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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

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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
5 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
10 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, acetonil 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-
15 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
20 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
25 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.

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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.

5 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,
10 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
15 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%
20 (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
25 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
30 a flavoring agent (e.g., a flavoring compound produced by combining an iron complex or

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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).

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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
5 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.

10 *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 Bgl1 (BioGrammatics, Inc.) was transformed with linearized plasmids, and stable integrants were selected by antibiotic resistance.

15 **TABLE 1**

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

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

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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 decolorized and retained the low temperature denaturation property.

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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

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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 decolorized and retained the low temperature denaturation property.

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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 H₂SO₄ 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 H₂SO₄ 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 H₂SO₄ 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.

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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%
5 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,
10 fatty, beefy, nutty, sulfur, metallic, buttery, sweet, savory, and umami.

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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

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Preparation of flavor infused fat replica

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

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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.

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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 MGI) 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)

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- 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)

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
10 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.
15 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
20 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

25 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®

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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, <i>E. coli</i>)	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

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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, <i>E. coli</i>)	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 1×10^{-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

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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.

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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
5 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,
10 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
15 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.
20 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

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

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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
5 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
10 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
15 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.

20

EXAMPLE 17

Preparation of meat dough containing lutein

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

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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
5 (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
10 KP26MIXER 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
15 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.

20

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
5 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
10 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
15 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.

20 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,

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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.

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Compounds	Peak Area		
	Lutein Amount	Norm	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			
3,3-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-ethylidihydro-			
2(5H)-Furanone, 5-ethyl-			

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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.

5

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
10 (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
15 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
20 described by GCO as being grainy and oxidized notes.

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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

5
10
15
20

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

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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

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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	Precursor mix 2
	(mM)	(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

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EXAMPLE 22**Preparation of bloody agar**

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

5

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.

15

EXAMPLE 23

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

20

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

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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 $2.5 \times 10^{-5} \%$, butyrolactone was added to a final concentration of $2.5 \times 10^{-8} \%$, and δ -tetradecalactone was added to a final concentration $5 \times 10^{-9} \%$. 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

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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 conglucinin 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.

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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%,
5 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
10 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
15 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.

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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

5 [§]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.

10

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.

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EXAMPLE 28**Assembly and cooking of burger**

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

5

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

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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

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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

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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
5 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
10 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
15 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
20 bitterness. The lactones also increased desired flavors of creamy, buttery, caramelized, fatty, fresh, and fruity.

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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^{-8}	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

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Butyrolactone	2.5×10^{-8}	5-Ethyl-4-hydroxy-2-methyl-3(2H)-furanone	2.5×10^{-6}	<i>γ-Octalactone</i>	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
<i>Delta-dodecalactone</i>	1×10^{-5}					60%	Buttery, fatty/oily, sl beefy	Grain
<i>4-hydroxy-2,5-dimethyl-3(2H)-furanone</i>	1×10^{-5}					60%	Sl sweet/caramelized	Grain
<i>γ-Octalactone</i>	1×10^{-5}					80%	Fatty, sweet/caramelized	Grain
Butyrolactone	2.5×10^{-8}					83%	Bright/sharp/bitter, sweet, fatty	Eggy

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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

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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-dimethylundecane, 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

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

EXAMPLE 34

5 **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.

10 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
15 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
20 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

25 **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
30 2% of the DI water, (ii) – (v) a slurry, cooked or not cooked, of either honeydew melon

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(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, α -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 panellists 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

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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.

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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

5 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.

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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.

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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

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(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:

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(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.

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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

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(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,

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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.

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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.

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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.

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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, bacteriochlorophyll, 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

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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-ethylidihydro-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.

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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-ethylidihydro-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.

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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

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group consisting of tetrahydro-6-methyl-2H-pyran-2-one, delta-octalactone, 5-ethylidihydro-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-methyldihydrofuran-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.

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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.

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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

26 May 2015

Date of mailing of the international search report

28 AUG 2015

Name and mailing address of the ISA/US

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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 Patent Application Fee Transmittal				
Application Number:		15678342		
Filing Date:		16-Aug-2017		
Title of Invention:		EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST		
First Named Inventor/Applicant Name:		Smita Shankar		
Filer:		M. Angela Parsons/Kirsten Neuman		
Attorney Docket Number:		38767-0048002		
Filed as Large Entity				
Filing Fees for Utility under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Miscellaneous:				
SUBMISSION- INFORMATION DISCLOSURE STMT	1806	1	240	240
Total in USD (\$)				240

Electronic Acknowledgement Receipt	
EFS ID:	33950861
Application Number:	15678342
International Application Number:	
Confirmation Number:	1018
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:	38767-0048002
Receipt Date:	09-OCT-2018
Filing Date:	16-AUG-2017
Time Stamp:	11:07:34
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$ 240
RAM confirmation Number	100918INTEFSW00019676061050
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	Transmittal.pdf	72083	no	1
			c7a5b378ba7d03266645535aed8f8025d1c20e8b		
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
2	Information Disclosure Statement (IDS) Form (SB08)	PTO_1449.pdf	128116	no	1
			23d7dd7f796a7d7ee46e607f965199cf58a18549		
Warnings:					
Information:					
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3	Foreign Reference	WO2015153666.pdf	4239746	no	95
			dd533a2627e72a51277fad8900957465bdb8dc06		
Warnings:					
Information:					
4	Non Patent Literature	Krainer_2015.pdf	812849	no	9
			f8856e181fb28566698835f8b87500245dd99b7e		
Warnings:					
Information:					
5	Non Patent Literature	Liu_2014.pdf	1340871	no	8
			a378a0613196d7c57d864fd6b409cf5496ff601		
Warnings:					
Information:					
6	Non Patent Literature	Fraser_2018.pdf	1590629	no	22
			f5dd39b1808c2a6b4d0a7d24d387bd3aa625f03d		

Warnings:					
Information:					
7	Fee Worksheet (SB06)	fee-info.pdf	30563	no	2
			dd08304a75ef36f8b5e12be6231c7470ebd159a2		
Warnings:					
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Total Files Size (in bytes):				8214857	
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. <u>New Applications Under 35 U.S.C. 111</u> 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. <u>National Stage of an International Application under 35 U.S.C. 371</u> 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. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.					



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/678,342	08/16/2017	Smita Shankar	38767-0048002	1018

26191	7590	10/11/2018
FISH & RICHARDSON P.C. (TC)		
PO BOX 1022		
MINNEAPOLIS, MN 55440-1022		

EXAMINER	
KETTER, JAMES S	

ART UNIT	PAPER NUMBER
1636	

NOTIFICATION DATE	DELIVERY MODE
10/11/2018	ELECTRONIC

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

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

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

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U.S. Patent and Trademark Office
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Alexandria, Virginia 22313-1450

APPLICATION NO./ CONTROL NO.	FILING DATE	FIRST NAMED INVENTOR/ PATENT IN REEXAMINATION	ATTORNEY DOCKET NO.
15/678,342	08/16/2017	Shankar et al.	38767-0048002

FISH & RICHARDSON P.C. (TC) PO BOX 1022 MINNEAPOLIS, MN 55440-1022		EXAMINER	
		JAMES S KETTER	
		ART UNIT	PAPER
		1636	20181006

DATE MAILED: _____

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Commissioner for Patents

Please find attached the corrected page 1 of the three-page IDS filed 8/16/2017.

/JAMES S KETTER/
Primary Examiner, Art Unit 1636

CORRECTED PAGE

Sheet 1 of 3

Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))	Attorney Docket No. 38767-0048002	Application No. 15/678,342
	Applicant Impossible Foods Inc.	
	Filing Date August 16, 2017	Group Art Unit

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.	4,683,195	07/28/1987	Mullis et al.			
	2.	4,683,202	07/28/1987	Mullis			
	3.	4,800,159	01/24/1989	Mullis et al.			
	4.	4,965,188	10/23/1990	Mullis et al.			
	5.	5,204,253	04/20/1993	Sanford et al.			
	6.	5,538,800	07/23/1996	Jin et al.			
	7.	6,013,863	01/11/2000	Lundquist et al.			
	8.	6,329,571	12/11/2001	Hiei			
	9.	2002/00194643 0194643 016675	12/19/2002	Merot Bertrand et al.			
	10.	2008/0166757	06/01/2008	Bron 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
	11.	WO 1998/012913	04/02/1998	WIPO				
	12.	WO 2004/057946	07/15/2004	WIPO				
	13.	WO 2004/099405	11/18/2004	WIPO				
	14.	WO 2012/083424	06/28/2012	WIPO				
	15.	WO 2013/010042	01/17/2013	WIPO				
	16.	WO 2014/110532	07/17/2014	WIPO				
	17.	WO 2014/110539	07/17/2014	WIPO				

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	18.	Chenna et al., "Multiple sequence alignment with the Clustal series of programs" Nucleic Acids Res., 31(13):3497-500 (2003)
	19.	Chiruvolu et al., "Recombinant protein production in an alcohol oxidase-defective strain of Pichia pastoris in fedbatch fermentations" Enzyme Microb. Technol., 21: 277-83 (1997)

Examiner Signature /JAMES S KETTER/	Date Considered ~~11/29/2017~~ 10/06/2018
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. /J.S.K/

Sheet 2 of 3

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 38767-0048002	Application No. 15/678,342
Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Impossible Foods Inc.	
		Filing Date August 16, 2017	Group Art Unit
(37 CFR §1.98(b))			

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	20.	Dayhoff et al., "A Model of Evolutionary Change in Proteins," Atlas of Protein Sequence and Structure, 5: 345-352 (1978)
	21.	GenBank Accession No. AB365355, "Candida boidinii TRM1 gene for Zn(II)2Cys6-type transcription factor Trm1, complete cds," dated January 24, 2008, 3 pages
	22.	GenBank Accession No. AB548760, "Candida boidinii TRM2 gene for C2H2-type transcription factor Trm2, complete cds," dated May 14, 2010, 3 pages
	23.	GenBank Accession No. ABD57365, "methanol expression regulator I [Komagataella pastoris]," dated March 4, 2006, 2 pages
	24.	GenBank Accession No. AEOI02000005, bases 858873 to 862352, "Ogataca parapolyomorpha DL-1 chr3, whole genome shotgun sequence," dated December 3, 2013, 186 pages
	25.	GenBank Accession No. BAF99700, "Zn(II)2Cys6-type transcription factor Trm1 [Candida boidinii]," dated January 23, 2008, 2 pages
	26.	GenBank Accession No. BAJ07608, "C2H2-type transcription factor Trm2 [Candida boidinii]," dated May 13, 2010, 2 pages
	27.	GenBank Accession No. DQ395124, "Pichia pastoris methanol expression regulator I gene, complete cds," dated March 4, 2006, 2 pages
	28.	GenBank Accession No. ESX01253, "Regulatory protein ADR1 [Ogataca parapolyomorpha DL-1]," dated December 2, 2013, 2 pages
	29.	GenBank Accession No. FJ752551, "Pichia pastoris dihydroxyacetone synthase 1 (DAS1) gene, complete cds" dated March 21, 2009, 2 pages
	30.	GenBank Accession No. X02425, "Hansenula polymorpha MOX gene for methanol oxidase" dated April 20, 1993, 3 pages
	31.	GenBank Accession No. YSAAOD1A, "Candida boidinii methanol oxidase (AOD1) gene, complete cds," dated April 27, 1993, 2 pages
	32.	Hargrove et al., "Characterization of recombinant soybean leghemoglobin a and apolar distal histidine mutants" J Mol. Biol. 266, 1032-1042 (1997)
	33.	Inan & Meagher, "Non-repressing carbon sources for alcohol oxidase (AOX1) promoter of Pichia pastoris" J. Biosci. Bioeng., 92:585-9 (2001)
	34.	International Search Report and Written Opinion in International Application No. PCT/US2014/055227, dated March 20, 2015, 20 pages
	35.	Jokipii-Lukkari et al., "Intrinsic non-symbiotic and truncated haemoglobins and heterologous Vitreoscilla haemoglobin expression in plants," Journal of Experimental Botany, 60(2):409-422 (January 2009)
	36.	Kranthi et al., "Identification of Mxr1p-binding sites in the promoters of genes encoding dihydroxyacetone synthase and peroxin 8 of the methylotrophic yeast Pichia pastoris" Yeast, 27:705-11 (2010)
	37.	Liachko & Dunham, "An autonomously replicating sequence for use in a wide range of budding yeasts" FEMS Yeast Res., 14:364-7 (2014)
	38.	Lloyd et al., "Targeted mutagenesis using zinc-finger nucleases in Arabidopsis" Proc. Natl. Acad. Sci. USA, 102:2232-2237 (2005)

Examiner Signature /JAMES S KETTER/	Date Considered 11/29/2017
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.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.S.K/

Substitute Disclosure Form

Sheet 3 of 3

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 38767-0048002	Application No. 15/678,342
Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))		Applicant Impossible Foods Inc.	
		Filing Date August 16, 2017	Group Art Unit

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	39.	Londer et al. "Production and preliminary characterization of a recombinant triheme cytochrome c(7) from Geobacter sulfurreducens in Escherichia coli." Biochem Biophys Act 1 1554(3): 202-211 ~~(2007)~~ (2002) .
	40.	Lutz et al., "A guide to choosing vectors for transformation of the plastid genome of higher plants" Plant Physiol. 145: 1201-1210 (2007)
	41.	Miele et al., "A GATA-type transcription factor regulates expression of the high-affinity iron uptake system in the methylotrophic yeast Pichia pastoris" Arch. Biochem. Biophys., 465: 172-9 (2007)
	42.	Nakagawa et al., "Alcohol oxidase hybrid oligomers formed in vivo and in vitro" Yeast, 15: 1223-30 (1999)
	43.	Qu et al., "Ectopic expression of the cotton non-symbiotic hemoglobin gene GhHb1 triggers defense responses and increases disease tolerance in Arabidopsis," Plant Cell Physiol., 47:1058-1068 (2006)
	44.	Raymond et al., "Development of the methylotrophic yeast Pichia methanolica for the expression of the 65 kilodalton isoform of human glutamate decarboxylase" Yeast, 14: 11-23 (1998)
	45.	Richards et al., "Construction of a GFP-BAR plasmid and its use for switchgrass transformation" Plant Cell. Rep. 20:48-20 54 (2001)
	46.	Sinagawa-Garcia et al., "Next generation synthetic vectors for transformation of the plastid genome of higher plants" Plant Afol Biol 70: 487-498 (2009)
	47.	Somleva et al., "Agrobacterium-Mediated Genetic Transformation of Switchgrass" Crop Sci. 42:2080-2087 (2002)
	48.	Supplementary European Search Report in International Application No. EP 14844701.4, dated January 27, 2017, 7 pages
	49.	Tanaka and Tanaka, "Tetrapyrrole biosynthesis in higher plants" Annu. Rev. Plant Biol. 58: 321-46 (2007)
	50.	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)
	51.	Townsend et al., "High-frequency modification of plant genes using engineered zinc-finger nucleases" Nature 459: 442-445 (2009)
	52.	UniprotKB Accession No. P02236, "Leghemoglobin C2," dated November 1, 1988, 9 pages
	53.	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)
	54.	Xie and Yang, "RNA-guided genome editing in plants using a CRISPR-Cas system" Mol. Plant 6: 1975-1983 (2013)
	55.	Zhang et al., "Transcription activator-like effector nucleases enable efficient plant genome engineering" Plant Physiology 161: 20-27 (2013)

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.S.K./

Examiner Signature /JAMES S KETTER/	Date Considered 11/29/2017
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

Attorney Docket No.: 38767-0048002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor :	Smita Shankar	Art Unit :	1636
Application No. :	15/678,342	Examiner :	James S. Ketter
Filed :	August 16, 2017	Conf. No. :	1018
Title :	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST		

MAIL STOP ISSUE FEE

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 after a Final Office Action or a Notice of Allowance, but before payment of the Issue Fee. Under 37 CFR § 1.97(e)(1), the undersigned certifies that each item of information contained in this Information Disclosure Statement was first cited in a Communication from a foreign Patent Office in a counterpart foreign application not more than three months prior to the filing of this Information Disclosure Statement.

Please apply all charges applicable under 37 C.F.R. § 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: /November 9, 2018/

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

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

61784443.doc

Sheet 1 of 1

Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))	Attorney Docket No. 38767-0048002	Application No. 15/678,342
	Applicant Impossible Foods Inc.	
	Filing Date August 16, 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	No

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	1.	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
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 Patent Application Fee Transmittal				
Application Number:		15678342		
Filing Date:		16-Aug-2017		
Title of Invention:		EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST		
First Named Inventor/Applicant Name:		Smita Shankar		
Filer:		M. Angela Parsons/Kirsten Neuman		
Attorney Docket Number:		38767-0048002		
Filed as Large Entity				
Filing Fees for Utility under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Miscellaneous:				
SUBMISSION- INFORMATION DISCLOSURE STMT	1806	1	240	240
Total in USD (\$)				240

Electronic Acknowledgement Receipt	
EFS ID:	34259138
Application Number:	15678342
International Application Number:	
Confirmation Number:	1018
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:	38767-0048002
Receipt Date:	09-NOV-2018
Filing Date:	16-AUG-2017
Time Stamp:	09:59:09
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$ 240
RAM confirmation Number	110918INTEFSW00007760061050
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Information Disclosure Statement (IDS) Form (SB08)	Transmittal.pdf	72016	no	1
			4ca1f16c6bc5dec48d011d13059b36f5a22b73dc		
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
2	Information Disclosure Statement (IDS) Form (SB08)	PTO_1449.pdf	123897	no	1
			e36fd9f25f908b5bdfa46b4a2fb6fad313c4d7f6		
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Information:					
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3	Non Patent Literature	Proulx_2006.pdf	3780409	no	5
			14ad119e38b88f96df17a78c360e7f3ea1bb359d		
Warnings:					
Information:					
4	Fee Worksheet (SB06)	fee-info.pdf	30562	no	2
			ba5eae94734993b4c3c6bcbf8e8a6c3f9e704bd5		
Warnings:					
Information:					
Total Files Size (in bytes):			4006884		

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

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PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web

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Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22314-1450

By fax, send to: (571) 273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

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26191 7590 8/15/2018

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P.O. BOX 1022
MINNEAPOLIS, MN 55440-1022

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)

(Signature)

(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/678,342	08/16/2017	Smita Shankar	38767-0048002	1018

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

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	LARGE	\$1000	\$0		\$1000	11/15/2018

EXAMINER	ART UNIT	CLASS-SUBCLASS
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KETTER, JAMES S.

1636

435-254230

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

- ☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
- ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

- (1) The names of up to 3 registered patent attorneys or agents OR, alternatively, 1 Fish & Richardson P.C.
- (2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed. 2 _____
- 3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

Impossible Foods Inc.

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Redwood City, CA

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government4a. Fees Submitted: ☒ Issue Fee ☐ Publication Fee (if required) ☐ Advance Order - # of Copies

4b. Method of Payment (Please first reapply any previously paid fee shown above):

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5. Change of Entity Status (from status indicated above)

☐ Applicant certifying micro entity status. See 37 CFR 1.29☐ Applicant asserting small entity status. See 37 CFR 1.27.☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid Certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

NOTE: If the application was previously under micro entity status, checking this box will be taken as a notification of loss of entitlement to micro entity status.

NOTE: Checking this box will be taken as a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature /M. Angela Parsons/Date /November 13, 2018/Typed or printed name M. Angela Parsons, Ph.D.Registration No. 44,282

PTOL-85 Part B (08-18). Approved for use through 1/31/2020

OMB 0651-0033

U.S. Patent and Trademark Office, U.S. DEPARTMENT OF COMMERCE

61747696.doc

Electronic Patent Application Fee Transmittal				
Application Number:		15678342		
Filing Date:		16-Aug-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:		38767-0048002		
Filed as Large Entity				
Filing Fees for Utility under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
UTILITY APPL ISSUE FEE	1501	1	1000	1000

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Extension-of-Time:				
Miscellaneous:				
Total in USD (\$)				1000

Electronic Acknowledgement Receipt	
EFS ID:	34286725
Application Number:	15678342
International Application Number:	
Confirmation Number:	1018
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/Alysha Claflin
Filer Authorized By:	M. Angela Parsons
Attorney Docket Number:	38767-0048002
Receipt Date:	13-NOV-2018
Filing Date:	16-AUG-2017
Time Stamp:	11:58:06
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$1000
RAM confirmation Number	111318INTEFSW00020440061050
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Post Allowance Communication - Incoming	Response.pdf	71362	no	1
			f00da6a263c424f584b104479e578f75359d1364		
Warnings:					
Information:					
2	Issue Fee Payment (PTO-85B)	PartB.pdf	160962	no	1
			1ac3097bdc70cb529c599ccfa5193ad0fc73494		
Warnings:					
Information:					
3	Fee Worksheet (SB06)	fee-info.pdf	30469	no	2
			26d4a3987ea2e6be11317e5b6d903632a58b7e5b		
Warnings:					
Information:					
Total Files Size (in bytes):			262793		
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. <u>New Applications Under 35 U.S.C. 111</u> 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. <u>National Stage of an International Application under 35 U.S.C. 371</u> 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. <u>New International Application Filed with the USPTO as a Receiving Office</u> 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.
15/678,342	08/16/2017	Smita Shankar	38767-0048002	1018

26191	7590	11/14/2018
FISH & RICHARDSON P.C. (TC)		
PO BOX 1022		
MINNEAPOLIS, MN 55440-1022		

EXAMINER	
KETTER, JAMES S	

ART UNIT	PAPER NUMBER
1636	

NOTIFICATION DATE	DELIVERY MODE
11/14/2018	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



UNITED STATES DEPARTMENT OF COMMERCE

U.S. Patent and Trademark Office

Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450

APPLICATION NO./ CONTROL NO.	FILING DATE	FIRST NAMED INVENTOR/ PATENT IN REEXAMINATION	ATTORNEY DOCKET NO.
15/678,342	08/16/2017	Shankar et al.	38767-0048002

FISH & RICHARDSON P.C. (TC) PO BOX 1022 MINNEAPOLIS, MN 55440-1022		EXAMINER	
		JAMES S KETTER	
		ART UNIT	PAPER
		1636	20181108

DATE MAILED: _____

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

Commissioner for Patents

Please find attached the signed IDS filed 10/19/2018, as well as the corrected IDS filed 12/4/2017.

/JAMES S KETTER/
Primary Examiner, Art Unit 1636

Response to Rule 312 Communication	Application No. 15/678,342	Applicant(s) Shankar et al.	
	Examiner JAMES S KETTER	Art Unit 1636	AIA Status Yes
-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address -- 1. ☐ The amendment filed on _____ under 37 CFR 1.312 has been considered, and has been: a) ☐ entered. b) ☐ entered as directed to matters of form not affecting the scope of the invention. c) ☐ disapproved because the amendment was filed after the payment of the issue fee. Any amendment filed after the date the issue fee is paid must be accompanied by a petition under 37 CFR 1.313(c)(1) and the required fee to withdraw the application from issue. d) ☐ disapproved. See explanation below. e) ☐ entered in part. See explanation below. <u>The IDS filed 10/9/2018 and the corrected IDS filed 12/4/2017 are attached.</u>			
/JAMES S KETTER/ Primary Examiner, Art Unit 1636			

Sheet 1 of 1

Substitute Disclosure Form Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 38767-0048002	Application No. 15/678,342
	Applicant Impossible Foods Inc.		
	Filing Date August 16, 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	No
	1.	WO 2015/153666	10/8/2015	WIPO				

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

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.S.K/

Examiner Signature /JAMES S KETTER/	Date Considered 11/08/2018
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

CORRECTED PAGE

Sheet 1 of 3

Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))	Attorney Docket No. 38767-0048002	Application No. 15/678,342
	Applicant Impossible Foods Inc.	
	Filing Date August 16, 2017	Group Art Unit

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.	4,683,195	07/28/1987	Mullis et al.			
	2.	4,683,202	07/28/1987	Mullis			
	3.	4,800,159	01/24/1989	Mullis et al.			
	4.	4,965,188	10/23/1990	Mullis et al.			
	5.	5,204,253	04/20/1993	Sanford et al.			
	6.	5,538,800	07/23/1996	Jin et al.			
	7.	6,013,863	01/11/2000	Lundquist et al.			
	8.	6,329,571	12/11/2001	Hiei			
	9.	2002/00194643 0194643 0166757	12/19/2002	Merot Bertrand et al.			
	10.	2008/0166757	06/01/2008	Bron 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
	11.	WO 1998/012913	04/02/1998	WIPO				
	12.	WO 2004/057946	07/15/2004	WIPO				
	13.	WO 2004/099405	11/18/2004	WIPO				
	14.	WO 2012/083424	06/28/2012	WIPO				
	15.	WO 2013/010042	01/17/2013	WIPO				
	16.	WO 2014/110532	07/17/2014	WIPO				
	17.	WO 2014/110539	07/17/2014	WIPO				

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	18.	Chenna et al., "Multiple sequence alignment with the Clustal series of programs" Nucleic Acids Res., 31(13):3497-500 (2003)
	19.	Chiruvolu et al., "Recombinant protein production in an alcohol oxidase-defective strain of Pichia pastoris in fedbatch fermentations" Enzyme Microb. Technol., 21: 277-83 (1997)

Examiner Signature /JAMES S KETTER/	Date Considered ~~11/29/2017~~ 10/06/2018
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. /J.S.K/

Sheet 2 of 3

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 38767-0048002	Application No. 15/678,342
Information Disclosure Statement by Applicant (Use several sheets if necessary)		Applicant Impossible Foods Inc.	
		Filing Date August 16, 2017	Group Art Unit
(37 CFR §1.98(b))			

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	20.	Dayhoff et al., "A Model of Evolutionary Change in Proteins," Atlas of Protein Sequence and Structure, 5: 345-352 (1978)
	21.	GenBank Accession No. AB365355, "Candida boidinii TRM1 gene for Zn(II)2Cys6-type transcription factor Trm1, complete cds," dated January 24, 2008, 3 pages
	22.	GenBank Accession No. AB548760, "Candida boidinii TRM2 gene for C2H2-type transcription factor Trm2, complete cds," dated May 14, 2010, 3 pages
	23.	GenBank Accession No. ABD57365, "methanol expression regulator I [Komagataella pastoris]," dated March 4, 2006, 2 pages
	24.	GenBank Accession No. AEOI02000005, bases 858873 to 862352, "Ogataca parapolyomorpha DL-1 chr3, whole genome shotgun sequence," dated December 3, 2013, 186 pages
	25.	GenBank Accession No. BAF99700, "Zn(II)2Cys6-type transcription factor Trm1 [Candida boidinii]," dated January 23, 2008, 2 pages
	26.	GenBank Accession No. BAJ07608, "C2H2-type transcription factor Trm2 [Candida boidinii]," dated May 13, 2010, 2 pages
	27.	GenBank Accession No. DQ395124, "Pichia pastoris methanol expression regulator I gene, complete cds," dated March 4, 2006, 2 pages
	28.	GenBank Accession No. ESX01253, "Regulatory protein ADR1 [Ogataca parapolyomorpha DL-1]," dated December 2, 2013, 2 pages
	29.	GenBank Accession No. FJ752551, "Pichia pastoris dihydroxyacetone synthase 1 (DAS1) gene, complete cds" dated March 21, 2009, 2 pages
	30.	GenBank Accession No. X02425, "Hansenula polymorpha MOX gene for methanol oxidase" dated April 20, 1993, 3 pages
	31.	GenBank Accession No. YSAAOD1A, "Candida boidinii methanol oxidase (AOD1) gene, complete cds," dated April 27, 1993, 2 pages
	32.	Hargrove et al., "Characterization of recombinant soybean leghemoglobin a and apolar distal histidine mutants" J Mol. Biol. 266, 1032-1042 (1997)
	33.	Inan & Meagher, "Non-repressing carbon sources for alcohol oxidase (AOX1) promoter of Pichia pastoris" J. Biosci. Bioeng., 92:585-9 (2001)
	34.	International Search Report and Written Opinion in International Application No. PCT/US2014/055227, dated March 20, 2015, 20 pages
	35.	Jokipii-Lukkari et al., "Intrinsic non-symbiotic and truncated haemoglobins and heterologous Vitreoscilla haemoglobin expression in plants," Journal of Experimental Botany, 60(2):409-422 (January 2009)
	36.	Kranthi et al., "Identification of Mxr1p-binding sites in the promoters of genes encoding dihydroxyacetone synthase and peroxin 8 of the methylotrophic yeast Pichia pastoris" Yeast, 27:705-11 (2010)
	37.	Liachko & Dunham, "An autonomously replicating sequence for use in a wide range of budding yeasts" FEMS Yeast Res., 14:364-7 (2014)
	38.	Lloyd et al., "Targeted mutagenesis using zinc-finger nucleases in Arabidopsis" Proc. Natl. Acad. Sci. USA, 102:2232-2237 (2005)

Examiner Signature /JAMES S KETTER/	Date Considered 11/29/2017
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.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.S.K/

Substitute Disclosure Form

Sheet 3 of 3

Substitute Disclosure Form	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 38767-0048002	Application No. 15/678,342
Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))		Applicant Impossible Foods Inc.	
		Filing Date August 16, 2017	Group Art Unit

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	39.	Londer et al. "Production and preliminary characterization of a recombinant triheme cytochrome c(7) from Geobacter sulfurreducens in Escherichia coli." Biochem Biophys Act 1 1554(3): 202-211 ~~(200)~~ (2002) .
	40.	Lutz et al., "A guide to choosing vectors for transformation of the plastid genome of higher plants" Plant Physiol. 145: 1201-1210 (2007)
	41.	Miele et al., "A GATA-type transcription factor regulates expression of the high-affinity iron uptake system in the methylotrophic yeast Pichia pastoris" Arch. Biochem. Biophys., 465: 172-9 (2007)
	42.	Nakagawa et al., "Alcohol oxidase hybrid oligomers formed in vivo and in vitro" Yeast, 15: 1223-30 (1999)
	43.	Qu et al., "Ectopic expression of the cotton non-symbiotic hemoglobin gene GhHb1 triggers defense responses and increases disease tolerance in Arabidopsis," Plant Cell Physiol., 47:1058-1068 (2006)
	44.	Raymond et al., "Development of the methylotrophic yeast Pichia methanolica for the expression of the 65 kilodalton isoform of human glutamate decarboxylase" Yeast, 14: 11-23 (1998)
	45.	Richards et al., "Construction of a GFP-BAR plasmid and its use for switchgrass transformation" Plant Cell. Rep. 20:48-20 54 (2001)
	46.	Sinagawa-Garcia et al., "Next generation synthetic vectors for transformation of the plastid genome of higher plants" Plant Afol Biol 70: 487-498 (2009)
	47.	Somleva et al., "Agrobacterium-Mediated Genetic Transformation of Switchgrass" Crop Sci. 42:2080-2087 (2002)
	48.	Supplementary European Search Report in International Application No. EP 14844701.4, dated January 27, 2017, 7 pages
	49.	Tanaka and Tanaka, "Tetrapyrrole biosynthesis in higher plants" Annu. Rev. Plant Biol. 58: 321-46 (2007)
	50.	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)
	51.	Townsend et al., "High-frequency modification of plant genes using engineered zinc-finger nucleases" Nature 459: 442-445 (2009)
	52.	UniprotKB Accession No. P02236, "Leghemoglobin C2," dated November 1, 1988, 9 pages
	53.	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)
	54.	Xie and Yang, "RNA-guided genome editing in plants using a CRISPR-Cas system" Mol. Plant 6: 1975-1983 (2013)
	55.	Zhang et al., "Transcription activator-like effector nucleases enable efficient plant genome engineering" Plant Physiology 161: 20-27 (2013)

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /J.S.K./

Examiner Signature /JAMES S KETTER/	Date Considered 11/29/2017
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

PTO/SB/30 (11-17)

Approved for use through 11/30/2020. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

**Request
for
Continued Examination (RCE)
Transmittal**Address to:
Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Application Number	15/678,342
Filing Date	August 16, 2017
First Named Inventor	Smita Shankar
Art Unit	1636
Examiner Name	James S. Ketter
Attorney Docket Number	38767-0048002

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.

Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO) on page 2.

1. **Submission required under 37 CFR 1.114** Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

- a. ☐ Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

- i. ☐ Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____

- ii. ☐ Other _____

- b. ☒ Enclosed

- i. ☐ Amendment/Reply

- iii. ☒ Information Disclosure Statement (IDS)

- ii. ☐ Affidavit(s)/ Declaration(s)

- iv. ☐ Other _____

2. **Miscellaneous**

Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a

- a. ☐ period of _____ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

- b. ☐ Other _____

3. **Fees**

The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.

The Director is hereby authorized to charge the following fees any underpayment of fees, or credit any overpayments to

- a. ☒ Deposit Account No. 06-1050

- i. ☒ RCE fee required under 37 CFR 1.17(e)

- ii. ☐ Extension of time fee (37 CFR 1.136 and 1.17)

- iii. ☒ Other any deficiencies

- b. ☐ Check in the amount of \$ _____ enclosed

- c. ☐ Payment by credit card (Form PTO-2038 enclosed)

- d. ☒ Payment by EFS-Web

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.**SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED**

Signature	/M. Angela Parsons/	Date	/December 6, 2018/
Name (Print/Type)	M. Angela Parsons, Ph.D.	Registration No.	44,282

CERTIFICATE OF MAILING OR TRANSMISSION

I hereby certify that this correspondence is being EFS-Web transmitted to the United States Patent and Trademark Office (USPTO), deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop RCE, Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450 or facsimile transmitted to the USPTO on the date shown below.

Signature	
Name (Print/Type)	
Date	

This collection of information is required by 37 CFR 1.114. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Mail Stop RCE, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Sheet 1 of 1

Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))	Attorney Docket No. 38767-0048002	Application No. 15/678,342
	Applicant Impossible Foods Inc.	
	Filing Date August 16, 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.	2011/0287467	11/24/2011	Crane 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
	2.	JPA 2008017774	1/31/2008	JP				
	3.	WO 2008/090211	7/31/2008	WIPO				

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	4.	Sasano et al., "Trm2p-dependent depression is essential for methanol-specific gene activation in the methylotrophic yeast <i>Candida boidinii</i> ", FEMS Yeast Res., Vol. 10, 535-544, 2010

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



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

Electronic Patent Application Fee Transmittal				
Application Number:		15678342		
Filing Date:		16-Aug-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:		38767-0048002		
Filed as Large Entity				
Filing Fees for Utility under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
PETITION FEE- 37 CFR 1.17(H) (GROUP III)	1464	1	140	140
RCE- 1ST REQUEST	1801	1	1300	1300
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Miscellaneous:				
Total in USD (\$)				1440



UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

Decision Date : December 7, 2018

In re Application of :

Smita Shankar

DECISION ON PETITION

UNDER CFR 1.313(c)(2)

Application No : 15678342

Filed : 16-Aug-2017

Attorney Docket No : 38767-0048002

This is an electronic decision on the petition under 37 CFR 1.313(c)(2), filed December 7, 2018, to withdraw the above-identified application from issue after payment of the issue fee.

The petition is **GRANTED**.

The above-identified application is withdrawn from issue for consideration of a submission under 37 CFR 1.114 (request for continued examination). See 37 CFR 1.313(c)(2).

Petitioner is advised that the issue fee paid in this application cannot be refunded. If, however, this application is again allowed, petitioner may request that it be applied towards the issue fee required by the new Notice of Allowance.

Telephone inquiries concerning this decision should be directed to the Patent Electronic Business Center (EBC) at 866-217-9197.

This application file is being referred to Technology Center AU 1636 for processing of the request for continuing examination under 37 CFR 1.114.

Office of Petitions

Electronic Acknowledgement Receipt	
EFS ID:	34502371
Application Number:	15678342
International Application Number:	
Confirmation Number:	1018
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:	38767-0048002
Receipt Date:	07-DEC-2018
Filing Date:	16-AUG-2017
Time Stamp:	11:45:10
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$ 1440
RAM confirmation Number	120718INTEFSW00012370061050
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Petition automatically granted by EFS	petition-request.pdf	31552	no	2
			f5e39f8b84b2af4f58214b26441ccc2decababf		
Warnings:					
Information:					
2	Quick Path Information Disclosure Statement	QPIDSCertReq.pdf	107009	no	1
			7756cd55ba9030d2491515bd65527bee9a0d1b40		
Warnings:					
Information:					
3	Request for Continued Examination (RCE)	RCE.pdf	117215	no	1
			4be768fa0a0ed1fa3beb8223ff96f92162801953		
Warnings:					
This is not a USPTO supplied RCE SB30 form.					
Information:					
4	Quick Path Information Disclosure Statement	ids.pdf	74691	no	2
			3db788bdc44f303e8b28098fe22869a1bd10a7b9		
Warnings:					
Information:					
5	Information Disclosure Statement (IDS) Form (SB08)	PTO1449.pdf	123983	no	1
			8a30f908fb209b61034788210c2a4681e62338b1		
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
6	Foreign Reference	JP2008017774.pdf	76264	no	1
			317bacb6ade93740452e9bf6143e8cc2111622e7		

Warnings:					
Information:					
7	Foreign Reference	WO2008090211A1_Part1.pdf	25900050 fd39443b2d4a0ce112eaa1d5cf8a3a6214c2d0b9	no	25
Warnings:					
Information:					
8	Foreign Reference	WO2008090211A1_Part2.pdf	4355691 29e06cbca97b56b69aa49d98cd735d4518ebaa69	no	5
Warnings:					
Information:					
9	Non Patent Literature	Sasano_2010.pdf	233342 584a429b0700b39d15c4b5f8a6a02f786c17937d	no	10
Warnings:					
Information:					
10	Fee Worksheet (SB06)	fee-info.pdf	32225 d033d7aad4e80ae4458eb0098f2a6fe997ba5169	no	2
Warnings:					
Information:					
Total Files Size (in bytes):			31052022		
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. <u>New Applications Under 35 U.S.C. 111</u> 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. <u>National Stage of an International Application under 35 U.S.C. 371</u> 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. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.					

Doc Code: PET.AUTO Document Description: Petition automatically granted by EFS-Web		PTO/SB/140 U.S. Patent and Trademark Office Department of Commerce
Electronic Petition Request	PETITION TO WITHDRAW AN APPLICATION FROM ISSUE AFTER PAYMENT OF THE ISSUE FEE UNDER 37 CFR 1.313(c)	
Application Number	15678342	
Filing Date	16-Aug-2017	
First Named Inventor	Smita Shankar	
Art Unit	1636	
Examiner Name	JAMES KETTER	
Attorney Docket Number	38767-0048002	
Title	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST	
An application may be withdrawn from issue for further action upon petition by the applicant. To request that the Office withdraw an application from issue, applicant must file a petition under this section including the fee set forth in § 1.17(h) and a showing of good and sufficient reasons why withdrawal of the application from issue is necessary. APPLICANT HEREBY PETITIONS TO WITHDRAW THIS APPLICATION FROM ISSUE UNDER 37 CFR 1.313(c). A grantable petition requires the following items: (1) Petition fee; and (2) One of the following reasons: (a) Unpatentability of one or more claims, which must be accompanied by an unequivocal statement that one or more claims are unpatentable, an amendment to such claim or claims, and an explanation as to how the amendment causes such claim or claims to be patentable; (b) Consideration of a request for continued examination in compliance with § 1.114 (for a utility or plant application only); or (c) Express abandonment of the application. Such express abandonment may be in favor of a continuing application, but not a CPA under 37 CFR 1.53(d).		
Petition Fee		
☐ Small Entity		
☐ Micro Entity		
☒ Regular Undiscounted		
Reason for withdrawal from issue		

☐ One or more claims are unpatentable ☒ Consideration of a request for continued examination (RCE) (List of Required Documents and Fees) ☐ Applicant hereby expressly abandons the instant application (any attorney/agent signing for this reason must have power of attorney pursuant to 37 CFR 1.32(b)).	
RCE request, submission, and fee. ☐ I certify, in accordance with 37 CFR 1.4(d)(4) that : ☐ The RCE request ,submission, and fee have already been filed in the above-identified application on ☒ Are attached.	
THIS PORTION MUST BE COMPLETED BY THE SIGNATORY OR SIGNATORIES I certify, in accordance with 37 CFR 1.4(d)(4) that I am: ☒ An attorney or agent registered to practice before the Patent and Trademark Office who has been given power of attorney in this application. ☐ An attorney or agent registered to practice before the Patent and Trademark Office, acting in a representative capacity. ☐ A sole inventor ☐ 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 ☐ A joint inventor; all of whom are signing this e-petition	
Signature	/M. Angela Parsons/
Name	M. Angela Parsons
Registration Number	44282

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
31 July 2008 (31.07.2008)

PCT

(10) International Publication Number
WO 2008/090211 A1

(51) International Patent Classification:

C12N 15/81 (2006.01) C07K 14/39 (2006.01)
C12N 15/31 (2006.01) C12P 21/02 (2006.01)

(74) Common Representative: NOVOZYMES A/S; Patents,
Krogshoejvej 36, DK-2880 Bagsvaerd (DK).

(21) International Application Number:

PCT/EP2008/050870

(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA,
CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE,
EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID,
IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC,
LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN,
MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH,
PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV,
SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN,
ZA, ZM, ZW.

(22) International Filing Date: 25 January 2008 (25.01.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

PA 2007 00122 26 January 2007 (26.01.2007) DK

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL,
NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG,
CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(71) Applicant (for all designated States except US):
NOVOZYMES A/S [DK/DK]; Krogshoejvej 36,
DK-2880 Bagsvaerd (DK).

(72) Inventors; and

(75) Inventors/Applicants (for US only): TSUTSUMI,
Noriko [JP/JP]; 3-2-16, Higashisugano, Ichikawa, Chiba
272-0823 (JP). TERUI, Yuji [JP/JP]; 16-5 Hakuraku,
Kanagawa, Yokohama, Kanagawa 221-0065 (JP). TAK-
AGI, Shinobu [JP/JP]; Maehara-nishi 1-31-1-708,
Funabashi, Chiba 274-0825 (JP). KONG, XiangYu
[CN/CN]; 6 Feng Qiao Lu, Building 3, Unit 1, Room
1906, Beijing 100070 (CN).

Published:

— with international search report

— with sequence listing part of description published sepa-
rately in electronic form and available upon request from
the International Bureau



WO 2008/090211 A1

(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.

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TITLE: METHOD FOR METHANOL INDEPENDENT INDUCTION FROM METHANOL INDUCIBLE PROMOTERS IN PICHIA.

Reference to sequence listing

- 5 This application contains a Sequence Listing in computer readable form. The computer readable form is incorporated herein by reference.

FIELD OF THE INVENTION

- 10 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

- 15 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.
- 20 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.

- 25 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).

- 30 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

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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-

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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.

5 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): 883-10 897).

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.

20 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.

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In another embodiment the expression of the positive regulator is controlled from an inducible promoter which is not methanol inducible.

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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.

5 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.

10 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.

15 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".

20 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.

25 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.

30 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.

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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:

5 Sequence 1: ACMSHTWGER-NL
 | | | | |
Sequence 2: HGWGEDANLAMNPS

10 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.

15 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).

20 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).

25 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).

30 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).

30 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

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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.

5 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 , ^3H , ^{35}S , biotin, or avidin).

10 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 immo-
15 bilized 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
20 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,
25 200 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
30 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).

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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.

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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-

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motor 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

5

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.

15

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.

20

Medium and assay

RD medium (1M sorbitol, 2% dextrose, 1.34% yeast nitrogen base, 4×10^{-5} % biotin, 0.005% L-glutamic acid, L-methionine, 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/mL zeocin 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 2 days 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.

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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.

- 5 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 μ mol inorganic phosphate per minute.

10

Phytase plate assay:

20 μ L 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.

15

PCR conditions

20

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 μ g of template DNA. Reaction conditions are, for example;

Temp (°C)	Time	Cycle
95	5 min	1
95	15 sec	35
48	30 sec	
68	3.5 min	
68	10 min	1

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Example 1: Constitutive expression of Prm1 in a strain expressing a phytase gene under DAS1 promoter controlCloning of TEF1 promoter and construction of pNo-TP10

- 5 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 cerevisiae*, *Candida albicans*, and *Hanseniaspora uvarum*, the following degenerated primes were designed.

TEF1(f); 5'-ttaaartaygcntgggt-3' (protein; FKYAWV) SEQ ID NO: 6 and 7

- 10 TEF5(r); 5'-arytgytcrtrgtgcatytc-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	30
55° C	1 min	
72° C	3 min	

15

- 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
- 20 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'-ggagtctcgaactccacaa-3' SEQ ID NO: 11

- 25 TEF-TSP3; 5'-agcgatgtcgatggtgatac-3' SEQ ID NO: 12

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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	5 min	1
42° C	1 min	
72° C	2 min	
94° C	30 sec	20
55° C	30 sec	
72° C	100 sec	
72° C	7 min	1

5

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	30 sec	30
58° C	30 sec	
72° C	100 sec	
72° C	7 min	1

10

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:

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temp	time	cycle
94° C	3 min	1
94° C	30 sec	30
65° C	30 sec	
72° C	100 sec	
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'- tacagggcgcggtgggatatcggatccagctcatctagga-3' (BamHI is underlined) (SEQ ID NO: 13)

primer2; 5'- tgaagatggatgggaatctcatatggttggcgaataactaaaatgtatgt-3' (SEQ ID NO: 14)

primer3; 5'- acatacatttagttattcgccaaccatatgagattcccatccatcttca-3' (SEQ ID NO: 15)

primer4; 5'- taattcgcgccgcctaggaaattctactcggtgacagcgccactcggg-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 fidelity mixture (Roche), 1X Expand high fidelity buffer (Roche), and 100 ng of genomic DNA. The PCR program was as shown below.

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temp	time	cycle
94° C	2 min	1
94° C	10 sec	11
50° C	30 sec	
68° C	3 min	
94° C	10 sec	20
50° C	30 sec	
68° C	3 min+20 sec/cycle	
68° C	7 min	1

- 5 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 fidelity (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	10 sec	11
58° C	30 sec	
68° C	3 min	
94° C	10 sec	20
58° C	30 sec	
68° C	3 min+20 sec/cycle	
68° C	7 min	1

- 10 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

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and the phytase gene was ligated with pGAPZ α 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 boidinii* (EMBL: AF086822). Around 1kb of 5'-untranslated 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'-ctcatatgttttgatgtttgatagttga-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'-actatttcgaaatgcctcctaaacatcggtg-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 μ L of reaction including 1X LA PCR buffer II (TAKARA), 2.5U of LA taq (TAKARA), 2.5mM MgCl₂, 2.5mM dNTP, 1 microM of each primer, 100 ng of genomic DNA with the following program:

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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 Sall restriction sites, resulting in pGPrm. The plasmid pGPrm carries the Prm1 gene under GAP promoter control and a zeo-
cin 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

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Example 2: Constitutive expression of Prm1 in the strain expressing a phytase gene under AOX1 promoter

Media:

5 MD (1.34% YNB, $4 \times 10^{-5}\%$ biotin, 2% dextrose)

BMSY (1% yeast extract, 2% peptone, 100mM potassium phosphate buffer, pH 6.0, 1.34% YNB, $4 \times 10^{-5}\%$ biotin, 1% sorbitol)

Vector:

10 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

15 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	CATTCTGTTCTCTCTCTTTTCCAAGGAAACACCTTC (SEQ ID NO: 26)
OAPhy-F	ggaaaagagaGAGGAACAGAATGGAATGAAGTTGG (SEQ ID NO: 27)
OPhy-Ca	AATT CTT ACTCGGTGACAGCGCACTC (SEQ ID NO: 28)
OPhy-Cb	CTT ACTCGGTGACAGCGCACTC (SEQ ID NO: 29)

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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
5 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
10 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
15 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-
20 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 ma-
25 ture 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 2nd 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
30 into pPIC-NoT vector at *Bam*HI and *Eco*RI 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

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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

5 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.

10 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

15

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:

20

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)

25

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)

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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);

- 5 JP13/HIS4-forward-NY: 5' CCCTGTATTAATCTCCCTTCATCAGAATTGGTTAATTGGTTG 3'
(SEQ ID NO: 36); and
JP15/HIS4-rev-NY: 5' TCTGGCGCAGTGGTCCAATCATCGATAAGCTTTAATGCGG 3'
(SEQ ID NO: 37)

- 10 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.

- 15 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:

- 20 A) A PCR that would give a product of 704 bp in case PRM1 was NOT deleted – us-
ing 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)

- 25 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);
30 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:

- 35 JP32/PRM1-3'-rev-test2: 5' CTCCCTCTCCAGCTGCTTCG 3' (SEQ ID NO: 42); and
JP34/HIS4-forw-test4: 5' CGGTGCCTGACTGCGTTAGC 3' (SEQ ID NO: 43)

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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.

5

A Prm1 gene deletion mutant of GS115, PFJo435, could not grow on MM (1.34% YNB, 4×10^{-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.

10

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

15 DAS40 and pNo-DP2 are described before in example 1.
Construction of pNori12 are shown below.

Construction of pNori12

20 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'-ataaatttgacagttaagtcgacctctgtaaattaattgataatttcaa-3' (SEQ ID NO: 44)

25 pr137; 5'-caatgatgatgatgatgatggtcgacgtttaaacttaataaaaagggaatttacaagcc-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 (Roche), 1X Expand high fidelity buffer (Roche), and 100ng of genomic DNA of GS115. The PCR program was as described below.

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temp	time	cycle
94° C	2 min	1
94° C	10 sec	11
55° C	30 sec	
68° C	3 min	
94° C	10 sec	20
55° C	30 sec	
68° C	3 min+20 sec/cycle	
68° C	7 min	1

The amplified 500bp of fragment was inserted in the Sall site of pGPm using In-Fusion PCR cloning kit (Clontech) to fuse it with the ORF of Pm, resulting in pNori11. In order to make the expression plasmid which has GAP-Pm 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 Pm1 gene under GAP promoter control, phytase gene under DAS promoter control, and a zeocin resistant gene as the selection marker.

10 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	ATTGAACAACCTATTTTCGAAACCATGAGCAATCTACCCCC (SEQ ID NO: 46)
Mxr-R	GAGTTTTTGTCTAGAAATGACACCACCATCTAGTCGG (SEQ ID NO: 47)

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PCR conditions

Temp (°C)	Time	Cycles
95	5 min	1
95	15 sec	35
48	30 sec	
68	3.5 min	
68	10 min	1

5 A 3.5 kbp PCR fragment digested with BstBI and XbaI was mixed with pGAPZ α 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.

10

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

15

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.

20

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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
5 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,
10 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.
- 15 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.
- 20 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
25 promoter is provided with additional binding sites for the positive regulator.

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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.
- 5 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 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
15 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
20 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]) 28 September 2000 (2000-09-28) examples 1-7, 12, 16-19 claims 26-29	1
A	EP 0 911 416 A2 (HOFFMANN LA ROCHE [CH]) DSM IP ASSETS BV [NL]) 28 April 1999 (1999-04-28) examples 1-3 claims 1-6	1
A	EP 0 299 108 A1 (RHEIN BIOTECH GES FUR BIOTECHN [DE] RHEIN BIOTECH GES FUER BIOTECH [DE]) 18 January 1989 (1989-01-18) cited in the application example 2 page 10, lines 22-30	1
-/-		

☒ 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 document but published on or after the international filing date

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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

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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

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Date of the actual completion of the international search

11 April 2008

Date of mailing of the international search report

25/04/2008

Name and mailing address of the ISA/

European Patent Office, P.B. 5518 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

van de Kamp, Mart

INTERNATIONAL SEARCH REPORT

International application No
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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) page 2, lines 8-26 page 10, lines 14-25 example 2	1
A	LIN-CEREGRINO G P ET AL: "Mxrip, a key regulator of the methanol utilization pathway and peroxisomal genes in Pichia pastoris" MOLECULAR AND CELLULAR BIOLOGY, vol. 26, no. 3, February 2006 (2006-02), pages 883-897, XP002438945 ISSN: 0270-7306 cited in the application abstract figures 2,3 page 895, left-hand column, lines 5-20 page 896, left-hand column, line 57 - right-hand column, line 16	11,12
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-/--

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

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
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Document code: WFEE

United States Patent and Trademark Office
Sales Receipt for Accounting Date: 12/10/2018

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/678,342	08/16/2017	Smita Shankar	38767-0048002	1018
26191 7590 12/13/2018 FISH & RICHARDSON P.C. (TC) PO BOX 1022 MINNEAPOLIS, MN 55440-1022 UNITED STATES OF AMERICA			EXAMINER LEITH, NANCY J	
			ART UNIT 1636	PAPER NUMBER
			NOTIFICATION DATE 12/13/2018	DELIVERY MODE 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



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FISH & RICHARDSON P.C. (TC) : NOTICE OF WITHDRAWAL
PO BOX 1022 : FROM ISSUE
MINNEAPOLIS MN MINNESOTA 55440-1022 : UNDER 37 CFR 1.313(b)

12/13/18

In re Application of:

Shankar, et al

Serial No. 15/678342

Filed: 08/16/2017

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

This application is being withdrawn to permit reopening of prosecution. See 37 CFR 1.313(b).

The above-identified application is hereby withdrawn from issue.

The issue fee is refundable upon written request. If, however, the application is again found allowable, the issue fee can be applied toward payment of the issue fee in the amount identified on the new Notice of Allowance and Issue Fee Due upon written request. This request and any balance due must be received on or before the due date noted in the new Notice of Allowance in order to prevent abandonment of the application.

Telephone inquiries should be directed to the SPE, Anne Gussow at (571)272-6047.

The above-identified application is being forwarded to the examiner for prompt appropriate action, including notifying applicant of the new status of this application.

A handwritten signature in black ink, appearing to read "W. Gary Jones", written over a horizontal line.

W. Gary Jones, Director
Technology Center 1600



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NOTICE OF ALLOWANCE AND FEE(S) DUE

26191 7590 12/26/2018
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

EXAMINER

LEITH, NANCY J

ART UNIT PAPER NUMBER

1636

DATE MAILED: 12/26/2018

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/678,342	08/16/2017	Smita Shankar	38767-0048002	1018

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

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1000	\$0.00	\$1000.00	\$0	03/26/2019

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

26191 7590 12/26/2018
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/678,342	08/16/2017	Smita Shankar	38767-0048002	1018

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

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1000	\$0.00	\$1000.00	\$0	03/26/2019

EXAMINER	ART UNIT	CLASS-SUBCLASS
LEITH, NANCY J	1636	435-254230

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,

1 _____

(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

2 _____

3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ☐ Individual ☐ Corporation or other private group entity ☐ Government4a. Fees submitted: ☐ Issue Fee ☐ Publication Fee (if required) ☐ Advance Order - # of Copies _____

4b. Method of Payment: (Please first reapply any previously paid fee shown above)

☐ Electronic Payment via EFS-Web ☐ Enclosed check ☐ Non-electronic payment by credit card (Attach form PTO-2038)☐ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. Change in Entity Status (from status indicated above)

☐ Applicant certifying micro entity status. See 37 CFR 1.29☐ Applicant asserting small entity status. See 37 CFR 1.27☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____

Date _____

Typed or printed name _____

Registration No. _____



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/678,342	08/16/2017	Smita Shankar	38767-0048002	1018
26191	7590	12/26/2018	EXAMINER	
FISH & RICHARDSON P.C. (TC)			LEITH, NANCY J	
PO BOX 1022			ART UNIT	
MINNEAPOLIS, MN 55440-1022			PAPER NUMBER	
			1636	
DATE MAILED: 12/26/2018				

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b) (Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

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

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

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulations.

Notice of Allowability	Application No. 15/678,342	Applicant(s) Shankar et al.	
	Examiner NANCY J. LEITH	Art Unit 1636	AIA Status Yes

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

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to the Information Disclosure Statements filed November 9, 2018 and December 7, 2018.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on ____.
2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on ____; the restriction requirement and election have been incorporated into this action.
3. ☒ The allowed claim(s) is/are 1-14 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.
4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
Certified copies:
 a) ☐ All b) ☐ Some *c) ☐ None of the:
 1. ☐ Certified copies of the priority documents have been received.
 2. ☐ Certified copies of the priority documents have been received in Application No. ____ .
 3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).
 * Certified copies not received: ____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date ____ .
Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).
6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892) 2. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 20181109 & 20181207. 3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material ____. 4. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____.	5. ☐ Examiner's Amendment/Comment 6. ☐ Examiner's Statement of Reasons for Allowance 7. ☐ Other _____.
--	---

/NANCY J LEITH/ Primary Examiner, Art Unit 1636	
--	--

Search Notes 	Application/Control No. 15/678,342	Applicant(s)/Patent Under Reexamination Shankar et al.
	Examiner NANCY J LEITH	Art Unit 1636

CPC - Searched*		
Symbol	Date	Examiner
No classified search made.	11/29/2017	JK
No classified search made.	06/08/2018	JK
No classified search made.	08/10/2018	JK

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
Inventors' names searched.	11/29/2017	JK
Priority checked.	11/29/2017	JK
BRS search attached,	11/29/2017	JK
[MEDLINE search terms and strategy as for BRS search.]	11/29/2017	JK
Updated from previous searches.	06/08/2018	JK
Updated from previous searches.	08/10/2018	JK

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
	Interference search part of BRS search (US-PGPUB).	08/10/2018	JK

/JAMES S KETTER/ Primary Examiner, Art Unit 1636	
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<i>Search Notes</i> 	Application/Control No. 15/678,342	Applicant(s)/Patent Under Reexamination Shankar et al.
	Examiner NANCY J LEITH	Art Unit 1636

/JAMES S KETTER/ Primary Examiner, Art Unit 1636	
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Issue Classification 	Application/Control No. 15/678,342	Applicant(s)/Patent Under Reexamination Shankar et al.
	Examiner NANCY J LEITH	Art Unit 1636

CPC						
Symbol					Type	Version
C12N	/	15	/	815	F	2013-01-01
C07K	/	14	/	39	I	2013-01-01
C12N	/	15	/	81	I	2013-01-01
C07K	/	14	/	415	I	2013-01-01
C07K	/	14	/	805	I	2013-01-01
C12N	/	1	/	16	I	2013-01-01
C12N	/	15	/	635	I	2013-01-01

CPC Combination Sets				
Symbol	Type	Set	Ranking	Version

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	14	
/NANCY J LEITH/ Primary Examiner, Art Unit 1636	13 December 2018	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

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Part of Paper No.: 20181213

Issue Classification 	Application/Control No. 15/678,342	Applicant(s)/Patent Under Reexamination Shankar et al.
	Examiner NANCY J LEITH	Art Unit 1636

INTERNATIONAL CLASSIFICATION			
CLAIMED			
C12N	/	15	/ 81
NON-CLAIMED			
/		/	

US ORIGINAL CLASSIFICATION	
CLASS	SUBCLASS

CROSS REFERENCES(S)						
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)					

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	14	
/NANCY J LEITH/ Primary Examiner, Art Unit 1636	13 December 2018	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

Receipt date: 11/09/2018

15/678,342 - GAU: 1636

Sheet 1 of 1

Substitute Disclosure Form U.S. Department of Commerce Patent and Trademark Office Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))	Attorney Docket No. 38767-0048002	Application No. 15/678,342
	Applicant Impossible Foods Inc.	
	Filing Date August 16, 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	No

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
/N. J. L./	1.	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 /NANCY J LEITH/	Date Considered 12/13/2018
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: 12/07/2018

15/678,342 - GAU: 1636

Sheet 1 of 1

Substitute Disclosure Form Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 38767-0048002	Application No. 15/678,342
		Applicant Impossible Foods Inc.	
		Filing Date August 16, 2017	Group Art Unit 1636

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
/N.J.L./	1.	2011/0287467	11/24/2011	Crane 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
/N.J.L./	2.	JPA 2008017774	1/31/2008	JP				
/N.J.L./	3.	WO 2008/090211	7/31/2008	WIPO				

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
/N.J.L./	4.	Sasano et al., "Trm2p-dependent depression is essential for methanol-specific gene activation in the methylotrophic yeast Candida boidinii", FEMS Yeast Res., Vol. 10, 535-544, 2010

Examiner Signature /NANCY J LEITH/	Date Considered 12/13/2018
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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UNITED STATES OF AMERICA



**Courtesy Reminder for
Application Serial No: 15/678,342**

Attorney Docket No: 38767-0048002
Customer Number: 26191
Date of Electronic Notification: 12/26/2018

This is a courtesy reminder that new correspondence is available for this application. If you have not done so already, please review the correspondence. The official date of notification of the outgoing correspondence will be indicated on the form PTOL-90 accompanying the correspondence.

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To view your correspondence online or update your email addresses, please visit us anytime at <https://sportal.uspto.gov/secure/myportal/privatepair>. If you have any questions, please email the Electronic Business Center (EBC) at EBC@uspto.gov or call 1-866-217-9197.

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Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web

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INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

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26191 7590 12/26/2018

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(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/678,342	08/16/2017	Smita Shankar	38767-0048002	1018

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

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	LARGE	\$1000	\$0		\$1000	03/26/2019

EXAMINER	ART UNIT	CLASS-SUBCLASS
LEITH, NANCY J.	1636	435-254230

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

- ☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
- ☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. Use of a Customer Number is required.

2. For printing on the patent front page, list

- (1) The names of up to 3 registered patent attorneys or agents OR, alternatively, 1 Fish & Richardson P.C.
- (2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed. 2 _____
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3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE
Impossible Foods Inc.(B) RESIDENCE: (CITY and STATE OR COUNTRY)
Redwood City, CAPlease check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government4a. Fees Submitted: ☒ Issue Fee ☐ Publication Fee (if required) ☐ Advance Order - # of Copies

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5. Change of Entity Status (from status indicated above)

- ☐ Applicant certifying micro entity status. See 37 CFR 1.29
- ☐ Applicant asserting small entity status. See 37 CFR 1.27.
- ☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid Certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

NOTE: If the application was previously under micro entity status, checking this box will be taken as a notification of loss of entitlement to micro entity status.

NOTE: Checking this box will be taken as a notification of loss of entitlement to small or micro entity status, as applicable.

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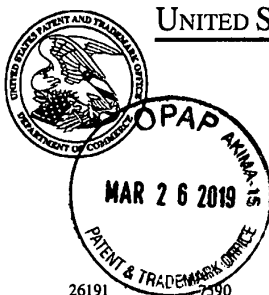
Authorized Signature /M. Angela Parsons/Date /March 26, 2019/Typed or printed name M. Angela Parsons, Ph.D.Registration No. 44,282

PTOL-85 Part B (08-18). Approved for use through 1/31/2020

OMB 0651-0033

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26191 7390 12/26/2018
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EXAMINER
LEITH, NANCY J

ART UNIT PAPER NUMBER

1636

DATE MAILED: 12/26/2018

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/678,342	08/16/2017	Smita Shankar	38767-0048002	1018

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

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1000	\$0.00	\$1000.00	\$0	03/26/2019

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

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II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

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Electronic Acknowledgement Receipt	
EFS ID:	34502371
Application Number:	15678342
International Application Number:	
Confirmation Number:	1018
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:	38767-0048002
Receipt Date:	07-DEC-2018
Filing Date:	16-AUG-2017
Time Stamp:	11:45:10
Application Type:	Utility under 35 USC 111(a)

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Payment Type	DA
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12/07/2018 INTEFSW 00012370 061050 15678342

02 FC:1801 1300.00 CR

Attorney Docket No.: 38767-0048002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor :	Smita Shankar	Art Unit :	1636
Application No. :	15/678,342	Examiner :	Nancy J. Leith
Filed :	August 16, 2017	Conf. No. :	1018
Title :	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST		

MAIL STOP ISSUE FEE

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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 after a Final Office Action or a Notice of Allowance, but before payment of the Issue Fee.

Certification under 37 CFR § 1.97(e)(1)

Each item of information contained in this Information Disclosure Statement was first cited in any Communication from a foreign Patent Office in a counterpart foreign application not more than three months prior to the filing of this Information Disclosure Statement.

Please apply any necessary 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: /March 26, 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

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Sheet 1 of 1

Substitute Disclosure Form Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 38767-0048002	Application No. 15/678,342
	Applicant Impossible Foods Inc.		
	Filing Date August 16, 2017	Group Art Unit 1636	

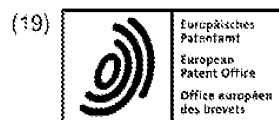
U.S. Patent Documents							
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Foreign Patent Documents or Published Foreign Patent Applications							
Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation Yes No
	1.	EP 2669375	12/4/2013	EP			

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
	2.	Mitrophanov, A. Y., and Groisman, E. A. "Positive feedback cellular control systems." BIOESSAYS, Vol. 30, No. 6, Pages 542-555, 2008
	3.	Mitrophanov et al., "Positive autoregulation shapes response timing and intensity in two component signal transduction systems." J MOL BIOL, Vol. 401, No. 4, Pages 671-680, 2010

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.	

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(11) **EP 2 669 375 A1**

(12) **EUROPEAN PATENT APPLICATION**
published in accordance with Art. 153(4) EPC

(43) Date of publication:
04.12.2013 Bulletin 2013/49

(51) Int Cl.:
C12N 15/09 ^(2006.01) **C07K 16/24** ^(2006.01)
C12N 1/19 ^(2006.01) **C12P 21/00** ^(2006.01)

(21) Application number: **12739392.4**

(86) International application number:
PCT/JP2012/051102

(22) Date of filing: **19.01.2012**

(87) International publication number:
WO 2012/102171 (02.08.2012 Gazette 2012/31)

(84) Designated Contracting States:
**AL AT BE BG CH CY CZ DE DK EE ES FI FR GB
GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO
PL PT RO RS SE SI SK SM TR**

(30) Priority: **27.01.2011 JP 2011015719**

(71) Applicant: **Kaneka Corporation**
Osaka-shi, Osaka 530-8288 (JP)

(72) Inventors:
• **NISHI Teruyuki**
Takasago-shi
Hyogo 676-8688 (JP)
• **ISHIGURO Kojiro**
Takasago-shi
Hyogo 676-8688 (JP)

(74) Representative: **HOFFMANN EITLE**
Patent- und Rechtsanwälte
Arabellastrasse 4
81925 München (DE)

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

(57) An object of the present invention is 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.

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Description

Technical Field

5 **[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

10

[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.

15

[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.

20

[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).

25

[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.

30

[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.

35

[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

40

Non-Patent Documents

[0008]

45

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

50

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.

55

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Means for Solving the Problem

[0010] The present invention includes the following inventions.

- 5 (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:
 - 10 (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;
 - 15 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:
 - 25 (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;
 - 30 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.
- 40 (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;
 - 45 (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.
- 50 (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;
 - 55 (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.

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(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 (pUCPgpaPaMPP1TmG418-introduced) and fully humanized anti-TNF- α Fab antibody; 3: a transformant of *Pichia angusta* expressing PpMPP1 (pUCPgpaPpMPP1TmG418-introduced) and fully humanized anti-TNF- α Fab antibody; 4: a transformant of *Pichia angusta* expressing Mut3 (pUCPgpaMut3TmG418-introduced) and fully humanized anti-TNF- α Fab antibody; 5: a transformant of *Pichia angusta* co-expressing PaMPP1 and Mut3 (pUCPgpaMut3PgpaPaMPP1TmG418-intro-

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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_

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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

5 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

10 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

15 hypothetical protein SNOG_09854 (*Phaeosphaeria nodorum* SN15), XP_001820352 hypothetical protein (*Aspergillus oryzae* R1B40), XP_001820847 hypothetical protein (*Aspergillus oryzae* R1B40), XP_001822149 hypothetical protein (*Aspergillus oryzae* R1B40), XP_001825067 hypothetical protein (*Aspergillus oryzae* R1B40), XP_001827368 hypothetical protein (*Aspergillus oryzae* R1B40), 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_

20 002145025 C6 transcription factor (ArcA), putative (*Penicillium marneffei* ATCC 18224), XP_002149655 conserved hypothetical protein (*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 trans-

25 scription 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 ZYRO0800242p (*Zygosaccharomyces rouxii*), XP_002498535 ZYRO08125 (*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_

30 002560015 Pc14g00200 (*Penicillium chrysogenum* Wisconsin 54-1255), XP_002563281 Pc20g07580 (*Penicillium chrysogenum* Wisconsin 54-1255), XP_002567816 Pc21g07780 (*Penicillium chrysogenum* Wisconsin 54-1255), XP_002628177 C6 zinc finger domain-containing protein (*Ajallomyces 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

35 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

40 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

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(*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_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_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 mameffei* 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 SOWgo), 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 Gaslp promoter, the GCR1 promoter, the GDH promoter, the GSH promoter, the HAR07 promoter, the

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5 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 Nii2p 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
10 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 PRA1 promoter, the Prm1 promoter, the PYC promoter, the RHO promoter, the SLA2
15 promoter, the SNF1 promoter, the STL1 promoter, the Swlp 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 Ynf1p promoter, the YNA1 promoter, the YNA2 promoter, the YNI1 promoter, the YNR1 promoter, the YNT1 promoter, the YPT1 promoter, or the Ypl7p promoter, and more preferably the AOX promoter, the DHAS promoter, the FMD promoter, or the MOX promoter.

20 **[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.

25 **[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.

30 **[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, Genetex).

35 **[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.

40 **[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.

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[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,

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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).

5 [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

10 with the GenBank Accession Number AY045886, 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 boldinii*.

[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

15 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,

20 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

25 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.

30 [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.

35 [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, Genetix).

[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.

40 [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.

45 [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

50 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.

55

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

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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 YEpl 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.

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[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 (MF α) 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 *NruI* site or the *EcoRV* 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.

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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* DH5 α 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 (MF α , 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* DH5 α 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 ID NOs: 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 MF α 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 MF α 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 MF α 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 MF α 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 MF α 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,

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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 MF α 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 of 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 pUCPgapMut3PgapPaMPP1 TmG418.

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

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

[0101] A gene fragment having the *NotI* recognition sequence on 5' terminal side of the PpMPP1 gene and the *Eco52I* 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 *NotI* and *Eco52I*, and it was then inserted into the *NotI* 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 *EcoRV* or *NruI* site in the MOX terminator gene or the *NotI* 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 μ l) 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 μ l 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 μ l of ice-cooled STM buffer at the end, and the resulting suspension was designated as a competent cell solution.

[0107] The competent cell solution (80 μ l) was mixed with 3 μ l 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 μ F, 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 angusta* 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 μ l) 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 μ l of a 1 M sorbitol solution, and

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the resulting suspension was designated as the competent cell solution.

[0110] The competent cell solution (50 μ l) was mixed with 3 μ l 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 μ F, 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 EcoRV 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 μ l) 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 μ l 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 μ l 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 μ l) was mixed with 3 μ l 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 μ F, 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 μ l) 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 μ l of a 1 M sorbitol solution, and the suspension

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was designated as the competent cell solution.

[0118] The competent cell solution (50 μ l) was mixed with 3 μ l 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 μ F, 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 (MaxiSorp, Nunc) at 50 μ l/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 μ l 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 μ l/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 μ l/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 μ l 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 μ l 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 (pUCPgPaMPP1TmG418-introduced) and fully humanized anti-TNF- α Fab antibody, the transformants expressing PpMPP1 (pUCPgPaMPP1TmG418-introduced) and fully humanized anti-TNF- α Fab antibody, and the transformants expressing Mut3 (pUCPgMut3TmG418-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 (pUCPgPaMPP1PgMut3PgPaMPP1TmG418) 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.

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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.

5 **[0126]** All publications, patents, and patent applications cited herein are incorporated herein by reference in their entirety.

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40 Ser Gly Lys Pro Lys Lys Glu Thr Pro Pro Ala Lys Arg Arg Val Arg
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Thr Gly Cys Leu Thr Cys Arg Arg Lys His Lys Lys Cys Asp Glu Asn
45 35 40 45

Arg Pro Lys Cys Asp Phe Cys Thr Ala Lys Gly Leu Glu Cys Val Trp
50 55 60

55 Pro Glu Glu Val Lys Lys Asn Val Phe Val Asn Asn Ser Phe Lys Asp
65 70 75 80

Phe Cys Phe Ser Gln Ser Lys His Arg Asp Arg Phe Arg Lys Pro Thr
85 90 95

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	Asp	Phe	Asp	Leu	Ser	Ser	Ser	Leu	Ser	Pro	Leu	Asp	Tyr	His	Leu	Gly	
				100					105						110		
5	Pro	Asp	Asp	Pro	Arg	Thr	Arg	Lys	Lys	Ala	Tyr	Ser	Leu	Asp	Val	Ala	
			115					120						125			
	Asp	His	Tyr	Leu	Ser	Ala	Phe	Asp	Gly	Tyr	Ser	Arg	Lys	Asn	Glu	Tyr	
10			130				135					140					
	Leu	Asp	Ala	Pro	Leu	Thr	Thr	Ala	Tyr	Pro	Leu	Leu	Glu	Thr	Ser	Glu	
			145			150					155					160	
15	Ser	Pro	Leu	Ser	Gly	Leu	Phe	Asn	Ala	Pro	Gln	Glu	Leu	Pro	Ser	Gln	
					165					170					175		
	Leu	Thr	Arg	Thr	Pro	Ser	Phe	Gly	Ala	Ser	Ser	Leu	Ser	Tyr	Phe	Asp	
20					180				185						190		
	Thr	Leu	Gln	Ser	Tyr	Ser	Met	Asn	Pro	Ser	Leu	Ser	Ser	Gly	Ser	Asn	
25			195					200					205				
	Asp	Lys	Ile	Ser	Thr	Ala	Ser	Ser	Val	Ser	Ser	Asp	Met	Ser	Leu	Ala	
			210				215					220					
30	Lys	Lys	Asp	Ala	Asp	Phe	Glu	His	Arg	Tyr	Ser	Phe	Ala	Glu	Pro	Gln	
			225			230					235					240	
	Lys	Lys	Ser	Lys	Phe	Gly	Leu	Asp	Tyr	Tyr	Asp	Leu	Pro	Asn	Thr	Leu	
35					245					250					255		
	Asn	Asp	Leu	Met	Cys	Leu	Phe	Ile	Ala	Thr	Lys	Asp	Ser	Thr	Ser	Gly	
				260				265						270			
40	Ser	Lys	Leu	Pro	Asn	Asn	Glu	Thr	Ile	Phe	Glu	Ile	Phe	Thr	Asp	Lys	
			275				280						285				
	Ser	Thr	Met	Glu	Ala	Pro	Ser	Ile	Asp	Asp	His	Glu	Met	Met	Leu	Leu	
45			290				295					300					
	Met	Glu	Thr	Tyr	Ile	Asp	Gln	Ile	Ala	His	Phe	Leu	Asn	Lys	Ser	Asp	
50					305		310				315					320	
	Glu	Thr	Gln	Cys	Asn	Ile	Phe	Met	Thr	Lys	Ile	Pro	Glu	Leu	Ala	His	
					325					330					335		
55	Arg	Phe	Pro	Ser	Leu	Lys	Tyr	Ala	Ile	Leu	Gly	Leu	Ala	Ser	Arg	His	
				340				345						350			

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	Leu	Glu	Lys	Val	Arg	Ser	Asp	Tyr	Ser	Gly	Glu	Lys	Thr	Leu	Gln	Tyr	
			355					360					365				
5	Tyr	Thr	Tyr	Ser	Leu	Gln	Gln	Leu	Ser	Met	Cys	Leu	Asn	Ser	Asp	Lys	
		370					375					380					
	Asn	Thr	Leu	Glu	Val	Val	Ala	Thr	Cys	Val	Leu	Leu	Cys	Tyr	Phe	Glu	
10		385				390					395					400	
	Ile	Leu	Ser	Thr	Asn	Pro	Asp	Ser	Trp	His	Ser	Arg	Leu	Glu	Asn	Cys	
					405					410					415		
15	Gly	Ser	Met	Leu	Lys	Val	Cys	His	Ile	Asn	Ala	Arg	Ser	Glu	Thr	Glu	
				420					425					430			
20	Leu	Glu	Lys	Ala	Leu	Phe	Trp	Ser	Phe	Ala	Ala	Leu	Asp	Ile	Gly	Cys	
			435					440					445				
	Cys	Ala	Val	Gly	Asp	Arg	Lys	Thr	Val	Val	Arg	Pro	Glu	Asp	Trp	Phe	
25		450					455					460					
	Gly	Thr	Asp	Ala	Pro	Ser	Ser	Pro	Thr	Glu	Thr	Gly	Thr	Ala	Leu	Gln	
		465				470					475					480	
30	Ser	Met	Leu	Tyr	Leu	Ser	Cys	Gln	Ile	Phe	Gln	Leu	Val	Ser	Ser	Asn	
				485						490					495		
	Asp	Asp	Gln	Ser	Glu	Phe	Asp	Ser	Cys	Trp	Lys	Ser	Leu	Trp	Lys	Gln	
35				500					505					510			
	Leu	Gly	Asp	Trp	Glu	Arg	Ala	Arg	Ser	Ser	Asn	Met	Arg	Gly	Phe	Cys	
			515					520					525				
40	Phe	Lys	Arg	Asp	Asn	Ser	Phe	Pro	Glu	Thr	Leu	Phe	Ser	Ser	Ser	Thr	
		530					535					540					
45	Ala	Leu	Cys	Ala	Asn	Gln	Leu	Phe	His	Thr	Cys	Cys	Ile	Leu	Met	Leu	
		545				550					555					560	
	Gln	Thr	Lys	Pro	Arg	Leu	Val	Lys	Leu	His	Glu	Pro	Glu	Lys	Gln	Glu	
50					565					570					575		
	Arg	Thr	Pro	Asp	Thr	Leu	Phe	Gln	Ser	Ala	Ile	Lys	Thr	Glu	Gln	Gly	
				580					585					590			
55	Glu	Ser	Val	Ser	Thr	Ser	Ser	Leu	Met	Ser	Arg	Ser	Gln	Ile	Trp	His	
			595					600					605				

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Ala Lys Gln Ile Ile Gly Ile Asn Val Cys Asn Met Lys Ser Ala Ser
610 615 620

5 Ser Arg Ser Leu Gly Cys Gln Ile Leu Ser Leu Gln Ala Ile Trp Ile
625 630 635 640

Ala Gly Lys Leu Ile Ser Ser Ala His Glu His Ser Ile Ile Leu Gln
10 645 650 655

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660 665 670

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50 Thr Leu Met Asn Lys Asp Ile Pro Ser Asn Ala Lys Gln Ala Lys Ser
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55 Lys Val Ser Ala Ala Pro Ala Lys Thr Gly Ser Lys Ser Ala Gly Gly
35 40 45

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	Ser	Gly	Asn	Asn	Asn	Ala	Ala	Pro	Val	Lys	Lys	Arg	Val	Arg	Thr	Gly	
	50						55					60					
5	Cys	Leu	Thr	Cys	Arg	Lys	Lys	His	Lys	Lys	Cys	Asp	Glu	Asn	Arg	Asn	
	65					70					75					80	
	Pro	Lys	Cys	Asp	Phe	Cys	Thr	Leu	Lys	Gly	Leu	Glu	Cys	Val	Trp	Pro	
10					85					90					95		
	Glu	Asn	Asn	Lys	Lys	Asn	Ile	Phe	Val	Asn	Asn	Ser	Met	Lys	Asp	Phe	
				100					105					110			
15	Leu	Gly	Lys	Lys	Thr	Val	Asp	Gly	Ala	Asp	Ser	Leu	Asn	Leu	Ala	Val	
		115						120					125				
	Asn	Leu	Gln	Gln	Gln	Gln	Ser	Ser	Asn	Thr	Ile	Ala	Asn	Gln	Ser	Leu	
20		130					135					140					
	Ser	Ser	Ile	Gly	Leu	Glu	Ser	Phe	Gly	Tyr	Gly	Ser	Gly	Ile	Lys	Asn	
25	145					150				155						160	
	Glu	Phe	Asn	Phe	Gln	Asp	Leu	Ile	Gly	Ser	Asn	Ser	Gly	Ser	Ser	Asp	
					165				170					175			
30	Pro	Thr	Phe	Ser	Val	Asp	Ala	Asp	Glu	Ala	Gln	Lys	Leu	Asp	Ile	Ser	
				180					185					190			
	Asn	Lys	Asn	Ser	Arg	Lys	Arg	Gln	Lys	Leu	Gly	Leu	Leu	Pro	Val	Ser	
35		195					200						205				
	Asn	Ala	Thr	Ser	His	Leu	Asn	Gly	Phe	Asn	Gly	Met	Ser	Asn	Gly	Lys	
40		210					215					220					
	Ser	His	Ser	Phe	Ser	Ser	Pro	Ser	Gly	Thr	Asn	Asp	Asp	Glu	Leu	Ser	
	225					230				235					240		
45	Gly	Leu	Met	Phe	Asn	Ser	Pro	Ser	Phe	Asn	Pro	Leu	Thr	Val	Asn	Asp	
				245					250					255			
	Ser	Thr	Asn	Asn	Ser	Asn	His	Asn	Ile	Gly	Leu	Ser	Pro	Met	Ser	Cys	
50				260					265					270			
	Leu	Phe	Ser	Thr	Val	Gln	Glu	Ala	Ser	Gln	Lys	Lys	His	Gly	Asn	Ser	
		275						280					285				
55	Ser	Arg	His	Phe	Ser	Tyr	Pro	Ser	Gly	Pro	Glu	Asp	Leu	Trp	Phe	Asn	

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[illegible]

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	Cys	Ile	Leu	Leu	Ser	Val	Phe	His	Ile	Met	Ser	Ser	Glu	Pro	Ser	Thr	
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5	Gln	Arg	Asp	Ile	Ile	Val	Ser	Leu	Ala	Lys	Tyr	Ile	Gln	Ala	Cys	Asn	
				565						570					575		
	Ile	Asn	Gly	Phe	Thr	Ser	Asn	Asp	Lys	Leu	Glu	Lys	Ser	Ile	Phe	Trp	
10				580					585					590			
	Asn	Tyr	Val	Asn	Leu	Asp	Leu	Ala	Thr	Cys	Ala	Ile	Gly	Glu	Glu	Ser	
			595					600					605				
15	Met	Val	Ile	Pro	Phe	Ser	Tyr	Trp	Val	Lys	Glu	Thr	Thr	Asp	Tyr	Lys	
		610					615					620					
	Thr	Ile	Gln	Asp	Val	Lys	Pro	Phe	Phe	Thr	Lys	Lys	Thr	Ser	Thr	Thr	
20		625				630					635					640	
	Thr	Asp	Asp	Asp	Leu	Asp	Asp	Met	Tyr	Ala	Ile	Tyr	Met	Leu	Tyr	Ile	
					645					650					655		
25	Ser	Gly	Arg	Ile	Ile	Asn	Leu	Leu	Asn	Cys	Arg	Asp	Ala	Lys	Leu	Asn	
				660					665					670			
	Phe	Glu	Pro	Lys	Trp	Glu	Phe	Leu	Trp	Asn	Glu	Leu	Asn	Glu	Trp	Glu	
30			675					680					685				
	Leu	Asn	Lys	Pro	Leu	Thr	Phe	Gln	Ser	Ile	Val	Gln	Phe	Lys	Ala	Asn	
35			690					695				700					
	Asp	Glu	Ser	Gln	Gly	Gly	Ser	Thr	Phe	Pro	Thr	Val	Leu	Phe	Ser	Asn	
		705			710						715					720	
40	Ser	Arg	Ser	Cys	Tyr	Ser	Asn	Gln	Leu	Tyr	His	Met	Ser	Tyr	Ile	Ile	
					725					730					735		
	Leu	Val	Gln	Asn	Lys	Pro	Arg	Leu	Tyr	Lys	Ile	Pro	Phe	Thr	Thr	Val	
45				740					745					750			
	Ser	Ala	Ser	Met	Ser	Ser	Pro	Ser	Asp	Asn	Lys	Ala	Gly	Met	Ser	Ala	
50			755					760					765				
	Ser	Ser	Thr	Pro	Ala	Ser	Asp	His	His	Ala	Ser	Gly	Asp	His	Leu	Ser	
			770				775					780					
55	Pro	Arg	Ser	Val	Glu	Pro	Ser	Leu	Ser	Thr	Thr	Leu	Ser	Pro	Pro	Pro	
		785				790					795					800	

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	Asn Ala Asn Gly Ala Gly Asn Lys Phe Arg Ser Thr Leu Trp His Ala	
	805 810 815	
5	Lys Gln Ile Cys Gly Ile Ser Ile Asn Asn Asn His Asn Ser Asn Leu	
	820 825 830	
10	Ala Ala Lys Val Asn Ser Leu Gln Pro Leu Trp His Ala Gly Lys Leu	
	835 840 845	
	Ile Ser Ser Lys Ser Glu His Thr Gln Leu Leu Lys Leu Leu Asn Asn	
	850 855 860	
15	Leu Glu Cys Ala Thr Gly Trp Pro Met Asn Trp Lys Gly Lys Glu Leu	
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20 25 30

Leu Gly Arg Glu Ile Leu Met Lys Pro Ser Asn Ser Ala Pro Pro Arg
35 40 45

25 Ile Ile Met Pro Ala Gly Ser Asp Gly Ser Ser Gly Val Val Leu Gln
50 55 60

30 Lys Lys Arg Arg Val Val Arg Ala Cys Asp Arg Cys Arg Lys Leu Lys
65 70 75 80

35 Ile Lys Cys Ser Gly Asp Leu Pro Cys Ile His Cys Thr Val Tyr Ser
85 90 95

Tyr Glu Cys Thr Tyr Asp Gln Pro Ser Arg Ala Lys Arg Lys Asn Thr
100 105 110

40 Thr Ala Asn Ala Gly Ser Pro Ser Gly Pro Ser Ala Asn Ser Gln His
115 120 125

45 Pro His Leu Pro Thr Asn Ser Leu Glu Val Met Ala Arg Met Ala Asp
130 135 140

Arg Leu Lys Leu Tyr Asp Asp Ile Leu His Arg Leu Leu Pro Asp Ile
145 150 155 160

50 Lys Leu Thr Asp Leu Asn Asp Asn Pro Lys Pro Ile Asn Pro Met Lys
165 170 175

55 Leu Leu Thr Ala Met Asn Lys Leu Arg Glu Lys Ser Cys Asp Glu Thr
180 185 190

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	Lys	Ser	Ser	Ser	Lys	Thr	Ile	Ala	Glu	Glu	Tyr	Glu	Arg	Leu	Pro	Asp	
				195				200					205				
5	Val	Pro	Leu	Pro	Thr	Leu	Pro	Ser	Val	Pro	Lys	Pro	Ala	Pro	Ser	Thr	
		210					215					220					
	Thr	Ala	Val	Lys	Thr	His	Glu	Ser	Pro	Ser	Glu	Arg	Ser	His	Phe	Cys	
10	225					230					235					240	
	Ser	Pro	His	Gly	Gly	Gly	Pro	His	Gln	Gly	Ile	Asp	Gly	Ser	Tyr	Glu	
				245						250					255		
15	Ser	Ser	Met	Gly	Lys	Glu	Ile	Lys	Ile	Ile	Leu	Pro	Ser	Arg	Glu	Val	
				260				265						270			
	Ala	Leu	Arg	Leu	Ile	Thr	Lys	Thr	Trp	Glu	Asn	Ala	Cys	Val	Leu	Phe	
20			275					280					285				
	Arg	Phe	Tyr	His	Arg	Pro	Ala	Phe	Ile	Glu	Asp	Leu	Asn	Glu	Leu	Tyr	
25		290					295					300					
	Asp	Thr	Asp	Pro	Ser	Gln	Tyr	Thr	Asn	Lys	Gln	Gln	Arg	Phe	Leu	Pro	
	305					310					315					320	
30	Leu	Val	Tyr	Ser	Val	Met	Ala	Cys	Gly	Ala	Leu	Phe	Leu	Lys	Ser	Asp	
					325					330					335		
	Glu	Gln	Thr	Asn	Gly	Ser	Lys	Asn	Gly	Glu	Pro	Thr	Pro	Glu	Asn	Glu	
35				340					345					350			
	Val	Phe	Glu	Asp	Glu	Gly	Tyr	Arg	Tyr	Phe	Ile	Ala	Ala	Arg	Lys	Leu	
40			355					360					365				
	Ile	Asp	Ile	Thr	Asp	Thr	Arg	Asp	Thr	Tyr	Gly	Ile	Gln	Thr	Ile	Val	
		370					375					380					
45	Met	Leu	Ile	Ile	Phe	Leu	Gln	Cys	Ser	Ala	Arg	Leu	Ser	Thr	Cys	Tyr	
	385					390					395					400	
	Ala	Tyr	Ile	Gly	Ile	Ala	Leu	Arg	Ala	Ala	Leu	Arg	Glu	Gly	Leu	His	
50				405						410				415			
	Arg	Lys	Leu	Asp	Tyr	Pro	Phe	Asn	Pro	Ile	Glu	Leu	Glu	Ile	Arg	Lys	
			420					425					430				
55	Arg	Leu	Phe	Trp	Thr	Ile	Tyr	Lys	Met	Asp	Ile	Tyr	Val	Asn	Thr	Met	

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	435		440		445
5	Leu Gly Leu Pro Arg Thr Ile Ser Glu Asp Asp Phe Asp Gln Asp Met				
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	Pro Ile Glu Leu Asp Asp Glu Asn Ile Thr Val Asp Gly Tyr Arg Phe				
	465		470		475
10	Glu Lys Gln Gly Asp Arg Leu Ser Ser Ser Gly Ile Ala Asn Ala His				
		485		490	495
15	Thr Arg Leu Ile Phe Ile Met Lys Lys Ile Val Thr Lys Leu Tyr Pro				
		500		505	510
	Ile Lys Pro Arg Lys Ile Glu Asn Gly Arg Arg Pro Glu Leu Met Thr				
20		515		520	525
	His Asp Ile Val Tyr Gln Leu Glu Ile Asp Leu Gln Asp Trp Met Asn				
	530		535		540
25	Ser Leu Pro Leu Glu Leu Lys Pro Gly Val Glu Pro Ser Ser Gln Tyr				
	545		550		555
	Leu Lys Ala Asn Arg Leu Leu His Ile Ser Tyr Leu His Val Lys Ile				
30		565		570	575
	Ile Leu Tyr Arg Pro Phe Ile His Tyr Leu Ser Ser Asp His Val Asn				
35		580		585	590
	Arg Pro Lys Ser Ser Asn Asp Leu Lys Cys Ile Glu Lys Ala Lys Asn				
		595		600	605
40	Cys Ile Asn Val Ala Arg Ile Val Val Lys Leu Ala Glu Asp Met Ile				
	610		615		620
	Asn Lys Asn Met Leu Ser Gly Ser Tyr Trp Phe Ser Ile Tyr Thr Ile				
45	625		630		635
	Phe Phe Ser Val Ala Cys Leu Val Tyr Tyr Val His Phe Ala Pro Pro				
		645		650	655
50	Val Thr Ser Ser Gly Asn Phe Asp Pro Glu Tyr Ile Ala Ile Lys Lys				
		660		665	670
55	Asp Ala Glu Ser Gly Lys Arg Val Leu Asp Ile Leu Lys Asp Ser Ser				
	675		680		685

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	Met	Ala	Ala	Arg	Arg	Thr	Tyr	Asn	Ile	Leu	Asn	Ala	Leu	Phe	Glu	Glu	
	690						695					700					
5	Leu	Asn	Arg	Lys	Thr	Ala	Asn	Ala	Ser	Ile	Ser	His	Glu	Pro	Pro	Asn	
	705					710					715					720	
	Ser	His	Phe	Leu	Thr	Pro	Gln	Val	Gly	Lys	Ser	Gln	Phe	Glu	Arg	Asp	
10					725					730					735		
	Asn	His	Lys	Lys	Asp	Gln	Met	Ala	Ile	Pro	Leu	Pro	Pro	Gln	Asn	Met	
				740					745					750			
15	Arg	Pro	Pro	Glu	Tyr	Val	Gln	Gln	Pro	Phe	Asn	Gly	Tyr	Ser	Lys	Met	
			755					760					765				
	Glu	Asn	Ser	Glu	Ile	Asn	Asn	Ile	Val	Asn	Gly	Val	Asn	Phe	Ile	Asp	
20		770					775					780					
	Gly	Val	Ser	Thr	Gly	Ile	Asn	Leu	Asn	Leu	Pro	Ser	Asn	Ser	Glu	Val	
25		785				790					795					800	
	Glu	Asp	Val	Pro	Ser	Ser	Ile	Ser	Pro	Pro	Asp	Gln	Asn	Ser	Lys	Tyr	
					805					810					815		
30	Lys	Tyr	Val	Pro	Gly	Pro	Met	Asp	Gln	Leu	Asp	Met	Lys	Ile	Phe	Gly	
				820					825					830			
	Arg	Phe	Leu	Pro	Pro	Tyr	Met	Leu	Asn	Ser	Asn	Gln	Gln	Ser	Thr	Asp	
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	Asn	Thr	Pro	Glu	Pro	Glu	Pro	Tyr	Pro	Glu	Gln	Tyr	Gln	Glu	Pro	Glu	
40		850					855					860					
	Asn	Glu	Pro	Gln	Asn	Gly	Thr	Ile	Phe	Gly	Ser	Ile	Pro	Ser	Gly	Leu	
	865					870					875					880	
45	Ser	Thr	Val	Ser	Gln	Ser	Gln	Gln	Asn	Ser	Lys	Gly	Phe	Thr	Gly	Leu	
					885					890					895		
	Pro	Pro	Asp	Tyr	Gln	Gly	Pro	Pro	Lys	Arg	Tyr	Asp	Phe	Glu	Asp	Met	
50				900					905					910			
	Phe	Tyr	Asn	Asp	Trp	Ser	Ala	Gly	Pro	Pro	Asn	Asp	Phe	Asn	Gln	Lys	
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55	Ser																

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EP 2 669 375 A1

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Claims

- 25 1. A yeast for transformation, wherein the capacity for target protein production is improved by high-level expression of an MPP1 homolog.
- 30 2. The yeast for transformation according to claim 1, wherein the MPP1 homolog is any of the polypeptides (a), (b), and (c) below:
- 35 (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.
- 40 3. The yeast for transformation according to claim 1, wherein the MPP1 homolog is partially composed of any of the polypeptides (g), (h) and (i) below:
- 45 (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.
- 50 50 55

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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 Pms1 promoter, the SNF1 promoter, the Swlp 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

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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.

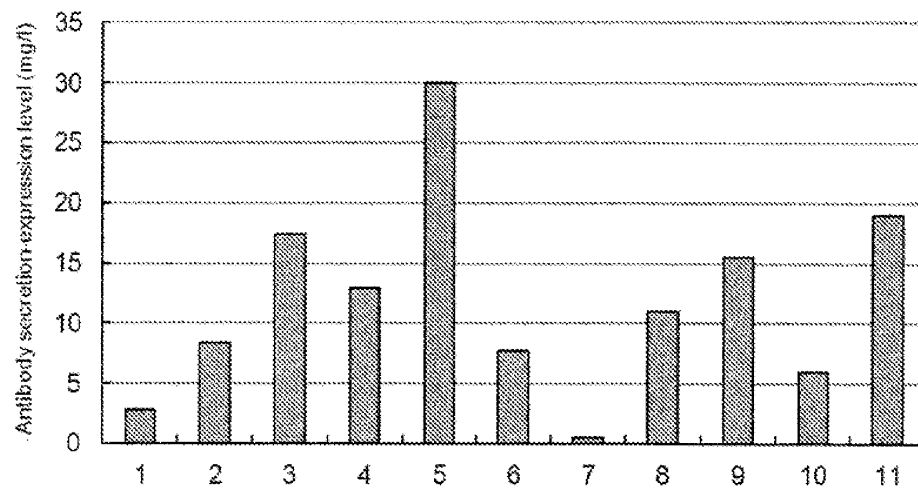
18. The method for producing a target protein according to claim 17, wherein culture is conducted using glucose, and/or glycerol, and/or methanol as a carbon source.

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.

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Fig. 1



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INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2012/051102

A. CLASSIFICATION OF SUBJECT MATTER

C12N15/09(2006.01)1, C07K16/24(2006.01)1, C12N1/19(2006.01)1, C12P21/00(2006.01)1

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)

CB/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-Reider 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., Trmlp, a Zn(II)2Cys6-Type Transcription Factor, Is a Master Regulator of Methanol-Specific Gene Activation in the Methylophilic 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

"Z" document member of the same patent family

Date of the actual completion of the international search

12 April, 2012 (12.04.12)

Date of mailing of the international search report

24 April, 2012 (24.04.12)

Name and mailing address of the ISA/
Japanese Patent Office

Authorized officer

Facsimile No.

Telephone No.

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

EP 2 669 375 A1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP2012/051162

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 Methylophilic Yeast <i>Candida boidinii</i> , 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 <i>Pichia pastoris</i> , Nature Biotechnology, 2009, Vol. 27, p. 561-566	1-20
A	E. Berardi and B. Tinto, <i>Pichia angusta</i> 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)

EP 2 669 375 A1

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]
- Transcriptional downregulation of peroxisome numbers affects selective peroxisome degradation in *Hansenula polymorpha*. *J. Biol. Chem.*, 2003, vol. 278, 40749-40756 [0017]
- GENETYX Ver. 10 [0050]
- Molecular Cloning. Cold Spring Harbor Laboratory Press, 1989 [0080]
- Current Protocols in Molecular Biology. Greene Publishing Associates and Wiley-Interscience [0080]

Electronic Patent Application Fee Transmittal				
Application Number:		15678342		
Filing Date:		16-Aug-2017		
Title of Invention:		EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST		
First Named Inventor/Applicant Name:		Smita Shankar		
Filer:		M. Angela Parsons/Kirsten Neuman		
Attorney Docket Number:		38767-0048002		
Filed as Large Entity				
Filing Fees for Utility under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Miscellaneous:				
SUBMISSION- INFORMATION DISCLOSURE STMT	1806	1	240	240
Total in USD (\$)				240

Electronic Acknowledgement Receipt	
EFS ID:	35530615
Application Number:	15678342
International Application Number:	
Confirmation Number:	1018
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:	38767-0048002
Receipt Date:	26-MAR-2019
Filing Date:	16-AUG-2017
Time Stamp:	13:58:58
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$ 240
RAM confirmation Number	032719INTEFSW00000277061050
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Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22314-1450

By fax, send to: (571) 273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

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26191 7590 12/26/2018

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MINNEAPOLIS, MN 55440-1022

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I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)

(Signature)

(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/678,342	08/16/2017	Smita Shankar	38767-0048002	1018

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

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	LARGE	\$1000	\$0		\$1000	03/26/2019

EXAMINER	ART UNIT	CLASS-SUBCLASS
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LEITH, NANCY J.

1636

435-254230

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

- ☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.
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2. For printing on the patent front page, list

- (1) The names of up to 3 registered patent attorneys or agents OR, alternatively, 1 Fish & Richardson P.C.
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- 3 _____

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(A) NAME OF ASSIGNEE

Impossible Foods Inc.

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Redwood City, CA

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government4a. Fees Submitted: ☒ Issue Fee ☐ Publication Fee (if required) ☐ Advance Order - # of Copies

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5. Change of Entity Status (from status indicated above)

☐ Applicant certifying micro entity status. See 37 CFR 1.29☐ Applicant asserting small entity status. See 37 CFR 1.27.☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid Certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

NOTE: If the application was previously under micro entity status, checking this box will be taken as a notification of loss of entitlement to micro entity status.

NOTE: Checking this box will be taken as a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature /M. Angela Parsons/Date /March 26, 2019/Typed or printed name M. Angela Parsons, Ph.D.Registration No. 44,282

PTOL-85 Part B (08-18). Approved for use through 1/31/2020

OMB 0651-0033

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EFS ID:	35536523
Application Number:	15678342
International Application Number:	
Confirmation Number:	1018
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:	38767-0048002
Receipt Date:	26-MAR-2019
Filing Date:	16-AUG-2017
Time Stamp:	17:52:20
Application Type:	Utility under 35 USC 111(a)

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/678,342	08/16/2017	Smita Shankar	38767-0048002	1018

26191	7590	04/03/2019
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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
04/03/2019	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.

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Notice of Allowability	Application No. 15/678,342	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--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to the Information Disclosure Statement filed March 26, 2019.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

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

3. ☒ The allowed claim(s) is/are 1-14 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

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

Certified copies:

a) ☐ All b) ☐ Some *c) ☐ None of the:

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

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

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

* Certified copies not received: _____ .

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____ .

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)	5. ☐ Examiner's Amendment/Comment
2. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date <u>20190326</u> .	6. ☐ Examiner's Statement of Reasons for Allowance
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.	7. ☐ Other _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____.	

/NANCY J LEITH/ Primary Examiner, Art Unit 1636	
--	--

Issue Classification 	Application/Control No. 15/678,342	Applicant(s)/Patent Under Reexamination Shankar et al.
	Examiner NANCY J. LEITH	Art Unit 1636

CPC						
Symbol					Type	Version
C12N	/	15	/	815	F	2013-01-01
C07K	/	14	/	39	I	2013-01-01
C12N	/	15	/	81	I	2013-01-01
C07K	/	14	/	415	I	2013-01-01
C07K	/	14	/	805	I	2013-01-01
C12N	/	1	/	16	I	2013-01-01
C12N	/	15	/	635	I	2013-01-01

CPC Combination Sets				
Symbol	Type	Set	Ranking	Version

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	14	
/NANCY J LEITH/ Primary Examiner, Art Unit 1636	01 April 2019	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

U.S. Patent and Trademark Office

Part of Paper No.: 20190401

Issue Classification 	Application/Control No. 15/678,342	Applicant(s)/Patent Under Reexamination Shankar et al.
	Examiner NANCY J. LEITH	Art Unit 1636

INTERNATIONAL CLASSIFICATION			
CLAIMED			
C12N	/	15	/ 81

NON-CLAIMED			
/		/	

US ORIGINAL CLASSIFICATION	
CLASS	SUBCLASS

CROSS REFERENCES(S)						
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)					

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	14	
/NANCY J LEITH/ Primary Examiner, Art Unit 1636	01 April 2019	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

Issue Classification 	Application/Control No. 15/678,342	Applicant(s)/Patent Under Reexamination Shankar et al.
	Examiner NANCY J. LEITH	Art Unit 1636

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

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	14	
/NANCY J LEITH/ Primary Examiner, Art Unit 1636	01 April 2019	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	NONE

Receipt date: 03/26/2019

15/678,342 - GAU: 1636

Sheet 1 of 1

Substitute Disclosure Form Information Disclosure Statement by Applicant (Use several sheets if necessary) (37 CFR §1.98(b))	U.S. Department of Commerce Patent and Trademark Office	Attorney Docket No. 38767-0048002	Application No. 15/678,342
	Applicant Impossible Foods Inc.		
	Filing Date August 16, 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
/N. J. L./	1.	EP 2669375	12/4/2013	EP			Yes No

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document
/N. J. L./	2.	Mitrophanov, A. Y., and Groisman, E. A. "Positive feedback cellular control systems." BIOESSAYS, Vol. 30, No. 6, Pages 542-555, 2008
/N. J. L./	3.	Mitrophanov et al., "Positive autoregulation shapes response timing and intensity in two component signal transduction systems." J MOL BIOL, Vol. 401, No. 4, Pages 671-680, 2010

Examiner Signature /NANCY J LEITH/	Date Considered 04/01/2019
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



UNITED STATES PATENT AND TRADEMARK OFFICE

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

APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/678,342	04/30/2019	10273492	38767-0048002	1018

26191 7590 04/10/2019
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b) (application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (<http://pair.uspto.gov>).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site <http://pair.uspto.gov> for additional applicants):

Smita Shankar, Millbrae, CA;
Impossible Foods Inc., Redwood City, CA;
Martin Andrew Hoyt, San Francisco, CA;

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 USA offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to encourage and facilitate business investment. To learn more about why the USA is the best country in the world to develop technology, manufacture products, and grow your business, visit SelectUSA.gov.

Attorney's Docket No.: 38767-0048002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Smita Shankar	Art Unit	:	1636
Patent No.	:	10,273,492	Examiner	:	Nancy J. Leith
Issue Date	:	April 30, 2019	Conf. No.	:	1018
Serial No.	:	15/678,342			
Filed	:	August 16, 2017			
Title	:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST			

Attention Certificate of Corrections Branch
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REQUEST FOR CERTIFICATE OF CORRECTION

Please issue a certificate of correction in accordance with the attached form.

One or more of the errors to be corrected were made by applicant. The fee of \$160 is being paid with this request.

Apply any other necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: May 18, 2022

/Lisa D. Fazzino/

Lisa D. Fazzino, Reg. No. 80,542

Customer Number 26191
Fish & Richardson P.C.
Telephone: +1 (612) 766-2032
Facsimile: (877) 769-7945

100663019.doc

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

Page 1 of 1

PATENT NO. : 10,273,492
APPLICATION NO.: 15/678,342
ISSUE DATE : April 30, 2019
INVENTOR(S) : Smita Shankar and Martin Andrew Hoyt

It is certified that an error appears or errors appear in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the cover page

On line 12 of "OTHER PUBLICATIONS", please delete "Chimvolu" and insert - - Chiruvolu - -, therefor.

In the claims

Claim 4: In column 77, line 65, please delete "porphogilinogen" and insert - - porphobilinogen - -, therefor.

MAILING ADDRESS OF SENDER (Please do not use customer number below):

Fish & Richardson P.C.
P.O. Box 1022
Minneapolis, MN 55440-1022

This collection of information is required by 37 CFR 1.322, 1.323, and 1.324. 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 1.0 hour 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: Attention Certificate of Corrections Branch, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and selection option2.

Electronic Patent Application Fee Transmittal				
Application Number:		15678342		
Filing Date:		16-Aug-2017		
Title of Invention:		EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST		
First Named Inventor/Applicant Name:		Smita Shankar		
Filer:		Lisa Fazzino/Emily Palmer		
Attorney Docket Number:		38767-0048002		
Filed as Large Entity				
Filing Fees for Utility under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
CERTIFICATE OF CORRECTION	1811	1	160	160

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Extension-of-Time:				
Miscellaneous:				
Total in USD (\$)				160

Electronic Acknowledgement Receipt	
EFS ID:	45743264
Application Number:	15678342
International Application Number:	
Confirmation Number:	1018
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	Lisa Fazzino/Emily Palmer
Filer Authorized By:	Lisa Fazzino
Attorney Docket Number:	38767-0048002
Receipt Date:	18-MAY-2022
Filing Date:	16-AUG-2017
Time Stamp:	12:25:46
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$ 160
RAM confirmation Number	E20225HC26053240
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Certificate of Correction	ReqCOC.pdf	128281	no	2
			d660032fcb857d8a2554b0c5c4b9f4b29e33f520		
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	37926	no	2
			164b3a3d19eb556e2fa4bb5d041a6f03cec7fbc		
Warnings:					
Information:					
Total Files Size (in bytes):			166207		
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. <u>New Applications Under 35 U.S.C. 111</u> 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. <u>National Stage of an International Application under 35 U.S.C. 371</u> 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. <u>New International Application Filed with the USPTO as a Receiving Office</u> 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.					