

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

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

DESCRIPTION OF DRAWINGS

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

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

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

Figure 4 are schematics showing plasmids pGAB and pMx354.

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

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

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

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

Figure 9 shows the production of LegH from the modified *P. pastoris* strains. Panel A is a SDS gel showing lysates from *P. pastoris* strains grown in shake flasks: 51, MXY0051; 118, MXY0118; 183, MXY0183. Panel B is a table comparing LegH production from strains MXY0118, MXY0183, and MXY0207.

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

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

15 DETAILED DESCRIPTION

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

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

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

5 Recombinant nucleic acid molecules used in the methods described herein are typically DNA, but RNA molecules can be used under the appropriate circumstances. As used herein, “exogenous” refers to any nucleic acid sequence that is introduced into the genome of a cell from an external source, where the external source can be the same or a different organism or a nucleic acid generated synthetically. For example, an exogenous nucleic acid can be a nucleic acid from
10 one microorganism (e.g., one genus or species of methylotrophic yeast) that is introduced into a different genus or species of methylotrophic yeast, however, an exogenous nucleic acid also can be a nucleic acid from a methylotrophic yeast that is introduced recombinantly into a methylotrophic yeast as an additional copy despite the presence of a corresponding native nucleic acid sequence. For example, *P. pastoris* contains an endogenous nucleic acid encoding a Mxr1
15 transcriptional activator; an additional *P. pastoris* Mxr1 nucleic acid (e.g., introduced recombinantly into *P. pastoris*) or modifying the endogenous *P. pastoris* Mxr1 nucleic acid is considered exogenous.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Pichia strains are able to grow on methanol as the sole carbon source. Methanol utilization is initiated by the conversion of methanol to formaldehyde by the action of alcohol
30 oxidase. The methylotrophic yeast, *Pichia pastoris*, contains two genes for alcohol oxidases,

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

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

As used herein, nucleic acids can include DNA and RNA, and includes nucleic acids that
25 contain one or more nucleotide analogs or backbone modifications. A nucleic acid can be single stranded or double stranded, which usually depends upon its intended use. Also provided are nucleic acids and polypeptides that differ from a given sequence. Nucleic acids and polypeptides can have at least 50% sequence identity (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%,
30 98%, or 99% sequence identity) to a given nucleic acid or polypeptide sequence.

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

The alignment of two or more sequences to determine percent sequence identity can be performed using the computer program ClustalW and default parameters, which allows alignments of nucleic acid or polypeptide sequences to be carried out across their entire length (global alignment). Chenna et al., 2003, *Nucleic Acids Res.*, 31(13):3497-500. ClustalW calculates the best match between a query and one or more subject sequences, and aligns them so that identities, similarities and differences can be determined. Gaps of one or more residues can be inserted into a query sequence, a subject sequence, or both, to maximize sequence alignments. For fast pairwise alignment of nucleic acid sequences, the default parameters can be used (i.e., word size: 2; window size: 4; scoring method: percentage; number of top diagonals: 4; and gap penalty: 5); for an alignment of multiple nucleic acid sequences, the following parameters can be used: gap opening penalty: 10.0; gap extension penalty: 5.0; and weight transitions: yes. For fast pairwise alignment of polypeptide sequences, the following parameters can be used: word size: 1; window size: 5; scoring method: percentage; number of top diagonals: 5; and gap penalty: 3. For multiple alignment of polypeptide sequences, the following parameters can be used: weight matrix: blosum; gap opening penalty: 10.0; gap extension penalty: 0.05; hydrophilic gaps: on; hydrophilic residues: Gly, Pro, Ser, Asn, Asp, Gln, Glu, Arg, and Lys; and residue-specific gap penalties: on. ClustalW can be run, for example, at the Baylor College of Medicine Search Launcher website or at the European Bioinformatics Institute website on the World Wide Web.

Changes can be introduced into a nucleic acid molecule, thereby leading to changes in the amino acid sequence of the encoded polypeptide. For example, changes can be introduced into nucleic acid coding sequences using mutagenesis (e.g., site-directed mutagenesis, PCR-mediated mutagenesis) or by chemically synthesizing a nucleic acid molecule having such changes. Such

nucleic acid changes can lead to conservative and/or non-conservative amino acid substitutions at one or more amino acid residues. A “conservative amino acid substitution” is one in which one amino acid residue is replaced with a different amino acid residue having a similar side chain (see, for example, Dayhoff et al. (1978, in *Atlas of Protein Sequence and Structure*, 5(Suppl. 3):345-352), which provides frequency tables for amino acid substitutions), and a non-conservative substitution is one in which an amino acid residue is replaced with an amino acid residue that does not have a similar side chain. Nucleic acid and/or polypeptide sequences may be modified as described herein to improve one or more properties including, without limitation, increased expression (e.g., transcription and/or translation), tighter regulation, deregulation, loss of catabolite repression, modified specificity, secretion, thermostability, solvent stability, oxidative stability, protease resistance, catalytic activity, and/or color.

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

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

Polypeptides can be purified from natural sources (e.g., a biological sample) by known methods such as DEAE ion exchange, gel filtration, and hydroxyapatite chromatography. A polypeptide also can be purified, for example, by expressing a nucleic acid in an expression

vector. In addition, a purified polypeptide can be obtained by chemical synthesis. The extent of purity of a polypeptide can be measured using any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

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

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

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

30 Nucleic acids can be detected using any number of amplification techniques (see, e.g., PCR Primer: A Laboratory Manual, 1995, Dieffenbach & Dveksler, Eds., Cold Spring Harbor

Laboratory Press, Cold Spring Harbor, NY; and U.S. Patent Nos. 4,683,195; 4,683,202; 4,800,159; and 4,965,188) with an appropriate pair of oligonucleotides (e.g., primers). A number of modifications to the original PCR have been developed and can be used to detect a nucleic acid.

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

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

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

hybridization, washing, and exposure conditions. Preferably, the target nucleic acids are on the same membrane.

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

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

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

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

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

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

EXAMPLES

PART A. Materials and Methods

Example 1—Polymerase Chain Reaction

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

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

Example 2—Plasmid Construction by Ligation

5 50-100 ng of restriction enzyme digested plasmid and 3X molar excess of PCR amplified inserts were incubated in the presence of T4 DNA ligase (New England Biolabs). Ligation was carried out at 16°C for greater than 2 hr. 2 µl of ligation reaction was transformed into DH10B electrocompetent *E. coli* cells

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

10 1.5-2 µl of ligation mixture was transformed into 20 µl of ElectroMax DH10B T1 Phage-Resistant Competent Cells (Invitrogen, Cat # 12033-015) by electroporation using ~~MicroPulser~~ MICROPULSER™ (BioRad) set at 1.7 kV using a 1 mm gap cuvette (BioRad, Cat # 165-2089); after a pulse 1 ml SOC was added to cells and cells were incubated at 37°C for 1 h with shaking at 15 200 rpm. 10 µl of recovery mixture was plated on LB agar plates containing ampicillin at a concentration of 100 µg/ml. Plates were incubated overnight at 37°C.

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

20 Plasmid DNA was digested with either PmeI restriction endonuclease (New England BioLabs, Cat # R0560L) in 1x ~~CutSmart~~ CUTSMART® Buffer for 1-4 hours at 37°C or SfiI restriction endonuclease in 1x ~~CutSmart~~ CUTSMART® Buffer for 1-4 hours at 50°C (New England BioLabs, Cat # R0123L). Linearized plasmid was gel purified from a 0.8% agarose gel using ~~ZymoClean~~ ZYMOCLEAN™ Gel DNA Recovery Kit (Zymo Research, Cat # D4002). DNA was eluted in 20 µl H₂O.

25

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

30 Selected strains of *P. pastoris* were grown to mid-exponential growth phase (~2 OD) in 25 ml YPD medium. Cells were collected by centrifugation at 930 x g for 15 minutes. The cell pellet was resuspended in 2 ml of a solution of 80% YPD and 200 mM HEPES, pH 6.8. 75 µl of 1 M DTT was added. The resuspended cell pellet was mixed at 100 rpm at 30°C for 25 minutes.

A 40 ml volume of ice cold, sterile water was added to the suspension, and the cells were collected by centrifugation at 1125 x g for 15 minutes and placed on ice. The cell pellet was resuspended in 40 ml ice cold water and collected as before for two additional wash steps. The cell pellet was then resuspended in 20 ml of ice cold 1 M sorbitol and collected by centrifugation as before. The final cell pellet was suspended in 0.3 ml ice cold, sterile sorbitol, aliquoted and frozen at -80°C.

Example 6—Transformation into *P. pastoris*

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

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

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

F&R Ref No.: 38767-0048US1

Example 8—Shake Flask Medium

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

BMY Media Components

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

10

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

Low-Osmolarity Medium for Shake Flask

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

5

Example 9—Fermentation Medium and Feeds

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

10

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

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

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

15

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

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

5

Vitamin solution recipe

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

Alternatively, trace metals PTM1 can be made as follows:

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

10

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

5

Glycerol feed solution

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

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

10

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

Seed Shake Flask Protocol

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

25

Fermentation Protocol

The fermentation medium and feeds were prepared as described herein. The initial volume should be about 40% of the maximum fermenter volume, e.g., 4 L, if the maximum working volume of the fermenter is 10 L. This is because the process will approach the maximum working volume by the end of the fermentation. The fermenter is inoculated with shake flask seed at 10% inoculum-fermenter ratio, e.g. if 4 L of initial media are present in the fermenter, the fermenter is inoculated with about 0.4 L of shake flask seed. The total volume in the fermenter at this point is referred to T0 volume, e.g. 4.4 L in this representative example. Process controls include the following: 30°C temperature; dissolved oxygen controlled by agitation-aeration cascade to maintain a 20% saturation set point; and pH controlled via addition of 28% NH₄OH, the set point will depend on the phase of the process.

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

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

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

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

PART B. Construction of Production Strains

Production Strain MXY0183

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

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

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

F&R Ref No.: 38767-0048US1

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

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

5

~~Phusion~~ **PHUSION®** High-Fidelity DNA Polymerase (New England BioLabs, Cat # M0530L) was used to amplify genes from genomic DNA. PCR products were obtained and purified using DNA Clean&Concentrator-5 (Cat # D4004) and DNA was eluted in 25 µl of H₂O. Vector DNA, pGAZ and pGAN, and PCR products were digested with BsaI (New England BioLabs, Cat # R0535S) in 50 µl reaction volume at 37°C.

10

Linearized vectors and digested PCR products were purified from 0.8% agarose gel using ~~Zymo clean~~ **ZYMOCLEAN™** Gel DNA Recovery Kit (Zymo Research Cat # D4002). DNA was eluted in 20 µl H₂O. Ligation reactions were set up in 10 µl at 16°C overnight using T4 DNA Ligase (New England BioLabs, Cat # M0202S).

15

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

Digested products were purified from 0.8% agarose gel using ZymoClean ZYMOCLEAN™ Gel DNA Recovery Kit (Zymo Research Cat # D4002). DNA was eluted in 20 µl H₂O.

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

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

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

15

F&R Ref No.: 38767-0048US1

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

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

Assembling Paox1_UPS_FDHTerm- Paox1_UPD_FDHTerm-Paox1_CPO_FDHTerm. on pGAN plasmid (pMx327)

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

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

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

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

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

Obtained PCR products were purified using DNA Clean&Concentrator-5 (Zymo Research, Cat # D4004) and DNA was eluted in 25 µl of H₂O.

F&R Ref No.: 38767-0048US1

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

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

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

10 Digested vector and PCR products were gel purified from 0.8% agarose using ~~ZymoClean~~ ZYMOCLEAN™ Gel DNA Recovery Kit (Zymo Research, Cat # D4002). DNA was eluted in 20 µl H₂O.

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

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

25 *Assembling Paox1_ALAsynthase_FDH1term.- Paox1_PPGoxidase_FDH1term- Paox1_Fc_FDH1term.- Paox1_PBD_FDH1term cassette (pMx330)*

a. PCR amplification of gene-cassettes:

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

30

F&R Ref No.: 38767-0048US1

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

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

5

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

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

10

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

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

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

15

~~Phusion~~ PHUSION® High-Fidelity DNA Polymerase (New England BioLabs, Cat # M0530L) was used to amplify DNA from plasmids. The PCR products were obtained and purified using DNA Clean&Concentrator-5 (Zymo Research, Cat # D4004) and DNA was eluted in 25 µl of H₂O.

5 b. Preparation of vectors

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

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

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

15 c. Making intermediate constructs and assembling a final cassette

Digested vector and PCR products were gel purified from 0.8% agarose using ~~ZymoClean~~ ZYMOCLEAN™ Gel DNA Recovery Kit (Zymo Research, Cat # D4002). DNA was eluted in 20 µl of H₂O.

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

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

30 1.5 µl of ligation mixture was transformed into 20 µl of ElectroMax DH10B T1 Phage-Resistant Competent Cells (Invitrogen, Cat # 12033-015) by electroporation using ~~MicroPulser~~ MICROPULSER™ (BioRad) set at 1.7 kV; cells were incubated at 37°C in 1 ml SOC for 1 h

with shaking at 200 rpm. 10 µl of recovery mixture was plated on LB agar plates containing ampicillin at a concentration of 100 µg/ml. Plates were incubated overnight at 37°C. Colonies were screened by colony PCR for the presence of the insert. The sequences of junctions between the vector and the inserts were confirmed.

5

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

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

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

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

A plasmid containing two copies of the soybean LegH gene (sequence optimized for *P. pastoris*; SEQ ID NO:3) under the control of the pAOX1 promoter designated pMX282 (Figure
20 2ii and Figure 3) was linearized using SfiI restriction enzyme. Linearized plasmid was purified from a 0.8% agarose gel as described and transformed into the *P. pastoris* strain containing ALAD, generating the strain MXY0118 (Figure 3). qPCR was used and determined that strain MXY0118 contained several copies of the LegH gene, likely due to concatamerization of the plasmid, pMX282, at the time of recombination.

25 Plasmid pMX327 (Figure 2iii and Figure 3) containing genes encoding Uroporphyrinogen III synthase (UPS), Uroporphyrinogen III decarboxylase (UPD) and Coproporphyrinogen III oxidase (CPO) (the enzymes catalyzing steps 4, 5 and 6, respectively) under control of the AOX1

promoter was linearized with the SfiI restriction endonuclease and introduced into MXY0118, yielding strain MXY0170 (Figure 3).

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

Production Strain MXY0207

Example 14—Construction of pGAB Expression Vector

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

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

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

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

The purified BSD PCR product and pGAZ vector were digested with 10 units each of the MluI and BbvCI restriction endonucleases (New England Biolabs, Ipswich, MA) for 1 hour at 37°C in 1x NEBuffer 3.1 (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, 100 µg/ml BSA, pH 7.9 @ 25°C). Digested DNA products were recovered by gel electrophoresis as described above.

5 The purified, MluI and BbvCI digested BSD product and pGAZ vector were incubated with 400 units of T4 DNA ligase (New England Biolabs) in 1x T4 DNA ligase reaction buffer (50 mM Tris-HCl, 10 mM MgCl₂, 1 mM ATP, 10 mM DTT, pH 7.5 @ 25°C) in a 20 µl reaction, at 16°C for 2 hours in a 20 µl reaction. Electrocompetent *E. coli* DH10B cells were transformed with 2 µl of the ligation reaction and antibiotic resistant transformants were selected on LSB agar
10 plates supplemented with 100 µg/µl ampicillin.

Example 15—Construction of Mxr1 Expression Vector

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

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

25 The amplified Mxr1 PCR product and the pGAB vector were digested with 10 units of NotI restriction endonuclease (New England Biolabs) for 1 hour at 37°C in 1x NEBuffer 3.1 (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, 100 µg/ml BSA, pH 7.9 @ 25°C). Following digestion, the NotI-digested pMx352 vector was treated with 5 units Antarctic phosphatase (New England Biolabs) for 15 minutes at 37°C in 1x Antarctic phosphatase buffer (50 mM Bis-Tris-
30 Propane-HCl, 1 mM MgCl₂, 0.1 mM ZnCl₂, pH 6 @ 25°C).

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

The NotI-digested fragment containing Mxr1 open reading frame was introduced into pGAB at a NotI site immediately downstream of the AOX1 promoter by ligation. A mixture containing 137 ng of NotI-digested DNA encoding the Mxr1 open reading frame and 60 ng of NotI-digested, phosphatase-treated pMx352 was incubate with 400 units of T4 DNA ligase (New
10 England Biolabs) in 1x T4 DNA ligase reaction buffer (50 mM Tris-HCl, 10 mM MgCl₂, 1 mM ATP, 10 mM DTT, pH 7.5 @ 25°C) in a 20 µl reaction, at 16°C, for 2 hours in a 20 µl reaction. Electrocompetent E. coli DH10B cells were transformed with 2 µl of the ligation reaction and antibiotic resistant transformants were selected on LSB agar plates supplemented with 100 µg/µl ampicillin. Plates were incubated overnight at 37°C. Colonies were screened for the presence of
15 the insert by PCR using primers MxO0495 and MxO0496. The sequence of the final vector was confirmed by DNA sequencing.

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

Example 16—Construction of Native Mxr1 Expression Vector

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

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

PCR products were obtained and purified using DNA Clean&Concentrator-5 and DNA was eluted in 12 μ l of H₂O. The purified PCR products were then combined and used as a
5 template for a subsequent round of PCR amplification using primers MxO0617 and MxO0618. The resulting PCR product was composed of the 3' end of the AOX1 promoter, followed by the Mxr1 open reading frame, the AOX1 terminator, a short linker sequence, and the 5' end of the AOX1 promoter. The PCR product was obtained and purified as described herein. The purified PCR product was cloned into the pCR™-Blunt II-TOPO® vector using the Zero Blunt® TOPO®
10 PCR Cloning Kit (Invitrogen, Cat # K2800-20) to create the pMX402 vector.

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

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

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

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

25

Production Strain MXY0291

Example 18—Construction of Strains MXY0213 and MXY0260

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

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

A plasmid containing a different *Pichia pastoris*-codon optimized variant of soybean LegH gene (variant 3; SEQ ID NO:5) under the control of the pAOX1 promoter designated pMX399 was used as a source of template for PCR amplification of the gene. The backbone from TOPO cloning plasmid pMX401 was PCR amplified. The insert and vector were assembled using ~~Gibson assembly~~ GIBSON ASSEMBLY® (NEB ~~Gibson assembly~~ GIBSON ASSEMBLY® kit) to generate plasmid pMX422. This plasmid was used as a template for a subsequent round of PCR amplification using primers MxO0617 and MxO0618 shown below.

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

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

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

Example 20—Curing Transformants of Plasmid Vectors Bearing Selection Markers

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

Production Strains MXY0330, MXY0333, and MXY0338

15 Example 21—Construction of Strain MXY0306

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

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

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

Example 22—Linear Constructs for Hybrid Promoter Strains

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

Primers used to amplify the linear constructs

Primer designation	Sequence	SEQ ID NO:
MXO0718	GAGCTTCTTCTACGGCCCCC	53
MXO0723	TCCAGCAGAGTAAAATTCCTAGGGAC	54
MXO0724	CTCTTTTAGGTTTAAAGTTGTGGGAACAGTAACA	55
MXO0729	GTGGGTGCTTCTTTGCGTGG	56
MXO0730	AGAATTGCCATCAAGAGACTCAGGACT	57
MXO0735	GATAGAGAGAAATCGCAAACCTTTGAGAGGAAG	58

15

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

Example 23—Nucleic Acid Sequences

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

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GCTTATTTGTCTGTCAGACATGTACTCGAGCATTGCTCGTCAGGAACACTTGAAACGACACGAA
 AGGTCTCACACCAAGGAGAAACCTTTCAGCTGCGGCATTGTTCTCGTAAATTCAGCCGTCGAGA
 TCTGTTATTGAGACATGCCCCAAAACCTGCACAGCAACTGCTCTGATGCGGCCATAACAAGACTAA
 5 GCGCAAGGCAACTCGTCGGTCTTCTAATGCCGCGGGTTCCATATCTGGTCTACTCCGGTGACA
 ACGCCAAATACTATGGGTACGCCCCGAAGATGGCGAGAAACGAAAAGTTCAGAACTGGCCGGCCG
 CCGGGACTCAAATGAACAGAACTGCAACTGCAACAACAACATCTACAGCAACAACCACAGTTGC
 AATACCAACAATCTCTTAAGCAGCATGAAAATCAAGTCCAGCAGCCTGATCAAGATCCATTGATA
 TCCCCGAGAATGCAATTATTCAATGATTCCAACCATCACGTAAACAATTTGTTTGATCTTGACT
 10 AAGAAGAGCTTCCTTCTCCGCCGTTAGTGGAATAATTATGCCCATTTATGTGAATAATTTTCAAC
 AAGATGCCTCTTCTACCAATCCAAATCAAGATTCAAATAATGCCGAATTTGAGAATATTGAATTT
 TCTACCCCAACAATGATGCCCGTTGAAGATGCTGAACTTGGATGAACAACATGGGTCCAATTCC
 GAACTTCTCTCTCGATGTGAACAGGAACATTGGTGATAGCTTTACAGATATACAACACAAGAATT
 CAGAGCCTATTATATCCGAACCGCCCCAAGGACACCGCTCCAAACGACAAGAAGTTGAATGGCTAC
 TCTTTTACGAAGCCCCCATCAAGCCATTAGAATCCCTATTTTCTGTGAGGAATACAAAGAGAAA
 15 CAAGTATAAAACAAATGACGACTCTCCAGACACCGTGGATAATAACTCCGCACCGGCTGCTAATA
 CCATTCAAGAAGTTGAGTCTTCTTTGAATGCATCCAAGAATTTTGGCTTGCCAAGTGGTTATTCC
 TTCTATGGTAATTTGGACCAACAGACTTTCTCTAACACGTTATCATGCACTTCTTCTAATGCCAC
 AATTTGCCCCATTCTACTCGATAACTCCATTAATAATAACTCCACTAGTGACGTGAGACCAGAAT
 TTAGAACACAAAGTGTACCTCTGAAATGAGTCAAGCCCCCTCCCCCTCTCAAAAAACAACCTCG
 20 AAATATTCACCGAAGTTCTTTTACCAGCAACATGCGGTCGTTTATTCACCTACGCTCTTTCCAA
 GTATCCTTTTATTGGTGTGCCCACTCCAACCTCTCCGGAGAACGAAAGACTAAATGAATATGCTG
 ATTCATTCACCAACCGTTTCTTAAATCATTATCCTTTCATACATGTCACGATTCTCAAAGAATAC
 TCCCTTTTCAAGGCAATTTTAGATGAGAATGAGTCGACTAAGAACTGGGAAAATAATCAGTTTTA
 CTTAGAGAACCAACGAATATCAATTGTTTGTCTTCTTCTTGGTGGCTACGATAGGTGCAGTAC
 25 TATCAAACAACAAAAGGATGCTTCGAATTTATACGAAGCTTCAAGGCGTTGTATTCATGTTTAC
 TTAGATTCCAGGAAAAAGATACCCACTTCCTTGTCGCAAAATAACAATGACTCTCCACTTTGGCT
 AATTCAATCCCTGACGTTATCTGTTATGTATGGGTATTTGCGGACAATGACATTAGTTTGAATG
 TCGTGATCAGACAAGTTAACGCACTTAATTCTCTGGTCAAGACTTCGGGCCCTGAATAGGACCTCA
 ATTATAGATCTTTTCAACATCAACAAACCTTTGGATAATGAACTCTGGAATCAATTCGTGAAAAT
 30 AGAGTCCACCGTAAGGACAATCCACACGATTTTTCAAATCAGTTCCAACCTAAGCGCCTTGTA
 ATATTATTCATCGTTGAAAATTGATGACCTAATGATTACTCTACCAGTTCCCAACAACACTTTGG
 CAAGCTGATTCTTTTGTGAAATTCAAAGTCTAAGTTACGGAAATCAGATCCCTTTTCAATATAC
 AAGAGTACTACAGAATTTGATTGATTACAATCAGCCATTGAGCGATGGAAAATTTTTGTATGAAA
 ACCATGTAAGTGAGTTTGGACTCATATGCCTACAGAATGGTCTACACCAATACAGCTATTTCCAA
 35 AAATTGACTGCTGTCAATAACAGAGAAGATGCGCTATTCACAAAGGTTGTTAATTCACCTCACAG
 TTGGGATAGGATGATTTGCAATTCTGATTTGTTTCCAAAGAAGATATATCAGCAGAGTTGCTTGA
 TTTTGGACTCAAAGTTGCTTAATAATTTTCTGATTGTCAAGAGCTCATTGAAAGTTTCGACCGGA
 GACGTTAGTTCTTTGAATAAGTTAAAAGAAAACGTGTGGCTTAAAACTGGAATCAAGTGTGTGC
 TATCTATTATAACAGCTTCATGAACATTCTGCTCCAGTATTCAAAGAAGTACAATGACATAG
 40 AGTTTGTGGATGACATGATTAATTTGAGTCTAATCATCATCAAGATTATGAACTCATTTTCTAT
 AACAAATGTCAAAGACAATTATGAGGATGAAAATGACTTCAAATTGCAAGAGTTAAATTTAACATT
 TGACAATTTTATGAGAAAATATCCTTGAATTTGACAATATATTTCGATATATTTTGTATGATCT
 ACAAGATAATTACCAATTACGAAAAGTTTATGAAGATCAAACACAAGTTAATTACTACAATTCT
 AATTCGAATATAAGCTTCTTGCATCATTTGCAACTCTCCTCGGTTATCAATAACACCCAAATGAA
 45 CCAGAATGATTATATGAAAACAGATATTGATGAAAAGCTTGATCAGCTTTTCCACATCTATCAA
 CATTTTCCGGCTGTATCTGGATTAGAAAAGTTTATGAAGTTCAAATTCACCTATCATGACTTT

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5 GAGACAGAGTTTTCAAGTCTCTCAATATCCAATATACTGAACACTCATGCTGCTTCTAACAATGA
CACAAATGCTGCTGATGCTATGAATGCCAAGGATGAAAAATATCTCCCACAACCTTTGAATAGCG
TATTACTTGCTGATGAAGGAAATGAAATTCCGGTCGTAATAACGATTGAGACCGCCTGTTTCATG
CTGAACGAGCTAATTAATTTTGAAGTAGGTTTGAAATTTCTCAAGATAGGTGAGTCATTTTTTGA
TTTCTTGTATGAGAATAACTACAAGTTCATCCACTTCAAAACTTAAATGACGGAATGTTCCACA
TCAGGATATACCTAGAAAACCGACTAGATGGTGGTGTCTAG

Mxr1 protein sequence (the underlined 6 amino acid at N-term introduced during cloning) (SEQ ID NO:2)
10 MRDRGRMSNLPPTFGSTRQSPEDQSPVPKELSFNGTTPSGKLRLFVCQCTRAFARQEHKLRHE
RSHTKEKPFSCGICSRKFSRRDLLLRHAQKLHSNCSDAAITRLRRKATRRESSNAAGSISGSTPVT
TPNTMGTPEDGEKRKVQKLAGRRDSNEQKLQLQQQHLQQQPQLQYQQSLKQHENQVQOPDQDPLI
SPRMQLFNDSNHHVNNLFDLGLRRASFSAVSGNNYAHYVNNFQQDASSTNPNQDSNNAEFENIEF
15 STPQMMPVEDAETWMNNMGPIPNFSLDVNRNIGDSFTDIQHKNSEPIISEPPKDTAPNDKKLNGY
SFYEAPIKPLESLFSVRNTRKNKYKTNDSDPTVDNNSAPAANTIQELESSLNASKNFCLPTGYS
FYGNLDQQTFSENTLSCTSSNATISPILLDNSINNSTSDVRPEFRTQSVTSEMSQAPPPPQKNNS
KYSTEVLFSTNMRSFIHYALSKYPFIGVPTPTLPENERLNEYADSFTNRFLNHYPFIHVTILKEY
SLFKAILDENESTKNWENNQFYLENQRISIVCLPLL VATIGAVLSNNKKDASNL YEASRRCIHVY
LDSRKKIPTSLSANNNDSPWLIIQSLTSLVMYGLFADNDISLNVVIRQVNALNSLVKTSGLNRTS
20 IIDLFNINKPLDNELWNQFVKIESTVRTIHTIFQISSNLSALYNIIPSLKIDDLMITLPVPTTLW
QADS FVKFKSLSYGNQIPFQYTRVLQNLIDYNQPLSDGKFLYENHVSEFGLICLQNLGHQYSYFQ
KLTAVNNREDALFTKVNSLSHSDRMISNSDLFPKKIYQQSCLILDSKLLNFLIVKSSSLKVSTG
DVSSLNKLKENVWLKNWNQVCAIYNSFMNIPAPSIQKKYNDIEFVDDMINLSLIIKIMKLIFY
NNVKDNYEDENDFKLQELNLTFDNFDEKISLNL TILFDIFLMIYKIIITNYEKFMKIKHKFNYYNS
25 NSNISFLHHFELSSVINNTQMNQNDYMKTDIDIDEKLDQLFHIYQTFFRLYLDLEKFMKFKFNHDF
ETEFSSLSISNINLTHAASNNDTNAADAMNAKDEKISPTTLNSVLLADEGNENSGRNNDSDRLF
LNELINFEVGLKFLKIGESFFDFLYENNYKFIHFKNLNDGMFHIRIYLENRLDGGV*

Pichia pastoris-Codon-optimized LegH nucleic acid sequence (SEQ ID NO:3)
30 ATGGGTGCTTTTACCGAGAAGCAGGAAGCACTTGTTCCTCTTCGTTCTGAAGCTTTTAAGGCTAA
CATCCCTCAATACTCTGTTGTGTTTTACACGTCCATTCTAGAAAAAGCTCCTGCTGCCAAGGACC
TCTTCTCTTTTCTGTCCAACGGTGTAGATCCATCCAATCCCAAATTAACAGGTCACGCTGAGAAA
TTGTTTCGGTTT TAGTCAGAGATAGCGCTGGACAATTGAAAGCAAATGGTACTGTGGTTGCTGATGC
TGCCTTGGGCAGCATCCATGCACAGAAGGCAATTACAGACCCACAATTTGTTGTTGTGAAGGAAG
35 CTCTGCTTAAAACTATAAAGGAAGCCGTCGGAGACAAATGGAGTGACGAGTTGTCATCAGCTTGG
GAGGTAGCTTATGATGAGTTGGCCGCAGCAATCAAAAAGGCATTCTAA

Pichia pastoris-Codon-optimized LegH amino acid sequence (SEQ ID NO:4)
40 MGAFTEKQEALVSSSF EAFKANIPQYSVVFYTSILEKAPAAKDLFSFLSNGVDPSNP KLTGHA EK
LFGLVRDSAGQLKANGTVVADAALGSIHAQKAITDPQFVVVKEALLKTIKEAVGDKWSELS SAW
EVAYDELA
IKKAF

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

F&R Ref No.: 38767-0048US1

TTTTCTCTTTCTTGTCTGAACGGCGTGGATCCCTCAAATCCTAAGCTGACTGGTCACGCCGAGAAG
CTTTTTGGTTTGGTCAGAGACAGCGCCGGACAGCTGAAAGCTAACGGTACAGTTGTGGCAGATGC
TGCCTTGGGATCTATACATGCACAAAAGGCTATCACCGACCCACAGTTTGTGGTTGTAAAAGAGG
CTCTACTCAAACTATCAAGGAAGCAGTTGGTGACAAATGGAGCGATGAATTGTCCAGTGCATGG
5 GAGGTCGCTTACGATGAGTTAGCTGCTGCAATCAAAAAGGCTTTCTAA

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

MGAFTEKQEALVSSSF EAFKANIPQYSVVFYTSILEKAPAAKDLFSFLSNGVDPSNPKLTGHA EK
LFGLVRDSAGQLKANGTVVADAALGSIHAQKAITDPQFVVVKEALLKTIKEAVGDKWSDELSSAW
10 EVAYDELAAAIKKAF

***Pichia pastoris* pAOX1 promoter (SEQ ID NO:7)**

GATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTTGCCATCCGACATCCACAGGTCCAT
TCTCACACATAAGTGCCAAACGCAACAGGAGGGGATACACTAGCAGCAGACCGTTGCAAACGCAG
15 GACCTCCACTCCTCTTCTCCTCAACACCCACTTTTGCCATCGAAAAACCAGCCCAGTTATTGGGC
TTGATTGGAGCTCGCTCATTCCAATTCCTTCTATTAGGCTACTAACACCATGACTTTATTAGCCT
GTCTATCCTGGCCCCCTGGCGAGGTTTCATGTTTGTATTTCGAATGCAACAAGCTCCGCATT
ACACCCGAACATCACTCCAGATGAGGGCTTTCTGAGTGTGGGTCAAATAGTTTCATGTTCCCCA
AATGGCCCAAACTGACAGTTTAAACGCTGTCTTGGAACCTAATATGACAAAAGCGTGATCTCAT
20 CCAAGATGAACTAAGTTTGGTTGCTTGAATGCTAACGGCCAGTTGGTCAAAAAGAACTTCCAA
AAGTCGGCATAACCGTTTGTCTTGTGTTGGTATTGATTGACGAATGCTCAAAAATAATCTCATTAAT
GCTTAGCGCAGTCTCTCTATCGCTTCTGAACCCCGGTGCACCTGTGCCGAAACGCAATGGGGAA
ACACCCGCTTTTTTGGATGATTATGCATTGTCTCCACATTGTATGCTTCCAAGATTCTGGTGGGAA
TACTGCTGATAGCCTAACGTTTCATGATCAAAATTTAACTGTTCTAACCCCTACTTGACAGCAATA
25 TATAAACAGAAGGAAGCTGCCCTGTCTTAAACCTTTTTTTTTTATCATCATATTAGCTTACTTTC
ATAATTGCGACTGGTTCCAATTGACAAGCTTTTGATTTTAAACGACTTTTAAACGACAACTTGAGAA
GATCAAAAACAACCTAATTATTCGAAACG

***Pichia pastoris* pGAP promoter (SEQ ID NO:8)**

CGACTATTATCGATCAATGAAATCCATCAAGATTGAAATCTTAAAATTGCCCTTTCACTTGACA
GGATCCTTTTTTGTAGAAATGTCTTGGTGTCTCGTCCAATCAGGTAGCCATCTCTGAAATATCT
GGCTCCGTTGCAACTCCGAACGACCTGCTGGCAACGTAAAATTTCTCCGGGGTAAAACCTTAAATGT
GGAGTAATGGAACCAGAAACGTCTCTTCCCTTCTCTCTCCTTCCACCGCCCGTTACCGTCCCTAG
GAAATTTTACTCTGCTGGAGAGCTTCTTCTACGGCCCCCTTGCAAGCAATGCTCTTCCCAGCATTA
35 CGTTGCGGGTAAAACGGAGGTGCTGTACCCGACCTAGCAGCCCAGGGATGGAAAAGTCCCGGCCG
TCGCTGGCAATAATAGCGGGCGGACGCATGTCATGAGATTATTGGAACCACCAGAATCGAATAT
AAAAGGCGAACACCTTTCCCAATTTTGGTTTCTCCTGACCCAAAGACTTTAAATTTAATTTATTT
GTCCCTATTTCAATCAATTGAACAACCTATCAAAACACG

***Pichia pastoris* pGCW14 promoter (SEQ ID NO:9)**

CAGGTGAACCCACCTAACTATTTTTTAACTGGGATCCAGTGAGCTCGCTGGGTGAAAGCCAACCAT
CTTTTGTTCGGGGAACCGTGCTCGCCCCGTAAAGTTAATTTTTTTTTTCCCGCGCAGCTTTAATC
TTTCGGCAGAGAAGGCGTTTTTCATCGTAGCGTGGGAACAGAATAATCAGTTCATGTGCTATACAG
GCACATGGCAGCAGTCACTATTTTGTCTTTTAACTTAAAGTCGTTTCATCAATCATTAAC TGACC
45 AATCAGATTTTTTGCATTTGCCACTTATCTAAAAATACTTTTGTATCTCGCAGATACGTT CAGTG
GTTTCCAGGACAACACCCAAAAAAGGTATCAATGCCACTAGGCAGTCGGTTTTATTTTTTGGTCA

F&R Ref No.: 38767-0048US1

CCCACGCAAAGAAGCACCCACCTCTTTTAGGTTTTAAGTTGTGGGAACAGTAACACCGCCTAGAG
CTTCAGGAAAAACCAGTACCTGTGACCGCAATTCACCATGATGCAGAATGTTAATTTAAACGAGT
GCCAAATCAAGATTTCAACAGACAAATCAATCGATCCATAGTTACCCATTCCAGCCTTTTCGTCG
TCGAGCCTGCTTCATTCCTGCCTCAGGTGCATAACTTTGCATGAAAAGTCCAGATTAGGGCAGAT
5 TTTGAGTTTAAATAGGAAATATAAACAAATATACCGCGAAAAAGGTTTGTATTATAGCTTTTCGC
CTGGTGCCGTACGGTATAAATACATACTCTCCTCCCCCCCCCTGGTTCTCTTTTCTTTTGTTACT
TACATTTTACCGTTCCGTCACTCGCTTCACTCAACAACAAAA

Pichia pastoris pTEF1 promoter (SEQ ID NO:10)
10 ATAAGTGTGCGCTCTTTTATCTGCCGCACTGCATGAGGTGTCCCCTTAGTGGGAAAGAGTACTGA
GCCAACCTGGAGGACAGCAAGGGAAAAATACCTACAACCTTGCTTCATAATGGTCGTAAAAACAA
TCCTTGTGCGATATAAGTGTGTAGACTGTCCCTTATCCTCTGCGATGTTCTTCTCTCAAAGTT
TGCGATTTCTCTCTATCAGAATTGCCATCAAGAGACTCAGGACTAATTTTCGAGTCCCACACGCA
CTCGTACATGATTGGCTGAAATTTCCCTAAAGAATTTCTTTTTCACGAAAAATTTTTTTTTTACAC
15 AAGATTTTCAGCAGATATAAATGGAGAGCAGGACCTCCGCTGTGACTCTTCTTTTTTTTCTTTT
ATTCTCACTACATACATTTTAGTTATTCGCCAAC

Heme biosynthesis enzyme 1- ALA Synthase (SEQ ID NO:11)
20 ATGGAGTTTGTGCGCCGTCAGTCCATGAATGCCTGTCCCTTTGTCAGGTCAACTTCTACCCACCA
TTTGAAGAAGTTGGCAGCAAACAGTTCTCTAGCTGCTACTGCTAGTCATTGTCCCGTGGTTGGCC
CTGCTCTCCAACAGCAGAGATACTACTCTCAACCTTCCAAGCCAGCCCAAGCCCAACCTCCGAC
ATTGCTACTGGGATCAAGAAGGATGTTTCTCCGATCCGATGGACTCTAATGAAACCGCCTTTGA
TTACAATGGAATGTATGAGTCTGATCTTGCGAATAAACGTAAAGATAACTCGTATCGTTATTTCA
ATAACATCAACCGTCTAGCCAAGGAGTTTCCCAAGGCACATCGCCAGACCGAAGATGACAAGGTG
25 ACCGTCTGGTGCTCTAACGACTACTTAGGAATGGGTAGGCATCCTGAGATTATCAAAACCATGAA
GGCTACCATGGACAAGTACGGTTCCGGAGCAGGAGGAAGTACGAGGTGATAACACG
CCGCTATCAATTTGGAAAGCGAGTTGGCTTGCTTGAAACAAGAAGGAAGCGGCTCTGGTGTTTTCA
TCATGTTTCATAGCTAACGATGCAATCATCTCGTTGTTGGGACAAAAAATCAAAATTTGGTCAT
TTTCTCTGACCAGTCGAATCATGCTTCCATGATATTGGGTGTGCGTAACTCCAAAGCGAAGAAGC
30 ACATCTTCAAGCACAACAATTTGAAGGATCTGGAGTCGCAGTTAGCTCAGTACCCCAAGTCGACT
CCTAAACTGATCGCCTTCGAGTCAGTTTACTCTATGTGTGGATCTGTGGCTCCCATTGAGAAGAT
TTGCGATTTGGCTAAAAGGTACGGTGCCCTCACCTTCTTGATGAAGTTCATGCTGTTGGAATGT
ATGGTCCTCATGGACAGGGTGTAGCTGAGCATTTGGACTTTGATCTGCATTTACAGTCTGGAATC
GCCAGTCCTAGCGTGGTGGACAAACGCACCATATTGGATCGTGTGACATGATTACTGGTACTTG
35 CGGAAAGTCATTTGGTACTGTTGGAGGTTACGTTGCTGGTAGTGCCAACCTAATTGATTGGTTAA
GATCCTATGCGCCAGGTTTCATTTTCACTACCACACTTCCCTCCTGCTATCATGGCTGGTACAGCC
ACTTCTGTTTCGTATTGTTAGGGCCGACATTGAGGCCCGTATCAAGCAACAGCTTAATACTCGCTA
CGTCAAAGACTCATTTGAAAACCTTGGTATTCCAGTCATTCCAAACCCAAGTCACATTGTTCCCTG
TTCTAGTTGGAAATGCTGCAGATGCCAAGAAGGCATCCGATATGTTAATGAACAAACACCGTATT
40 TATGTTCAAGCTATTAATACTACCCTACTGTGCCTGTGCGTGAAGAACGACTAAGGATTACTCCTAC
TCCAGGTCATGGAAAGGAGATTTGTGACCAGCTGATCAGCGCTGTCGACGATGTTTTTACTGAGC
TTAATTTACCAAGAATCAACAAATGGCAGTCCCAAGGTGGTCATTGCGGTGTTGGTGATGCTAAT
TACGTACCAGAACCCAATCTGTGGACTCAGGACCAGCTCAGCTTGACAAACCAAGACTTGCACTC
CAATGTGCACAACCCAGTGATTGAGCAGATCGAAACCTCATCAGGAGTCAGATTGTAG
45

F&R Ref No.: 38767-0048US1

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

ATGGTGCATAAGGCTGAATACTTGGACGACCACCAACTCAGATTTCCAGCATTCTTTTCAGGAGG
TTACAACCACCCATTACTTCGTGAATGGCAACATGAACGTCAACTCAACAAAAACATGTTTCATCT
TTCCCTGTTTGTACAGATCGACCAGACGAAGAAGAACTTATTCCTAGTCTACCTAATATCAAG
5 AGGTTTGGCGTTAACAAGTTGATTCCCTTATGTAGGAGGTTTGGTTTCCAAAGGATTGAGGGCGGT
GATCCTATTTGGTGTTCCTCTGAAGCCCGGTGTGAAAGATGAAGAAGGAACGGCCGCTGATGATC
CAGAGGGACCTGTTATCCAAGCCATCAAACACTTGAGAAAGAACTTTCTGACCTGTATATCATC
ACCGATGTCTGTCTATGTGAGTACACCAGCCATGGACATTGTGGAATACTATATGAGGATGGCAC
TATCAACAGAGAGCTCTCAGTCCGTCGTATTGCTGCTGTAGCTGTCAAATATGCTCAAGCTGGAG
10 CCAACTCTGTGGCTCCTTCTGATATGACTGACGGCAGAATAAGAGATATTAAAGAAGGCTTACTA
AGTGCAGGACTGGCACATAAAACGTTTGTATGTCCACGCTGCAAATTTCTCTGGTAATTTGTA
TGGCCCTTTTCAGAGATGCTGCAGGTTCCCTGTCCATCTCAAGGGGACAGAAAATGTTACCAGCTTC
CTTCTGGAGGAAAAGGGTTGGCCCATCGTGCTCTGATTCTGTGATATGAATGAAGGCACTGATGGA
ATTATTGTCAAACCATCTACATTCTATTTGGACATTGTGCTGATGCTTATCAGCTTTGTAAAGA
15 CTATCCTATCTGCTGTTACCAGGTTTCTGGAGAGTACGCCATGCTACATGCAGCGGCAGAGAAGA
ATATTGTTGATCTGAAATCAATCGCTTTTGAAGCTCATCAAGGATTCTTGCGGGCTGGAGCTCGT
TTAATCATTAGTTACTTTACCCCTGAATTCCTGGAGTGGTTATCTGAATGA

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

20 ATGAACCAATCGAACAGAGCGGACCCATTGATTGCAGTTCCTTGAAATTGGGGTCCCGAAAGTC
CGCTCTGGCTATAATCCAGGCAGAAATCGTCCGCCAATTGATATTGAAAGAATACCCTGAATTGG
AGACGAAGTTGGTCAGTGTGTCCACCCTGGGGGACCAAGTCCAGAATAAAGCACTTTTCACGTTT
GGAGGAAAATCTTTGTGGACCAAAGAAGTTGAGATGTTGTTGTTGGAGAGTGTGGGAGGATTTGA
CCAAATAGACATGATTGTACACTCGTTGAAAGACATGCCAACTCATTTACCAGACGAATTTGAGC
25 TGGGTTGCATTATTGAAAGAGAAGACCCTAGAGACGCTTTGGTCGTGCAAGATGGTTTATCTTAC
AAGTCATTGGCCGACCTTCCAGAGGGAGCTGTGGTCCGTACGTCTTCGGTTAGAAGATCGGCTCA
ACTACTGAAGAATTTCCCTCATCTGAAATTCAAATCTGTTAGAGGAAACCTTCAGACCAGACTAA
GAAAATTAGATGATCCAGATTCCGAGTACTGCTGTCTCCTCCTTGACAGCAGCCGGTTTAAATCAGG
ACAGGCTTACAACACAGAATTTCAATGTATTTGAACGACGATGTGATGTACCACTCCGTCGGACA
30 AGGAGCATTAGGAGTAGAGATCAGAAAAGGTGACCAATTCATGAAAATATCTGTGAAAAGATTG
GGCATAGAACCACCACCCTTCGTTGTCTTGCAGAGAGAGCACTGCTGAGATATCTAGAGGGAGGC
TGCTCGGTGCCAATTGGGGTCTCCACTATTTATAGCGAGGATACGAAGGAAGTTACCATGAACTC
CCTAGTCGTCAGTTGTAACGGTCTGACTCGGTAACAGAATCAATGACTGAAGTCGTGACTACTG
AAGAGCAAGCTGAAGATTTCCGTGAAAGGCTGGCCAGAAAGCTCATAGATCAAGGTGCGAAACGC
35 ATTCTTGACGAGATCAACTTCAACAAGATCAAAGAGATTAAGGAAGAGGGTTTACATTAA

Heme biosynthesis enzyme 4- Uroporphyrinogen III synthase (SEQ ID NO:14)

ATGCCAAAAGCCATTCTTCTGAAGAATAAACTACACCGAAGGATCCTTATCTGGAGAACTTCGT
AAGTAGTGGCTACTCGACCGATTTCTGTAACACTTTTAGATCATATTCACATGGAGAAATCTGAGA
40 TCATCGCATTTCTCAAGACTGACTACTTTTTGCATAAACTTTGGCGTTTATTATTACGTCCCAA
AGAGCTGTAGAAATGCTGAATGAGTGTATGCAAATACTGAGACGTAAGTATCTGAAATTACACA
AATCATCTATAGTAAACCTGTCTATACAGTTGGCCCTGCCACCTACAGAATACTTGCGGATGCTG
GCTTCGTGGATCTACGAGGCGGAGATAAGGCAGGAAACGGATCCATTCTAGCCCAGATAATTTTG
AATGATGACATTTTACACTGGAATTGAAGATTCTGACAAGCATATAACGTTTTTACGGGAGAAAC
45 AAGGAGAGACATAATTCCTAAATGTTTACTCTCTAACAACCTTCAACTTTACGAAAAGATTGTCT
ACAAGACTCTTCTAGGGATGATATCGTGACTAGATTCAAGTCTGCCGTTGACAGCATCGACCAA

F&R Ref No.: 38767-0048US1

TCGCAAAGAAGTTCCAGTTGGGTGGTCTTCTTTTCGCCTCAAGGAACAGAGGACATTGTAACGTA
TCTTCAACACACCAAAGACCAATTTAATATTGCATCTATCGGGCCAACCACAGAAAAATACCTTC
TAAGCAAAAACCTGAAACCAAAAGTTGTGGCACCTAAGCCAGAGCCTATCTCTTTACTATTGTCT
ATACAAAAAGTGCACTAA

5

Heme biosynthesis enzyme 5- Uroporphyrinogen III decarboxylase (SEQ ID NO:15)

ATGAGTAGATTTCCAGAACTGAAGAATGACCTTATTTTAAGGGCAGCTCGTGTTGAAAAAGTTGA
ACGTCCCCCAATATGGATTATGAGACAGGCCGGAAGATATCTTCCGGAGTACCATGAGGTCAAAG
GAGGTAGGGACTTCTTTGAACTTGCAGGGATGCTGAGATTGCTTCTGAAATTACTATCCAGCCG
10 ATTACGCATTTTGACGGTCTGATCGATGCAGCTATTATCTTCAGTGATATCTTGGTGATTCTCTCA
AGCTATGGGCATGGAAGTTAAGATGGTGGACAAAGTTGGCCACAGTTCCCCAATCCGCTAAGAA
AACCGTCTGACTTGGATCATTTGAAAAAGACGTTGACGTTTGAAGGAACTCGATTGGGCCTTC
AAAGCTATCTCATTGACCAGAAAAAACTCAATGGACGAGTGCCTTTGCTTGGATTTTGTGGTGC
TCCTTGGACTCTACTGGTTTATATGACTGAAGGAGGCGGTACCAAGATGTTTCGATTTGCAAAAG
15 AGTGGATCTACAAGTTTACCAAGGAATCTCATCAATTACTCCAACAGATCACTGACGTTGCAGTT
GAATTCTTAGCTCAGCAAGTTGTTGCAGGTGCCCAAATGTTACAAGTTTTTGAATCTTGGGGCGG
TGAATTGGGGCCTGATGAATTTCGATGAGTTTTCTTTGCCTTATTTGAGACAGATTTCTCTAAAC
TTCCCTGAGGTTGAAGGAACCTTGAATCACAGAGAATGTTCCCATAACTGTCTTTGCTAAAGGC
TCTTGGTACGCCTTGGAGCAATTGTGCGACAGTGTTATGATGTTGTCTCGTTGGATTGTTTATT
20 CCGTCCAAGTGATGCTGTCCAGATTGCTAACGGAAGAATCGCATTGCAAGGTAATCTTGACCCTG
GAACCATGTACGGCTCCAAAGAAACCATTTCCAAGAAAGTGGACAAAATGATCAAGGGTTTGGT
GGAGGAAAGCAAACTACATAATTAATTTGGACACGGCACTCATCCATTATGGATCCAGAACA
GATCAGATGGTTCTTACAAGAATGTCATCGCATTTGGATCTCAATAG

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

ATGGCCATCGACTCTGATATCAATCTAAGCTCTCCCAATGATTCCATCCGTCAAAGGATGTTCGA
GCTTATCCAGCGGAAGCAACTCGAAATTGTGCGTGCATTGGAGGCAATTGAAGGAAACGATACCA
AATTTCTGTTCTGATTCTTGGGAAAGAGGAGCCGAAGGTGGAGGAGGAAGATCTATGCTTATTCAA
GATGGAAGAGTGTTTGAAAAGGCTGGTGTAATATTTCCAAGGTTTCATGGCGTATTGCCTCCTCA
30 AGCTGTGAGCCAGATGAGAAATGACCACTCCAAGCTAGATCTGCCTGCGGGAACCTCTCTGAAGT
TCTTTGCCTGTGGGCTTTTCGTTGGTCATTTCATCCCCATAATCCCCATGCTCCAACCTACCCATCTG
AATTATCGCTACTTCGAACTTGGGATGAACTGGAAAGCCTCACACCTGGTGGTTTGGGGGCGG
TGCTGATTTAACGCCTTCGTACCTGTATCCCGAGGATGCCAAGCAATTCCATCAAGCCCATAAGG
ATGCCCTGGACAAACACGATGTTAGCTTGTACCCGAGATTCAAAAAGTGGTGTGATGAATACTTT
35 CTGATCAAACATCGAAATGAACTAGAGGTATTGGGGGTATTTTCTTTGATGATTTTGACGAGTT
TGATGCTGAGAGGTCCCTGAAGTTGGTTGAAGATTGTTTCAATGCTTTCTTGGAAATCTTATCCCG
CTATCACTCGAAAAAGGATGGACACCCCTTCAACTGATGCTGAGAAGAAGTGGCAACAAATTAGA
AGAGGAAGATATGTCGAATTCAACTTAGTATTGGATAGAGGTACTCAATTTGGTTTGAGAACGCC
TGGATCTCGTGTTGAAAGTATTTTGATGTCGTTGCCAAGAACAGCTGGTTGGGTCTATGATCATC
40 ATCCAGAGCCTGGCTCCAGAGAAGAGGAGTTATTGCAGGTACTACAAAATCCTATTGAATGGGTA
TGA

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

ATGCTGAAAAGTCTTGCACCAAATTCCTCAATTGCCGTTTTAGGTTTCAGGGATATCTGGATTGAC
45 TTTCAGCTTTTTTTTTGAATCGGTTGCGTCCCGATGTTAAGATCCATATCTTTGAAAAATCCAAGC
AGGTTGGAGGATGGATCAGATCAGAAGAGCATGAACTTTTCATTTTGAAAAGGGACCCAGAAGT

F&R Ref No.: 38767-0048US1

TTGAGAGGCACAAATACGGGTACCTTGATGTTGTTGGATCTTCTTACCAAGATAGGAGCAAATGA
 CAAGGTCCTGGGACTGCACAAAGATTCTCTTGCTAATAAAAAGTATCTGTTGTCCCGTTCTCAG
 ATGTTTCACGGAAACAACGCAAAGCTTCTTCAAGTGCCACAGGATTTTCTGTTGTAAAGTTC
 ATGTTTGACCCGTTGTCTAAGGATCTCATTCTCGGTCTTTTGAAAGAACCATGGCAACCAAAATT
 5 AAAGTATTCAGATGAGTCGGTTGACCATTTTTTCAACAGAAGATTTGCTACCAAACTATCAGAGA
 ATATCGTCAGCGCAATTGTGCATGGAATCTATGCGGGCGACGTGAAGAAGTTAAGTGTGAAAGCC
 ATCTTCCCTAGGCTCCCTGAGATGGAACAGGAAAGTGGCTCTATTATAAGGTATATGATCGCCCA
 ATACAGGACAAAAAAGAACGTCAAACAAAAAGTTGACCCTTTTTTGGCAGATTATGAAAAATTGA
 TCGGTACATCTTTGAGTTTCAAAAATATTTCTTTGTTTCTGAAAACTTTCCCATGCTGAGTTTT
 10 CAGGGTGGACTACAGAACTTCCCATCTCATTGAAGAACCATTTATCACAGATTGAAAACATCAA
 GTTTTCATTTTGACAGCAAAATCAAAAACATTGCTTTGGAGAGCGGTAAGGTGGCATTGACTGACC
 ATGATCAGGTTTATCTTGTGACCATGTGAGATCTACCATTAATACCAACGAATTGGCCAAAATC
 ATTTACCCCGTTGTTCCAAGTTCTACTAAGAAAAAATCCGTTTTCAAATCCAAAGCGAATGGCCC
 AGGGCTGGTCAAATGTTTGAGCTGGCTACACTATACAAATATACTAATGTGCAACATTTATATAC
 15 CTAAGCACGTCTCAAAATCTATCACCGGATTTGGATACTTGGTTCCTCGATCAATGTCTTCTCAG
 GCATCCAAACTTCTCGGTGTCATATTTGACTCAGACATCGAGACTGCAATGACTCCTAATTTTAC
 AGAGGCCAACATTACGGCGATAAACAGTAACTCTGCATCTCCCAAGCAACTCCAAAAGTTTTCTG
 ACCAATTCGTCAATAATGATCTCCCTAAATACACCAAGTTGACGCTAATGCTTGGAGGTCATTAT
 CTCAAGTCGGAGGCAGACATGCCCCGTTCCGCAGAGAGTAAACATGCTGTCAAGGCGATTCTGTC
 20 AAATCACCTGAATATTGATCTAGATGAGTTTGCATCTTTGCCAGACTTCAAGATGGAAATCACCA
 AGATCCCCAACTGCATTCCCCAATATGAAGTTGGGTATCTTGATCTCAAGAGAAAGGTTTCAAT
 GCAGCCTCCAAAGAGTTCAACGACCAATAAGTTTTGGAGGCATGGCATTGTTGATGGTGTGGG
 GATCCCTGACTGTGTCCAGAATGCATTCAAAGATTTCGGCTACCCTCAGTGGCATTAA

 25 **Heme biosynthesis enzyme 8- Ferrochelatase (SEQ ID NO:18)**
 ATGCTTAACCGTCGTTTCCAATCTACCGTGTCTCGAGTCTGAACAAGGGCACTGGAATAGTGTT
 CATGAATATGGGTGGTCCCTCCACTGTCAAGGAAACCTATGACTTTTTTATTTTCGTCTTTTCTCGG
 ACGGAGATTTAATCCCGTTTGGCAGATTTTCAAGAACATCCTGGCCCGCTTCATTGCAAGTAGAAGA
 ACACCCAAAATTGAATCCTACTACAAAGCTATCGGAGGTGGGTCTCCTATCCGAAAGTGGTCTGA
 30 ATACCAGAGTTCTAAACTATGTGAAAAATTAGACATTATCAGTCCACAATCGGCTCCTCATAAGC
 CTTATGTTGCCTTCAGATACGCTAATCCTCTCACTGAAGATACTTTACAAAAGATGAAAAATGAT
 GGAATTACTAAGGCCATTGCCTTTTCTCAATATCCGCAATTTAGTTATTCAACCACCGGATCATC
 GATTAACGAACTTTACAGGCAATCGAAAATTTTGGACCCTGATCAATCTATTAATGGACAGTTA
 TAGATCGCTGGCCTGACCACCCAGCCTTAGTTAAAACCTTTTCGAGCTCATATCAAAGATACTCTA
 35 AACAGATTCAAACTGAAAATGGACTGACTGACACAAAAGACGTGTCCTCCAATTCAGTGCTCA
 TTCTTTACCAATGGATATTGTCAATAAAGGAGATTTCGTATCCTGCAGAAGTCGCAGCGAGTGTCT
 TTGCCATTATGAAAGAACTTAACTTCTCAAATCCTTATAAAATTAACCTGGCAATCACAGGTTGGC
 CCAAAGCCTTGGCTGGGTGCTCAAACCTGAAAAAATTACCAAGCAGCTAGCATCCAGTGATGTTCC
 TGGAGTCGTTTTGGTTCCTATTGCCTTTACCTCTGATCATATTGAAACTCTCCATGAACTGGATA
 40 TTGAACTGATTCAAGAACTACCTAATCCTTCAAAAGTAAAGCGAGTTGAATCGTTGAACGGAGAC
 CAAACTTTCATTGACTCCTTGGCAGAACTAGTGAAGAGTCACATTGATTGGAAGGTTGTATTTTC
 CAACCAGTTGCCATTGGATTCCATGCTGGGAGTAGTGTGAGATAATTCCCTCACAGATCCAAAAG
 AGTTTTTCAGAGCCCATTGA

Part C. Results and Discussion

Example 24— Characterization of Strain MXY0183

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

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

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

20

Example 25— Characterization of Strain MXY0207

Experiments were then performed to determine the benefits of overexpressing the
transcriptional activator, Mxr1, in the presence of the genes encoding the 8 enzymes involved in
heme biosynthesis. Strain MXY0183, which contains >10 copies of the LegH sequence and the
25 genes encoding the 8 enzymes involved in heme biosynthesis, and sister strains MXY0206 and
MXY0207, which contain >10 copies of the LegH sequence, the genes encoding the 8 enzymes
involved in heme biosynthesis, and the Mxr1 transcriptional activator, were grown in shake flask
cultures in the presence of glycerol, which is a repressing carbon source for these strains.
Photographs of the shake flask cultures after 48 hr are shown in Figure 10A, and photographs of
30 the pellets from cells grown on BMY media for 48 hours with no additional source of carbon are

shown in Figure 10B; these experiments demonstrated that significant expression of transgenes (e.g. heme enzymes) under the control of the AOX1 promoter occurs in the absence of an inducing carbon source when a repressing carbon source is consumed in the growth medium of a strain in which Mxr1 is also expressed from the AOX1 promoter. The relative yield of heme-loaded LegH, when shake flask cultures were grown in the absence of induction agent, is shown in Figure 10C. These experiments demonstrated that significant production of a recombinant, heme-loaded protein is accomplished from AOX1 promoter-driven transgenes in the absence of methanol induction in Pichia strains in which Mxr1 expression is also driven by the AOX1 promoter.

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

Example 26—Characterization of Strain MXY0291

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

Example 27—Characterization of Hybrid Promoter Strains

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

F&R Ref No.: 38767-0048US1

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

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

EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST

TECHNICAL FIELD

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

BACKGROUND

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

SUMMARY

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

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

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

In some embodiments, the exogenous nucleic acid encoding a transcriptional activator comprises a Mxr1 sequence from *Pichia pastoris*, a Adr1 sequence from *Hansenula polymorpha*, a Trm1 sequence from *Candida boidinii*, and a Trm2 sequence from *Candida boidinii*. A representative nucleic acid encoding a transcriptional activator is shown in DQ395124. A
5 representative transcriptional activator has an amino acid sequence shown in ABD57365.

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

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

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

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

F&R Ref No.: 38767-0048US1

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

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

DESCRIPTION OF DRAWINGS

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

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

Figure 3 is a schematic showing the generation of the production strains, MXY0183 and MXY0207, from the parent strain, Bgl1.

Figure 4 are schematics showing plasmids pGAB and pMx354.

Figure 5 is a schematic showing the generation of the antibiotic selection free production strains, MXY0291 and MXY0338, from the parent strain, Bgl1.

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

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

F&R Ref No.: 38767-0048US1

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

Figure 9 shows the production of LegH from the modified *P. pastoris* strains. Panel A is a SDS gel showing lysates from *P. pastoris* strains grown in shake flasks: 51, MXY0051; 118, MXY0118; 183, MXY0183. Panel B is a table comparing LegH production from strains MXY0118, MXY0183, and MXY0207.

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

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

15 DETAILED DESCRIPTION

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

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

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

5 Recombinant nucleic acid molecules used in the methods described herein are typically DNA, but RNA molecules can be used under the appropriate circumstances. As used herein, “exogenous” refers to any nucleic acid sequence that is introduced into the genome of a cell from an external source, where the external source can be the same or a different organism or a nucleic acid generated synthetically. For example, an exogenous nucleic acid can be a nucleic acid from
10 one microorganism (e.g., one genus or species of methylotrophic yeast) that is introduced into a different genus or species of methylotrophic yeast, however, an exogenous nucleic acid also can be a nucleic acid from a methylotrophic yeast that is introduced recombinantly into a methylotrophic yeast as an additional copy despite the presence of a corresponding native nucleic acid sequence. For example, *P. pastoris* contains an endogenous nucleic acid encoding a Mxr1
15 transcriptional activator; an additional *P. pastoris* Mxr1 nucleic acid (e.g., introduced recombinantly into *P. pastoris*) or modifying the endogenous *P. pastoris* Mxr1 nucleic acid is considered exogenous.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Pichia strains are able to grow on methanol as the sole carbon source. Methanol utilization is initiated by the conversion of methanol to formaldehyde by the action of alcohol
30 oxidase. The methylotrophic yeast, *Pichia pastoris*, contains two genes for alcohol oxidases,

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

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

As used herein, nucleic acids can include DNA and RNA, and includes nucleic acids that
25 contain one or more nucleotide analogs or backbone modifications. A nucleic acid can be single stranded or double stranded, which usually depends upon its intended use. Also provided are nucleic acids and polypeptides that differ from a given sequence. Nucleic acids and polypeptides can have at least 50% sequence identity (e.g., at least 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%,
30 98%, or 99% sequence identity) to a given nucleic acid or polypeptide sequence.

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

The alignment of two or more sequences to determine percent sequence identity can be performed using the computer program ClustalW and default parameters, which allows alignments of nucleic acid or polypeptide sequences to be carried out across their entire length (global alignment). Chenna et al., 2003, *Nucleic Acids Res.*, 31(13):3497-500. ClustalW calculates the best match between a query and one or more subject sequences, and aligns them so that identities, similarities and differences can be determined. Gaps of one or more residues can be inserted into a query sequence, a subject sequence, or both, to maximize sequence alignments. For fast pairwise alignment of nucleic acid sequences, the default parameters can be used (i.e., word size: 2; window size: 4; scoring method: percentage; number of top diagonals: 4; and gap penalty: 5); for an alignment of multiple nucleic acid sequences, the following parameters can be used: gap opening penalty: 10.0; gap extension penalty: 5.0; and weight transitions: yes. For fast pairwise alignment of polypeptide sequences, the following parameters can be used: word size: 1; window size: 5; scoring method: percentage; number of top diagonals: 5; and gap penalty: 3. For multiple alignment of polypeptide sequences, the following parameters can be used: weight matrix: blosum; gap opening penalty: 10.0; gap extension penalty: 0.05; hydrophilic gaps: on; hydrophilic residues: Gly, Pro, Ser, Asn, Asp, Gln, Glu, Arg, and Lys; and residue-specific gap penalties: on. ClustalW can be run, for example, at the Baylor College of Medicine Search Launcher website or at the European Bioinformatics Institute website on the World Wide Web.

Changes can be introduced into a nucleic acid molecule, thereby leading to changes in the amino acid sequence of the encoded polypeptide. For example, changes can be introduced into nucleic acid coding sequences using mutagenesis (e.g., site-directed mutagenesis, PCR-mediated mutagenesis) or by chemically synthesizing a nucleic acid molecule having such changes. Such

nucleic acid changes can lead to conservative and/or non-conservative amino acid substitutions at one or more amino acid residues. A “conservative amino acid substitution” is one in which one amino acid residue is replaced with a different amino acid residue having a similar side chain (see, for example, Dayhoff et al. (1978, in *Atlas of Protein Sequence and Structure*, 5(Suppl. 3):345-352), which provides frequency tables for amino acid substitutions), and a non-conservative substitution is one in which an amino acid residue is replaced with an amino acid residue that does not have a similar side chain. Nucleic acid and/or polypeptide sequences may be modified as described herein to improve one or more properties including, without limitation, increased expression (e.g., transcription and/or translation), tighter regulation, deregulation, loss of catabolite repression, modified specificity, secretion, thermostability, solvent stability, oxidative stability, protease resistance, catalytic activity, and/or color.

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

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

Polypeptides can be purified from natural sources (e.g., a biological sample) by known methods such as DEAE ion exchange, gel filtration, and hydroxyapatite chromatography. A polypeptide also can be purified, for example, by expressing a nucleic acid in an expression

vector. In addition, a purified polypeptide can be obtained by chemical synthesis. The extent of purity of a polypeptide can be measured using any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

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

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

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

30 Nucleic acids can be detected using any number of amplification techniques (see, e.g., PCR Primer: A Laboratory Manual, 1995, Dieffenbach & Dveksler, Eds., Cold Spring Harbor

Laboratory Press, Cold Spring Harbor, NY; and U.S. Patent Nos. 4,683,195; 4,683,202; 4,800,159; and 4,965,188) with an appropriate pair of oligonucleotides (e.g., primers). A number of modifications to the original PCR have been developed and can be used to detect a nucleic acid.

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

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

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

hybridization, washing, and exposure conditions. Preferably, the target nucleic acids are on the same membrane.

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

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

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

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

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

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

EXAMPLES

PART A. Materials and Methods

Example 1—Polymerase Chain Reaction

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

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

Example 2—Plasmid Construction by Ligation

5 50-100 ng of restriction enzyme digested plasmid and 3X molar excess of PCR amplified inserts were incubated in the presence of T4 DNA ligase (New England Biolabs). Ligation was carried out at 16°C for greater than 2 hr. 2 µl of ligation reaction was transformed into DH10B electrocompetent *E. coli* cells

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

10 1.5-2 µl of ligation mixture was transformed into 20 µl of ElectroMax DH10B T1 Phage-Resistant Competent Cells (Invitrogen, Cat # 12033-015) by electroporation using MICROPULSER™ (BioRad) set at 1.7 kV using a 1 mm gap cuvette (BioRad, Cat # 165-2089); after a pulse 1 ml SOC was added to cells and cells were incubated at 37°C for 1 h with shaking at 15 200 rpm. 10 µl of recovery mixture was plated on LB agar plates containing ampicillin at a concentration of 100 µg/ml. Plates were incubated overnight at 37°C.

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

20 Plasmid DNA was digested with either PmeI restriction endonuclease (New England BioLabs, Cat # R0560L) in 1x CUTSMART® Buffer for 1-4 hours at 37°C or SfiI restriction endonuclease in 1x CUTSMART® Buffer for 1-4 hours at 50°C (New England BioLabs, Cat # R0123L). Linearized plasmid was gel purified from a 0.8% agarose gel using ZYMOCLEAN™ Gel DNA Recovery Kit (Zymo Research, Cat # D4002). DNA was eluted in 20 µl H₂O.

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

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

collected by centrifugation at 1125 x g for 15 minutes and placed on ice. The cell pellet was resuspended in 40 ml ice cold water and collected as before for two additional wash steps. The cell pellet was then resuspended in 20 ml of ice cold 1 M sorbitol and collected by centrifugation as before. The final cell pellet was suspended in 0.3 ml ice cold, sterile sorbitol, aliquoted and
5 frozen at -80°C.

Example 6—Transformation into *P. pastoris*

30-100 ng of linearized plasmid DNA was transformed into 30 µl of electrocompetent *P. pastoris* cells using a 1 mm gap GenePulser cuvette (BioRad) with a GenePulser (BioRad) set at
10 1.15 kV. 1 ml of YPD/1M sorbitol was added and mixed at a 1:1 ratio to the cells. The cells were allowed to recover for 3 h at 30°C with shaking at 100 rpm. 100 µl of the recovery mixture was plated on a YPD plate containing the appropriate antibiotic, and the rest of the cells were plated on a YPD plate with the appropriate antibiotic. Plates were incubated at 30°C for 48 hours. Primary transformation plates were streaked onto YPD plates with appropriate antibiotic, and
15 plates were incubated for 48 h at 30°C. Individual clones were patched onto YPD plates with antibiotics and the patches were used to do colony PCR or gDNA prep to confirm integration into the chromosome and to grow the strains in shake flasks for further analysis.

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

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

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Example 8—Shake Flask Medium

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

BMY Media Components

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

10

F&R Ref No.: 38767-0048US1

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

Low-Osmolarity Medium for Shake Flask

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

5

Example 9—Fermentation Medium and Feeds

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

10

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

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

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

15

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

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

5

Vitamin solution recipe

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

Alternatively, trace metals PTM1 can be made as follows:

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

10

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

5

Glycerol feed solution

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

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

10

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

Seed Shake Flask Protocol

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

25

Fermentation Protocol

The fermentation medium and feeds were prepared as described herein. The initial volume should be about 40% of the maximum fermenter volume, e.g., 4 L, if the maximum working volume of the fermenter is 10 L. This is because the process will approach the maximum working volume by the end of the fermentation. The fermenter is inoculated with shake flask seed at 10% inoculum-fermenter ratio, e.g. if 4 L of initial media are present in the fermenter, the fermenter is inoculated with about 0.4 L of shake flask seed. The total volume in the fermenter at this point is referred to T0 volume, e.g. 4.4 L in this representative example. Process controls include the following: 30°C temperature; dissolved oxygen controlled by agitation-aeration cascade to maintain a 20% saturation set point; and pH controlled via addition of 28% NH₄OH, the set point will depend on the phase of the process.

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

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

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

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

PART B. Construction of Production Strains

Production Strain MXY0183

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

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

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

F&R Ref No.: 38767-0048US1

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

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

5

PHUSION® High-Fidelity DNA Polymerase (New England BioLabs, Cat # M0530L) was used to amplify genes from genomic DNA. PCR products were obtained and purified using DNA Clean&Concentrator-5 (Cat # D4004) and DNA was eluted in 25 µl of H₂O. Vector DNA, pGAZ and pGAN, and PCR products were digested with BsaI (New England BioLabs, Cat # R0535S) in 50 µl reaction volume at 37°C.

10

Linearized vectors and digested PCR products were purified from 0.8% agarose gel using ZYMOCLEAN™ Gel DNA Recovery Kit (Zymo Research Cat # D4002). DNA was eluted in 20 µl H₂O. Ligation reactions were set up in 10 µl at 16°C overnight using T4 DNA Ligase (New England BioLabs, Cat # M0202S).

15

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

25

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Digested products were purified from 0.8% agarose gel using ZYMOCLEAN™ Gel DNA Recovery Kit (Zymo Research Cat # D4002). DNA was eluted in 20 µl H₂O.

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

5 1.5 µl of ligation mixture was transformed into 20 µl of ElectroMax DH10B T1 Phage-Resistant Competent Cells (Invitrogen, Cat # 12033-015) by electroporation using MICROPULSER™ (BioRad) set at 1.7 kV; cells were incubated at 37°C in 1 ml SOC for 1 h with shaking at 200 rpm. 10 µl of recovery mixture was plated on LB agar plates containing ampicillin at concentration 100 µg/ml. Plates were incubated overnight at 37°C. Colonies were
10 screened by colony PCR for the presence of the insert. The sequences of the genes were confirmed.

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

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

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

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Assembling Paox1_UPS_FDHTerm- Paox1_UPD_FDHTerm-Paox1_CPO_FDHTerm. on pGAN plasmid (pMx327)

pGAN-CPGoxidase (pMx312) was used as a vector to clone the UPS and UPD cassettes.

UPG III synthase cassette was PCR amplified from pMx310 with primers to AOX1 promoter /

- 5 FDH1 terminator containing NheI and SphI recognition sites for restriction endonucleases correspondingly:

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

UPG III decarboxylase cassette was PCR amplified from pMx311 with primers to AOX1

- 10 promoter / FDH1 terminator containing SphI and AgeI recognition sites for restriction endonucleases correspondingly:

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

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

Obtained PCR products were purified using DNA Clean&Concentrator-5 (Zymo Research, Cat # D4004) and DNA was eluted in 25 µl of H₂O.

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

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

F&R Ref No.: 38767-0048US1

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

5 Digested vector and PCR products were gel purified from 0.8% agarose using ZYMOCLEAN™ Gel DNA Recovery Kit (Zymo Research, Cat # D4002). DNA was eluted in 20 µl H₂O.

Three way ligation between UPG III synthase cassette digested with NheI-SphI, UPG III decarboxylase cassette digested with SphI-AgeI and a vector digested with NheI-AgeI was set up in 10 µl at 16°C overnight using T4 DNA Ligase (New England BioLabs, Cat # M0202S).
10 1.5 µl of ligation mixture was transformed into 20 µl of ElectroMax DH10B T1 Phage-Resistant Competent Cells (Invitrogen, Cat # 12033-015) by electroporation using MICROPULSER™ (BioRad) set at 1.7 kV; cells were incubated at 37°C in 1 ml SOC for 1 h with shaking at 200 rpm. 10 µl of recovery mixture was plated on LB agar plates containing ampicillin at a concentration of 100 µg/ml. Plates were incubated overnight at 37°C. Colonies
15 were screened by colony PCR for the presence of the insert. The sequences of the junctions between vector and inserts were confirmed.

Assembling Paox1_ALAsynthase_FDHIterm.- Paox1_PPGoxidase_FDHIterm- Paox1_Fc_FDHIterm.- Paox1_PBD_FDHIterm cassette (pMx330)

20 a. PCR amplification of gene-cassettes:

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

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

25

F&R Ref No.: 38767-0048US1

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

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

5

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

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

10

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

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

15 PHUSION® High-Fidelity DNA Polymerase (New England BioLabs, Cat # M0530L) was used to amplify DNA from plasmids. The PCR products were obtained and purified using DNA Clean&Concentrator-5 (Zymo Research, Cat # D4004) and DNA was eluted in 25 µl of H₂O.

b. Preparation of vectors

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

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

pGAG-ALAsyn-PBD (pMx332) was digested in 1x CUTSMART® Buffer with XhoI (New England BioLabs, Cat # R0146S) and AgeI-HF (New England BioLabs, Cat # R3552S) 10 overnight at 37°C.

c. Making intermediate constructs and assembling a final cassette

Digested vector and PCR products were gel purified from 0.8% agarose using Gel DNA Recovery Kit (Zymo Research, Cat # D4002). DNA was eluted in 20 µl of H₂O.

pGAZ-PBD (pMx321) vector, digested with NheI-XhoI restriction endonucleases, was 15 ligated with ALAsynthase cassette PCR product digested with the same enzymes in 10 µl reaction at 16°C overnight using T4 DNA Ligase (New England BioLabs, Cat # M0202S) to yield a pGAZ-ALAsyn.-PBD plasmid (pMx328).

pGAG-ALAsyn-PBD (pMx332) digested with XhoI and AgeI-HF restriction endonucleases was ligated with PPGoxidase cassette and Ferrochelatase cassette PCR products 20 digested with XhoI, AflII and AflII, AgeI-HF restriction endonucleases correspondingly in a three way ligation reaction using T4 DNA Ligase (New England BioLabs, Cat # M0202S) to yield a pGAG-ALAsynthase_PPGoxidase_Fc_PBD (pMx330).

1.5 µl of ligation mixture was transformed into 20 µl of ElectroMax DH10B T1 Phage-Resistant Competent Cells (Invitrogen, Cat # 12033-015) by electroporation using 25 MICROPULSER™ (BioRad) set at 1.7 kV; cells were incubated at 37°C in 1 ml SOC for 1 h with shaking at 200 rpm. 10 µl of recovery mixture was plated on LB agar plates containing ampicillin at a concentration of 100 µg/ml. Plates were incubated overnight at 37°C. Colonies were screened by colony PCR for the presence of the insert. The sequences of junctions between the vector and the inserts were confirmed.

30

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

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

Genome

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

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

 A plasmid containing two copies of the soybean LegH gene (sequence optimized for *P. pastoris*; SEQ ID NO:3) under the control of the pAOX1 promoter designated pMX282 (Figure
15 2ii and Figure 3) was linearized using SfiI restriction enzyme. Linearized plasmid was purified from a 0.8% agarose gel as described and transformed into the *P. pastoris* strain containing ALAD, generating the strain MXY0118 (Figure 3). qPCR was used and determined that strain MXY0118 contained several copies of the LegH gene, likely due to concatamerization of the plasmid, pMX282, at the time of recombination.

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

25 Genes encoding ALA synthase (ALAS), Protoporphyrin III oxidase (PPO), Ferrochelatase (FC) and Porphobilinogen deaminase (PBD) (the enzymes catalyzing steps 1, 7, 8 and 3, respectively) from the *P. pastoris* genome were assembled on plasmid pMX330 (Figure 2iv and

Figure 3). pMX330 was linearized with the SfiI restriction endonuclease and transformed into MXY0170, leading to the generation of strain MXY0183 (Figure 3). The genotype of MXY0183 was confirmed using PCR and qPCR.

5 **Production Strain MXY0207**

Example 14—Construction of pGAB Expression Vector

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

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

15

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

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

The purified BSD PCR product and pGAZ vector were digested with 10 units each of the MluI and BbvCI restriction endonucleases (New England Biolabs, Ipswich, MA) for 1 hour at 37°C in 1x NEBuffer 3.1 (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, 100 µg/ml BSA, pH
25 7.9 @ 25°C). Digested DNA products were recovered by gel electrophoresis as described above.

The purified, MluI and BbvCI digested BSD product and pGAZ vector were incubated with 400 units of T4 DNA ligase (New England Biolabs) in 1x T4 DNA ligase reaction buffer (50

mM Tris-HCl, 10 mM MgCl₂, 1 mM ATP, 10 mM DTT, pH 7.5 @ 25°C) in a 20 µl reaction, at 16°C for 2 hours in a 20 µl reaction. Electrocompetent *E. coli* DH10B cells were transformed with 2 µl of the ligation reaction and antibiotic resistant transformants were selected on LSB agar plates supplemented with 100 µg/µl ampicillin.

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Example 15—Construction of Mxr1 Expression Vector

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

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

The amplified Mxr1 PCR product and the pGAB vector were digested with 10 units of
20 NotI restriction endonuclease (New England Biolabs) for 1 hour at 37°C in 1x NEBuffer 3.1 (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, 100 µg/ml BSA, pH 7.9 @ 25°C). Following digestion, the NotI-digested pMx352 vector was treated with 5 units Antarctic phosphatase (New England Biolabs) for 15 minutes at 37°C in 1x Antarctic phosphatase buffer (50 mM Bis-Tris-Propane-HCl, 1 mM MgCl₂, 0.1 mM ZnCl₂, pH 6 @ 25°C).

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

The NotI-digested fragment containing Mxr1 open reading frame was introduced into pGAB at a NotI site immediately downstream of the AOX1 promoter by ligation. A mixture containing 137 ng of NotI-digested DNA encoding the Mxr1 open reading frame and 60 ng of NotI-digested, phosphatase-treated pMx352 was incubate with 400 units of T4 DNA ligase (New England Biolabs) in 1x T4 DNA ligase reaction buffer (50 mM Tris-HCl, 10 mM MgCl₂, 1 mM ATP, 10 mM DTT, pH 7.5 @ 25°C) in a 20 µl reaction, at 16°C, for 2 hours in a 20 µl reaction. Electrocompetent E. coli DH10B cells were transformed with 2 µl of the ligation reaction and antibiotic resistant transformants were selected on LSB agar plates supplemented with 100 µg/µl ampicillin. Plates were incubated overnight at 37°C. Colonies were screened for the presence of the insert by PCR using primers MxO0495 and MxO0496. The sequence of the final vector was confirmed by DNA sequencing.

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

Example 16—Construction of Native Mxr1 Expression Vector

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

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

PCR products were obtained and purified using DNA Clean&Concentrator-5 and DNA was eluted in 12 µl of H₂O. The purified PCR products were then combined and used as a template for a subsequent round of PCR amplification using primers MxO0617 and MxO0618. The resulting PCR product was composed of the 3' end of the AOX1 promoter, followed by the Mxr1 open reading frame, the AOX1 terminator, a short linker sequence, and the 5' end of the AOX1 promoter. The PCR product was obtained and purified as described herein. The purified PCR product was cloned into the pCRTM-Blunt II-TOPO[®] vector using the Zero Blunt[®] TOPO[®] PCR Cloning Kit (Invitrogen, Cat # K2800-20) to create the pMX402 vector.

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

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

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

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

Production Strain MXY0291

25 Example 18—Construction of Strains MXY0213 and MXY0260

Figure 5 shows the steps taken to construct antibiotic marker free strain MXY0213 that contains 7 enzymes of the heme biosynthetic pathway. A linear piece of DNA containing variant Mxr1 (6 extra amino acids at N terminus) under pAOX1, with homology to the pAOX1 promoter on each end, was introduced using co-transformation (Figure 5 and Figure 6i). This linear Mxr1 expression cassette was simultaneously introduced into *Pichia* strain MXY213 with the pIL75

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F&R Ref No.: 38767-0048US1

plasmid by transformation. The pIL75 vector carries a panARS autonomous replication sequence (Liachko & Dunham, 2014, FEMS Yeast Res., 14:364-7), which allows for maintenance of the plasmid vector without integration into the genome of the transformed cells, and a kanMX marker for selection of transformants with the antibiotic G418. Transformed cells were selected on media supplemented with G418 for the presence of kanMX marker on the pIL75 plasmid. *Pichia* transformants were screened by colony PCR for transformants that took up both the pIL75 plasmid and had correctly integrated the Mxr1 expression cassette.

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

A plasmid containing a different *Pichia pastoris*-codon optimized variant of soybean LegH gene (variant 3; SEQ ID NO:5) under the control of the pAOX1 promoter designated pMX399 was used as a source of template for PCR amplification of the gene. The backbone from TOPO cloning plasmid pMX401 was PCR amplified. The insert and vector were assembled using GIBSON ASSEMBLY® (NEB GIBSON ASSEMBLY® kit) to generate plasmid pMX422. This plasmid was used as a template for a subsequent round of PCR amplification using primers MxO0617 and MxO0618 shown below.

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

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

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

Example 20—Curing Transformants of Plasmid Vectors Bearing Selection Markers

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

Production Strains MXY0330, MXY0333, and MXY0338**Example 21—Construction of Strain MXY0306**

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

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

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

Example 22—Linear Constructs for Hybrid Promoter Strains

F&R Ref No.: 38767-0048US1

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

Primers used to amplify the linear constructs

Primer designation	Sequence	SEQ ID NO:
MXO0718	GAGCTTCTTCTACGGCCCCC	53
MXO0723	TCCAGCAGAGTAAAATTCCTAGGGAC	54
MXO0724	CTCTTTTAGGTTTAAAGTTGTGGGAACAGTAACA	55
MXO0729	GTGGGTGCTTCTTTGCGTGG	56
MXO0730	AGAATTGCCATCAAGAGACTCAGGACT	57
MXO0735	GATAGAGAGAAATCGCAAACCTTGAGAGGAAG	58

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

Example 23—Nucleic Acid Sequences

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

F&R Ref No.: 38767-0048US1

GGCGCAAGGCAACTCGTCGGTCTTCTAATGCCGCGGGTTCCATATCTGGTTCTACTCCGGTGACA
ACGCCAAATACTATGGGTACGCCCCGAAGATGGCGAGAAACGAAAAGTTCAGAAACTGGCCGGCCG
CCGGGACTCAAATGAACAGAACTGCAACTGCAACAACAACATCTACAGCAACAACCACAGTTGC
AATACCAACAATCTCTTAAGCAGCATGAAAATCAAGTCCAGCAGCCTGATCAAGATCCATTGATA
5 TCCCCGAGAATGCAATTATTCAATGATTCCAACCATCACGTAAACAATTTGTTTGATCTTGACT
AAGAAGAGCTTCCTTCTCCGCGGTTAGTGGAAATAATTATGCCCATTTGAGAATATTTCAAC
AAGATGCCTCTTCTACCAATCCAAATCAAGATTCAAATAATGCCGAATTTGAGAATATTTGAATTT
TCTACCCCAACAAATGATGCCCGTTGAAGATGCTGAACTTTGGATGAACAACATGGGTCCAATTC
GAACTTCTCTCTCGATGTGAACAGGAACATTGGTGATAGCTTTACAGATATACAACACAAGAATT
10 CAGAGCCTATTATATCCGAACCGCCCAAGGACACCGCTCCAAACGACAAGAAGTTGAATGGCTAC
TCTTTTTACGAAGCCCCCATCAAGCCATTAGAATCCCTATTTTCTGTGAGGAATACAAAGAGAAA
CAAGTATAAAACAAATGACGACTCTCCAGACACCGTGGATAATAACTCCGCACCGGCTGCTAATA
CCATTCAAGAACTTGAGTCTTCTTTGAATGCATCCAAGAATTTTTGCTTGCCAAGTGGTTATTCC
TTCTATGGTAATTTGGACCAACAGACTTTCTCTAACACGTTATCATGCACTTCTTCTAATGCCAC
15 AATTTGCGCCATTCTACTCGATAACTCCATTAATAATAACTCCACTAGTGACGTGAGACCAGAAT
TTAGAACACAAAGTGTCACCTCTGAAATGAGTCAAGCCCCCTCCCCCTCTCAAAAAACAACTCG
AAATATTCCACCGAAGTTCTTTTTACCAGCAACATGCGGTCGTTTATTCACTACGCTCTTTCCAA
GTATCCTTTTTATTGGTGTGCCCACTCCAACCTCTCCGAGAAACGAAAGACTAAATGAATATGCTG
ATTCATTACCAACCGTTTCTTAAATCATTATCCTTTCATACATGTCACGATTCTCAAAGAATAC
20 TCCCTTTTCAAGGCAATTTTAGATGAGAATGAGTCGACTAAGAACTGGGAAAATAATCAGTTTTA
CTTAGAGAACCAACGAATATCAATTGTTTGTCTTCTCTTTGGTGGCTACGATAGGTGCAGTAC
TATCAAACAACAAAAGGATGCTTCGAATTTATACGAAGCTTCAAGGCGTTGTATTATGTTTAC
TTAGATTCCAGGAAAAAGATACCCACTTCCTTGTCGCAATAACAATGACTCTCCACTTTGGCT
AATTCATCCCTGACGTTATCTGTTATGTATGGGTATTTGCGGACAATGACATTAGTTTGAATG
25 TCGTGATCAGACAAGTTAACGCACTTAATTCTCTGGTCAAGACTTCGGGCTGAATAGGACCTCA
ATTATAGATCTTTTCAACATCAACAAACCTTTGGATAATGAACTCTGGAATCAATTCGTGAAAAT
AGAGTCCACCGTAAGGACAATCCACACGATTTTTCAAATCAGTTCCAACCTTAAGCGCCTTGTACA
ATATTATTCCATCGTTGAAAATTGATGACCTAATGATTACTCTACCAGTTCCCAACACTTTGG
CAAGCTGATTCTTTTGTGAAATTCAAAGTCTAAGTTACGGAAATCAGATCCCTTTTCAATATAC
30 AAGAGTACTACAGAATTTGATTGATTACAATCAGCCATTGAGCGATGGAAAATTTTTGTATGAAA
ACCATGTAAGTGAGTTTGGACTCATATGCCTACAGAATGGTCTACACCAATACAGCTATTTCCAA
AAATTGACTGCTGTCAATAACAGAGAAGATGCGCTATTACAAAGGTTGTTAATTCACCTCACAG
TTGGGATAGGATGATTTTGAATTTCTGATTTGTTTCCAAAGAAGATATATCAGCAGAGTTGCTTGA
TTTTGGACTCAAAGTTGCTTAATAATTTCTGATTGTCAAGAGCTCATTGAAAGTTTCGACCGGA
35 GACGTTAGTTCTTTGAATAAGTTAAAAGAAAACGTGTGGCTTAAAACTGGAATCAAGTGTGTGC
TATCTATTATAACAGCTTCATGAACATTCTGCTCCAGTATTCAAAGAAGTACAATGACATAG
AGTTTGTGGATGACATGATTAATTTGAGTCTAATCATCATCAAGATTATGAACTCATTTTCTAT
AACAATGTCAAAGACAATTATGAGGATGAAAATGACTTCAAATTGCAAGAGTTAAATTTAACATT
TGACAATTTTGATGAGAAAATATCCTTGAATTTGACAATATTATTCGATATATTTTTGATGATCT
40 ACAAGATAATTACCAATTACGAAAAGTTTATGAAGATCAAACACAAGTTTAATTACTACAATTCT
AATTCGAATATAAGCTTCTTGCATCATTTTGAACCTCTCCTCGGTTATCAATAACACCCAAATGAA
CCAGAATGATTATATGAAAACAGATATTGATGAAAAGCTTGATCAGCTTTTCCACATCTATCAA
CATTTTTCCGGCTGTATCTGGATTTAGAAAAGTTTATGAAGTTCAAATTCACCTATCATGACTTT
GAGACAGAGTTTTCAAGTCTCTCAATATCCAATATACTGAACACTCATGCTGCTTCAACAATGA
45 CACAAATGCTGCTGATGCTATGAATGCCAAGGATGAAAAATATCTCCCACTTTGAATAGCG
TATTACTTGCTGATGAAGGAAATGAAAATTCGGTCTGTAATAACGATTACAGACCGCCTGTTTCATG

F&R Ref No.: 38767-0048US1

CTGAACGAGCTAATTAATTTTGAAGTAGGTTTGAAATTTCTCAAGATAGGTGAGTCATTTTTTTGA
TTTCTTGTATGAGAATAACTACAAGTTCATCCACTTCAAAAACCTTAAATGACGGAATGTTCCACA
TCAGGATATACCTAGAAAACCGACTAGATGGTGGTGTCTAG

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

MRDRGRMSNLPPTFGSTRQSPEDQSPVPKELS FNGTTPSGKLRLFVCQTCTRAFARQEH LKRHE
RSHTKEKPFSCGICSRKFSRRDLLLRHAQKLHSNCSDAAITRLRRKATRSSNAAGSISGSTPVT
TPNTMGTPEDGEKRKVQKLGRRRDSNEQKLQLQQQHLQQQPQLQYQQSLKQHENQVQPDQDPLI
10 SPRMQLFNDSNHHVNNLFDLGLRRASFSAVSGNNYAHYVNNFQQDASSTNPNQDSNNAEFENIEF
STPQMMPVEDAETWMNNMGPIPNFSLDVNRNIGDSFTDIQHKNSEPIISEPPKDTAPNDKKLNGY
SFYEAPIKPLESLFSVRNTRKNKYKTNDSDPTVDNNSAPAANTIQELESSLNASKNFCLPTGYS
FYGNLDQQTFSENTLSCTSSNATISPILLDNSINNNSTSDVRPEFRTQSVTSEMSQAPPPPQKNNS
KYSTEVLFSTNMRSFIHYALSKYPFIGVPTPTLPENERLNEYADSFTNRFLNHYPFIHVTILKEY
15 SLFKAILDENESTKNWENNQFYLENQRISIVCLPLL VATIGAVLSNNKKDASNL YEASRRCIHVY
LDSRKKIPTSLSANNNDSPLWLIQSLTSLVMYGLFADNDISLNVVIRQVNALNSLVKTSGLNRTS
IIDLFNINKPLDNELWNQFVKIESTVRTIHTIFQISSNLSALYNIIPSLKIDDLMITLPVPTTLW
QADS FVKFKSLSYGNQIPFQYTRVLQNLIDYNQPLSDGKFLYENHVSEFGLICLQNGLHQYSYFQ
KLTAVNNREDALFTKVNSLHSDRMISNSDLFPKKIYQQSCLILDSKLLNFLIVKSSLKVSTG
20 DVSSLNKLKENVWLKNWNQVCAIYYNSFMNIPAPSIQKKYNDIEFVDDMINLSLIIKIMKLI FY
NNVKDNYEDENDFKLQELNLTFDNFDEKISLNL TILFDIFLMIYKIIITNYEKFMKIKHKFNYYNS
NSNISFLHHFELSSVINNTQMNQNDYMKTDID EKLDQLFHIYQTFFRLYLDLEKFMKFKFN YHDF
ETEFSSLSISNILNTHAASNNDTNAADAMNAKDEKISPTTLNSVLLADEGNENSGRNNDSDRLFM
LNELINFVGLKFLKIGESFFDFLYENNYKFIHFKNLNDGMFHIRIYLENRLDGGV*

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

ATGGGTGCTTTTACCGAGAAGCAGGAAGCACTTGTTTCTCTTCGTTCTGAAGCTTTTAAGGCTAA
CATCCCTCAATACTCTGTTGTGTTTTACACGTCCATTCTAGAAAAAGCTCCTGCTGCCAAGGACC
TCTTCTCTTTTCTGTCCAACGGTGTAGATCCATCCAATCCCAAATTAACAGGTCACGCTGAGAAA
30 TTGTTTCGTTTTAGTCAGAGATAGCGCTGGACAATTGAAAGCAAATGGTACTGTGGTTGCTGATGC
TGCCTTGGGCAGCATCCATGCACAGAAGGCAATTACAGACCCACAATTTGTTGTTGTGAAGGAAG
CTCTGCTTAAACTATAAAGGAAGCCGTCGGAGACAAATGGAGTGACGAGTTGTCATCAGCTTGG
GAGGTAGCTTATGATGAGTTGGCCGCAGCAATCAAAAAGGCATTCTAA

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

MGAFTKQEALVSSSF EAFKANIPQYSVVFYTSILEKAPAAKDLFSFLSNGVDPSNPKLTGHAEK
LFGLVRDSAGQLKANGTVVADAALGSIHAQKAITDPQFVVVKEALLKTIKEAVGDKWSDELSSAW
EVAYDELAAA
IKKAF

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

ATGGGTGCATTTACAGAAAAACAAGAGGCTTTAGTATCCTCATCTTTTGAAGCTTTCAAAGCCAA
TATTCCTCAATACTCCGTTGTTTTCTATACGTCCATTTTGGAAAAGGCTCCAGCAGCTAAGGACC
TTTTCTCTTTCTTGTCGAACGGCGTGGATCCCTCAAATCCTAAGCTGACTGGTCACGCCGAGAAG
45 CTTTTTGGTTTGGTCAGAGACAGCGCCGACAGCTGAAAGCTAACGGTACAGTTGTGGCAGATGC
TGCCCTTGGGATCTATACATGCACAAAAGGCTATCACCGACCCACAGTTTGTGGTTGTAAAAGAGG

Motif Exhibit 1040, Page 1389 of 1537

F&R Ref No.: 38767-0048US1

TCGAGCCTGCTTCATTCCTGCCTCAGGTGCATAACTTTGCATGAAAAGTCCAGATTAGGGCAGAT
TTTGAGTTTAAAATAGGAAATATAAACAAATATACCGCGAAAAAGGTTTGTATAGCTTTTCGC
CTGGTGCCGTACGGTATAAATACATACTCTCCTCCCCCCCCCTGGTTCTCTTTTCTTTTGTACT
TACATTTTACCGTTCCGTCACTCGCTTCACTCAACAACAAA

5

Pichia pastoris pTEF1 promoter (SEQ ID NO:10)

ATAACTGTCGCCTCTTTTATCTGCCGCACTGCATGAGGTGTCCCCTTAGTGGGAAAGAGTACTGA
GCCAACCCTGGAGGACAGCAAGGGAAAAATACCTACAACCTTGCTTCATAATGGTCGTAAAAACAA
TCCTTGTCGGATATAAGTGTTGTAGACTGTCCCTTATCCTCTGCGATGTTCTTCTCTCAAAGTT
TGCGATTTCTCTCTATCAGAATTGCCATCAAGAGACTCAGGACTAATTTTCGCAGTCCCACACGCA
CTCGTACATGATTGGCTGAAATTTCCCTAAAGAATTTCTTTTTCACGAAAATTTTTTTTTTACAC
AAGATTTTTCAGCAGATATAAATGGAGAGCAGGACCTCCGCTGTGACTCTTCTTTTTTTTCTTTT
ATTCTCACTACATACATTTTAGTTATTCGCCAAC

10

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

ATGGAGTTTGTGCGCCGTCAGTCCATGAATGCCTGTCCCTTTGTCAGGTCAACTTCTACCCACCA
TTTGAAGAAGTTGGCAGCAAACAGTTCTCTAGCTGCTACTGCTAGTCATTGTCCCGTGGTTGGCC
CTGCTCTCCAACAGCAGAGATACTACTCTCAACCTTCCAAGCCAGCCCAAGCCCAACCTCCGAC
ATTGCTACTGGGATCAAGAAGGATGTTTCTCCGATCCGTATGGACTCTAATGAAACCGCCTTTGA
TTACAATGGAATGTATGAGTCTGATCTTGCGAATAAACGTAAAGATAACTCGTATCGTTATTTCA
ATAACATCAACCGTCTAGCCAAGGAGTTTCCCAAGGCACATCGCCAGACCGAAGATGACAAGGTG
ACCGTCTGGTGCTCTAACGACTACTTAGGAATGGGTAGGCATCCTGAGATTATCAAAACCATGAA
GGCTACCATGGACAAGTACGGTTCCGGAGCAGGAGGAAGTGAAGCAATTGCAGGTGATAACACG
CCGCTATCAATTTGGAAAGCGAGTTGGCTTGCTTGAACAAGAAGGAAGCGGCTCTGGTGTTTTCA
TCATGTTTTCATAGCTAACGATGCAATCATCTCGTTGTTGGGACAAAAATCAAAAATTTGGTCAT
TTTCTCTGACCAGTCGAATCATGCTTCCATGATATTGGGTGTGCGTAACTCCAAAGCGAAGAAGC
ACATCTTCAAGCACAACAATTTGAAGGATCTGGAGTCGCAGTTAGCTCAGTACCCCAAGTCGACT
CCTAAACTGATCGCCTTTCGAGTCAGTTTACTCTATGTGTGGATCTGTGGCTCCCATTGAGAAGAT
TTGCGATTTGGCTAAAAGGTACGGTGCCCTCACCTTCTTGGATGAAGTTCATGCTGTTGGAATGT
ATGGTCCTCATGGACAGGGTGTAGCTGAGCATTTGGACTTTGATCTGCATTTACAGTCTGGAATC
GCCAGTCCTAGCGTGGTGGACAAACGCACCATATTGGATCGTGTGACATGATTACTGGTACTTG
CGGAAAGTCATTTGGTACTGTTGGAGGTTACGTTGCTGGTAGTGCCAACCTAATTGATTGGTTAA
GATCCTATGCGCCAGGTTTCATTTTCACTACCACACTTCTCCTGCTATCATGGCTGGTACAGCC
ACTTCTGTTTCGTATTGTTAGGGCCGACATTGAGGCCCCGATCAAGCAACAGCTTAATACTCGCTA
CGTCAAAGACTCATTTGAAAACCTTGGTATTCCAGTCATTCCAAACCCAAAGTCACATTGTTCCCTG
TTCTAGTTGGAAATGCTGCAGATGCCAAGAAGGCATCCGATATGTTAATGAACAAACACCGTATT
TATGTTCAAGCTATTAACCTACTGTGCCTGTGCGTGAAGAACGACTAAGGATTACTCCTAC
TCCAGGTCATGGAAAGGAGATTTGTGACCAGCTGATCAGCGCTGTCGACGATGTTTTTACTGAGC
TTAATTTACCAAGAATCAACAAATGGCAGTCCCAAGGTGGTCATTGCGGTGTTGGTGATGCTAAT
TACGTACCAGAACCCAATCTGTGGACTCAGGACCAGCTCAGCTTGACAAACCAAGACTTGCACTC
CAATGTGCACAACCCAGTGATTGAGCAGATCGAAACCTCATCAGGAGTCAGATTGTAG

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

ATGGTGCATAAGGCTGAATACTTGGACGACCACCCAACCTCAGATTTCCAGCATTTCTTTCAGGAGG
TTACAACCACCCATTACTTTCGTGAATGGCAACATGAACGTCAACTCAACAAAAACATGTTTCATCT

45

F&R Ref No.: 38767-0048US1

TTCCCCCTGTTTGTACAGATCGACCAGACGAAGAAGAACTTATTCCTAGTCTACCTAATATCAAG
AGGTTTGGCGTTAACAAGTTGATTCCTTATGTAGGAGGTTTGGTTTCCAAAGGATTGAGGGCGGT
GATCCTATTTGGTGTTCCTCTGAAGCCCGGTGTGAAAGATGAAGAAGGAACGGCCGCTGATGATC
CAGAGGGACCTGTTATCCAAGCCATCAAACACTTGAGAAAGAACTTTCTGACCTGTATATCATC
5 ACCGATGTCTGTCTATGTGAGTACACCAGCCATGGACATTGTGGAATACTATATGAGGATGGCAC
TATCAACAGAGAGCTCTCAGTCCGTCGTATTGCTGCTGTAGCTGTCAAATATGCTCAAGCTGGAG
CCAACCTCTGTGGCTCCTTCTGATATGACTGACGGCAGAATAAGAGATATTAAAGAAGGCTTACTA
AGTGCAGGACTGGCACATAAAACGTTTGTATGTCCACGCTGCAAAATTCTCTGGTAATTTGTA
TGGCCCTTTTCAGAGATGCTGCAGGTTTCTGTCCATCTCAAGGGGACAGAAAATGTTACCAGCTTC
10 CTTCTGGAGGAAAAGGGTTGGCCCATCGTGCTCTGATTCTGTGATATGAATGAAGGCACTGATGGA
ATTATTGTCAAACCATCTACATTCTATTTGGACATTGTCTGCTGATGCTTATCAGCTTTGTAAAGA
CTATCCTATCTGCTGTTACCAGGTTTCTGGAGAGTACGCCATGCTACATGCAGCGGCAGAGAAGA
ATATTGTTGATCTGAAATCAATCGCTTTTGAAGCTCATCAAGGATTCTTGCGGGCTGGAGCTCGT
TTAATCATTAGTTACTTTACCCCTGAATTCCTGGAGTGTTATCTGAATGA

15

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

ATGAACCAAATCGAACAGAGCGGACCCATTGATTGCAGTTCCTTGAAATTGGGGTCCCCGAAAGTC
CGCTCTGGCTATAATCCAGGCAGAAATCGTCCGCCAATTGATATTGAAAGAATACCCTGAATTGG
AGACGAAGTTGGTCAGTGTGTCCACCCTGGGGGACCAAGTCCAGAATAAAGCACTTTTCACGTTT
20 GGAGGAAAATCTTTGTGGACCAAGAAGCTTGAGATGTTGTTGTTGGAGAGTGTGGGAGGATTTGA
CCAAATAGACATGATTGTACACTCGTTGAAAGACATGCCAACTCATTTACCAGACGAATTTGAGC
TGGGTTGCATTATTGAAAGAGAAGACCCTAGAGACGCTTTGGTCGTGCAAGATGGTTTATCTTAC
AAGTCATTGGCCGACCTTCCAGAGGGAGCTGTGGTCCGTACGTCCTTCGGTTAGAAGATCGGCTCA
ACTACTGAAGAATTTCCCTCATCTGAAATTCAAATCTGTTAGAGGAAACCTTCAGACCAGACTAA
25 GAAAATTAGATGATCCAGATTCCGAGTACTGCTGTCTCCTCCTTGCAGCAGCCGGTTTAAATCAGG
ACAGGCTTACAACACAGAATTTCAATGTATTTGAACGACGATGTGATGTACCACTCCGTCGGACA
AGGAGCATTAGGAGTAGAGATCAGAAAAGGTGACCAATTCATGAAAAATATCTGTGAAAAGATTG
GGCATAGAACCACCACCCTTCGTTGTCTTGCAGAGAGAGCACTGCTGAGATATCTAGAGGGAGGC
TGCTCGGTGCCAATTGGGGTCTCCACTATTTATAGCGAGGATACGAAGGAACTTACCATGAACTC
30 CCTAGTCGTCAGTTGTAACGGTCGTGACTCGGTAACAGAATCAATGACTGAAGTCGTGACTACTG
AAGAGCAAGCTGAAGATTTTCGGTGAAAGGCTGGCCCAAGCTCATAGATCAAGGTGCGAAACGC
ATTCTTGACGAGATCAACTTCAACAAGATCAAAGAGATTAAGGAAGAGGGTTTACATTAA

Heme biosynthesis enzyme 4- Uroporphyrinogen III synthase (SEQ ID NO:14)

ATGCCAAAAGCCATTCTTCTGAAGAATAAACTACACCGAAGGATCCTTATCTGGAGAACTTCGT
AAGTAGTGGCTACTCGACCGATTTCTGTACCACTTTTAGATCATATTCACATGGAGAAATCTGAGA
TCATCGCATTCTCAAGACTGACTACTTTTTTGCATAAACTTTGGCGTTTATTATTACGTCCCAA
AGAGCTGTAGAAATGCTGAATGAGTGTATGCAAATACTGAGACGTACTGATCCTGAAATTACACA
AATCATCTATAGTAAACCTGTCTATACAGTTGGCCCTGCCACCTACAGAATACTTGCGGATGCTG
40 GCTTCGTGGATCTACGAGGCGGAGATAAGGCAGGAAACGGATCCATTCTAGCCCAGATAATTTTG
AATGATGACATTTACACTGGAATTGAAGATTCTGACAAGCATATAACGTTTTTACGGGAGAAAC
AAGGAGAGACATAATTCCCAAATGTTTACTCTCTAACAACCTTCAACTTTACGAAAAGATTGTCT
ACAAGACTCTTCCTAGGGATGATATCGTGACTAGATTCAAGTCTGCCGTTGACAGCATCGACCAA
TCGCAAGAAGTTCCAGTTGGGTGGTCTTCTTTTCGCCTCAAGGAACAGAGGACATTGTAACGTA
45 TCTTCAACACACCAAAGACCAATTTAATATTGCATCTATCGGGCCAACACAGAAAAATACCTTC

F&R Ref No.: 38767-0048US1

TAAGCAAAAACCTGAAACCAAAAGTTGTGGCACCTAAGCCAGAGCCTATCTCTTTACTATTGTCT
ATACAAAAAGTGCACTAA

Heme biosynthesis enzyme 5- Uroporphyrinogen III decarboxylase (SEQ ID NO:15)

5 ATGAGTAGATTTCCAGAACTGAAGAATGACCTTATTTTAAGGGCAGCTCGTGGTGAAAAAGTTGA
ACGTCCCCCAATATGGATTATGAGACAGGCCGGAAGATATCTTCCGGAGTACCATGAGGTCAAAG
GAGGTAGGGACTTCTTTGAACTTGCAGGGATGCTGAGATTGCTTCTGAAATTACTATCCAGCCG
ATTACGCATTTTGACGGTCTGATCGATGCAGCTATTATCTTCAGTGATATCTTGGTGATTCTCTCA
AGCTATGGGCATGGAAGTTAAGATGGTGGACAAAGTTGGCCACAGTTCCCCAATCCGCTAAGAA
10 AACCGTCTGACTTGGATCATTGAAAAAAGACGTTGACGTTTGAAGGAACCTCGATTGGGCCTTC
AAAGCTATCTCATTGACCAGAAAAAACTCAATGGACGAGTGCCTTTGCTTGGATTTTGTGGTGC
TCCTTGGACTCTACTGGTTTATATGACTGAAGGAGGCGGTACCAAGATGTTTCGATTTGCAAAAG
AGTGGATCTACAAGTTTACCAAGGAATCTCATCAATTACTCCAACAGATCACTGACGTTGCAGTT
GAATTCTTAGCTCAGCAAGTTGTTGCAGGTGCCCAAATGTTACAAGTTTTTGAATCTTGGGGCGG
15 TGAATTGGGGCCTGATGAATTTCGATGAGTTTTCTTTGCCTTATTTGAGACAGATTTCTCTAAAC
TTCCCCTGAGGTTGAAGGAACCTGGAATCACAGAGAATGTTCCCATAACTGTCTTTGCTAAAGGC
TCTTGGTACGCCTTGGAGCAATTGTGCGACAGTGGTTATGATGTTGTCTCGTTGGATTGGTTATT
CCGTCCAAGTGATGCTGTCCAGATTGCTAACGGAAGAATCGCATTGCAAGGTAATCTTGACCCTG
GAACCATGTACGGCTCCAAAGAAACCATTTCCAAGAAAGTGGACAAAATGATCAAGGGTTTTGGT
20 GGAGGAAAGCAAACTACATAATTAATTTTGACACGGCAGTCAATCCATTATGGATCCAGAACA
GATCAGATGGTTCTTACAAGAATGTCATCGCATTTGGATCTCAATAG

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

25 ATGGCCATCGACTCTGATATCAATCTAAGCTCTCCCAATGATTCATCCGTCAAAGGATGTTCTGA
GCTTATCCAGCGGAAGCAACTCGAAATTGTGCGTGCATTGGAGGCAATTGAAGGAAACGATACCA
AATTTTCGTTCTGATTCTTGGGAAAAGAGGAGCCGAAGGTGGAGGAGGAAGATCTATGCTTATTCAA
GATGGAAGAGTGTTTGAAAAGGCTGGTGTAATATTTCCAAGGTTTCATGGCGTATTGCCTCCTCA
AGCTGTGAGCCAGATGAGAAATGACCACTCCAAGCTAGATCTGCCTGCGGGAACCTCTCTGAAGT
TCTTTGCCTGTGGGCTTTTCGTTGGTCATTTCATCCCATTAATCCCATGCTCCAACCTACCCATCTG
30 AATTATCGCTACTTCGAACTTGGGATGAACTGGAAAGCCTCACACCTGGTGGTTTGGGGGCGG
TGCTGATTTAACGCCTTCGTACCTGTATCCCGAGGATGCCAAGCAATTCCATCAAGCCCATAGG
ATGCCCTGGACAAACACGATGTTAGCTTGTACCCGAGATTCAAAAAGTGGTGTGATGAATACTTT
CTGATCAAACATCGAAATGAACTAGAGGTATTGGGGGTATTTTCTTTGATGATTTTGACGAGTT
TGATGCTGAGAGGTCCCTGAAGTTGGTTGAAGATTGTTTCAATGCTTTCTTGGAAATCTTATCCCG
35 CTATCACTCGAAAAAGGATGGACACCCCTTCAACTGATGCTGAGAAGAAGTGGCAACAAATTAGA
AGAGGAAGATATGTCGAATTCAACTTAGTATTGGATAGAGGTACTCAATTTGGTTTGAGAACGCC
TGGATCTCGTGTTGAAAGTATTTTGATGTGCGTTGCCAAGAACAGCTGGTTGGGTCTATGATCATC
ATCCAGAGCCTGGCTCCAGAGAAGAGGAGTTATTGCAGGTACTACAAAATCCTATTGAATGGGTA
TGA

40

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

45 ATGCTGAAAAGTCTTGACCAAAATTCCTCAATTGCCGTTTTAGGTTTCAGGGATATCTGGATTGAC
TTTCAGCTTTTTTTTTGAATCGGTTGCGTCCCGATGTTAAGATCCATATCTTTGAAAAATCCAAGC
AGGTTGGAGGATGGATCAGATCAGAAGAGCATGAACTTTTCATTTTGAAAAGGGACCCAGAAGT
TTGAGAGGCACAAATACGGGTACCTTGATGTTGTTGGATCTTCTTACCAAGATAGGAGCAAATGA
CAAGGTCCTGGGACTGCACAAAGATTCTCTTGCTAATAAAAAGTATCTGTTGTCCCGTTCTCAG

F&R Ref No.: 38767-0048US1

ATGTTTCACGGAAACAACGCAAAGCTTCTTCAAGTGCCACAGGATTTTCAGCTCTTTTGTAAAGTTC
ATGTTTGACCCGTTGTCTAAGGATCTCATTCTCGGTCTTTTGAAAGAACCATGGCAACCAAAATT
AAAGTATTCAGATGAGTCGGTTGACCATTTTTTCAACAGAAGATTTGCTACCAAACTATCAGAGA
ATATCGTCAGCGCAATTGTGCATGGAATCTATGCGGGCGACGTGAAGAAGTTAAGTGTGAAAGCC
5 ATCTTCCCTAGGCTCCCTGAGATGGAACAGGAAAGTGGCTCTATTATAAGGTATATGATCGCCCA
ATACAGGACAAAAAGAACGTCAAACAAAAAGTTGACCCTTTTTTGGCAGATTATGAAAAATTGA
TCGGTACATCTTTGAGTTTCAAAAATATTTCTTTGTTTTCTGAAAACTTTCCCATGCTGAGTTTT
CAGGGTGGACTACAGAACTTCCCATCTCATTGAAGAACCATTTATCACAGATTGAAAAACATCAA
GTTTCATTTTGACAGCAAAATCAAAAACATTGCTTTGGAGAGCGGTAAGGTGGCATTGACTGACC
10 ATGATCAGGTTTATCTTGTGACCATGTGAGATCTACCATTAATACCAACGAATTGGCCAAAATC
ATTTACCCCGTTGTTCCAAGTTCTACTAAGAAAAAATCCGTTTTCAAATCCAAAGCGAATGGCCC
AGGGCTGGTCAAATGTTTGAGCTGGCTACACTATACAAATATACTAATGTGCAACATTTATATAC
CTAAGCACGTCTCAAATCTATCACCGGATTTGGATACTTGGTTCCTCGATCAATGTCTTCTCAG
GCATCCAACTTCTCGGTGTCATATTTGACTCAGACATCGAGACTGCAATGACTCCTAATTTTAC
15 AGAGGCCAACATTACGGCGATAAACAGTAACTCTGCATCTCCCAAGCAACTCCAAAAGTTTTCTG
ACCAATTCGTCAATAATGATCTCCCTAAATACACCAAGTTGACGCTAATGCTTGGAGGTCATTAT
CTCAAGTCGGAGGCAGACATGCCCGGTTCCGCAGAGAGTAAACATGCTGTCAAGGCGATTCTGTC
AAATCACCTGAATATTGATCTAGATGAGTTTGCATCTTTGCCAGACTTCAAGATGGAAATCACCA
AGATCCCCAACTGCATTCCCCAATATGAAGTTGGGTATCTTGATCTCAAGAGAAAGGTTTCAGAAT
20 GCAGCCTCCAAAGAGTTCAACGACCAATAAGTTTTGGAGGCATGGCATTGTTGGTGTGGG
GATCCCTGACTGTGTCCAGAATGCATTCAAAGATTTCGGCTACCCTCAGTGGCATTAA

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

ATGCTTAACCGTCGTTTCCAATCTACCGTGTCTCGAGTCTGAACAAGGGCACTGGAATAGTGTT
25 CATGAATATGGGTGGTCCCTCCACTGTCAAGGAAACCTATGACTTTTTATTTTCGTCTTTTCTCGG
ACGGAGATTTAATCCCGTTTGGCAGATTTCAGAACATCCTGGCCCGCTTCATTGCAAGTAGAAGA
ACACCCAAAATTGAATCCTACTACAAAGCTATCGGAGGTGGGTCTCCTATCCGAAAGTGGTCTGA
ATACCAGAGTTCTAAACTATGTGAAAAATTAGACATTATCAGTCCACAATCGGCTCCTCATAAGC
CTTATGTTGCCTTCAGATACGCTAATCCTCTCACTGAAGATACTTTACAAAAGATGAAAAATGAT
30 GGAATTACTAAGGCCATTGCCTTTTCTCAATATCCGCAATTTAGTTATTCAACCACCGGATCATC
GATTAACGAACTTTACAGGCAATCGAAAATTTTGGACCCTGATCAATCTATTAAATGGACAGTTA
TAGATCGCTGGCCTGACCACCCAGCCTTAGTTAAAACCTTTCGCAGCTCATATCAAAGATACTCTA
AACAGATTCAAACTGAAAATGGACTGACTGACACAAAAGACGTCGTCTCCAATTCAGTGCTCA
TTCTTTACCAATGGATATTGTCAATAAAGGAGATTTCGTATCCTGCAGAAGTCGCAGCGAGTGTCT
35 TTGCCATTATGAAAGAACTTAACTTCTCAAATCCTTATAAATTAACCTGGCAATCACAGGTTGGC
CCAAAGCCTTGGCTGGGTGCTCAAACCTGAAAAAATTACCAAGCAGCTAGCATCCAGTGATGTTCC
TGGAGTCGTTTTGGTTCCTATTGCCTTTACCTCTGATCATATTGAACTCTCCATGAACTGGATA
TTGAACTGATTCAAGAACTACCTAATCCTTCAAAGTAAAGCGAGTTGAATCGTTGAACGGAGAC
CAAACCTTTCATTGACTCCTTGGCAGAAGTAGTGAAGAGTCACATTGATTGCAAGGTTGTATTTTC
40 CAACCAGTTGCCATTGGATTCCATGCTGGGAGTAGTGTGAGATAATTCCCTCACAGATCCAAAAG
AGTTTTTCAGAGCCCATTGA

Part C. Results and Discussion

Example 24— Characterization of Strain MXY0183

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

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

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

20

Example 25— Characterization of Strain MXY0207

Experiments were then performed to determine the benefits of overexpressing the
transcriptional activator, Mxr1, in the presence of the genes encoding the 8 enzymes involved in
heme biosynthesis. Strain MXY0183, which contains >10 copies of the LegH sequence and the
25 genes encoding the 8 enzymes involved in heme biosynthesis, and sister strains MXY0206 and
MXY0207, which contain >10 copies of the LegH sequence, the genes encoding the 8 enzymes
involved in heme biosynthesis, and the Mxr1 transcriptional activator, were grown in shake flask
cultures in the presence of glycerol, which is a repressing carbon source for these strains.
Photographs of the shake flask cultures after 48 hr are shown in Figure 10A, and photographs of
30 the pellets from cells grown on BMY media for 48 hours with no additional source of carbon are

shown in Figure 10B; these experiments demonstrated that significant expression of transgenes (e.g. heme enzymes) under the control of the AOX1 promoter occurs in the absence of an inducing carbon source when a repressing carbon source is consumed in the growth medium of a strain in which Mxr1 is also expressed from the AOX1 promoter. The relative yield of heme-loaded LegH, when shake flask cultures were grown in the absence of induction agent, is shown in Figure 10C. These experiments demonstrated that significant production of a recombinant, heme-loaded protein is accomplished from AOX1 promoter-driven transgenes in the absence of methanol induction in Pichia strains in which Mxr1 expression is also driven by the AOX1 promoter.

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

Example 26—Characterization of Strain MXY0291

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

Example 27—Characterization of Hybrid Promoter Strains

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

F&R Ref No.: 38767-0048US1

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

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

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Application Number:	15573300
International Application Number:	
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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
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Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		RespNFOA.pdf	152983	yes	13
			692995d6aab327ad0ae57ca69615ee6ffdb58724		

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Document Description			Start	End	
Applicant Arguments/Remarks Made in an Amendment			8	13	
Claims			3	7	
Specification			2	2	
Amendment/Req. Reconsideration-After Non-Final Reject			1	1	
Warnings:					
Information:					
2	Specification	SubSpec_markedup.pdf	296138	no	48
			5b369ad3b7b1641618116679f0def9151c51ff0d		
Warnings:					
Information:					
3	Specification	SubSpec_clean.pdf	295576	no	48
			0bc49c60037afd2b790c821c242fba18b434983		
Warnings:					
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Total Files Size (in bytes):			744697		
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.					

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 8 of 13

Attorney Docket: 38767-0048US1

REMARKS

Applicants respectfully request entry of the amendments and remarks submitted herein. Claims 1, 10, 11, 12, 15, 17, 20, and 30 have been amended herein, and claims 8, 14, 19, 31, 33-36 and 38 have been canceled without prejudice to continued prosecution.

Claims 1-7, 9-13, 15-18, 30, 32, and 37 are currently pending, and claims 20-29 are withdrawn. Reconsideration of the pending application is respectfully requested.

Objections to the Specification

According to the Examiner, the specification is objected to because the Tables at pages 16, 18-21, 24-29, 31, 33, and 35-37 should be numbered consecutively.

Applicants thank the Examiner for the suggestion regarding the consecutive numbering of the Tables, but Applicants respectfully decline. There is no rule that Tables within the specification are required to appear in order.

According to the Examiner, the use of trade names or marks used in commerce should be capitalized wherever they appear.

Applicants submit herewith a Substitute Specification, both a Clean Copy and a Marked-Up Copy, in which the trade names identified by the Examiner have been capitalized. In view of the amendments herein, Applicants respectfully request that the objections to the specification be withdrawn.

Claim Objections

According to the Examiner, claims 8, 12, and 17 are objected to because of the following informalities:

- at claim 8, the acronyms Mxr1, Adr1, Trm1, and Trm2 should be spelled out at their first occurrences, with the acronym provided in parentheses.

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 9 of 13

Attorney Docket: 38767-0048US1

- at claim 12, the acronyms AOD1, MOX, MOD1, DHAS, FLD1, and PEX8 should be spelled out at their first occurrences, with the acronym provided in parentheses.
- at claim 17, the acronyms ALA, UPGIII, CPG, and PPG should be spelled out at their first occurrences, with the acronym provided in parentheses.

Without acquiescing to the Examiner's objections, Applicants have amended the relevant claims herein. In view of the amendments herein, Applicants respectfully request that the objections to the claims be withdrawn.

Claim Rejections - 35 U.S.C. § 112

Claims 10-11, 19, and 30-38 are rejected under 35 U.S.C. § 112(b) or 35 U.S.C. § 112 (pre-AIA), second paragraph, as the Examiner asserted that those claims are indefinite for the reasons set forth in the OA at pages 4 – 5.

Applicants have amended claims 10 and 11 to refer to Gi numbers. We believe it would be appreciated by the Examiner that the Gi number remains the same, even if the actual sequence is revised.

Applicants have canceled claim 19, thereby rendering this rejection moot.

Applicants have amended claim 30 to remove the parenthesis. Claim 30 now refers to “globin family PF00042,” which would be understood to refer to globins that fall within the PF00042 family of proteins.

In view of the amendments and remarks herein, Applicants respectfully request that the rejection of the pending claims under 35 U.S.C. § 112(b) or 35 U.S.C. § 112 (pre-AIA), second paragraph, be withdrawn.

Claim Rejections - 35 U.S.C. § 102

Claims 1-5, 7, 12-14, and 19 are rejected under 35 U.S.C. § 102(a)(1) and § 102(a)(2) as being anticipated by Zhou et al. (WO 2014/008729), as evidenced by US 2015/0299716, which is a National Stage Application of the '729 publication and also qualifies as a 35 U.S.C. § 102(a)(2) reference. All citation numbering refers to the '716 publication. According to the Examiner, Zhou discloses a methylotrophic yeast that comprises an exogenous nucleic acid

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 10 of 13

Attorney Docket: 38767-0048US1

encoding the transcriptional activator Mit1 operably linked to a methanol-inducible promoter (OA at page 6).

Without acquiescing to the Examiner's rejection, Applicants have amended independent claim 1 as indicated herein (i.e., to require the presence of a Mxr1 transcriptional activator sequence). Zhou does not teach or suggest using a Mxr1 transcriptional activator sequence and, therefore, Zhou does not anticipate the pending claims. In view of the amendments and remarks herein, Applicants respectfully request that the rejection of the pending claims under 35 U.S.C. § 102 be withdrawn.

Claim Rejections - 35 U.S.C. § 103

Claim 1-19, 30-33, 35, and 37 are rejected under 35 U.S.C. § 103 as being unpatentable over Zhou (as evidenced by US 2015/0299716) in view of Varadan et al. (WO 2015/153666) and Lin-Cereghino et al. (2006, Mol. Cell. Biol., 26(3):883-897). According to the Examiner,

It would have been obvious to one with ordinary skill in the art before the effective filing date of the claimed invention provide Zhou's methylotrophic yeast comprising sequences encoding Varadan's leghemoglobin and polypeptides involved in heme biosynthesis under the control of Lin-Cereghino's mxr1 transcriptional activator and the AOX1 promoter of each of Zhou, Varadan, and Lin-Cereghino in the *Pichia pastoris*, as disclosed as being a system for producing recombinant proteins because these systems are well-known in the recombinant protein production art. As such, it would be well within the purview of one ordinarily skilled in the art to use the components of Zhou and Lin-Cereghino in a yeast comprising leghemoglobin and polypeptides involved in heme biosynthesis, and combining these elements would be predictable and have a reasonable expectation of success, whether the components are in a replication-competent plasmid or stably integrated into the genome of the yeast cell.

OA at page 9. This rejection is respectfully traversed as follows.

The Supreme Court, in *KSR Int'l Co. v. Teleflex Inc.* (127 S. Ct. 1727, 1741 (2007)), stated that

a patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art. Although common sense directs one to look with care at a patent application that claims as innovation the combination of two known devices according to their established functions, it can be important to identify a reason that would have prompted a person of ordinary skill in the

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 11 of 13

Attorney Docket: 38767-0048US1

relevant field to combine the elements in the way the claimed new invention does. This is so because inventions in most, if not all, instances rely upon building blocks long since uncovered, and claimed discoveries almost of necessity will be combinations of what, in some sense, is already known.

As an initial matter, the present application and the Varadan et al. application were, not later than the effective filing date of the claimed invention in the present application, owned by Impossible Foods, Inc. Impossible Foods, Inc. is the named Applicant on both applications. Therefore, Applicants invoke the prior art exception under 35 U.S.C. § 102(b)(2)(C), and the subject matter disclosed in Varadan et al. cannot be used in a rejection under 35 U.S.C. § 103.

In the absence of Varadan, Zhou and Lin-Cereghino cannot support an obviousness rejection of the pending claims. Accordingly, in view of the amendments and remarks herein, Applicants respectfully request that the rejection of the pending claims under 35 U.S.C. § 103 be withdrawn.

Claims 34, 36, and 38 are rejected under 35 U.S.C. § 103 as being unpatentable over Zhou, Varadan, and Lin-Cereghino, and further in view of Liu et al. (2014, Metab. Eng., 21:9-16). This rejection is respectfully traversed as follows.

As set forth above, independent claims 1 and 30 are novel and nonobvious over the cited references. It is well known that, if an independent claim is nonobvious under 35 U.S.C. § 103, then any claim depending therefrom is nonobvious. *In re Fine*, 837 F.2d 1071, 5 USPQ2d 1596 (Fed. Cir. 1988) and cited in MPEP § 2143.03.

In view of the amendments and remarks herein, Applicants respectfully request that the rejection of the pending claims under 35 U.S.C. § 103 be withdrawn.

Double Patenting

Claims 1-19 and 30-18 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-21 of U.S. Patent No. 9,938,327.

Applicants submit herewith an e-Terminal Disclaimer to terminally disclaim over the indicated issued patent.

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 12 of 13

Attorney Docket: 38767-0048US1

Claims 1-19 and 30-18 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-14 of U.S. Patent No. 10,273,492.

Applicants submit herewith an e-Terminal Disclaimer to terminally disclaim over the indicated patent.

Claims 1-19 and 30-38 provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 17-26 of co-pending Application No. 16/123,121.

Applicants respectfully request that this provisional rejection be held in abeyance until allowable subject matter is found. At that time, Applicants will consider submitting an appropriate Terminal Disclaimer.

Request for Rejoinder

Claims 20-29 were withdrawn as directed to a non-elected invention following the Restriction Requirement mailed April 8, 2019 and Applicants' election of June 6, 2019. Since independent claim 1 should be allowable in view of the amendments and remarks herein, and since claim 20 has been amended to be commensurate in scope with independent claim 1, Applicants respectfully request that claims 20-29 be rejoined and allowed pursuant to MPEP § 821.04.

CONCLUSION

Applicants respectfully request that claims 1-7, 9-13, 15-18, 20-30, 32 and 37 be allowed. If a telephone call to the undersigned would expedite prosecution, the Examiner is encouraged to do so.

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 13 of 13

Attorney Docket: 38767-0048US1

This Response to Office Action is being filed before the expiration of the three-month Shortened Statutory Period for reply, and the fees for the Terminal Disclaimers are being submitted herewith. Please apply any charges, or credits, to Deposit Account No. 06-1050, referencing the above-indicated Attorney Docket Number.

Respectfully submitted,

Date: October 15, 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

62019365.doc

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 3 of 13

Attorney Docket: 38767-0048US1

Amendments to Claims:

Please amend claims 1, 10, 11, 12, 15, 17, 20, and 30 as follows and please cancel claims 8, 14, 19, 31, 33-36 and 38 without prejudice to continued prosecution. The claims and their status are shown below.

1. (Currently Amended) A methylotrophic yeast cell comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises a first ~~[[an]]~~ exogenous nucleic acid encoding a methanol expression regulator 1 (Mxr1) transcriptional activator operably linked to at least one methanol-inducible promoter element comprising a sequence to which the Mxr1 transcriptional activator binds, wherein the recombinant nucleic acid comprises a second exogenous nucleic acid encoding a heterologous polypeptide operably linked to at least one methanol-inducible promoter element.

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

3. (Previously Presented) The yeast cell of claim 1, wherein the methylotrophic yeast cell is of a *Pichia* yeast.

4. (Previously Presented) The yeast cell of claim 1, wherein the cell is a *Pichia* cell.

5. (Previously Presented) The yeast cell of claim 1, wherein the cell is a *Pichia pastoris* cell.

6. (Previously Presented) The yeast cell of claim 1, wherein the recombinant nucleic acid molecule is stably integrated into the genome of the methylotrophic yeast cell.

7. (Previously Presented) The yeast cell of claim 1, wherein the recombinant nucleic acid molecule is extrachromosomally expressed from a replication-competent plasmid.

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 4 of 13

Attorney Docket: 38767-0048US1

8. (Canceled)

9. (Previously Presented) The yeast cell of claim 1, wherein the exogenous nucleic acid encoding a transcriptional activator comprises a Mxr1 sequence from *Pichia pastoris*.

10. (Currently Amended) The yeast cell of claim 1, wherein the transcriptional activator comprises a sequence shown in GenPept Gi 88866603 ~~GenBank Accession No. ABD57365~~.

11. (Currently Amended) The yeast cell of claim 10, wherein the transcriptional activator is encoded by the nucleic acid sequence shown in GenBank Gi 88866602 ~~GenBank Accession No. DQ395124~~.

12. (Currently Amended) The yeast cell of claim 1, wherein the at least one methanol-inducible promoter element is selected from the group consisting of an alcohol oxidase 1 (AOX1) promoter element from *Pichia pastoris*, an alcohol oxidase (AOD1) promoter element from *Candida boidinii*, a methanol oxidase (MOX) promoter element from *Hansenula polymorpha*, a methanolica alcohol oxidase (MOD1) promoter element from *Pichia methanolica*, a dihydroxyacetone synthase (DHAS) promoter element from *Pichia pastoris*, a formaldehyde dehydrogenase (FLD1) promoter element from *Pichia pastoris*, and a peroxin 8 (PEX8) promoter element from *Pichia pastoris*.

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

14. (Canceled)

15. (Currently Amended) The yeast cell of claim 1 [[14]], wherein the heterologous nucleic acid encodes at least one polypeptide involved in the biosynthesis of an iron co-factor.

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 5 of 13

Attorney Docket: 38767-0048US1

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

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

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

19. (Canceled)

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

providing the yeast cell of claim 1;

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

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

thereby expressing the heterologous polypeptide.

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

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 6 of 13

Attorney Docket: 38767-0048US1

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

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

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

25. (Withdrawn) The method of claim 20, wherein the introducing step comprises a technique selected from the group consisting of transduction, electroporation, biolistic particle delivery, and chemical transformation.

26. (Withdrawn) The method of claim 20, wherein the conditions under which the cells are cultured comprises the addition of iron or a pharmaceutically or metabolically acceptable salt thereof.

27. (Withdrawn) The method of claim 20, wherein the culturing step comprises culturing the cell in the presence of methanol.

28. (Withdrawn) The method of claim 20, wherein the culturing step comprises culturing the cell in the absence of methanol.

29. (Withdrawn) The method of claim 20, wherein the culturing step comprises culturing the cell in the absence of selection.

30. (Currently Amended) A methylotrophic *Pichia* yeast cell comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises:
a nucleic acid encoding a Mxr1 transcriptional activator sequence from *P. pastoris*;

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 7 of 13

Attorney Docket: 38767-0048US1

a nucleic acid encoding a member of the globin family [([)]PF00042([)]]; and
a nucleic acid encoding at least one polypeptide involved in heme biosynthesis,
wherein each nucleic acid is operably linked to a methanol-inducible promoter element,
wherein the methanol-inducible promoter element comprises at least one sequence to which a
Mxr1 transcriptional activator binds.

31. (Canceled)

32. (Previously Presented) The yeast cell of claim 30, wherein the member of the globin family is leghemoglobin.

33-36. (Canceled)

37. (Previously Presented) The yeast cell of claim 31, wherein the methanol-inducible promoter element is an alcohol oxidase I (AOX1) promoter element from *P. pastoris*.

38. (Canceled)

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 2 of 13

Attorney Docket: 38767-0048US1

Amendments to the Specification:

Please replace the specification on file with the enclosed Substitute Specification – Clean Copy.

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. 38767-0048US1	Application No. 15/573,300
	Applicant Impossible Foods Inc.	
	Filing Date November 10, 2017	Group Art Unit 1636

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.	6,261,827	7/17/2001	Susan L. Elrod			
	2.	2011/0287467	11/24/2011	Brian R. Crane			
	3.	2019/0292217	9/26/2019	Simon Christopher Davis			
	4.	2019/0292555	9/26/2019	Simon Christopher Davis			

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
	5.	GenBank Accession No. AF066054.1, "Pichia pastoris formaldehyde dehydrogenase (FLD1) gene, complete cds," dated September 17, 1998, 2 pages
	6.	GenBank Accession No. AY288296.1, "Pichia pastoris 3-phosphoglycerate kinase (PGK1) gene, complete cds," dated July 22, 2005, 2 pages
	7.	GenBank Accession No. CAY70887.1, "Hypothetical protein PAS_chr3_0836 [Komagataella phaffii GS115]," dated February 27, 2015, 2 pages
	8.	GenBank Accession No. U62648.1, "Pichia pastoris glyceraldehyde-3-phosphate dehydrogenase (GAP) gene, complete cds," dated March 7, 1997, 2 pages
	9.	GenBank Accession No. XM_002490678.1, "Komagataella phaffii GS115 Hypothetical protein (PAS_chr1-4_0586), partial Mrna," dated July 22, 2009, 2 pages
	10.	Hoffman, et al., "Identification of rate-limiting steps in yeast heme biosynthesis," Biochem. Biophys. Res. Commun., 2003, 310(4):1247-1253
	11.	PCT International Search Report and Written Opinion in International Appln. No. PCT/US2016/031797, dated 9/20/2016, 9 pages
	12.	Supplementary European Search Report and Opinion in EP Appln. No. EP 16793420.7, dated 8/29/2018, 8 pages

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

Attorney Docket No.: 38767-0048US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

MAIL STOP AMENDMENT

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

INFORMATION DISCLOSURE STATEMENT

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

This statement is being filed after a first Office Action on the merits, but before receipt of a Final Office Action or a Notice of Allowance.

Please apply any necessary 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: October 16, 2019

/M. Angela Parsons/

M. Angela Parsons
Reg. No. 44,282

Customer Number 26191
Fish & Richardson P.C.
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62133481.doc

Electronic Patent Application Fee Transmittal				
Application Number:		15573300		
Filing Date:		10-Nov-2017		
Title of Invention:		EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST		
First Named Inventor/Applicant Name:		Smita Shankar		
Filer:		M. Angela Parsons/Emma Johnson		
Attorney Docket Number:		38767-0048US1		
Filed as Large Entity				
Filing Fees for U.S. National Stage under 35 USC 371				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
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:	37477455
Application Number:	15573300
International Application Number:	
Confirmation Number:	4807
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	M. Angela Parsons/Kristyn Waldhauser
Filer Authorized By:	M. Angela Parsons
Attorney Docket Number:	38767-0048US1
Receipt Date:	16-OCT-2019
Filing Date:	10-NOV-2017
Time Stamp:	17:37:39
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$ 240
RAM confirmation Number	E20190FH38048762
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	Non Patent Literature	EPSearchReport_16793420-7.pdf	679640	no	8
			6ce0d31c7ec01a78c1e3b08acb2aca8398b01c1b		
Warnings:					
Information:					
2	Non Patent Literature	GENBANK_AF066054.pdf	839537	no	2
			206cbf6ed95f7ee9e72bfb80a1d2fcefb1721e72		
Warnings:					
Information:					
3	Non Patent Literature	GENBANK_AY288296.pdf	957719	no	2
			1fdd0a0535ab199917b1a94ceb17b033e4a81d29		
Warnings:					
Information:					
4	Non Patent Literature	GENBANK_CAY70887.pdf	764728	no	2
			62a389ee5be5d3d8f418864e4cae0ad2a263ed58		
Warnings:					
Information:					
5	Non Patent Literature	GENBANK_U62648.pdf	651589	no	2
			3fe5d47b17367356f67c69da1bd933451a36b0ca		
Warnings:					
Information:					
6	Non Patent Literature	GENBANK_XM_002490678.pdf	371207	no	1
			098f885562fd7e04d070debeedcaab3d73f5dfec		
Warnings:					
Information:					

7	Non Patent Literature	HOFFMAN_IdentificationofRate-Limiting.pdf	9876619 60fe43c7ef484947a40a5988eb61df7ea9563a4c	no	7
Warnings:					
Information:					
8	Non Patent Literature	PCTISR_PCTUS2016031797.pdf	10022042 539a05ca0363efd79cf59fa091f9ee88cda942fc	no	9
Warnings:					
Information:					
9	Information Disclosure Statement (IDS) Form (SB08)	IDS.pdf	1163638 e2d461b0756330e7283138a184ecc37bde2d7f80	no	2
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
10	Fee Worksheet (SB06)	fee-info.pdf	30622 331009caa862bf8447bbddf2864a9f31c038cd1c	no	2
Warnings:					
Information:					
Total Files Size (in bytes):			25357341		
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.					

PCT/US2016/031797 20.09.2016

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 387670048WO1	FOR FURTHER ACTION	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. PCT/US16/31797	International filing date (<i>day/month/year</i>) 11 May 2016 (11.05.2016)	(Earliest) Priority Date (<i>day/month/year</i>) 11 May 2015 (11.05.2015)
Applicant IMPOSSIBLE FOODS INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 4 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the language, the international search was carried out on the basis of:

- ☒ the international application in the language in which it was filed.
☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

b. ☐ This international search report has been established taking into account the rectification of an obvious mistake authorized by or notified to this Authority under Rule 91 (Rule 43.6bis(a)).

c. ☒ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, see Box No. I.

2. ☒ Certain claims were found unsearchable (see Box No. II).

3. ☐ Unity of invention is lacking (see Box No. III).

4. With regard to the title,

- ☒ the text is approved as submitted by the applicant.
☐ the text has been established by this Authority to read as follows:

5. With regard to the abstract,

- ☒ the text is approved as submitted by the applicant.
☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the drawings,

- a. the figure of the drawings to be published with the abstract is Figure No. 2
☐ as suggested by the applicant.
☒ as selected by this Authority, because the applicant failed to suggest a figure.
☐ as selected by this Authority, because this figure better characterizes the invention.
- b. ☐ none of the figures is to be published with the abstract.

PCT/US2016/031797 20.09.2016

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/31797

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
- a. ☐ forming part of the international application as filed:
☐ in the form of an Annex C/ST.25 text file.
☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☒ furnished subsequent to the international filing date for the purposes of international search only:
☒ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

PCT/US2016/031797 20.09.2016

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US16/31797

A. CLASSIFICATION OF SUBJECT MATTER IPC(8) - C07K 14/39; C12N 15/00, 15/81; C12R 1/84 (2016.01) CPC - C07K 14/39; C12N 15/00, 15/81; C12R 1/84 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) Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched IC: C07K 14/39; C12N 1/19, 15/00, 15/29, 15/81; C12P 21/00; C12R 1/00, 1/84 (2016.01) CPC: C07K 14/39; C12N 15/00, 15/81, 15/815; C12P 21/00; C12R 1/00, 1/84 Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); EBSCO Discovery; PubMed; Google; Google Scholar; Google Patents; KEYWORDS: methylophilic yeast, Pichia, pastoris, Mxr1, leghemoglobin, heme		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2014/008729 A1 (EAST CHINA UNIVERSITY OF SCIENCE AND TECHNOLOGY) January 16, 2014; page 2, paragraph 15; page 4, paragraph 6; page 6, paragraph 6; page 7, paragraph 13; page 8, paragraphs 11-12; claim 1	1-2, 3/1-2, 4/1-2
Y	(LIN-CEREGHINO, GP et al.) Mxr1p, A Key Regulator Of The Methanol Utilization Pathway And Peroxisomal Genes In Pichia Pastoris. Molecular and Cellular Biology. February 2006, Vol. 26, No. 3; pages 883-897; abstract; page 883, column 2, paragraph 2; page 884, column 2, paragraph 2; page 886, column 2, paragraph 2; page 889, column 1, paragraph 3; DOI: 10.1128/MCB.26.3.883-897.2006	30-32, 33/30-32, 34/30-32
Y	IMPOSSIBLE FOODS. GRAS Notification For Soybean Leghemoglobin Protein Derived From Pichia Pastoris. Publication [online]: 14 September 2014 [retrieved 24 August 2016]. Retrieved from the Internet: <URL: http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm427026.pdf >	30-32, 33/30-32, 34/30-32
Y	EP 2058398 A1 (NIPRO COPORATION) May 13, 2009; abstract	30-32, 33/30-32, 34/30-32
☐ 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 "G" document member of the same patent family		
Date of the actual completion of the international search 29 August 2016 (29.08.2016)		Date of mailing of the international search report 20 SEP 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300		Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

PCT/US2016/031797 20.09.2016

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US16/31797

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.: 5-29, 35-38
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)) (January 2015)

PCT/US2016/031797 20.09.2016

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43bis.1)

To: M. Angela Parsons
Fish & Richardson P.C.
P.O. Box 1022
Minneapolis, Minnesota 55440-1022
United States of America

Date of mailing (day/month/year) 20 SEP 2016

Applicant's or agent's file reference
387670048WO1

FOR FURTHER ACTION

See paragraph 2 below

International application No.

PCT/US16/31797

International filing date (day/month/year)

11 May 2016 (11.05.2016)

Priority date (day/month/year)

11 May 2015 (11.05.2015)

International Patent Classification (IPC) or both national classification and IPC

IPC(8) - C07K 14/39; C12N 15/00, 15/81; C12R 1/84 (2016.01)

CPC - C07K 14/39; C12N 15/00, 15/81; C12R 1/84

Applicant IMPOSSIBLE FOODS INC.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/
Mail Stop PCT, Attn: ISA/US
Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-8300

Date of completion of this opinion

29 August 2016 (29.08.2016)

Authorized officer

Shane Thomas

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

PCT/US2016/031797 20.09.2016

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US16/31797

Box No. I Basis of this opinion

1. With regard to the language, this opinion has been established on the basis of:
 - ☒ the international application in the language in which it was filed.
 - ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).
2. ☐ This opinion has been established taking into account the rectification of an obvious mistake authorized by or notified to this Authority under Rule 91 (Rule 43bis.1(a)).
3. ☒ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this opinion has been established on the basis of a sequence listing:
 - a. ☐ forming part of the international application as filed:
 - ☐ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☒ furnished subsequent to the international filing date for the purposes of international search only:
 - ☒ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
4. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
5. Additional comments:

PCT/US2016/031797 20.09.2016

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US16/31797

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 5-29, 35-38

because:

☐ the said international application, or the said claims Nos. _____ relate to the following subject matter which does not require an international search (*specify*):

☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 5-29, 35-38 are so unclear that no meaningful opinion could be formed (*specify*):

Claims Nos.: 5-29, 35-38 are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed (*specify*):

☒ no international search report has been established for said claims Nos. 5-29, 35-38

☐ a meaningful opinion could not be formed without the sequence listing; the applicant did not, within the prescribed time limit:

☐ furnish a sequence listing in the form of an Annex C/ST.25 text file, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.

☐ furnish a sequence listing on paper or in the form of an image file complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.

☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13ter.1(a) or (b).

☐ See Supplemental Box for further details.

PCT/US2016/031797 20.09.2016

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US16/31797

Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims	30-32, 33/30-32, 34/30-32	YES
	Claims	1-2, 3/1-2, 4/1-2	NO
Inventive step (IS)	Claims	NONE	YES
	Claims	1-2, 3/1-2, 4/1-2, 30-32, 33/30-32, 34/30-32	NO
Industrial applicability (IA)	Claims	1-2, 3/1-2, 4/1-2, 30-32, 33/30-32, 34/30-32	YES
	Claims	NONE	NO

2. Citations and explanations:

Claims 1-2, 3/1-2, and 4/1-2 lack novelty under PCT Article 33(2) as being anticipated by WO 2014/008729 A1 (EAST CHINA UNIVERSITY OF SCIENCE AND TECHNOLOGY) (hereinafter 'China').

As per claim 1, China discloses a methylotrophic yeast cell (abstract) comprising a recombinant nucleic acid molecule (page 6, paragraph 6), wherein the recombinant nucleic acid molecule comprises an exogenous nucleic acid encoding a transcriptional activator (an exogenous gene encoding a methanol inducible transcription factor 1 (Mit1) polypeptide (a transcriptional activator); page 4, paragraph 6; page 7, paragraph 13; claim 1) operably linked to at least one methanol-inducible promoter element (operatively connected (linked) to a methanol inducible promoter; claim 1).

As per claim 2, China discloses the yeast cell of claim 1, and China further discloses wherein the methylotrophic yeast cell is of a yeast selected from the group consisting of *Candida* (page 2, paragraph 15), *Hansenula* (page 2, paragraph 15), *Pichia* (page 2, paragraph 15) and *Torulopsis* (page 2, paragraph 15).

As per claims 3/1-2, China discloses the yeast cell of claims 1-2, and China further discloses wherein the methylotrophic yeast cell is of a *Pichia* yeast (page 2, paragraph 15).

As per claims 4/1-2, China discloses the yeast cell of claims 1-2, and China further discloses wherein the cell is a *Pichia* cell (abstract; page 8, paragraphs 11-12).

Claims 30-32, 33/30-32, and 34/30-32 lack an inventive step under PCT Article 33(3) as being obvious over the publication entitled, 'Mxr1p, A Key Regulator Of The Methanol Utilization Pathway And Peroxisomal Genes In *Pichia pastoris*', by Lin-Cereghino, GP et al. (hereinafter 'Lin-Cereghino') in further view of the publication entitled, 'GRAS Notification for Soybean Leghemoglobin Protein Derived from *Pichia pastoris*' by Impossible Foods Inc. (hereinafter 'Impossible'), and EP 2058398 A1 (Nipro Corporation) (hereinafter 'Nipro').

As per claim 30, Lin-Cereghino discloses a methylotrophic *Pichia* yeast cell (*Pichia pastoris* that grows on methanol (a methylotrophic *Pichia* yeast cell); abstract) comprising a recombinant nucleic acid molecule (abstract; page 886, column 2, paragraph 2), wherein the recombinant nucleic acid molecule comprises: a nucleic acid encoding a Mxr1 transcriptional activator sequence from *P. pastoris* (a nucleic acid encoding MXR1; abstract; page 886, column 2, paragraph 2). Lin-Cereghino does not disclose a nucleic acid encoding a member of the globin family (PF00042); and a nucleic acid encoding at least one polypeptide involved in heme biosynthesis. Impossible discloses a nucleic acid encoding a member of the globin family (PF00042) (a leghemoglobin protein derived from *Pichia pastoris* (a member of the globin family (PF00042)); page 1, paragraph 1). It would have been obvious to a person of ordinary skill in the art, at the time the invention was made, to have modified the methylotrophic *Pichia* yeast cell, as previously disclosed by Lin-Cereghino, to include a nucleic acid encoding a member of the globin family (PF00042), as previously disclosed by Impossible, as the previous disclosure by Lin-Cereghino includes a method to improve growth of *Pichia* yeast cell on a growth medium to enhance the production of recombinant proteins (Lin-Cereghino; abstract; page 883, column 2, paragraph 2). Impossible discloses a soybean protein produced in *Pichia* yeast cells (Impossible, page 1, paragraph 1), and a combination of these references would have resulted in a superior methylotrophic *Pichia* yeast cell for producing a member of the globin family. Nipro discloses a nucleic acid encoding at least one polypeptide involved in heme biosynthesis (a DNA encoding a human hemoglobin α -chain (Hba), (a nucleic acid encoding at least one polypeptide involved in heme biosynthesis) which is under regulation of a promoter functional in a yeast of the genus *Pichia*; abstract). It would have been obvious to a person of ordinary skill in the art, at the time the invention was made, to have modified the methylotrophic *Pichia* yeast cell, as previously disclosed by Lin-Cereghino, to include a nucleic acid encoding at least one polypeptide involved in heme biosynthesis, as previously disclosed by Nipro, as the previous disclosure by Lin-Cereghino includes a method to improve growth of *Pichia* yeast cell on a growth medium to enhance the production of recombinant proteins (Lin-Cereghino; abstract; page 883, column 2, paragraph 2). Nipro discloses a heme protein produced in *Pichia* yeast cells (Nipro, abstract), and a combination of these references would have resulted in a superior methylotrophic *Pichia* yeast cell for producing a polypeptide involved in heme biosynthesis.

---Continued Within the Next Supplemental Box---

PCT/US2016/031797 20.09.2016

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US16/31797

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.

Continuation of:

----Continued from Box V: Citations and Explanations----

As per claim 31, Lin-Cereghino, Impossible, and Nipro, in combination, disclose the yeast cell of claim 30, and Lin-Cereghino further discloses wherein the nucleic acid encoding the Mxr1 transcriptional activator sequence is operably linked to a methanol-inducible promoter element (Mxr1p specifically binds to AOX1 5' promoter sequences (a methanol-inducible promoter); page 884, column 1, paragraph 2).

As per claim 32, Lin-Cereghino, Impossible, and Nipro, in combination, disclose the yeast cell of claim 30, but Lin-Cereghino does not disclose wherein the member of the globin family is leghemoglobin. Impossible discloses wherein the member of the globin family is leghemoglobin (a leghemoglobin protein derived from *Pichia pastoris*; page 1, paragraph 1). It would have been obvious to a person of ordinary skill in the art, at the time the invention was made, to have modified the methylotrophic *Pichia* yeast cell, as previously disclosed by Lin-Cereghino, to include wherein the member of the globin family is leghemoglobin, as previously disclosed by Impossible, as the previous disclosure by Lin-Cereghino includes a method to improve growth of *Pichia* yeast cell on a growth medium to enhance the production of recombinant proteins (Lin-Cereghino; abstract; page 883, column 2, paragraph 2), Impossible discloses a soybean protein produced in *Pichia* yeast cells (Impossible, page 1, paragraph 1), and a combination of these references would have resulted in a superior methylotrophic *Pichia* yeast cell for producing a member of the globin family.

As per claims 33/30-32, Lin-Cereghino, Impossible, and Nipro, in combination, disclose the yeast cell of claims 30-32, but Lin-Cereghino does not disclose wherein the nucleic acid encoding the member of the globin family is operably linked to a methanol-inducible promoter element. Impossible discloses wherein the nucleic acid encoding the member of the globin family is operably linked to a methanol-inducible promoter element (the LegH plasmid was integrated into the genome of Bg10 cells at the AOX1 promoter (a methanol-inducible promoter element); page 8, paragraph 2). It would have been obvious to a person of ordinary skill in the art, at the time the invention was made, to have modified the methylotrophic *Pichia* yeast cell, as previously disclosed by Lin-Cereghino, to include wherein the nucleic acid encoding the member of the globin family is operably linked to a methanol-inducible promoter element, as previously disclosed by Impossible, as the previous disclosure by Lin-Cereghino includes a method to improve growth of *Pichia* yeast cell on a growth medium to enhance the production of recombinant proteins (Lin-Cereghino; abstract; page 883, column 2, paragraph 2), Impossible discloses a soybean protein produced in *Pichia* yeast cells (Impossible, page 1, paragraph 1), and a combination of these references would have resulted in a superior methylotrophic *Pichia* yeast cell for producing a member of the globin family.

As per claims 34/30-32, Lin-Cereghino, Impossible, and Nipro, in combination, disclose the yeast cell of claims 30-32, and Lin-Cereghino further discloses wherein the nucleic acid is operably linked to a constitutive promoter element (the promoter region from the GAP gene (a constitutive promoter) is fused to sequence; page 889, column 1, paragraph 3). Lin-Cereghino does not disclose wherein the nucleic acid encoding the member of the globin family is operably linked. Impossible discloses wherein the nucleic acid encoding the member of the globin family is operably linked (the LegH plasmid was integrated into the genome of Bg10 cells at the AOX1 promoter; page 8, paragraph 2). It would have been obvious to a person of ordinary skill in the art, at the time the invention was made, to have modified the methylotrophic *Pichia* yeast cell, as previously disclosed by Lin-Cereghino, to include wherein the nucleic acid encoding the member of the globin family is operably linked, as previously disclosed by Impossible, as the previous disclosure by Lin-Cereghino includes a method to improve growth of *Pichia* yeast cell on a growth medium to enhance the production of recombinant proteins (Lin-Cereghino; abstract; page 883, column 2, paragraph 2), Impossible discloses a soybean protein produced in *Pichia* yeast cells (Impossible, page 1, paragraph 1), and a combination of these references would have resulted in a superior methylotrophic *Pichia* yeast cell for producing a member of the globin family.

Claims 1-2, 3/1-2, 4/1-2, 30-32, 33/30-32, and 34/30-32 have industrial applicability as defined by PCT Article 33(4) because the subject matter can be made or used in industry.



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/573,300	11/10/2017	Smita Shankar	38767-0048US1	4807
26191	7590	11/25/2019	EXAMINER	
FISH & RICHARDSON P.C. (TC)			LEITH, NANCY J	
PO BOX 1022			ART UNIT	PAPER NUMBER
MINNEAPOLIS, MN 55440-1022			1636	
			NOTIFICATION DATE	DELIVERY MODE
			11/25/2019	ELECTRONIC

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

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

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

PATDOCTC@fr.com

Application/Control Number: 15/573,300
Art Unit: 1636

Page 2

DETAILED ACTION

Notice of Pre-AIA or AIA Status

The present application, filed on or after March 16, 2013, is being examined under the first inventor to file provisions of the AIA.

Applicants' reply to the July 16, 2019 Office Action, filed October 16, 2019, is acknowledged. Applicants cancel claims 14, 19, 31, 33-35, and 38. Claims 20-29 remain withdrawn from further consideration as being drawn to a nonelected invention. Applicants amend claims 1, 10-12, 15, 17-18, and 30, as well as withdrawn claim 20. Claims 1-13, 15-18, 30, 32, and 37 are under examination.

Any objection or rejection of record in the previous Office Action, mailed July 16, 2019, which is not addressed in this action has been withdrawn in light of Applicants' amendments and/or arguments.

This action is FINAL.

Information Disclosure Statement

The Information Disclosure Statements filed July 12, 2019 and October 16, 2019 have been considered.

Claim Rejections - 35 USC § 112

The following is a quotation of 35 U.S.C. 112(b):

(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 10-11 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the

Application/Control Number: 15/573,300
Art Unit: 1636

Page 3

inventor or a joint inventor, or for pre-AIA the applicant regards as the invention. **These rejections are maintained.**

Claim 10 attempts to set forth a sequence for the transcriptional activator by use of a GenBank Accession Number. While inclusion of the GenBank Accession Number qualifies as an intent to incorporate a specific sequence, this Accession Number fails to uniquely identify the specific sequence intended to be encompassed by the claims and is an improper incorporation by reference because sequences of GenBank are subject to change over time. However, because there is a single version of this sequence, Applicants can provide the specific sequence in a sequence listing and list the SEQ. ID. NO. in the claim.

Claim 11 depends from claim 10, and is therefore included in this rejection.

Claim 11 attempts to set forth a sequence for the transcriptional activator by use of a GenBank Accession Number. While inclusion of the GenBank Accession Number qualifies as an intent to incorporate a specific sequence, this Accession Number fails to uniquely identify the specific sequence intended to be encompassed by the claims and is an improper incorporation by reference because sequences of GenBank are subject to change over time. However, because there is a single version of this sequence, Applicants can provide the specific sequence in a sequence listing and list the SEQ. ID. NO. in the claim.

Double Patenting

Applicant is advised that should claim 3 be found allowable, claim 4 will be objected to under 37 CFR 1.75 as being a substantial duplicate thereof. When two claims in an application are duplicates or else are so close in content that they both cover the same thing, despite a slight difference in wording, it is proper after allowing one claim to object to the other as being a substantial duplicate of the allowed claim. See MPEP § 706.03(k).

Application/Control Number: 15/573,300
Art Unit: 1636

Page 4

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the “right to exclude” granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(I)(1) - 706.02(I)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission.

Application/Control Number: 15/573,300
Art Unit: 1636

Page 5

For more information about eTerminal Disclaimers, refer to

www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

Claims 1-13, 15-18, 30, 32, and 37 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 17-26 of copending Application No.16/123,121 (reference application). This rejection is maintained.

Although the claims at issue are not identical, they are not patentably distinct from each other because the '121 application claims a methylotrophic yeast cell comprising a transcriptional activator operably linked to a promoter element. The instant application also claims a methylotrophic yeast cell comprising a transcriptional activator operably linked to a promoter element. It would have been obvious to one with ordinary skill in the art before the effective filing date of the claimed invention to use a methanol-inducible promoter for the '121 application's promoter element because this is a well-known promoter and because the promoter is inducible, provides control over the yeast cell protein production system.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

Response to Amendments and Arguments

Regarding the maintained rejections under 35 U.S.C. § 112(b)/second paragraph, Applicants assert that because the GenBank numbers do not change even if the versions are altered, the use of these numbers to claim a sequence is acceptable. However, it is noted that the versions CAN change over time, even if the accession numbers do not change. Therefore, while there is only a single version of the sequences at this time, the metes and bounds of the claims can change over time, should any other versions be deposited with GenBank. However, as stated above, because there is a single version of the sequences of claims 10 and 11, Applicants may provide these sequences in a Sequence Listing,

Application/Control Number: 15/573,300
Art Unit: 1636

Page 6

and amend the claims to claim the sequences by their Sequence ID numbers. Until Applicants make such amendments, these rejections are maintained.

Regarding the rejections under 35 U.S.C. §§ 102(a)(1)/102(a)(2) and 103 are withdrawn in light of Applicants' amendments, arguments, and the invocation of the prior art exception under 35 U.S.C. §102(b)(2)(C) relating to Varadan. Because Varadan is no longer available prior art, these rejections are withdrawn.

The Terminal Disclaimer over U.S. Patent Nos. 9, 938,327 and 10,273,492, filed October 16, 2019 are approved. Applicants request that the obviousness-type double patenting rejection over U.S. Patent Application No. 16/123,121 be held in abeyance until allowable subject matter is indicated. Therefore, this rejection is maintained.

Conclusion

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

Application/Control Number: 15/573,300
Art Unit: 1636

Page 7

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

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

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

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

NANCY J. LEITH
Primary Examiner
Art Unit 1636

/NANCY J LEITH/
Primary Examiner, Art Unit 1636

Search Notes 	Application/Control No. 15/573,300	Applicant(s)/Patent Under Reexamination Shankar et al.
	Examiner NANCY .J LEITH	Art Unit 1636

CPC - Searched*		
Symbol	Date	Examiner

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
Inventor Search (PALM)	07/09/2019	NJL
EAST Search (USPat, USPub, EPO, JPO, Derwent), text search only , see search history	07/09/2019	NJL
STN Search (Biosis, Embase, Medline), see search history	07/09/2019	NJL
Reviewed prosecution histories of U.S. Patent Nos. 9,938,327 and 10,273,492 and U.S. Patent Application No. 16/123,121	07/09/2019	NJL
Inventor Search (PALM), updated	11/20/2019	NJL
EAST Search (USPat, USPub, EPO, JPO, Derwent), updated, text search only, see search history	11/20/2019	NJL
STN Search (Biosis, Embase, Medline), updated, see search history	11/20/2019	NJL

/NANCY J LEITH/ Primary Examiner, Art Unit 1636	
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<i>Search Notes</i> 	Application/Control No. 15/573,300	Applicant(s)/Patent Under Reexamination Shankar et al.
	Examiner NANCY .J LEITH	Art Unit 1636

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/NANCY J LEITH/ Primary Examiner, Art Unit 1636	
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<i>Index of Claims</i> 	Application/Control No. 15/573,300	Applicant(s)/Patent Under Reexamination Shankar et al.
	Examiner NANCY J. LEITH	Art Unit 1636

✓	Rejected
=	Allowed

-	Cancelled
÷	Restricted

N	Non-Elected
I	Interference

A	Appeal
O	Objected

CLAIMS										
☐ Claims renumbered in the same order as presented by applicant					☐ CPA		☐ T.D.		☐ R.1.47	
CLAIM		DATE								
Final	Original	04/02/2019	07/11/2019	11/20/2019						
	1	÷	✓	✓						
	2	÷	✓	✓						
	3	÷	✓	✓						
	4	÷	✓	✓						
	5	÷	✓	✓						
	6	÷	✓	✓						
	7	÷	✓	✓						
	8	÷	✓	✓						
	9	÷	✓	✓						
	10	÷	✓	✓						
	11	÷	✓	✓						
	12	÷	✓	✓						
	13	÷	✓	✓						
	14	÷	✓	-						
	15	÷	✓	✓						
	16	÷	✓	✓						
	17	÷	✓	✓						
	18	÷	✓	✓						
	19	÷	✓	-						
	20	÷	N	N						
	21	÷	N	N						
	22	÷	N	N						
	23	÷	N	N						
	24	÷	N	N						
	25	÷	N	N						
	26	÷	N	N						
	27	÷	N	N						
	28	÷	N	N						
	29	÷	N	N						
	30	÷	✓	✓						
	31	÷	✓	-						
	32	÷	✓	✓						
	33	÷	✓	-						
	34	÷	✓	-						
	35	÷	✓	-						
	36	÷	✓	-						
	37	÷	✓	✓						
	38	÷	✓	-						

Receipt date: 07/12/2019

15/573,300 - 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. 38767-0048US1	Application No. 15/573,300
	Applicant Impossible Foods Inc.	
	Filing Date November 10, 2017	Group Art Unit 1636

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
/N. J. L./	1.	8,236,528	08/07/2012	Takagi et al.			

Foreign Patent Documents or Published Foreign Patent Applications								
Examiner Initial	Desig. ID	Document Number	Publication Date	Country or Patent Office	Class	Subclass	Translation	
							Yes	No

Other Documents (include Author, Title, Date, and Place of Publication)		
Examiner Initial	Desig. ID	Document

Examiner Signature /NANCY J LEITH/	Date Considered 11/19/2019
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
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

BIB DATA SHEET

CONFIRMATION NO. 4807

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
15/573,300	11/10/2017	435	1636	38767-0048US1
RULE				
APPLICANTS Impossible Foods Inc., Redwood City, CA;				
INVENTORS Smita Shankar, Millbrae, CA; Martin Andrew Hoyt, San Francisco, CA;				
** CONTINUING DATA ***** This application is a 371 of PCT/US2016/031797 05/11/2016 which claims benefit of 62/313,491 03/25/2016 and claims benefit of 62/236,506 10/02/2015 and claims benefit of 62/222,388 09/23/2015 and claims benefit of 62/220,366 09/18/2015 and claims benefit of 62/203,052 08/10/2015 and claims benefit of 62/185,921 06/29/2015 and claims benefit of 62/183,074 06/22/2015 and claims benefit of 62/159,899 05/11/2015				
** FOREIGN APPLICATIONS *****				
** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ** 01/25/2018				
Foreign Priority claimed ☐ Yes ☒ No 35 USC 119(a-d) conditions met ☐ Yes ☒ No Verified and Acknowledged <u>/NANCY J LEITH/</u> Examiner's Signature		☐ Met after Allowance Initials	STATE OR COUNTRY CA	SHEETS DRAWINGS 11
			TOTAL CLAIMS 38	INDEPENDENT CLAIMS 2
ADDRESS FISH & RICHARDSON P.C. (TC) PO BOX 1022 MINNEAPOLIS, MN 55440-1022 UNITED STATES				
TITLE EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST				
FILING FEE RECEIVED 3060	FEES: Authority has been given in Paper No. _____ to charge/credit DEPOSIT ACCOUNT No. _____ for following:		☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit	

Receipt date: 10/16/2019

15/573,300 - 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. 38767-0048US1	Application No. 15/573,300
	Applicant Impossible Foods Inc.		
	Filing Date November 10, 2017	Group Art Unit 1636	

U.S. Patent Documents							
Examiner Initial	Desig. ID	Document Number	Publication Date	Patentee	Class	Subclass	Filing Date If Appropriate
	1.	6,261,827	7/17/2001	Susan L. Elrod			
	2.	2011/0287467	11/24/2011	Brian R. Crane			
	3.	2019/0292217	9/26/2019	Simon Christopher Davis			
	4.	2019/0292555	9/26/2019	Simon Christopher Davis			

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
	5.	GenBank Accession No. AF066054.1, "Pichia pastoris formaldehyde dehydrogenase (FLD1) gene, complete cds," dated September 17, 1998, 2 pages
	6.	GenBank Accession No. AY288296.1, "Pichia pastoris 3-phosphoglycerate kinase (PGK1) gene, complete cds," dated July 22, 2005, 2 pages
	7.	GenBank Accession No. CAY70887.1, "Hypothetical protein PAS_chr3_0836 [Komagataella phaffii GS115]," dated February 27, 2015, 2 pages
	8.	GenBank Accession No. U62648.1, "Pichia pastoris glyceraldehyde-3-phosphate dehydrogenase (GAP) gene, complete cds," dated March 7, 1997, 2 pages
	9.	GenBank Accession No. XM_002490678.1, "Komagataella phaffii GS115 Hypothetical protein (PAS_chr1-4_0586), partial Mrna," dated July 22, 2009, 2 pages
	10.	Hoffman, et al., "Identification of rate-limiting steps in yeast heme biosynthesis," Biochem. Biophys. Res. Commun., 2003, 310(4):1247-1253
	11.	PCT International Search Report and Written Opinion in International Appln. No. PCT/US2016/031797, dated 9/20/2016, 9 pages
	12.	Supplementary European Search Report and Opinion in EP Appln. No. EP 16793420.7, dated 8/29/2018, 8 pages

Examiner Signature /NANCY J LEITH/	Date Considered 11/19/2019
EXAMINER: Initials citation considered. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.	

Substitute Disclosure Form:
ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /N.J.L/

Receipt date: 10/16/2019

15/573,300 - GAU: 1636

Attorney Docket No.: 38767-0048US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

MAIL STOP AMENDMENT

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

INFORMATION DISCLOSURE STATEMENT

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

This statement is being filed after a first Office Action on the merits, but before receipt of a Final Office Action or a Notice of Allowance.

Please apply any necessary 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: October 16, 2019

/M. Angela Parsons/

M. Angela Parsons
Reg. No. 44,282

Customer Number 26191
Fish & Richardson P.C.
Telephone: +1 (612) 638-5707
Facsimile: (877) 769-7945

62133481.doc

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /N.J.L./

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	625	(yeast\$5 or candid\$9 or hansenu19 or pichia\$9 or torulopsis\$9 or pastoris\$9 or ppastoris\$9) same (transcri\$9 (2a) activat\$8) same (promoter\$6) same((methanol\$3 or ch3\$4) near3 induc\$9)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2019/11/20 10:03
L2	21	(yeast\$5 or candid\$9 or hansenu19 or pichia\$9 or torulopsis\$9 or pastoris\$9 or ppastoris\$9) same (transcri\$9 near2 activat\$8) same (promoter\$6) same((methanol\$3 or ch3\$4) near3 induc\$9)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2019/11/20 10:03
L3	7841	(yeast\$5 or candid\$9 or hansenu19 or pichia\$9 or torulopsis\$9 or pastoris\$9 or ppastoris\$9) same (transcri\$9 near2 activat\$8) same (promoter\$6)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2019/11/20 10:03
L4	15787	(yeast\$5 or candid\$9 or hansenu19 or pichia\$9 or torulopsis\$9 or pastoris\$9 or ppastoris\$9) same (transcri\$9 near2 activat\$8)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2019/11/20 10:04
L5	114	l4 and (promoter\$6 same((methanol\$3 or ch3\$4) near3 induc\$9))	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2019/11/20 10:04
L6	14	l5 and mxr1\$3	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2019/11/20 10:05
L7	60	l5 and ((alcohol\$3 near2 oxidas\$5) or aox1\$3)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2019/11/20 10:06
L8	61	l6 or l7	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2019/11/20 10:06
L9	49	l8 and @ad<="20150510"	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2019/11/20 10:07
L10	317	SHANKAR-S.in. or SHANKAR-S-B.in. or SHANKAR-S-D.in. or SHANKAR-S-H.in. or SHANKAR-S-J.in. or SHANKAR-S-K.in. or	US-PGPUB; USPAT;	OR	OFF	2019/11/20 10:12

EAST Search History

		SHANKAR-S-K-A.in. or SHANKAR-S-L.in. or SHANKAR-S-M.in. or SHANKAR-S-N.in. or SHANKAR-S-O-PATHMASUNTHARAM-J.in. or SHANKAR-S-O-P-J.in. or SHANKAR-S-R.in. or SHANKAR-S-S.in. or SHANKAR-S-S-B.in. or SHANKAR-S-S-V.in. or SHANKAR-S-T.in. or SHANKAR-S-U.in. or SHANKAR-S-V.in. or SHANKAR-SMITA.in. or HOYT-M.in. or HOYT-M-A.in. or HOYT-MARTIN-ANDREW.in.	EPO; JPO; DERWENT			
L11	104	IMPOSSIBLE.as. or IMPOSSIBLEBURGER.as. or IMPOSSIBLE-FOODS-INC.as. or IMPOSSIBLE-FOOD-INC.as.	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2019/11/20 10:14
L12	418	I10 or I11	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2019/11/20 10:14
L13	7	I1 and I12	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2019/11/20 10:14

11/ 20/ 2019 10:14:47 AM

C:\Users\hleith\Documents\EAST\Workspaces\15573300.wsp

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FILE 'BIOSIS, EMBASE, MEDLINE' ENTERED AT 10:24:14 ON 20 NOV 2019
L1      15 SEA FILE=MFE SPE=ON ABB=ON PLU=ON (YEAST? OR CANDID? OR
        HANSENUL? OR PICHIA? OR PASTORI? OR PPASTORI?) (L) (TRANSCRI?
        (2A) ACTIVAT?) (L) (PROMOTER?) (L) ((METHANOL? OR CH3?) (3A)
        INDUC?)
L2      19068 SEA FILE=MFE SPE=ON ABB=ON PLU=ON (YEAST? OR CANDID? OR
        HANSENUL? OR PICHIA? OR PASTORI? OR PPASTORI?) (L) (TRANSCRI?
        (2A) ACTIVAT?)
L3      15 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L2 AND (PROMOTER? (L)
        ((METHANOL? OR CH3?) (3A) INDUC?))
L4      15 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L1 OR L3
L5      12 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L2 AND MXR1?
L6      27 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L4 OR L5
L7      28 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L2 AND ((ALCOHOL? (2A)
        OXIDAS?) OR AOX1?)
L8      45 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L6 OR L7
L9      19 DUPLICATE REMOVE L8 (26 DUPLICATES REMOVED)
L*** DEL 16 S L6 OR L7
L*** DEL 15 S L6 OR L7
L*** DEL 15 S L6 OR L7
L10     10 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L9 AND PY<2016
        D L10 1-10
        E SHANKAR S/ AU
L11     1538 SEA FILE=MFE SPE=ON ABB=ON PLU=ON ("SHANKAR S"/ AU OR
        "SHANKAR S BARANI"/ AU OR "SHANKAR S C"/ AU OR "SHANKAR S DR"/ AU
        OR "SHANKAR S DR PROF"/ AU OR "SHANKAR S G"/ AU OR "SHANKAR S G
        V"/ AU OR "SHANKAR S G VIJAY"/ AU OR "SHANKAR S GOKUL"/ AU OR
        "SHANKAR S GOWRI"/ AU OR "SHANKAR S J"/ AU OR "SHANKAR S JAI"/ AU
        OR "SHANKAR S K"/ AU OR "SHANKAR S K DR"/ AU OR "SHANKAR S K DR
        PROF"/ AU OR "SHANKAR S K PROF"/ AU OR "SHANKAR S K PROF DR"/ AU
        OR "SHANKAR S KARTHI K"/ AU OR "SHANKAR S KRUPA"/ AU OR "SHANKAR
        S L"/ AU OR "SHANKAR S L R"/ AU OR "SHANKAR S L RAVI"/ AU OR
        "SHANKAR S M"/ AU OR "SHANKAR S M DR"/ AU OR "SHANKAR S P"/ AU OR
        "SHANKAR S P DR PROF"/ AU OR "SHANKAR S PRABHU"/ AU OR "SHANKAR
        S PROF"/ AU OR "SHANKAR S R"/ AU OR "SHANKAR S R MADHAN"/ AU OR
        "SHANKAR S RAJA"/ AU OR "SHANKAR S RAVI"/ AU OR "SHANKAR S S"/ AU
        OR "SHANKAR S SHARATH"/ AU OR "SHANKAR S SHIV"/ AU OR "SHANKAR S
        SHI VA"/ AU OR "SHANKAR S V"/ AU OR "SHANKAR S VI DYA"/ AU OR
        "SHANKAR S VIJAY"/ AU)
        E SHANKAR SMI TA/ AU
L12     22 SEA FILE=MFE SPE=ON ABB=ON PLU=ON "SHANKAR SMI TA"/ AU
        E HOYT M AU
L13     236 SEA FILE=MFE SPE=ON ABB=ON PLU=ON ("HOYT M"/ AU OR "HOYT M
        A"/ AU OR "HOYT M ANDREW"/ AU OR "HOYT MARTIN"/ AU OR "HOYT
        MARTIN A"/ AU OR "HOYT MARTIN ANDREW"/ AU)
L14     1794 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L11 OR L12 OR L13
        E IMPOSSIBLE/ CS
L15     52 SEA FILE=MFE SPE=ON ABB=ON PLU=ON (IMPOSSIBLE/ CS OR
        "IMPOSSIBLE FOODS IMPOSSIBLEFOODS COM REDWOOD CITY 94063 CA
        USA"/ CS OR "IMPOSSIBLE FOODS INC"/ CS OR "IMPOSSIBLE FOODS INC
        400 SAGINAW DR REDWOOD CITY CA 94063 USA"/ CS OR "IMPOSSIBLE
        FOODS INC REDWOOD CITY CA 94063 USA"/ CS OR "IMPOSSIBLE FOODS
        INC REDWOOD CITY CA USA"/ CS OR "IMPOSSIBLE FOODS INC THE BOARD
        OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY"/ CS OR
        "IMPOSSIBLE FOODS INC, 400 SAGINAW DRIVE, REDWOOD CITY, CA,
        94063"/ CS OR "IMPOSSIBLE FOODS INC, 525 CHESAPEAKE DRIVE,
        REDWOOD CITY, CA, 94063"/ CS OR "IMPOSSIBLE FOODS INC, REDWOOD
        CITY, CA"/ CS OR "IMPOSSIBLE FOODS INC, REDWOOD CITY, CA,
        94063"/ CS OR "IMPOSSIBLE FOODS REDWOOD CITY CA UNITED STATES
        OF AMERICA"/ CS OR "IMPOSSIBLE FOODS STANFORD CA USA"/ CS OR
        "IMPOSSIBLE FOODS, INC, REDWOOD CITY, CA, 94063"/ CS OR
        "IMPOSSIBLE FOODS, REDWOOD CITY, CA"/ CS OR "IMPOSSIBLE FOODS,
        REDWOOD CITY, CA, 94063"/ CS)
L16     1844 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L14 OR L15
L17     0 SEA FILE=MFE SPE=ON ABB=ON PLU=ON L1 AND L16
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Doc Code: DIST.E.FILE Document Description: Electronic Terminal Disclaimer - Filed		PTO/SB/25 U.S. Patent and Trademark Office Department of Commerce
Electronic Petition Request	TERMINAL DISCLAIMER TO OBIATE A PROVISIONAL DOUBLE PATENTING REJECTION OVER A PENDING "REFERENCE" APPLICATION	
Application Number	15573300	
Filing Date	10-Nov-2017	
First Named Inventor	Smita Shankar	
Attorney Docket Number	38767-0048US1	
Title of Invention	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST	
☒ Filing of terminal disclaimer does not obviate requirement for response under 37 CFR 1.111 to outstanding Office Action		
☒ This electronic Terminal Disclaimer is not being used for a Joint Research Agreement.		
Owner	Percent Interest	
Impossible Foods Inc.	100%	
The owner(s) of percent interest listed above in the instant application hereby disclaims, except as provided below, the terminal part of the statutory term of any patent granted on the instant application which would extend beyond the expiration date of the full statutory term of any patent granted on pending reference Application Number(s) 16123121 filed on 09/06/2018 as the term of any patent granted on said reference application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending reference application. The owner hereby agrees that any patent so granted on the instant application shall be enforceable only for and during such period that it and any patent granted on the reference application are commonly owned. This agreement runs with any patent granted on the instant application and is binding upon the grantee, its successors or assigns. In making the above disclaimer, the owner does not disclaim the terminal part of any patent granted on the instant application that would extend to the expiration date of the full statutory term of any patent granted on said reference application, "as the term of any patent granted on said reference application may be shortened by any terminal disclaimer filed prior to the grant of any patent on the pending reference application," in the event that any such patent granted on the pending reference application: expires for failure to pay a maintenance fee, is held unenforceable, is found invalid by a court of competent jurisdiction, is statutorily disclaimed in whole or terminally disclaimed under 37 CFR 1.321, has all claims canceled by a reexamination certificate, is reissued, or is in any manner terminated prior to the expiration of its full statutory term as shortened by any terminal disclaimer filed prior to its grant.		
☒ Terminal disclaimer fee under 37 CFR 1.20(d) is included with Electronic Terminal Disclaimer request.		

☐ I certify, in accordance with 37 CFR 1.4(d)(4), that the terminal disclaimer fee under 37 CFR 1.20(d) required for this terminal disclaimer has already been paid in the above-identified application.	
Applicant claims the following fee status:	
☐ Small Entity ☐ Micro Entity ☒ Regular Undiscounted	
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.	
THIS PORTION MUST BE COMPLETED BY THE SIGNATORY OR SIGNATORIES 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 is of record in this application Registration Number <u>44282</u>	
☐ 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 request	
Signature	/M. Angela Parsons/
Name	M. Angela Parsons

*Statement under 37 CFR 3.73(b) is required if terminal disclaimer is signed by the assignee (owner).
Form PTO/SB/96 may be used for making this certification. See MPEP § 324.

Electronic Patent Application Fee Transmittal				
Application Number:		15573300		
Filing Date:		10-Nov-2017		
Title of Invention:		EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST		
First Named Inventor/Applicant Name:		Smita Shankar		
Filer:		M. Angela Parsons/Judith Witzel		
Attorney Docket Number:		38767-0048US1		
Filed as Large Entity				
Filing Fees for U.S. National Stage under 35 USC 371				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
STATUTORY OR TERMINAL DISCLAIMER	1814	1	160	160
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				

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

Doc Code: DISQ.E.FILE
Document Description: Electronic Terminal Disclaimer – Approved

Application No.: 15573300

Filing Date: 10-Nov-2017

Applicant/Patent under Reexamination: Shankar

Electronic Terminal Disclaimer filed on January 23, 2020

☒ APPROVED

This patent is subject to a terminal disclaimer

☐ DISAPPROVED

Approved/Disapproved by: Electronic Terminal Disclaimer automatically approved by EFS-Web

U.S. Patent and Trademark Office

Electronic Acknowledgement Receipt	
EFS ID:	38369213
Application Number:	15573300
International Application Number:	
Confirmation Number:	4807
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	M. Angela Parsons/Judith Witzel
Filer Authorized By:	M. Angela Parsons
Attorney Docket Number:	38767-0048US1
Receipt Date:	23-JAN-2020
Filing Date:	10-NOV-2017
Time Stamp:	11:53:13
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$ 160
RAM confirmation Number	E20201MB53129164
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	Terminal Disclaimer-Filed (Electronic)	eTerminal-Disclaimer.pdf	34021	no	2
			cde315b1cac375ef0b907678538b1ea982a1cd358		
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30725	no	2
			b7224dae99efb818cd041462a358d5fd2fc8dcb6		
Warnings:					
Information:					
Total Files Size (in bytes):			64746		
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.					

Attorney Docket No.: 38767-0048US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

MAIL STOP AF

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

RESPONSE TO FINAL OFFICE ACTION MAILED NOVEMBER 25, 2019

In response to the Final Office Action mailed November 25, 2019, please consider the following reply.

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 2 of 10

Attorney Docket: 38767-0048US1

Amendments to the Specification:

Please replace the Sequence Listing currently in the application with the enclosed Sequence Listing.

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 3 of 10

Attorney Docket: 38767-0048US1

Amendments to Claims:

Please cancel claim 3 without prejudice to continued prosecution, and please amend claims 10 and 11 as follows. The claims and their status are shown below.

1. (Previously Presented) A methylotrophic yeast cell comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises a first exogenous nucleic acid encoding a methanol expression regulator 1 (Mxr1) transcriptional activator operably linked to at least one methanol-inducible promoter element comprising a sequence to which the Mxr1 transcriptional activator binds, wherein the recombinant nucleic acid comprises a second exogenous nucleic acid encoding a heterologous polypeptide operably linked to at least one methanol-inducible promoter element.

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

3. (Canceled)

4. (Previously Presented) The yeast cell of claim 1, wherein the cell is a *Pichia* cell.

5. (Previously Presented) The yeast cell of claim 1, wherein the cell is a *Pichia pastoris* cell.

6. (Previously Presented) The yeast cell of claim 1, wherein the recombinant nucleic acid molecule is stably integrated into the genome of the methylotrophic yeast cell.

7. (Previously Presented) The yeast cell of claim 1, wherein the recombinant nucleic acid molecule is extrachromosomally expressed from a replication-competent plasmid.

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 4 of 10

Attorney Docket: 38767-0048US1

8. (Canceled)

9. (Previously Presented) The yeast cell of claim 1, wherein the exogenous nucleic acid encoding a transcriptional activator comprises a Mxr1 sequence from *Pichia pastoris*.

10. (Currently Amended) The yeast cell of claim 1, wherein the transcriptional activator comprises a sequence shown in SEQ ID NO: 59 ~~GenPept Gi 88866603~~.

11. (Currently Amended) The yeast cell of claim 10, wherein the transcriptional activator is encoded by the nucleic acid sequence shown in SEQ ID NO: 60 ~~GenBank Gi 88866602~~.

12. (Previously Presented) The yeast cell of claim 1, wherein the at least one methanol-inducible promoter element is selected from the group consisting of an alcohol oxidase 1 (AOX1) promoter element from *Pichia pastoris*, an alcohol oxidase (AOD1) promoter element from *Candida boidinii*, a methanol oxidase (MOX) promoter element from *Hansenula polymorpha*, a methanolic alcohol oxidase (MOD1) promoter element from *Pichia methanolica*, a dihydroxyacetone synthase (DHAS) promoter element from *Pichia pastoris*, a formaldehyde dehydrogenase (FLD1) promoter element from *Pichia pastoris*, and a peroxin 8 (PEX8) promoter element from *Pichia pastoris*.

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

14. (Canceled)

15. (Previously Presented) The yeast cell of claim 1, wherein the heterologous nucleic acid encodes at least one polypeptide involved in the biosynthesis of an iron co-factor.

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

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 5 of 10

Attorney Docket: 38767-0048US1

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

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

19. (Canceled)

20. (Withdrawn—Previously Presented) A method for expressing a heterologous polypeptide in a cell, the method comprising:

providing the yeast cell of claim 1;

and

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

thereby expressing the heterologous polypeptide.

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

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

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

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 6 of 10

Attorney Docket: 38767-0048US1

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

25. (Withdrawn) The method of claim 20, wherein the introducing step comprises a technique selected from the group consisting of transduction, electroporation, biolistic particle delivery, and chemical transformation.

26. (Withdrawn) The method of claim 20, wherein the conditions under which the cells are cultured comprises the addition of iron or a pharmaceutically or metabolically acceptable salt thereof.

27. (Withdrawn) The method of claim 20, wherein the culturing step comprises culturing the cell in the presence of methanol.

28. (Withdrawn) The method of claim 20, wherein the culturing step comprises culturing the cell in the absence of methanol.

29. (Withdrawn) The method of claim 20, wherein the culturing step comprises culturing the cell in the absence of selection.

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

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

pastoris;

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

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

wherein each nucleic acid is operably linked to a methanol-inducible promoter element, wherein the methanol-inducible promoter element comprises at least one sequence to which a Mxr1 transcriptional activator binds.

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 7 of 10

Attorney Docket: 38767-0048US1

31. (Canceled)

32. (Previously Presented) The yeast cell of claim 30, wherein the member of the globin family is leghemoglobin.

33-36. (Canceled)

37. (Previously Presented) The yeast cell of claim 31, wherein the methanol-inducible promoter element is an alcohol oxidase I (AOX1) promoter element from *P. pastoris*.

38. (Canceled)

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 8 of 10

Attorney Docket: 38767-0048US1

REMARKS

Applicants respectfully request entry of the amendments and remarks submitted herein. Claims 10 and 11 have been amended as discussed below, and claim 3 has been canceled without prejudice to continued prosecution. In addition, the specification has been amended to incorporate an updated Sequence Listing, as explained in more detail below. Although the present application is After Final, the present amendments should be entered because they are believed to put the claims in condition for allowance.

Claims 1, 2, 4-7, 9-13, 15-18, 30, 32 and 37 are currently pending¹, and claims 20-29 are withdrawn. Reconsideration of the pending application is respectfully requested.

Sequence Listing

Applicants electronically submit herewith an updated Sequence Listing and have amended the specification to incorporate the enclosed updated Sequence Listing into the above-referenced application. The enclosed Sequence Listing, submitted as an ASCII text file, complies with the requirements under both 37 CFR §1.821(c) and 37 CFR §1.821(e). The enclosed Sequence Listing fulfills the requirements under 37 CFR §1.821 - §1.825 and does not introduce any new matter into the application.

Claim Rejections - 35 U.S.C. § 112

Claims 10-11 are rejected under 35 U.S.C. § 112(b) or 35 U.S.C. § 112 (pre-AIA), second paragraph, as the Examiner asserted that those claims are indefinite.

Without acquiescing to the Examiner's rejection, Applicants have added the appropriate sequences to the Sequence Listing in the present application and amended claims 10 and 11 to

¹ The Examiner indicated in the Office Action Summary and on page 2 of the Office Action mailed November 25, 2019 that claim 8 also is pending. Claim 8 was canceled, however, in the Response to Office Action filed on October 16, 2019.

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 9 of 10

Attorney Docket: 38767-0048US1

refer to the sequence identifier instead of a database Accession number. In addition, the specification has been amended to incorporate the enclosed updated Sequence Listing.

In view of these amendments and remarks, Applicants respectfully request that the rejection of claims 10-11 under 35 U.S.C. § 112(b) or 35 U.S.C. § 112 (pre-AIA), second paragraph, be withdrawn.

The Double Patenting Objection / Rejection

The Examiner advised Applicant that should claim 3 be found allowable, claim 4 will be objected to under 37 CFR § 1.75 as being a substantial duplicate thereof. OA at page 3.

Without acquiescing to the Examiner's potential objection, Applicants have canceled claim 3 without prejudice to continued prosecution. Accordingly, Applicants respectfully submit that any potential double patenting rejection against claim 3 or claim 4 is now moot.

Claims 1-13, 15-18, 30, 32, and 37 are provisionally rejected on the ground of non-statutory double patenting as being unpatentable over claims 17-26 of co-pending Application No. 16/123,121.

Using the language provided in MPEP 717.02(b)(III), Applicants herewith submit that the present application, U.S. Application No. 15/573,300, and co-pending reference application, U.S. Application No. 16/123,121, were, at the time the invention described in the pending claims was effectively filed, owned by Impossible Foods, Inc.

Based on common ownership, Applicants submit herewith a Terminal Disclaimer in compliance with 37 CFR § 1.321(c), and respectfully request that the provisional rejection on the grounds of non-statutory double patenting be withdrawn.

Request for Rejoinder

Claims 20-29 were withdrawn as directed to a non-elected invention following the Restriction Requirement mailed April 8, 2019 and Applicants' election of June 6, 2019. Since independent claim 1 should be allowable, and since claim 20 previously was amended to be commensurate in scope with independent claim 1, Applicants respectfully request that claims 20-29 be rejoined and allowed pursuant to MPEP § 821.04.

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 10 of 10

Attorney Docket: 38767-0048US1

CONCLUSION

Applicants respectfully request that claims 1, 2, 4-7, 9-13, 15-18, 20-30, 32 and 37 be allowed. If a telephone call to the undersigned would expedite prosecution, the Examiner is encouraged to do so.

The present Response to Final Office Action is being submitted within the 2-month early period for Reply. Therefore, no fees are believed due. If this is incorrect, please apply any charges, or credits, to Deposit Account No. 06-1050, referencing the above-indicated Attorney Docket Number.

Respectfully submitted,

Date: January 23, 2020

/M. Angela Parsons/
M. Angela Parsons, Ph.D.
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62186089.doc

Electronic Acknowledgement Receipt	
EFS ID:	38376588
Application Number:	15573300
International Application Number:	
Confirmation Number:	4807
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	M. Angela Parsons/Judith Witzel
Filer Authorized By:	M. Angela Parsons
Attorney Docket Number:	38767-0048US1
Receipt Date:	23-JAN-2020
Filing Date:	10-NOV-2017
Time Stamp:	11:57:25
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment		no			
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Response After Final Action			1	1	
Specification			2	2	
Claims			3	7	
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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.					

DocCode – SEQ.TXT

SCORE Placeholder Sheet for IFW Content

Application Number: 15573300

Document Date: 01/23/2020

The presence of this form in the IFW record indicates that the following document type was received in electronic format on the date identified above. This content is stored in the SCORE database.

Since this was an electronic submission, there is no physical artifact folder, no artifact folder is recorded in PALM, and no paper documents or physical media exist. The TIFF images in the IFW record were created from the original documents that are stored in SCORE.

- Sequence Listing

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Form Revision Date: March 1, 2019

PTO/SB/06 (09-11)
Approved for use through 1/31/2014. OMB 0651-0032
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 displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875				Application or Docket Number 15/573,300		Filing Date 11/10/2017		☐ To be Mailed	
ENTITY: ☒ LARGE ☐ SMALL ☐ MICRO									
APPLICATION AS FILED - PART I									
FOR		(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA		RATE (\$)	FEE (\$)			
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))		N/A	N/A		N/A				
☐ SEARCH FEE (37 CFR 1.16(k), (l), or (m))		N/A	N/A		N/A				
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))		N/A	N/A		N/A				
TOTAL CLAIMS (37 CFR 1.16(i))		minus 20 =	*		x \$80 =				
INDEPENDENT CLAIMS (37 CFR 1.16(h))		minus 3 =	*		x \$420 =				
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))		If the specification and drawings exceed 100 sheets of paper, the application size fee due is \$310 (\$155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).							
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))									
* If the difference in column 1 is less than zero, enter "0" in column 2.					TOTAL				
APPLICATION AS AMENDED - PART II									
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	Independent (37 CFR 1.16(h))	* 2	Minus	*** 3	= 0	x \$460 =	0		
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	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))								
						TOTAL ADD'L FEE	0		
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						TOTAL ADD'L FEE			
* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.						LIE			
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".						/VICTOR BARLOW/			
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".									
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.									

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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

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Sequence Listing was accepted.
If you need help call the Patent Electronic Business Center at (866)
217-9197 (toll free).
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His Ser Asn Cys Ser Asp Ala Ala Ile Thr Arg Leu Arg Arg Lys Ala
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Ser Asn Met Arg Ser Phe Ile His Tyr Ala Leu Ser Lys Tyr Pro Phe
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Ile Gly Val Pro Thr Pro Thr Leu Pro Glu Asn Glu Arg Leu Asn Glu
485 490 495

Tyr Ala Asp Ser Phe Thr Asn Arg Phe Leu Asn His Tyr Pro Phe Ile
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His Val Thr Ile Leu Lys Glu Tyr Ser Leu Phe Lys Ala Ile Leu Asp
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Glu Asn Glu Ser Thr Lys Asn Trp Glu Asn Asn Gln Phe Tyr Leu Glu
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565 570 575

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Gln Ser Leu Thr Leu Ser Val Met Tyr Gly Leu Phe Ala Asp Asn Asp
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Ile Asn Lys Pro Leu Asp Asn Glu Leu Trp Asn Gln Phe Val Lys Ile
660 665 670

Glu Ser Thr Val Arg Thr Ile His Thr Ile Phe Gln Ile Ser Ser Asn
675 680 685

Leu Ser Ala Leu Tyr Asn Ile Ile Pro Ser Leu Lys Ile Asp Asp Leu
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Met Ile Thr Leu Pro Val Pro Thr Thr Leu Trp Gln Ala Asp Ser Phe
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Thr Arg Val Leu Gln Asn Leu Ile Asp Tyr Asn Gln Pro Leu Ser Asp
740 745 750

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Leu Gln Asn Gly Leu His Gln Tyr Ser Tyr Phe Gln Lys Leu Thr Ala
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Ile Tyr Gln Gln Ser Cys Leu Ile Leu Asp Ser Lys Leu Leu Asn Asn
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Phe Leu Ile Val Lys Ser Ser Leu Lys Val Ser Thr Gly Asp Val Ser
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Ser Leu Asn Lys Leu Lys Glu Asn Val Trp Leu Lys Asn Trp Asn Gln
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Leu Ser Leu Ile Ile Ile Lys Ile Met Lys Leu Ile Phe Tyr Asn Asn
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965 970 975

Ser Asn Ile Ser Phe Leu His His Phe Glu Leu Ser Ser Val Ile Asn
980 985 990

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995 1000 1005

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Tyr Leu Asp Leu Glu Lys Phe Met Lys Phe Lys Phe Asn Tyr His
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Met Asn Ala Lys Asp Glu Lys Ile Ser Pro Thr Thr Leu Asn Ser
1070 1075 1080

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Lys Leu Phe Gly Leu Val Arg Asp Ser Ala Gly Gln Leu Lys Ala Asn
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Gly Thr Val Val Ala Asp Ala Ala Leu Gly Ser Ile His Ala Gln Lys
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100 105 110

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Phe
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ggtacagttg tggcagatgc tgccttggga tctatacatg caaaaaggc taccaccgac



UNITED STATES PATENT AND TRADEMARK OFFICE

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

NOTICE OF ALLOWANCE AND FEE(S) DUE

26191 7590 02/04/2020
FISH & RICHARDSON P.C. (TC)
PO BOX 1022
MINNEAPOLIS, MN 55440-1022

EXAMINER	
LEITH, NANCY J	
ART UNIT	PAPER NUMBER
1636	

DATE MAILED: 02/04/2020

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
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15/573,300	11/10/2017	Smita Shankar	38767-0048US1	4807
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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	\$0.00	\$1000	05/04/2020

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 02/04/2020
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.
15/573,300	11/10/2017	Smita Shankar	38767-0048US1	4807

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	\$0.00	\$1000	05/04/2020

EXAMINER	ART UNIT	CLASS-SUBCLASS
LEITH, NANCY J	1636	435-069100

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. _____



UNITED STATES PATENT AND TRADEMARK OFFICE

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

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/573,300	11/10/2017	Smita Shankar	38767-0048US1	4807
26191	7590	02/04/2020	EXAMINER	
FISH & RICHARDSON P.C. (TC)			LEITH, NANCY J	
PO BOX 1022				
MINNEAPOLIS, MN 55440-1022			ART UNIT	PAPER NUMBER
			1636	
DATE MAILED: 02/04/2020				

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.

<i>Notice Requiring Inventor's Oath or Declaration</i>	Application No. 15/573,300	Applicant(s) Smita Shankar	
	Examiner LEITH, NANCY J	Art Unit 1636	

This notice is an attachment to the Notice of Allowability (PTOL-37), or the Notice of Allowability For A Design Application (PTOL-37D).

An inventor's oath or declaration in compliance with 37 CFR 1.63 or 1.64 executed by or with respect to each inventor has not yet been submitted.

An oath or declaration in compliance with 37 CFR 1.63, or a substitute statement in compliance with 37 CFR 1.64, executed by or with respect to each inventor (for any inventor for which a compliant oath, declaration, or substitute statement has not yet been submitted) **MUST** be filed no later than the date on which the issue fee is paid. See 35 U.S.C. 115(f). Failure to timely comply will result in ABANDONMENT of this application.

A properly executed inventor's oath to declaration has not been received for the following inventor(s):

If applicant previously filed one or more oaths, declarations, or substitute statements, applicant may have received an informational notice regarding deficiencies therein.

The following deficiencies are noted:

INFORMAL ACTION PROBLEMS

- Properly executed inventor's oath or declaration for the following inventor(s) has not been submitted: **Smita Shankar** and **Martin Andrew Hoyt**

Questions relating to this Notice should be directed to the Application Assistance Unit at 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 regulation.

Notice of Allowability	Application No. 15/573,300	Applicant(s) Shankar et al.	
	Examiner NANCY J. LEITH	Art Unit 1636	AIA (FITF) Status Yes

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

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 Amendment and Terminal Disclaimer filed January 23, 2020.
☐ 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 See Continuation Sheet. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov**.
4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
Certified copies:
a) ☐ All b) ☐ Some *c) ☐ None of the:
1. ☐ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. ____.
3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).
* Certified copies not received: ____.

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

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

Attachment(s)

1. ☐ Notice of References Cited (PTO-892) 2. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date ____. 3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material ____. 4. ☒ Interview Summary (PTO-413), Paper No./Mail Date. <u>20200127</u> .	5. ☒ Examiner's Amendment/Comment 6. ☒ Examiner's Statement of Reasons for Allowance 7. ☐ Other _____.
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/NANCY J LEITH/ Primary Examiner, Art Unit 1636	
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Continuation Sheet (PTOL-37)

Application No. **15/573,300**

Continuation of 3. The allowed claim(s) is/are: 1-2,4-7,9-13,15-18,20-30,32 and 37

Application/Control Number: 15/573,300
Art Unit: 1636

Page 2

EXAMINER'S AMENDMENT

An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in an interview with Dr. M. Angela Parsons on January 28, 2019.

The application has been amended as follows:

10. (Currently Amended) The yeast cell of claim 1, wherein the transcriptional activator comprises ~~[[a]]~~ the sequence shown in SEQ ID NO: 59.

15. (Currently Amended) The yeast cell of claim 1, wherein the heterologous nucleic acid encodes at least one polypeptide involved in ~~the~~ biosynthesis of an iron co-factor.

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

providing the yeast cell of claim 1; and
culturing the cell under conditions ~~suited~~ suitable for expression of the recombinant nucleic acid molecule,
thereby expressing the heterologous polypeptide.

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

Application/Control Number: 15/573,300
Art Unit: 1636

Page 3

22. (Original) The method of claim 20, wherein the heterologous polypeptide is a plant hemoglobin.
23. (Original) The method of claim 22, wherein the plant hemoglobin is leghemoglobin.
24. (Original) The method of claim 23, wherein the leghemoglobin has a sequence selected from the group consisting of SEQ ID NO:4 and SEQ ID NO:6.
25. (Original) The method of claim 20, wherein the introducing step comprises a technique selected from the group consisting of transduction, electroporation, biolistic particle delivery, and chemical transformation.
26. (Currently Amended) The method of claim 20, wherein the conditions under which the ~~cells are~~ cell is cultured comprises ~~the~~ addition of iron or a pharmaceutically or metabolically acceptable salt thereof.
27. (Currently Amended) The method of claim 20, wherein the culturing step comprises culturing the cell in the ~~present~~ presence of methanol.
28. (Original) The method of claim 20, wherein the culturing step comprises culturing the cell in the absence of methanol.
29. (Original) The method of claim 20, wherein the culturing step comprises culturing the cell in the absence of selection.

Application/Control Number: 15/573,300
Art Unit: 1636

Page 4

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

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

a nucleic acid encoding a member of the globin family PF00042; and a nucleic acid encoding at least one polypeptide involved in heme biosynthesis, wherein each nucleic acid is operably linked to a methanol-inducible promoter element, wherein the methanol-inducible promoter element comprises at least one sequence to which a Mxr1 transcriptional activator binds.

37. (Currently Amended) The yeast cell of claim [[31]] 30, wherein the methanol-inducible promoter element is an alcohol oxidase 1 (AOX1) promoter element from *P. pastoris*.

REASONS FOR ALLOWANCE

The following is an examiner's statement of reasons for allowance:

The prior art fails to disclose or suggest a methylotrophic yeast cell comprising a methanol expression regulator 1 (Mxr1) transcriptional activator, a methanol-inducible promoter element to which the Mxr1 transcriptional activator binds, and a nucleic acid encoding a heterologous polypeptide. The yeast can be *Pichia pastoris*. The prior art fails to disclose or suggest a method of producing a polypeptide by culturing the methylotrophic yeast under conditions for expressing the polypeptide, the polypeptide can be a member of the heme family.

The Terminal Disclaimer over U.S. Patent Application Publication No. 16/123,121, filed January 23, 2019, is approved.

Application/Control Number: 15/573,300
Art Unit: 1636

Page 5

Claims 20-29 are rejoined with allowed claims 1-2, 4-7, 9-13, 15-18, 32, and 37, and are also allowed.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled “Comments on Statement of Reasons for Allowance.”

Conclusion

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

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

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

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <https://ppair-my.uspto.gov/pair/PrivatePair>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a

Application/Control Number: 15/573,300
Art Unit: 1636

Page 6

USPTO Customer Service Representative or access to the automated information system, call 800-786-

9199 (IN USA OR CANADA) or 571-272-1000.

NANCY J. LEITH
Primary Examiner
Art Unit 1636

/NANCY J LEITH/
Primary Examiner, Art Unit 1636

<i>Examiner-Initiated Interview Summary</i>	Application No. 15/573,300	Applicant(s) Shankar et al.	
	Examiner NANCY J LEITH	Art Unit 1636	AIA (FITF) Status Yes

All participants (applicant, applicant's representative, PTO personnel):

(1) Nancy J. Leith. (3) ____.

(2) Dr. M. Angela Parsons. (4) ____.

Date of Interview: 28 January 2020.

Type: ☒ Telephonic ☐ Video Conference
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.
If Yes, brief description: ____.

Issues Discussed ☐ 101 ☐ 112 ☐ 102 ☐ 103 ☒ Others
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: 1-2,4-7,9-13,15-18,20-30,32 and 37.

Identification of prior art discussed: N/A.

Substance of Interview
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

Dr. Parsons authorized the changes to the claims, as set forth in the Examiner's Amendment.

Applicant recordation instructions: It is not necessary for applicant to provide a separate record of the substance of interview.

Examiner recordation instructions: Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/NANCY J LEITH/ Primary Examiner, Art Unit 1636	
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<i>Search Notes</i> 	Application/Control No. 15/573,300	Applicant(s)/Patent Under Reexamination Shankar et al.
	Examiner NANCY J LEITH	Art Unit 1636

CPC - Searched*		
Symbol	Date	Examiner

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.

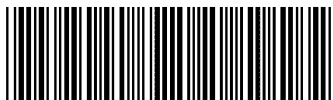
/NANCY J LEITH/ Primary Examiner, Art Unit 1636	
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Search Notes 	Application/Control No. 15/573,300	Applicant(s)/Patent Under Reexamination Shankar et al.
	Examiner NANCY J LEITH	Art Unit 1636

Search Notes		
Search Notes	Date	Examiner
Inventor Search (PALM)	07/09/2019	NJL
EAST Search (USPat, USPub, EPO, JPO, Derwent), text search only , see search history	07/09/2019	NJL
STN Search (Biosis, Embase, Medline), see search history	07/09/2019	NJL
Reviewed prosecution histories of U.S. Patent Nos. 9,938,327 and 10,273,492 and U.S. Patent Application No. 16/123,121	07/09/2019	NJL
Inventor Search (PALM), updated	11/20/2019	NJL
EAST Search (USPat, USPub, EPO, JPO, Derwent), updated, text search only, see search history	11/20/2019	NJL
STN Search (Biosis, Embase, Medline), updated, see search history	11/20/2019	NJL
Inventor Search (PALM), updated	01/27/2020	NJL
EAST Search (USPat, USPub, EPO, JPO, Derwent), updated, text search only see search history	01/27/2020	NJL
STN Search (Biosis, Embase, Medline), updated, see search history	01/27/2020	NJL
Reviewed Sequence Search Results in SCORE	01/28/2020	NJL

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
Text Search Only	EAST Interference Search (USPat, USPub), see search history	01/27/2020	NJL
	Reviewed Interference Sequence Search Results in SCORE	01/28/2020	NJL

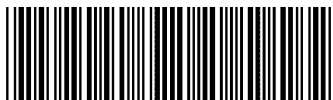
/NANCY J LEITH/ Primary Examiner, Art Unit 1636	
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Issue Classification 	Application/Control No. 15/573,300	Applicant(s)/Patent Under Reexamination Shankar et al.
	Examiner NANCY J. LEITH	Art Unit 1636

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	Version
	/		/		

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	28	
/NANCY J LEITH/ Primary Examiner, Art Unit 1636	28 January 2020	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	None

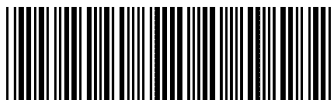
Issue Classification 	Application/Control No. 15/573,300	Applicant(s)/Patent Under Reexamination Shankar et al.
	Examiner NANCY J. LEITH	Art Unit 1636

INTERNATIONAL CLASSIFICATION			
CLAIMED			
C12N15/81	/	15	/ 81
C07K14/39	/	14	/ 39
C07K14/415	/	14	/ 415
C07K14/805	/	14	/ 805
C12N1/16	/	1	/ 16
C12N15/63	/	15	/ 63
NON-CLAIMED			
/	/		

US ORIGINAL CLASSIFICATION	
CLASS	SUBCLASS
435	69.1

CROSS REFERENCES(S)						
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)					
435	69.6	70.1	71.1	440	483	254.2
435	254.23					

NONE		Total Claims Allowed:	
(Assistant Examiner)		28	
(Date)			
/NANCY J LEITH/		28 January 2020	
Primary Examiner, Art Unit 1636		O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)		1	None
(Date)			

<i>Issue Classification</i> 	Application/Control No. 15/573,300	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
1	1	8	10		19	24	28	28	37						
2	2	9	11	16	20	25	29		38						
	3	10	12	17	21	26	30								
3	4	11	13	18	22		31								
4	5		14	19	23	27	32								
5	6	12	15	20	24		33								
6	7	13	16	21	25		34								
	8	14	17	22	26		35								
7	9	15	18	23	27		36								

NONE (Assistant Examiner) _____ (Date) _____		Total Claims Allowed: 28	
/NANCY J LEITH/ Primary Examiner, Art Unit 1636 (Primary Examiner) _____ (Date) _____		28 January 2020 O.G. Print Claim(s) 1	O.G. Print Figure None

(FILE 'HOME' ENTERED AT 10:19:23 ON 27 JAN 2020)

FILE 'BIOSIS, EMBASE, MEDLINE' ENTERED AT 10:19:33 ON 27 JAN 2020

L1 15 S (YEAST? OR CANDID? OR HANSENUL? OR PICHIA? OR TORULOPSIS? OR
L2 19217 S (YEAST? OR CANDID? OR HANSENUL? OR PICHIA? OR TORULOPSIS? OR
L3 15 S L2 AND (PROMOTER? (L) ((METHANOL? OR CH3?) (3A) INDUC?))
L4 12 S L2 AND MXR1?
L5 28 S L2 AND ((ALCOHOL? (2A) OXIDAS?) OR AOX1?)
L6 45 S L3 OR L4 OR L5
L7 19 DUPLICATE REMOVE L6 (26 DUPLICATES REMOVED)
L8 10 S L7 AND PY<2016
E SHANKAR S/AU
L9 1547 S E3-E41
E SHANKAR SMITA/AU
L10 49 S E3-E4
E HOYT M/AU
L11 334 S E3-E11,E18-E20
E IMPOSSIBLE/CS/
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L13 53 S E3-E18
L14 1979 S L9 OR L10 OR L11 OR L12 OR L13
L15 0 S L2 AND L14
L16 34 S L14 AND (HEME? OR HEMOGLOB? OR HAEME? OR HAEMOGLOB? OR LEGHEM
L17 20 DUPLICATE REMOVE L16 (14 DUPLICATES REMOVED)

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	626	(yeast\$5 or candid\$9 or hansenu19 or pichia\$9 or torulopsis\$9 or pastoris\$9 or ppastoris\$9) same (transcri\$9 (2a) activat\$8) same (promoter\$6) same((methanol\$3 or ch3\$4) near3 induc\$9)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 09:53
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L4	15895	(yeast\$5 or candid\$9 or hansenu19 or pichia\$9 or torulopsis\$9 or pastoris\$9 or ppastoris\$9) same (transcri\$9 near2 activat\$8)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 09:58
L5	18	"I5" and (promoter\$6 same((methanol\$3 or ch3\$4) near3 induc\$9))	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 09:58
L6	2	"I6" and mxr1\$3	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 09:58
L7	114	I4 and (promoter\$6 same((methanol\$3 or ch3\$4) near3 induc\$9))	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 09:59
L8	14	I7 and mxr1\$3	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 09:59
L9	60	I7 and ((alcohol\$3 near2 oxidas\$5) or aox1\$3)	US-PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 09:59
L10	61	I8 or I9	US-PGPUB; USPAT;	OR	OFF	2020/01/27 09:59

EAST Search History

			EPO; JPO; DERWENT			
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L13	107	IMPOSSIBLE.as. or IMPOSSIBLEBURGER.as. or IMPOSSIBLE- FOODS-INC.as. or IMPOSSIBLE-FOOD- INC.as.	US- PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 09:59
L14	427	l12 or l13	US- PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 10:00
L15	7	l4 and l14	US- PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 10:00
L16	15	((yeast\$5 or candid\$9 or hansenu19 or pichia\$9 or torulopsis\$9 or pastoris\$9 or ppastoris\$9) same (transcri\$9 (2a) activat\$8) same (promoter\$6) same((methanol\$3 or ch3\$4) near3 induc\$9)).clm.	US- PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 10:00
L18	5	((yeast\$5 or candid\$9 or hansenu19 or pichia\$9 or torulopsis\$9 or pastoris\$9 or ppastoris\$9) same (transcri\$9 near2 activat\$8) same (promoter\$6) same((methanol\$3 or ch3\$4) near3 induc\$9).clm.)	US- PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 10:01
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L29	626	(yeast\$5 or candid\$9 or hansenu19 or pichia\$9 or torulopsis\$9 or pastoris\$9 or ppastoris\$9) same (transcri\$9 (2a) activat\$8) same (promoter\$6) same((methanol\$3 or ch3\$4) near3 induc\$9)	US- PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 10:33
L30	21	(yeast\$5 or candid\$9 or hansenu19 or pichia\$9 or torulopsis\$9 or pastoris\$9 or	US- PGPUB;	OR	OFF	2020/01/27 10:33

EAST Search History

		ppastoris\$9) same (transcri\$9 near2 activat\$8) same (promoter\$6) same((methanol\$3 or ch3\$4) near3 induc\$9)	USPAT; EPO; JPO; DERWENT			
L31	7886	(yeast\$5 or candid\$9 or hansenu\$9 or pichia\$9 or torulopsis\$9 or pastoris\$9 or ppastoris\$9) same (transcri\$9 near2 activat\$8) same (promoter\$6)	US- PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 10:33
L32	15896	(yeast\$5 or candid\$9 or hansenu\$9 or pichia\$9 or torulopsis\$9 or pastoris\$9 or ppastoris\$9) same (transcri\$9 near2 activat\$8)	US- PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 10:33
L33	115	l32 and (promoter\$6 same((methanol\$3 or ch3\$4) near3 induc\$9))	US- PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 10:34
L34	39	l32 and mxr1\$3	US- PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 10:34
L35	14	l33 and mxr1\$3	US- PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 10:34
L36	61	l33 and ((alcohol\$3 near2 oxidas\$5) or aox1\$3)	US- PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 10:34
L37	62	l35 or l36	US- PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 10:35
L38	50	l37 and @ad<="20150510"	US- PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 10:35
L39	7	l14 and l37	US- PGPUB; USPAT; EPO; JPO; DERWENT	OR	OFF	2020/01/27 10:36

1/ 27/ 2020 10:39:52 AM

C:\Users\hleith\Documents\EAST\Workspaces\15573300.wsp

EAST Search History

EAST Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L17	15	((yeast\$5 or candid\$9 or hansenu19 or pichia\$9 or torulopsis\$9 or pastoris\$9 or ppastoris\$9) same (transcri\$9 (2a) activat\$8) same (promoter\$6) same((methanol\$3 or ch3\$4) near3 induc\$9)).clm.	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:00
L19	5	((yeast\$5 or candid\$9 or hansenu19 or pichia\$9 or torulopsis\$9 or pastoris\$9 or ppastoris\$9) same (transcri\$9 near2 activat\$8) same (promoter\$6) same((methanol\$3 or ch3\$4) near3 induc\$9)).clm.	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:01
L20	65	((yeast\$5 or candid\$9 or hansenu19 or pichia\$9 or torulopsis\$9 or pastoris\$9 or ppastoris\$9) same (transcri\$9 near2 activat\$8) same (promoter\$6)).clm.	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:02
L22	249	((yeast\$5 or candid\$9 or hansenu19 or pichia\$9 or torulopsis\$9 or pastoris\$9 or ppastoris\$9) same (transcri\$9 near2 activat\$8)).clm.	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:03
L23	13901	l17 or l19 or l20 or "l21"	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:03
L24	259	l17 or l19 or l20 or l22	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:03
L25	20	l24 and (promoter\$6 same((methanol\$3 or ch3\$4) near3 induc\$9)).clm.	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:04
L26	7	l25 and mxr1\$3.clm.	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:04
L27	18	l25 and ((alcohol\$3 near2 oxidas\$5) or aox1\$3).clm.	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:04
L28	7	l26 and l27	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:04
L40	15	((yeast\$5 or candid\$9 or hansenu19 or pichia\$9 or torulopsis\$9 or pastoris\$9 or ppastoris\$9) same (transcri\$9 (2a) activat\$8) same (promoter\$6) same((methanol\$3 or ch3\$4) near3 induc\$9)).clm.	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:36
L41	5	((yeast\$5 or candid\$9 or hansenu19 or pichia\$9 or torulopsis\$9 or pastoris\$9 or ppastoris\$9) same (transcri\$9 near2 activat\$8) same (promoter\$6) same((methanol\$3 or ch3\$4) near3 induc\$9)).clm.	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:36

EAST Search History

L42	65	((yeast\$5 or candid\$9 or hansenul\$9 or pichia\$9 or torulopsis\$9 or pastoris\$9 or ppastoris\$9) same (transcri\$9 near2 activat\$8) same (promoter\$6)).clm.	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:37
L43	249	((yeast\$5 or candid\$9 or hansenul\$9 or pichia\$9 or torulopsis\$9 or pastoris\$9 or ppastoris\$9) same (transcri\$9 near2 activat\$8)).clm.	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:37
L44	259	l40 or l41 or l42 or l43	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:37
L45	20	l44 and (promoter\$6 same((methanol\$3 or ch3\$4) near3 induc\$9)).clm.	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:37
L46	7	l45 and mxr1\$3).clm.	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:38
L47	2	"l35" and ((alcohol\$3 near2 oxidas\$5) or aox1\$3).clm.	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:38
L48	0	"l45" and ((alcohol\$3 near2 oxidas\$5) or aox1\$3).clm.	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:38
L49	18	l45 and ((alcohol\$3 near2 oxidas\$5) or aox1\$3).clm.	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:39
L50	7	l46 and l49	US-PGPUB; USPAT	OR	OFF	2020/01/27 10:39

1/ 27/ 2020 10:40:07 AM

C:\Users\hleith\Documents\EAST\Workspaces\15573300.wsp

OK TO ENTER: /N.J.L./

Attorney Docket No.: 38767-0048US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

MAIL STOP AF

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

RESPONSE TO FINAL OFFICE ACTION MAILED NOVEMBER 25, 2019

In response to the Final Office Action mailed November 25, 2019, please consider the following reply.



UNITED STATES PATENT AND TRADEMARK OFFICE

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

BIB DATA SHEET

CONFIRMATION NO. 4807

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.	
15/573,300	11/10/2017	435	1636	38767-0048US1	
RULE					
APPLICANTS Impossible Foods Inc., Redwood City, CA;					
INVENTORS Smita Shankar, Millbrae, CA; Martin Andrew Hoyt, San Francisco, CA;					
** CONTINUING DATA ***** This application is a 371 of PCT/US2016/031797 05/11/2016 which claims benefit of 62/313,491 03/25/2016 and claims benefit of 62/236,506 10/02/2015 and claims benefit of 62/222,388 09/23/2015 and claims benefit of 62/220,366 09/18/2015 and claims benefit of 62/203,052 08/10/2015 and claims benefit of 62/185,921 06/29/2015 and claims benefit of 62/183,074 06/22/2015 and claims benefit of 62/159,899 05/11/2015					
** FOREIGN APPLICATIONS *****					
** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ** 01/25/2018					
Foreign Priority claimed ☐ Yes ☒ No		STATE OR COUNTRY CA	SHEETS DRAWINGS 11	TOTAL CLAIMS 38	INDEPENDENT CLAIMS 2
35 USC 119(a-d) conditions met ☐ Yes ☒ No					
Verified and Acknowledged		/NANCY J LEITH/ Examiner's Signature	Initials		
ADDRESS FISH & RICHARDSON P.C. (TC) PO BOX 1022 MINNEAPOLIS, MN 55440-1022 UNITED STATES					
TITLE EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST					
FILING FEE RECEIVED 3060	FEES: Authority has been given in Paper No. _____ to charge/credit DEPOSIT ACCOUNT No. _____ for following:		☐ All Fees		
			☐ 1.16 Fees (Filing)		
			☐ 1.17 Fees (Processing Ext. of time)		
			☐ 1.18 Fees (Issue)		
			☐ Other _____		
☐ Credit					

TRANSMITTAL FOR POWER OF ATTORNEY TO ONE OR MORE REGISTERED PRACTITIONERS

Application Number	15/573,300		
Filing Date	November 10, 2017		
First Named Inventor	Smita Shankar		
Title	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST		
Art Unit	1636		
Examiner Name	Nancy J. Leith		
Attorney Docket Number	38767-0048US1		
SIGNATURE of Applicant or Patent Practitioner			
Signature	/Kimberly M. Maize, Reg. No. 76,370/	Date (Optional)	March 11, 2020
Name	Kimberly M. Maize	Registration Number	76,370
Title (if Applicant is a juristic entity)			
Applicant Name (if Applicant is a juristic entity)			
NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. If more than one applicant, use multiple forms.			
☐ *Total of _____ forms are submitted.			

62244885.doc

PTO/AIA/82B(07-13)

Approved for use through 11/30/2014. OMB 0551-0035
U.S. Patent and Trademark Office, U.S. DEPARTMENT OF COMMERCE

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POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

☒ I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above:

26191

OR

☐ I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter to:

☐ The address associated with the above-mentioned Customer Number

OR

☒ The address associated with Customer Number: **26191**

OR

Firm or Individual Name			
Address			
City	State	Zip	
Country			
Telephone	Email		

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

Impossible Foods Inc.

☐ Inventor or Joint Inventor (title not required below)

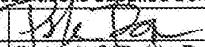
☐ Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

☒ Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

☐ Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature		Date (Optional)	4/30/2014
Name	Patrick O'Reilly Brown M.D., Ph.D.		
Title	CEO, Impossible Foods Inc.		

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

☐ *Total of _____ forms are submitted.

This collection of information is required by 37 CFR 1.131, 1.32, and 1.33. 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 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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



Electronic Acknowledgement Receipt	
EFS ID:	38835043
Application Number:	15573300
International Application Number:	
Confirmation Number:	4807
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	Kimberly M. Maize/Nikki Krahler
Filer Authorized By:	Kimberly M. Maize
Attorney Docket Number:	38767-0048US1
Receipt Date:	11-MAR-2020
Filing Date:	10-NOV-2017
Time Stamp:	14:49:28
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment		no			
File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Power of Attorney	38767-0048US1_POA.pdf	2171571	no	2
			ded4a943e8bf4899d01ef9dd0a1767df3db010c8		
Warnings:					

Information:	
Total Files Size (in bytes):	2171571
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 NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/573,300	11/10/2017	Smita Shankar	38767-0048US1

CONFIRMATION NO. 4807

POA ACCEPTANCE LETTER

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



Date Mailed: 03/17/2020

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 03/11/2020.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at (571) 272-4000 or (571) 272-4200 or 1-888-786-0101.

/nbekele/

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.

DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN APPLICATION DATA SHEET (37 CFR 1.76)

Title of Invention	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
---------------------------	---

As the below named inventor, I hereby declare that:

This declaration ☐ The attached application, or is directed to:

☒ United States application or PCT international application number 15/573,300 filed on November 10, 2017

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001 by fine or imprisonment of not more than five (5) years, or both.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Smita Shankar Date (Optional) : Apr 22, 2020

Signature: Smita Shankar

DocuSigned by: 2A75368907841A...

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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

**DECLARATION (37 CFR 1.63) FOR UTILITY OR DESIGN APPLICATION USING AN
APPLICATION DATA SHEET (37 CFR 1.76)**

Title of Invention	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
-------------------------------	--

As the below named inventor, I hereby declare that:

This declaration ☐ The attached application, or
is directed to:

☒ United States application or PCT international application number 15/573,300
filed on November 10, 2017

The above-identified application was made or authorized to be made by me.

I believe that I am the original inventor or an original joint inventor of a claimed invention in the application.

I hereby acknowledge that any willful false statement made in this declaration is punishable under 18 U.S.C. 1001
by fine or imprisonment of not more than five (5) years, or both.

WARNING:

Petitioner/applicant is cautioned to avoid submitting personal information in documents filed in a patent application that may contribute to identity theft. Personal information such as social security numbers, bank account numbers, or credit card numbers (other than a check or credit card authorization form PTO-2038 submitted for payment purposes) is never required by the USPTO to support a petition or an application. If this type of personal information is included in documents submitted to the USPTO, petitioners/applicants should consider redacting such personal information from the documents before submitting them to the USPTO. Petitioner/applicant is advised that the record of a patent application is available to the public after publication of the application (unless a non-publication request in compliance with 37 CFR 1.213(a) is made in the application) or issuance of a patent. Furthermore, the record from an abandoned application may also be available to the public if the application is referenced in a published application or an issued patent (see 37 CFR 1.14). Checks and credit card authorization forms PTO-2038 submitted for payment purposes are not retained in the application file and therefore are not publicly available.

LEGAL NAME OF INVENTOR

Inventor: Martin Andrew Hoyt Date (Optional): Apr 22, 2020
DocuSigned by:

Signature: Martin Hoyt
E8C9E196D551463...

Note: An application data sheet (PTO/SB/14 or equivalent), including naming the entire inventive entity, must accompany this form or must have been previously filed. Use an additional PTO/AIA/01 form for each additional inventor.

This collection of information is required by 35 U.S.C. 115 and 37 CFR 1.63. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 1 minute to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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6224888.doc

Electronic Acknowledgement Receipt	
EFS ID:	39260978
Application Number:	15573300
International Application Number:	
Confirmation Number:	4807
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	Kimberly M. Maize/Nikki Krahler
Filer Authorized By:	Kimberly M. Maize
Attorney Docket Number:	38767-0048US1
Receipt Date:	24-APR-2020
Filing Date:	10-NOV-2017
Time Stamp:	16:08:16
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment		no			
File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Oath or Declaration filed	38767-0048US1_ExecutedDeclarationx.pdf	147940 cd0e5ef6ccb53ed3d69bac155d46959d2c46ee27	no	2
Warnings:					

Information:	
Total Files Size (in bytes):	147940
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.	

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 2

Attorney Docket 38767-0048US1

Amendments to the Claims:

This listing of claims replaces all prior versions and listings of claims in the application:

Listing of Claims:

1. (Currently Amended) A methylotrophic yeast cell comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises a first exogenous nucleic acid encoding a methanol expression regulator 1 (Mxr1) transcriptional activator operably linked to at least one methanol-inducible promoter element comprising a sequence to which the Mxr1 transcriptional activator binds, wherein the recombinant nucleic acid molecule comprises a second exogenous nucleic acid encoding a heterologous polypeptide operably linked to at least one methanol-inducible promoter element.

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

3. (Canceled)

4. (Previously presented) The yeast cell of claim 1, wherein the cell is a *Pichia* cell.

5. (Previously presented) The yeast cell of claim 1, wherein the cell is a *Pichia pastoris* cell.

6. (Previously presented) The yeast cell of claim 1, wherein the recombinant nucleic acid molecule is stably integrated into the genome of the methylotrophic yeast cell.

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 3

Attorney Docket 38767-0048US1

7. (Previously presented) The yeast cell of claim 1, wherein the recombinant nucleic acid molecule is extrachromosomally expressed from a replication-competent plasmid.

8. (Canceled)

9. (Previously presented) The yeast cell of claim 1, wherein the exogenous nucleic acid encoding a transcriptional activator comprises a Mxr1 sequence from *Pichia pastoris*.

10. (Previously presented) The yeast cell of claim 1, wherein the transcriptional activator comprises the sequence shown in SEQ ID NO: 59.

11. (Previously presented) The yeast cell of claim 10, wherein the transcriptional activator is encoded by the nucleic acid sequence shown in SEQ ID NO: 60.

12. (Previously presented) The yeast cell of claim 1, wherein the at least one methanol-inducible promoter element is selected from the group consisting of an alcohol oxidase 1 (AOX1) promoter element from *Pichia pastoris*, an alcohol oxidase (AOD1) promoter element from *Candida boidinii*, a methanol oxidase (MOX) promoter element from *Hansenula polymorpha*, a methanolic alcohol oxidase (MOD1) promoter element from *Pichia methanolica*, a dihydroxyacetone synthase (DHAS) promoter element from *Pichia pastoris*, a formaldehyde dehydrogenase (FLD1) promoter element from *Pichia pastoris*, and a peroxin 8 (PEX8) promoter element from *Pichia pastoris*.

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

14. (Canceled)

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 4

Attorney Docket 38767-0048US1

15. (Currently Amended) The yeast cell of claim 1, wherein the ~~heterologous~~second
exogenous nucleic acid encodes at least one heterologous polypeptide involved in biosynthesis of
an iron co-factor.

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

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

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

19. (Canceled)

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

providing the yeast cell of claim 1;

and

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

thereby expressing the heterologous polypeptide.

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

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 5

Attorney Docket 38767-0048US1

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

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

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

25. (Previously Presented) The method of claim 20, wherein the introducing step comprises a technique selected from the group consisting of transduction, electroporation, biolistic particle delivery, and chemical transformation.

26. (Previously Presented) The method of claim 20, wherein the conditions under which the cell is cultured comprises addition of iron or a pharmaceutically or metabolically acceptable salt thereof.

27. (Previously Presented) The method of claim 20, wherein the culturing step comprises culturing the cell in the presence of methanol.

28. (Previously Presented) The method of claim 20, wherein the culturing step comprises culturing the cell in the absence of methanol.

29. (Previously Presented) The method of claim 20, wherein the culturing step comprises culturing the cell in the absence of selection.

30. (Previously presented) A methylotrophic *Pichia* yeast cell comprising a recombinant nucleic acid molecule, wherein the recombinant nucleic acid molecule comprises:
a nucleic acid encoding a Mxr1 transcriptional activator sequence from *P. pastoris*;

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 6

Attorney Docket 38767-0048US1

a nucleic acid encoding a member of globin family PF00042; and
a nucleic acid encoding at least one polypeptide involved in heme biosynthesis,
wherein each nucleic acid is operably linked to a methanol-inducible promoter element,
wherein the methanol-inducible promoter element comprises at least one sequence to which a
Mxr1 transcriptional activator binds.

31. (Canceled)

32. (Previously presented) The yeast cell of claim 30, wherein the member of the globin
family is leghemoglobin.

33-36. (Canceled)

37. (Previously presented) The yeast cell of claim 30, wherein the methanol-inducible
promoter element is an alcohol oxidase I (AOX1) promoter element from *P. pastoris*.

38. (Canceled)

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 7

Attorney Docket 38767-0048US1

REMARKS

Claims 1, 2, 4-7, 9-13, 15-18, 20-30, 32, and 37 were allowed. Applicant has herein amended claims 1 and 15 to correct inadvertent errors that were not previously recognized. The amended claims do not introduce any new matter or require a further search or examination. The amended claims are patentable for at least the reasons provided in the Notice of Allowance.

Applicant asks that all claims be allowed in view of the amendment to the claims. In addition, please apply any other necessary charges or credits to Deposit Account 06-1050, referencing the above attorney docket number.

Respectfully submitted,

Date: May 4, 2020

/Kimberly M. Maize, Reg. No. 76,370/

Kimberly M. Maize
Reg. No. 76,370

Customer Number 26191
Fish & Richardson P.C.
Telephone: +1 (612) 638-5707
Facsimile: (877) 769-7945

62311872.doc

Electronic Patent Application Fee Transmittal				
Application Number:		15573300		
Filing Date:		10-Nov-2017		
Title of Invention:		EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST		
First Named Inventor/Applicant Name:		Smita Shankar		
Filer:		Kimberly M. Maize/Judith Witzel		
Attorney Docket Number:		38767-0048US1		
Filed as Large Entity				
Filing Fees for U.S. National Stage under 35 USC 371				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
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:	39345332
Application Number:	15573300
International Application Number:	
Confirmation Number:	4807
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	Kimberly M. Maize/Judith Witzel
Filer Authorized By:	Kimberly M. Maize
Attorney Docket Number:	38767-0048US1
Receipt Date:	04-MAY-2020
Filing Date:	10-NOV-2017
Time Stamp:	17:08:52
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$ 1000
RAM confirmation Number	E202054H09180400
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	RespNOA.pdf	76408	no	2
			cad57af1a52b5e1673fc021fd53695a9e21b3909		
Warnings:					
Information:					
2	Issue Fee Payment (PTO-85B)	PartB.pdf	161911	no	1
			4b9584c006cdd5c9f249d49b2bd110d7def90b76		
Warnings:					
Information:					
3		312Amendment.pdf	107341	yes	7
			4f405e5612c373b2a907431d23c6a97abddadfb5		
	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Amendment after Notice of Allowance (Rule 312)		1	1	
	Claims		2	6	
	Applicant Arguments/Remarks Made in an Amendment		7	7	
Warnings:					
Information:					
4	Fee Worksheet (SB06)	fee-info.pdf	30752	no	2
			ea2ff18561fd222ed5ddec6e3dfeda66a022be0d		
Warnings:					
Information:					
Total Files Size (in bytes):			376412		

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

New Applications Under 35 U.S.C. 111

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

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

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

New International Application Filed with the USPTO as a Receiving Office

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

First Named Inventor : Smita Shankar
Application No. : 15/573,300
Filed : November 10, 2017
Page : 2 of 2

Attorney Docket: 38767-0048US1

some of the features recited in the dependent claim and its base claim(s), which combination of features may not include all of the limitations identified in the Examiner's reasons for allowance.

Respectfully submitted,

Date: April 4, 2020

/Kimberly M. Maize, Reg. No. 76,370/

Kimberly M. Maize
Reg. No. 76,370

Customer Number 26191
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PART B - FEE(S) TRANSMITTAL

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

By mail, send to: Mail Stop ISSUE FEE
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.

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

26191 7590 02/04/2020

FISH & RICHARDSON P.C.
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.
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15/573,300 11/10/2017 Smita Shankar 38767-0048US1 4807

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
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nonprovisional LARGE \$1000 \$0 \$1000 05/04/2020

EXAMINER	ART UNIT	CLASS-SUBCLASS
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LEITH, NANCY J. 1636 435-069100

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, 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

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. 06-1050

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 /Kimberly M. Maize, Reg. No. 76,370/Date May 4, 2020Typed or printed name Kimberly M. MaizeRegistration No. 76,370

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

62311943.doc



UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/573,300	11/10/2017	Smita Shankar	38767-0048US1	4807
26191 7590 05/11/2020 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
			05/11/2020	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

Response to Rule 312 Communication	Application No. 15/573,300	Applicant(s) Shankar et al.	
	Examiner NANCY J. LEITH	Art Unit 1636	AIA (FITF) Status Yes
-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address -- 1. ☒ The amendment filed on <u>04 May 2020</u> 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. _____			
/NANCY J LEITH/ Primary Examiner, Art Unit 1636		/HEATHER CALAMITA/ Supervisory Patent Examiner, Art Unit 1636 (acting)	

Attorney's Docket No.: 38767-0048US1

OK TO ENTER: /N.J.L./

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor :	Smita Shankar	Art Unit :	1636
Application No. :	15/573,300	Examiner :	Nancy J. Leith
Filed :	November 10, 2017	Confirmation No. :	4807
		Notice of Allowance Date:	2/4/2020
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

AMENDMENT AFTER ALLOWANCE
PURSUANT TO 37 C.F.R. §1.312

Please amend the application as indicated on the following pages. This amendment is being filed concurrently with the payment of the issue fee.



UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/573,300	06/23/2020	10689656	38767-0048US1	4807

26191 7590 06/03/2020

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 41 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):

Impossible Foods Inc., Redwood City, CA;
Smita Shankar, Millbrae, 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.: 38767-0048US1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor	:	Smita Shankar	Art Unit	:	1636
Patent No.	:	10,689,656	Examiner	:	Nancy J. Leith
Issue Date	:	June 23, 2020	Conf. No.	:	4807
Serial No.	:	15/573,300			
Filed	:	November 10, 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.

All errors to be corrected were made in printing by the Patent and Trademark Office, and
no fee is believed to be due.

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

Respectfully submitted,

Date: August 18, 2020

/Kimberly M. Maize, Reg. No. 76,370/

Kimberly M. Maize
Reg. No. 76,370

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

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PTO/SB/44 (09-07)
Approved for use through 08/31/2013. OMB 0651-0033
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(Also Form PTO-1050)

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

Page 1 of 1

PATENT NO. : 10,689,656
APPLICATION NO.: 15/573,300
ISSUE DATE : June 23, 2020
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:

In Column 90 Line 12 In Claim 26, delete "Mxrl1" and insert –Mxr1–, 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.

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Electronic Acknowledgement Receipt	
EFS ID:	40311520
Application Number:	15573300
International Application Number:	
Confirmation Number:	4807
Title of Invention:	EXPRESSION CONSTRUCTS AND METHODS OF GENETICALLY ENGINEERING METHYLOTROPHIC YEAST
First Named Inventor/Applicant Name:	Smita Shankar
Customer Number:	26191
Filer:	Kimberly M. Maize/Emily Palmer
Filer Authorized By:	Kimberly M. Maize
Attorney Docket Number:	38767-0048US1
Receipt Date:	18-AUG-2020
Filing Date:	10-NOV-2017
Time Stamp:	11:14:33
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment		no			
File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Request for Certificate of Correction	ReqForCOC.pdf	134493	no	2
			ac0df670d5334b0d0ea9f9cd1846bd9aace1fdc2		
Warnings:					

Information:	
Total Files Size (in bytes):	134493
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
CERTIFICATE OF CORRECTION

PATENT NO. : 10,689,656 B2
APPLICATION NO. : 15/573300
DATED : June 23, 2020
INVENTOR(S) : Smita Shankar and Martin Andrew Hoyt

Page 1 of 1

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

In the Claims

Column 90 Line 12 Claim 26, delete "Mxrl1" and insert --Mxr1--, therefor.

Signed and Sealed this
Fifteenth Day of September, 2020



Andrei Iancu
Director of the United States Patent and Trademark Office