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(72) Feltaláló(k):
WALKER, Keith A., San Diego, CA 92130 (US)
KNUTH, Mark E., Poway, CA 92064 (US)
FONG, Noel M., San Diego, CA 92122 (US)
BEETHAM, Peter R., Carlsbad, CA 92011 (US)

(73) Jogosult(ak):
Nucelis LLC, San Diego, CA 92121 (US)

(74) Képviselő:
SBGK Szabadalmi Ügyvivői Iroda, Budapest

(54) **Eljárások és készítmények szkvalén előállítására élesztő alkalmazásával**

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(54) **METHODS AND COMPOSITIONS FOR PRODUCING SQUALENE USING YEAST**

VERFAHREN UND ZUSAMMENSETZUNGEN ZUR HERSTELLUNG VON SQUALEN MIT HEFE

PROCÉDÉS ET COMPOSITIONS DE PRODUCTION DE SQUALÈNE À L'AIDE DE LEVURE

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(73) Proprietor: **Nucelis Inc.**
San Diego, CA 92121 (US)

(72) Inventors:
- **WALKER, Keith A.**
  San Diego, CA 92130 (US)
- **KNUTH, Mark E.**
  Poway, CA 92064 (US)
- **FONG, Noel M.**
  San Diego, CA 92122 (US)
- **BEETHAM, Peter R.**
  Carlsbad, CA 92011 (US)

(74) Representative: **Zimmermann & Partner Patentanwälte mbB**
Postfach 330 920
80069 München (DE)

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Description

FIELD OF THE INVENTION

[0001] Disclosed are methods and compositions for producing isoprenoids such as squalene using yeast.

BACKGROUND OF THE INVENTION

[0002] The following description of the background of the invention is provided simply as an aid in understanding the invention and is not admitted to describe or constitute prior art to the invention.

[0003] Isoprenoids, such as squalene, are commercially important types of lipids. They have excellent lubricity, oxidative stability, low pour points, low freezing points, high flash points, and facile biodegradability. Squalene is currently produced by extraction from olive oil or cold water shark liver oil at a high unit cost. Because of the high unit cost, economically feasible uses for squalene and squalane (the fully hydrogenated derivative of squalene) are in small market applications such as watch lubricants,
pharmaceuticals/nutraceuticals, cosmetics, perfumes and as chemical intermediates for high-value products.

[0004] There exist, however, significant potential markets for biodegradable lubricants, lubricant additives, and hydraulic fluids. Biodegradability of these products is particularly important for environmentally sensitive applications, such as agricultural applications, or where considerable lubricant or hydraulic fluids may be lost to the environment. The potential markets for biodegradable lubricants, lubricant additives, and hydraulic fluids are quite large, estimated to be on the order of five million metric tons per annum.

[0005] Biodegradable lubricants, lubricant additives, and hydraulic fluids derived from vegetable and animal fats and oils are available, but they have drawbacks. They typically solidify at relatively high temperatures (i.e., they solidify in cold weather) and have flash points that are too low for use in hot conditions, (i.e., they break down or combust under normal hot engine conditions).

[0006] Thus, a cost effective method of production of squalene is desired that would allow for large-scale manufacturing and widespread use of squalene and squalane in biodegradable lubricants, lubricant additives, and hydraulic fluids.

[0007] Chang et al., (Appl. Microbiol. Biotechnol., 2008, 78, 963-72) discloses the discovery of a wild type yeast, *Pseudozyma* sp. JCC207, that produces "a large amount of squalene and several polyunsaturated fatty acids." Chang *et al.* describe isolating *Pseudozyma* sp. JCC207 from seawater collected near Guam, USA, and are unsure whether *Pseudozyma* sp. JCC207 is a new species or a variant of *P. regulosa* or *P. aphidis.* In the article, "the efficiency of squalene production [of *Pseudozyma* sp. JCC207] was investigated under different conditions."

[0008] Dow AgroSciences LLC, *Using Yeast Fermentation to Product Cost-effective and Biodegradable Lubricants*, http://statusreports.atp.nist.gov.reports/95-01-0148PDF.pdf, discloses that "[t]he company proposed to use genetic engineering to alter the metabolic characteristics of an oleaginous (oily) yeast to increase the yeast's ability to produce isoprenes through biosynthesis." Specifically, four enzymes were targeted: ACCase, hydroxymethylglutaryl CoA reductasc (HMGR), squalene synthetase, and squalene epoxidase.

[0009] U.S. Patent No. 5,460,949 discloses "[a] method increasing the accumulation of squalene and specific sterols in yeast." In particular, it is disclosed that "[s]qualene and sterol accumulation is increased by increasing the expression level of a gene encoding a polypeptide having the HMG-CoA reductase activity."

[0010] WO 2008/130372 A9 relates to methods for the biological production of certain sterol compounds, and to systems for producing oleaginous yeasts or fungi that are capable of producing certain sterol compounds.

SUMMARY OF THE INVENTION

[0011] Subject matter of the present invention is a composition as defined in claim 1, and a method of producing squalene as defined in claim 2. The dependent claims relate to particular embodiments thereof.

[0012] One aspect of the present invention accordingly relates to a composition comprising a genetically converted yeast. The genetically converted yeast expresses one or more modified enzymes having one or more designed mutations, wherein the one or more designed mutations are at defined positions within said enzyme. The one or more modified enzymes comprise squalene epoxidase, and the squalene epoxidase has reduced activity and/or expression. The yeast produces increased quantities of squalene as compared to the native yeast, and it is a *Yarrowia lipolytica* strain selected from the group consisting of ATCC 90812, ATCC MYA-2613, or Yeastern polg.

[0013] Another aspect of the present invention relates to a method of producing squalene by a genetically converted yeast. The method comprises increasing or decreasing activity or expression of one or more enzymes in the isoprenoid biosynthesis pathway. The enzyme activity or expression is increased or decreased by one or more designed mutations, wherein the one or more designed mutations are at defined positions within said enzyme. The one or more modified enzymes comprise squalene epoxidase, and the squalene epoxidase has reduced activity and/or expression. The yeast

produces increased quantities of squalene as compared to the native yeast, and it is a *Yarrowia lipolytica* strain selected from the group consisting of ATCC 90812, ATCC MYA-2613, or Yeastern polg.

[0014] According to a particular embodiment, the genetically converted yeast is derived from an oleaginous yeast.

[0015] According to a particular embodiment, the one or more modified enzymes further comprise a modified enzyme selected from the group consisting of acetyl-CoA carboxylase ("ACCase"), HMG-CoA reductase, squalene synthase, ATP citrate lyase, ATP citrate synthase, mevalonate kinase, glycerol kinase and 5-aminolevulinate synthase.

[0016] According to a particular embodiment, the activity or expression is reduced to about 90%; or about 80%; or about 70%; or about 60%; or about 50%; or about 40%; or about 30%; or about 20%; or about 10%; or about 5% of the activity or expression of the corresponding native yeast.

[0017] According to a particular embodiment, the activity or expression is between 90-95%; or 80-90%; or 70-80%; 60-70%; or 50-60%; or 40-50%; or about 30-40%; or about 20-30%; about 10-20%; or about 5-10%; or about 2-5% of the activity or expression of the corresponding native yeast.

[0018] According to a particular embodiment, the yeast is the Yeastern polg strain of *Yarrowia lipolytica.*

[0019] According to a particular embodiment, an antifungal agent is present in the composition or is added to the yeast in the method. For example, an antifungal agent is present in the composition or is added to the yeast in the method at a concentration between 0.5 to 100 μg/ml, between 1 to 25 μg/ml, or between 10 to 15 μg/ml. According to a particular embodiment, the antifungal agent may be an allylamine antifungal agent. For example, the antifungal agent may be an allylamine antifungal agent present at a concentration between 0.5 to 100 μg/ml, such as between 1 to 25 μg/ml, or between 10 to 15 μg/ml. According to a particular embodiment, the antifungal agent may be terbinafine. For example, the terbinafine may be present at a concentration between 0.5 to 100 μg/ml, such as between 1 to 25 μg/ml, or between 10 to 15 μg/ml.

[0020] According to a particular embodiment, the method comprises cultivating the yeast with an antifungal agent; wherein the yeast is the Yeastern polg strain of *Yarrowia lipolytica* and wherein the antifungal agent is terbinafine. For example, the terbinafine may be present at a concentration of about 12.5 μg/ml or greater.

[0021] As disclosed herein, increased amounts of an isoprenoid (for example, squalene) produced by a genetically converted or non-genetically converted yeast may be the result of mutating, modifying and/or altering the activity of one or more enzymes within the isoprenoid biosynthesis pathway. For example acetyl-CoA carboxylase (or "ACCase"), HMG-CoA reductase, squalene epoxidase, squalene synthase, ATP citrate synthase, mcvalonatc kinase (e.g., *Y. lipolytica* mevalonate kinase (Genolevures YALIOB16038g)), glycerol kinase (e.g., *Y. lipolytica* glycerol kinase (Genolevures YALI0F00484g)) and/or 5-aminolevulinate synthase (e.g., encoded by *Saccharomyces cerevisiae* HEM1 gene) may be modified, mutated or have altered activity.

[0022] As disclosed herein, a genetically converted yeast expressing a modified enzyme may be produced by introducing a mutation in the enzyme through use of a gene repair oligonucleobase as described herein. Such methods may include introducing a gene repair oligonucleobase containing a specific mutation for a target gene of interest into a yeast cell by any of a number of methods well-known in the art (e.g., electroporation, LiOAc, biolistics, spheroplasting, and/or *Agrobacterium* (see, for example, McClelland, C.M., Chang, Y.C., and Kwon-Chung, K.J. (2005) Fungal Genetics and Biology 42:904-913) and identifying a cell having the mutated enzyme.

[0023] As disclosed herein, a method of producing isoprenoids, preferably squalene, may include providing a genetically converted or non-genetically converted yeast as described herein and extracting squalene from the yeast. The method may include exposing yeast (either genetically converted or non-genetically converted) to an antifungal agent (for example, an allylamine antifungal agent such as terbinafine) and extracting squalene from the yeast. The method may include exposing a genetically converted yeast such as described herein to an antifungal agent (for example, an allylamine antifungal agent such as terbinafine) and extracting squalene from the yeast. The method may include exposing a non-genetically converted yeast such as described herein to an antifungal agent (for example, an allylamine antifungal agent such as terbinafine) and extracting squalene from the yeast.

[0024] In the methods and compositions disclosed herein that include an antifungal agent (for example, an allylamine antifungal agent such as terbinafine), the antifungal agent (for example terbinafine or other antifungal agent) may be added or present in a concentration at or above about 1 μg/ml; or about 5 μg/ml; or about 10 μg/ml; or about 11 μg/ml; or about 12 μg/ml; or about 12.5 μg/ml; or about 13 μg/ml; or about 15 μg/ml; or about 16 μg/ml; or about 20 μg/ml; or about 25 μg/ml; or about 30 μg/ml; or about 40 μg/ml; or about 50 μg/ml or greater. In the methods and compositions disclosed herein that include an antifungal agent (for example, an allylamine antifungal agent such as terbinafine), the antifungal agent (for example terbinafine or other antifungal agent) may be added or present in a concentration between about 0.5 to 100 μg/ml; or 0.5 to 50 μg/ml; or 1 to 50 μg/ml; or 5 to 50 μg/ml; or 8 to 50 μg/ml; or 10 to 50 μg/ml; or 12 to 50 μg/ml; or 15 to 50 μg/ml; or 15 to 50 μg/ml; or 25 to 50 μg/ml; or 1 to 25 μg/ml; or 5 to 25 μg/ml; or 10 to 25 μg/ml; or 10 to 20 μg/ml; or 10 to 15 μg/ml.

[0025] Disclosed herein is a genetically converted yeast that produces isoprenoids. In certain examples, the genetically converted yeast produces squalene.

[0026] Further disclosed herein is a genetically converted yeast, wherein the yeast is genetically converted such that

it produces increased levels of squalene as compared to the corresponding native yeast. In certain examples, the genetically converted yeast expresses one or more modified enzymes having one or more mutations. In certain examples the expression level of one or more enzymes in the genetically converted yeast is increased or decreased relative to the corresponding native yeast. In related examples, the genetically converted yeast expresses one or more modified enzymes having one or more mutations and the expression level of one or more enzymes in the genetically converted yeast is increased or decreased relative to the corresponding native yeast. In certain preferred examples a genetically converted yeast as disclosed herein is genetically converted by introducing a mutation into an enzyme using a gene repair oligobase. In some examples a genetically converted yeast as disclosed herein is genetically converted by introducing one or more mutations at or around the translation start site of a gene encoding an enzyme to increase or decrease expression of the enzyme, for example, as described in US Patent Application Nos. 10/411,969 and 11/625,586. In certain examples, the enzyme modified in a genetically converted yeast as disclosed herein includes one or more enzymes selected from the group consisting of acetyl-CoA carboxylase (or "ACCase"), HMG-CoA reductase, squalene epoxidase, squalene synthase, ATP citrate lyase, ATP citrate synthase, mevalonate kinase (e.g., *Y. lipolytica* mevalonate kinase (Genolevures YALI0B16038g)), glycerol kinase (e.g., *Y. lipolytica* glycerol kinase (Genolevures YALI0F00484g) and 5-aminolevulinate synthase.

[0027] A nucleobase comprises a base, which is a purine, pyrimidine, or a derivative or analog thereof. Nucleosides are nucleobases that contain a pentosefuranosyl moiety, e.g., an optionally substituted riboside or 2'-deoxyriboside. Nucleosides can be linked by one of several linkage moieties, which may or may not contain phosphorus. Nucleosides that are linked by unsubstituted phosphodiester linkages are termed nucleotides. "Nucleobases" as used herein include peptide nucleobases, the subunits of peptide nucleic acids, and morpholine nucleobases as well as nucleosides and nucleotides.

[0028] An oligonucleobase is a polymer of nucleobases, which polymer can hybridize by Watson-Crick base pairing to a DNA having the complementary sequence. An oligonucleobase chain has a single 5' and 3' terminus, which are the ultimate nucleobases of the polymer. A particular oligonucleobase chain can contain nucleobases of all types. An oligonucleobase compound is a compound comprising one or more oligonucleobase chains that are complementary and hybridized by Watson-Crick base pairing. Nucleobases are either deoxyribo-type or ribo-type. Ribo-type nucleobases are pentosefuranosyl containing nucleobases wherein the 2' carbon is a methylene substituted with a hydroxyl, alkyloxy or halogen. Deoxyribo-type nucleobases are nucleobases other than ribo-type nucleobases and include all nucleobases that do not contain a pentosefuranosyl moiety.

[0029] An oligonucleobase strand generically includes both oligonucleobase chains and segments or regions of oligonucleobase chains. An oligonucleobase strand has a 3' end and a 5' end. When an oligonucleobase strand is coextensive with a chain, the 3' and 5' ends of the strand are also 3' and 5' termini of the chain.

[0030] The term "gene repair oligonucleobase" is used herein to denote oligonucleobases, including mixed duplex oligonucleotides, non-nucleotide containing molecules, single stranded oligodeoxynucleotides and other gene repair molecules as described in detail below.

[0031] In some examples, a genetically converted yeast or non-genetically converted yeast as disclosed herein is derived from an oleaginous yeast. In certain preferred examples, a genetically converted yeast or non-genetically converted yeast as disclosed herein is derived from a yeast selected from the group consisting of *Cryptococcus curvatus, Yarrowia lipolytica*, *Rhodotorula glutinus*, and *Rhodosporidium toruloides.* In some preferred examples, the genetically converted yeast or non-genetically converted yeast is derived from a yeast selected from the group consisting of *Cryptococcus curvatus*, *Yarrowia lipolytica,* and *Rhodotorula glutinus.* In related examples, the genetically converted yeast or non-genetically converted yeast is derived from a yeast selected from the group consisting of *Cryptococcus curvatus,* and *Rhodotorula glutinus.* In certain preferred examples, the genetically converted yeast or non-genetically converted yeast is not derived from *Yarrowia lipolytica*. The genetically converted yeast used in the methods and compositions according to the present invention is a *Yarrowia lipolytica* strain selected from the group consisting of ATCC 90812, ATCC MYA-2613, and Yeastern polg.

[0032] In certain preferred examples, an enzyme that is modified in a genetically converted yeast as disclosed herein is acetyl-CoA carboxylase (or "ACCase"). In some preferred examples, acetyl-CoA carboxylase in a genetically converted yeast is modified such that its activity and/or expression is decreased relative to the corresponding native yeast; or such that the activity and/or expression is eliminated. In other examples, the acetyl-CoA carboxylase may be modified so that its substrate selectivity is altered. In some preferred examples, the genetically converted yeast is modified such that the activity and/or expression of acetyl-CoA carboxylase is reduced relative to the corresponding native yeast but the activity is not eliminated. In some preferred examples, the genetically converted yeast is modified such that the activity and/or expression of acetyl-CoA carboxylase in the genetically converted yeast is reduced to about 90%; or about 80%; or about 70%; or about 60%; or about 50%; or about 40%; or about 30%; or about 20%; or about 10%; or about 5% of the activity and/or expression of the corresponding native yeast. In related examples, the genetically converted yeast is modified such that the activity and/or expression of acetyl-CoA carboxylase in the genetically converted yeast is between about 90-95%; or about 80-90%; or about 70-80%; or about 60-70%; or about 50-60%; or about 40-50%; or about

30-40%; or about 20-30%; or about 10-20%; or about 5-10%; or about 2-5% of the activity and/or expression of the corresponding native yeast.

[0033] In certain preferred examples, an enzyme that is modified in a genetically converted yeast as disclosed herein is HMG-CoA reductase. In some preferred examples, HMG-CoA reductase in a genetically converted yeast is modified such that its activity and/or expression is increased relative to the corresponding native yeast. In other examples, the HMG-CoA reductase may be modified so that it substrate selectivity is altered. In certain preferred examples, the genetically converted yeast is modified such that the activity and/or expression of HMG-CoA reductase in the genetically converted yeast is increased to at least 1.2-fold; or 1.5-fold; or 2-fold; or 3-fold; or 4-fold; or 5-fold; or 10-fold; or 15-fold; or 20-fold; or 50-fold; or 100-fold; or 1,000-fold; or 10,000-fold; or 100,000-fold; or 1,000,000-fold higher than the activity and/or expression of the corresponding native yeast.

[0034] An enzyme that is modified in the genetically converted yeast used in the methods and compositions according to the present invention is squalene epoxidase. In some preferred examples, squalene epoxidase in a genetically converted yeast is modified such that its activity and/or expression is decreased relative to the corresponding native yeast; or such that the activity and/or expression is eliminated. In other examples, the squalene epoxidase may be modified so that its substrate selectivity is altered. In some preferred examples, the
genetically converted yeast is modified such that the activity and/or expression of squalene epoxidase is reduced relative to the corresponding native yeast but the activity is not eliminated. In certain examples, the squalene epoxidase is modified to include one or more mutations or homologs of one or more mutations associated with increased sensitivity to terbinafine. In certain examples, the yeast is not *Saccharomyces cerevisiae* and the squalene epoxidase is modified to include the homologs of one or more of the following mutations associated with increased sensitivity to terbinafine in the *Saccharomyces cerevisiae* ERG1 gene: G30S, L37P, and R269G (see, e.g., Turnowsky, 2005, 2007 and 2008). In certain examples, the yeast is Y. lipolytica and the squalene epoxidase is modified to include the homologs of one or more of the following mutations associated with increased sensitivity to terbinafine in the *Saccharomyces cerevisiae* ERG1 gene: G30S, L37P, and R269G (see, e.g., Turnowsky, 2005, 2007 and 2008). In some examples, the yeast squalene epoxidase gene is modified as described herein by synthesis and replacement of the wild-type gene or by introduction of mutations by RTDS. In some preferred examples, the genetically converted yeast is modified such that the activity and/or expression of squalene epoxidase in the genetically converted yeast is reduced to about 90%; or about 80%; or about 70%; or about 60%; or about 50%; or about 40%; or about 30%; or about 20%; or about 10%; or about 5% of the activity and/or expression of the corresponding native yeast. In related examples, the genetically converted yeast is modified such that the activity and/or expression of squalene epoxidase in the genetically converted yeast is between about 90-95%; or about 80-90%; or about 70-80%; or about 60-70%; or about 50-60%; or about 40-50%; or about 30-40%; or about 20-30%; or about 10-20%; or about 5-10%; or about 2-5% of the activity and/or expression of the corresponding native yeast.

[0035] In certain preferred examples, an enzyme that is modified in a genetically converted yeast as disclosed herein is squalene synthase. In some preferred examples, squalene synthase in a genetically converted yeast is modified such that its activity and/or expression is increased relative to the corresponding native yeast. In other examples, the squalene synthase may be modified so that it substrate selectivity is altered. In certain preferred examples, the genetically converted yeast is modified such that the activity and/or expression of squalene synthase in the genetically converted yeast is increased to at least 1.2-fold; or 1.5-fold; or 2-fold; or 3-fold; or 4-fold; or 5-fold; or 10-fold; or 15-fold; or 20-fold; or 50-fold; or 100-fold; or 1,000-fold; or 10,000-fold; or 100,000-fold; or 1,000,000-fold higher than the activity and/or expression of the corresponding native yeast.

[0036] In certain preferred examples, an enzyme that is modified in a genetically converted yeast as disclosed herein is ATP citrate lyase. In some examples, either or both subunits of ATP citrate lyase genes (for example, *Yarrowia lipolytica* ATP citrate lyase; Genoleveres YALI0D24431g and YALI0E34793g) are modified as described herein. In certain examples, the activity of ATP citrate lyase in a modified yeast is increased by the insertion and/or heterologous expression of an animal ATP lyase gene which comprises a single subunit holoenzyme. In some preferred examples, ATP citrate lyase in a genetically converted yeast is modified such that its activity and/or expression is increased relative to the corresponding native yeast. In certain preferred examples, the genetically converted yeast is modified such that the activity and/or expression of ATP citrate lyase in the genetically converted yeast is increased to at least 1.2-fold; or 1.5-fold; or 2-fold; or 3-fold; or 4- fold; or 5- fold; or 10-fold; or 10-fold; or 20-fold; or 50-fold; or 100-fold; or 1,000-fold; or 10,000-fold; or 100,000-fold; or 1,000,000-fold higher than the activity and/or expression of the corresponding native yeast.

[0037] In certain examples of the compositions and methods disclosed herein, the enzyme that is modified in a genetically converted yeast or non-genetically converted yeast is ATP citrate synthase. Preferably, its activity and/or expression is increased relative to the corresponding native yeast.

[0038] In certain preferred examples, an enzyme that is modified in a genetically converted yeast as disclosed herein is mevalonate kinase (e.g., *Y .lipolytica* mevalonate kinase (Genolevures YALI0B16038g)). In some preferred examples, nevalonate kinase (e.g., *Y. lipolytica* mevalonate kinase (Genolevures YALI0B16038g)) in a genetically converted yeast

is modified such that its activity and/or expression is increased relative to the corresponding native yeast. In certain preferred examples, the genetically converted yeast is modified such that the activity and/or expression of mevalonate kinase (e.g., *Y. lipolytica* mevalonate kinase (Genolevures YALI0B16038g)) in the genetically converted yeast is increased to at least 1.2-fold; or 1.5-fold; or 2-fold; or 3-fold; or 4-fold; or 5-fold; or 10-fold; or 15-fold; or 20-fold; or 50-fold; or 100-fold; or 1,000-fold; or 10,000-fold; or 100,000-fold; or 1,000,000-fold higher than the activity and/or expression of the corresponding native yeast.

[0039] In certain preferred examples, an enzyme that is modified in a genetically converted yeast as disclosed herein is glycerol kinase (e.g., *Y. lipolytica* glycerol kinase (Genolevures YALI0F00484g)). In some preferred examples, glycerol kinase (e.g., *Y .lipolytica* glycerol kinase (Genolevures YALI0F00484g)) in a genetically converted yeast is modified such that its activity and/or expression is increased relative to the corresponding native yeast. In certain preferred examples, the genetically converted yeast is modified such that the activity and/or expression of mevalonate kinase glycerol kinase (e.g., *Y. lipolytica* glycerol kinase (Genolevures YALI0F00484g)) in the genetically converted yeast is increased to at least 1.2-fold; or 1.5-fold; or 2-fold; or 3-fold; or 4-fold; or 5-fold; or 10-fold; or 15-fold; or 20-fold; or 50-fold; or 100-fold; or 1,000-fold; or 10,000-fold; or 100,000-fold; or 1,000,000-fold higher than the activity and/or expression of the corresponding native yeast.

[0040] In certain examples of the compositions and methods disclosed herein, the enzyme that is modified in a genetically converted yeast or non-genetically converted yeast is 5-aminolevulinate synthase (e.g., encoded by *Saccharomyces cerevisiae* HEM1 gene). Preferably, its activity and/or expression is increased relative to the corresponding native yeast.

[0041] In certain preferred examples disclosed herein, the converted
yeast is a genetically converted yeast; in other preferred examples, the genetically converted yeast is a transgenic yeast. Further examples are a yeast that includes both transgenic and genetic alterations.

[0042] In certain examples, disclosed are compositions that include a yeast (for example a genetically converted yeast such as disclosed herein or a non-genetically converted yeast) wherein at least 10% of the total lipid content is squalene; or at least 20% of the total lipid content is squalene; or at least 25% of the total lipid content is squalene; or at least 28% of the total lipid content is squalene; or at least 30% of the total lipid content is squalene; or at least 32% of the total lipid content is squalene; or at least 35% of the total lipid content is squalene; or at least 37% of the total lipid content is squalene; or at least 38% of the total lipid content is squalene; or at least 40% of the total lipid content is squalene; or at least 42% of the total lipid content is squalene; or at least 45% of the total lipid content is squalene; or at least 47% of the total lipid content is squalene; or at least 50% of the total lipid content is squalene; or at least 52% of the total lipid content is squalene; or at least 55% of the total lipid content is squalene; or at least 57% of the total lipid content is squalene; or at least 60% or more of the total lipid content is squalene.

[0043] The phrase "genetically converted yeast" or "genetically altered yeast" as used herein refers to a yeast having one or more genetic modifications, such as transgenes and/or modified enzymes which contain one or more designed mutation(s). Such designed mutations may result in a modified enzyme having an activity that is different from the native enzyme. Such differences can include differences in substrate specificity or level of activity. As used herein, a "transgenic yeast" is one type of a "genetically converted yeast".

[0044] The term "native yeast" as used herein refers to a yeast that is not genetically converted (i.e., transgenic or genetically altered). Native yeasts include wild type yeasts as well as yeasts that have been selectively bred to attain particular characteristics.

[0045] The phrase "transgenic yeast" refers to a yeast having a gene from another yeast species or non-yeast species. Such a gene may be referred to as a "transgene."

[0046] As used herein the term "target gene" refers to the gene encoding the enzyme to be modified.

[0047] The phrase "oleaginous yeast" refers to a yeast that contains at least about 20% cell dry weight (cdw) lipid extractable from the organism. The capacity to accumulate levels of lipid at least about 20% cdw is not confined to a particular genus; greater than about 20% cdw lipid has been reported in *Lipomyces lipofer*, *L. starkeyi*, *L. tetrasporus*, *Candida lipolytica*, *C. diddensiae*, *C. paralipolytica*, *C. curvata*, *Cryptococcus albidus*, *Cryptococcus laumentii*, *Geotrichum candidum*, *Rhodotorula graminus, Trichosporon pullulans, Rhodosporidium toruloides*, *Rhodotorula glutinus, Rhodotorula gracilis*, and *Yarrowia lipolytica.* See, e.g., Tatsumi, et al. U.S. Pat. No. 4,032,405, and Rattray, Microbial Lipids, Vol. 1 (1998).

[0048] The term "about" as used herein means in quantitative terms plus or minus 10%. For example, "about 3%" would encompass 2.7-3.3% and "about 10%" would encompass 9-11%.

[0049] Unless otherwise indicated, any percentages stated herein are percent by weight.

[0050] Other features and advantages of the invention will be apparent from the following description of the preferred embodiments and from the claims.

DETAILED DESCRIPTION OF THE INVENTION

Types of Yeast.

[0051] The compositions and methods as disclosed herein can be based on any of a number of yeast species or strains. In certain examples, the yeast is an oleaginous yeast. For example the yeast may be *Cryptococcus curvatus* (for example ATCC 20508), *Yarrowia lipolytica* (for example ATCC 20688 or ATCC 90811), *Rhodotorula glutinus* (for example ATCC 10788 or ATCC 204091), and *Rhorosporidium toruloides.* The inventors have discovered that, relative to certain other yeast (such as *Yarrowia lipolytica*), *Cryptococcus curvatus* and *Rhodotorula glutinis* grow to very high cell densities on a wide variety of substrates, and produce large amounts of total lipid under many culture conditions. Accordingly, in certain examples *Cyptococcus curvatus* and *Rhodotorula glutinis* may be particularly advantageous for the compositions and methods as disclosed herein. There are many genetic tools (for example, transformation protocols, selectable markers) that are well developed and specific for *Yarrowia lipolytica*; as such in some embodiments *Yarrowia lipolytica* may be particularly advantageous for the compositions and methods as disclosed herein. In the compositions and methods according to the present invention, the genetically converted yeast is a Yarrowia lipolytica strain selected from the group consisting of ATCC 90812, ATCC MYA-2613, or Yeastern polg.

Gene repair oligonucleobases

[0052] The methods disclosed herein may be practiced with
"gene repair oligonucleobases" having the conformations and chemistries as described in detail below. The "gene repair oligonucleobases" include mixed duplex oligonucleotides, non-nucleotide containing molecules, single stranded oligodeoxynucleotides and other gene repair molecules described in the below noted patents and patent publications. The "gene repair oligonucleobases" have also been described in published scientific and patent literature using other names including "recombinagenic oligonucleobases;" "RNA/DNA chimeric oligonucleotides;" "chimeric oligonucleotides;" "mixed duplex oligonucleotides (MDONs);" "RNA DNA oligonucleotides (RDOs);" "gene targeting oligonucleotides;" "genoplasts;" "single stranded modified oligonucleotides;" "Single stranded oligodeoxynucleotide mutational vectors;" "duplex mutational vectors;" and "heteroduplex mutational vectors."

[0053] Oligonucleobases having the conformations and chemistries described in U.S. Pat. No. 5,565,350 by Kmiec (Kmiec I) and U.S. Pat. No. 5,731,181 by Kmiec (Kmiec II)
are suitable for use as "gene repair oligonucleobases" .

[0054] The gene repair oligonucleobases in Kmiec I and/or Kmiec II contain two complementary strands, one of which contains at least one segment of RNA-type nucleotides (an "RNA segment") that are base paired to DNA-type nucleotides of the other strand.

[0055] Kmiec II discloses that purine and pyrimidine base-containing non-nucleotides can be substituted for nucleotides. Additional gene repair molecules that can be used
are described in U.S. Pat. Nos. 5,756,325; 5,871,984; 5,760,012; 5,888,983; 5,795,972; 5,780,296; 5,945,339; 6,004,804; and 6,010,907 and in International Patent No. PCT/US00/23457; and in International Patent Publication Nos. WO 98/49350; WO 99/07865; WO 99/58723; WO 99/58702; and WO 99/40789.

[0056] In one example, the gene repair oligonucleobase is a mixed duplex oligonucleotide in which the RNA-type nucleotides of the mixed duplex oligonucleotide are made RNase resistant by replacing the 2'-hydroxyl with a fluoro, chloro or bromo functionality or by placing a substituent on the 2'-O. Suitable substituents include the substituents taught by the Kmiec II. Alternative substituents include the substituents taught by U.S. Pat. No. 5,334,711 (Sproat) and the substituents taught by patent publications EP 629 387 and EP 679 657 (collectively, the Martin Applications).

[0057] As used herein, a 2'-fluoro, chloro or bromo derivative of a ribonucleotide or a ribonucleotide having a 2'-OH substituted with a substituent described in the Martin Applications or Sproat is termed a "2'-Substituted Ribonucleotide." As used herein the term "RNA-type nucleotide" means a 2'-hydroxyl or 2'-Substituted Nucleotide that is linked to other nucleotides of a mixed duplex oligonucleotide by an unsubstituted phosphodiester linkage or any of the non-natural linkages taught by Kmiec I or Kmiec II. As used herein the term "deoxyribo-type nucleotide" means a nucleotide having a 2'-H, which can be linked to other nucleotides of a gene repair oligonucleobase by an unsubstituted phosphodiester linkage or any of the non-natural linkages taught by Kmiec I or Kmiec II.

[0058] In a particular example disclosed herein, the gene repair oligonucleobase is a mixed duplex oligonucleotide that is linked solely by unsubstituted phosphodiester bonds. In alternative examples, the linkage is by substituted phosphodiesters, phosphodiester derivatives and non-phosphorus-based linkages as taught by Kmiec II. In yet another example, each RNA-type nucleotide in the mixed duplex oligonucleotide is a 2'-Substituted Nucleotide. Particular preferred examples of 2'-Substituted Ribonucleotides are 2'-fluoro, 2'-methoxy, 2'-propyloxy, 2'-allyloxy, 2'-hydroxylethyloxy, 2'-methoxyethyloxy, 2'-fluoropropyloxy and 2'-trifluoropropyloxy substituted ribonucleotides. More preferred examples of 2'-Substituted Ribonucleotides are 2'-fluoro, 2'-methoxy, 2'-methoxyethyloxy, and 2'-allyloxy substituted nucleotides.

In another example, the mixed duplex oligonucleotide is linked by unsubstituted phosphodiester bonds.

[0059] Although mixed duplex oligonucleotides having only a single type of 2'-substituted RNA-type nucleotide are more conveniently synthesized, the methods disclosed herein can be practiced with mixed duplex oligonucleotides having two or more types of RNA-type nucleotides. The function of an RNA segment may not be affected by an interruption caused by the introduction of a deoxynucleotide between two RNA-type trinucleotides, accordingly, the term RNA segment encompasses such as "interrupted RNA segment." An uninterrupted RNA segment is termed a contiguous RNA segment. In an alternative example, an RNA segment can contain alternating RNase-resistant and unsubstituted 2'-OH nucleotides. The mixed duplex oligonucleotides preferably have fewer than 100 nucleotides and more preferably fewer than 85 nucleotides, but more than 50 nucleotides. The first and second strands are Watson-Crick base paired. In one example, the strands of the mixed duplex oligonucleotide are covalently bonded by a linker, such as a single stranded hexa, penta or tetranucleotide so that the first and second strands are segments of a single oligonucleotide chain having a single 3' and a single 5' end. The 3' and 5' ends can be protected by the addition of a "hairpin cap" whereby the 3' and 5' terminal nucleotides are Watson-Crick paired to adjacent nucleotides. A second hairpin cap can, additionally, be placed at the junction between the first and second strands distant from the 3' and 5' ends, so that the Watson-Crick pairing between the first and second strands is stabilized.

[0060] The first and second strands contain two regions that are homologous with two fragments of the target gene, i.e., have the same sequence as the target gene. A homologous region contains the nucleotides of an RNA segment and may contain one or more DNA-type nucleotides of connecting DNA segment and may also contain DNA-type nucleotides that are not within the intervening DNA segment. The two regions of homology are separated by, and each is adjacent to, a region having a sequence that differs from the sequence of the target gene, termed a "heterologous region." The heterologous region can contain one, two or three mismatched nucleotides. The mismatched nucleotides can be contiguous or alternatively can be separated by one or two nucleotides that are homologous with the target gene. Alternatively, the heterologous region can also contain an insertion or one, two, three or of five or fewer nucleotides. Alternatively, the sequence of the mixed duplex oligonucleotide may differ from the sequence of the target gene only by the deletion of one, two, three, or five or fewer nucleotides from the mixed duplex oligonucleotide. The length and position of the heterologous region is, in this case, deemed to be the length of the deletion, even though no nucleotides of the mixed duplex oligonucleotide are within the heterologous region. The distance between the fragments of the target gene that are complementary to the two homologous regions is identically the length of the heterologous region when a substitution or substitutions is intended. When the heterologous region contains an insertion, the homologous regions are thereby separated in the mixed duplex oligonucleotide farther than their complementary homologous fragments are in the gene, and the converse is applicable when the heterologous region encodes a deletion.

[0061] The RNA segments of the mixed duplex oligonucleotides are each a part of a homologous region, i.e., a region that is identical in sequence to a fragment of the target gene, which segments together preferably contain at least 13 RNA-type nucleotides and preferably from 16 to 25 RNA-type nucleotides or yet more preferably 18-22 RNA-type nucleotides or most preferably 20 nucleotides. In one example, RNA segments of the homology regions are separated by and adjacent to, i.e., "connected by" an intervening DNA segment. In one example, each nucleotide of the heterologous region is a nucleotide of the intervening DNA segment. An intervening DNA segment that contains the heterologous region of a mixed duplex oligonucleotide is termed a "mutator segment."

[0062] In another example disclosed herein, the gene repair oligonucleobase is a single stranded oligodeoxynucleotide mutational vector (SSOMV), which is disclosed in International Patent Application PCT/US00/23457, U.S. Pat. Nos. 6,271,360, 6,479,292, and 7,060,500 . The sequence of the SSOMV is based on the same principles as the mutational vectors described in U.S. Pat. Nos. 5,756,325; 5,871,984; 5,760,012; 5,888,983; 5,795,972; 5,780,296; 5,945,339; 6,004,804; and 6,010,907 and in International Publication Nos. WO 98/49350; WO 99/07865; WO 99/58723; WO 99/58702; and WO 99/40789. The sequence of the SSOMV contains two regions that are homologous with the target sequence separated by a region that contains the desired genetic alteration termed the mutator region. The mutator region can have a sequence that is the same length as the sequence that separates the homologous regions in the target sequence, but having a different sequence. Such a mutator region can cause a substitution. Alternatively, the homologous regions in the SSOMV can be contiguous to each other, while the regions in the target gene having the same sequence are separated by one, two or more nucleotides. Such a SSOMV causes a deletion from the target gene of the nucleotides that are absent from the SSOMV. Lastly, the sequence of the target gene that is identical to the homologous regions may be adjacent in the target gene but separated by one two or more nucleotides in the sequence of the SSOMV. Such an SSOMV causes an insertion in the sequence of target gene.

[0063] The nucleotides of the SSOMV are deoxyribonucleotides that are linked by unmodified phosphodiester bonds except that the 3' terminal and/or 5' terminal internucleotide linkage or alternatively the two 3' terminal and/or 5' terminal internucleotide linkages can be a phosphorothioate or phosphoamidate. As used herein an internucleotide linkage is the linkage between nucleotides of the SSOMV and does not include the linkage between the 3' end nucleotide or 5' end nucleotide and a blocking substituent, see supra. In a specific example the length of the SSOMV is between 21 and 55 deoxynucleotides and the lengths of the homology regions are, accordingly, a total length of at least 20 deoxynucle-

otides and at least two homology regions should each have lengths of at least 8 deoxynucleotides.

[0064] The SSOMV can be designed to be complementary to either the coding or the non-coding strand of the target gene. When the desired mutation is a substitution of a single base, it is preferred that both the mutator nucleotide be a pyrimidine. To the extent that is consistent with achieving the desired functional result it is preferred that both the mutator nucleotide and the targeted nucleotide in the complementary strand be pyrimidines. Particularly preferred are SSOMV that encode transversion mutations, i.e., a C or T mutator nucleotide is mismatched, respectively, with a C or T nucleotide in the complementary strand.

[0065] In addition to the oligodeoxynucleotide the SSOMV can contain a 5' blocking substituent that is attached to the 5' terminal carbons through a linker. The chemistry of the linker is not critical other than its length, which should preferably be at least 6 atoms long and that the linker should be flexible. A variety of non-toxic substituents such as biotin, cholesterol or other steroids or a non-intercalating cationic fluorescent dye can be used. Particularly preferred as reagents to make SSOMV are the reagents sold as Cy3™ and Cy5™ by Glen Research, Sterling Va., which are blocked phosphoroamidites that upon incorporation into an oligonucleotide yield 3,3,3',3'-tetramethyl N,N'-isopropyl substituted indomonocarbocyanine and indodicarbocyanine dyes, respectively. Cy3 is the most preferred. When the indocarbocyanine is N-oxyalkyl substituted it can be conveniently linked to the 5' terminal of the oligodeoxynucleotide through as a phosphodiester with a 5' terminal phosphate. The chemistry of the dye linker between the dye and the oligodeoxynucleotide is not critical and is chosen for synthetic convenience. When the commercially available Cy3 phosphoramidite is used as directed the resulting 5' modification consists of a blocking substituent and linker together which are a N-hydroxypropyl, N'-phosphatidylpropyl 3,3,3',3'-tetramethyl indomonocarbocyanine.

[0066] In one preferred example, the indocarbocyanine dye is tetra substituted at the 3 and 3' positions of the indole rings. Without limitations as to theory these substitutions prevent the dye from being an intercalating dye. The identity of the substituents as these positions are not critical. The SSOMV can in addition have a 3' blocking substituent. Again the chemistry of the 3' blocking substituent is not critical.

Heterologous Expression

[0067] In certain examples, heterologous expression is used to express foreign genes or extra copies of endogenous genes in yeast (for example, *Yarrowia lipolytica*). Heterologous expression in yeast can be performed using methods well known in the art. Expression of foreign genes or extra copies of endogenous genes in yeast using heterologous expression may involve use of a vector that includes (a) promoter sequences for transcriptional initiation, (b) terminator sequences for termination of transcription, and (c) a selectable marker. Heterologous expression and expression vectors may be as described, for example, in Madzak, C., Gaillardin, C., and Beckerich, J-M., 2004 Heterologous Protein Expression and Secretion in the Non-Conventional Yeast Yarrowia lipolytica: a review, Journal of Biotechnology 109:63-81. In certain examples of the compositions and methods disclosed herein, the vector is pYLEX1 (Yeastern). A non-limiting list of selectable marker genes that may be used includes *ura3, lys5, trp1, leu2*, *ade1, E.coli hph* encoding hygromycin resistance, and *SUC2* from *Saccharomyces cerevisiae.* A non-limiting list of promoters that may be used includes pLEU2, pXPR2, pPOX2, pPOT1,pICL1, pG3P, pMTP, pTEF, and pRPS7. In certain examples, the promoter is the hp4d promoter, which is a strong, constitutive hybrid promoter (U.S. Patent 6,083,717 issued Jul.4,2000). A non-limiting list of terminator sequences that may be used includes *XPR2t, LIP2t,* and *PHO5t.*

[0068] In certain examples, one or more of *Yarrowia lipolytica LYS1 (Genolevures* YALI0B15444g), *TRP1 (Genolevures* YALIOB07667g), and *ADE1 (Genolevures* YALI0E33033g) genes are used as selectable markers. In certain examples, one or more of *Yarrowia lipolytica* URA3 (GenBank: U40564.1) or LEU2 (Genoluveres YALI0C00407) genes are used as selectable markers.

[0069] In certain examples, an integrative expression vector includes one or more promoters and/or terminator sequences selected from the group consisting of *Yarrowia lipolytica* glycolytic pathway genes, alkane or glycerol utilization genes, *XPR2, ACC1, HMG1*, *ERG1,* and *ERG9.*

[0070] In certain examples of one or both subunits of *Yarrowia lipolytica* ATP citrate lyase (Genoleveres YALI0D24431g and YALI0E34793g) in *Yarrowia lipolytica* are overexpressed.

Modified enzymes

[0071] A modified or mutated enzyme of the present disclosure can be modified or mutated by base pair changes, insertions, substitutions, and the like.

[0072] The genes encoding enzymes involved in the fatty acid biosynthesis pathway and isoprenoid biosynthesis pathway are the preferred targets for mutation. In some examples, the target gene encodes an acyl CoA carboxylase. In other examples, the target gene encodes an HMG-CoA reductase. In other examples, the target gene encodes a squalene epoxidase. In other examples, the target gene encodes a squalene synthase. In certain examples, the target gene encodes ATP citrate lyase. Mutations can be designed that reduce or eliminate the activity of an enzyme, enhance

the activity of an enzyme, or that alter the activity of the enzyme (e.g., change the substrate selectivity).

[0073] In wild-type oleaginous yeast, acetyl-CoA is extensively channeled into fatty acid biosynthesis via acetyl-CoA carboxylase (ACCase). Thus in order to increase the amount of acetyl-CoA available for squalene synthesis, it is desirable to reduce the enzymatic expression or specific activity of ACCase. An exemplary gene sequence for ACCase is the ACC1 gene in *Saccharomyces cerevisiae* as shown in accession number Z71631. Accordingly in certain examples, reduced intracellular activities of ACCase, the enzyme at the branch point between mevalonate biosynthesis and triglyceride biosynthesis will decrease the amount of acetyl-CoA partitioned for oil synthesis, thereby increasing its availability to the isoprene pathway.

[0074] HMG-CoA reductase activity is the rate-limiting enzyme for isoprene biosynthesis. Exemplary gene sequences for HMG-CoA reductase include the HMG1 and HMG1 genes in *Sacchanomyces cerevisiae* as shown in accession numbers NC_001145 and NC_001144, respectfully. Accordingly, in certain examples, HMG-CoA reductase activity will be increased by modifying the HMGR gene to increase transcription, stabilize the resultant protein, and/or reduce product feedback inhibition.

[0075] Decreasing ACCase activity and/or increasing HMG-CoA reductase activity in a yeast can create a core isoprenoid production organism capable of producing a number of related isoprenoid products by the manipulation of subsequent enzymes in the pathway.

[0076] Squalene epoxidase catalyzes the first committed step of sterol biosynthesis. An exemplary gene sequence for Squalene epoxidase is the ERG1 gene in *Saccharomyces cerevisiae* as shown in accession number NC_001139. Accordingly, in certain examples, squalene epoxidase activity, sensitivity to inhibitors and/or expression will be attenuated in a yeast, for example by catalytically important residues in the enzyme's amino acid sequence.

[0077] Squalene synthase catalyzes the synthesis of squalene by condensing two c15 isoprene precursors (farnesyl diphosphate (FPP)). An exemplary gene sequence for squalene synthase is the ERG9 gene in *Saccharomyces cerevisiae* as shown in accession number NC_001140. Accordingly, in certain examples, squalene synthase activity and/or expression will be increased in a yeast.

[0078] ATP citrate lyase (E.C. 4.1.3.8) catalytically cleaves citrate to produce acetyl CoA and oxaloacetate. Acetyl CoA can be used by ACCase for fatty acid biosynthesis or by acetyl CoA acetyl transferase for the production of isoprenes and derivatives such as squalene.

[0079] Mevalonate kinase is the first enzyme after HMG-CoA Reductase in the mevalonate pathway, and catalyzes the conversion of Mevalonate to Mevalonate-5-phosphate. Accordingly, in certain examples, mevalonate kinase activity and/or expression levels will be increased in yeast, for example, by changing catalytically important residues in the enzyme's amino acid sequence or increasing its gene dosage or transcript levels.

[0080] Glycerol kinase catalyzes the transfer of a phosphate from ATP to glycerol to form glycerol phosphate. Accordingly, in certain examples, glycerol kinase activity and/or expression levels will be increased in yeast, for example by changing catalytically important residues in the enzyme's amino acid sequence or increasing its gene dosage or transcript levels.

[0081] The result of the metabolic changes in certain examples will be to channel carbon from acetyl-CoA to squalene, and attenuate major competitive pathways for this carbon stream, resulting in a significant increase of squalene produced.

Delivery of gene repair oligonucleobases into yeast cells

[0082] Any commonly known method can be used in the methods disclosed herein to transform a yeast cell with a gene repair oligonucleobase. Exemplary methods include the use of electroporation, LiOAc, biolistics, spheroplasting, and/or *Agrobacterium* (see, for example, McClelland, C.M., Chang, Y.C., and Kwon-Chung, K.J. (2005) Fungal Genetics and Biology 42:904-913).

[0083] In certain examples, a gene repair oligonucleobase is introduced into a yeast cell by electroporation. In some examples, a gene repair oligonucleobase is introduced into a yeast cell that has been chemically treated with PEG (3350 or 4000 mw) and/or Lithium Acetate by electroporation. In certain examples, a gene repair oligonucleobase is introduced into a yeast cell using PEG (3350 or 4000 mw) and/or Lithium Acetate.

[0084] Specific conditions for using microcarriers in the methods disclosed herein are described in International Publication WO 99/07865, US09/129,298. For example, ice cold microcarriers (60 mg/mL), mixed duplex oligonucleotide (60 mg/mL), 2.5 M $CaCl_2$ and 0.1 M spermidine are added in that order; the mixture gently agitated, e.g., by vortexing, for 10 minutes and let stand at room temperature for 10 minutes, whereupon the microcarriers are diluted in 5 volumes of ethanol, centrifuged and resuspended in 100% ethanol. Exemplary concentrations of the components in the adhering solution include 8-10 $\mu g/\mu L$ microcarriers, 14-17 $\mu g/\mu L$ mixed duplex oligonucleotide, 1.1-1.4 M $CaCl_2$ and 18-22 mM spermidine. In one example, the component concentrations are 8 $\mu g/\mu L$ microcarriers, 16.5 $\mu g/\mu L$ mixed duplex oligonucleotide, 1.3 M $CaCl_2$ and 21 mM spermidine.

[0085] In some examples, gene repair oligonucleobases can be delivered to the yeast cell by electroporation, according to techniques well known to those skilled in the art. (See, e.g. Becker, D. M., and Guarente, L. High Efficiency Trans-

formation of Yeast by Electroporation. Methods in Enzymology, vol. 194, section [12] pp. 182-186. 1991. Elsevier Academic Press, London.

Selection of yeast having the desired modified enzyme

[0086] Yeast expressing the modified enzyme can be identified through any of a number of means. In one method, a co-conversion strategy using gene repair oligonucleobases (GRONs) to target both a selectable conversion (i.e., a marker) and a non-selectable conversion (e.g., a target gene of interest) in the same experiment. In this way, the cells to which GRONs were not delivered or were unable to transmit the conversions specified by the GRON would be eliminated. Since delivery of GRONs targeting unrelated genes is not expected to be selective, at some frequency, a colony with a successfully selected conversion would also be expected to have a conversion in one of the other targeted genes. Conversion events would be resolved by single nucleotide polymorphism (SNP) analysis.

[0087] Thus, genomic DNA is extracted from yeast and screening of the individual DNA samples using a SNP detection technology, e.g., allele-specific Polymerase Chain Reaction (ASPCR), for each target. To independently confirm the sequence change in positive yeast, the appropriate region of the target gene may be PCR amplified and the resulting amplicon either sequenced directly or cloned and multiple inserts sequenced.

[0088] Alternatively, the incorporation of the mutation into the gene of interest can be identified by any of a number of molecular biology techniques designed to detect single nucleotide mutations in extracted nucleic acid (e.g., amplification methods such as PCR and single nucleotide primer extension analysis). Larger mutations can be detected by amplification and sequencing of the region of the target gene to be mutated.

[0089] Alternatively, yeast or yeast cells containing the modified enzyme can be identified by, for example, analysis of the composition of isoprenoids produced by the yeast. Thus, the yeast can be grown and oils extracted and analyzed using methods known in the art (e.g., gas chromatography or HPLC).

EXAMPLES

Example 1. *Cryptococcus curvatus* and *Rhodotorula glutinis* transformation systems. (not according to the present invention)

[0090] To create a *Cryptococcus curvatus* (ATCC strain 20508) and *Rhodotorula glutinis* (ATCC strains 10788 and 204091) transformation system, a KANMX expression cassette (promoter-gene-terminator) which confers kanamycin resistance to *S. cerevisiae* is used as a selectable marker to convert the strains from kanamycin sensitivity to resistance (See *e.g.,* Baudin, A., et al. (1993) Nucleic Acids Research (21) 3329-3330). The strains are transformed with the expression cassette alone, as well as KANMX ligated to restriction fragments of a plasmid reported in *R. glutinus* (See *e.g* Oloke, J.K., and Glick, B.R. (2006) African Journal of Biotechnology 5(4):327-332) containing DNA origins of replication. DNA is introduced into *C. curvatus* and *R. glutinis* by electroporation, LiOAc, biolistics, spheroplasting, and/or *Agrobacterium* (McClelland, C.M., Chang, Y.C., and Kwon-Chung, K.J. (2005) Fungal Genetics and Biology 42:904-913).

Example 2. Selectable Markers. (not according to the present invention)

[0091] To generate uracil auxotrophic mutants in *Cyptococcus curvatus* and *Rhodotorula glutinis,* cells were grown in minimal media containing anti-metabolite 5-fluoroorotic acid to select for resistant mutants with lesions in the ura3 or ura5 genes. 33 stable 5-FOA^R colonies of *Cryptococcus curvatus* and 20 stable 5-FOA^R colonies of *Rhodotorula glutinis* were banked. Wild type URA3 genes from both *Cryptococcus curvatus* and *Rhodotorula glutinis* are cloned and the mutant ura3 genes in the 5-FOA^R isolates are sequenced.

[0092] Other auxotrophic markers are cloned by functional complementation in *Saccharomyces cerevisiae* (See Ho, Y.R., and Chang, M.C. (1988) Chinese Journal of Microbiology and Immunology 21(1):1-8). Genomic and/or cDNA libraries are constructed from *Cryptococcus curvatus* and *Rhodotorula glutinis* for ligation into a uracil-selectable *Saccharomyces* expression vector for transformation into strain YPH500 (*MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 hits3-Δ200 leu2-Δ1)* to select for lysine, adenine, tryptophan, histidine, and leucine prototrophs. From these prototrophs, the corresponding genes for LYS2, ADE2, TRP1, HIS3, and LEU2 are sequenced from the genomic or cDNA insert.

Example 2. Gene Manipulation in Yeast using RTDS technology. (not according to the present invention)

[0093] The alleles of the leu2, lys5 and ura3 genes from *Yarrowia lipolytica* strain ATCC 90811 (leu2-35 lys5-12 ura3-18 XPR2B) were cloned by PCR and their sequences compared to the wild type alleles to identify differences.

[0094] For ura3, differences were found at positions 1365 (A→G mutation, resulting in a silent change of AAA→AAG coding for lysine), 1503 (AAGAA extra sequences in ATCC 90811 which results in a frame change, but which comes

back in frame at 1511 resulting in 7 additional amino acids, after which the sequence continues as the YL URA3 in GenBank), 1511 (extra T in ATCC 90811), and 1978 (C→T mutation, leading to a stop mutation truncating the protein 24 amino acids short of the carboxy terminus). A GRON oligonucleotide was designed to restore prototrophy by converting STOP(TGA)→R (CGA) to yield 264R based on YlUra3 - YLU40564 amino acid numbering. The GRONs used are YlUra31264/C/40/5'Cy3/3'idC, which has the sequence
VCGAGGTCTGTACGGCCAGAACCGAGATCCTATTGAGGAGGH, and
YlUra31264/NC/40/5'Cy3/3'idC, which has the sequence
VCCTCCTCAATAGGATCTCGGTTCTGGCCGTACAGACCTCGH,where V=CY3;
H=3'DMT dC CPG. 10, 30, and 50 μg of each of the GRONs were transformed into *Yarrowia lipolytica* strain ATCC 90811 using a Lithium acetate-based method, and plated onto ura- 2% glucose. A total of 82 ura+ colonies were obtained with the GRON designed using the coding strand and 6 colonies with the GRON designed using the non-coding strand, demonstrating the strand bias common in transforming with gap-repair oligonucleotides. Sequencing of 18 of the coding-strand transformants demonstrated the intended change in 17 of the clones.

[0095] For LEU2 differences were found at positions 1710 (extra C absent leading to a frame shift and premature protein termination); 1896 (extra T); 2036 (T→A mutation, located after the stop codon); 2177 (extra T in missing, located after stop codon).

[0096] For LEU2 differences were found at positions 1092 (G→A TCG→TCA, a conservative substitution (Serine)); 1278 (G→A CAG→CAA, a conservative substitution (Glutamine)); 1279 (G→A GGT→ATT, changing V→I).

[0097] Accordingly, the mutations can be used for various purposes, for example to convert prototrophic yeast to become auxotrophic and vice versa.

[0098] A similar strategy for demonstrating the effectiveness of RTDS technology in *Cyptococcus curvatus* and *Rhodotorula glutinis* is performed as described for *Yarrowia lipolytica* in which ura3 mutations are corrected to restore prototrophy.

[0099] In certain examples, the effectiveness of RTDS in *Y. lipolytica* may be demonstrated by integrating a mutated version of the *E. coli* hygromycin gene into its genome. This version of the gene, which harbors a point mutation at G34T, encodes an E12STOP change such that the natural hygromycin sensitivity of *Y. lipolytica* is not affected. Transformation with a GRON correcting this mutation will confer resistance of the *Y. lipolytica* strain, for example, up to 1000 ug/ml of hygromycin. Double mutations in the hygromycin resistance (HGH) gene are also constructed, comprising of G34T A37T (E12ASTOP K13STOP) which may be corrected by a single GRON, and G34T T149G (E12STOP Y46 STOP) which may be corrected by 2 GRONS.

[0100] To testing GRON activity in *Yarrowia,* the natural sensitivity of wild-type *Yarrowia lipolytica* to the aminoglycoside antibiotic hygromycin B was used. Hygromycin B (hmB) is an aminocyclitol antibiotic produced by *Streptomyces hygroscopicus* which inhibits protein synthesis in both procaryotes and eucaryotes by interfering with ribosomal translocation and with aminoacyltRNA recognition. Resistance can be conferred by introduction of the *hph* gene (also known as *aph(4))* from *E. coli* (GENBANK V01499) which encodes an aminocyclitol phoshotransferase that inactivates hygromycin B by covalent addition of a phosphate group to the 4-position of the cyclitol ring. *Yarrowia lipolytica* strain Polg (*Mat a ura3-302: :URA3 leu2-270 xpr2-322 axp-2* from Yeastern) was transformed with the *E. coli hph* gene containing either a single (E12stop from G34T) or double mutation E12stopK13stop (G34T.A37T) mutations cloned into vector pyLEX1-2u-ura3-13, putting the gene under control of the hpd4 promoter and XPR2 terminator. The linearized vector was integrated into the genome upon selection for restoration of prototrophy conferred by the LEU2 marker. The resultant strains harbor disabled versions of the hygromycin phosphotransferase gene (hence hygromycin sensitive), and were converted with the following GRONs restoring either G34T or G34T.A37T to wild type (hygromycin resistant).

GRONs for restoring E12stop to wild type (T34G)

HPH2/C/42/5'Cy3/3'idC
5'Cy3-GAACTCACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAG-3'idC
HPH2/NC/42/5'Cy3/3'idC
5'Cy3-HCTTTTCGATCAGAAACTTCTCGACAGACGTCGCGGTGAGTTC-3'idC

GRONs for restoring E12stopK13stop to wild type (T34G T37A)

HPH3/C/43/5'Cy3/3'idC
5'Cy3-CTCACCGCGACGTCTGTCGAGAAGTTTCTGATCGAAAAGTTCG-3'idC
HPH3/NC/43/5'Cy3/3'idC
5'Cy3-CGAACTTTTCGATCAGAAACTTCTCGACAGACGTCGCGGTGAG-3'idC

[0101] 30μg of the indicated GRON was used to convert the single- or double- hph mutant strain in replicate (x6),

pooled, and an aliquot plated onto YEPD plates containing 100-1000 μg/ml hygromycin to optimize the signal-to-noise ratio. With both strains, significant numbers of putatively converted colonies were obtained at any given hygromycin concentration above the 'No DNA' control, with a strong bias toward the non-coding GRON strand in both cases. Taken together, these results suggest GRON conversion of the hygromycin phosphotransferase gene target in *Yarrowia lipolytica,* and further that conversion of two mutations (T34G T37A) can be accomplished using a single GRON. DNA sequencing is performed to confirm restoration of the wild-type genotype.

Strain	DNA	Colonies on 100 μg/ml Hygromycin	Colonies on 200 μg/ml Hygromycin	Colonies on 400 μg/ml Hygromycin	Colonies on 600 μg/ml Hygromycin	Colonies on 800 μg/ml Hygromycin	Colonies on 1000 μg/ml Hygromycin
E12stop	No DNA	0	0	1	1	0	0
E12stop	30μg coding strand	0	1	6	1	1	0
E12stop	30μg non-coding strand	20	21	21	12	10	8
E12stopK13stop	No DNA	0	0	4	1	0	0
E12stopK13stop	30μg coding strand	2	2	1	2	0	2
E12stopK13stop	30μg non-coding strand	5	3	5	7	8	8

Example 3. Cloning of target genes. (not according to the present invention)

[0102] The sequences for ACCase, HMGR, squalene synthase and squalene epoxidase, available in the NCBI database from *Saccharomyces* and other yeasts, are used as a source of PCR primers and the corresponding genes are cloned from *Cryptococcus curvatus* and *Rhodotorula glutinis* along with their corresponding regulatory regions (promoters, terminators). To identify 'up' and 'down' promoter mutations that increase or decrease transcription, respectively, the promoters for these four genes are cloned with a relatively error-prone DNA polymerase to generate point mutations in the promoters, and these fragments are cloned into plasmids fused with Green Fluorescent Protein (GFP) or beta-galactosidase reporter genes for testing *in vitro* in *S. cerevisiae* or *E. coli.* Promoter "up" mutations are reintroduced into the HMGR and squalene synthase genomic sequences by RTDS, while "down" promoter mutations are being made in the genomic ACCase and squalene epoxidase sequences. The promoters from essential genes (e.g. GAPDH, actin) in *R. glutinis* and *C. curvatus* are cloned for use in heterologous gene expression. Primers for PCR cloning are designed from homology to these genes in *S. cerevisiae.*

Example 4. Manipulation of target for increased squalene production.

[0103] **ACCase.** The number of copies of the ACCase gene is determined in *R. glutinis* and C. *curvatus* and other yeasts. RTDS is utilized to reduce ACCase expression by introducing stop codons immediately after the translational start site in any extra copies.

[0104] **Squalene Epoxidase.** Similarly, an increase in squalene accumulation in *S. cerevisiae* has been achieved by disruption of one copy of the squalene epoxidase in the diploid. Kamimura, N., Hidaka, M., Masaki, H., and Uozumi, T. (1994) Appl. Microb. Biotech. 42: 353-357. The number of copies of squalene epoxidase in *R. glutinis* and *C. curvatus* and other yeasts is determined, and RTDS is used to create or insert a stop codon immediately after the translational start site in extra copies beyond the first one.

[0105] In some examples, Squalene epoxidase activity is attenuated by addition of terbinafine (an inhibitor of Squalene epoxidase) to the media. In certain examples, amino acid changes to the Squalene epoxidase are made to increase the sensitivity of Squalene epoxidase to terbinafine (for example amino acid changes homologous to G30S, L37P, and R269G mapped on *Saccharomyces* ERG1). In some examples, the amino acid changes are made by gene synthesis and replacement of the wild-type gene with the mutant version by homologous recombination. In other examples, the changes are introduced into the wild-type gene by RTDS.

[0106] **HMGR.** Both *Saccharomyces cerevisiae* and mammalian HMGR enzymes contain amino acid sequences in their linker regions which are present in many short-lived proteins that are subject to rapid intracellular turnover in eukaryotes (see Rogers, S., Wells, R., and Rechsteiner, M. (1986) Science 234: 364-368; and Chun, K.T., and Simoni, R.D. (1991) J. Biol. Chem. 267(6): 4236-4246). Similar sequences, if present, are identified in the HMGR genes in *Y. lipolytica, R. glutinis* and/or *C. curvatus,* and eliminated using RTDS to reduce HMGR protein turnover. Such similar sequences have been found in the *S. cerevisiae* squalene synthase gene, and it is also determined if such sequences are present in the squalene synthase genes in *Y. lipolytica, R. glutinis* and/or *C. curvatus.* The sequences, if present in *Y. lipolytica, R. glutinis* and/or *C. curvatus* squalene synthase, are also eliminated using RTDS to reduce protein turnover.

[0107] HMGR in *S. cerevisiae* comprises two highly conserved domains, of which the N-terminal 552 amino acids are responsible for membrane association. Overexpression of the truncated HMG1 protein containing only the C-terminal catalytic portion led a 40-fold increase of HMG-CoA activity in *S. cerevisiae* with an increased accumulation of squalene to 5.5% of dry matter (Polakowski, T., Stahl, U., and Lang, C. (1998) Appl. Microbiol. Biotech. 49:66-71). It is determined if *Y, lipolytica, R. glutinis* and *C. curvatus* HMGR proteins have a similar structure, and, if so, fragments having only the soluble catalytic domain may be expressed.

[0108] The protein structure and DNA sequence of HMGR is highly conserved between eukaryotes from fungi to mammals, with a membrane-associated N-terminal domain and catalytic C-terminal domain. The boundary between the two domains can be mapped to a region of amino acids 500-600 in the *Yarrowia lipolytica HMG1* gene (Genelouvres *Yarrowia lipolytica* YALI0E04807g) where the hydrophobicity plot transitions from hydrophobic to hydrophilic. Resides 548 and 544 are chosen from evaluation of the hydrophobicity plot of *Yarrowia lipolytica HMG1,* and its homology to the N-termini of the truncated *Saccharomyces cerevisiae* (Donald, K.A.G., et al, 1997. Appl. Environ. Micro. 63(9): 3341-3344) and *Candida utilis* (Shimada, H. et al, 1998. Appl. Environ. Micro. 64(7):2676-2680) proteins. Accordingly, in one example, amino acids 548-1000 of the C-terminal domain of *Yarrowia lipolytica HMG1 I* is expressed; in a second example amino acids 544-1000 of the C-terminal domain of *Yarrowia lipolytica HMG1 I* is expressed. In related examples, amino acids 543-1000 of the C-terminal domain of *Yarrowia lipolytica HMG1 I* is expressed; or amino acids 545-1000 of the C-terminal domains of *Yarrowia lipolytica HMG1 I* is expressed; or amino acids 546-1000 of the C-terminal domains of *Yarrowia lipolytica HMG1 I is* expressed; or amino acids 547-1000 of the C-terminal domains of *Yarrowia lipolytica HMG1 I* is expressed; or amino acids 549-1000 of the C-terminal domains of *Yarrowia lipolytica HMG1 I* is expressed.

[0109] Expression of the 457 amino-acid C-terminal catalytic domain of HMGR (residues 543-1000) in *Y. lipolytica*

strain Polg yielded 2% squalene/total lipid compared to 0% in the control strain containing the vector alone in experiments using shakeflasks. The process is repeated and expanded using fermenters.

[0110] In Syrian hamsters, activity of the HMGR catalytic domain is down-modulated by phosphorylation by an AMP-dependent kinase (Omkumar, R.V., Darnay, B.G., and Rodwell, V.W. (1994) J. Biol. Chem. 269:6810-6814), and a similar mode of regulation has been described in *S. cerevisiae.* It is determined if the HMGR proteins in *R. glutinis, C. curvatus* and other yeasts are similarly regulated, and if so, RTDS is employed to eliminate the phosphorylation site.

[0111] **Squalene synthase.** Squalene synthase in mammalian systems is coordinately regulated on the transcriptional level along with HMG-CoA synthase and farnesyl diphosphate synthase by SREBPs (sterol regulatory element binding proteins) (Szkopinsda, A., Swiezewska, E., and Karst, F (2000) Biochem. Biophys. Res. Comm. 267:473-477). SREBPs exist in three forms, of which one binds the squalene synthase promoter. It is determined if such transcription factors and/or binding sites are present on the squalene synthase promoter in *R. glutinis, C.curvatus* and other yeasts, and, if present, RTDS is used to make changes to such transcription factors and/or binding sites that enhance transcription of squalene synthase.

[0112] Overexpression of the *Y. lipolytica* Squalene Synthase in *Y. lipolytica* strain Polg yielded 2% squalene/total lipid compared to 0% in the control strain containing the vector alone using shakeflasks. The process is repeated and expanded using fermenters.

Example 5. Growth Conditions for *Cryptococcus curvatus.* (not according to the present invention)

[0113] *Cryptococcus curvatus* growth was evaluated to determine the best carbon sources to maximize its cell mass in culture. In a Yeast Extract-based rich media (10 g/L yeast extract, 20 g/L peptone), *C. curvatus* grew well in 2-20% w/v glucose, achieving a maximal level of 55 g/L cell dry weight (CDW) at 16% w/v glucose and above after 4 days. Similarly, *C. curvatus* grew in the same media with 3-12% w/v glycerol, achieving a CDW of 40 g/L in 12% w/v glycerol after 5 days. *C. curvatus* was also grown in Biodiesel glycerol (Imperial Western Products, Coachella, CA) up to 3.5% w/v, resulting in 23 g/L CDW.

Example 6. Environmental manipulation of target genes fon increased squalene production.

[0114] Environmental manipulations are tested to increase the net yield of squalene. These include (a) inhibiting ACCase expression and/or activity with oleic acid, olive or other vegetable oil(s), inositol, choline, soraphen, fluazifop, and clethodim or other ACCase inhibiting herbicides, (b) inhibiting squalene epoxidase expression and/or activity with terbinafine, tolnaftate, and ergosterol or other squalene epoxidase inhibiting fungicides, (c) manipulating the C/N ratio in glycerol-based media (in the starting media or by add-ins), (d) varying the nitrogen source in the media (organic vs. inorganic vs. simple/complex), (e) varying carbon addition regimes (e.g. batch vs. feeding), (f) examining the effect of depleting nutrients other than carbon source, (g) varying the carbon source to include mixtures of sugars, sugar alcohols, alcohols, polyalcohols, and organic acids, (h) selecting for growth on HMGR-inhibitory compounds such as lovastatin or other statin-type inhibitors, and (i) selecting for high oil production in culture using lipophillic dyes or stains and/or by analyzing for extractable lipids using, for example, gravimetric or gas chromatographic methods.

[0115] For example, *Yarrowia lipolytica* ATCC 90904 was cultivated in high Carbon/Nitrogen ratio media (C/N = 420, Li, Y-H., Liu, B., Zhao, Z-B., and Bai, F-W. 2006 "Optimized Culture Medium and Fermentation Conditions for Lipid Production by Rhodosporidium toruloides" Chinese Journal of Biotechnology 22(4): 650-656) (hereinafter "CYM001 Media") supplemented with 0 to 50 μg/ml terbinafine at 30'C, 300 rpm for 120 h. Concentrations of 12.5μg/ml or higher of terbinafine resulted in up to 18.5% of total lipid as squalene.

[0116] Various *Yarrowia lipolytica* strains are used for lipid and squalene production including ATCC 20688, ATCC 90811, ATCC 90904, ATCC 90812, ATCC MYA-2613, and Yeastern polg. For example, *Yarrowia lipolytica* strain *polg* (Yeastern) was cultivated in high Carbon/Nitrogen ratio media (C/N = 420, Li, Y-H., Liu, B., Zhao, Z-B., and Bai, F-W. 2006 "Optimized Culture Medium and Fermentation Conditions for Lipid Production by Rhodosporidium toruloides" Chinese Journal of Biotechnology 22(4): 650-656) (hereinafter "CYM001 Media") supplemented with 0 to 50 μg/ml terbinafine at 30'C, 300 rpm for 120 h. Concentrations of 12.5μg/ml or higher of terbinafine resulted in up to 38 % of total lipid as squalene and Values of total lipid/Cell Dry weight of up to 51 % were achieved.

[0117] In another example, *Yarrowia lipolytica* ATCC 90904 was cultivated in CYM001 media supplemented with 0 to 50 μg/ml Oleic acid at 30'C, 300 rpm for 120 h. Supplementation with 10μl/ml Oleic acid was found to improve lipid accumulation 10-fold in lipid/CDW (cell dry weight) over no supplementation.

[0118] In a further example, *Yarrowia lipolytica* ATCC 90904 was cultivated in CYM001 media supplemented with 0 to 200 μM clethodim at 30'C, 300 rpm for 120 h. Supplementation of 200 μM clethodim resulted in a 60-fold increase in the yield (mg) of squalene per 60-ml flask.

[0119] Increased oxygen has been shown to cause the differential regulation of HMG1 and HMG2 in *S. cerevisiae,* resulting in rapid degradation of HMG2 and increased expression of HMG1 under aerobic conditions (Casey, W.M.,

Keesler, G.A., Parks, L.W. (1992) J. Bact. 174:7283-7288). It is determined if the number of HMGR genes in our oleaginous yeasts is affected by oxygen and, if so, their expression and activity is manipulated in the fermenter by altering oxygen levels.

[0120] Starting with "CYM001 Media" (Li, Y-H., Liu, B., Zhao, Z-B., and Bai, F-W. (2006) Chinese Journal of Biotechnology 22(4):650-656), various components and concentrations of components are changed (including the addition of new components) to improve cell growth, percent total lipid content/unit mass of cells, and percent squalene/total lipid. Media components that are evaluated include: carbon sources: glycerol, glucose, nitrogen sources: ammonium compounds, nitrates, amino acids, mineral salts: potassium, magnesium, sodium, iron, manganese, zinc, calcium, copper, yeast extract, lipid precursors and lipid synthesis affectors: terbinafine, clethodim, oleic acid, palmitoleic acid, linoleic acid, linolenic acid and antifoaming agents. Other factors that are evaluated include: percent inoculum, elapsed fermentation time, temperature, pH, back pressure, dissolved oxygen (DO), feed composition, feed strategy and agitation strategy.

Example 7. Strain Selection. (not according to the present invention)

[0121] Traditional strain selection methods are used in oleaginous yeasts to increase their net squalene productivity. Strains mutagenized by UV, nitrosoguanidine, or ethane methyl sulfonate are screened and/or selected for increased squalene accumulation. Strains are also subjected to iterative selection pressure, such as repeated passage on YEP (15 g/L yeast extract, 5 g/L peptone) media containing 3% glycerol or media containing lovastatin and other known HMGR inhibitors. Strains are also subjected to repeated passage on CYM001 Media containing varying amounts of glycerol and/or glucose or media containing lovastatin and/or other known HMGR inhibitors, and/or squalene synthase inhibitors to obtain spontaneous mutants with increased HMGR and/or squalene synthase activity. Such mutations may be in HMGR, squalene synthase, or other genes ("secondary site mutations").

[0122] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0123] The inventions illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising," "including," "containing," etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed.

[0124] Thus, it should be understood that although the invention has been specifically disclosed by preferred embodiments and optional features, modification, improvement and variation of the inventions embodied therein herein disclosed may be resorted to by those skilled in the art, and that such modifications, improvements and variations are considered to be within the scope of this invention. The materials, methods, and examples provided here are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention.

Claims

1. A composition comprising a genetically converted yeast, wherein said genetically converted yeast expresses one or more modified enzymes having one or more designed mutations, said one or more modified enzymes comprising squalene epoxidase, wherein said squalene epoxidase has reduced activity and/or expression,
   wherein said one or more designed mutations are at defined positions within said enzyme,
   wherein said yeast produces increased quantities of squalene as compared to the native yeast, and
   wherein the yeast is a *Yarrowia lipolytica* strain selected from the group consisting of ATCC 90812, ATCC MYA-2613, or Yeastern polg.

2. A method of producing squalene by a genetically converted yeast, said method comprising
   increasing or decreasing activity or expression of one or more enzymes in the isoprenoid biosynthesis pathway, said one or more enzymes comprising squalene epoxidase, wherein said squalene epoxidase has reduced activity and/or expression,
   wherein said enzyme activity or expression is increased or decreased by one or more designed mutations,
   wherein said one or more designed mutations are at defined positions within said enzyme,
   wherein said genetically converted yeast produces increased quantities of squalene as compared to the native yeast and
   wherein the yeast is a *Yarrowia lipolytica* strain selected from the group consisting of ATCC 90812, ATCC MYA-2613, or Yeastern polg.

3. The compositions or methods according to claim 1 or 2, wherein said genetically converted yeast is derived from an oleaginous yeast.

4. The compositions or methods according to any of the preceding claims, further comprising a modified enzyme selected from the group consisting of acetyl-CoA carboxylase ("ACCase"), HMG-CoA reductase, squalene synthase, ATP citrate lyase, ATP citrate synthase, mevalonate kinase, glycerol kinase and 5-aminolevulinate synthase.

5. The compositions or methods according to any of the preceding claims, wherein said activity or expression is reduced to 90%; or 80%; or 70%; or 60%; or 50%; or 40%; or 30%; or 20%; or 10%; or 5% of the activity or expression of the corresponding native yeast.

6. The compositions or methods according to any of the preceding claims, wherein said activity or expression is between 90-95%; or 80-90%; or 70-80%; 60-70%; or 50-60%; or 40-50%; or 30-40%; or 20-30%; 10-20%; or 5-10%; or 2-5% of the activity or expression of the corresponding native yeast.

7. The compositions or methods of any of the preceding claims, wherein the yeast is the Yeastern polg strain of *Yarrowia lipolytica.*

8. The compositions or methods of any of the preceding claims, wherein an antifungal agent is present in the composition or is added to the yeast in the method; or wherein an antifungal agent is present in the composition or is added to the yeast in the method at a concentration between 0.5 to 100 μg/ml, between 1 to 25 μg/ml, or between 10 to 15 μg/ml.

9. The compositions or methods of any of the preceding claims, wherein an antifungal agent is present in the composition or is added to the yeast in the method; and wherein the antifungal agent is an allylamine antifungal agent; or wherein an antifungal agent is present in the composition or is added to the yeast in the method; and wherein the antifungal agent is an allylamine antifungal agent at a concentration between 0.5 to 100 μg/ml, between 1 to 25 μg/ml, or between 10 to 15 μg/ml.

10. The compositions or methods of any of the preceding claims, wherein an antifungal agent is present in the composition or is added to the yeast in the method; and wherein the antifungal agent is terbinafine, or is terbinafine at a concentration between 0.5 to 100 μg/ml, between 1 to 25 μg/ml, or between 10 to 15 μg/ml.

11. The method of any of the preceding claims, said method comprising
cultivating said yeast with an antifungal agent; wherein the yeast is the Yeastern polg strain of *Yarrowia lipolytica* and wherein the antifungal agent is terbinafine.

12. The method of claim 11, wherein the antifungal agent is terbinafine at a concentration or 12.5 μg/ml or greater.

Patentansprüche

1. Zusammensetzung, umfassend eine genetisch konvertierte Hefe, wobei die genetisch konvertierte Hefe ein oder mehrere modifizierte Enzyme mit einer oder mehreren konzipierten Mutationen exprimiert, wobei das eine oder die mehreren modifizierten Enzyme Squalenepoxidase umfassen, wobei die Squalenepoxidase eine reduzierte Aktivität und/oder Expression aufweist,
wobei die eine oder mehreren konzipierten Mutationen sich an definierten Positionen innerhalb des Enzyms befinden,
wobei die Hefe im Vergleich zu der nativen Hefe erhöhte Mengen an Squalen produziert, und
wobei die Hefe ein *Yarrowia lipolytica*-Stamm ausgewählt aus der Gruppe bestehend aus ATCC 90812, ATCC MYA-2613, oder Yeastern po1g ist.

2. Verfahren zur Herstellung von Squalen durch eine genetisch konvertierte Hefe, das Verfahren umfassend
Erhöhen oder Herabsetzen der Aktivität oder Expression von einem oder mehreren Enzymen in dem Isoprenoid-Biosyntheseweg, wobei das eine oder die mehreren Enzyme Squalenepoxidase umfassen, wobei die Squalenepoxidase eine reduzierte Aktivität und/oder Expression aufweist,
wobei die Enzym-Aktivität oder -Expression durch eine oder mehrere konzipierte Mutationen erhöht oder herabgesetzt ist,
wobei die eine oder mehreren konzipierten Mutationen sich an definierten Positionen innerhalb des Enzyms befinden,
wobei die genetisch konvertierte Hefe im Vergleich zu der nativen Hefe erhöhte Mengen an Squalen produziert und

wobei die Hefe ein *Yarrowia lipolytica*-Stamm ausgewählt aus der Gruppe bestehend aus ATCC 90812, ATCC MYA-2613, oder Yeastern po1g ist.

3. Zusammensetzungen oder Verfahren gemäß Anspruch 1 oder 2, wobei die genetisch konvertierte Hefe von einer öligen Hefe abgeleitet ist.

4. Zusammensetzungen oder Verfahren gemäß einem der vorhergehenden Ansprüche, ferner umfassend ein modifiziertes Enzym ausgewählt aus der Gruppe bestehend aus Acetyl-CoA-Carboxylase ("ACCase"), HMG-CoA-Reduktase, Squalen-Synthase, ATP-Citrat-Lyase, ATP-Citrat-Synthase, Mevalonat-Kinase, Glycerol-Kinase und 5-Aminolevulinat-Synthase.

5. Zusammensetzungen oder Verfahren gemäß einem der vorhergehenden Ansprüche, wobei die Aktivität oder Expression auf 90%; oder 80%, oder 70%; oder 60%; oder 50%; oder 40%; oder 30%; oder 20%; oder 10%; oder 5% der Aktivität oder Expression der korrespondierenden nativen Hefe herabgesetzt ist.

6. Zusammensetzungen oder Verfahren gemäß einem der vorhergehenden Ansprüche, wobei die Aktivität oder Expression zwischen 90-95%; oder 80-90%; oder 70-80%; 60-70%; oder 50-60%; oder 40-50%; oder 30-40%; oder 20-30%; 10-20%; oder 5-10%; oder 2-5% der Aktivität oder Expression der korrespondierenden nativen Hefe liegt.

7. Zusammensetzungen oder Verfahren gemäß einem der vorhergehenden Ansprüche, wobei die Hefe der Yeastern po1g-Stamm der *Yarrowia lipolytica* ist.

8. Zusammensetzungen oder Verfahren gemäß einem der vorhergehenden Ansprüche, wobei ein Antimykotikum in der Zusammensetzung vorhanden ist oder zu der Hefe in dem Verfahren hinzugefügt wird; oder wobei ein Antimykotikum in einer Konzentration zwischen 0,5 bis 100 μg/ml, zwischen 1 bis 25 μg/ml, oder zwischen 10 bis 15 μg/ml in der Zusammensetzung vorhanden ist oder zu der Hefe in dem Verfahren hinzugefügt wird.

9. Zusammensetzungen oder Verfahren gemäß einem der vorhergehenden Ansprüche, wobei ein Antimykotikum in der Zusammensetzung vorhanden ist oder zu der Hefe in dem Verfahren hinzugefügt wird; und wobei das Antimykotikum ein Allylamin-Antimykotikum ist; oder wobei ein Antimykotikum in der Zusammensetzung vorhanden ist oder zu der Hefe in dem Verfahren hinzugefügt wird; und wobei das Antimykotikum ein Allylamin-Antimykotikum in einer Konzentration zwischen 0,5 bis 100 μg/ml, zwischen 1 bis 25 μg/ml, oder zwischen 10 bis 15 μg/ml ist.

10. Zusammensetzungen oder Verfahren gemäß einem der vorhergehenden Ansprüche, wobei ein Antimykotikum in der Zusammensetzung vorhanden ist oder zu der Hefe in dem Verfahren hinzugefügt wird; und wobei das Antimykotikum Terbinafin ist; oder Terbinafin in einer Konzentration zwischen 0,5 bis 100 μg/ml, zwischen 1 bis 25 μg/ml, oder zwischen 10 bis 15 μg/ml ist.

11. Verfahren gemäß einem der vorhergehenden Ansprüche, das Verfahren umfassend
Kultivieren der Hefe mit einem Antimykotikum; wobei die Hefe der Yeastern po1g-Stamm der *Yarrowia lipolytica* ist und wobei das Antimykotikum Terbinafin ist.

12. Verfahren gemäß Anspruch 11, wobei das Antimykotikum Terbinafin in einer Konzentration von 12,5 μg/ml oder mehr ist.

Revendications

1. Composition comprenant une levure transformée génétiquement, dans laquelle ladite levure transformée génétiquement exprime une ou plusieurs enzymes modifiées ayant une ou plusieurs mutations créées, lesdites une ou plusieurs enzymes modifiées comprenant la squalène époxidase, la squalène époxidase ayant une activité et/ou une expression diminuée,
dans laquelle lesdites une ou plusieurs mutations créées sont en des positions définies au sein de ladite enzyme, dans laquelle ladite levure produit des quantités accrues de squalène comparé à la levure native, et
dans laquelle la levure est une souche de *Yarrowia lipolytica* sélectionnée dans le groupe consistant en ATCC 90812, ATCC MYA-2613 ou Yeastern polg.

2. Procédé de production de squalène par une levure transformée génétiquement, ledit procédé comprenant

l'augmentation ou la diminution de l'activité ou de l'expression d'une ou plusieurs enzymes dans la voie de biosynthèse des isoprénoïdes, lesdites une ou plusieurs enzymes comprenant la squalène époxidase, la squalène époxidase ayant une activité et/ou une expression diminuée,
dans lequel ladite activité ou expression enzymatique est augmentée ou diminuée par une ou plusieurs mutations créées,
dans lequel lesdites une ou plusieurs mutations créées sont en des positions définies au sein de ladite enzyme,
dans lequel ladite levure transformée génétiquement produit des quantités accrues de squalène comparé à la levure native et
dans lequel la levure est une souche de *Yarrowia lipolytica* sélectionnée dans le groupe consistant en ATCC 90812, ATCC MYA-2613 ou Yeastern polg.

3. Compositions ou procédés selon la revendication 1 ou 2, dans lesquels ladite levure transformée génétiquement est issue d'une levure oléagineuse.

4. Compositions ou procédés selon l'une quelconque des revendications précédentes, comprenant en outre une enzyme modifiée sélectionnée dans le groupe consistant en l'acétyl-CoA carboxylase ("ACCase"), la HMG-CoA réductase, la squalène synthase, l'ATP citrate lyase, l'ATP citrate synthase, la mévalonate kinase, la glycérol kinase et la 5-aminolévulinate synthase.

5. Compositions ou procédés selon l'une quelconque des revendications précédentes, dans lesquels ladite activité ou expression est diminuée à 90 % ; ou 80 % ; ou 70 % ; ou 60 % ; ou 50 % ; ou 40 % ; ou 30 % ; ou 20 % ; ou 10 % ; ou 5 % de l'activité ou de l'expression de la levure native correspondante.

6. Compositions ou procédés selon l'une quelconque des revendications précédentes, dans lesquels ladite activité ou expression se situe entre 90 et 95 % ; ou 80 et 90 % ; ou 70 et 80 % ; 60 et 70 % ; ou 50 et 60 % ; ou 40 et 50 % ; ou 30 et 40 % ; ou 20 et 30 % ; 10 et 20 % ; ou 5 et 10 % ; ou 2 et 5 % de l'activité ou de l'expression de la levure native correspondante.

7. Compositions ou procédés selon l'une quelconque des revendications précédentes, dans lesquels la levure est la souche Yeastern polg de *Yarrowia lipolytica.*

8. Compositions ou procédés selon l'une quelconque des revendications précédentes, dans lesquels un agent antifongique est présent dans la composition ou est ajouté à la levure dans le procédé ; ou dans lesquels un agent antifongique est présent dans la composition ou est ajouté à la levure dans le procédé à une concentration entre 0,5 et 100 μg/ml, entre 1 et 25 μg/ml ou entre 10 et 15 μg/ml.

9. Compositions ou procédés selon l'une quelconque des revendications précédentes, dans lesquels un agent antifongique est présent dans la composition ou est ajouté à la levure dans le procédé ; et dans lesquels l'agent antifongique est un agent antifongique allylamine ; ou dans lesquels un agent antifongique est présent dans la composition ou est ajouté à la levure dans le procédé ; et dans lesquels l'agent antifongique est un agent antifongique allylamine à une concentration entre 0,5 et 100 μg/ml, entre 1 et 25 μg/ml ou entre 10 et 15 μg/ml.

10. Compositions ou procédés selon l'une quelconque des revendications précédentes, dans lesquels un agent antifongique est présent dans la composition ou est ajouté à la levure dans le procédé ; et dans lesquels l'agent antifongique est la terbinafine, ou est la terbinafine à une concentration entre 0,5 et 100 μg/ml, entre 1 et 25 μg/ml ou entre 10 et 15 μg/ml.

11. Procédé selon l'une quelconque des revendications précédentes, ledit procédé comprenant la culture de ladite levure avec un agent antifongique ; dans lequel la levure est la souche Yeastern polg de *Yarrowia lipolytica* et dans lequel l'agent antifongique est la terbinafine.

12. Procédé selon la revendication 11, dans lequel l'agent antifongique est la terbinafine à une concentration de 12,5 μg/ml ou plus.

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Eljárások és készítmények szkvalén előállítására élesztő alkalmazásával

SZABADALMI IGÉNYPONTOK

1. Készítmény, amely genetikailag átalakított élesztőt tartalmaz, ahol a szóban forgó genetikailag átalakított élesztő egy vagy több módosított enzimet expresszál, amelyek egy vagy több tervezett mutációval rendelkeznek, a szóban forgó egy vagy több módosított enzim a szkvalén epoxidáz, ahol a szóban forgó szkvalén epoxidáznak csökkent az aktivitása és/vagy az expressziója,

ahol a szóban forgó egy vagy több tervezett mutáció a szóban forgó enzimben előre meghatározott pozícióban van,

ahol a szóban forgó élesztő a természetes élesztőhöz viszonyítva fokozott mennyiségű szkvalént termel, és

ahol a szóban forgó élesztő a *Yarrowia lipolytica* törzs, amelyet az ATCC 90812, ATCC MYA-
2613, vagy Yeastern polg csoportból választhatunk ki.

2. Eljárás szkvalén előállítására genetikailag átalakított élesztővel, azzal jellemezve, hogy a következő lépéseket tartalmazza:

megnöveljük vagy csökkentjük egy vagy több, az izoprenoid bioszintézis útban levő enzim aktivitását vagy expresszióját, a szóban forgó egy vagy több enzim tartalmazza a szkvalén epoxidázt, ahol a szóban forgó szkvalén epoxidáznak csökkent az aktivitása és/vagy az expressziója,

ahol a szóban forgó enzimaktivitást vagy expressziót egy vagy több tervezett mutáció növeli vagy csökkenti,

ahol az egy vagy több tervezett mutáció a szóban forgó enzim meghatározott pontjaiban van,

ahol a szóban forgó élesztő a natív élesztőhöz viszonyítva fokozott mennyiségű szkvalént termel, és

ahol a szóban forgó élesztő a *Yarrowia lipolytica* törzs, amelyet az ATCC 90812, ATCC MYA-
2613, vagy Yeastern polg csoportból választhatunk ki.

3. Az 1. vagy 2. igénypont szerinti készítmények vagy eljárások, ahol a szóban forgó genetikailag átalakított élesztő egy olajos élesztőből származik.

4. Az előző igénypontok bármelyike szerinti készítmények vagy eljárások, amelyek tartalmaznak még egy módosított enzimet, amelyet a következő csoportból választhatunk ki: acetil-CoA

karboxiláz ("ACCase"), HMG-CoA-reduktáz, szkvalén-szintáz, ATP-citrát-liáz, ATP-citrát-szintáz, mevalonát-kináz, glicerin-kináz és 5-aminolevulinát-szintáz.

5. Az előző igénypontok bármelyike szerinti készítmények vagy eljárások, ahol a szóban forgó aktivitás vagy expresszió a megfelelő természetes élesztő aktivitásának vagy expressziójának 90%-a; vagy 80%-a; vagy 70%-a; vagy 60%-a; vagy 50%-a; vagy 40%-a; vagy 30%-a; vagy 20%-a; vagy 10%-a; vagy 5%-a.

6. Az előző igénypontok bármelyike szerinti készítmények vagy eljárások, ahol a szóban forgó aktivitás vagy expresszió a megfelelő természetes élesztő aktivitásának vagy expressziójának 90-95%-a, vagy 80-90%-a, vagy 70-80%-a; 60-70%; vagy 50-60%-a; vagy 40-50%-a; vagy 30-40%-a; vagy 20-30%-a; 10-20%; vagy 5-10%-a, vagy 2-5%-a.

7. Az előző igénypontok bármelyike szerinti készítmények vagy eljárások, ahol az élesztő a *Yarrowia lipolytica* Yeastern polg törzse.

8. Az előző igénypontok bármelyike szerinti készítmények vagy eljárások, ahol egy gombaellenes szer van a készítményben, vagy az eljárásban azt adjuk az élesztőhöz; vagy ahol egy gombaellenes szer van jelen a készítményben, vagy az eljárásban ezt adjuk az élesztőhöz 0,5-100 μg /ml, 1-25 μg/ml, vagy 10-15 μg/ml közötti koncentrációban.

9. Az előző igénypontok bármelyike szerinti készítmények vagy eljárások, ahol egy gombaellenes szer van a készítményben, vagy az eljárásban azt adjuk az élesztőhöz; és ahol a gombaellenes szer egy allilamin gombaellenes szer; vagy ahol egy gombaellenes szer van a készítményben, vagy az eljárásban azt adjuk az élesztőhöz; és ahol a gombaellenes szer egy allilamin gombaellenes szer 0,5-100 μg/ml, 1-25 μg/ml, vagy 10-15 μg/ml közötti koncentrációban.

10. Az előző igénypontok bármelyike szerinti készítmények vagy eljárások, ahol egy gombaellenes szer van a készítményben, vagy az eljárásban azt adjuk az élesztőhöz; és ahol a gombaellenes szer terbinafin, vagy terbinafin 0,5-100 μg/ml, 1-25 μg/ml, vagy 10-15 μg/ml közötti koncentrációban.

11. Az előző igénypontok bármelyike szerinti eljárás, azzal jellemezve, hogy az eljárás a következő lépéseket tartalmazza:

a szóban forgó élesztőt egy gombaellenes szerrel tenyésztjük; ahol az élesztő a *Yarrowia lipolytica* Yeastern polg törzse, és ahol a gombaellenes szer terbinafin.

12. A 11. igénypont szerinti eljárás, azzal jellemezve, hogy a gombaellenes szer terbinafin 12,5 μg/ml vagy nagyobb koncentrációban.