter, the DHAS promoter, the AOX promoter, and the GAP promoter.

**[0068]** When a carbon source is glucose or glycerol, examples of promoters for MPP1 homolog gene expression include, but are not particularly limited to, the GAP promoter, the TEF promoter, the LEU2 promoter, the URA3 promoter, the ADE promoter, the ADH1 promoter, and the PGK1 promoter.

**[0069]** For transcription factor gene expression, a promoter that can induce gene expression with a selected carbon source may adequately be used, and the promoters for MPP1 homolog gene expression exemplified above can also serve as promoters for transcription factor gene expression.

**[0070]** The "target protein expression vector" of the present invention comprises a "target protein expression cassette" having a promoter activated by the MPP1 homolog, and preferably the methanol-inducible promoter, in the 5' side of the target protein gene and the termination codon and/or the terminator sequence in the 3' side of the target protein gene. When the expression vector is to be integrated into the chromosome, the vector preferably comprises a nucleotide sequence having 50% or higher sequence identity with a 50-bp or longer nucleotide sequence of the yeast chromosome.

**[0071]** When a target protein is to be secreted from the yeast, a nucleotide sequence encoding a signal sequence may be introduced into the 5' end of the target protein gene. A nucleotide sequence encoding a signal sequence is not particularly limited, provided that such nucleotide sequence encodes a signal sequence that can be secreted and expressed by yeast. Examples thereof include nucleotide sequences encoding signal sequences of the mating factor α (MFα) of *Saccharomyces cerevisiae*, acid phosphatase (PHO1) of *Pichia angusta*, acid phosphatase (PHO1) of *Pichia pastoris*, invertase (SUC2) of *Saccharomyces cerevisiae*, PLB1 of *Saccharomyces cerevisiae*, bovine serum albumin (BSA), human serum albumin (HSA), and immunoglobulin.

**[0072]** The "target protein expression cassette", "MPP1 homolog gene expression cassette", "transcription factor gene expression cassette", and the like of the present invention may be contained in a single type of vector. In such a case, specifically, a yeast host and a transformant are produced simultaneously.

**[0073]** When the MPP1 homolog gene, the transcription factor gene, and the target protein-encoding gene are introduced into yeast, use of selection marker genes, such as the auxotrophic complementary gene or drug tolerant gene, together with the MPP1 homolog gene expression cassette, the transcription factor gene expression cassette, and the target protein gene cassette, is preferable. A selection marker is not particularly limited. Auxotrophic complementary genes, such as the URA3 gene, the LEU2 gene, the ADE1 gene, or the HIS4 gene, can be selected by restoration of the prototrophic phenotypes of the uracil-, leucine-, adenine-, or histidine-auxotrophs, respectively. Drug tolerant genes, such as the G418 tolerant gene, the zeocin tolerant gene, or the hygromycin tolerant gene, can be selected based on the tolerance in a medium containing G418, zeocin, or hygromycin, respectively. The auxotrophic selection marker used when preparing a yeast host cannot be used, unless the selection marker is not destroyed. In such a case, the selection marker may be restored by methods known in the art.

**[0074]** When the MPP1 homolog gene or transcription factor gene is to be integrated into the chromosome, it is preferable to prepare such genes so as to have DNA comprising homologous regions of the yeast chromosome. The MPP1 homolog expression cassette or transcription factor gene expression cassette may be integrated into the chromosome via non-specific integration without using homologous regions, integration using a single homologous region, or double-integration using two homologous regions.

**[0075]** Regarding homologous regions, there may be 50% or higher sequence identity between the nucleotide sequence of the chromosome and that of the expression vector. While the length thereof is not particularly limited, it is preferably 50 bp or longer. Sequence identity between homologous regions is more preferably 70% or higher, further preferably 80% or higher, still further preferably 90% or higher, and most preferably 100%.

**[0076]** In the case of integration using a homologous region, for example, a homologous region of the plasmid vector is cleaved at one or more sites with restriction enzymes, so as to locate the homologous region at the end of a linear expression vector. Thus, transformation can be achieved. In the case of homologous integration into the MOX terminator, for example, a linear expression vector obtained by cleaving the MOX terminator sequence at the *Nru*I site or the *Eco*RV site may be used to integrate the expression cassette into the MOX terminator.

**[0077]** The number of copies of expression cassettes per chromosome is not particularly limited. The location in the chromosome at which the MPP1 homolog gene or transcription factor gene expression vector has been integrated is not particularly limited, provided that the MPP1 homolog gene or the transcription factor gene is produced in the strain. When two or more copies of expression vectors are integrated into a transformant, a plurality of expression vectors may be integrated thereinto at the same position, or they may be integrated at different positions one by one.

**[0078]** Methods for preparing the target protein gene, the MPP1 homolog gene, or the transcription factor gene of the present invention are not particularly limited. Such genes may be prepared via PCR or other techniques from microorganism cells, yeast cells, insect cells, plant cells, mammalian animal cells, or phage display libraries. Alternatively, the target gene may be fully synthesized based on such nucleotide sequence. For example, a DNA primer designed based on the MPP1 homolog gene is synthesized, chromosome DNA is isolated from a yeast having the MPP1 homolog gene, and PCR is carried out using the chromosome DNA as a template and the above DNA primer. Thus, the target gene can be obtained.

**[0079]** The target protein gene, MPP1 homolog gene, or transcription factor gene on the chromosome can be modified by introducing a gene into a host chromosome via homologous recombination, site-directed mutagenesis, or other techniques. For example, the protein gene can be introduced into the chromosome so as to increase the number of copies, a promoter located upstream of the protein may be substituted with a more potent promoter, or a codon can be modified to become more suitable as a host of the protein gene.

Examples

**[0080]** Hereafter, the present invention is described in greater detail with reference to the examples, although the present invention is not limited thereto. The recombinant DNA technology employed in the examples below is specifically described in the following books: Molecular Cloning 2nd Edition, Cold Spring Harbor Laboratory Press, 1989; and Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley-Interscience.

**[0081]** Plasmids obtained in the examples below were amplified using transformants obtained with the use of *E. coli* DH5α competent cells (Takara Bio Inc.) under the designated conditions.

**[0082]** The MOX promoter (SEQ ID NO: 1), the MOX terminator (SEQ ID NO: 2), and the LEU2 gene (SEQ ID NO: 3) used when constructing an antibody expression vector were prepared by PCR using, as a template, genomic DNA of *Pichia angusta* 8V. The AOX promoter (SEQ ID NO: 4) was prepared by PCR using genomic DNA of *Pichia pastoris* X-33 as a template. The mating factor α pre-pro signal gene (MFα, SEQ ID NO: 5) was prepared by PCR using genomic DNA of *Saccharomyces cerevisiae* S288c as a template.

**[0083]** The antibody gene was prepared by PCR using, as templates, the L-chain gene (SEQ ID NO: 6) and the Fd-chain gene (SEQ ID NO: 7) (JP 2009-082033 A) chemically synthesized based on the disclosed sequence information of the fully humanized anti-TNF-α antibody (adalimumab; HUMIRA®).

**[0084]** In addition, the GAP promoter used for vector construction (SEQ ID NO: 8), the G418 tolerant gene regulated by the GAP promoter (SEQ ID NO: 9), the PaMPP1 gene (SEQ ID NO: 10), the PpMPP1 gene (SEQ ID NO: 11), and the Mut3 gene (SEQ ID NO: 12) were obtained by PCR using, for example, genomic DNA of *Pichia angusta* NCYC495, genomic DNA of *Pichia pastoris* X-33, or an adequate commercially available vector as templates.

**[0085]** PCR was carried out using the Prime STAR HS DNA Polymerase (Takara Bio Inc.) in accordance with the manufacturer's instructions. Genomic DNAs were prepared from yeasts using Dr. GenTLE® (Takara Bio Inc.) in accordance with the manufacturer's instructions.

**[0086]** Plasmids were prepared by transformation into *E. coli* DH5α competent cells (Takara Bio Inc.). Plasmids were prepared from plasmid-carrying strains using the QIAprep spin miniprep kit (QIAGEN).

(Example 1) Construction of fully humanized anti-TNF-α Fab antibody expression vector

**[0087]** A gene fragment (SEQ ID NO: 13) having the *Hind*III-*Not*I-*Bam*HI-*Spe*I-*Mun*I-*Bgl*II-*Xba*I-*Eco*RI multicloning sites was fully synthesized, and the resultant was inserted into the *Hind*III and *Eco*RI sites of pUC19 to prepare pUC-1. Also, a gene fragment having the *Hind*III recognition sequences on both sides of the LEU2 gene was prepared by PCR using the primers 1 and 2 (SEQ ID NOs: 14 and 15), the fragment was treated with *Hind*III, and it was then inserted into the *Hind*III site of pUC-1 to prepare pUC-2. Subsequently, a gene fragment having the *Bam*HI recognition sequences on both sides of the MOX promoter was prepared by PCR using the primers 3 and 4 (SEQ IDs NO: 16 and 17), the fragment was treated with *Bam*HI, and it was then inserted into the *Bam*HI site of pUC-2 to prepare pUC-Pmox. A gene fragment having the *Eco*RI recognition sequences on both sides of the AOX promoter was prepared by PCR using the primers 5 and 6 (SEQ ID NOs: 18 and 19), the fragment was treated with *Eco*RI, and it was then inserted into the *Mun*I site of pUC-2 to prepare pUC-Paox.

**[0088]** A gene fragment having the *Mun*I recognition sequences on both sides of the MOX promoter was prepared by PCR using the primers 7 and 8 (SEQ ID NOs: 20 and 21), the fragment was treated with *Mun*I, and it was then inserted into the *Mun*I site of pUC-Pmox to prepare pUC-PmoxPmox. A gene fragment having the *Bam*HI recognition sequences on both sides of the AOX promoter was prepared by PCR using the primers 9 and 10 (SEQ ID NOs: 22 and 23), the fragment was treated with *Bam*HI, and it was then inserted into the *Bam*HI site of pUC-Paox to prepare pUC-PaoxPaox. A gene fragment having the *Xba*I recognition sequences on both sides of the MOX promoter was prepared by PCR using the primers 11 and 12 (SEQ ID NOs: 24 and 25), the fragment was treated with *Xba*I, and it was then inserted into the *Xba*I sites of pUC-PmoxPmox and pUC-PaoxPaox to prepare pUC-PmoxPmoxTm and pUC-PaoxPaoxTm.

**[0089]** A gene fragment having the *Spe*I recognition sequence in a site upstream of MFα was prepared by PCR using the primers 13 and 14 (SEQ ID NOs: 26 and 27). A gene fragment having the 19-bp 3'-terminal fragment of MFα in a site upstream of the L-chain and the *Spe*I recognition sequence in a site downstream of the L-chain was prepared by PCR using the primers 15 and 16 (SEQ ID NOs: 28 and 29). These gene fragments were mixed to prepare a template, and PCR was carried out using the resulting template and the primers 13 and 16 to prepare a gene fragment having *Spe*I recognition sequences on both sides of a fusion gene of MFα and the L-chain. The resulting gene fragment was treated with *Spe*I and inserted into the *Spe*I sites of pUC-PmoxPmoxTm and pUC-PaoxPaoxTm to prepare pUC-Pmox-LPmoxTm and pUC-PaoxLPaoxTm.

**[0090]** Separately, a gene fragment having the *Bgl*II recognition sequence in a site upstream of MFα was prepared by PCR using the primers 17 (SEQ ID NO: 30) and 14. A gene fragment having the 19-bp 3'-terminal fragment of MFα in a site upstream of the Fd-chain and the *Bgl*II recognition sequence in a site downstream of the Fd-chain was prepared by PCR using the primers 18 and 19 (SEQ ID NOs: 31 and 32). These gene fragments were mixed to prepare a template,

and PCR was carried out using the resulting template and the primers 17 and 19 to prepare a gene fragment having the *Bgl*II recognition sequences on both sides of a fusion gene of MFα and the Fd-chain. The resulting gene fragment was treated with *Bgl*II and inserted into the *Bgl*II sites of pUC-PmoxLPmoxTm and pUC-PaoxLPaoxTm to prepare pUC-PmoxLPmoxFdTm and pUC-PaoxLPaoxFdTm.

**[0091]** pUC-PmoxLPmoxFdTm is designed to allow expression of the L-chain and the Fd-chain of the Fab antibody under the control of the MOX promoter. pUC-PaoxLPaoxFdTm is designed to allow expression of the L-chain and the Fd-chain of the Fab antibody under the control of the AOX promoter.

(Example 2) Construction of PAMPP1 expression vector

**[0092]** A gene fragment having the *Xba*I recognition sequences on both sides of the MOX terminator was prepared by PCR using the primers 11 and 12, the fragment was treated with *Xba*I, and it was then inserted into the *Xba*I site of pUC-1 prepared in Example 1 to prepare pUCTm.

**[0093]** Subsequently, a gene fragment having the *Eco*RI recognition sequences on both sides of the GAP promoter was prepared by PCR using the primers 20 and 21 (SEQ ID NOs: 33 and 34), the fragment was treated with *Eco*RI, and it was then inserted into the *Mun*I site of pUCTm to prepare pUCPgapTm. Subsequently, a gene fragment having the *Eco*RI recognition sequences on both sides of the G418 tolerant gene controlled by the GAP promoter was prepared by PCR using the primers 20 and 22 (SEQ ID NO: 35), the fragment was treated with *Eco*RI, and it was then inserted into the *Eco*RI site of pUCPgapTm to prepare pUCPgapTmG418. Subsequently, a gene fragment having the *Bam*HI recognition sequences on both sides of the PaMPP1 gene was prepared by PCR using the primers 23 and 24 (SEQ ID NOs: 36 and 37), the fragment was treated with *Bam*HI, and it was then inserted into the *Bgl*II site of pUCPgapTmG418 to construct pUCPgapPaMPP1 TmG418.

**[0094]** This expression vector is designed to allow expression of PaMPP1 (SEQ ID NO: 38) under the control of the GAP promoter.

(Example 3) Construction of PpMPP1 expression vector

**[0095]** A gene fragment having the *Bam*HI recognition sequences on both sides of the PpMPP1 gene was prepared by PCR using the primers 25 and 26 (SEQ ID NOs: 39 and 40), the fragment was treated with *Bam*HI, and it was then inserted into the *Bgl*II site of pUCPgapTmG418 to construct pUCPgapPpMPP1TmG418.

**[0096]** This expression vector is designed to allow expression of PpMPP1 (SEQ ID NO: 41) under the control of the GAP promoter.

(Example 4) Construction of Mut3 expression vector

**[0097]** A gene fragment having the *Eco*RI recognition sequences on both sides of the G418 tolerant gene controlled by the GAP promoter was prepared by PCR using the primers 20 and 22, the fragment was treated with *Eco*RI, and it was then inserted into the *Eco*RI site of pUCTm to prepare pUCTmG418. Subsequently, a gene fragment having the *Spe*I recognition sequence on 5' terminal side of the Mut3 gene and *Bam*HI recognition sequence on 3' terminal side the Mut3 gene was prepared by PCR using the primers 27 and 28 (SEQ ID NOs: 42 and 43), the fragment was treated with *Spe*I and *Bam*HI, and the resultant was inserted into the *Spe*I and *Bgl*II sites of pUCTmG418 to construct pUCMut3TmG418. Subsequently, a gene fragment having the *Bam*HI recognition sequences on both sides of the GAP promoter was prepared by PCR using the primers 29 and 30 (SEQ ID NOs: 44 and 45), the fragment was treated with *Bam*HI, and it was then inserted into the *Bam*HI site of pUCMut3TmG418 to construct pUCPgapMut3TmG418.

**[0098]** This expression vector is designed to allow expression of Mut3 (SEQ ID NO: 46) under the control of the GAP promoter.

(Example 5) Construction of PaMPP1/Mut3 co-expression vector

**[0099]** A gene fragment having the *Bam*HI recognition sequence on 5' terminal side of the Mut3 gene and the *Spe*I recognition sequence on 3' terminal side of the Mut3 gene was prepared by PCR using the primers 31 and 32 (SEQ ID NOs: 47 and 48), the fragment was treated with *Bam*HI and *Spe*I, and the resultant was inserted into the *Bam*HI and *Spe*I sites of pUCPgapPaMPP1TmG418 obtained in Example 2 to construct pUCMut3PgapPaMPP1TmG418. Subsequently, a gene fragment having the *Bam*HI recognition sequences on both sides of the GAP promoter was prepared by PCR using the primers 29 and 30, the fragment was treated with *Bam*HI, and it was then inserted into the *Bam*HI site of pUCMut3PgapPaMPP1TmG418 to construct pUCPgapMut3PgapPaMPP 1 TmG418.

**[0100]** This expression vector is designed to allow co-expression of PaMPP1 and Mut3 under the control of the GAP promoter.

(Example 6) Construction of PaMPP1/Mut3/PpMPP1 co-expression vector

**[0101]** A gene fragment having the *Not*I recognition sequence on 5' terminal side of the PpMPP1 gene and the *Eco*52I recognition sequence on 3' terminal side of the PpMPP1 gene was prepared by PCR using pUCPgapPpMPP1TmG418 obtained in Example 3 as a template and the primers 33 and 34 (SEQ ID NOs: 51 and 52). The fragment was treated with *Not*I and *Eco*52I, and it was then inserted into the *Not*I site of pUCPgapMut3PgapPaMPP1TmG418 obtained in Example 5 to construct pUCPgapPpMPP1PgapMut3PgapPaMPP1TmG418.

**[0102]** This expression vector is designed to allow co-expression of PaMPP1, Mut3, and PpMPP1 under the control of the GAP promoter.

(Example 7) Preparation of MPP1 homolog-expressing host yeast using MPP1 homolog expression vector

**[0103]** *E. coli* strains were transformed with the use of pUCPgapPaMPP1TmG418, pUCPgapPpMPP1TmG418, pUCPgapMut3TmG418, pUCPgapMut3PgapPaMPP1TmG418, and pUCPgapPpMPP1PgapMut3PgapPaMPP1TmG418 constructed in Examples 2 to 6, the resulting transformants were cultured in 5 ml of 2YT medium (1.6% Bacto Tryptone (Difco), 1.0% Bacto Yeast Extract (Difco), and 0.5% NaCl), and pUCPgapPaMPP1TmG418, pUCPgapPpMPP1TmG418, pUCPgapMut3TmG418, pUCPgapMut3PgapPaMPP1TmG418, and pUCPgapPpMPP1PgapMut3PgapPaMPP1TmG418 were obtained from the resulting strains using the QIAprep spin miniprep kit (QIAGEN).

**[0104]** The plasmids were cleaved at the *Eco*RV or *Nru*I site in the MOX terminator gene or the *Not*I site in the multicloning site to prepare linearized plasmids. With the use of such linearized pUCPgapPaMPP1TmG418, pUCPgapPpMPP1TmG418, pUCPgapMut3TmG418, pUCPgapMut3PgapPaMPP1TmG418, and pUCPgapPpMPP1PgapMut3PgapPaMPP1TmG418, *Pichia angusta* and *Pichia pastoris* were subjected to transformation in the manner described below.

**[0105]** The leucine-auxotrophs of *Pichia angusta* NCYC495 were inoculated into 3 ml of YPD medium (1% Bacto Yeast Extract (Difco), 2% peptone, and 2% glucose) and subjected to shake culture at 37°C overnight to obtain a pre-culture solution. The obtained pre-culture solution (500 μl) was inoculated into 50 ml of YPD medium, shake culture was conducted at 37°C until OD 600 reached 1 to 1.5, the culture product was centrifuged at 3,000 x g and 20°C for 10 minutes to harvest cells, and the cells were resuspended in 10 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 250 μl of 1M DTT (final concentration: 25 mM).

**[0106]** The suspension was incubated at 37°C for 15 minutes, the cells were harvested by centrifugation at 3,000 x g and 20°C for 10 minutes, and the resultant was washed with 50 ml of STM buffer (270 mM sucrose, 10 mM Tris-HCl, and 1 mM magnesium chloride; pH 7.5), which had been ice-cooled in advance. After the cells were harvested by centrifugation at 3,000 x g and 4°C for 10 minutes, the cells were washed again with 25 ml of ice-cooled STM buffer, followed by centrifugation at 3,000 x g and 4°C for 10 minutes to harvest cells. The cells were suspended in 250 μl of ice-cooled STM buffer at the end, and the resulting suspension was designated as a competent cell solution.

**[0107]** The competent cell solution (60 μl) was mixed with 3 μl of a solution of linearized pUCPgapPaMPP1TmG418, pUCPgapPpMPP1TmG418, pUCPgapMut3TmG418, pUCPgapMut3PgapPaMPP1TmG418, or pUCPgapPpMPP1PgapMut3PgapPaMPP1TmG418, the mixtures were transferred into an electroporation cuvette (a disposable cuvette; electrode gap: 2 mm; BM Equipment Co., Ltd.) and pulsed at 7.5 kV/cm, 10 μF, and 900 Ω, the resultant was suspended in 1 ml of YPD medium, and the suspension was then allowed to stand at 37°C for 1 hour. Thereafter, cells were harvested by centrifugation at 3,000 x g at room temperature for 5 minutes, the harvested cells were suspended in 1 ml of YNBMSG medium (0.17% yeast nitrogen base w/o amino acid and ammonium sulfate (Difco) and 0.1% sodium glutamate), and the cells were harvested from the suspension again by centrifugation at 3,000 x g at room temperature for 5 minutes. The cells were resuspended in an adequate amount of YNBMSG medium, the resulting cell suspension was applied to the YNBMSGG418 selection agar plate (0.17% yeast nitrogen base w/o amino acid and ammonium sulfate (Difco), 0.1% sodium glutamate, 1.5% agarose, 2% glucose, 0.05% G418 disulfate, and 100 mg/l leucine), and the cells grown as a result of stationary culture at 37°C for 3 days were selected. Thus, 5 types of *Pichia angustan MPP1 homolog*-expressing host yeast cells were obtained.

**[0108]** The leucine-auxotrophs of *Pichia pastoris* X-33 were inoculated into 3 ml of YPD medium (1% Bacto Yeast Extract (Difco), 2% peptone, and 2% glucose) and subjected to shake culture at 30°C overnight to obtain a pre-culture solution. The obtained pre-culture solution (500 μl) was inoculated into 100 ml of YPD medium, shake culture was conducted at 30°C until OD 600 reached 1 to 1.5, cells were harvested by centrifugation at 1,500 x g and 4°C for 5 minutes, and the harvested cells were resuspended in 100 ml of sterile water.

**[0109]** The suspension was centrifuged at 1,500 x g and 4°C for 5 minutes to harvest cells, and the harvested cells were resuspended in 50 ml of sterile water. The suspension was centrifuged at 1,500 x g and 4°C for 5 minutes to harvest cells, and the cells were resuspended in 4 ml of a 1 M sorbitol solution. The resulting suspension was centrifuged at 1,500 x g and 4°C for 5 minutes to harvest cells, the cells were resuspended in 200 μl of a 1 M sorbitol solution, and

the resulting suspension was designated as the competent cell solution.

**[0110]** The competent cell solution (50 μl) was mixed with 3 μl of a solution of linearized pUCPgapPaMPP1TmG418, pUGPgapPpMPP1TmG418, pUCPgapMut3TmG418, or pUCPgapMut3PgapPaMPP1TmG418, the mixtures were transferred into an electroporation cuvette (a disposable cuvette; electrode gap: 2 mm; BM Equipment Co., Ltd.) and pulsed at 7.5 kV/cm, 25 μF, and 200 Ω, the resulting cells were suspended in 1 ml of a 1 M sorbitol solution, the resulting cell suspension was applied to the YNBMSGG418 selection agar plate (0.17% yeast nitrogen base w/o amino acid and ammonium sulfate (Difco), 0.1% sodium glutamate, 1.5% agarose, 2% glucose, 0.05% G418 disulfate, and 100 mg/l leucine), and the cells grown as a result of stationary culture at 30°C for 3 days were selected. Thus, 4 types of *Pichia pastoris* MPP1 homolog-expressing host yeast cells were obtained.

(Example 8) Preparation of transformant expressing MPP1 homolog and fully humanized anti-TNF-α Fab antibody using fully humanized anti-TNF-α Fab antibody expression vector

**[0111]** *E. coli* strains were transformed with the use of pUC-PmoxLPmoxFdTm and pUC-PaoxLPaoxFdTm constructed in Example 1, the transformants were cultured in 5 ml of 2YT medium (1.6% Bacto Tryptone (Difco), 1.0% Bacto Yeast Extract (Difco), and 0.5% NaCl), and pUC-PmoxLPmoxFdTm and pUC-PaoxLPaoxFdTm were obtained from the cells using the QIAprep spin miniprep kit (QIAGEN).

**[0112]** The plasmids were cleaved at the *Eco*RV site in the MOX terminator gene to prepare linearized plasmids. With the use of such linearized pUC-PmoxLPmoxFdTm and pUC-PaoxLPaoxFdTm, *Pichia angusta* was transformed with pUC-PmoxLPmoxFdTm, and *Pichia pastoris* was transformed with pUC-PaoxLPaoxFdTm in the manner described below.

**[0113]** The 5 types of *Pichia angustan MPP1 homolog*-expressing host yeast cells obtained in Example 7 or the leucine-auxotrophs of *Pichia angusta* NCYC495 were inoculated into 3 ml of YPD medium (1% Bacto Yeast Extract (Difco), 2% peptone, and 2% glucose) and subjected to shake culture at 37°C overnight to obtain a pre-culture solution. The obtained pre-culture solution (500 μl) was inoculated into 50 ml of YPD medium, shake culture was conducted at 37°C until OD 600 reached 1 to 1.5, cells were harvested by centrifugation at 3,000 x g and 20°C for 10 minutes, and the harvested cells were resuspended in 10 ml of 50 mM potassium phosphate buffer (pH 7.5) containing 250 μl of 1M DTT (final concentration: 25 mM).

**[0114]** The suspension was incubated at 37°C for 15 minutes, followed by centrifugation at 3,000 x g and 20°C for 10 minutes to harvest cells and washing with 50 ml of STM buffer (270 mM sucrose, 10 mM Tris-HCl, 1 mM magnesium chloride; pH 7.5), which had been ice-cooled in advance. After the wash solution was centrifuged at 3,000 x g and 4°C for 10 minutes to harvest cells, the cells were washed again with 25 ml of ice-cooled STM buffer, followed by centrifugation at 3,000 x g and 4°C for 10 minutes to harvest cells. The cells were suspended in 250 μl of ice-cooled STM buffer at the end, and the resulting suspension was designated as a competent cell solution.

**[0115]** The competent cell solution (60 μl) was mixed with 3 μl of a solution of linearized pUC-PmoxLPmoxFdTm, the mixture was transferred into an electroporation cuvette (a disposable cuvette; electrode gap: 2 mm; BM Equipment Co., Ltd.) and pulsed at 7.5 kV/cm, 10 μF, and 900 Ω, the resulting cells were suspended in 1 ml of YPD medium, and the suspension was then allowed to stand at 37°C for 1 hour. Thereafter, the suspension was centrifuged at 3,000 x g at room temperature for 5 minutes to harvest cells, the cells were suspended in 1 ml of YNBMSG medium (0.17% yeast nitrogen base w/o amino acid and ammonium sulfate (Difco) and 0.1% sodium glutamate), and the resultant was centrifuged again at 3,000 x g at room temperature for 5 minutes to harvest cells. The cells were resuspended in an adequate amount of YNBMSG medium, transformants of *Pichia angustan MPP1 homolog*-expressing host yeast cells were applied to the YNBMSGG418 selection agar plate (0.17% yeast nitrogen base w/o amino acid and ammonium sulfate (Difco), 0.1% sodium glutamate, 1.5% agarose, 2% glucose, and 0.05% G418 disulfate), the leucine-auxotrophic transformants of *Pichia angusta* NCYC495 were applied to the YNB selection agar plate (0.67% yeast nitrogen base w/o amino acid (Difco), 1.5% agarose, and 2% glucose), and the cells grown as a result of stationary culture at 37°C for 3 days were selected. Thus, transformants of *Pichia angusta* expressing MPP1 homolog and fully humanized anti-TNF-α Fab antibody and transformants of *Pichia angusta* expressing fully humanized anti-TNF-α Fab antibody were obtained.

**[0116]** The four types of MPP1 homolog-expressing host yeast cells of *Pichia pastoris* obtained in Example 7 or the leucine-auxotrophs of *Pichia pastoris* X-33 were inoculated into 3 ml of YPD medium (1% Bacto Yeast Extract (Difco), 2% peptone, and 2% glucose) and subjected to shake culture at 30°C overnight to obtain a pre-culture solution. The obtained pre-culture solution (500 μl) was inoculated into 100 ml of YPD medium, shake culture was conducted at 30°C until OD 600 reached 1 to 1.5, cells were harvested by centrifugation at 1,500 x g and 4°C for 5 minutes, and the harvested cells were resuspended in 100 ml of sterile water.

**[0117]** The suspension was centrifuged at 1,500 x g and 4°C for 5 minutes to harvest cells, and the cells were resuspended in 50 ml of sterile water. The suspension was centrifuged at 1,500 x g and 4°C for 5 minutes to harvest cells, and the cells were resuspended in 4 ml of a 1 M sorbitol solution. The resulting suspension was centrifuged at 1,500 x g and 4°C for 5 minutes to harvest cells, the cells were resuspended in 200 μl of a 1 M sorbitol solution, and the suspension

was designated as the competent cell solution.

**[0118]** The competent cell solution (50 μl) was mixed with 3 μl of a solution of linearized pUC-PaoxLPaoxFdTm, the mixture was transferred into an electroporation cuvette (a disposable cuvette; electrode gap: 2 mm, BM Equipment Co., Ltd.) and pulsed at 7.5 kV/cm, 25 μF, and 200 Ω, the resulting cells were suspended in 1 ml of a 1 M sorbitol solution, the transformants of *Pichia pastoris* (as MPP1 homolog-expressing host cells) were applied to the YNBMSGG418 selection agar plate (0.17% yeast nitrogen base w/o amino acid and ammonium sulfate (Difco), 0.1% sodium glutamate, 1.5% agarose, 2% glucose, and 0.05% G418 disulfate), the leucine-auxotrophic transformants of *Pichia pastoris* were applied to the YNB selection agar plate (0.67% yeast nitrogen base w/o amino acid (Difco), 1.5% agarose, and 2% glucose), and the strains grown as a result of stationary culture at 30°C for 3 days were selected. Thus, transformants of *Pichia pastoris* expressing MPP1 homolog and fully humanized anti-TNF-α Fab antibody and transformants of *Pichia pastoris* expressing fully humanized anti-TNF-α Fab antibody were obtained.

(Example 9) Culture of transformants expressing MPP1 homolog and fully humanized anti-TNF-α Fab antibody

**[0119]** The transformants expressing MPP1 homolog and fully humanized anti-TNF-α Fab antibody and the transformants expressing fully humanized anti-TNF-α Fab antibody obtained in Example 8 were inoculated into 2 ml of BMGY medium (1% Bacto Yeast Extract, 2% peptone, 0.34% yeast nitrogen base w/o amino acid and ammonium sulfate (Difco), 1% ammonium sulfate, 0.4 mg/l biotin, 100 mM potassium phosphate (pH 6.0), and 2% glycerol) and subjected to shake culture at 37°C and 120 rpm for 72 hours, and the culture supernatant was then recovered by centrifugation at 15,000 rpm and 4°C for 1 minute.

(Example 10) Measurement of amount of fully humanized anti-TNF-α Fab antibody secretion by ELISA

**[0120]** The amount of the anti-TNF-α Fab antibody secreted and expressed in the culture supernatant of each of the transformants expressing MPP1 homolog and fully humanized anti-TNF-α Fab antibody was measured by sandwich ELISA (i.e., enzyme-linked immunosorbent assay) in the manner described below. The goat anti-human IgG (Fab-specific) antibody (SIGMA) diluted 2,500-fold with an immobilization buffer (a 0.1 N sodium bicarbonate solution; pH 9.6) was applied to an ELISA plate (MaxiSoap, Nunc) at 50 μl/well, and the plate was subjected to incubation at 4°C overnight.

**[0121]** After the completion of incubation, the solution was removed from the wells, the wells were blocked with 250 μl of Immunoblock (Dainippon Sumitomo Pharma Co., Ltd.), and the plate was allowed to stand at room temperature for 1 hour. The product was washed three times with PBST buffer (8 g/l sodium chloride, 0.2 g/l potassium chloride, 1.15 g/l monosodium phosphate (anhydrous), 0.2 g/l potassium dihydrogen phosphate (anhydrous), and 0.1 % Tween 20), and serial dilutions of the standard Fab antibody (Anti-Human IgG Fab, Rockland) the culture supernatants were added at 50 μl/well, and the reaction was allowed to proceed at room temperature for 1 hour.

**[0122]** After the plate was washed twice with PBST buffer, a secondary antibody solution diluted 8,000-fold with PBST buffer (secondary antibody: Anti-human IgG (Fab-specific)-HRP conjugate antibody (SIGMA)) was added at 50 μl/well, and the reaction was allowed to proceed at room temperature for 1 hour. After the plate was washed four times with PBST buffer, 100 μl of TMB-1 Component Microwell Peroxidase Substrate SureBlue (KPL) was added, and the reaction solution was allowed to stand at room temperature for 20 minutes. The reaction was terminated with the addition of 100 μl of TMB Stop Solution (KPL), and the absorbance at 450 nm was then measured using a microplate reader (BenchMark Plus, Bio-Rad). The Fab antibody in the culture supernatant was quantified using the calibration curve for the standard Fab antibody. The amount of secretory expression of the anti-TNF-α Fab antibody determined by the above method is shown in Fig. 1.

**[0123]** As a result, the amounts of antibody secretory expression attained with the transformants expressing PaMPP1 (pUCPgapPaMPP1TmG418-introduced) and fully humanized anti-TNF-α Fab antibody, the transformants expressing PpMPP1 (pUCPgapPpMPP1TmG418-introduced) and fully humanized anti-TNF-α Fab antibody, and the transformants expressing Mut3 (pUCPgapMut3TmG418-introduced) and fully humanized anti-TNF-α Fab antibody were found to be apparently higher than those attained with the host strains (i.e., transformants expressing fully humanized anti-TNF-α Fab antibody).

**[0124]** Further, the amounts of antibody secretory expression attained with the transformants co-expressing PaMPP1/Mut3 (pUCPgMut3PgMPP1TmG418-introduced) and fully humanized anti-TNF-α Fab antibody were significantly higher than those attained with the hosts (i.e., transformants expressing fully humanized anti-TNF-α Fab antibody) and those attained with the transformants expressing the PaMPP1 (pUCPgMPP1TmG418-introduced) and fully humanized anti-TNF-α Fab antibody. Since the amounts of antibody secretory expression attained with the transformants co-expressing PaMPP1/Mut3/PpMPP1 (pUCPgapPpMPP1PgapMut3PgapPaMPP1TmG418) and fully humanized anti-TNF-α Fab antibody were lower than those attained with the transformants co-expressing PaMPP1/Mut3 and fully humanized anti-TNF-α Fab antibody, a combination of PaMPP1 with Mut3 was found to be effective.

Industrial Applicability

**[0125]** The present invention can be used in the field of production of proteins and, in particular, in relation to antibodies used for treatment of diseases.

**[0126]** All publications, patents, and patent applications cited herein are incorporated herein by reference in their entirety.

SEQUENCE LISTING

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35 40 45

Arg Pro Lys Cys Asp Phe Cys Thr Ala Lys Gly Leu Glu Cys Val Trp
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Pro Glu Glu Val Lys Lys Asn Val Phe Val Asn Asn Ser Phe Lys Asp
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Phe Cys Phe Ser Gln Ser Lys His Arg Asp Arg Phe Arg Lys Pro Thr
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Thr Leu Gln Ser Tyr Ser Met Asn Pro Ser Leu Ser Ser Gly Ser Asn
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Phe Lys Arg Asp Asn Ser Phe Pro Glu Thr Leu Phe Ser Ser Ser Thr
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Glu Ser Val Ser Thr Ser Ser Leu Met Ser Arg Ser Gln Ile Trp His
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## Claims

1. A yeast for transformation, wherein the capacity for target protein production is improved by high-level expression of an MPP1 homolog.

2. The yeast for transformation according to claim 1, wherein the MPP1 homolog is any of the polypeptides (a), (b), and (c) below:

(a) a polypeptide consisting of the amino acid sequence as shown in SEQ ID NO: 38;
(b) a polypeptide consisting of an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 38 by substitution, deletion, insertion, and/or addition of one or a plurality of amino acids;
(c) a polypeptide consisting of an amino acid sequence having 85% or higher sequence identity with the amino acid sequence as shown in SEQ ID NO: 38;
or any of the polypeptides (d), (e) and (f) below:
(d) a polypeptide consisting of the amino acid sequence as shown in SEQ ID NO: 41;
(e) a polypeptide consisting of an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 41 by substitution, deletion, insertion, and/or addition of one or a plurality of amino acids; or
(f) a polypeptide consisting of an amino acid sequence having 85% or higher sequence identity with the amino acid sequence as shown in SEQ ID NO: 41.

3. The yeast for transformation according to claim 1, wherein the MPP1 homolog is is partially composed of any of the polypeptides (g), (h) and (i) below:

(g) a polypeptide consisting of the amino acid sequence as shown in SEQ ID NO: 49;
(h) a polypeptide consisting of an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 49 by substitution, deletion, insertion, and/or addition of one or a plurality of amino acids;
(i) a polypeptide consisting of an amino acid sequence having 85% or higher sequence identity with the amino acid sequence as shown in SEQ ID NO: 49;
or any of the polypeptides (j), (k), and (l) below:
(j) a polypeptide consisting of the amino acid sequence as shown in SEQ ID NO: 50;
(k) a polypeptide consisting of an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 50 by substitution, deletion, insertion, and/or addition of one or a plurality of amino acids; or
(l) a polypeptide consisting of an amino acid sequence having 85% or higher sequence identity with the amino acid sequence as shown in SEQ ID NO: 50.

4. The yeast for transformation according to any of claims 1 to 3, wherein the transcription factors are expressed at high levels.

5. The yeast for transformation according to claim 4, wherein the transcription factor acts additively or synergistically with the MPP1 homolog.

6. The yeast for transformation according to claim 4 or 5, wherein the transcription factor is any of the polypeptides (m), (n), and (o) below:

(m) a polypeptide consisting of the amino acid sequence as shown in SEQ ID NO: 46;
(n) a polypeptide consisting of an amino acid sequence derived from the amino acid sequence as shown in SEQ ID NO: 46 by substitution, deletion, insertion, and/or addition of one or a plurality of amino acids; and
(o) a polypeptide consisting of an amino acid sequence having 85% or higher sequence identity with the amino acid sequence as shown in SEQ ID NO: 46.

7. The yeast for transformation according to any of claims 1 to 6, wherein the gene consisting of any of the nucleotide sequences (p), (q), (r), and (s) is expressed at high levels:

(p) a nucleotide sequence encoding the MPP 1 homolog;
(q) the nucleotide sequence as shown in SEQ ID NO: 10 or 11;
(r) a nucleotide sequence hybridizing under stringent conditions to a nucleotide sequence complementary to the nucleotide sequence as shown in SEQ ID NO: 10 or 11; and
(s) a nucleotide sequence having 85% or higher sequence identity with the nucleotide sequence as shown in SEQ ID NO: 10 or 11.

8. The yeast for transformation according to claim 7, wherein the gene consisting of any of the nucleotide sequences (t), (u), (v), and (w) below is expressed at high levels:

(t) a nucleotide sequence encoding a transcription factor;
(u) a nucleotide sequence as shown in SEQ ID NO: 12;
(v) a nucleotide sequence hybridizing under stringent conditions to a nucleotide sequence complementary to the nucleotide sequence as shown in SEQ ID NO: 12; and
(w) a nucleotide sequence having 85% or higher sequence identity with the nucleotide sequence as shown in SEQ ID NO: 12.

9. The yeast for transformation according to any of claims 1 to 8, wherein the expression level of the target protein is controlled by a promoter that is activated by the MPP 1 homolog.

10. The yeast for transformation according to claim 9, wherein the promoter is a methanol-inducible promoter.

11. The yeast for transformation according to claim 10, wherein the methanol-inducible promoter is selected from the group consisting of the AMO promoter, the AOX1 promoter, the AOX2 promoter, the CAT promoter, the DAK promoter, the DAO promoter, the DHAS promoter, the FAO promoter, the FDH promoter, the FGH promoter, the FMD promoter, the GAP promoter, the MOX promoter, the MPP1 promoter, the Mut3 promoter, the Mxr1 promoter, the PER6 promoter, the PER10 promoter, the Pex3 promoter, the Pex5 promoter, the Pex8 promoter, the Pex10 promoter, the PHO1 promoter, the Prm1 promoter, the SNF1 promoter, the Swi/p promoter, the Trm1 promoter, the Trm2 promoter, the YNA1 promoter, and the YNA2 promoter.

12. The yeast for transformation according to claim 10, wherein the methanol-inducible promoter is selected from the group consisting of the AOX promoter, the DHAS promoter, the FMD promoter, and the YNA2 promoter.

13. The yeast for transformation according to any of claims 1 to 12, which is a transformant of the methanol-assimilating yeast.

14. The yeast for transformation according to claim 13, wherein the methanol-assimilating yeast is selected from the group consisting of a yeast of the genus *Pichia*, a yeast of the genus *Candida*, and a yeast of the genus *Torulopsis*.

15. The yeast for transformation according to claim 14, wherein the yeast of the genus *Pichia* is selected from the group

consisting of *Pichia angusta*, *Pichia pastoris*, *Pichia methanolica*, and *Pichia minuta*.

**16.** A method for producing a target protein, **characterized by** comprising using the yeast for transformation according to any of claims 1 to 15.

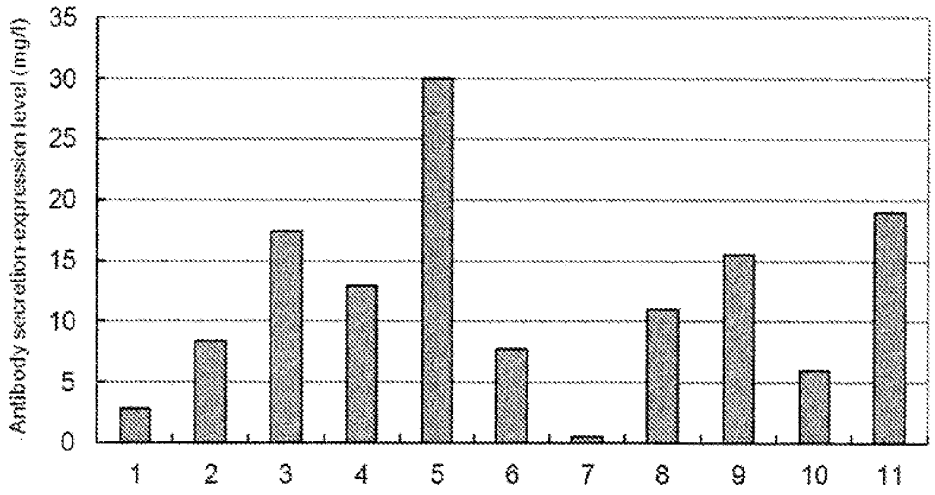
**17.** The method for producing a target protein according to claim 16, wherein the yeast for transformation according to any of claims 1 to 15 is cultured.

**18.** The method for producing a target protein according to claim 17, wherein culture is conducted using glucose, and/or glycerol, and/or methanol as a carbon source.

**19.** The method for producing a target protein according to claim 17, wherein culture is conducted using glucose, or glycerol, or methanol as a carbon source.

**20.** A target protein produced by the method according to any of claims 16 to 19.

Fig. 1


Antibody secretion expression level (mg/l)
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
11

## INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2012/051102

A. CLASSIFICATION OF SUBJECT MATTER
*C12N15/09*(2006.01)i, *C07K16/24*(2006.01)i, *C12N1/19*(2006.01)i, *C12P21/00*(2006.01)i

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)
C12N15/09, C07K16/24, C12N1/19, C12P21/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

| | | | |
|---|---|---|---|
| Jitsuyo Shinan Koho | 1922-1996 | Jitsuyo Shinan Toroku Koho | 1996-2012 |
| Kokai Jitsuyo Shinan Koho | 1971-2012 | Toroku Jitsuyo Shinan Koho | 1994-2012 |

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA/BIOSIS/MEDLINE/WPIDS(STN), JSTPlus/JMEDPlus/JST7580(JDreamII)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | Tim van Zutphen et al., Adaptation of Hansenula polymorpha to methanol: a transcriptome analysis, BMC Genomics, 2010, Vol. 11, No. 1 | 1-20 |
| Y | Adriana Nivea Leao-Helder et al., Transcriptional Down-regulation of Peroxisome Numbers Affects Selective Peroxisome Degradation in Hansenula polymorpha, J. Biol. Chem., 2003, Vol. 278, No. 42, p. 40749-40756 | 1-20 |
| Y | Yu Sasano et al., Trm1p, a Zn(II)2Cys6-Type Transcription Factor, Is a Master Regulator of Methanol-Specific Gene Activation in the Methylotrophic Yeast Candida boidinii, Eukaryot Cell, 2008, Vol. 7, No. 3, p. 527-536 | 1-20 |

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

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| Date of the actual completion of the international search<br>12 April, 2012 (12.04.12) | Date of mailing of the international search report<br>24 April, 2012 (24.04.12) |
| Name and mailing address of the ISA/<br>Japanese Patent Office<br><br>Facsimile No. | Authorized officer<br><br>Telephone No. |

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**INTERNATIONAL SEARCH REPORT**

International application No.
PCT/JP2012/051102

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | Hiroya Yurimoto, Molecular Basis of Methanol-Inducible Gene Expression and Its Application in the Methylotrophic Yeast Candida boidinii, Bioscience, Biotechnology, and Biochemistry, 2009, Vol. 73, No. 4, p. 793-800 | 1-20 |
| X | JP 2009-82033 A (Kaneka Corp.), 23 April 2009 (23.04.2009), claim 10 (Family: none) | 20 |
| A | Kristof De Schutter et al., Genome sequence of the recombinant protein production host Pichia pastoris, Nature Biotechnology, 2009, Vol. 27, p. 561-566 | 1-20 |
| A | E. Berardi and B. Tinto, Pichia angusta transcriptional activator Mut3p (MUT3) gene, complete cds, GenBank, ACCESSION:AY046886, VERSION:AY046886.1, GI:21913143, 2002 | 1-20 |

Form PCT/ISA/210 (continuation of second sheet) (July 2009)

## REFERENCES CITED IN THE DESCRIPTION

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### Patent documents cited in the description


- JP 2011015719 A **[0011]**
- JP 2009082033 A **[0083]**


### Non-patent literature cited in the description


- **GELLISSEN, G.** *Appl. Microbiol. Biotechnol.*, December 2000, vol. 54 (6), 741-50 **[0008]**
- **V., GENU et al.** *Eur. J. Biochem.*, 2003, vol. 270, 2467-2475 **[0008]**
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- Molecular Cloning. Cold Spring Harbor Laboratory Press, 1989 **[0080]**
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Espacenet

# Bibliographic data: JP2008017774 (A) — 2008-01-31

## PRODUCTION OF RECOMBINANT HUMAN HEMOGLOBIN BY USING PICHIA YEAST

**Inventor(s):** NAKASHIRO KEISUKE; HOASHI YOHEI; KAI TOSHIYA; UNO KIMIYUKI; ODAGIRI MASAKI ± (NAKASHIRO KEISUKE, ; HOASHI YOHEI, ; KAI TOSHIYA, ; UNO KIMIYUKI, ; ODAGIRI MASAKI)

**Applicant(s):** NIPRO CORP ± (NIPRO CORP)

**Classification:** **- international:** ***C12N15/09; C12P21/02;*** *C12R1/84*
**- cooperative:**

**Application number:** JP20060192673 20060713 Global Dossier

**Priority number (s):** JP20060192673 20060713

### Abstract of JP2008017774 (A)

PROBLEM TO BE SOLVED: To provide a safe gene-recombinant type hemoglobin having functions and properties similar to the hemoglobin originated from human blood, and carrying no risk of contamination by a virus. ;SOLUTION: The method for producing a functional recombinant human hemoglobin includes culturing a yeast of the genus Pichia transformed by an expression vector containing a DNA encoding the human hemoglobin [alpha] chain (Hb[alpha]) under control of a promotor functionable in the Pichia yeast, and an expression vector containing a DNA encoding the human hemoglobin [beta] chain (Hb[beta]) under control of a promotor functionable in the Pichia yeast in a medium, and collecting the human hemoglobin from the obtained culture product. The expression vector contains AOX1 promotor, the DNA encoding the Hb[beta], a terminator functionable in the Pichia yeast, the AOX1 promotor, the DNA encoding the Hb[alpha] and a terminator functionable in the Pichia yeast in order. ;COPYRIGHT: (C)2008,JPO&INPIT

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(54) Title: METHOD FOR METHANOL INDEPENDENT INDUCTION FROM METHANOL INDUCIBLE PROMOTERS IN PICHIA

(57) Abstract: The present invention relates to a method for producing a polypeptide in a methylotrophic yeast host cell, wherein expression of the polypeptide is controlled by a methanol inducible promoter, comprising: i) expression of a positive regulator from a non-native promoter, said positive regulator activating transcription from the methanol inducible promoter, and ii) no addition of methanol.

# TITLE: METHOD FOR METHANOL INDEPENDENT INDUCTION FROM METHANOL INDUCIBLE PROMOTERS IN PICHIA.

## Reference to sequence listing

This application contains a Sequence Listing in computer readable form. The computer readable form is incorporated herein by reference.

## FIELD OF THE INVENTION

The present invention relates to a method for producing a polypeptide in a methylotrophic yeast host cell, wherein expression of the polypeptide is controlled by a methanol inducible promoter.

## BACKGROUND OF THE INVENTION

Eukaryotic organisms are widely used in industry as host cells for producing polypeptide for, *e.g.*, pharmaceutical and industrial applications. The ability to manipulate gene transcription and expression gives the basis for providing higher production yields.

Conventionally, maximal expression of a gene in a eukaryotic organism is achieved by amplifying in the chromosome an expression cassette containing a single promoter operably linked to a gene encoding the polypeptide of interest and an amplifier selective marker.

Controlled expression is often desirable. In methylotrophic yeast it has been known for long that certain promoters are dependent on the presence of methanol in the growth medium for the induction of transcription. This induction by methanol requires the presence of additional factors, however, the exact mechanism of action for such factors have not been elucidated. Examples of positive factors known from yeast include Mxr1p, described as a key positive regulator required for methanol utilization in *Pichia pastoris* (Lin-Cereghino *et al.*, 2006, Mol Cell Biol 26(3): 883-897).

Examples of these methanol dependent promoters have been described in several yeast cells belonging to the group of yeast known as methylotrophic yeast. The promoters controlling expression of the enzymes involved in methanol metabolism in these organisms are particularly strong, and these promoters are generally used to control the heterologous expression of proteins in yeast. However, the specific carbon source used for the cultivation of these host cells has an enormous influence on the regulation of methanol metabolism promoters. Methanol and glycerol are considered as adequate substrates for methylotrophic yeast expression systems, while glucose has been considered inadequate (EP 299108). It is

therefore desirable if expression from the known methanol metabolism promoters can be made less dependent on the substrate.

## SUMMARY OF THE INVENTION

The invention provides a method for producing a polypeptide in a methylotrophic yeast host cell, wherein expression of the polypeptide is controlled by a methanol inducible promoter, comprising: i) expression of a positive regulator from a non-native promoter, said positive regulator activating transcription from the methanol inducible promoter, and ii) no addition of methanol. The invention further discloses a method for increasing the expression level of a heterologous polypeptide under the control of a methanol inducible promoter, comprising providing constitutive expression of the prm1 gene.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the controlled expression of polypeptides from methanol inducible promoters. Examples of these promoters have been described in several yeast cells belonging to the group of yeast known as methylotrophic yeast. In the context of the present invention a methylotrophic yeast is defined as a group of yeast which can utilize methanol as a sole carbon source for their growth. The promoters for the enzymes involved in methanol metabolism in these organisms are particularly strong, and these promoters (methanol metabolism promoters) are generally used to control the heterologous expression of proteins in yeast.

Known members of methylotrophic yeast host cells belong to the genera selected from the group consisting of *Pichia, Hansenula, Candida, Torulopsis.* According to the invention the *Pichia* host cell can in one embodiment be selected from the group consisting of *P. pastoris, P. methanolica, P. angusta, P. thermomethanolica*. The *Hansenula* or *Candida* host cells can be selected from the group consisting of *H. polymorpha*, and *C. boidinii.*

Several promoters have previously been isolated and described in the literature from which the expression of heterologous polypeptides can be controlled by the addition of methanol to the growth medium. Such promoters include but are not limited to *e.g.* the AOX1 promoter (Alcohol Oxidase promoter), DHAS promoter (or DAS promoter) (dihydroxyacetone synthase promoter), FDH promoter (or FMDH promoter) (formate dehydrogenase promoter), MOX promoter (Methanol Oxidase promoter), AOX2 promoter, ZZA1, PEX5-, PEX8-, PEX14-promoter. Particularly the promoters useful in the present invention are promoters for enzymes involved in the methanol metabolism. Such promoters may be isolated from yeasts by the skilled person using conventional techniques. All of the above mentioned promoters have already been isolated from particular members of the group of methylotrophic yeast and by homology searches corresponding promoters from other members can easily be identi-

fied. This is further illustrated by the examples included herein. More particularly the promoters are selected from the group consisting of the formate dehydrogenase (FMD or FMDH) promoter, the methanol oxidase (MOX) promoters, the dihydroxyacetone synthase (DAS or DHAS) promoter or the alcohol oxidase (AOX1) promoter.

Normally all of the above promoters require the presence of methanol for their induction. This induction by methanol requires the presence of additional factors (such as transcription factors), however, the exact mechanism of action for such factors have not been elucidated. In yeast *e.g.* Mxr1p, has been described as a key positive regulator required for methanol utilization in *Pichia pastoris* (Lin-Cereghino *et al.*, 2006, Mol Cell Biol 26(3): 883-897).

The inventors of the present invention have discovered that the controlled expression of a single positive factor, encoded by the Prm1 gene from *Pichia pastoris*, as described herein, can be sufficient in order to induce transcription from several methanol inducible promoters without the need for methanol in the growth medium. As disclosed herein this principle has been demonstrated using the Prm1 protein as a model protein for the positive activator and using the AOX1 or the DAS promoters for the controlled expression of a reporter polypeptide. The results obtained have shown that it is possible to induce the AOX1 or the DAS promoters simply by controlling the expression of the prm1 gene and without the presence of methanol in the growth medium.

In one embodiment of the invention the positive regulator is expressed constitutively from a suitable promoter. Preferably the promoter is not the native promoter meaning that the promoter controlling the expression of the positive regulator is different from the promoter normally controlling the expression. In the context of the present invention such preferred promoters are termed "non-native". The promoter could still be native to the host organism but it will be foreign in the context of the gene in question, e.g. the prm1 gene. In one particular embodiment the promoter is selected from the group consisting of the GAP promoter (glyceraldehyde-3-phosphate dehydrogenase promoter), the TEF1 promoter (Translational elongation factor EF-1 alpha promoter), and the PGK promoter (phosphoglycerate kinase promoter). The host cell according to the invention would normally express the positive regulator from an endogenous gene present on the chromosome in addition to the expression controlled by the non-native promoter as described above. In a further embodiment the endogenous copy of the gene encoding the positive regulator could be inactivated, e.g. by deletion, or the normal promoter controlling the endogenous copy of the gene could be replaced by the chosen non-native promoter.

In another embodiment the expression of the positive regulator is controlled from an inducible promoter which is not methanol inducible.

The positive regulator according to the invention is in one embodiment Prm1 as described herein. In one particular embodiment Prm1 comprises the amino acid sequence of SEQ ID NO: 1 or an allelic variant thereof; or a fragment thereof that has regulator activity.

In another particular embodiment, Prm1 consists of the amino acid sequence of SEQ ID NO: 1 or an allelic variant thereof; or a fragment thereof; or a substitution, deletion or addition of one or several amino acids that has regulator activity.

The term "polypeptide fragment" is defined herein as a polypeptide having one or more amino acids deleted from the amino and/or carboxyl terminus of SEQ ID NO: 1 or a homologous sequence thereof, wherein the fragment has regulator activity.

The term "allelic variant" denotes herein any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. An allelic variant of a polypeptide is a polypeptide encoded by an allelic variant of a gene.

The relatedness between two amino acid sequences such as *e.g.* between two functional homologues is described by the parameter "identity".

For purposes of the present invention, the alignment of two amino acid sequences is determined by using the Needle program from the EMBOSS package (http://emboss.org) version 2.8.0. The Needle program implements the global alignment algorithm described in Needleman, S. B. and Wunsch, C. D. (1970) J. Mol. Biol. 48, 443-453. The substitution matrix used is BLOSUM62, gap opening penalty is 10, and gap extension penalty is 0.5.

The degree of identity between an amino acid sequence of the present invention ("invention sequence"; *e.g.* amino acids 1 to 989 of SEQ ID NO: 1 and a different amino acid sequence ("foreign sequence") is calculated as the number of exact matches in an alignment of the two sequences, divided by the length of the "invention sequence" or the length of the "foreign sequence", whichever is the shortest. The result is expressed in percent identity.

An exact match occurs when the "invention sequence" and the "foreign sequence" have identical amino acid residues in the same positions of the overlap (in the alignment example below this is represented by "|"). The length of a sequence is the number of amino acid residues in the sequence.

In the purely hypothetical alignment example below, the overlap is the amino acid sequence "HTWGER-NL" of Sequence 1; or the amino acid sequence "HGWGEDANL" of Sequence 2. In the example a gap is indicated by a "-".

Hypothetical alignment example:

```
Sequence 1:     ACMSHTWGER-NL

                    | |||  ||

Sequence 2:         HGWGEDANLAMNPS
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In one embodiment according to the invention the functional homologue of Prm1 is at least 70 % identical to SEQ ID NO: 1, particularly at least 80 % identical to SEQ ID NO: 1, particularly at least 85% identical to SEQ ID NO: 1, particularly at least 90%, more particularly at least 95%, most particularly at least 98% identical to SEQ ID NO: 1.

In another embodiment the functional homologue of Prm1 is encoded by a polynucleotide which hybridizes under at least low stringency conditions with (i) nucleotides 1 to 2970 of SEQ ID NO: 2, or (ii) a complementary strand of (i).

In another embodiment the functional homologue of Prm1 is encoded by a polynucleotide which hybridizes under at least medium stringency conditions with (i) nucleotides 1 to 2970 of SEQ ID NO: 2, or (ii) a complementary strand of (i).

In another embodiment the functional homologue of Prm1 is encoded by a polynucleotide which hybridizes under at least high stringency conditions with (i) nucleotides 1 to 2970 of SEQ ID NO: 2, or (ii) a complementary strand of (i).

In another embodiment the functional homologue of Prm1 is encoded by a polynucleotide which hybridizes under very high stringency conditions with (i) nucleotides 1 to 2970 of SEQ ID NO: 2, or (ii) a complementary strand of (i).

The positive regulator according to the invention may also be a functional homologue of Prm1 isolated from other yeast cells. Such functional homologues can be isolated starting from the sequence shown in SEQ ID NO: 2, *e.g.* by using SEQ ID NO: 2 or a fragment thereof, to design a nucleic acid probe to identify and clone DNA encoding polypeptides having regulator activity from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic or cDNA of the genus or species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 14, preferably at least 25, more

preferably at least 35, and most preferably at least 70 nucleotides in length. It is, however, preferred that the nucleic acid probe is at least 100 nucleotides in length. For example, the nucleic acid probe may be at least 200 nucleotides, preferably at least 300 nucleotides, more preferably at least 400 nucleotides, or most preferably at least 500 nucleotides in length. Even longer probes may be used, *e.g.*, nucleic acid probes which are at least 600 nucleotides, at least preferably at least 700 nucleotides, more preferably at least 800 nucleotides, or most preferably at least 900 nucleotides in length. Both DNA and RNA probes can be used. The probes are typically labelled for detecting the corresponding gene (for example, with $^{32}P$, $^{3}H$, $^{35}S$, biotin, or avidin).

A genomic DNA or cDNA library prepared from such other organisms may, therefore, be screened for DNA which hybridizes with the probes described above and which encodes a polypeptide having regulator activity. Genomic or other DNA from such other organisms may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA which is homologous with SEQ ID NO: 2 or a subsequence thereof, the carrier material is used in a Southern blot.

For purposes of the present invention, hybridization indicates that the nucleotide sequence hybridizes to a labelled nucleic acid probe corresponding to the nucleotide sequence shown in SEQ ID NO: 2, its complementary strand, or a subsequence thereof, under very low to very high stringency conditions. Molecules to which the nucleic acid probe hybridizes under these conditions can be detected using X-ray film.

For long probes of at least 100 nucleotides in length, very low to very high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 µg/ml sheared and denatured salmon sperm DNA, and either 25% formamide for very low and low stringencies, 35% formamide for medium and medium-high stringencies, or 50% formamide for high and very high stringencies, following standard Southern blotting procedures for 12 to 24 hours optimally.

For long probes of at least 100 nucleotides in length, the carrier material is finally washed three times each for 15 minutes using 2X SSC, 0.2% SDS preferably at least at 45°C (very low stringency), more preferably at least at 50°C (low stringency), more preferably at least at 55°C (medium stringency), more preferably at least at 60°C (medium-high stringency), even more preferably at least at 65°C (high stringency), and most preferably at least at 70°C (very high stringency).

In a particular embodiment, the wash is conducted using *0.2X* SSC, 0.2% SDS preferably at least at 45°C (very low stringency), more preferably at least at 50°C (low stringency), more preferably at least at 55°C (medium stringency), more preferably at least at 60°C (medium-high stringency), even more preferably at least at 65°C (high stringency), and most preferably at least at 70°C (very high stringency). In another particular embodiment, the wash is conducted using *0.1X* SSC, 0.2% SDS preferably at least at 45°C (very low stringency), more preferably at least at 50°C (low stringency), more preferably at least at 55°C (medium stringency), more preferably at least at 60°C (medium-high stringency), even more preferably at least at 65°C (high stringency), and most preferably at least at 70°C (very high stringency).

As described above the positive regulator according to the invention may also be a functional homologue of Prm1 isolated from other yeast cells. According to one embodiment of the invention one such candidate could be Mut3 encoded by the mut3 gene from *Hansenula polymorpha* (syn. *Pichia angusta*). In the examples provided herein Prm1 has been overproduced in *Pichia pastoris*. It is however possible that the same effect can be obtained by overproducing Mut3 in *Pichia* or Prm1 in *Hansenula* or Mut3 in *Hansenula*. This has not been tested.

Therefore in a further embodiment of the invention the positive regulator is Mut3.

An increase in the level of positive regulator present in the host cell can also be provided by simply having multiple copies of the gene encoding the regulator present in the host cell.

Even though the exact mechanism of action of the positive regulator, Prm1, has not been elucidated it is most likely that the regulator will bind to the promoter region of the methanol inducible promoter. In one particular embodiment of the invention the methanol inducible promoter controlling the expression of a heterologous polypeptide is therefore provided with additional binding sites for the positive regulator thereby increasing the positive effect of the regulator.

The positive effect provided by increasing the level of positive regulator in the cell could be dependent on the presence other factors or it could be independent. One such other factor could be the Mxr1 protein (Lin-Cereghino *et al.*, 2006, Mol Cell Biol 26(3): 883-897). Therefore in a further embodiment according to the invention expression of the mxr1 gene is also controlled by a non-native promoter, particularly the promoter selected from the group consisting of the GAP promoter, TEF1 promoter, and PGK promoter.

In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods well known in the art. For example, the cell may be cultivated by shake flask cultivation, and small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (*e.g.*, in catalogues of the American Type Culture Collection). If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell lysates.

The polypeptides may be detected using methods known in the art that are specific for the polypeptides. These detection methods may include use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. For example, an enzyme assay may be used to determine the activity of the polypeptide as described herein.

The resulting polypeptide may be recovered using methods known in the art. For example, the polypeptide may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation.

The polypeptides produced by the present invention may be purified by a variety of procedures known in the art including, but not limited to, chromatography (*e.g.*, ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (*e.g.*, preparative isoelectric focusing), differential solubility (*e.g.*, ammonium sulfate precipitation), SDS-PAGE, or extraction (see, *e.g.*, Protein Purification, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

In one particular embodiment the polypeptide produced from the host cell is heterologous to the host cell. In another embodiment the polypeptide is homologous to the host cell.

The effects observed in the present invention are illustrated in the examples below showing that expression from selected promoters can be observed without the addition of methanol to the growth medium. Addition of methanol could possibly further enhance the observed effect of increasing the level of Prm1 in the host cell. A further embodiment of the invention is therefore a method as described in all embodiments above for increasing the expression level of a heterologous polypeptide under the control of a methanol inducible pro-

moter in which method methanol is present in the medium, and Prm1 is expressed at increased levels compared to a wild type cell. Preferably expression of the prm1 gene is constitutive.

## EXAMPLES

### Materials and methods

Strains and plasmids

*Pichia pastoris* GS115 (genotype:*his4*, Invitrogen™) was used as the host strain for protein expression. *E. coli* DH5alpha (Invitrogen™), TOP10 (Invitrogen™) or XL10 (Stratagene™) were used as cloning hosts in construction of the expression vectors. The plasmids pPIC9K and pGAPZα (Invitrogen™) were used for the construction of expression plasmids and pCR2.1-TOPO (Invitrogen™), pT7Blue (Novagen™) were used for sub-cloning of PCR fragments.

Transformation of *Pichia pastoris*:

*Pichia pastoris* strains are transformed by electroporation according to the manufacturer's protocol (Invitrogen, Cat. #K1710-01). Competent cells are prepared as described and stored in 40 µl aliquots at -70°C. Linearized plasmid DNA (500ng) is mixed with 40 µl of competent cells and stored on ice for 5 min. Cells are transferred to an ice-cold 0.2 cm electroporation cuvette. Transformation is performed using a BioRad™ GenePulser II. Parameters used were 1500 V, 25 µF and 200 Ω. Immediately after pulsing, cells are suspended in 1 ml of ice cold 1 M sorbitol. The mixtures are plated on the relevant selection plates.

Medium and assay

RD medium (1M sorbitol, 2% dextrose, 1.34% yeast nitrogen base, $4x10^{-5}$%biotin, 0.005% L-glutamic acid, L-methinonine, L-lysine, L-leucine, and L-isoleucine, 2% agar noble) was used for the regeneration of transformants from *Pichia pastoris* GS115 with his4 selection. In case of the selection with zeocin resistance, YPD agar (1% yeast extract, 2% peptone, 2% glucose, 2% bacto agar) supplemented with 1M sorbitol and 100 micro g/mLzeocin was used for the regeneration. YPD (1% yeast extract, 2% peptone and 2% glucose), YPGly (1% yeast extract, 2% peptone and 2% glycerol) were used for the protein expression with *Pichia pastoris*. When necessary, methanol was added to the culture broth after 1 day and 2days cultivation at the final concentration 1% or 2% (v/v). *E.coli* strains were cultivated with LB medium (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl) with relevant antibiotics.

Phytase assay method:

7.5 mM of sodium phytate dissolved in the acetate buffer pH 5.5 is mixed with 1/2 volume of enzyme sample solution in the same acetate buffer containing 0.01% Tween 20. After the incubation at 37°C for 30 minutes, the stop reagent containing 20mM ammonium heptamolybdate and 0.06% ammonium vanadate dissolved in 10.8% nitric acid is added to generate yellow complex with released inorganic phosphate. The amount of released phosphate is measured photometrically as the absorbance at 405 nm. One phytase unit is defined as the amount of enzyme to release 1 µmol inorganic phosphate per minute.

Phytase plate assay:

20 µL of supernatant from the culture broth after 2-5 days incubation of the transformants is applied into a 4 mm hole punched in the following plate: 1% agarose plate containing 0.1 M Sodium acetate (pH 4.5) and 0.1 % Inositol Hexaphosphoric acid. The plate is incubated at 37°C over night and a buffer consisting of 1M $CaCl_2$ and 0.2M Sodium acetate (pH 4.5) is poured over the plate. The plate is left at room temperature for 1 h and the phytase activity identified as a clear zone.

PCR conditions

PCR reaction was typically carried out in the following or equivalently conditions: Reaction mixtures contained 2mM dNTP, 10 pmol of forward and reverse primer, 2.8 unit of Expand high fidelity mixture (Roche), 1X Expand high fidelity buffer (Roche), and 100 µg of template DNA. Reaction conditions are, for example;

| Temp (°C) | Time | Cycle |
|---|---|---|
| 95 | 5 min | 1 |
| 95<br>48<br>68 | 15 sec<br>30 sec<br>3.5 min | 35 |
| 68 | 10 min | 1 |

**Example 1: Constitutive expression of Prm1 in a strain expressing a phytase gene under DAS1 promoter control**

Cloning of TEF1 promoter and construction of pNo-TP10

Cloning of TEF1 (Translation Elongation Factor1) promoter from *Pichia pastoris* was carried out by the following procedure. By alignment of the homologous regions of the TEF1 proteins from the yeasts *Saccharomyces cervisiae*, *Candida albicanse*, and *Hanseniaspora uvarum*, the following degenerated primes were designed.

TEF1(f); 5'-ttyaartaygcntgggt-3' (protein; FKYAWV) SEQ ID NO: 6 and 7

TEF5(r); 5'-arytgytcrtgrtgcatytc-3' (protein; EMHHEQL) SEQ ID NO: 8 and 9

PCR was carried out using 50 microL of reaction including 4mM dNTP, 10 microM of each primer, 1unit of Taq polymerase (Roche), 1X Taq buffer (Roche), and 100 ng of genomic DNA of GS115 DNA. The PCR conditions were as shown below.

| temp | time | cycle |
|---|---|---|
| 94° C | 1 min | |
| 55° C | 1 min | 30 |
| 72° C | 3 min | |

The amplified 0.7 kbp fragment was purified and sub-cloned into TA-cloning vector, pTBlue7. The resulting plasmid was used for the sequence determination. The obtained DNA sequence and presumed amino acid sequence are shown in SEQ ID NO: 3 and SEQ ID NO: 4 respectively. Since the obtained sequences had similarity to known TEF1 proteins, cloning of the upstream region (SEQ ID NO: 5) comprising the promoter sequence was carried out using DNA walking Speed Up premix kit (Seegene, K1501) together with the following specific primers.

TEF-TSP1; 5'-tgacggtaacgtggtacttt-3' SEQ ID NO: 10

TEF-TSP2; 5'-ggagtctcgaacttccacaa-3' SEQ ID NO: 11

TEF-TSP3; 5'-agcgatgtcgatggtgatac-3' SEQ ID NO: 12

PCR was carried out using the 50 microL of reaction mixture including 4mM dNTP, 10 microM of TEF-TSP1 primer, 2.5 microM of DW-ACP2 primer in the kit, 1unit of Taq polymerase (Roche), 1X Taq-buffer (Roche), and 100 ng of genomic DNA. The PCR program was as shown below.

| temp | time | cycle |
|---|---|---|
| 94° C<br>42° C<br>72° C | 5 min<br>1 min<br>2 min | 1 |
| 94° C<br>55° C<br>72° C | 30 sec<br>30 sec<br>100 sec | 20 |
| 72° C | 7 min | 1 |

A second PCR was carried out using 20 microL of reaction mixture including 2 mM dNTP, 10 microM of TEF-TSP2 primer, 10 microM of DW-ACPN primer from the kit, 0.4 units of Taq polymerase (Roche), 1X Taq-buffer (Roche), and 5 micro L of the purified PCR product from the first round with the following program:

| temp | time | cycle |
|---|---|---|
| 94° C | 3 min | 1 |
| 94° C<br>58° C<br>72° C | 30 sec<br>30 sec<br>100 sec | 30 |
| 72° C | 7 min | 1 |

A third PCR was carried out using 50 microL of reaction including 2mM dNTP, 10 microM of TEF-TSP3 primer, 10 microM of Universal primer from the kit, 1 unit of Taq polymerase (Roche), 1X Taq-buffer (Roche), and 1 micro L of 10 times diluted PCR product from the second round with the following program:

| temp | time | cycle |
|---|---|---|
| 94° C | 3 min | 1 |
| 94° C<br>65° C<br>72° C | 30 sec<br>30 sec<br>100 sec | 30 |
| 72° C | 7 min | 1 |

After three times nested PCR, a 1.2 kbp fragment was amplified. This fragment was sub-cloned into pT7Blue and the sequence was determined. The obtained sequence is shown in SEQ ID NO: 5. The 1.2 kbp region upstream of TEF1 ORF was used as TEF1 promoter for further experiment.

The plasmid pNo-TP10 is an expression plasmid for bacterial phytase under TEF1 promoter and carries a zeocin resistant marker gene. Construction of pNo-TP10 was carried out as described. The TEF1 promoter and the codon optimized phytase gene was cloned from genomic DNA of *Pichia pastoris* A94 (see Example 2) using the following primers. Primer 1 and 2 were used for the amplification of TEF1 promoter and primer3 and 4 were used for phytase gene.

primer1; 5'- tacagggcgcgtggggatatcggatccagctcatctaggga-3' (BamHI is underlined) (SEQ ID NO: 13)

primer2; 5'- tgaagatggatgggaatctcatatggttggcgaataactaaaatgtatgt-3' (SEQ ID NO: 14)

primer3; 5'- acatacattttagttattcgccaaccatatgagattcccatccatcttca-3' (SEQ ID NO: 15)

primer4; 5'- taattcgcggccgccctagggaattcttactcggtgacagcgcactcggg-3' (EcoRI is underlined) (SEQ ID NO: 16)

PCR was carried out using 50 microL reaction including 2mM dNTP, 10 microM of each primer, 2.8 unit of Expand high fiderlity mixture (Roche), 1X Expand high fidelity buffer (Roche), and 100 ng of genomic DNA. The PCR program was as shown below.

| temp | time | cycle |
|---|---|---|
| 94° C | 2 min | 1 |
| 94° C<br>50° C<br>68° C | 10 sec<br>30 sec<br>3 min | 11 |
| 94° C<br>50° C<br>68° C | 10 sec<br>30 sec<br>3 min+20 sec/cycle | 20 |
| 68° C | 7 min | 1 |

An amplified 1.2 kbp fragment of the TEF1 promoter and a 1.5 kbp fragment containing the phytase gene including secretion signal were purified using gel extraction and they were subjected to a second round of PCR with primer1 and primer4 in order to fuse these fragments using the overlap generated in the first PCR. PCR was carried out using the 50 microL of reaction including 2mM dNTP, 10 microM of each primer, 2.8 unit of Expand high fiderlity (Roche), 1X Expand high fidelity buffer (Roche), and 1µl of the purified TEF1 promoter and 1µl of the purified phytase gene. The PCR program was as shown below.

| temp | time | cycle |
|---|---|---|
| 94° C | 2 min | 1 |
| 94° C<br>58° C<br>68° C | 10 sec<br>30 sec<br>3 min | 11 |
| 94° C<br>58° C<br>68° C | 10 sec<br>30 sec<br>3 min+20 sec/cycle | 20 |
| 68° C | 7 min | 1 |

The amplified 2.7 kbp fragment was sub-cloned into pT7Blue and the resultant plasmid was designated pT12-8. The BamHI - EcoRI fragment from pT12-8 including the TEF1 promoter

and the phytase gene was ligated with pGAPZα cut with BglII and EcoRI to result in pNo-TP10.

Cloning of DAS1 promoter and construction of pNo-DP3

The DAS1 promoter is known to be strongly inducible by methanol in *Candida boidinii* (Yurimoto,H. Komeda,T. Lim,C.R. Nakagawa,T. Kondo,K. Kato,N. Sakai,Y.; Biochim. Biophys. Acta 1493(1-2):56-63(2000).) The DAS1 gene encoding dihydroxyacetone synthase was found in the genome sequence of *Pichia pastoris* by blast search as the homologue to the DAS1 gene of *Candida boindinii* (EMBL: AF086822). Around 1kb of 5'-untlanslated region of the DAS1 gene was isolated as a promoter region from genomic DNA of *Pichia pastoris* by PCR using the primers shown below;

primer43; 5'-ttttggtcatgcatgacgtcatagggagaaaaaccgagac-3' (NsiI site is underlined) (SEQ ID NO: 17)

primer44; 5'-ctcatatgttttgatgtttgatagtttga-3' (NdeI site is underlined) (SEQ ID NO: 18)

The amplified 1kb fragment was sub-cloned into the NsiI/NdeI sites of pCR2.1-TOPO and used for the sequence determination of this fragment (SEQ ID NO: 19). The 1kb of DAS1 promoter region was excised as an NsiI/NdeI fragment from pCR2.1-TOPO and ligated with pNo-TP10 digested with NsiI/NdeI generating pNo-DP2. To change the marker gene from zeocin resistant gene to His4 gene, a 1.4 kb AatII fragment containing the DAS1 promoter and a part of bacterial phytase gene from pNo-DP2 was ligated to a 9.0 kb of AatII fragment from pPICNoT-G01651 (See Example 2) containing the rest of phytase gene and the His4 gene and bacterial vector. The generated plasmid was named pNo-DP3.

Cloning of Prm1 and construction of pGPrm

A gene, which we have named, Prm1 (positive regulator for methanol), and which encodes a novel positive regulator of methanol inducible promoters, was isolated from genomic DNA of *Pichia pastoris* by PCR using the primers shown below;

primer 32; 5'-actatttcgaaatgcctcctaaacatcggctg-3', (BstBI site is underlined) (SEQ ID NO: 20)

primer 33; 5'-gtcgacttaactgcaaaatttattg-3' (SalI is underlined) (SEQ ID NO: 21)

PCR was carried out using the 50 μL of reaction including 1X LA PCR buffer II (TAKARA), 2.5U of LA taq (TAKARA), 2.5mM $MgCl_2$, 2.5mM dNTP, 1 microM of each primer, 100 ng of genomic DNA with the following program:

| temp | time | cycles |
|---|---|---|
| 94° C | 30 sec | 30 |
| 55° C | 30 sec | |
| 72° C | 4 min | |

The obtained 1kb of fragment was sub-cloned into pCR2.1-TOPO. After confirmation of the sequence, a 1 kbp fragment comprising the Prm1 gene (SEQ ID NO: 2) was cloned into pGAPZα (Invitrogen) using the BstBI and SalI restriction sites, resulting in pGPrm. The plasmid pGPrm carries the Prm1 gene under GAP promoter control and a zeocin resistance gene as the selection marker.

Constitutive expression of Prm1 in DAS40, a strain expressing phytase under DAS1 promoter

The plasmid pNo-DP3 was transformed into *Pichia pastoris* GS115 with his4 selection. The generated transformants were re-isolated and tested for phytase expression using YPD medium with methanol addition. One transformant, DAS40, was selected as the strain which showed phytase activity in presence of methanol.

The plasmid pGPrm was transformed into the *Pichia pastoris* DAS40, and transformants were isolated on zeocin plates. Generated transformants were cultivated in YPD medium at 30°C for 2days with agitation, and phytase activity in the supernatant of the culture broth was measured by phytase assay. The results are shown in the table below.

| Strain | Relative phytase activity |
|---|---|
| Transformant A | 44.2 |
| Transformant B | 13.3 |
| Transformant C | 7.6 |
| DAS40 | 1 |

**Example 2: Constitutive expression of Prm1 in the strain expressing a phytase gene under AOX1 promoter**

**Media:**

MD (1.34% YNB, $4X10^{-5}$% biotin, 2% dextrose)

BMSY (1% yeast extract, 2% peptone, 100mM potassium phosphate buffer, pH 6.0, 1.34% YNB, $4X10^{-5}$% biotin, 1% sorbitol)

**Vector:**

pPIC-NoT, *Pichia pastoris* expression vector under AOX1 promoter, which was derived by eliminating the alpha-secretion signal from pPIC9K (Invitrogen™).

To create pPIC-NoT vector plasmid pPIC9K was digested with BamHI and EcoRI, and the digested major fragment was isolated from agarose gel. A synthetic DNA fragment containing BamHI and EcoRI sites were created by annealing the following two oligoes:

NoT-1 P-GATCCTACGTAGCTGAG (SEQ ID NO: 22 ) and

NoT-2 P-AATTCTCAGCTACGTAG (SEQ ID NO: 23 )

The above synthetic DNA fragment was ligated into the digested pPIC9K plasmid, and the resulting vector pPIC-NoT was verified by sequencing.

**PCR Primers:**

| Oligo Name | Oligo Seq |
|---|---|
| OA-Na | GATCCAA**ACC**ATGAGATTCCCATCCATCTTCACTG (SEQ ID NO: 24) |
| OA-Nb | CAA**ACC**ATGAGATTCCCATCCATCTTCACTG (SEQ ID NO: 25) |
| OAPhy-R | CATTCTGTTCCTCTCTCTTTTCCAAGGAAACACCTTC (SEQ ID NO: 26) |
| OAPhy-F | ggaaaagagaGAGGAACAGAATGGAATGAAGTTGG (SEQ ID NO: 27) |
| OPhy-Ca | AATTC**TTA**CTCGGTGACAGCGCACTC (SEQ ID NO: 28) |
| OPhy-Cb | C**TTA**CTCGGTGACAGCGCACTC (SEQ ID NO: 29) |

**Plasmid:**

pPICNoT-G01651

A codon optimized *Citrobacter braakii* phytase gene was used in pPICNoT-G01651 in order to increase the expression yield of the cb phytase in *Pichia pastoris*. The wild type *Citrobacter braakii* phytase gene was modified based on *P. pastoris*-preferred codon usage, by means of replacing rare codons, eliminating repetitive AT and decreasing the GC content. The designed sequence was also analyzed to avoid potential intron.

The modified phytase genes (G01651) fused to a modified alpha-factor secretion signal sequence were designed based on the codon bias of *P. pastoris*. The *P. pastoris* codon usage table is from www.kazusa.jp as well as Zhao *et al*, 2000 (Zhao X, Huo KK, Li YY. Synonymous condon usage in Pichia pastoris. Chinese Journal of Biotechnology, 2000, 16(3): 308-311). Rare codons for arginine were eliminated. Besides substitution of rare codons, the total G+C content was decreased below 50%, and AT-rich regions were modified to avoid premature termination. In addition, cryptic introns within modified coding regions were eliminated as described in co-pending Danish patent application PA 2006 01042 / NZ10978.000-DK. The synthetic gene sequence is shown in SEQ ID NO: 30 (complete ORF without signal sequence).

The expression plasmid pPICNoT-G01651 was generated as described below:

The PCR fragment encoding the mature form of cb phytase (SEQ ID NO: 30) fused in-frame with optimized α-factor signal peptide (SEQ ID NO: 31) was created by overlap extension PCR method as follows: the fragment I containing α -factor signal peptide was amplified from pJ2:G01468 plasmid (pJ2:G01468 was generated by DNA2.0, and contains the mature form of plectasin fused with α-factor secretion signal which was modified based on *P. pastoris* codon usage) with specific primers OA-Na and OAPhy-R, while the fragment II encoding mature phytase was amplified from plasmid pJ2:G01651 (generated by DNA2.0 and contains the synthetic phytase gene encoding mature form of C. braakii phytase) using specific primers OAPhy-F and OPhy-Ca. Then fragment I and II were mixed and used as a template for 2$^{nd}$ step PCR amplification with specific primers OA-Na/b and OPhy-Ca/b to obtain the targeted PCR fragment. The DNA fragment was purified by gel extraction kit then sub-cloned into pPIC-NoT vector at *BamH*I and *EcoR*I sites. The resulting expression construct was confirmed by sequencing.

**Expression test in a 3ml scale:**

Expression test of the selected transformants was done in a 3ml scale using 24-deep well plates (Whatman, UK). Each transformant was grown in BMSY media for 2.5 days at

28°C with vigorous shaking (200rpm); then 300 µl 0.5% methanol was added to each well every day for 4 days to induce heterogeneous gene expression. Samples of medium culture were taken daily during induction, stored at -20°C for phytase activity assay.

Constitutive expression of Prm1 in A94, a strain expressing phytase under AOX1 promoter

The expression plasmid pPICNoT-G01651 was transformed into *Pichia pastoris* GS115 with *his4* selection, and generated transformants on MD agar with 1M sorbitol were re-isolated and tested for phytase expression in 3 ml scale. One isolate, A94, was selected as the strain which shows phytase activity in presence of methanol. The plasmid pGPrm was subsequently transformed into A94 and transformants were isolated with zeocin resistance. Isolated transformants were cultivated in YPD medium at 30°C for 2 days with agitation, and phytase activities in the culture supernatants were measured by phytase assay. The results are shown in the table below.

| Strain | Relative phytase activity |
|---|---|
| Transformant 1 | 1.86 |
| Transformant 2 | 2.57 |
| Transformant 3 | 3.0 |
| A94 | 1 |

**Example 3: Gene disruption of Prm1 in *Pichia pastoris* GS115 and derived strains**

A 1571 bp gene fragment 5' to the PRM1 gene was amplified by PCR using the primers:

JP23/PRM1-5'-forw-NY: 5' GCGCGAATTCCACAGGGCTTGCTAAGAAATC 3' (SEQ ID NO: 32); and
JP26/PRM1-5'-rev - NY: 5' GAAGGGAGATTAATACAGGGC 3' (SEQ ID NO: 33)

A 1473 bp gene fragment 3' to the PRM1 gene was amplified by PCR using the primers:

JP17/PRM1-3'-forw-NY: 5' GATTGGACCACTGCGCCAGATAC 3' (SEQ ID NO: 34); and
JP19/PRM1-3'-rev-NY: 5' GCGCGTCGACCCACCCGAGGATAAGAAGG 3' (SEQ ID NO: 35)

A 3382 bp fragment containing the HIS4 gene including promoter and terminator was amplified by PCR using the primers below (in each end the fragment contains a 20 bp overlap to PRM1 5' and 3' regions, respectively, intended for SOE-PCR);

JP13/HIS4-forward-NY: 5' CCCTGTATTAATCTCCCTTCATCAGAATTGGTTAATTGGTTG 3' (SEQ ID NO: 36); and
JP15/HIS4-rev-NY: 5' TCTGGCGCAGTGGTCCAATCATCGATAAGCTTTAATGCGG 3' (SEQ ID NO: 37)

The three above described gene fragments are fused in the following order: PRM1-5'+ HIS4 + PRM1-3') using SOE-PCR (Splicing by Overlapping Extension PCR) creating a prm1 deletion fragment having the selectable HIS4 marker flanked by the 5' prm1 fragment and the 3' prm1 fragment.

The prm1 deletion fragment was subsequently transformed into GS115 (having a his4-minus phenotype) which allows for selection of transformants on a minimal medium without histidine. A strain deleted for the prm1 gene, PFJo435, was characterized by PCR analysis. Three different PCR's were run in order to verify a correct prm1-deletion:

A) A PCR that would give a product of 704 bp in case PRM1 was NOT deleted – using primers:

JP58/PRM1-orf-forw-test7: 5' CTGGAGCAGAGTATACAGCC 3' (SEQ ID NO: 38); and
JP59/PRM1-orf-rev-test8: 5' CTCAATAAATGCGGGTCTGTG 3' (SEQ ID NO: 39)

B) A deletion specific PCR that would give a product of 1950 bp in case of PRM1 deletion – using primers:

JP31/PRM1-5'-forw-test1: 5' CCTGGTTGATCAGCTCCACC 3' (SEQ ID NO: 40); and
JP33/HIS4-rev-test3: 5' CCCGTCAAGTCAGCGTAATGC 3' (SEQ ID NO: 41)

C) A deletion specific PCR that would give a product of 1550 bp in case of PRM1 deletion – using primers:

JP32/PRM1-3'-rev-test2: 5' CTCCCTCTCCAGCTGCTTCG 3' (SEQ ID NO: 42); and
JP34/HIS4-forw-test4: 5' CGGTGCCTGACTGCGTTAGC 3' (SEQ ID NO: 43)

Strain PFJo435 did not result in a PCR product from PCR A, but resulted in the predicted deletion specific PCR product from B and C – showing that PRM1 has been deleted in PFJo435.

A Prm1 gene deletion mutant of GS115, PFJo435, could not grow on MM (1.34% YNB, $4X10^{-5}$% biotin, 2% methanol), while a wild type strain, GS115H, showed nice growth after 2 days cultivation at 30°C. The expression levels from the AOX or DAS promoters in the presence of methanol, e.g. measured as the phytase gene expression under the control of the DAS promoter or AOX promoter in DAS40 or A94 derivatives, were reduced in the Prm1 deleted derivatives. The reduced activity is attributed, to some extent by the exogenous Prm1 gene. The results on the DAS promoter are shown below.

| Strain construction | phytase yield with methanol (relative) | phytase yield with glucose (relative) |
|---|---|---|
| PFJo435 / pNo-DP2 (DAS-phytase) | 0.015 | 0.9 |
| PFJo435 / pNori12 (DAS-phytase + GAP-Prm) | 0.43 | 3.7 |
| DAS 40 | 1 | 1 |

DAS40 and pNo-DP2 are described before in example 1.

Construction of pNori12 are shown below.

Construction of pNori12

For the co-expression of the phytase gene from the DAS promoter and the Prm 1 gene from the GAP promoter, pNori12 was constructed.

To replace the terminator part, the original terminator of Prm was cloned using following primers:

pr136; 5'-ataaattttgacagttaagtcgacctctgtaaattaattgataatttcaa-3' (SEQ ID NO: 44)

pr137; 5'-caatgatgatgatgatggtcgacgtttaaacttaattaaaagggaaatttacaagcc-3' (SEQ ID NO: 45)

PCR was carried out using a total of 50microL reaction mixture including 2mM dNTP, 10 microM of each primer, 2.8unit of Expand high fidelity plus (Rosche), 1X Expand high fidelity buffer (Rosche), and 100ng of genomic DNA of GS115. The PCR program was as described below.

| temp | time | cycle |
|---|---|---|
| 94° C | 2 min | 1 |
| 94° C<br>55° C<br>68° C | 10 sec<br>30 sec<br>3 min | 11 |
| 94° C<br>55° C<br>68° C | 10 sec<br>30 sec<br>3 min+20 sec/cycle | 20 |
| 68° C | 7 min | 1 |

The amplified 500bp of fragment was inserted in the SalI site of pGPrm using In-Fusion PCR cloning kit (Clontech) to fuse it with the ORF of Prm, resulting in pNori11. In order to make the expression plasmid which has GAP-Prm and DAS-phytase, the 3354 bp of SnaBI-XmaI fragment which carries a phytase expression cassette containing DAS promoter was isolated from pNo-DP2 and ligated to 5323bp of XmaI-PmeI fragment of pNori11, resulting in pNori12. The plasmid pNori12 carries the Prm1 gene under GAP rpmoter control, phytase gene under DAS promoter control, and a zeocin resistant gene as the selection marker.

**Example 4: Constitutive expression of Mxr1**

Mxr1, a gene from *Pichia pastoris* reported to be a positive regulator for methanol induction ( G. P. Lin-Cereghino et al.;MOLECULAR AND CELLULAR BIOLOGY, Feb. 2006, p. 883–897 Vol. 26, No. 3 ) , was isolated from genomic DNA of *Pichia pastoris* by PCR using the primers Mxr-F and Mxr-R. The sequence of the primers and the PCR conditions are shown below.

PCR primers:

| Oligo name | Oligo Seq |
|---|---|
| Mxr-F | ATTGAACAACTATTTCGAAACCATGAGCAATCTACCCCC (SEQ ID NO: 46) |
| Mxr-R | GAGTTTTTTGTTCTAGAATGACACCACCATCTAGTCGG (SEQ ID NO: 47) |

PCR conditions

| Temp (°C) | Time | Cycles |
|---|---|---|
| 95 | 5 min | 1 |
| 95<br>48<br>68 | 15 sec<br>30 sec<br>3.5 min | 35 |
| 68 | 10 min | 1 |

A 3.5 kbp PCR fragment digested with BstBI and XbaI was mixed with pGAPZα digested with the same enzymes followed by ligation. The resulting plasmid pGMxr carries the Mxr1 gene under GAP promoter control and a zeocin resistance gene as the transformation marker. The plasmid pGMxr was transformed into *Pichia pastoris* DAS40 and/or A94. The transformants were isolated on zeocin plates. Generated transformants were cultivated in YPD medium at 30°C for 2 days with agitation, and phytase activity in the supernatant of culture broth was measured. The effect of constitutive expression of the Mxr 1 gene was observed as increased phytase activity in YPD medium in the transformants after introduction of pGMxr.

| Transformant | pGMxr | phytase activity by plate assay |
|---|---|---|
| Mxr#23 / DAS40 | Yes | + |
| DAS40 | No | - |

| Transformant | pGMxr | Phytase yield (relative) |
|---|---|---|
| AMxr2 / A94 | Yes | 7.6 |
| A94 | No | 1 |

The plasmid pGMxr is modified to replace the selection marker gene with e.g. ura3, ade2, arg4, hygromycin resistant gene, resulting in pGMxr-m. Furthermore, the promoter region can be replaced with another promoter such as PGK1 promoter or TEF1 promoter, giving pPMxr-m or pTMxr-m. The plasmid pGMxr-m, pPMxr-m or pTMxr-m is transformed into *Pichia pastoris* Transformant A or Transformant 3 with necessary modification for the transformation. Newly generated transformants are cultivated in YPD medium at 30°C for 2 days with agitation, and phytase activity in the supernatant of culture broth is measured.

## CLAIMS

1. A method for producing a polypeptide in a methylotrophic yeast host cell, wherein expression of the polypeptide is controlled by a methanol inducible promoter, comprising: i) expression of a positive regulator from a non-native promoter, said positive regulator activating transcription from the methanol inducible promoter, and ii) no addition of methanol.

2. The method according to claim 1, wherein the methylotrophic yeast host cell is selected from the group consisting of Pichia, Hansenula, Candida, and Torulopsis

3. The method according to claims 1 or 2, wherein the methanol inducible promoter is selected from the group consisting of the AOX1 promoter, DHAS promoter, FDH promoter, FMDH promoter, MOX promoter, AOX2 promoter, ZZA1, PEX5-, PEX8-, PEX14-promoter.

4. The method according to any of the preceding claims, wherein expression of the positive regulator is constitutive.

5. The method according to claim 1, wherein the positive regulator is Prm1 shown in SEQ ID NO: 1 or a functional homologue thereof.

6. The method according to claim 5, wherein the functional homologue is at least 85% identical to SEQ ID NO: 1, preferably at least 90%, more preferably at least 95%, most preferably at least 98% identical to SEQ ID NO: 1.

7. The method according to any of claims 4-6, wherein the positive regulator is under the control of the GAP promoter, TEF1 promoter or PGK promoter.

8. The method according to claim 1, wherein expression of the positive regulator is inducible.

9. The method according to any of the preceding claims, wherein the gene encoding the positive regulator is present in multicopy.

10. The method according to any of the claims 1-9, wherein the methanol inducible promoter is provided with additional binding sites for the positive regulator.

11. The method according to any of the preceding claims, wherein the mxr1 gene is controlled by a non-native promoter.

12. The method according to claim 11, wherein mxr1 is under the control of the GAP promoter, TEF1 promoter, or PGK promoter.

13. The method according claim 2, wherein the *Pichia* host cell is selected from the group consisting of *P. pastoris, P. methanolica, P. angusta, P. thermomethanolica*.

14. The method according to claim 2, wherein the *Hansenula* host cell is selected from the group consisting of *Hansenula polymorpha*.

15. The method according to claim 2, wherein the *Candida* host cell is selected from the group consisting of *Candida boidinii*.

16. The method according to any of the preceding claims, wherein the promoter controlling the endogenous copy of the positive regulator is also replaced by a non-native constitutive promoter.

17. The method according to any of the preceding claims, wherein the polypeptide is heterologous to the host cell.

18. The method according to any of the claims 1-16, wherein the polypeptide is homologous to the host cell.

19. A method for increasing the expression level of a heterologous polypeptide under the control of a methanol inducible promoter, comprising providing constitutive expression of the prm1 gene.

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/050870

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/81 C12N15/31 C07K14/39 C12P21/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12P C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, FSTA, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | WO 00/56903 A (ZYMOGENETICS INC [US])<br>28 September 2000 (2000-09-28)<br>examples 1-7,12,16-19<br>claims 26-29 | 1 |
| A | EP 0 911 416 A2 (HOFFMANN LA ROCHE [CH]<br>DSM IP ASSETS BV [NL])<br>28 April 1999 (1999-04-28)<br>examples 1-3<br>claims 1-6 | 1 |
| A | EP 0 299 108 A1 (RHEIN BIOTECH GES FUR<br>BIOTECHN [DE] RHEIN BIOTECH GES FUER<br>BIOTECH [DE]) 18 January 1989 (1989-01-18)<br>cited in the application<br>example 2<br>page 10, lines 22-30 | 1 |

-/--

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 11 April 2008 | 25/04/2008 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | van de Kamp, Mart |

Form PCT/ISA/210 (second sheet) (April 2005)

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/050870

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | WO 03/014363 A1 (BIOANALISI CT SUD S N C [IT]; ROSSOLINI GIAN MARIA [IT]; RICCIO MARIA) 20 February 2003 (2003-02-20)<br>page 2, lines 8-26<br>page 10, lines 14-25<br>example 2 | 1 |
| A | LIN-CEREGHINO G P ET AL: "Mxr1p, a key regulator of the methanol utilization pathway and peroxisomal genes in Pichia pastoris"<br>MOLECULAR AND CELLULAR BIOLOGY,<br>vol. 26, no. 3, February 2006 (2006-02),<br>pages 883-897, XP002438945<br>ISSN: 0270-7306<br>cited in the application<br>abstract<br>figures 2,3<br>page 895, left-hand column, lines 5-20<br>page 896, left-hand column, line 57 - right-hand column, line 16 | 11,12 |
| A | EP 0 340 986 A2 (CHIRON CORP [US])<br>8 November 1989 (1989-11-08)<br>examples I-III | 11,12 |
| A | WO 03/095653 A1 (RHEIN BIOTECH PROZ & PROD GMBH [DE]; SUCKOW MANFRED [DE])<br>20 November 2003 (2003-11-20)<br>pages 15-19 | |
| A | WO 90/03431 A (SALK INST BIOTECH IND [US])<br>5 April 1990 (1990-04-05)<br>example 1<br>claims 1-4 | 1 |
| A | NAKANO ET AL: "Effects of methanol feeding methods on chimeric alpha-amylase expression in continuous culture of Pichia pastoris"<br>JOURNAL OF BIOSCIENCE AND BIOENGINEERING,<br>vol. 101, no. 3, March 2006 (2006-03),<br>pages 227-231, XP005446006<br>ISSN: 1389-1723<br>abstract | 1 |

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Form PCT/ISA/210 (continuation of second sheet) (April 2005)

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/050870

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| 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, vol. 177, no. 1, 24 October 1996 (1996-10-24), pages 163-167, XP004043391 ISSN: 0378-1119 abstract | 1 |
| A | HARTNER F S ET AL: "Regulation of methanol utilisation pathway genes in yeasts." MICROBIAL CELL FACTORIES 2006, vol. 5, no. 39, 14 December 2006 (2006-12-14), pages 1-21, XP002438946 ISSN: 1475-2859 the whole document table 2 table 3 page 12, right-hand column, line 44 - page 14, left-hand column, line 31 | 1 |

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2008/050870

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 0056903 A | 28-09-2000 | AU 3910000 A | 09-10-2000 |
| EP 0911416 A2 | 28-04-1999 | EP 1659173 A1 | 24-05-2006 |
| EP 0299108 A1 | 18-01-1989 | AT 105858 T | 15-06-1994 |
| | | AU 2443488 A | 03-05-1990 |
| | | DE 3789866 D1 | 23-06-1994 |
| | | DE 3789866 T2 | 22-09-1994 |
| | | ES 2056799 T3 | 16-10-1994 |
| | | JP 2124093 A | 11-05-1990 |
| | | JP 3072993 B2 | 07-08-2000 |
| WO 03014363 A1 | 20-02-2003 | AT 372385 T | 15-09-2007 |
| | | EP 1414979 A1 | 06-05-2004 |
| | | IT MI20011728 A1 | 06-02-2003 |
| | | JP 2004538004 T | 24-12-2004 |
| | | US 2004241829 A1 | 02-12-2004 |
| EP 0340986 A2 | 08-11-1989 | AT 123521 T | 15-06-1995 |
| | | CA 1324969 C | 07-12-1993 |
| | | DE 68922932 D1 | 13-07-1995 |
| | | DE 68922932 T2 | 19-10-1995 |
| | | DK 221589 A | 07-11-1989 |
| | | ES 2072900 T3 | 01-08-1995 |
| | | JP 2104291 A | 17-04-1990 |
| | | JP 3074174 B2 | 07-08-2000 |
| | | JP 3484386 B2 | 06-01-2004 |
| | | JP 2000300277 A | 31-10-2000 |
| | | JP 3561513 B2 | 02-09-2004 |
| | | JP 2003116525 A | 22-04-2003 |
| | | JP 2004081222 A | 18-03-2004 |
| WO 03095653 A1 | 20-11-2003 | AU 2003240610 A1 | 11-11-2003 |
| | | CA 2485119 A1 | 20-11-2003 |
| | | DE 10220894 A1 | 27-11-2003 |
| | | EP 1504103 A1 | 09-02-2005 |
| | | KR 20050025180 A | 11-03-2005 |
| | | US 2006089492 A1 | 27-04-2006 |
| WO 9003431 A | 05-04-1990 | AU 4332589 A | 18-04-1990 |
| | | DK 55191 A | 24-05-1991 |
| | | EP 0436625 A1 | 17-07-1991 |
| | | JP 4501662 T | 26-03-1992 |

Form PCT/ISA/210 (patent family annex) (April 2005)

Attorney's Docket No.: 38767-0048US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | | | | | |
|---|---|---|---|---|---|
| First Named Inventor | : | Smita Shankar | Art Unit | : | 1636 |
| Application No. | : | 15/573,300 | Examiner | : | Nancy J. Leith |
| Filed | : | November 10, 2017 | Conf. No. | : | 4807 |
| Title | : | EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST | | | |

**Mail Stop Amendment**
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RESPONSE TO RESTRICTION REQUIREMENT

In response to the Restriction Requirement mailed April 8, 2019, Applicants elect Group I, claims 1-19 and 30-38, for examination. This election is made without traverse.

This Response to Restriction Requirement is being filed within the two-month Shortened Statutory Period for reply. 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.

Respectfully submitted,

Date: /June 6, 2019/

/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

61888037.doc

# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 36217885 |
| **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 Wasilkus |
| **Filer Authorized By:** | M. Angela Parsons |
| **Attorney Docket Number:** | 38767-0048US1 |
| **Receipt Date:** | 06-JUN-2019 |
| **Filing Date:** | 10-NOV-2017 |
| **Time Stamp:** | 07:22:22 |
| **Application Type:** | U.S. National Stage under 35 USC 371 |

## Payment information:

| | |
|---|---|
| Submitted with Payment | no |

## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Response to Election / Restriction Filed | Reply.pdf | 73065<br>de0d241eaadbb0eff1e154c83312a05bd712276c | no | 1 |

**Warnings:**

| Information: | |
|---|---|
| Total Files Size (in bytes): | 73065 |

**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.**

Attorney Docket No.: 38767-0048US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | | | | | |
|---|---|---|---|---|---|
| First Named Inventor | : | Smita Shankar | Art Unit | : | 1636 |
| Application No. | : | 15/573,300 | Examiner | : | Nancy J. Leith |
| Filed | : | November 10, 2017 | Conf. No. | : | 4807 |
| Title | : | EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST | | | |

**MAIL STOP AMENDMENT**
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

Please consider the documents listed on the disclosure form submitted with this statement. Copies of the documents are being submitted with this statement to the extent required under 37 CFR § 1.98(a)(2).

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 applicable under 37 CFR § 1.17(p), 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: July 12, 2019

/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

61995350.doc

Sheet 1 of 1

| Substitute Disclosure Form<br>(37 CFR §1.98(b)) | U.S. Department of Commerce<br>Patent and Trademark Office | Attorney Docket No.<br>38767-0048US1 | Application No.<br>15/573,300 |
|---|---|---|---|
| **Information Disclosure Statement by Applicant**<br>(Use several sheets if necessary) | | Applicant<br>Impossible Foods Inc. | |
| | | Filing Date<br>November 10, 2017 | Group Art Unit<br>1636 |

**U.S. Patent Documents**

| Examiner Initial | Desig. ID | Document Number | Publication Date | Patentee | Class | Subclass | Filing Date If Appropriate |
|---|---|---|---|---|---|---|---|
| | 1. | 8,236,528 | 08/07/2012 | Takagi 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 | No |

**Other Documents (include Author, Title, Date, and Place of Publication)**

| Examiner Initial | Desig. ID | Document |
|---|---|---|

| Examiner Signature | Date Considered |
|---|---|
| EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. | |

Substitute Disclosure Form

# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 36567094 |
| **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/Kirsten Neuman |
| **Filer Authorized By:** | M. Angela Parsons |
| **Attorney Docket Number:** | 38767-0048US1 |
| **Receipt Date:** | 12-JUL-2019 |
| **Filing Date:** | 10-NOV-2017 |
| **Time Stamp:** | 12:22:09 |
| **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 | Information Disclosure Statement (IDS) Form (SB08) | Transmittal.pdf | 72656<br>f34eac7ea711a624663466cb30c45121dbf4cc2b | no | 1 |

**Warnings:**

| **Information:** | | | | | |
|---|---|---|---|---|---|
| This is not an USPTO supplied IDS fillable form | | | | | |
| 2 | Information Disclosure Statement (IDS) Form (SB08) | PTO_1449.pdf | 121163<br>3b5fb17ffd036491974abc9461ede84e8fa9ae5f | no | 1 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| This is not an USPTO supplied IDS fillable form | | | | | |
| | | | **Total Files Size (in bytes):** | 193819 | |

**This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.**

**New Applications Under 35 U.S.C. 111**

**If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.**

**National Stage of an International Application under 35 U.S.C. 371**

**If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.**

**New International Application Filed with the USPTO as a Receiving Office**

**If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.**



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 15/573,300 | 11/10/2017 | Smita Shankar | 38767-0048US1 | 4807 |

26191 7590 07/16/2019
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

| EXAMINER | |
|---|---|
| LEITH, NANCY J | |

| ART UNIT | PAPER NUMBER |
|---|---|
| 1636 | |

| NOTIFICATION DATE | DELIVERY MODE |
|---|---|
| 07/16/2019 | ELECTRONIC |

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

PATDOCTC@fr.com

| *Office Action Summary* | **Application No.** 15/573,300 | **Applicant(s)** Shankar et al. | |
|---|---|---|---|
| | **Examiner** NANCY J. LEITH | **Art Unit** 1636 | **AIA (FITF) Status** Yes |

*-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --*

**Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

1)☑ Responsive to communication(s) filed on 06 July 2019.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims***

5) ☑ Claim(s) 1-38 is/are pending in the application.

5a) Of the above claim(s) 20-29 is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1-19 and 30-38 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

**Application Papers**

10)☑ The specification is objected to by the Examiner.

11)☑ The drawing(s) filed on 10 November 2017 is/are: a)☑ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

**Priority under 35 U.S.C. § 119**

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

**Certified copies:**

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

1) ☑ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date See Continuation Sheet.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

**Continuation Sheet (PTOL-326)** **Application No.** 15/573,300

Continuation of Attachment(s) 2) Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date: 20180413; 20180619; 20180706; 20181116; 20190604

**DETAILED ACTION**

***Notice of Pre-AIA or AIA Status***

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

***Election/Restrictions***

Applicant's election without traverse of Group I (claims 1-19 and 30-38) in the reply filed on June 6, 2019 is acknowledged.

Claims 20-29 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected invention, there being no allowable generic or linking claim. Election was made **without** traverse in the reply filed on June 6, 2019.

Claims 1-19 and 30-38 are under examination.

***Information Disclosure Statement***

The Information Disclosure Statements filed April 13, 2018; June 19, 2018; July 6, 2018; November 16, 2018; and June 4, 2019 have been considered.

***Specification***

**The disclosure is objected to because of the following informalities:**

The Tables at pages 16, 18-21, 24-29, 31, 33, and 35-37 should be numbered consecutively.

The use of the terms PHUSION® at page 16, lines 8 and 10; page 24, line 3; page 26, line 12; and page 28, line 11; CUTSMART® at page 17, line 5; page 26, lines 16 and 19; page 27, line 1; page 28, line 15; and page 29, line 1 ; ZYMOCLEAN™ at page 17, line 6; page 24, lines 9 and 27; page 29, line 5; page 31, line 16; page 32, line 24; and page 34, line 10; ZEOCIN™ at page 23, line 20 and page 31, line 4;

MICROPULSER™ at page 25, line 4; page 27, line 10; and page 29, line 17; SYBR™ at page 31, line 14 and page 32, line 22; and GIBSION ASSEMBLY® at page 35, lines 4-5; which are trade names or marks used in commerce, has been noted in this application. They should be capitalized wherever they appear and be accompanied by the generic terminology.

Although the use of trade names and marks used in commerce (i.e., trademarks, service marks, certification marks, and collective marks) are permissible in patent applications, the proprietary nature of the marks should be respected and every effort made to prevent their use in any manner which might adversely affect their validity as commercial marks.

Appropriate correction is required.

### *Claim Objections*

**Claims 8, 12, and 17 are objected to because of the following informalities:**

At claim 8, lines 2-4, the acronyms Mxr1, Adr1, Trm1, and Trm2 should be spelled out at their first occurrences, with the acronym provided in parentheses.

At claim 12, lines 3-6, the acronyms AOD1, MOX, MOD1, DHAS, FLD1, and PEX8 should be spelled out at their first occurrences, with the acronym provided in parentheses.

At claim 17, lines 2-4, the acronyms ALA, UPGIII, CPG, and PPG should be spelled out at their first occurrences, with the acronym provided in parentheses.

Appropriate correction is required.

### *Claim Rejections - 35 USC § 112*

The following is a quotation of 35 U.S.C. 112(b):

> (b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

**Claims 10-11, 19, and 30-38 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.**

Claim 10 attempts to set forth a sequence for the transcriptional activator by use of a GenBank Accession Number. While inclusion of the GenBank Accession Number qualifies as an intent to incorporate a specific sequence, this Accession Number fails to uniquely identify the specific sequence intended to be encompassed by the claims and is an improper incorporation by reference because sequences of GenBank are subject to change over time. However, because there is a single version of this sequence, Applicants can provide the specific sequence in a sequence listing and list the SEQ. ID. NO. in the claim.

Claim 11 depends from claim 10, and is therefore included in this rejection.

Claim 11 attempts to set forth a sequence for the transcriptional activator by use of a GenBank Accession Number. While inclusion of the GenBank Accession Number qualifies as an intent to incorporate a specific sequence, this Accession Number fails to uniquely identify the specific sequence intended to be encompassed by the claims and is an improper incorporation by reference because sequences of GenBank are subject to change over time. However, because there is a single version of this sequence, Applicants can provide the specific sequence in a sequence listing and list the SEQ. ID. NO. in the claim.

At claim 19, it is not clear if the yeast cell lacks heterologous sequences for selection in its genome prior to transformation, or if this means that such heterologous sequences are not added at the

time of any transformation. For purposes of examination, this is interpreted as the methylotrophic yeast not including the selection sequences in the genome, prior to transformation.

At claim 30, line 5, it is not clear if the globin family is limited to the PG00042 globin family listed in the parentheses, or if any globin family is encompassed by the claims. For the purposes of examination, this is interpreted as including any member of any globin family.

Claims 31-38 depend from claim 30, and are therefore included in this rejection.

In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

***Claim Rejections - 35 USC § 102***

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

> A person shall be entitled to a patent unless –
>
> (a)(1) the claimed invention was patented, described in a printed publication, or in public use, on sale or otherwise available to the public before the effective filing date of the claimed invention.
>
> (a)(2) the claimed invention was described in a patent issued under section 151, or in an application for patent published or deemed published under section 122(b), in which the patent or application, as the case may be, names another inventor and was effectively filed before the effective filing date of the claimed invention.

**Claims 1-5, 7, 12-14, and 19 are rejected under 35 U.S.C. 102(a)(1) and 102(a)(2) as being anticipated by Zhou *et al.* (PCT Patent Application Publication No. WO 2014/008729, published January 16, 2014, and cited in the Information Disclosure Statement filed April 13, 2018), as evidenced by U.S. Patent Application Publication No. 2015/0299716, published October 25, 2015, filed January 9,**

**2015, which is a National Stage Application of the '729 publication, and also qualifies as a 35 U.S.C. 102(a)(2) reference. All citation numbering refers to the U.S. '716 publication.**

Zhou discloses a methylotrophic yeast that comprises an exogenous nucleic acid encoding the transcriptional activator Mit1 operably linked to a methanol-inducible promoter (abstract and paragraphs [0002], [0013], and [0017]). Zhou discloses that the methanol-inducible promoter may be the AOX1 promoter, a DHAS promoter, a MOX promoter, an AOD1 promoter, a PEX8 promoter from methylotrophic yeasts including *Pichia pastoris*, *Hansenula*, *Candida*, or *Torulopsis* (paragraphs [0002] and [0017]-[0018]). Zhou discloses that the yeast cell can include a recombinant nucleic acid molecule in a replication-competent plasmid (paragraph [0077]). Zhou discloses that an exogenous (*i.e.*, heterologous) polypeptide can be expressed in the methylotrophic yeast (paragraph [0066]). Zhou discloses that the plasmid can include heterologous selection sequences, which is interpreted as the yeast cell lacking heterologous sequences for selection (paragraph [0079]).

Zhou discloses each and every limitation of claims 1-5, 7, 12-14, and 19, and therefore anticipates claims 1-5, 7, 12-14, and 19.

***Claim Rejections - 35 USC § 103***

The following is a quotation of 35 U.S.C. 103 which forms the basis for all obviousness rejections set forth in this Office action:

> A patent for a claimed invention may not be obtained, notwithstanding that the claimed invention is not identically disclosed as set forth in section 102, if the differences between the claimed invention and the prior art are such that the claimed invention as a whole would have been obvious before the effective filing date of the claimed invention to a person having ordinary skill in the art to which the claimed invention pertains. Patentability shall not be negated by the manner in which the invention was made.

The factual inquiries set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966), that are applied for establishing a background for determining obviousness under 35 U.S.C. 103 are summarized as follows:

1. Determining the scope and contents of the prior art.

2. Ascertaining the differences between the prior art and the claims at issue.

3. Resolving the level of ordinary skill in the pertinent art.

4. Considering objective evidence present in the application indicating obviousness or nonobviousness.

This application currently names joint inventors. In considering patentability of the claims the examiner presumes that the subject matter of the various claims was commonly owned as of the effective filing date of the claimed invention(s) absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and effective filing dates of each claim that was not commonly owned as of the effective filing date of the later invention in order for the examiner to consider the applicability of 35 U.S.C. 102(b)(2)(C) for any potential 35 U.S.C. 102(a)(2) prior art against the later invention.

**Claim 1-19, 30-33, 35, and 37 are rejected under 35 U.S.C. 103 as being unpatentable over Zhou *et al.* (PCT Patent Application Publication No. WO 2014/008729, published January 16, 2014, and cited in the Information Disclosure Statement filed April 13, 2018), as evidenced by U.S. Patent Application Publication No. 2015/0299716, published October 25, 2015, filed January 9, 2015, which is a National Stage Application of the '729 publication, and also qualifies as a 35 U.S.C. 102(a)(2) reference in view of Varadan *et al.* (PCT Patent Application No. WO 2015/153666, published October 8, 2015, claiming priority to U.S. Provisional Patent Application Nos. 61/973,181 and 62/058,230, filed March 31, 2014 and October 1, 2014, respectively, and cited in the Information Disclosure Statement filed November 16, 2018) and Lin-Cereghino *et al.* (26(3) Molecular and Cellular Biology 883-897 (2006), and cited in the Information Disclosure Statement filed April13, 2018).**

Zhou discloses a methylotrophic yeast that comprises an exogenous nucleic acid encoding the transcriptional activator Mit1 operably linked to a methanol-inducible promoter (abstract and

paragraphs [0002], [0013], and [0017]). Zhou discloses that the methanol-inducible promoter may be the AOX1 promoter, a DHAS promoter, a MOX promoter, an AOD1 promoter, a PEX8 promoter from methylotrophic yeasts including *Pichia pastoris*, *Hansenula*, *Candida*, or *Torulopsis* (paragraphs [0002] and [0017]-[0018]). Zhou discloses that the yeast cell can include a recombinant nucleic acid molecule in a replication-competent plasmid (paragraph [0077]). Zhou discloses that an exogenous (*i.e.*, heterologous) polypeptide can be expressed in the methylotrophic yeast (paragraph [0066]). Zhou discloses that the plasmid can include heterologous selection sequences, which is interpreted as the yeast cell lacking heterologous sequences for selection (paragraph [0079]).

Zhou fails to disclose or suggest that the transcriptional activator is Mxr1 from *Pichia pastoris*. Zhou fails to disclose or suggest that the recombinant nucleic acid molecules comprises nucleic acid sequences encoding a member of the globin family or encoding a polypeptide involved in heme biosynthesis. Zhou fails to explicitly disclose or suggest integration of the recombinant molecule into the genome of the yeast.

Varadan discloses methylotrophic yeasts including Pichia pastoris that comprise the nucleic acid encoding a globin molecule and a molecule involved in heme biosynthesis, which may be ALA synthase, ADA dehydratase, porphobilinogen deaminase, uroporphyrinogen III synthase, uroporphyrinogen III decarboxylase, or uroporphyrinogen III synthase, coproporphyrinogen oxidase, protoporphyrinogen III oxidase, and ferrochelatase (pages 36-37, Table 1). Varadan discloses that the promoter may be an AOX1 promoter (page 36, lines 10-14). Varadan discloses that the globin can be leghemoglobin or a variety of other known hemoglobins (page 6, lines 4-7 and page 15, lines 20-25).

Lin-Cereghino discloses that the transcriptional activator Mxr1 is a regulator if methanol utilization in *Pichia pastoris* (abstract). Lin-Cereghino discloses plasmids comprising the Mxr1 transcriptional activator under the control of the AOX1 promoter or the PEX8 promoter (page 885, column 1, final full paragraph). Lin-Cereghino discloses that the nucleic acids may be stably integrated

into a locus in wild-type and mxr1 mutant strains (paragraph bridging pages 891-893). Lin-Cereghino discloses the sequences of GenBank Accession Nos. ABD57365 and DQ395124 (page 888). Lin-Cereghino discloses that Pichia pastoris is a major system for the production of recombinant proteins (page 896, column 2, final paragraph).

It would have been obvious to one with ordinary skill in the art before the effective filing date of the claimed invention provide Zhou's methylotrophic yeast comprising sequences encoding Varadan's leghemoglobin and polypeptides involved in heme biosynthesis under the control of Lin-Cereghino's mxr1 transcriptional activator and the AOX1 promoter of each of Zhou, Varadan, and Lin-Cereghino in the *Pichia pastoris*, as disclosed as being a system for producing recombinant proteins because these systems are well-known in the recombinant protein production art. As such, it would be well within the purview of one ordinarily skilled in the art to use the components of Zhou and Lin-Cereghino in a yeast comprising leghemoglobin and polypeptides involved in heme biosynthesis, and combining these elements would be predictable and have a reasonable expectation of success, whether the components are in a replication-competent plasmid or stably integrated into the genome of the yeast cell.

**Claims 34, 36, and 38 are rejected under 35 U.S.C. 103 as being unpatentable over Zhou, Varadan, and Lin-Cereghino, as applied to claims 1-19, 30-33, 35, and 37 above, and further in view of Liu et al. (21 Metabolic Engineering 9-16 (2014), and cited in the Information Disclosure Statement filed November 16, 2018).**

Zhou, Varadan, and Lin-Cereghino disclose and suggest a methylotrophic yeast comprising a transcriptional activator and a methanol-inducible promoter for the production of leghemoglobin, as discussed above.

Zhou, Varadan, and Lin-Cereghino fail to disclose or suggest the use of a constitutive promoter such as TEF1.

Liu discloses the production of hemoglobin in yeast cells (abstract). Liu discloses that the production of globin polypeptides can be under the control of the constitutive promoter TEF1 (page 10, column 2, second full paragraph).

It would have been obvious to one with ordinary skill in the art before the effective filing date of the claimed invention to substitute Liu's constitutive TEF1 promoter for the inducible AOX1 promoter of Zhou, Varadan, and Lin-Cereghino because the use of the constitutive promoter provides for a constant production from the claimed yeast cell, without requiring the induction of the methanol-inducible promoters of Zhou, Varadan, and Lin-Cereghino. Because both inducible and constitutive promoter systems are well known to those skilled in the art, one of ordinary skill in the art would have a predictable and reasonable expectation of success in substituting one well-known promoter system for another well-known promoter system, as both are used for polypeptide (globin) production in yeast cell.

***Double Patenting***

Applicant is advised that should claim 3 be found allowable, claim 4 will be objected to under 37 CFR 1.75 as being a substantial duplicate thereof. When two claims in an application are duplicates or else are so close in content that they both cover the same thing, despite a slight difference in wording, it is proper after allowing one claim to object to the other as being a substantial duplicate of the allowed claim. *See* MPEP § 706.03(k).

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference

claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

**Claims 1-19 and 30-18 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-21 of U.S. Patent No. 9,938,327.**

Although the claims at issue are not identical, they are not patentably distinct from each other because the ‘327 patent claims a methylotrophic *Pichia* yeast cell comprising a leghemoglobin encoding

nucleic acid, a *Pichia* promoter element, and an Mxr1 transciptional activator. The instant application claims a methylotrophic yeast cell comprising a transcriptional activator operably linked to a methanol-inducible promoter element. The '327 patent claims therefore provide for each element of the instant application's claims.

**Claims 1-19 and 30-18 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-14 of U.S. Patent No. 10,273,492.**

Although the claims at issue are not identical, they are not patentably distinct from each other because the '492 patent claims a methylotrophic *Pichia* yeast cell comprising a heme biosynthesis encoding nucleic acid, a *Pichia* promoter element, and an Mxr1 transciptional activator. The instant application claims a methylotrophic yeast cell comprising a transcriptional activator operably linked to a methanol-inducible promoter element. The '492 patent claims therefore provide for each element of the instant application's claims.

**Claims 1-19 and 30-38 provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 17-26 of copending Application No.16/123,121 (reference application).**

Although the claims at issue are not identical, they are not patentably distinct from each other because the '121 application claims a methylotrophic yeast cell comprising a transciptional activator operably linked to a promoter element. The instant application also claims a methylotrophic yeast cell comprising a transcriptional activator operably linked to a promoter element. It would have been obvious to one with ordinary skill in the art before the effective filing date of the claimed invention to use a methanol-inducible promoter for the '121 application's promoter element because this is a well-

known promoter and because the promoter is inducible, provides control over the yeast cell protein production system.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

***Conclusion***

Any inquiry concerning this communication or earlier communications from the examiner should be directed to NANCY J LEITH whose telephone number is (313)446-4874. The examiner can normally be reached on Monday - Thursday 8:00 AM - 6:30 PM.

Examiner interviews are available via telephone, in-person, and video conferencing using a USPTO supplied web-based collaboration tool. To schedule an interview, applicant is encouraged to use the USPTO Automated Interview Request (AIR) at http://www.uspto.gov/interviewpractice.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, HEATHER CALAMITA can be reached on (571) 272-2876. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

NANCY J. LEITH
Primary Examiner
Art Unit 1636

/NANCY J LEITH/
Primary Examiner, Art Unit 1636

| ***Notice of References Cited*** | Application/Control No. 15/573,300 | Applicant(s)/Patent Under Reexamination Shankar et al. | |
|---|---|---|---|
| | Examiner NANCY J. LEITH | Art Unit 1636 | Page 1 of 1 |

**U.S. PATENT DOCUMENTS**

| * | | Document Number Country Code-Number-Kind Code | Date MM-YYYY | Name | CPC Classification | US Classification |
|---|---|---|---|---|---|---|
| * | A | US-10273492-B2 | 04-2019 | Shankar; Smita | C07K14/39 | 1/1 |
| | B | | | | | |
| | C | | | | | |
| | D | | | | | |
| | E | | | | | |
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**FOREIGN PATENT DOCUMENTS**

| * | | Document Number Country Code-Number-Kind Code | Date MM-YYYY | Country | Name | CPC Classification |
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| | P | | | | | |
| | Q | | | | | |
| | R | | | | | |
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**NON-PATENT DOCUMENTS**

| * | | Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages) |
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| | X | |

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

| Index of Claims | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 15/573,300 | Shankar et al. |
| | Examiner | Art Unit |
| | NANCY J. LEITH | 1636 |

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|---|---|---|---|---|---|---|---|
| ✓ | Rejected | - | Cancelled | N | Non-Elected | A | Appeal |
| = | Allowed | ÷ | Restricted | I | Interference | O | Objected |

**CLAIMS**

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

| CLAIM | | DATE | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Final | Original | 04/02/2019 | 07/11/2019 | | | | | | | |
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| | 37 | ÷ | ✓ | | | | | | | |
| | 38 | ÷ | ✓ | | | | | | | |

| Search Notes | Application/Control No. | Applicant(s)/Patent Under Reexamination |
|---|---|---|
| | 15/573,300 | Shankar et al. |
| | **Examiner** | **Art Unit** |
| | NANCY J. LEITH | 1636 |

| CPC - Searched* | | |
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| **Symbol** | **Date** | **Examiner** |
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| CPC Combination Sets - Searched* | | |
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| **Symbol** | **Date** | **Examiner** |
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| US Classification - Searched* | | | |
|---|---|---|---|
| **Class** | **Subclass** | **Date** | **Examiner** |
| | | | |

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

| Search Notes | | |
|---|---|---|
| **Search Notes** | **Date** | **Examiner** |
| Inventor Search (PALM) | 07/09/2019 | NJL |
| EAST Search (USPat, USPub, EPO, JPO, Derwent), text search only, see search history | 07/09/2019 | NJL |
| STN Search (Biosis, Embase, Medline), see search history | 07/09/2019 | NJL |
| Reviewed prosecution histories of U.S. Patent Nos. 9,938,327 and 10,273,492 and U.S. Patent Application No. 16/123,121 | 07/09/2019 | NJL |

| Interference Search | | | |
|---|---|---|---|
| **US Class/CPC Symbol** | **US Subclass/CPC Group** | **Date** | **Examiner** |
| | | | |

| /NANCY J LEITH/<br>Primary Examiner, Art Unit 1636 | |
|---|---|

Receipt date: 11/16/2018

15/573,300 - GAU: 1636

Sheet 1 of 1

| Substitute Disclosure Form (37 CFR §1.98(b)) | U.S. Department of Commerce Patent and Trademark Office | Attorney Docket No. | Application No. |
|---|---|---|---|
| **Information Disclosure Statement by Applicant** (Use several sheets if necessary) | | 38767-0048US1 | 15/573,300 |
| | | Applicant: Impossible Foods Inc. | |
| | | Filing Date: November 10, 2017 | Group Art Unit: 1636 |

**U.S. Patent Documents**

| Examiner Initial | Desig. ID | Document Number | Publication Date | Patentee | Class | Subclass | Filing Date If Appropriate |
|---|---|---|---|---|---|---|---|

**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 |
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| Examiner Signature | Date Considered |
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EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

Receipt date: 06/04/2019

15/573,300 - GAU: 1636

Sheet 1 of 1

| Substitute Disclosure Form / U.S. Department of Commerce Patent and Trademark Office | Attorney Docket No. | Application No. |
|---|---|---|
| **Information Disclosure Statement by Applicant** (Use several sheets if necessary) (37 CFR §1.98(b)) | 38767-0048US1 | 15/573,300 |
| | Applicant: Impossible Foods Inc. | |
| | Filing Date: November 10, 2017 | Group Art Unit: 1636 |

**U.S. Patent Documents**

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| Examiner Initial | Desig. ID | Document Number | Publication Date | Country or Patent Office | Class | Subclass | Translation Yes | Translation No |
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| Examiner Signature | Date Considered |
|---|---|
| /NANCY J LEITH/ | 07/09/2019 |

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

Sheet 1 of 1

| Substitute Disclosure Form / U.S. Department of Commerce Patent and Trademark Office | Attorney Docket No. | Application No. |
|---|---|---|
| **Information Disclosure Statement by Applicant** (Use several sheets if necessary) (37 CFR §1.98(b)) | 38767-0048US1 | 15/573,300 |
| | 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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**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 |
|---|---|---|---|---|---|---|---|---|
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**Other Documents (include Author, Title, Date, and Place of Publication)**

| Examiner Initial | Desig. ID | Document |
|---|---|---|
| /N.J.L/ | 2. | GenBank Accession No. AAA33980, "leghemoglobin [Glycine max]," April 1993, 1 page |

| Examiner Signature | Date Considered |
|---|---|
| /NANCY J LEITH/ | 07/09/2019 |

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

**EAST Search History**

**EAST Search History (Prior Art)**

| Ref # | Hits | Search Query | DBs | Default Operator | Plurals | Time Stamp |
|---|---|---|---|---|---|---|
| L1 | 20 | (yeast$5 or candida$9 or hansenula$9 or pichia$9 or torulopsis$9 or pastoris$9 or ppastoris$9) same (transcri$9 near3 activat$7) same (promoter$6) same ((methanol$3 or ch3$4) near3 induc$9) | US-PGPUB; USPAT; EPO; JPO; DERWENT | OR | OFF | 2019/07/09 15:44 |
| L2 | 20 | (yeast$5 or saccharo$9 or cerevis$8 or scerevis$8 or candida$9 or hansenula$9 or pichia$9 or torulopsis$9 or pastoris$9 or ppastoris$9) same (transcri$9 near3 activat$7) same (promoter$6) same ((methanol$3 or ch3$4) near3 induc$9) | US-PGPUB; USPAT; EPO; JPO; DERWENT | OR | OFF | 2019/07/09 15:46 |
| L3 | 16752 | (yeast$5 or saccharo$9 or cerevis$8 or scerevis$8 or candida$9 or hansenula$9 or pichia$9 or torulopsis$9 or pastoris$9 or ppastoris$9) same (transcri$9 near3 activat$7) | US-PGPUB; USPAT; EPO; JPO; DERWENT | OR | OFF | 2019/07/09 15:50 |
| L4 | 121 | l3 and (promoter$6 same ((methanol$3 or ch3$4) near3 induc$9)) | US-PGPUB; USPAT; EPO; JPO; DERWENT | OR | OFF | 2019/07/09 15:50 |
| L5 | 14 | l4 and mxr1$2 | US-PGPUB; USPAT; EPO; JPO; DERWENT | OR | OFF | 2019/07/09 15:59 |
| L6 | 62 | l4 and ((alcohol$3 near2 oxidas$5) or aox1$2) | US-PGPUB; USPAT; EPO; JPO; DERWENT | OR | OFF | 2019/07/09 16:00 |
| L7 | 63 | l5 or l6 | US-PGPUB; USPAT; EPO; JPO; DERWENT | OR | OFF | 2019/07/09 16:01 |
| L8 | 7 | l4 and (leghemoglob$8 or legh$3) | US-PGPUB; USPAT; EPO; JPO; DERWENT | OR | OFF | 2019/07/09 16:24 |
| L9 | 63 | l7 or l8 | US-PGPUB; USPAT; EPO; JPO; DERWENT | OR | OFF | 2019/07/09 16:25 |
| L10 | 51 | l9 and @ad<="20150510" | US-PGPUB; USPAT; EPO; JPO; | OR | OFF | 2019/07/09 16:26 |

EAST Search History

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|---|---|---|---|---|---|---|
| | | | DERWENT | | | |
| L11 | 306 | SHANKAR-S.in. or SHANKAR-S-B.in. or SHANKAR-S-D.in. or SHANKAR-S-H.in. or SHANKAR-S-J.in. or SHANKAR-S-K.in. or SHANKAR-S-K-A.in. or SHANKAR-S-L.in. or SHANKAR-S-.in. or SHANKAR-S-N.in. or SHANKAR-S-O-PATHMASUNTHARAM-J.in. or SHANKAR-S-O-P-J.in. or SHANKAR-S-R.in. or SHANKAR-S-S.in. or SHANKAR-S-S-B.in. or SHANKAR-S-S-V.in. or SHANKAR-S-T.in. or SHANKAR-S-U.in. or SHANKAR-S-V.in. or SHANKAR-SMITA.in. or HOYT-M.in. or HOYT-M-A.in. or HOYT-MARTIN-ANDREW.in. | US-PGPUB; USPAT; EPO; JPO; DERWENT | OR | OFF | 2019/07/09 16:47 |
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| L13 | 334 | l11 or l12 | US-PGPUB; USPAT; EPO; JPO; DERWENT | OR | OFF | 2019/07/09 16:48 |
| L14 | 7 | l2 and l13 | US-PGPUB; USPAT; EPO; JPO; DERWENT | OR | OFF | 2019/07/09 16:48 |
| L15 | 7 | l3 and l13 | US-PGPUB; USPAT; EPO; JPO; DERWENT | OR | OFF | 2019/07/09 16:48 |
| L16 | 7 | l14 or l15 | US-PGPUB; USPAT; EPO; JPO; DERWENT | OR | OFF | 2019/07/09 16:59 |

**7/9/2019 4:59:31 PM**

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# BIB DATA SHEET

**CONFIRMATION NO. 4807**

| SERIAL NUMBER | FILING or 371(c) DATE<br>RULE | CLASS | GROUP ART UNIT | ATTORNEY DOCKET NO. |
|---|---|---|---|---|
| 15/573,300 | 11/10/2017 | 435 | 1636 | 38767-0048US1 |

**APPLICANTS**
Impossible Foods Inc., Redwood City, CA;

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

**** CONTINUING DATA ***************************
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 ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ****
01/25/2018

| Foreign Priority claimed ☐ Yes ☑ No<br>35 USC 119(a-d) conditions met ☐ Yes ☑ No<br>Verified and Acknowledged /NANCY J LEITH/ Examiner's Signature | ☐ Met after Allowance<br>Initials | STATE OR COUNTRY | SHEETS DRAWINGS | TOTAL CLAIMS | INDEPENDENT CLAIMS |
|---|---|---|---|---|---|
| | | CA | 11 | 38 | 2 |

**ADDRESS**
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022
UNITED STATES

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

| FILING FEE RECEIVED | FEES: Authority has been given in Paper | |
|---|---|---|
| 3060 | No._____ to charge/credit DEPOSIT ACCOUNT<br>No._____ for following: | ☐ All Fees<br>☐ 1.16 Fees (Filing)<br>☐ 1.17 Fees (Processing Ext. of time)<br>☐ 1.18 Fees (Issue)<br>☐ Other _____<br>☐ Credit |

BIB (Rev. 05/07).

STN Express Query Summary

(FILE 'HOME' ENTERED AT 17:52:41 ON 09 JUL 2019)

FILE 'BIOSIS, EMBASE, MEDLINE' ENTERED AT 17:52:49 ON 09 JUL 2019

L1 22548 SEA FILE=MFE SPE=ON ABB=ON PLU=ON (YEAST? OR SACCHARO? OR CEREVIS? OR SCEREVIS? OR CANDID? OR HANSENUL? OR PICHIA? OR TORULOP? OR PASTORIS? OR PPASTORIS?) (L) (TRANSCRI? (3A) ACTIVAT?)

L2 15 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L1 AND (PROMOTER? (L) ((METHANOL? OR CH3?) (3A) INDUC?))

L3 931 SEA FILE=MFE SPE=ON ABB=ON PLU=ON (YEAST? OR SACCHARO? OR CEREVIS? OR SCEREVIS? OR CANDID? OR HANSENUL? OR PICHIA? OR TORULOP? OR PASTORIS? OR PPASTORIS?) (L) PROMOTER? (L) ((METHANOL? OR CH3?) (3A) INDUC?)

L4 25 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L3 AND MXR1?

L5 756 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L3 AND ((ALCOHOL? (2A) OXIDAS?) OR AOX1?)

L6 0 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L5 AND (LEGHEMOGLOB? OR LEGH?)

L7 731 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L3 AND (((ALCOHOL? (2A) OXIDAS?) OR AOX1?) (4A) PROMOTER?)

L8 585 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L3 AND ((ALCOHOL? (2A) OXIDAS? (2A) 1) OR AOX1?) (4A) PROMOTER?\

L9 585 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L3 AND (((ALCOHOL? (2A) OXIDAS? (2A) 1) OR AOX1?) (4A) PROMOTER?)

L10 197 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L9 AND METHYLOTROPH?

L11 0 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L10 AND (GLOBIN? OR HEMOGLOBIN? OR LEGHEMOGLOBIN? OR LEGH?)

L12 0 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L10 AND HEME?

L13 230 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L2 OR L4 OR L10

L14 106 DUPLICATE REMOVE L13 (124 DUPLICATES REMOVED)

L*** DEL 77 S L2 OR L4 OR L10

L*** DEL 83 S L2 OR L4 OR L10

L*** DEL 70 S L2 OR L4 OR L10

L15 81 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L14 AND PY<2016

D L15 1-81

E SHANKAR S/AU

L16 1521 SEA FILE=MFE SPE=ON ABB=ON PLU=ON ("SHANKAR S"/AU OR "SHANKAR S C"/AU OR "SHANKAR S DR"/AU OR "SHANKAR S DR PROF"/AU OR "SHANKAR S G"/AU OR "SHANKAR S G V"/AU OR "SHANKAR S G VIJAY"/AU OR "SHANKAR S GOKUL"/AU OR "SHANKAR S GOWRI"/AU OR "SHANKAR S J"/AU OR "SHANKAR S JAI"/AU OR "SHANKAR S K"/AU OR "SHANKAR S K DR"/AU OR "SHANKAR S K DR PROF"/AU OR "SHANKAR S K PROF"/AU OR "SHANKAR S K PROF DR"/AU OR "SHANKAR S KARTHIK"/AU OR "SHANKAR S KRUPA"/AU OR "SHANKAR S L"/AU OR "SHANKAR S L R"/AU OR "SHANKAR S L RAVI"/AU OR "SHANKAR S M"/AU OR "SHANKAR S M DR"/AU OR "SHANKAR S P"/AU OR "SHANKAR S P DR PROF"/AU OR "SHANKAR S PRABHU"/AU OR "SHANKAR S PROF"/AU OR "SHANKAR S R"/AU OR "SHANKAR S R MADHAN"/AU OR "SHANKAR S RAJA"/AU OR "SHANKAR S RAVI"/AU OR "SHANKAR S S"/AU OR "SHANKAR S SHARATH"/AU OR "SHANKAR S SHIV"/AU OR "SHANKAR S SHIVA"/AU OR "SHANKAR S V"/AU OR "SHANKAR S VIDYA"/AU OR "SHANKAR S VIJAY"/AU)

E SHANKAR SMITA/AU

L17 22 SEA FILE=MFE SPE=ON ABB=ON PLU=ON "SHANKAR SMITA"/AU

E HOYT M/AU

L18 234 SEA FILE=MFE SPE=ON ABB=ON PLU=ON ("HOYT M"/AU OR "HOYT M A"/AU OR "HOYT M ANDREW"/AU OR "HOYT MARTIN"/AU OR "HOYT MARTIN A"/AU OR "HOYT MARTIN ANDREW"/AU)

E IMPOSSIBLE/CS

L19 49 SEA FILE=MFE SPE=ON ABB=ON PLU=ON (IMPOSSIBLE/CS OR "IMPOSSIBLE FOODS IMPOSSIBLEFOODS COM REDWOOD CITY 94063 CA USA"/CS OR "IMPOSSIBLE FOODS INC"/CS OR "IMPOSSIBLE FOODS INC 400 SAGINAW DR REDWOOD CITY CA 94063 USA"/CS OR "IMPOSSIBLE FOODS INC REDWOOD CITY CA 94063 USA"/CS OR "IMPOSSIBLE FOODS

INC REDWOOD CITY CA USA"/CS OR "IMPOSSIBLE FOODS INC THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY"/CS OR "IMPOSSIBLE FOODS INC., 400 SAGINAW DRIVE, REDWOOD CITY, CA, 94063"/CS OR "IMPOSSIBLE FOODS INC., 525 CHESAPEAKE DRIVE, REDWOOD CITY, CA, 94063"/CS OR "IMPOSSIBLE FOODS INC., REDWOOD CITY, CA"/CS OR "IMPOSSIBLE FOODS INC., REDWOOD CITY, CA, 94063"/CS OR "IMPOSSIBLE FOODS REDWOOD CITY CA UNITED STATES OF AMERICA"/CS OR "IMPOSSIBLE FOODS STANFORD CA USA"/CS OR "IMPOSSIBLE FOODS, INC, REDWOOD CITY, CA, 94063"/CS OR "IMPOSSIBLE FOODS, REDWOOD CITY, CA"/CS OR "IMPOSSIBLE FOODS, REDWOOD CITY, CA, 94063"/CS)

L20 1822 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L16 OR L17 OR L18 OR L19

L21 0 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L1 AND L20

L22 34 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L20 AND (HEME? OR GLOBIN? OR HEMOGLOB? OR LEGHEMOGLOB? OR LEGH?)

L23 20 DUPLICATE REMOVE L22 (14 DUPLICATES REMOVED)

D L23 1-20

Receipt date: 07/06/2018

15/573,300 - GAU: 1636

Sheet 1 of 1

| Substitute Disclosure Form / U.S. Department of Commerce Patent and Trademark Office | Attorney Docket No. | Application No. |
|---|---|---|
| **Information Disclosure Statement by Applicant** (Use several sheets if necessary) (37 CFR §1.98(b)) | 38767-0048US1 | 15/573,300 |
| | 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 |
|---|---|---|---|---|---|---|---|
| | -- | -- | -- | -- | | | |

**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 |
|---|---|---|---|---|---|---|---|---|
| | -- | -- | -- | -- | | | | |

**Other Documents (include Author, Title, Date, and Place of Publication)**

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| /N.J.L/ | 1. | [No Author Listed] Impossible Foods Inc. "GRAS Notification for Soybean Leghemoglobin Protein Derived from Pichia Pastoris." GRAS notice 000737, Retrieved from internet <URL:https://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm, 1063 pages 2017 |

| Examiner Signature | Date Considered |
|---|---|
| /NANCY J LEITH/ | 07/09/2019 |

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

Receipt date: 04/13/2018

15/573,300 - GAU: 1636



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| **Information Disclosure Statement by Applicant** (Use several sheets if necessary) | | 38767-0048US1 | 15/573,300 |
| | | Applicant: Impossible Foods Inc. | |
| | | Filing Date: November 10, 2017 | Group Art Unit: ~~1636~~ 1636 |

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| Examiner Initial | Desig. ID | Document Number | Publication Date | Country or Patent Office | Class | Subclass | Translation Yes | Translation No |
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| Examiner Signature | Date Considered |
|---|---|
| /NANCY J LEITH/ | 07/09/2019 |

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /N.J.L/

Receipt date: 04/13/2018 15/573,300 - GAU: 1636



| Substitute Disclosure Form (37 CFR §1.98(b)) | U.S. Department of Commerce Patent and Trademark Office | Attorney Docket No. | Application No. |
|---|---|---|---|
| **Information Disclosure Statement by Applicant** (Use several sheets if necessary) | | 38767-0048US1 | 15/573,300 |
| | | Applicant: Impossible Foods Inc. | |
| | | 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 |
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| Examiner Signature | Date Considered |
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| /NANCY J LEITH/ | 07/09/2019 |

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|---|---|---|
| Examiner Initial | Desig. ID | Document |
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| Examiner Signature | Date Considered |
|---|---|
| /NANCY J LEITH/ | 07/09/2019 |

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /N.J.L/

Receipt date: 04/13/2018 15/573,300 - GAU: 1636



| Substitute Disclosure Form / U.S. Department of Commerce Patent and Trademark Office | Attorney Docket No. | Application No. |
|---|---|---|
| **Information Disclosure Statement by Applicant** (Use several sheets if necessary) | 38767-0048US1 | 15/573,300 |
| | Applicant: Impossible Foods Inc. | |
| (37 CFR §1.98(b)) | Filing Date: November 10, 2017 | Group Art Unit: 1656 |

| Other Documents (include Author, Title, Date, and Place of Publication) | | |
|---|---|---|
| Examiner Initial | Desig. ID | Document |
| | 62. | Tovkach et al., "A toolbox and procedural notes for characterizing novel zinc finger nucleases for genome editing in plant cells" Plant J, 57: 747-757 25 (2009) |
| | 63. | Townsend et al., "High-frequency modification of plant genes using engineered zinc-finger nucleases" Nature 459: 442-445 (2009) |
| | 64. | UniprotKB Accession No. P02236, "Leghemoglobin C2," dated November 1, 1988, 9 pages |
| | 65. | Wu et al., "Efficient production of a functional single-chain antidigoxin antibody via an engineered Bacillus subtilis expression-secretion system" Bio/Technology (NY) 11(1): 71-76 (1993) (abstract only) |
| | 66. | Xie and Yang, "RNA-guided genome editing in plants using a CRISPR-Cas system" Mal. Plant 6: 1975-1983 (2013) |
| | 67. | Zhang et al., "Transcription activator-like effector nucleases enable efficient plant genome engineering" Plant Physiology 161: 20-27 (2013) |

| Examiner Signature | Date Considered |
|---|---|
| /NANCY J LEITH/ | 07/09/2019 |

EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

Substitute Disclosure Form

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /N.J.L/

PTO/SB/06 (09-11)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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| PATENT APPLICATION FEE DETERMINATION RECORD<br>Substitute for Form PTO-875 | Application or Docket Number<br>15/573,300 | Filing Date<br>11/10/2017 | ☐ To be Mailed |
|---|---|---|---|

**ENTITY:** ☑ LARGE ☐ SMALL ☐ MICRO

## APPLICATION AS FILED - PART I

| FOR | (Column 1)<br>NUMBER FILED | (Column 2)<br>NUMBER EXTRA | | RATE ($) | FEE ($) |
|---|---|---|---|---|---|
| ☐ BASIC FEE<br>(37 CFR 1.16(a), (b), or (c)) | N/A | N/A | | N/A | |
| ☐ SEARCH FEE<br>(37 CFR 1.16(k), (i), or (m)) | N/A | N/A | | N/A | |
| ☐ EXAMINATION FEE<br>(37 CFR 1.16(o), (p), or (q)) | N/A | N/A | | N/A | |
| TOTAL CLAIMS<br>(37 CFR 1.16(i)) | minus 20 = | * | | x $80 = | |
| INDEPENDENT CLAIMS<br>(37 CFR 1.16(h)) | minus 3 = | * | | x $420 = | |
| ☐ APPLICATION SIZE FEE (37 CFR 1.16(s)) | If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s). | | | | |
| ☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j)) | | | | | |
| * If the difference in column 1 is less than zero, enter "0" in column 2. | | | | TOTAL | |

## APPLICATION AS AMENDED - PART II

| | | (Column 1) | | (Column 2) | (Column 3) | | | |
|---|---|---|---|---|---|---|---|---|
| AMENDMENT | 10/16/2019 | CLAIMS REMAINING AFTER AMENDMENT | | HIGHEST NUMBER PREVIOUSLY PAID FOR | PRESENT EXTRA | | RATE ($) | ADDITIONAL FEE ($) |
| | Total<br>(37 CFR 1.16(i)) | * 29 | Minus | ** 38 | = 0 | | x $100 = | 0 |
| | Independent<br>(37 CFR 1.16(h)) | * 2 | Minus | *** 3 | = 0 | | x $460 = | 0 |
| | ☐ Application Size Fee (37 CFR 1.16(s)) | | | | | | | |
| | ☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j)) | | | | | | | |
| | | | | | | | TOTAL ADD'L FEE | 0 |

| | | (Column 1) | | (Column 2) | (Column 3) | | | |
|---|---|---|---|---|---|---|---|---|
| AMENDMENT | | CLAIMS REMAINING AFTER AMENDMENT | | HIGHEST NUMBER PREVIOUSLY PAID FOR | PRESENT EXTRA | | RATE ($) | ADDITIONAL FEE ($) |
| | Total<br>(37 CFR 1.16(i)) | * | Minus | ** | = | | x $0 = | |
| | Independent<br>(37 CFR 1.16(h)) | * | Minus | *** | = | | x $0 = | |
| | ☐ Application Size Fee (37 CFR 1.16(s)) | | | | | | | |
| | ☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j)) | | | | | | | |
| | | | | | | | TOTAL ADD'L FEE | |

| | |
|---|---|
| * If the entry in column 1 is less than the entry in column 2, write "0" in column 3. | LIE |
| ** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20". | /YOLANDA CHADWICK/ |
| *** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3". | |
| The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1. | |

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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| **Doc Code: DIST.E.FILE**<br>**Document Description: Electronic Terminal Disclaimer - Filed** | PTO/SB/26<br>U.S. Patent and Trademark Office<br>Department of Commerce |
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| | |
|---|---|
| Electronic Petition Request | **TERMINAL DISCLAIMER TO OBVIATE A DOUBLE PATENTING REJECTION OVER A "PRIOR" PATENT** |
| Application Number | 15573300 |
| Filing Date | 10-Nov-2017 |
| First Named Inventor | Smita Shankar |
| Attorney Docket Number | 38767-0048US1 |
| Title of Invention | EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST |

☒ Filing of terminal disclaimer does not obviate requirement for response under 37 CFR 1.111 to outstanding Office Action

☒ This electronic Terminal Disclaimer is not being used for a Joint Research Agreement.

| Owner | Percent Interest |
|---|---|
| Impossible Foods, Inc. | 100% |

The owner(s) with percent interest listed above in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of prior patent number(s)

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In making the above disclaimer, the owner does not disclaim the terminal part of the term of any patent granted on the instant application that would extend to the expiration date of the full statutory term of the prior patent, "as the term of said prior patent is presently shortened by any terminal disclaimer," in the event that said prior patent later:
- expires for failure to pay a maintenance fee;
- is held unenforceable;
- is found invalid by a court of competent jurisdiction;
- is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321;
- has all claims canceled by a reexamination certificate;
- is reissued; or
- is in any manner terminated prior to the expiration of its full statutory term as presently shortened by any terminal disclaimer.

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| (●) Terminal disclaimer fee under 37 CFR 1.20(d) is included with Electronic Terminal Disclaimer request.<br><br>( ) I certify, in accordance with 37 CFR 1.4(d)(4), that the terminal disclaimer fee under 37 CFR 1.20(d) required for this terminal disclaimer has already been paid in the above-identified application. | |
| Applicant claims the following fee status:<br><br>( ) Small Entity<br><br>( ) Micro Entity<br><br>(●) Regular Undiscounted<br><br>I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. | |
| THIS PORTION MUST BE COMPLETED BY THE SIGNATORY OR SIGNATORIES<br><br>I certify, in accordance with 37 CFR 1.4(d)(4) that I am:<br><br>(●) An attorney or agent registered to practice before the Patent and Trademark Office who is of record in this application<br><br>Registration Number 44282<br><br>( ) A sole inventor<br><br>( ) A joint inventor; I certify that I am authorized to sign this submission on behalf of all of the inventors as evidenced by the power of attorney in the application<br><br>( ) A joint inventor; all of whom are signing this request | |
| Signature | /M. Angela Parsons/ |
| Name | M. Angela Parsons |

*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner).
Form PTO/SB/96 may be used for making this certification. See MPEP § 324.

# 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:** | | | | |
| STATUTORY OR TERMINAL DISCLAIMER | 1814 | 1 | 160 | 160 |
| **Pages:** | | | | |
| **Claims:** | | | | |
| **Miscellaneous-Filing:** | | | | |
| **Petition:** | | | | |
| **Patent-Appeals-and-Interference:** | | | | |
| **Post-Allowance-and-Post-Issuance:** | | | | |

| Description | Fee Code | Quantity | Amount | Sub-Total in USD($) |
|---|---|---|---|---|
| **Extension-of-Time:** | | | | |
| **Miscellaneous:** | | | | |
| | | **Total in USD ($)** | | **160** |

Doc Code: DISQ.E.FILE
Document Description: Electronic Terminal Disclaimer – Approved

Application No.: 15573300

Filing Date: 10-Nov-2017

Applicant/Patent under Reexamination: Shankar

Electronic Terminal Disclaimer filed on October 16, 2019

☒ APPROVED

**This patent is subject to a terminal disclaimer**

☐ DISAPPROVED

Approved/Disapproved by: Electronic Terminal Disclaimer automatically approved by EFS-Web

U.S. Patent and Trademark Office

# Electronic Acknowledgement Receipt

| | |
|---|---|
| **EFS ID:** | 37475448 |
| **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:** | 16-OCT-2019 |
| **Filing Date:** | 10-NOV-2017 |
| **Time Stamp:** | 15:56:53 |
| **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 | $160 |
| RAM confirmation Number | E20190FF56516459 |
| Deposit Account | |
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## File Listing:

| Document Number | Document Description | File Name | File Size(Bytes)/ Message Digest | Multi Part /.zip | Pages (if appl.) |
|---|---|---|---|---|---|
| 1 | Terminal Disclaimer-Filed (Electronic) | eTerminal-Disclaimer.pdf | 33533<br>f6f4dca3511a429d0d4d89df4b341aef872f4b31 | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| 2 | Fee Worksheet (SB06) | fee-info.pdf | 30725<br>3739fb9a238cab112887fd2f50cbb44c0b095a22 | no | 2 |
| **Warnings:** | | | | | |
| **Information:** | | | | | |
| | | **Total Files Size (in bytes):** | 64258 | | |

**This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.**

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**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.**

Attorney Docket No.: 38767-0048US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | | | | | |
|---|---|---|---|---|---|
| First Named Inventor | : | Smita Shankar | Art Unit | : | 1636 |
| Application No. | : | 15/573,300 | Examiner | : | Nancy J. Leith |
| Filed | : | November 10, 2017 | Conf. No. | : | 4807 |
| Title | : | EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST | | | |

**Mail Stop Amendment**
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

RESPONSE TO OFFICE ACTION MAILED JULY 16, 2019

In response to the Office Action mailed July 16, 2019, please consider the following reply.

F&R Ref No.: 38767-0048US1

# 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.