

PRODUCTION OF FATTY ALCOHOLS IN RHODOSPORIDIUM

SEQUENCE LISTING

[0001] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is entitled 2577258PCTSequenceListing.txt, created on 24 January 2018 and is 112 kb in size. The information in the electronic format of the Sequence Listing is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] The present invention relates to the field of fungal production fatty alcohols. More specifically, the present invention relates to the production of fatty alcohols in *Rhodospiridium*.

[0003] The publications and other materials used herein to illuminate the background of the invention or provide additional details respecting the practice, are incorporated by reference, and for convenience are respectively grouped in the Bibliography.

[0004] The term “lipid” is scientifically used to define fatty acids and their naturally-occurring derivatives (esters or amides) (Ratledge and Wilkinson, 1988a) as compared to its solubility property-related definition in general textbooks (Gunstone et al., 2012). Although the dominant oil and fat supplier in the world is derived from agricultural products, animal and marine sources, an increasing space is required for the potential production of fuels and chemical materials from microorganisms due to the depletion of oil resources and global warming. Furthermore, value-added single cell oil (SCO) produced from sustainable feedstock led to the comparably economic consideration of microbial factory with cheap plant products.

[0005] Lipid-producing microorganisms that can accumulate more than 20% or more of their biomass as lipids are termed as oleaginous, and there are very limited number of oleaginous microorganisms, e.g. only 25 oleaginous yeasts among 600 different yeast species (Ratledge and Wilkinson, 1988b). Triacylglycerides (TAGs) and steryl esters (SEs) are the major neutral lipids that represent the most important storage form of energy for eukaryotic cells (Beopoulos et al., 2012; Coleman and Lee, 2004; Czabany et al., 2007; Lung and Weselake, 2006; Sorger and Daum, 2003). Acyl-CoA:diacylglycerol acyltransferase (DGAT, EC 2.3.1.20), a transmembrane enzyme, acts in the final and committed step of TAG biosynthesis (Fig. 1), which has been proposed to be the rate-limiting enzyme in lipid accumulation (Yen et al., 2008). TAG can also be formed by an acyl-CoA-independent pathway, in which the phospholipid:diacylglycerol acyltransferase (PDAT, EC 2.3.1.158) is responsible for TAG biosynthesis using phosphatidylcholine as the acyl donor (Dahlqvist et al., 2000; Yoon et al., 2012). Recently,

another two groups of DGAT, a bifunctional DGAT/wax ester synthase (ADP1) and a soluble form of DGAT, have been found in *Acinetobacter calcoaceticus* and peanut cotyledons for biosynthesis of wax (Kalscheuer and Steinbuchel, 2003) and 3-acetyl-1,2-diacyl-sn-glycerols (acTAG, an unusual TAG) (Chi et al., 2014), respectively. DGAT1 and DGAT2 are two of the enzymes DGATs responsible for the main part of TAG biosynthesis. Interestingly, DGAT2 family members don not show DNA or protein sequence similarities with DGAT1, and a comparative genomics study suggests that the DGAT1 and DGAT2 gene families are separated through evolution (Turchetto-Zolet et al., 2011).

[0006] *Rhodospiridium toruloides* (alias *Rhodotorula glutinis* or *Rhodotorula gracilis*), a member of *Pucciniomycotina* subphylum in the *Basidiomycota*, has been considered as an oleaginous and carotenogenic yeast (Ratledge and Wynn, 2002; Sampaio et al., 2003). *R. toruloides* is able to be cultured to extremely high cell density (>100 g/l dry cell mass) and accumulate more than 60% biomass as triglycerides, making it a good host for the production of oil for biodiesel and many other applications from biomass (Liu et al., 2009; Turcotte and Kosaric, 1988; Zhao et al., 2010). Moreover, *R. toruloides* can accumulate high amount of carotenoids. Recently, the carotenoid biosynthesis pathway and enzymes has been clarified in our lab (our submitted manuscript), which made it possible for either metabolic engineering to improve the yields of certain carotenoid compositions or application of the pigmentation as an easily traceable genetic marker as other carotenogenic fungi (Youssar and Avalos, 2007).

[0007] As widely used in versatile industrial products, oleochemicals are a class of lipid-derived aliphatic molecules (Biermann et al., 2011), which was dominantly produced from inexpensive lipid sources such as plant oils and animal fats recently (Pfleger et al., 2015). One alternative route for the bioconversion of the cheap oleochemical feedstocks to oleochemicals is to develop microbial biocatalysts through metabolic engineering (Keasling, 2010). Fatty alcohol, an important raw materials and consumer product with 2-3 times more expensive than free fatty acids and biodiesel (Pfleger et al., 2015), can be widely applied in detergent, lubricant, plastics and cosmetics industrials (Biermann et al., 2011; Noweck and Grafahrend, 2000). Similar as other oleochemicals, fatty alcohols are traditionally produced by chemical hydration method (Carlsson et al., 2011). Recently, the rising environmental concerns of chemical production method resulted in the enzymatic production of fatty alcohols in genetically engineering microbial hosts, where fatty acid acyl-CoA reductases (FARs) catalyze the reduction of different acyl-CoA molecules to the corresponding medium/long-chain alcohols (C8-C18). Two classes of FARs, alcohol- and aldehyde-forming, have been identified in many organisms. Recently,

microbial production of fatty alcohols through metabolic engineering has been successfully achieved in *Escherichia coli* (Haushalter et al., 2015; Liu et al., 2013a; Youngquist et al., 2013; Zheng et al., 2012) and *Saccharomyces cerevisiae* (Tang and Chen, 2015). Very recently, oleaginous yeasts *R. toruloides* was successfully developed for the production of fatty alcohols by metabolic engineering, where the highest titer ever reported to date (8 g/L) was achieved by fed-batch fermentation (Fillet et al., 2015).

[0008] It is desired to develop oleaginous yeast with increased production of fatty alcohols.

SUMMARY OF THE INVENTION

[0009] The present invention relates to the field of fungal production of fatty alcohols. More specifically, the present invention relates to the production of fatty alcohols in *Rhodospiridium*.

[0010] Thus, in one aspect, the present invention provides a genetically modified host cell having down-regulation of four host cell triacylglycerol (TAG) synthases. In some embodiments, the host cell TAG synthases are type 1 acyl-CoA:diacylglycerol acyltransferase (Dga1), type 2 phospholipid:diacylglycerol acyltransferase (Lro1), acyl-CoA:sterol acyltransferase (sterol ester synthase, Are1), and type 3 soluble acyltransferase (Dga3). In some embodiments, the genetically modified host cell further has an overexpression of a heterologous fatty acyl-CoA reductase (FAR1; sometimes also referred to herein as FAR). In some embodiments, the coding sequence for FAR1 is codon modified for expression in the host cell. In some embodiments, the genetically modified host cell contains multiple copies of *FAR1*. In some embodiments, each copy of *FAR1* is under control of the same or different promoters. In some embodiments, the genetically modified host cell further has down regulation of a host cell acyl-CoA oxidase 1 (Pox1).

[0011] In some embodiments, the genetically modified host cell comprises nucleic acid constructs, each comprising a nucleic acid sequence for down-regulating each of the TAG synthases described herein. In some embodiments, the genetically modified host cell comprises knocked-out host cell TAG synthases described herein. In some embodiments, the genetically modified host cell comprises a nucleic acid construct comprising a nucleic acid sequence for down-regulating a host cell Pox1. In some embodiments, the genetically modified host cell comprises a knocked-out host cell *Pox1*. In some embodiments, the genetically modified host cell comprises a nucleic acid construct comprising a promoter operatively linked to a heterologous nucleic acid sequence encoding FAR1. In some embodiments, the nucleic acid construct comprises multiple copies of *FAR1* operatively linked to the same or different

promoters. In some embodiments, the nucleic acid construct comprises three copies of *FARI* operatively linked to different promoters.

[0012] In some embodiments, the host cell is a cell of a *Rhodospiridium* species or a *Rhodotorula* species. In some embodiments, the host cell is a cell of a strain of *Rhodospiridium toruloides*. In some embodiments, the host cell is *R. toruloides* strain ATCC 10657.

[0013] In a second aspect, the present invention provides a method for producing fatty alcohols. In some embodiments, the method comprises growing the genetically modified host cells described herein in or on a suitable medium for growth of the genetically modified host cell and for production of fatty alcohols. In some embodiments, the genetically modified host cells are cultured in a culture medium described herein. In some embodiments, the genetically modified host cells are grown in a conical flask containing a culture medium described herein. In some embodiments the genetically modified host cells are cultured in the conical flasks at a temperature as described herein. In some embodiments, the conical flasks are shaken at a rate as described herein. In some embodiments, the genetically modified host cells are grown in a bioreactor containing a culture medium described herein.

BRIEF DESCRIPTION OF THE FIGURES

[0014] Fig. 1 shows a scheme of triacylglycerol biosynthesis pathway in fungi. Abbreviations: LPA, lysophosphatidic acid; PA, phosphatidic acid; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; G3P, sn-glycerol-3-phosphate; MAG, sn-1-monoacylglycerol; DAG, sn-1,2-diacylglycerol; TAG, triacylglycerol; FA, fatty acid, LPAT, acyl-CoA:lysophosphatidic acid acyltransferase; PAP, phosphatidate phosphatase; DGAT, acyl-CoA:diacylglycerol acyltransferase; MGAT, acyl-CoA:monoacylglycerol acyltransferase; DGTA, diacylglycerol transacylase; PDAT, phospholipid:diacylglycerol acyltransferase.

[0015] Figs. 2A and 2B show DGAT encoding genes and deletions in *R. toruloides*. Fig. 2A: Schematic diagram of DGAT encoding genes. Fig. 2B: Southern blot analysis of DGAT gene deletion mutants.

[0016] Figs. 3A and 3B show a bioinformatic characterization of DGATs in *R. toruloides*. Fig. 3A: Phylogenetic tree of DGATs from various species. Abbreviation of species: Ac, *Acinetobacter calcoaceticus*; Ah, *Arachis hypogaea* (peanut); At, *Arabidopsis thaliana*; Hs, *Homo sapiens*; Pv, *Phaseolus vulgaris*; Rc, *Ricinus communis* (castor bean); Rt, *Rhodospiridium toruloides*; Sc, *Saccharomyces cerevisiae*; Yl, *Yarrowia lipolytica*; Vf, *Vernicia fordii* (Tung tree). Fig. 3B: Predicted transmembrane domains of Dga1, Lro1, Are1 and

Dga3 from *R. toruloides*. The TMHMM web tools of the Center for Biological Sequence Analysis, Technical University of Denmark TMHMM Server plots the probability of the *R. toruloides* DGATs forming a transmembrane helix (0-1.0 on the y-axis).

[0017] Fig. 4 shows the fold change in expression level of genes involved in lipid biosynthesis pathway between growth and lipid-inducing medium at different time points. *R. toruloides* Cells were cultured in YPD till exponential phase (OD~10, time-point 0 h) and cells were pelleted, water-washed and resuspended to YNBD (YNB w/o aa or ammonium sulfate plus 50 g/L glucose) for 24 h. Total RNAs at 0, 12 and 24 h were extracted, treated with DNaseI and cDNAs were synthesized as the template.

[0018] Figs. 5A-5C show lipid profiles of DGAT mutants. Fig. 5A: Cell dry biomass of *R. toruloides* wild type (WT) and DGAT single and multiple gene deletion mutants. Fig. 5B: Relative lipid yields in *R. toruloides* WT and DGAT mutant strains. Lipid yield in WT strain was normalized to 100%. Fig. 5C: Fatty acid profiles in *R. toruloides* WT and DGAT mutant strains. (D) Lipid profiling of DGAT mutants through thin-layer chromatography. Cells were cultured in GJ2013 medium for 5 days with rotary shaking. The loading amount of lipids for TLC separation is 100 µg. All tests were performed by biological triplicates. Abbreviation of DGAT mutants: *dl* – $\Delta dga1\Delta lro1$; *da* – $\Delta dga1\Delta are1$; *dla* – $\Delta dga1\Delta lro1\Delta are1$; *dlad* – $\Delta dga1\Delta lro1\Delta are1\Delta dga3$. Abreviation of lipid components: SE – sterol ester; TAG – triacylglycerol; FFA – free fatty acids; DAG – diacylglycerol; MAG: monoacylglycerol; PL – polar lipids.

[0019] Fig. 6 shows lipid profiling of DGAT mutants through thin-layer chromatography. Cells were cultured in GJ2013 medium for 5 days with rotary shaking. The loading amount of lipids for TLC separation is 100 µg. All tests were performed by biological triplicates. Abbreviation of DGAT mutants is as in Figs. 5A-5C. Systematic names of fatty acids: C14:0 - Myristic acid; C16:0 - Palmitic acid; C16:1 - Palmitoleic acid; C18:0 - Stearic acid; C18:1 - Oleic acid; C18:2 - Linoleic acid; C18:3 - α -Linolenic acid; C20:0 - Arachidic acid; C22:0 - Behenic acid; C24:0 - Lignoceric acid.

[0020] Fig. 7 shows the formation of lipid bodies in different DGAT mutants. All cells cultured in MinRL3 medium for 5 days was used for the microscopic observation. The exposure time for DIC and BODIPY staining fluorescent microscopy was 1/15 s and 1/4 s, respectively, except the observation of some strains under the exposure time of 1 s as shown in the figures. Bar represents 10 µm. Abbreviations: DIC - differential interference contrast microscopy; BODIPY – fluorescent microscopy with excitation and emission wavelength of 488 and 509 nm,

respectively (eGFP channel); TEM – transmission electron microscopy. In TEM figures, L – lipid body; M – mitochondria; P – peroxisome.

[0021] Figs. 8A-8D show the overexpression of *DGA1* and *LRO1* in the null mutants. Fig. 8A: Effects of overexpression of *DGA1* in $\Delta dga1$ ($\Delta dga1C$) and quadruple mutant *dlad* (*dlad-DGA1*) on lipid production. Fig. 8B: Effects of overexpression of *LRO1* in quadruple mutant *dlad* (*dlad-LRO1*) on lipid production. Fig. 8C: Effects of overexpression of *DGA1* and *LRO1* in the quadruple mutant *dlad* on the formation of lipid bodies. Fig. 8D: Effects of Dga1 and Lro1 on the formation of lipid body. All cells cultured in MinRL3 medium for 5 days was used for the lipid extraction, TLC separation and microscopic observation. The loading amount of lipids for TLC separation is 100 μ g. The exposure time for DIC and BODIPY staining fluorescent microscopy was 1/15 and 1/4 second, respectively, except the extension exposure time of 1 second on *dlad* series mutants as shown in the figure. Bar represents 10 μ m. Abbreviations of DGAT mutants: *dlad* – $\Delta dga1\Delta lro1\Delta are1\Delta dga3$; *dlad-DGA1* – $\Delta dga1\Delta lro1\Delta are1\Delta dga3-P_{GPD1}::DGA1::T35S^{CAR2}$; *dlad-LRO1* – $\Delta dga1\Delta lro1\Delta are1\Delta dga3-P_{GPD1}::LRO1::T35S^{CAR2}$; *Adga1C* – $\Delta dga1-P_{GPD1}::DGA1::T35S^{CAR2}$; *ALRO1C* – $\Delta lro1-P_{GPD1}::LRO1::T35S^{CAR2}$. Abbreviations of lipid components: SE – sterol ester; TAG – triacylglycerol; FFA – free fatty acids; DAG – diacylglycerol; MAG: monoacylglycerol; PL – polar lipids. Abbreviations of Microscopy mode: DIC - differential interference contrast microscopy; BODIPY – fluorescent microscopy with excitation and emission wavelength of 488 and 509 nm, respectively (eGFP channel).

[0022] Figs. 9A-9C show that Dga3 is a bifunctional enzyme, acyltransferase and Δ^1 -pyrroline-5-carboxylate dehydrogenase. Fig. 9A: Effects of Dga3 on the formation of lipid body. DGA3 was constitutively expressed under the strong promoter *GPD1* and reintroduced back into the quadruple mutant *dlad*. The exposure time for DIC and BODIPY staining fluorescent microscopy was 1/15 and 1/4 second, respectively, except the extension exposure time of 1 second on *dlad* series mutants as shown in the figure. Bar represents 10 μ m. Abbreviations of DGAT mutants: *dlad* – $\Delta dga1\Delta lro1\Delta are1\Delta dga3$; *dlad-DGA3* – $\Delta dga1\Delta lro1\Delta are1\Delta dga3-P_{GPD1}::DGA3::T35S^{CAR2}$. (B) Drop assay of cell growth by serial dilution. A minimum medium (YNB) was used by supplementation of different carbon and nitrogen sources. Fig. 9B: *In vitro* enzyme assay of Δ^1 -pyrroline-5-carboxylate dehydrogenase. Fig. 9C: Proline metabolism pathway.

[0023] Figs. 10A-10C show metabolic engineering of *R. toruloides* to produce fatty alcohols. Fig. 10A: Triple gene overexpression cassette (FAR1 x3) in plasmid pKCGPTFAR1. Fig. 10B:

GC-MS separation and qualitative analysis of fatty alcohols and wax esters. Fig. 10C: Microscopic observation of lipid body formation through BODIPY staining. The best fatty alcohol producer *dladpFAR1* was cultured in different media for 5 days, stained with BODIPY and observed under microscopy.

[0024] Figs. 11A and 11B show metabolic engineering of fatty alcohol production in *R. toruloides*. Fig. 11A: Schematic diagram of metabolic engineering route for the production of fatty alcohol in *R. toruloides*. Fig. 11B: Fatty alcohol titers in different engineering mutants. All cells were cultured in GJm3 medium by shaking flask fermentation at 28°C, 280 rpm for 5 days. Abbreviations: *RtCE-nls* – Codon-optimized gene encoding a fusion protein of bacteriophage P1 Cre recombinase (*C*), human estrogen receptor α (*E*) and Simian virus 40 large antigen nuclear localization signal sequence (*nls*), this gene was regulated under the binding of estrogen and used for the excision of selection marker cassette by Cre/loxP recombination technique. $\Delta ku70$ – Deletion of KU70 gene to generate an elevated homologous recombination strain by defects in non-homologous end joining pathway. $\Delta fad2$ – Deletion of $\Delta 12,15$ bifunctional fatty acid desaturase gene. $\Delta dga1\Delta lro1\Delta are1\Delta dga3$ – Quadruple DGAT gene deletion mutant (lipid-less). $\Delta pox1$ – Deletion of peroxisomal acyl-CoA oxidase 1 gene to block the degradation of fatty acids through β -oxidation pathway.

[0025] Figs. 12A and 12B show an optimization of fatty alcohol production. Fig. 12A: TLC separation of extracted fatty alcohols from inside and outside of the cells (Intra- and Extra-, respectively). Lipid std is a mixture of TAG (10 mg/ml), 1,3-DAG, 1,2-DAG, 1-MAG and free fatty acids (each 1 mg/ml) as indicated in Fig. 8A. Fal/WE std is a mixture of palmityl palmitate (carbon 32 wax ester) and hexadecanol (carbon 17 fatty alcohol), each of 5 mg/ml. The amount of sample loading is 5 μ l each and developing system is hexane:ethyl ether:acetic acid = 80:20:1. Fig. 12B: Quantitation and profiling of extracted fatty alcohols after cultured in media with different carbon and nitrogen sources. SY – sucrose/yeast extract, DY – glucose/yeast extract, SC – sucrose/corn steep liquid, DY – glucose/corn steep liquid. C18:0-OH, C18:1-OH and C16:0-OH represents stearyl alcohol, oleyl alcohol and palmityl alcohol, respectively.

DETAILED DESCRIPTION

[0026] The present invention relates to the field of fungal production of α -eleosteric acid. More specifically, the present invention relates to the production of α -eleosteric acid in *Rhodospiridium*.

[0027] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention belongs.

[0028] The term “about” or “approximately” means within a statistically meaningful range of a value. Such a range can be within an order of magnitude, preferably within 50%, more preferably within 20%, more preferably still within 10%, and even more preferably within 5% of a given value or range. The allowable variation encompassed by the term “about” or “approximately” depends on the particular system under study, and can be readily appreciated by one of ordinary skill in the art.

[0029] A "control" or "control fungus" or "control fungal cell" provides a reference point for measuring changes in phenotype of a subject fungus or fungal cell in which genetic alteration, such as transformation, has been effected as to a polynucleotide of interest. A subject fungus or fungal cell may be descended from a fungus or fungal cell so altered and will comprise the alteration.

[0030] A control fungus or fungal cell may comprise, for example: (a) a wild-type fungus or fungal cell, i.e., of the same genotype as the starting material for the genetic alteration which resulted in the subject fungus or fungal cell; (b) a fungus or fungal cell of the same genotype as the starting material but which has been transformed with a null construct (i.e., with a construct which has no known effect on the trait of interest, such as a construct comprising a marker gene); (c) a fungus or fungal cell genetically identical to the subject fungus or fungal cell but which is not exposed to conditions or stimuli that would induce expression of the polynucleotide of interest or (d) the subject fungus or fungal cell itself, under conditions in which the polynucleotide of interest is not expressed.

[0031] “Constitutive promoter” refers to a promoter which is capable of causing a gene to be expressed in most cell types at most. A “strong constitutive promoter” refers to a constitutive promoter that drives the expression of a mRNA to the top 10% of any mRNA species in any given cell.

[0032] A "dsRNA" or “RNAi molecule,” as used herein in the context of RNAi, refers to a compound, which is capable of down-regulating or reducing the expression of a gene or the activity of the product of such gene to an extent sufficient to achieve a desired biological or physiological effect. The term "dsRNA" or “RNAi molecule,” as used herein, refers to one or more of a dsRNA, siRNA, shRNA, ihpRNA, synthetic shRNA, miRNA.

[0033] The term "down regulated," as it refers to genes inhibited by the subject RNAi method, refers to a diminishment in the level of expression of a gene(s) in the presence of one or

more RNAi construct(s) when compared to the level in the absence of such RNAi construct(s). The term "down regulated" is used herein to indicate that the target gene expression is lowered by 1-100%. For example, the expression may be reduced by about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99%.

[0034] The term "expression" with respect to a gene sequence refers to transcription of the gene and, as appropriate, translation of the resulting mRNA transcript to a protein. Thus, as will be clear from the context, expression of a protein coding sequence results from transcription and translation of the coding sequence.

[0035] As used herein, "gene" refers to a nucleic acid sequence that encompasses a 5' promoter region associated with the expression of the gene product, any intron and exon regions and 3' or 5' untranslated regions associated with the expression of the gene product.

[0036] As used herein, "genotype" refers to the genetic constitution of a cell or organism.

[0037] The term "heterologous" or "exogenous" when used with reference to portions of a nucleic acid indicates that the nucleic acid comprises two or more subsequences that are not found in the same relationship to each other in nature. For instance, the nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid, e.g., a promoter from one source and a coding region from another source. Similarly, a heterologous or exogenous protein indicates that the protein comprises two or more subsequences that are not found in the same relationship to each other in nature (e.g., a fusion protein).

[0038] "Inducible promoter" refers to a promoter which is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. The inducer can be a chemical agent such as a protein, metabolite, growth regulator, herbicide or phenolic compound or a physiological stress, such as that imposed directly by heat, cold, salt or toxic elements or indirectly through the action of a pathogen or disease agent such as a virus or other biological or physical agent or environmental condition.

[0039] "Introduced" in the context of inserting a nucleic acid fragment (e.g., a recombinant DNA construct) into a cell, means "transfection" or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid fragment into a eukaryotic or prokaryotic cell where the nucleic acid fragment may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

[0040] “Knock-out” or “knockout” as used herein refers to a gene that is or has been made inoperative. Knock-out or gene knock-out refers to an inhibition or substantial suppression of endogenous gene expression either by a transgenic or a non-transgenic approach. For example, knock-outs can be achieved by a variety of approaches including transposons, retrotransposons, deletions, substitutions, mutagenesis of the endogenous coding sequence and/or a regulatory sequence such that the expression is substantially suppressed; and any other methodology that suppresses the activity of the target of interest.

[0041] “Operable linkage” or “operably linked” or “operatively linked” as used herein is understood as meaning, for example, the sequential arrangement of a promoter and the nucleic acid to be expressed and, if appropriate, further regulatory elements such as, for example, a terminator, in such a way that each of the regulatory elements can fulfill its function in the recombinant expression of the nucleic acid to make dsRNA. This does not necessarily require direct linkage in the chemical sense. Genetic control sequences such as, for example, enhancer sequences, can also exert their function on the target sequence from positions which are somewhat distant, or indeed from other DNA molecules (cis or trans localization). Preferred arrangements are those in which the nucleic acid sequence to be expressed recombinantly is positioned downstream of the sequence which acts as promoter, so that the two sequences are covalently bonded with one another. Regulatory or control sequences may be positioned on the 5’ side of the nucleotide sequence or on the 3’ side of the nucleotide sequence as is well known in the art.

[0042] “Over-expression” or “overexpression” refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal, control or non-transformed organisms.

[0043] As used herein, “phenotype” refers to the detectable characteristics of a cell or organism, which characteristics are the manifestation of gene expression.

[0044] The terms “polynucleotide,” “nucleic acid” and “nucleic acid molecule” are used interchangeably herein to refer to a polymer of nucleotides which may be a natural or synthetic linear and sequential array of nucleotides and/or nucleosides, including deoxyribonucleic acid, ribonucleic acid, and derivatives thereof. It includes chromosomal DNA, self-replicating plasmids, infectious polymers of DNA or RNA and DNA or RNA that performs a primarily structural role. Unless otherwise indicated, nucleic acids or polynucleotide are written left to right in 5’ to 3’ orientation, Nucleotides are referred to by their commonly accepted single-letter codes. Numeric ranges are inclusive of the numbers defining the range.

[0045] The terms “polypeptide,” “peptide,” and “protein” are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. Amino acids may be referred to by their commonly known three-letter or one-letter symbols. Amino acid sequences are written left to right in amino to carboxy orientation, respectively. Numeric ranges are inclusive of the numbers defining the range.

[0046] “Promoter” refers to a nucleic acid fragment capable of controlling transcription of another nucleic acid fragment.

[0047] “Promoter functional in a fungus” is a promoter capable of controlling transcription in fungal cells whether or not its origin is from a fungal cell.

[0048] “Recombinant” refers to an artificial combination of two otherwise separated segments of sequence, e.g., by chemical synthesis or by the manipulation of isolated segments of nucleic acids by genetic engineering techniques. “Recombinant” also includes reference to a cell or vector, that has been modified by the introduction of a heterologous nucleic acid or a cell derived from a cell so modified, but does not encompass the alteration of the cell or vector by naturally occurring events (e.g., spontaneous mutation, natural transformation/transduction/transposition) such as those occurring without deliberate human intervention.

[0049] “Recombinant DNA construct” refers to a combination of nucleic acid fragments that are not normally found together in nature. Accordingly, a recombinant DNA construct may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that normally found in nature. The terms “recombinant DNA construct” and “recombinant construct” are used interchangeably herein. A suppression DNA construct, used herein, is a type of recombinant DNA construct. In several embodiments described herein, a recombinant DNA construct may also be considered an “over expression DNA construct.”

[0050] “Regulatory sequences” refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include, but are not limited to, promoters, translation leader sequences, introns, and polyadenylation recognition sequences. The terms “regulatory sequence” and “regulatory element” are used interchangeably herein.

[0051] “Stable transformation” refers to the introduction of a nucleic acid fragment into a genome of a host organism resulting in genetically stable inheritance. Once stably transformed, the nucleic acid fragment is stably integrated in the genome of the host organism and any subsequent generation.

[0052] “Transformation” as used herein refers to both stable transformation and transient transformation.

[0053] A “transformed cell” is any cell into which a nucleic acid fragment (e.g., a recombinant DNA construct) has been introduced.

[0054] “Transgenic fungus” includes reference to a fungus which comprises within its genome a heterologous polynucleotide. For example, the heterologous polynucleotide is stably integrated within the genome such that the polynucleotide is passed on to successive generations. The heterologous polynucleotide may be integrated into the genome alone or as part of a recombinant DNA construct. “Transgenic fungus” also includes reference to fungi which comprise more than one heterologous polynucleotide within their genome. A “transgenic fungus” encompasses all descendants which continue to harbor the foreign DNA.

[0055] Sequence alignments and percent identity calculations may be determined using a variety of comparison methods designed to detect homologous sequences including, but not limited to, the Megalign® program of the LASERGENE® bioinformatics computing suite (DNASTAR® Inc., Madison, WI). Unless stated otherwise, multiple alignment of the sequences provided herein were performed using the Clustal V method of alignment (Higgins and Sharp (1989) *CABIOS*. 5:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments and calculation of percent identity of protein sequences using the Clustal V method are KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. For nucleic acids these parameters are KTUPLE=2, GAP PENALTY=5, WINDOW=4 and DIAGONALS SAVED=4. After alignment of the sequences, using the Clustal V program, it is possible to obtain “percent identity” and “divergence” values by viewing the “sequence distances” table on the same program; unless stated otherwise, percent identities and divergences provided and claimed herein were calculated in this manner.

[0056] Alternatively, the Clustal W method of alignment may be used. The Clustal W method of alignment (described by Higgins and Sharp, *CABIOS*. 5:151-153 (1989); Higgins, D. G. et al., *Comput. Appl. Biosci.* 8:189-191 (1992)) can be found in the MegAlign™ v6.1 program of the LASERGENE® bioinformatics computing suite (DNASTAR® Inc., Madison,

Wis.). Default parameters for multiple alignment correspond to GAP PENALTY=10, GAP LENGTH PENALTY=0.2, Delay Divergent Sequences=30%, DNA Transition Weight=0.5, Protein Weight Matrix=Gonnet Series, DNA Weight Matrix=IUB. For pairwise alignments the default parameters are Alignment=Slow-Accurate, Gap Penalty=10.0, Gap Length=0.10, Protein Weight Matrix=Gonnet 250 and DNA Weight Matrix=IUB. After alignment of the sequences using the Clustal W program, it is possible to obtain “percent identity” and “divergence” values by viewing the “sequence distances” table in the same program.

[0057] The term “under stringent conditions” means that two sequences hybridize under moderately or highly stringent conditions. More specifically, moderately stringent conditions can be readily determined by those having ordinary skill in the art, e.g., depending on the length of DNA. The basic conditions are set forth by Sambrook et al., *Molecular Cloning: A Laboratory Manual*, third edition, chapters 6 and 7, Cold Spring Harbor Laboratory Press, 2001 and include the use of a prewashing solution for nitrocellulose filters 5xSSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0), hybridization conditions of about 50% formamide, 2xSSC to 6xSSC at about 40-50 °C (or other similar hybridization solutions, such as Stark’s solution, in about 50% formamide at about 42 °C) and washing conditions of, for example, about 40-60 °C, 0.5-6xSSC, 0.1% SDS. Preferably, moderately stringent conditions include hybridization (and washing) at about 50 °C and 6xSSC. Highly stringent conditions can also be readily determined by those skilled in the art, e.g., depending on the length of DNA.

[0058] Generally, such conditions include hybridization and/or washing at higher temperature and/or lower salt concentration (such as hybridization at about 65 °C, 6xSSC to 0.2xSSC, preferably 6xSSC, more preferably 2xSSC, most preferably 0.2xSSC), compared to the moderately stringent conditions. For example, highly stringent conditions may include hybridization as defined above, and washing at approximately 65-68 °C, 0.2xSSC, 0.1% SDS. SSPE (1xSSPE is 0.15 M NaCl, 10 mM NaH₂PO₄, and 1.25 mM EDTA, pH 7.4) can be substituted for SSC (1xSSC is 0.15 M NaCl and 15 mM sodium citrate) in the hybridization and washing buffers; washing is performed for 15 minutes after hybridization is completed.

[0059] It is also possible to use a commercially available hybridization kit which uses no radioactive substance as a probe. Specific examples include hybridization with an ECL direct labeling & detection system (Amersham). Stringent conditions include, for example, hybridization at 42 °C for 4 hours using the hybridization buffer included in the kit, which is supplemented with 5% (w/v) Blocking reagent and 0.5 M NaCl, and washing twice in 0.4% SDS, 0.5xSSC at 55 °C for 20 minutes and once in 2xSSC at room temperature for 5 minutes.

[0060] Thus, in one aspect, the present invention provides a genetically modified host cell having down-regulation of four host cell triacylglycerol (TAG) synthases. In some embodiments, the host cell TAG synthases are type 1 acyl-CoA:diacylglycerol acyltransferase (Dga1), type 2 phospholipid:diacylglycerol acyltransferase (Lro1), acyl-CoA:sterol acyltransferase (sterol ester synthase, Are1), and type 3 soluble acyltransferase (Dga3). In some embodiments, the genetically modified host cell further has an overexpression of a heterologous fatty acyl-CoA reductase (FAR1). In some embodiments, the coding sequence for FAR1 is codon modified for expression in the host cell. In some embodiments, the genetically modified host cell contains multiple copies of *FAR1*. In some embodiments, each copy of *FAR1* is under control of the same or different promoters. In some embodiments, the genetically modified host cell further has down regulation of a host cell acyl-CoA oxidase 1 (Pox1).

[0061] In some embodiments, the genetically modified host cell comprises nucleic acid constructs, each comprising a nucleic acid sequence for down-regulating each of the TAG synthases described herein. In some embodiments, the genetically modified host cell comprises knocked-out host cell TAG synthases described herein. In some embodiments, the genetically modified host cell comprises a nucleic acid construct comprising a nucleic acid sequence for down-regulating a host cell Pox1. In some embodiments, the genetically modified host cell comprises a knocked-out host cell *Pox1*. In some embodiments, the genetically modified host cell comprises a nucleic acid construct comprising a promoter operatively linked to a heterologous nucleic acid sequence encoding FAR1. In some embodiments, the nucleic acid construct comprises multiple copies of *FAR1* operatively linked to the same or different promoters, such as those described herein. In some embodiments, the nucleic acid construct comprises three copies of *FAR1* operatively linked to different promoters, such as those described herein.

[0062] In some embodiments, the invention provides a genetically modified fungal cell useful for producing fatty alcohols that comprises (a) a nucleic acid construct that overexpresses a heterologous fatty acyl-CoA reductase (FAR1); (b) either (i) a nucleic acid construct for down-regulating a host cell type 1 acyl-CoA:diacylglycerol acyltransferase (Dga1) or (ii) a knocked-out host cell *Dga1* gene or a combination of (i) and (ii); (c) either (i) a nucleic acid construct for down-regulating a host cell type 2 phospholipid:diacylglycerol acyltransferase (Lro1) or (ii) a knocked-out host cell *Lro1* gene or a combination of (i) and (ii); (d) either (i) a nucleic acid construct for down-regulating a host cell acyl-CoA:sterol acyltransferase (sterol ester synthase,

Are1) or (ii) a knocked-out host cell *Are1* gene or a combination of (i) and (ii); and (e) either (i) a nucleic acid construct for down-regulating a host cell type 3 soluble acyltransferase (*Dga3*) or (ii) a knocked-out host cell *Dga3* gene or a combination of (i) and (ii). In some embodiments, the genetically modified fungal cell further comprises (f) either (i) a nucleic acid construct for down-regulating a host cell acyl-CoA oxidase 1 (*Pox1*) or (ii) a knocked-out host cell *Pox 1* gene or a combination of (i) and (ii). In some embodiments, the genetic background of the genetically modified host cells is FAD^+ , $FAR1^+$, $\Delta dga1$, $\Delta ro1$, $\Delta are1$, $\Delta dga3$ and optionally $\Delta pox1$.

[0063] In some embodiments, the heterologous *FAR1* coding sequence(s) is (are) operatively linked to a strong constitutive promoter. In some embodiments, suitable strong constitutive promoters are described in WO 2012/169969, incorporated by reference herein in its entirety, which describes several polynucleotide sequences derived from the upstream region of *glyceraldehyde phosphate dehydrogenase* gene (*GPD1*), *translation initiation factor* gene (*TEF1*), and putative *stearoyl-CoA-delta 9-desaturase* gene (*FAD1*) of selected fungal species that are able to function as a strong promoter of gene expression in *Pucciniomycotina* and *Ustilaginomycotina* subphyla, including *Rhodosporidium*. In some embodiments, suitable strong constitutive promoters are described in WO 2014/142747, incorporated by reference herein in its entirety, which describes several polynucleotide sequences that function as strong promoters of gene expression in *Rhodosporidium*, *Rhodotorula*, *Sporobolomyces*, *Pseudozyma* and *Ustilago* genera. In some embodiments, suitable strong constitutive promoters are described in copending U.S. patent application No. 62/292,030 filed on 5 February 2016, incorporated herein by reference in its entirety, which describes intron-containing promoters that are capable of driving strong expression of RNA or proteins in species of the *Rhodosporidium* or *Rhodotorula* genera.

[0064] In some embodiments, the strong constitutive promoter is the *RtPLN1_{in}* promoter. In some embodiments the *RtPLN1_{in}* promoter comprises the sequence set forth in SEQ ID NO:15. In some embodiments, the strong constitutive promoter is the *RtGPD1* promoter. In some embodiments the *RtGPD1* promoter comprises the sequence set forth in SEQ ID NO:16. In some embodiments, the strong constitutive promoter is the *RtTEF1_{in}* promoter. In some embodiments, the *RtTEF1_{in}* promoter comprises the sequence set forth in SEQ ID NO:17. In some embodiments, each copy of the *FAR1* coding sequence is overexpressed by the same strong constitutive promoter, such as those described herein. In some embodiments, each copy of *FAR1* coding sequences is overexpressed by the strong constitutive *RtPLN1_{in}* promoter (SEQ

ID NO:15). In some embodiments, each copy of the FAR1 coding sequence is overexpressed by the strong constitutive *RtGPD1* promoter (SEQ ID NO:16). In some embodiments, each copy of the FAR1 coding sequence is overexpressed by the strong constitutive *RtTEF1_{in}* promoter (SEQ ID NO:17).

[0065] In some embodiments, multiple copies of the *FAR1* gene are present in the genetically modified host cell. In some embodiments, three copies of the *FAR1* gene are present in the genetically modified host cell. In some embodiments, each copy of the *FAR1* gene is operatively linked to a different strong constitutive promoter, such as those described herein. In some embodiments the different promoters are selected from by the strong constitutive *RtPLN1_{in}* promoter (SEQ ID NO:15), the strong constitutive *RtGPD1* promoter (SEQ ID NO:16), or the strong constitutive *RtTEF1_{in}* promoter (SEQ ID NO:17).

[0066] In some embodiments, the heterologous FAR1 is derived from *Marinobacter aquaeolei*. In some embodiments, the coding sequence is codon optimized for the host fungal cell. In some embodiments, the FAR1 derived from *M. aquaeolei* has the sequence set forth in SEQ ID NO:14. In some embodiments, the coding sequence for the FAR1 derived from *M. aquaeolei* is set forth in SEQ ID NO:13. In some embodiments, heterologous FAR1 is a fatty acyl-CoA reductase derived from any fungal species and codon optimized for strong expression in *Rhodospordium* species.

[0067] In some embodiments, the host cell type 1 acyl-CoA:diacylglycerol acyltransferase (*Dga1*) has the amino acid sequence set forth in SEQ ID NO:6. In some embodiments, *Dga1* is encoded by the nucleotide sequence set forth in SEQ ID NO:5. In some embodiments, the genomic sequence for *Dga1* is set forth in SEQ ID NO:1.

[0068] In some embodiments, the host cell type 2 phospholipid:diacylglycerol acyltransferase (*Lro1*) has the amino acid sequence set forth in SEQ ID NO:8. In some embodiments, *Lro1* is encoded by the nucleotide sequence set forth in SEQ ID NO:7. In some embodiments, the genomic sequence for *Lro1* is set forth in SEQ ID NO:2.

[0069] In some embodiments, the host cell acyl-CoA:sterol acyltransferase (sterol ester synthase, *Are1*) has the amino acid sequence set forth in SEQ ID NO:10. In some embodiments, *Are1* is encoded by the nucleotide sequence set forth in SEQ ID NO:9. In some embodiments, the genomic sequence for *Are1* is set forth in SEQ ID NO:3.

[0070] In some embodiments, the host cell type 3 soluble acyltransferase (*Dga3*) has the amino acid sequence set forth in SEQ ID NO:12. In some embodiments, *Dga3* is encoded by the

nucleotide sequence set forth in SEQ ID NO:11. In some embodiments, the genomic sequence for *Dga3* is set forth in SEQ ID NO:4.

[0071] In some embodiments, the host cell acyl-CoA oxidase 1 (*Pox1*) has the amino acid sequence set forth in SEQ ID NO:61. In some embodiments, *Pox1* is encoded by the nucleotide sequence set forth in SEQ ID NO:60. In some embodiments, the genomic sequence for *Pox1* is set forth in SEQ ID NO:62.

[0072] In some embodiments, the expression of the host cell type 1 acyl-CoA:diacylglycerol acyltransferase (*Dga1*) gene or production of its protein is reduced (down-regulated) or knocked-out by anti-sense expression, co-suppression, dsRNA, ribozymes, microRNA, RNAi, genome editing, targeted promoter inactivation, site-directed mutagenesis and knock-outs. In some embodiments, the expression of the host cell type 2 phospholipid:diacylglycerol acyltransferase (*Lro1*) gene or production of its protein is reduced (down-regulated) or knocked-out by anti-sense expression, co-suppression, dsRNA, ribozymes, microRNA, RNAi, genome editing, targeted promoter inactivation, site-directed mutagenesis and knock-outs. In some embodiments, the expression of the host cell acyl-CoA:sterol acyltransferase (sterol ester synthase, *Are1*) gene or production of its protein is reduced (down-regulated) or knocked-out by anti-sense expression, co-suppression, dsRNA, ribozymes, microRNA, RNAi, genome editing, targeted promoter inactivation, site-directed mutagenesis and knock-outs. In some embodiments, the expression of the host cell type 3 soluble acyltransferase (*Dga3*) gene or production of its protein is reduced (down-regulated) or knocked-out by anti-sense expression, co-suppression, dsRNA, ribozymes, microRNA, RNAi, genome editing, targeted promoter inactivation, site-directed mutagenesis and knock-outs.

[0073] Such techniques are described in U.S. Patent Nos. 7,312,323 and references cited therein. For example, reduction might be accomplished, for example, with transformation of a fungal host cell to comprise a promoter and other 5' and/or 3' regulatory regions described herein linked to an antisense nucleotide sequence, hairpin, RNA interfering molecule, double stranded RNA, microRNA or other nucleic acid molecule, such that tissue-preferred expression of the molecule interferes with translation of the mRNA of the native DNA sequence or otherwise inhibits expression of the native target gene in fungal cells. For further description of RNAi techniques or microRNA techniques, see, e.g., U.S. Patent Nos. 5,034,323; 6,326,527; 6,452,067; 6,573,099; 6,753,139; and 6,777,588. See also International Publication Nos. WO 97/01952, WO 98/36083, WO 98/53083, WO 99/32619 and WO 01/75164; and U.S. Patent Application Publication Nos. 2003/0175965, 2003/0175783, 2003/0180945, 2004/0214330,

2005/0244858, 2005/0277610, 2006/0130176, 2007/0265220, 2008/0313773, 2009/0094711, 2009/0215860, 2009/0308041, 2010/0058498 and 2011/0091975. See also International Publication No. WO 2016/159887. RNAi molecules or microRNA molecules (referred to collectively herein as RNAi molecules) can be prepared by the skilled artisan using techniques well known in the art, including techniques for the selection and testing of RNAi molecules and microRNA molecules that are useful for down regulating a target gene. See, for example, Wesley et al. (2001)], Mysara et al. (2011), and Yan et al. (2012).

[0074] Knockouts of the genes described herein are accomplished using conventional techniques well known to skilled artisan, for example, by using homologous recombination which may be enhanced by the use of a non-homologous end-joining (NHEJ) mutant (Koh et al., 2014) or by using the CRISPR-CAS9 system (Ran et al., 2013).

[0075] In some embodiments, the host cell is a cell of a *Rhodospiridium* species or a *Rhodotorula* species. In some embodiments, the host cell is a cell of a strain of *Rhodospiridium toruloides*. In some embodiments, a nucleic acid construct is stably integrated in the genome of the fungal cell. In other embodiments, the fungal cell is part of a composition also comprising a culture medium. In some embodiments, the host cell is *R. toruloides* strain ATCC 10657.

[0076] In a second aspect, the present invention provides a method for producing fatty alcohols. In some embodiments, the method comprises growing the genetically modified host cells described herein in or on a suitable medium for growth of the genetically modified host cell and for production of the desired terpene described herein. In some embodiments, the genetically modified host cells are cultured in a culture medium described herein. In some embodiments, the genetically modified host cells are grown in a conical flask containing a culture medium described herein. In some embodiments the genetically modified host cells are cultured in the conical flasks at about 20 °C to about 32 °C, preferably at about 25 °C to about 30 °C, more preferably at about 28 °C. In some embodiments, the conical flasks are shaken at about 100 rpm to about 300 rpm, preferably at about 150 rpm to about 300 rpm, more preferably about 250 rpm to about 280 rpm., more preferable about 280 rpm. In some embodiments, the medium is GJm3 or DYM1.

[0077] In some embodiments, the GJm3 medium comprises per litre: 70 g glucose, 2.5 g yeast extract, 0.4 g KH₂PO₄, 1.5 g MgSO₄·7H₂O, and 10 ml trace element solution. In some embodiments, the trace element solution comprises per litre: 4.0 g CaCl₂·2H₂O, 0.55 g FeSO₄·7H₂O, 0.52 g citric acid·H₂O, 0.1 g ZnSO₄·7H₂O, 0.076 g MnSO₄·H₂O, and 0.1 ml smoked H₂SO₄. In some embodiments the pH of the GJm3 medium is from about 5.5 to about

6.5, preferably from about 5.8 to about 6.2, more preferably about 6.0. In some embodiments, the DYM1 medium comprises per litre: 100 g glucose, 22.5 g yeast extract, 0.75 g K_2HPO_4 , 0.7 g NH_4NO_3 , 0.4 g $MgSO_4 \cdot 7H_2O$, and 0.4 g $CaCl_2 \cdot 2H_2O$. In some embodiments the pH of the DYM1 medium is from about 4.5 to about 5.5, preferably from about 4.8 to about 5.2, more preferably about 5.0.

[0078] In some embodiments, the genetically modified host cells are grown in a bioreactor containing a culture medium described herein. In some embodiments, the culture medium is the DYM1 medium described herein. In some embodiments, the genetically modified host cells from a seed culture in YPD broth (1% yeast extract, 2% peptone, 2% glucose) are inoculated into a bioreactor at a dilution rate of about 1% to about 30%, preferably about 5% to about 20%, more preferably about 10%. In some embodiments, the fermentation medium is kept at about 20 °C to about 32 °C, preferably at about 25 °C to about 30 °C, more preferably at about 30 °C. In some embodiments, the fermentation medium is kept at a pH of about 4.5 to about 5.5, preferably about 4.8 to about 5.2, more preferably about 5. In some embodiments, the pH is maintained by the addition of 12.5% NH_4OH solution. In some embodiments, the fermentation medium is kept at a pO_2 of about 10% to about 50%, preferably about 10% to about 40%, more preferably about 30%. In some embodiments, the pO_2 is sustained above 30% of air saturation by adapting the stirrer speed. In some embodiments, the bioreactor is aerated at about 2.5 L/min to about 4 L/min, preferably from about 2.8 L/min to about 3.2 L/min, more preferably about 3 L/min (1.5 vvm). In some embodiments, glucose concentration in the medium was kept at about 40 g/L to about 60 g/L by feeding glucose (about 800 g/L). In some embodiments, glucose feeding was stopped after 100 h to about 140 h, preferably about 110 h to about 130 h, more preferably about 120 h of cultivation to achieve its full consumption.

[0079] In some embodiments, fatty alcohols can be produced having from 4-6 carbons to as many as 22-26 carbons, including the commercially important lauryl (C14:0), stearyl (C18:0) and oleyl (C18:1) alcohols. In some embodiments, the amount of fatty alcohols produced in shaking flask cultures in accordance with the present invention ranges in the amount of about 1.5 g/L to about 2.5 g/L, typically about 2.0 g/L. In some embodiments, the amount of fatty alcohols produced in 2 L bioreactor cultures in accordance with the present invention ranges in the amount of about 11.0 g/L to about 13.0 g/L, typically about 12.0 g/L.

[0080] In preparing nucleic acid constructs for use in the present invention, the various DNA fragments may be manipulated, so as to provide for the DNA sequences in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be

employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g. transitions and transversions may be involved.

[0081] Nucleic acids of the present invention may also be synthesized, either completely or in part, especially where it is desirable to provide fungi-preferred sequences, by methods known in the art. Thus, all or a portion of the nucleic acids of the present invention may be synthesized using codons preferred by a selected host. Species-preferred codons may be determined, for example, from the codons used most frequently in the proteins expressed in a particular host species. Other modifications of the nucleotide sequences may result in mutants having slightly altered activity.

[0082] One or more nucleic acid constructs may be introduced directly into a fungal cell using techniques such as electroporation, DNA particle bombardment. Alternatively, the nucleic acid constructs may be combined with suitable T-DNA flanking regions and introduced into an *Agrobacterium tumefaciens* host, which will deliver the gene cassette into the fungal genome. Thus, any method, which provides for effective transformation/transfection of fungi may be employed. See, for example, U.S. Patent Nos. 7,241,937, 7,273,966 and 7,291,765 and U.S. Patent Application Publication Nos. 2007/0231905 and 2008/0010704 and references cited therein. See also, International Published Application Nos. WO 2005/103271 and WO 2008/094127 and references cited therein. See also International Publication No. WO 2016/159887.

[0083] The transformed fungi are transferred to standard growing media (e.g., solid or liquid nutrient media, grain, vermiculite, compost, peat, wood, wood sawdust, straw, etc.) and grown or cultivated in a manner known to the skilled artisan.

[0084] After the polynucleotide is stably incorporated into transformed fungi, it can be transferred to other fungi by sexual crossing. Any of a number of standard breeding techniques can be used, depending upon the species to be crossed.

[0085] It may be useful to generate a number of individual transformed fungi with any recombinant construct in order to recover fungi free from any positional effects. It may also be preferable to select fungi that contain more than one copy of the introduced polynucleotide construct such that high levels of expression of the recombinant molecule are obtained.

[0086] It may be desirable to produce fungal lines that are homozygous for a particular gene if possible in the particular species. In some species this is accomplished by the use

monosporous cultures. By using these techniques, it is possible to produce a haploid line that carries the inserted gene and then to double the chromosome number either spontaneously or by the use of colchicine. This gives rise to a fungus that is homozygous for the inserted gene, which can be easily assayed for if the inserted gene carries with it a suitable selection marker gene for detection of fungi carrying that gene. Alternatively, fungi may be self-fertilized, leading to the production of a mixture of spores that consists of, in the simplest case, three types, homozygous (25%), heterozygous (50%) and null (25%) for the inserted gene. Although it is relatively easy to score null fungi from those that contain the gene, it is possible in practice to score the homozygous from heterozygous fungi by Southern blot analysis in which careful attention is paid to the loading of exactly equivalent amounts of DNA from the mixed population, and scoring heterozygotes by the intensity of the signal from a probe specific for the inserted gene. It is advisable to verify the results of the Southern blot analysis by allowing each independent transformant to self-fertilize, since additional evidence for homozygosity can be obtained by the simple fact that if the fungi was homozygous for the inserted gene, all of the subsequent fungal lines from the selfed individual will contain the gene, while if the fungus was heterozygous for the gene, the generation grown from the selfed seed will contain null fungal lines. Therefore, with simple selfing one can select homozygous fungal lines that can also be confirmed by Southern blot analysis.

[0087] Creation of homozygous parental lines makes possible the production of hybrid fungus and spores that will contain a modified protein component. Transgenic homozygous parental lines are maintained with each parent containing either the first or second recombinant DNA sequence operably linked to a promoter. Also incorporated in this scheme are the advantages of growing a hybrid crop, including the combining of more valuable traits and hybrid vigor.

[0088] The practice of the present invention employs, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA, genetics, immunology, cell biology, cell culture and transgenic biology, which are within the skill of the art. See, e.g., Maniatis *et al.*, 1982, *Molecular Cloning* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook *et al.*, 1989, *Molecular Cloning*, 2nd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Sambrook and Russell, 2001, *Molecular Cloning*, 3rd Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); Ausubel *et al.*, 1992), *Current Protocols in Molecular Biology* (John Wiley

& Sons, including periodic updates); Glover, 1985, *DNA Cloning* (IRL Press, Oxford); Russell, 1984, *Molecular biology of plants: a laboratory course manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.); Anand, *Techniques for the Analysis of Complex Genomes*, (Academic Press, New York, 1992); Guthrie and Fink, *Guide to Yeast Genetics and Molecular Biology* (Academic Press, New York, 1991); Harlow and Lane, 1988, *Antibodies*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York); *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); Riott, *Essential Immunology*, 6th Edition, Blackwell Scientific Publications, Oxford, 1988; Fire et al., *RNA Interference Technology: From Basic Science to Drug Development*, Cambridge University Press, Cambridge, 2005; Schepers, *RNA Interference in Practice*, Wiley-VCH, 2005; Engelke, *RNA Interference (RNAi): The Nuts & Bolts of siRNA Technology*, DNA Press, 2003; Gott, *RNA Interference, Editing, and Modification: Methods and Protocols (Methods in Molecular Biology)*, Human Press, Totowa, NJ, 2004; Sohail, *Gene Silencing by RNA Interference: Technology and Application*, CRC, 2004.

EXAMPLES

[0089] The present invention is described by reference to the following Examples, which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized.

EXAMPLE 1

Materials and Methods

[0090] *Strains, media, and culture conditions:* *R. toruloides* strain ATCC 10657 was obtained from ATCC (USA). *R. toruloides* was cultured at 28°C in YPD broth (1% yeast extract, 2% peptone, 2% glucose, w/v) or on potato-dextrose agar (PDA). *A. tumefaciens* strain AGL2 (Cai et al., 2009) was cultured at 28°C in either liquid or solid 2YT medium (1.6% tryptone, 1%

yeast extract, 0.5% NaCl). *Escherichia coli* XL1-Blue was cultured in Luria-Bertani (LB) broth or on LB agar and used for routine DNA manipulations.

[0091] Lipid accumulation medium MinRL2 was prepared as reported previously (Liu et al., 2015). Lipid accumulation medium GJm3 was prepared as described previously (Jin et al., 2013) with some modifications. Briefly, GJm3 medium (per litre) contains 70 g glucose, 2.5 g yeast extract, 0.4 g KH_2PO_4 , 1.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 ml trace element solution, pH6.0. Trace element solution (per litre): 4.0 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.55 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.52 g citric acid $\cdot \text{H}_2\text{O}$, 0.1 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.076 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.1 ml smoked H_2SO_4 .

[0092] Fatty alcohol production medium SC, SY, DC and DY modified from the PSC medium (Fillet et al., 2015). SC (PSC) medium contains (per litre) 100 g sucrose, 22.5 g corn steep liquid, 0.75 g K_2HPO_4 , 0.7 g NH_4NO_3 , 0.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, pH5.0. SY medium is similar as SC medium except the replacement of corn steep liquid by yeast extract. DC and DY medium is similar as SC and SY medium, respectively, except the replacement of sucrose by glucose.

[0093] *Bioinformatics:* Putative *R. toruloides* homologues were identified through BLAST search (NCBI, USA) of the *Rhodotorula glutinis* ATCC 204091 genome database (whole-genome shotgun project PID-59971 by Mississippi State University) (Paul et al., 2014). The phylogenetic tree was constructed by MEGA version 6 program (<http://colon://www.megasoftware.net/>) using Neighbor-Joining algorithm and tested by Bootstrapping. The structural organization of the DGAT genes was determined after alignment of genomic DNA and cDNA sequences. Transmembrane structures were predicted using the transmembrane prediction server TMHMM-2.0 (<http://colon://www.cbs.dtu.dk/services/TMHMM/>) with the complete protein sequences.

[0094] *Plasmid constructs:* Microorganisms and plasmids used are listed in Table 1.

TABLE 1
Microorganisms and Plasmids

Plasmid/Strain	Plasmid/genotype	Reference
<i>Plasmids</i>		
pKO1	Binary vector used for gene deletion constructs	Herein
pKODGA1	Deletion of <i>DGA1</i>	Herein
pKOLRO1	Deletion of <i>LRO1</i>	Herein
pKOARE1	Deletion of <i>ARE1</i>	Herein
pKODGA3	Deletion of <i>DGA3</i>	Herein

pKC2DGA1	Overexpression of <i>DGA1</i> driven under <i>GPD1</i> promoter	Herein
pKC2LRO1	Overexpression of <i>LRO1</i> driven under <i>GPD1</i> promoter	Herein
pKC2ARE1	Overexpression of <i>ARE1</i> driven under <i>GPD1</i> promoter	Herein
pKC2DGA3	Overexpression of <i>DGA3</i> driven under <i>GPD1</i> promoter	Herein
pKC2DL	Overexpression of <i>DGA1</i> and <i>LRO1</i> driven under <i>GPD1</i> promoter	Herein
pGEX-6P-1	GST-fusion protein expression system	GE Healthcare
pGEXDGA3	<i>E. coli</i> expression of GST-Dga3 fusion protein	Herein
pKC2FAR1	Overexpression of <i>RtFAR1</i> driven under <i>GPD1</i> promoter	Herein
pKT13FAR1	Overexpression of <i>RtFAR1</i> driven under <i>TEF1in</i> promoter	Herein
pKP4FAR1	Overexpression of <i>RtFAR1</i> driven under <i>PLN1in</i> promoter	Herein
pKPGPTFAR1	Overexpression of <i>RtFAR1</i> (3x) driven under <i>GPD1</i> , <i>TEF1in</i> and <i>PLN1in</i> promoter	Herein
<i>E. coli</i> strains		
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI^qΔM15 Tn10</i> (Tet ^r)], plasmid cloning host	Agilent
BL21(DE3)	F ⁻ <i>ompT gal dcm lon hsdS_B(f_B⁻ m_B⁻) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])</i> , protein expression host	Novagen
BL21(DE3)/pGEX DGA3	<i>E. coli</i> engineering strain with heterologous expression of GST-Dga3 fusion protein	Herein
<i>A. tumefaciens</i> strain		
AGL1	T-DNA host	(Lazo et al., 1991)
<i>R. toruloides</i> strains		
ATCC 10657	<i>MATA</i> , source strain	ATCC
Rt1ck	<i>MATA</i> , Δ <i>ku70::hpt^R-ex:loxP</i> , wild-type	(Koh et al., 2014)
Δ <i>dga1</i>	<i>MATA</i> Δ <i>ku70Δdga1::hpt^R</i>	Herein
Δ <i>lro1</i>	<i>MATA</i> Δ <i>ku70Δlro1::hpt^R</i>	Herein
Δ <i>are1</i>	<i>MATA</i> Δ <i>ku70Δare1::hpt^R</i>	Herein
Δ <i>dga3</i>	<i>MATA</i> Δ <i>ku70Δdga3::hpt^R</i>	Herein
<i>dl</i>	<i>MATA</i> Δ <i>ku70Δdga1Δlro1::hpt^R</i>	Herein
<i>da</i>	<i>MATA</i> Δ <i>ku70Δdga1Δare1::hpt^R</i>	Herein
<i>dla</i>	<i>MATA</i> Δ <i>ku70Δdga1Δlro1Δare1::hpt^R</i>	Herein
<i>dlad</i>	<i>MATA</i> Δ <i>ku70Δdga1Δlro1Δare1Δdga3::hpt^R</i>	Herein
Δ <i>dga1C</i>	<i>MATA</i> Δ <i>ku70Δdga1::hpt^R-ex Δcar2::P_{GPD1}-DGA1-T_{35S}</i>	Herein
Δ <i>lro1C</i>	<i>MATA</i> Δ <i>ku70Δlro1::hpt^R-ex Δcar2::P_{GPD1}-LRO1-T_{35S}</i>	Herein
<i>dlad-DGA1</i>	<i>MATA</i> Δ <i>ku70Δdga1Δlro1Δare1Δdga3::hpt^R-ex Δcar2::P_{GPD1}-DGA1-T_{35S}</i>	Herein
<i>dlad-LRO1</i>	<i>MATA</i> Δ <i>ku70Δdga1Δlro1Δare1Δdga3::hpt^R-ex Δcar2::P_{GPD1}-LRO1-T_{35S}</i>	Herein
<i>dlad-ARE1</i>	<i>MATA</i> Δ <i>ku70Δdga1Δlro1Δare1Δdga3::hpt^R-ex:loxP P_{GPD1}-ARE1-T_{35S}::CAR2</i>	Herein
<i>dlad-DGA3</i>	<i>MATA</i> Δ <i>ku70Δdga1Δlro1Δare1Δdga3::hpt^R-ex Δcar2::P_{GPD1}-DGA3-T_{35S}</i>	Herein

<i>dlad-DL</i>	<i>MATA Δku70Δdga1Δlro1Δare1Δdga3::hpt^R-ex Δcar2::P_{GPD1}-DGA1-T_{35S}-P_{GPD1}-LRO1-T_{35S}</i>	Herein
<i>FAR1</i>	<i>MATA Δku70::hpt^R-ex Δcar2::P_{GPD1}-RtFAR1-T_{35S}-P_{PLN1in}-FAR1-T_{35S}-P_{TEF1in}-FAR1-T_{35S}</i>	Herein
<i>fad2FAR1</i>	<i>MATA Δku70Δfad2::hpt^R-ex Δcar2::P_{GPD1}-RtFAR1-T_{35S}-P_{PLN1in}-FAR1-T_{35S}-P_{TEF1in}-FAR1-T_{35S}</i>	Herein
<i>dladFAR1</i>	<i>MATA Δku70Δdga1Δlro1Δare1Δdga3::hpt^R-ex Δcar2::P_{GPD1}-RtFAR1-T_{35S}-P_{PLN1in}-FAR1-T_{35S}-P_{TEF1in}-FAR1-T_{35S}</i>	Herein
<i>dladpFAR1</i>	<i>MATA Δku70Δdga1Δlro1Δare1Δdga3Δpox1::hpt^R-ex Δcar2::P_{GPD1}-RtFAR1-T_{35S}-P_{PLN1in}-FAR1-T_{35S}-P_{TEF1in}-FAR1-T_{35S}</i>	Herein
<i>dladpFAR1</i>	<i>MATA Δku70Δdga1Δlro1Δare1Δdga3Δpox1Δfad2::hpt^R-ex Δcar2::P_{GPD1}-FAR1-T_{35S}-P_{PLN1in}-RtFAR1-T_{35S}-P_{TEF1in}-FAR1-T_{35S}</i>	Herein

Notes: *hpt^R*: hygromycin resistant gene cassette P_{GPD1-3}-*HPT-3*-T_{35S}
ex: excision of selection marker cassette *hpt^R*

[0095] Oligonucleotides used are listed in Table 2. All restriction and modification enzymes were from New England Biolabs (NEB, USA).

TABLE 2
Oligonucleotides

Name	Sequence (5'-3') (SEQ ID NO:)	RE site*
Rt113	CCGCCAATAACCTCACCTCAG (18)	
Rt114	GGCGATGGGAGCGTAGAATAC (19)	
LRO1L-Sf	AAAGAGCTCACTCACTGGCCTCCTCGTTC (20)	SacI
LRO1L-Br	AAAGGATCCACGTTGAGAGCGGAGAGGGAA (21)	BamHI
LRO1R-Hf	TTTAAGCTTGGACCAACGACTGCAGACCAT (22)	HindIII
LRO1R-Str	TTTAGGCCTGCCCAACCCGAGAATGAGCTT (23)	StuI
ARE1L-Sf	AAAGAGCTCATTGACCCTGCGTGTATGC (24)	SacI
ARE1L-Br	AAAGGATCCGCTCTTGAGTGCTCCGACGAAG (25)	BamHI
ARE1R-Hf	TCGTACGTTCTTGTTTCAGCG (26)	HindIII
ARE1R-Str	TTTAGGCCTCGCCTCTACCTCACTCACGT (27)	StuI
MFE1L-Sf	AAAGAGCTCGCGGAACAGGAGAACAAGGAG (28)	SacI
MFE1L-Br	AAAGGATCCGCTCACGTCAACACTCCCAAA (29)	BamHI
MFE1R-Hf	TTTAAGCTTAGAACCACTCGACCGTCTTCA (30)	HindIII
MFE1R-Str	TTTAGGCCTCTATCGACCTCTCCCAAGCC (31)	StuI
DGA3L-Sf	TTTAGGCCTCCAGATCAGGGTGAGTCGT (32)	StuI
DGA3L-Hr	TTTAAGCTTCTTGTTGGTTGTGGGGCAT (33)	HindIII
DGA3R-Bf	AAAGGATCCCTCACTGGCTGCATCTTCTCG (34)	BamHI
DGA3R-Sr	GTGTGCGATGACTGTGTGGT (35)	SacI

Rt123	CTGTAGAGGAGCTGCAGGATCAA (36)	
Rt124	CGAGCTTGATGAATCCTTTTCGT (37)	
LRO1f	TGCAGCTCCCTTCTTTTCGCTC (38)	
LRO1r	ATGCTGTGCGAGACGAGAACC (39)	
ARE1f	CTCGCACCCACTCTTCTTCCG (40)	
ARE1r	TTCCGATCCGCAAACCTCGTC (41)	
MFE1f	AACCAGGACACGATGGGCTTG (42)	
MFE1r	TCCTTCTCAGTCGCGCCAATG (43)	
DGA3f	CATGTTGCGCGGTCTCCACTT (44)	
DGA3r	CCTGCTTGGCCTTCTCAACGT (45)	
Rt347Nf	TTTCCATGGGCCAGCAGGCGACG (46)	Exp <i>DGA1</i>
Rt348Evr	TTTGATATCGTCCGTTGCGAGGAGTCA (47)	
Rt349Bsf	TTTTTCATGAGCACAGTACGGAGGCGCA (48)	Exp <i>LRO1</i>
Rt350Pmr	TTTGTTTAAACAGGCGTACAGGGTGGGTCAGC (49)	
Rt351Nf	TTTCCATGGCCTCGCTAGACCCGCCA (50)	Exp <i>ARE1</i>
Rt352Evr	TTTGATATCTGCGCCCTGAGCTCAGTAC (51)	
Rt471Bsf	TTTTTCATGACCGTCTCGACGAACGCTATC (52)	Exp <i>DGA3</i>
Rt472Evr	TTTGATATCTCCATTTAATCGCGCTGGTTC (53)	
Rt550Gf1	AGATTGTCGTTTTCCCGCCTTCAGTTTTTTACTAGTGGACGGCTTG (54)	
Rt551Gr2	GTCGCACCCCAAACGATGCTGAAGGCTCGCAAACATGCTAATTCGGGG GATCTGGATTTTAGTAC (55)	
Rt533F2f	GCGAGCCTTCAGCATCGTTTGGGGTGCGACCTAGTCACGCCTCTGTGA CTCGGTACG (56)	
Rt534F2r	CAATCAAGATGTCGTTGTGCTAGTGTACGCAAACATGCTAATTCGGGG GATCTGGATTTTAGTAC (57)	
Rt552Tf5	GCGTACACTAGCACAAACGACATCTTGATTGCTAGTGACGCGAAGCGG TAGAAG (58)	
Rt553Tr6	CGATACTCTCAAGGTCAGCTCGAATTGTTTAAACATGCTAATTCG (59)	

Note: * Restriction enzyme recognition site

[0096] All gene disruption was performed by ATMT using the binary vectors where the entire coding regions were replaced by the hygromycin resistant gene expression cassette. For deletion of *DGA1*, a 2.8 kb 5'-phosphorylated *DGA1* DNA fragment amplified using *R. toruloides* ATCC 10657 genomic DNA by oligos Rt113/114 was ligated to SacI (blunt-ended) and PmeI double digested pEX2 vector (Liu et al., 2011) to create the intermediate plasmid pEX2DGA1. pEX2DGA1 was double digested with SpeI (blunt-ended) and SmaI, and inserted with BamHI/HindIII-digested (blunt-ended) codon-optimized hygromycin selection cassette from pDXP795hptR ($P_{GPD1}::hpt-3::T_{nos}$) (Koh et al., 2014) to create the plasmid pKODGA1,

where P_{GPD1} , *hpt-3* and T_{nos} represents the promoter of endogenic glyceraldehyde 3-phosphate dehydrogenase, the codon-optimized gene of hygromycin phosphotransferase and the terminator of *A. tumefaciens* nopaline synthase gene, respectively (Liu et al., 2013b). For deletion of *LRO1*, left and right homology flanking fragment (~0.9 kb each) was amplified with oligo pairs LRO1L-Sf/LRO1L-Br and LRO1R-Hf/LRO1R-Str, respectively. A four-fragment ligation was performed with SacI/PmeI-digested pEX2 vector, SacI/BamHI-digested left flanking fragment, BamHI/HindIII-digested codon-optimized hygromycin selection cassette and HindIII/StuI-digested right flanking fragment to create plasmid pKOLRO1. A similar strategy was applied to construct pKOARE1 and pKODGA3. For construction of pKOARE1, oligo pairs ARE1L-Sf/ARE1L-Br and ARE1R-Hf/ARE1R-Str and restriction enzymes SacI/BamHI and HindIII/StuI were used to amplify and digest the left and right flanking DNA fragments of *ARE1* (0.9 kb each), respectively. pKODGA3 was constructed using oligo pairs DGA3L-Stf/DGA3L-Hr and DGA3R-Bf/DGA3R-Sr and restriction enzymes StuI/HindIII and BamHI/SacI.

[0097] For gene overexpressions, the entire open reading frame of *DGA1*, *LRO1* and *DGA3* was generated by reverse transcription-PCR using oligo pair Rt347Nf/Rt348Evr, Rt349Bsf/Rt350Pmr and Rt471Bsf/Rt472Evr, respectively. The 1.0 kb, 2.2 kb and 1.7 kb PCR products of *DGA1*, *LRO1* and *DGA3* were double digested with NcoI/EcoRV, BspHI/PmeI and BspHI/EcoRV, and cloned into the NcoI and EcoRV sites of pKCL2 (Liu et al., 2015) to create plasmid pKC2DGA1, pKC2LRO1 and pKC2DGA3, respectively.

[0098] For metabolic engineering of fatty alcohol production, the gene encoding *M. aquaeolei* VT8 fatty acid reductase (Maqu_2220, GenBank accession number YP_959486.1) (Hofvander et al., 2011) was codon-optimized based on the codon bias of *R. toruloides* and commercially synthesized (RtFAR1, Genscript, USA). The NcoI-EcoRV double digested RtFAR1 DNA fragment was firstly cloned to the same sites of vector pKCL2, pKCLT23 and pKCLP4 to create plasmid pKC2FAR1, pKCT2FAR1 and pKCP4FAR1 driven under the strong constitutive promoter of *GPD1* (Liu et al., 2013b) (SEQ ID NO:16), translation elongation factor 1 α gene (P_{TEF1in} ; SEQ ID NO:17) and the lipid production-correlated perilipin gene (P_{PLN1in} ; SEQ ID NO:15), respectively. The fatty acid reductase expression cassette $P_{GPD1}::RtFAR1::T_{35S}$, $P_{TEF1in}::RtFAR1::T_{35S}$ and $P_{PLN1in}::RtFAR1::T_{35S}$ was amplified using the template of above plasmid and oligo pair Rt550Gf1/Rt551Gr2, Rt533F2f/Rt534F2r and Rt552Tf5/Rt553Tr6, respectively, where T_{35S} represents the terminator of Cauliflower mosaic virus (CaMV) 35S gene. The final engineering plasmid pKCGPTFAR1 was created by assembly of above three RtFAR1 expression cassettes in the SpeI-PmeI-linearized vector pKCL2 by Gibson Assembly

(NEBuilder kit, Bio-Rad laboratories, USA) and sequencing confirmed using BigDye Terminator chemistry (Applied Biosystem, ThermoFisher Scientific, USA).

[0099] *Transformation and fungal colony PCR:* The binary vectors were electroporated into AGL1 and transformed to *R. toruloides* through *Agrobacterium tumefaciens*-mediated transformation (ATMT) as previously described (Liu et al., 2013b). For gene deletion analysis, positive T-DNA-tagging transformants were selected on YPD medium supplemented with 300 µg/ml cefotaxime and 150 µg/ml hygromycin, and screening for candidate gene deletion mutants were initiated by fungal colony PCR (Liu et al., 2013b) and verified by Southern blot analysis. For *in vivo* gene expression analysis, the *CAR2*-locus integrated transformants were easily selected by visual screening for albino mutants on the selection agar medium as reported previously (Liu et al., 2015).

[0100] *Extraction of genomic DNA and total RNA:* Genomic DNA and total RNA of *R. toruloides* were extracted as described previously (Liu et al., 2015). The concentrations of DNA or RNA samples were determined with NanoDrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, USA) and the integrity of the extracted nucleic acids were qualified by agarose gel electrophoresis.

[0101] *Southern blot analysis:* For Southern blot analysis, genomic DNA (5 µg) was digested with appropriate restriction enzyme and separated by electrophoresis in 0.8% agarose gel. Southern hybridization was performed using DIG High Prime DNA Labeling and Detection Starter Kit according to manufacturer's instruction (Roche Diagnostics, Indiana, USA), and the DIG-labelled probe was the flanking DNA fragment as indicated in Fig. 2A.

[0102] *Quantitative reverse transcription PCR:* To remove the trace DNA, total RNA was treated with DNase I (Roche Diagnostics) followed by precipitation with ethanol. cDNA was synthesized using the iScript™ Reverse Transcription Supermix for RT (Bio-Rad, USA) and real-time PCR was conducted in ABI PRISM 7900HT Sequence Detection System (Life Technologies, USA) using the ABI SYBR® Select Master Mix (Life Technologies, USA). Real-time PCR conditions were as follows: an initial 50°C for 2 min and 95°C denaturation step for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min. Triplicates were used for all qRT-PCR analyses. The data was acquired using the software SDS 2.4 (Applied Biosystems, Life Technologies, USA) and relative gene expression was calculated by RQ Manager software (version 1.2.1, Applied Biosystems) using the reference gene *ACT1* (GenBank accession number KR183696) and $2^{-\Delta\Delta C_t}$ method.

[0103] *Lipid extraction:* Total crude lipid was extracted by acid-heating procedure as previously reported (Elsey et al., 2007) with some modifications. After calculating dry cell biomass, 10 mg cells were mixed with 500 μ l 4 M HCl and lysed in a boiled water bath for 15 min. After frozen at -20°C for 1 h, the cell lyses were mixed with 0.2 mg pentadecanoic acid (C15:0, internal standard for the subsequent GC analysis) and 1.0 ml of lipid extraction solvent (chloroform:methanol = 2:1). The chloroform phase was removed to a new tube and crude lipid was gravimetrically quantified as the total lipid amounts after vacuum dry (Concentrator, Eppendorf, USA).

[0104] *Thin layer chromatography:* The lipid composition of extracted lipids were separated and analyzed by TLC as reported previously by (Athenstaedt, 2011) with some modifications. Aliquots of the crude lipids (5 μ L) were applied to Silica Gel 60 plates (Merck, Germany) by the CAMAG 5 Nano-Applicator (CAMAG, Muttenz, Switzerland) and chromatograms were developed in a two-step system, petroleum ester/ethyl acetate/acetic acid (25:25:1, v/v/v) and petroleum ester/ethyl acetate (49:1, v/v), and visualized using MnCl_2 -methanol-sulfuric acid buffer (Athenstaedt, 2011). A mixture of oleic acid, sterol, tri-, di- and mono-acylglycerol from Sigma-Aldrich including oleic acid (Sigma catalog No.), sterol (), glyceryl trioleate (92860), 1,2-dioleoyl-sn-glycerol (D0138), glyceryl 1,3-distearate (D8269) and 1-oleoyl-rac-glycerol (M7765), was used as the lipid standard. The separated lipid classes were visualized by dipping the plates into solution into a solution of 10% (w/v) copper (II)-sulphate-pentahydrate in 8% (v/v) phosphoric acid, drying at 100°C and heating at 170°C until ashed spots appeared. Photographs were taken using a CAMAG TLC Scanner. The amounts of different lipid components were measured on the basis of the band intensity through a web-based TLC analysis software JustQuantify (version 2.0, Sweday, Södra Sandby, Sweden).

[0105] *Lipid staining and fluorescent microscopy:* The BODIPY (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4-diazas-indacene) was used for staining lipid bodies according to the method adapted from Nile Red staining as reported by Yang *et al.* (Yang et al., 2012) with some modifications. Briefly, BODIPY (Life Technologies, USA) was dissolved in DMSO to a final concentration of 0.1 mg/ml. A 200 μ l aliquot of culture was washed twice with PBS and resuspended in 1 ml PBS supplemented with 6 μ l 0.1 mg/ml BODIPY and stained in the dark for 10 min before visualization. Colonies were observed using a Nikon SMZ 800 fluorescence microscope equipped with Plan Apo WD70 objective (Nikon, Tokyo, Japan) and a GFP-L filter (GFP Band pass, Ex 480/40 DM 505 BA 510). Images were acquired with a Nikon DS-5M camera.

[0106] *Cell biomass determination:* Cell biomass (dry cell weight) was determined by drying the water-washed cell pellet in a 70°C oven until constant weight was reached.

[0107] *Quantification of residual glucose:* Residual glucose in cell culture was quantified by HPLC (Shimadzu, Japan). Fermentation samples were filtered through a 0.2 µm membrane and run through a 300 x 7.0 mm Aminex HPX-87H column (Bio-Rad, USA) at a constant flow rate of 0.7 ml min⁻¹ using 5 mM sulfuric acid as the mobile phase. The column was maintained at 50°C and glucose was detected with a Refractive Index Detector (Shimadzu, Japan). Concentration of residual glucose in the cell culture was determined using calibration curve built with the standard glucose aqueous solution.

[0108] *Fatty acid composition analysis:* Gas-lipid chromatography-Mass spectrometry (GCMS) was used for fatty acid profiling. Preparation of fatty acid methyl esters (FAMES) and GCMS analyses were performed as described previously (Voelker and Davies, 1994) with some modifications. Lipids were dissolved in 300 µl petroleum ether-benzene (1:1, v/v), mixed with 0.4 M KOH in methanol, and keep room temperature for 2 hr. FAMES were extracted after addition of 1 ml of water. 1 µl of FAMES after 10-fold dilution in methanol was injected to a HP-88 fused silica capillary column (30-m length, 0.25-µm diameter, and 0.25-mm film thickness, Agilent J&W Scientific, Folsom, CA, USA) and separated in a GCMS (QP2010, SHIMIDZU, Japan). The running conditions were typically 42.3 ml/min nitrogen flow, 150°C for starting temperature (3 min), a 15-min ramp to 240°C, and holding at 240°C for 7 min. The mass spectrometry peaks were identified by searching against Shimadzu NIST08 compound library and quantified as percentages of total fatty acids (%TFA).

[0109] *Fatty alcohol production:* Lipid accumulation medium GJm3 was firstly used for fatty alcohol production unless indicated otherwise. Medium DYM1 was finally optimized for the production of fatty alcohol. Flask production was performed in 250 mL-flasks containing 50 mL of medium and cultured at 28°C, 280 rpm for 5 days. Fed-batch fermentation was performed in a Biostat[®] B fermentor (Sartorius AG, Göttingen, Germany) equipped with a 2-litre jacketed vessel. DYM1 medium (1.0 L) was added to the reactor and sterilized in autoclave (121 °C, 20 min). Once the medium was cooled down culture conditions were set to 30°C, pH 5 and 3 L/min aeration (1.5 vvm). The reactor was inoculated with 100 mL from a seed culture in YPD broth. The pH was maintained by adding 12.5% NH₄OH solution and *p*O₂ was sustained above 30% of air saturation by adapting the stirrer speed. Sugar concentration in the broth was kept at 40 to 60 g/L by feeding glucose (800 g/L). After 120 h of cultivation glucose feeding was stopped to achieve its full consumption.

[0110] *Extraction, identification and quantification of fatty alcohol:* Fatty alcohol was extracted and identified as described previously (Fillet et al., 2015) with some modifications. Briefly, cell culture (1 ml) was transferred in an Ependorf centrifuge tube and centrifuged at 10,000 rpm for 3 min to separate the supernatant broth and cell pellet. The supernatant was mixed with 20 mg heptadecanal as the internal standard (ISTD) and extracted with 500 μ L of ethyl acetate for three times. The organic phases were combined and dried with Na_2SO_4 power to obtain the extracellular fatty alcohols. To extract intracellular fatty alcohols, cell pellet was washed with PBS buffer and resuspended with above lipid extraction solvent (chloroform:methanol = 2:1) and 20 mg heptadecanal (ISTD). Cellular lysis was achieved with the addition of 2 volume of glass beads (0.4 mm in diameter, Sigma-Aldrich) followed by twice bead beating (40 s/round, FastPrep-24™ 5G, MP Biochemicals, Eindhoven, Netherlands). The organic phase was extracted, dried with Na_2SO_4 power and regarded as the intracellular fatty alcohols.

[0111] Fatty alcohols were qualified and quantified using above GCMS system (QP2010, SHIMIDZU, Japan) equipped with the DB-5HT capillary column (30-m length, 0.25- μ m diameter, 0.1-mm film thickness, Agilent J&W Scientific, Folsom, CA, USA). The running conditions were 42.3 ml/min nitrogen flow, 150°C for starting temperature (5 min), a 20-min ramp to 350°C, and holding at 350°C for 10 min. Mass spectrometer were set to solvent delay (5 min), electron impact ionization (70 eV) and dwell time (100 ms). Chromatograms were registered by SCAN mode (mass range 50-500 m/z). The mass spectrometry peaks were identified by searching against Shimadzu NIST08 compound library and quantified by the internal standard (hexadecanol, C17-OH).

[0112] *Microscopy:* A Nikon Eclipse 80i microscope equipped with a CFI Plan Apochromat objective lens (Nikon, Japan) was used for fluorescence and differential interference contrast (DIC) images of yeast cells, and images were acquired with a DS camera and ACT-2U software (Nikon, Japan). The BODIPY (4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene) was used for staining lipid bodies according to the method adapted from Nile Red staining as reported by Yang *et al.* (Yang et al., 2012) with some modifications. Briefly, BODIPY (Life Technologies, USA) was dissolved in DMSO to a final concentration of 0.1 mg/ml. A 200 μ L aliquot of culture was washed twice with PBS and resuspended in 1 ml PBS supplemented with 6 μ L 0.1 mg/ml BODIPY and stained in the dark for 10 min before visualization. A fluorescein filter under emission and excitation filter of 488 and 509 nm, was used to observe the fluorescence of yeast cells stained by BODIPY.

[0113] For electron microscopy, preparations were fixed with 1% glutaraldehyde and 1% OsO₄ in 0.1 M Phosphate buffer (pH7.2). Fixed preparations were dehydrated through a graded ethanol series and embedded in Epon 812 resin. Sections were sequentially stained with 4% uranyl acetate and 0.4% lead citrate and viewed in a transmission electron microscopy (TEM, JEM-2010, JEOL, Japan)

[0114] *Sequences*: The genomic nucleotide sequences of *DGA1*, *LRO1*, *ARE1* and *DGA3*, are set forth in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3 and SEQ ID NO:4, respectively. The coding nucleotide sequences of *DGA1*, *LRO1*, *ARE1*, *DGA3* and *RtFAR1* are set forth in SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11 and SEQ ID NO:13, respectively. The amino acid sequences of *DGA1*, *LRO1*, *ARE1*, *DGA3* and *RtFAR1* are set forth in SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12 and SEQ ID NO:14, respectively.

EXAMPLE 2

Diacylglycerol Acyltransferases in *R. toruloides*

[0115] In oleaginous yeasts, diacylglycerol acyltransferases catalyse the last and committed step in lipid biosynthesis in *R. toruloides*, and are essential for further engineering process. To uncover the enzymes involved in this step in *R. toruloides*, homologous searches (tBLASTn, NCBI) were performed using DGATs from *S. cerevisiae* and *Yarrowia lipolytica* as the queries and the genome sequence of *Rhodotorula glutinis* ATCC 204091 (Paul et al., 2014) as the target. A unique *R. toruloides* ortholog of acyl-CoA-dependent diacylglycerol acyltransferase (EC 2.3.1.20) was found sharing 40% and 51% identity to *S. cerevisiae* Dga1p (GenBank accession number NP_014888.1) (Sorger and Daum, 2002) or *Yarrowia lipolytica* Dga1p (CAG80304.1) (Athenstaedt, 2011), respectively. The putative *DGA1* coding sequence (CDS) spans 1,855 nt in the scaffold No.13 (AEVR02000013) of genome. Transcriptome analyses revealed a transcript of 1,257-nt mRNA including a 33-nt 5'UTR and 177-nt 3'UTR (untranslated region) as identified by (data not shown). *DGA1* gene is composed of 11 exons separated by 10 introns, encoding a 348-aa protein showing high homologous to other diacylglycerol O-acyltransferases with the signature DGAT motif (pfam03982), and the highest identity to the brown mold *Wallemia sebi* DGAT (XP_006957543.1, 66% identity in protein sequence).

TABLE 3

Gene Annotations

Gene	Scaffold No.	CDS Length (nt)	Transcript length (nt)	5'UTR (nt)	3'UTR (nt)	Exon number	Protein (aa)
<i>DGAI</i>	13	1,855	1,257	33 ^a	177 ^a	11	348
<i>LROI</i>	15	3,024	2,336	53 ^a	72 ^a	15	736
<i>ARE1</i>	1	2,788	2150	6 ^a	110 ^a	10	678
<i>DGA3</i>	3	2,250	1,929	110	151 ^a	9	555
<i>RtFAR1</i>	- ^b	1,549	1,549	- ^b	- ^b	1	513

^a Transcriptomics data^b not available according to the synthesized gene

[0116] Phospholipid:diacylglycerol acyltransferase (EC 2.3.1.158) from either *S. cerevisiae* (Sc Lro1p, NP_014405.1) (Oelkers et al., 2000) or *Yarrowia lipolytica* (Yl Lro1p, XP_504038.1) (Athenstaedt, 2011) have the same ortholog in *R. toruloides* that is also localized on scaffold #13, closely adjacent (~32.7 kb) to the putative *DGAI* gene. The putative *LROI* spans 3,024 nt in the genome, transcribing a 2,336-nt mRNA including a 53-nt 5'UTR and 72-nt 3'UTR (transcriptome analysis). *LROI* gene is composed of 15 exons separated by 14 introns. *LROI* encodes a 736-aa protein showing highly homologous to other LCAT motif (pfam02450)-containing phosphatidylcholine-sterol O-acyltransferases with the highest identity to *U. maydis* Um00322 (XP_756469.1, 54% identity).

[0117] *S. cerevisiae* acyl-CoA:sterol acyltransferase (EC 2.3.1.26, Are1p and Are2p, YCR048W and YNR019W, GenBank acc. no. NP_009978.1 and NP_014416.1, respectively) have a single ortholog in *R. toruloides* (EGU12278.1, *ARE1*) localized on the scaffold No.1. *R. toruloides* putative *ARE1* CDS spans 2,788 nt in the genome, transcribing a 2,150-nt mRNA including a 6-nt 5'UTR and 110-nt 3'UTR (transcriptome analysis). *ARE1* gene is composed of 10 exons that are separated by 9 introns, and the encoding 678-aa protein (Are1) has a signature MBOAT motif (membrane-bound O-acyltransferase family, pfam03062) and exhibits highly homologous to sterol O-acyltransferase from other Pucciniomycotina species, in which the highest identity is that from *R. toruloides* strain CECT 1137 and NP11 (BAN63763.1 and EMS22447.1, 96% and 95% identity, respectively).

[0118] In *R. toruloides*, a soluble DGAT located in the 10 S cytosolic TAG biosynthetic complex was found and functionally identified recently (Rani et al., 2013). However, only a partial sequence released (ABC41546.1, 221 aa in length) with truncation in its N-terminus.

Here, the full length of soluble DGAT gene (termed as *DGA3*) was identified through homologous searches (BLASTn, NCBI). *DGA3* spans 2,250 nt in the genome sequencing scaffold No.3, transcribing a 1,929-nt mRNA that contains a 110-nt 5'UTR (RACE analysis) and 151-nt 3'UTR (transcriptome analysis). *DGA3* gene is composed of 9 exons that are separated by 8 introns, and the encoding 555-aa protein (Dga3) exhibiting strongly homologous to aldehyde dehydrogenase family members (pfam00171) among which it shows the highest identity to *R. toruloides* NP11 (EMS23644.1, 98%). The reported partial protein sequence (ABC41546.1) is located in the C-terminus of Dga3, ranging from 335 aa to 555 aa. Five-aa differences between the partial sequence from *R. glutinis* MTCC 1151 (Gangar et al., 2001; Rani et al., 2013) and the full sequence from *R. toruloides*, where Q₃₃₅, K₃₃₆, C₃₃₇, Y₄₄₈ and F₄₅₃ in *R. toruloides* Dga3 as compared to A₁, R₂, G₃, F₁₁₄ and Y₁₁₉ in the partial sequence of Dga3 from *R. glutinis* MTCC 1151, respectively.

[0120] A phylogenetic tree of DGATs from various species was generated (Fig. 3A). The dendrogram has six main branches such as DGAT1, DGAT2, DGAT3, PDAT ARE1/2 and WSD, where Rt Dga1, Lro1 and Dga3 are clearly clustered into DGAT2, PDAT and DGAT3 group, respectively. *R. toruloides* genome sequence does not have any orthologs of *Y. lipolytica* YlDga2 or any other DGAT1 family members that was essential in TAG biosynthesis (Beopoulos et al., 2012) (Fig. 3A). Surprisingly, Rt Are1 shows weak homology to Yl Dga2p and was clustered into the DGAT1 group rather than the Are1/2 group (Fig. 3A). Hence, it suggests the possible DGAT1 activity of Are1.

[0121] To further identify the DGATs in *R. toruloides*, the transmembrane structures were predicted. Obviously, Lro1 and Dga3 are soluble proteins, while Dga1 and Are1 are both transmembrane proteins with multiple transmembrane regions (Fig. 3B). Dga1 has two highly probable transmembrane regions, which agrees well with DGAT2 from other species (Turchetto-Zolet et al., 2011). Are1 has 7 highly probable transmembrane regions, exhibiting a conserved distribution on the protein chain as its ortholog in *S. cerevisiae* other than DGAT1 from other species (Turchetto-Zolet et al., 2011), which was further demonstrated as an acyl-CoA dependent sterol acyltransferase.

EXAMPLE 3

Transcriptional Regulation of DGATs During Lipid Accumulation

[0122] To investigate the transcriptional regulation of different DGATs in *R. toruloides* during lipid accumulation, nitrogen sources were depleted in the media and mRNA levels were

quantified using qPCR analysis. Results showed that all DGAT mRNAs were significantly increased after 24 h starvation in nitrogen level (Fig. 4). Induction of *DGAI* and *LROI* occurred after 12 h nitrogen starvation, while decreases in mRNA levels could be observed in *AREI* and *DGA3* (Fig. 4). These suggest the transcriptional up-regulation of all DGAT genes during lipid accumulation.

EXAMPLE 4

Lack of DGATs Lead to Serious Lipid Production Defects in *R. toruloides*

[0123] To further functionally identify the effects of *R. toruloides* acyltransferases on lipid accumulation and lipid storage, single gene deletion mutants (*Δdga1*, *Δlro1*, *Δare1* and *Δdga3*) were generated through homologous recombination (Fig. 2A) and confirmed by Southern blot analyses (Fig. 2B). Lack of *DGAI* or *LROI* led to serious cell growth defects (Fig. 5A). Lack of *DGAI* resulted in dramatic decreases in lipid production by only 24% yields (Fig. 5B). However, lack of *LROI*, *AREI* or *DGA3* could improve lipid yields by 113%, 146% and 132%, respectively (Fig. 5B). Stacking deletions of *DGAI* and *LROI* in the double gene deletion mutant *Δdga1Δlro1* (*dl*) resulted in 5.7% lipid yields as compared to WT strain. It was surprised to see the increasing lipid yields when deletion of *AREI* in the background of *Δdga1* (another double gene deletion mutant *Δdga1Δare1*, *da*) from 24% to 29% of the yields in WT (Fig. 5B). However, deletion of *AREI* in the background of *dl* (the triple gene deletion mutant *Δdga1Δlro1Δare1*, *dla*) could further decrease the lipid yields to only 3.7% left (Fig. 5B). Furthermore, deletion of *DGA3* in the background of *dla* (quadruple gene deletion mutant *Δdga1Δlro1Δare1Δdga3*, *dlad*) alternately increased lipid yields from 3.7% to 8.5% (Fig. 5B). Taken together, these suggest that the three DGATs such as Dga1, Lro1 and Are1, would be essential for lipid accumulation in *R. toruloides*, while Dga3 is functionally unidentified.

[0124] To investigate the effects of different DGATs on lipid components, especially triacylglycerol (TAG) and steryl ester (SE), lipids were separated and quantified by TLC analysis. When comparing the four DGAT mutants, only *Δdga1* caused severe decrease (57.83%, Table 3) in triacylglycerol (TAG) yields (Fig. 5C), while other DGAT mutants showed 15~18% increase as compared to WT (Fig. 5C, Table 3), indicating an important role of Dga1 in TAG biosynthesis. As well agreement with above results, further stacking deletion of *LROI* and *AREI* in the double and triple gene deletion mutant, *dl* and *dla*, respectively, resulted in a gradual decrease in TAG level (Fig. 5C, Table 3). However, deletion of *AREI* in *dga1* null mutant (*dl*) showed little effects on TAG levels as compared to *Δdga1* (57.8% to 52.1%, Fig. 5C

and Table 3), indicating little effects of *Are1* on TAG production. As agreement with the total lipid production results, further deletion of *DGA3* resulted in the increase in TAG level and absolute elimination in steryl ester level (Fig. 5C). Hence, the triple deletion mutant (*dla*) could produce the lowest levels of TAG and the highest levels of free fatty acids among all strains tested (Fig. 5C). The fatty acid profiles in *Dgat* mutants are shown in Fig. 6.

[0125] *DGAI* showed little effects on steryl ester (SE) production. Lack of *ARE1* resulted in an obvious decrease (61%) in steryl ester level (Fig. 5C). However, lack of *LRO1* and *DGA3* could significantly enhance the yields of steryl ester by 1.5 and 2 fold, respectively (Fig. 5C, Table 3). Taken together, these suggest a direct role of *Are1*, indirect role of *Lro1* and *Dga3*, while no obvious role of *Dga1* in the biosynthesis of SE.

EXAMPLE 5

Lack of DGATs Affect the Formation of Lipid Bodies

[0126] Lipid body formation is essential for the biosynthesis of intracellular lipids. BODIPY, a highly lipophilic, electrically neutral bright green fluorescent dye, is used as an alternative to stain lipid bodies (Szymanski et al., 2007). High molar extinction coefficient, high oil/water partition coefficient, sharp emission bands, exceptional photo-chemical stability by maintaining fluorescence efficacy through high resistance to photo-bleaching, are among many other advantages of using BODIPY as a lipid stain (Govender et al., 2012). Here, the BODIPY staining coupled with fluorescent microscopy and TEM was used for the identification of lipid bodies. In well agreement with above results, lack of *DGAI* seriously reduced both the number and size of lipid bodies, where little differences could be observed if lacks of the other three single genes (Fig. 7). Formation of lipid bodies was gradually decreased in the double mutant (*da* and *dl*) and triple mutant (*dla*), and few lipid bodies could be observed in either triple mutant (*dla*) or quadruple mutant (*dlad*) (Fig. 7). TEM showed the similar results as the fluorescent microscopic observation by BODIPY-staining (Fig. 7). Furthermore, enlarged and more peroxisomes could be observed during the process of multiple gene deletions (Fig. 7). Together of the studies of lipid production, lipid profiles and lipid body formations, it suggests the essential and different roles of the four DGATs in *R. toruloides*.

EXAMPLE 6

Dga1 and Lro1 are Two Main Acyltransferases for the Biosynthesis of TAG

[0127] Bioinformatical analysis revealed that Dga1 and Lro1 would be the two main DGATs in *R. toruloides* (Fig. 3A), the terminal and only committed step in triacylglycerol biosynthesis. The essential effects of Dga1 and Lro1 on TAG biosynthesis were investigated by overexpression of their encoding genes. *DGAI* and *LROI* were both driven under the strong and constitutive promoter of *GPD1* (Liu et al., 2013b). As compared to WT, although the transcriptional up-regulation of either gene could not fully but partially complement the defects of lipid production in the quadruple mutant *dlad* (Fig. 8A). Due to the inverse effects of *LROI* on lipid production as compared to *DGAI* (Fig. 5B), it is not surprised to observe that overexpression of *LROI* in its null mutant (*Δlro1*) affected little in lipid yields (data not shown). Furthermore, constitutive expression of *DGAI* in its null mutant (*Δdga1*) could lead to an obvious improvement in either TAG or steryl ester levels (Fig. 8B), indicating the pivotal but not enough role of the sole *DGAI* gene in the biosynthesis of lipids, and the complex role of *DGAI* transcript levels on lipid production because of its driven by the *GPD1* promoter. A stacking overexpression of both genes could restore 90% lipid yields in the quadruple mutant *dlad* (Fig. 8C), suggesting the key roles of Dga1 and Lro1 in lipid biosynthesis in *R. toruloides*.

EXAMPLE 7

Dga3 Is a Bifunctional Enzyme

[0128] Previous studies revealed that *Rhodotorula glutinis* Dga3 belonged to the soluble DGAT3 class of acyl-CoA-dependent diacylglycerol acyltransferase, heterologous expression of which could rescue the lipid production and growth defects of *S. cerevisiae* lipid-deficient quadruple mutant H1246 in oleate-containing medium (Rani et al., 2013; Raychaudhuri et al., 2003). To our surprise, lack of Dga3 affected little on either TAG production or lipid body formation in *R. toruloides* (Fig. 5B, 5C and 7, Table 3), indicating the hidden role of Dga3 on lipid biosynthesis by the existence of the other three acyltransferases. To demonstrate this hypothesis, we introduced a constitutive expressed *DGA3* back into the *CAR2*-locus in the quadruple mutant *dlad* and determine its role in lipid production. BODIPY staining revealed that overexpression of *DGA3* could partially restore the formation of lipid bodies in the acyltransferase-disrupted mutant (Fig. 9A). Lipid profiling and quantification confirmed the TAG production were improved by the existence of Dga3 (data not shown). Hence, *in vivo* analysis revealed that Dga3 has the activity of acyltransferase.

[0129] To uncover why oleaginous yeast *R. toruloides* behave a soluble acyltransferase for TAG biosynthesis, we tried to re-evaluate its intracellular function. Surprisingly, bioinformatics analysis revealed that Dga3 is the unique ortholog of *S. cerevisiae* Δ^1 -pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12, Put2p, NP_011902.1, 46.1% identity), catalyzing the oxidation of pyrroline-5-carboxylate to glutamate in proline utilization pathway (Brandriss, 1983; Krzywicki and Brandriss, 1984). This suggests a potential function of Dga3 in proline utilization pathway.

[0130] Drop assay showed the growth defects of *dga3* null mutant ($\Delta dga3$) if cultured in media with the sole nitrogen source of proline, where cell propagation of $\Delta dga3$ was completely eliminated if proline was used as the unique carbon and nitrogen source (Fig. 9B). However, supplementation of an alternative nitrogen source (urea) or the enzymatic products of Put2p (glutamate or arginine) (Brandriss and Magasanik, 1979) showed little growth defects on $\Delta dga3$ (Fig. 9A). Collectively, *R. toruloides* Dga3 is a bi-functional enzyme, acyltransferase and Δ^1 -pyrroline-5-carboxylate dehydrogenase.

EXAMPLE 8

Metabolic Engineering of *R. toruloides* to Produce Fatty Alcohol

[0131] To identify the industrial application of lipid-less quadruple mutant *dlad*, as a principle of proof, fatty alcohols, one of the important oleochemicals, was designed to be produced in the oleaginous yeast *R. toruloides*. Till now, the most efficient fatty alcohol conversion enzyme is fatty acyl-CoA reductase from *M. aquaeolei* VT8 (Maqu_2220) (Liu et al., 2013a; Willis et al., 2011). A new Maqu_2220 encoding gene was designed based on the codon bias of *R. toruloides* (designated RtFAR1; SEQ ID NO:13), driven under three strong endogenic promoters with different regulatory profiles such as the lipid accumulation-correlated promoter of perilipin gene (*PLN1in*; SEQ ID NO:15) and two constitutive promoters of glyceraldehyde-3-phosphate dehydrogenase gene (*GPD1*) (Liu et al., 2013b; SEQ ID NO:16) and elongation factor 1 α gene (*TEF1in*; SEQ ID NO:17) (Fig. 10A). The triple fused RtFAR1 expression cassettes were integrated into the *CAR2*-locus of *R. toruloides* wild type and *dlad* quadruple mutant strains, in which the position effects of ectopic integrations were eliminated (Liu et al., 2015). TLC separation and GC-MS analysis confirmed the formation of fatty alcohols, dominantly in C16-OH (palmityl alcohol) and C18-OH (stearyl alcohol), with small amount of C18:1-OH (oleyl alcohol) (Fig. 11B and Fig. 10B). Surprisingly, fatty alcohols produced in *R. toruloides* ATCC 10657 were dominantly distributed within the cells (Fig. 11B), against dominant extracellular distribution in another *R. toruloides* strain, CECT 13085 (Fillet et

al., 2015). Under shaking flask fermentation in GJm3 medium, a simple and excellent lipid-producing medium routinely used in our lab, the titer of fatty alcohol from strain ATCC 10657 were lower than that from strain CECT 13085 (Fillet et al., 2015), about 0.3 g/L (Fig. 11B). As expected, quadruple disruption of DGAT genes could significantly improve the yields of fatty alcohols by 5 fold (0.8 g/L, Fig. 11B). Supprisingly, simple disruption of $\Delta 12,15$ -bifunctional fatty acid desaturase gene (*FAD2*, our unpublished data) could also result in a similar yield (Fig. 11B). Further block of fatty acid β -oxidation pathway by disruption of the most effective peroxisomal acyl-CoA oxidase isozyme (*Pox1*, our unpublished data) resulted in 1.8-fold improvement in fatty alcohol titer, reaching 1.4 g/L (Fig. 11B). However, the combined disruption of DGAT genes, *POX1* and *FAD2* in the sextuple mutant (*dga1lro1are1dga3pox1fad2, dladpf*) led to a significant decrease in fatty alcohol titer, even lower than the use of WT as the host strain (Fig. 11B).

[0132] To clarify why different strain of *R. toruloides* produced different titers of fatty alcohol and to further improve the yields, we optimized the media with the best engineering strain *dladpFAR1*. Based on the basal media used previously (Fillet et al., 2015), we firstly studied the effects of different carbon (glucose and sucrose) and organic nitrogen sources (yeast extract and corn steep liquid). Four media, named as SY, DY, SC and DC, showed significant differences on fatty alcohol production (Fig. 12A and 12B). As compared to the low effects of carbon source, nitrogen source affected greatly on product titers, in which two yeast extract-containing media, SY and DY, resulted in extremely high titer of fatty alcohol (11.5 and 12.4 g/L, respectively), and 37% and 30% of which is secreted into the media, respectively (Fig. 9B). Production in medium DY also achieved the highest productivity of 0.1 g/L/h, occupied about 42% of cell dry weight (data not shown), and BODIPY staining and lipid profiling showed that the residual cells still kept a low lipid content state (Fig. 10C).

BIBLIOGRAPHY

- [0133]** Athenstaedt, K., 2011. YALI0E32769g (*DGA1*) and YALI0E16797g (*LRO1*) encode major triacylglycerol synthases of the oleaginous yeast *Yarrowia lipolytica*. *Biochimica et biophysica acta*. 1811, 587-96.
- [0134]** Beopoulos, A., Haddouche, R., Kabran, P., Dulermo, T., Chardot, T., Nicaud, J. M., 2012. Identification and characterization of *DGA2*, an acyltransferase of the *DGAT1* acyl-CoA:diacylglycerol acyltransferase family in the oleaginous yeast *Yarrowia lipolytica*. *New insights into the storage lipid metabolism of oleaginous yeasts*. *Applied microbiology and biotechnology*. 93, 1523-37.

- [0135] Biermann, U., Bornscheuer, U., Meier, M. A., Metzger, J. O., Schafer, H. J., 2011. Oils and fats as renewable raw materials in chemistry. *Angew Chem Int Ed Engl.* 50, 3854-71.
- [0136] Brandriss, M. C., 1983. Proline utilization in *Saccharomyces cerevisiae*: analysis of the cloned PUT2 gene. *Mol Cell Biol.* 3, 1846-56.
- [0137] Brandriss, M. C., 1987. Evidence for positive regulation of the proline utilization pathway in *Saccharomyces cerevisiae*. *Genetics.* 117, 429-35.
- [0138] Brandriss, M. C., Magasanik, B., 1979. Genetics and physiology of proline utilization in *Saccharomyces cerevisiae*: enzyme induction by proline. *Journal of bacteriology.* 140, 498-503.
- [0139] Cai, L., Sun, L., Fu, L., Ji, L., 2009. media compositions, selection methods and agrobacterium strains for transformation of plants. PCT patent. WO 2011068468 A1.
- [0140] Carlsson, A. S., Yilmaz, J. L., Green, A. G., Stymne, S., Hofvander, P., 2011. Replacing fossil oil with fresh oil - with what and for what? *Eur J Lipid Sci Technol.* 113, 812-831.
- [0141] Chi, X., Hu, R., Zhang, X., Chen, M., Chen, N., Pan, L., Wang, T., Wang, M., Yang, Z., Wang, Q., Yu, S., 2014. Cloning and functional analysis of three diacylglycerol acyltransferase genes from peanut (*Arachis hypogaea* L.). *PLoS One.* 9, e105834.
- [0142] Coleman, R. A., Lee, D. P., 2004. Enzymes of triacylglycerol synthesis and their regulation. *Progress in lipid research.* 43, 134-76.
- [0143] Czabany, T., Athenstaedt, K., Daum, G., 2007. Synthesis, storage and degradation of neutral lipids in yeast. *Biochimica et biophysica acta.* 1771, 299-309.
- [0144] Dahlqvist, A., Ståhl, U., Lenman, M., Banas, A., Lee, M., Sandager, L., Ronne, H., Stymne, S., 2000. Phospholipid: diacylglycerol acyltransferase: an enzyme that catalyzes the acyl-CoA-independent formation of triacylglycerol in yeast and plants. *Proceedings of the National Academy of Sciences.* 97, 6487-6492.
- [0145] Elsey, D., Jameson, D., Raleigh, B., Cooney, M. J., 2007. Fluorescent measurement of microalgal neutral lipids. *J Microbiol Methods.* 68, 639-42.
- [0146] Fillet, S., Gibert, J., Suarez, B., Lara, A., Ronchel, C., Adrio, J. L., 2015. Fatty alcohols production by oleaginous yeast. *Journal of industrial microbiology & biotechnology.* 42, 1463-72.
- [0147] Gangar, A., Karande, A. A., Rajasekharan, R., 2001. Isolation and localization of a cytosolic 10 S triacylglycerol biosynthetic multienzyme complex from oleaginous yeast. *The Journal of biological chemistry.* 276, 10290-8.
- [0148] Govender, T., Ramanna, L., Rawat, I., Bux, F., 2012. BODIPY staining, an alternative to the Nile Red fluorescence method for the evaluation of intracellular lipids in microalgae. *Bioresource Technology.* 114, 507-511.
- [0149] Gunstone, F. D., Harwood, J. L., Dijkstra, A. J., 2012. The lipid handbook with CD-ROM. CRC Press.
- [0150] Haushalter, R. W., Groff, D., Deutsch, S., The, L., Chavkin, T. A., Brunner, S. F., Katz, L., Keasling, J. D., 2015. Development of an orthogonal fatty acid biosynthesis system in *E. coli* for oleochemical production. *Metab Eng.* 30, 1-6.

- [0151] Hofvander, P., Doan, T. T., Hamberg, M., 2011. A prokaryotic acyl-CoA reductase performing reduction of fatty acyl-CoA to fatty alcohol. *FEBS Lett.* 585, 3538-43.
- [0152] Jin, G., Zhang, Y., Shen, H., Yang, X., Xie, H., Zhao, Z. K., 2013. Fatty acid ethyl esters production in aqueous phase by the oleaginous yeast *Rhodospiridium toruloides*. *Bioresour Technol.* 150, 266-70.
- [0153] Kalscheuer, R., Steinbuchel, A., 2003. A novel bifunctional wax ester synthase/acyl-CoA:diacylglycerol acyltransferase mediates wax ester and triacylglycerol biosynthesis in *Acinetobacter calcoaceticus* ADP1. *The Journal of biological chemistry.* 278, 8075-82.
- [0154] Keasling, J. D., 2010. Manufacturing molecules through metabolic engineering. *Science.* 330, 1355-8.
- [0155] Koh, C. M., Liu, Y., Du, M., Ji, L., 2014. Molecular characterization of *KU70* and *KU80* homologues and exploitation of a *KU70*-deficient mutant for improving gene deletion frequency in *Rhodospiridium toruloides*. *BMC Microbiology.* 14, 50.
- [0156] Krzywicki, K. A., Brandriss, M. C., 1984. Primary structure of the nuclear *PUT2* gene involved in the mitochondrial pathway for proline utilization in *Saccharomyces cerevisiae*. *Mol Cell Biol.* 4, 2837-42.
- [0157] Lazo, G. R., Stein, P. A., Ludwig, R. A., 1991. A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. *Biotechnology (N Y).* 9, 963-7.
- [0158] Liu, A., Tan, X., Yao, L., Lu, X., 2013a. Fatty alcohol production in engineered *E. coli* expressing *Marinobacter* fatty acyl-CoA reductases. *Applied microbiology and biotechnology.* 97, 7061-71.
- [0159] Liu, H., Zhao, X., Wang, F., Li, Y., Jiang, X., Ye, M., Zhao, Z. K., Zou, H., 2009. Comparative proteomic analysis of *Rhodospiridium toruloides* during lipid accumulation. *Yeast.* 26, 553-66.
- [0160] Liu, Y., Koh, C. M., Sun, L., Hlaing, M. M., Du, M., Peng, N., Ji, L., 2013b. Characterization of glyceraldehyde-3-phosphate dehydrogenase gene *RtGPD1* and development of genetic transformation method by dominant selection in oleaginous yeast *Rhodospiridium toruloides*. *Appl Microbiol Biotechnol.* 97, 719-29.
- [0161] Liu, Y., Koh, C. M., Sun, L., Ji, L., 2011. Tartronate semialdehyde reductase defines a novel rate-limiting step in assimilation and bioconversion of glycerol in *Ustilago maydis*. *PLoS One.* 6, e16438.
- [0162] Liu, Y., Koh, C. M. J., Ngoh, S. T., Ji, L., 2015. Engineering an efficient and tight d-amino acid-inducible gene expression system in *Rhodospiridium/Rhodotorula* species. *Microbial Cell Factories.* 14, 170-185.
- [0163] Lung, S. C., Weselake, R. J., 2006. Diacylglycerol acyltransferase: a key mediator of plant triacylglycerol synthesis. *Lipids.* 41, 1073-88.
- [0164] Mysara, M. et al. (2011). MysiRNA-designer: a workflow for efficient siRNA design. *PLOS one* 6(10):e25642.
- [0165] Noweck, K., Grafahrend, W., Fatty alcohols. *Ullmann's encyclopedia of industrial chemistry.* Wiley-VCH KGaA, Weinheim, 2000.
- [0166] Oelkers, P., Tinkelenberg, A., Erdeniz, N., Cromley, D., Billheimer, J. T., Sturley, S. L., 2000. A lecithin cholesterol acyltransferase-like gene mediates diacylglycerol esterification in yeast. *The Journal of biological chemistry.* 275, 15609-12.

- [0167] Paul, D., Magbanua, Z., Arick, M., 2nd, French, T., Bridges, S. M., Burgess, S. C., Lawrence, M. L., 2014. Genome Sequence of the Oleaginous Yeast *Rhodotorula glutinis* ATCC 204091. Genome announcements. 2.
- [0168] Pfleger, B. F., Gossing, M., Nielsen, J., 2015. Metabolic engineering strategies for microbial synthesis of oleochemicals. Metab Eng. 29, 1-11.
- [0169] Ran, F. Ann, et al. (2013). Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell 154:1380-1389.
- [0170] Rani, S. H., Saha, S., Rajasekharan, R., 2013. A soluble diacylglycerol acyltransferase is involved in triacylglycerol biosynthesis in the oleaginous yeast *Rhodotorula glutinis*. Microbiology. 159, 155-66.
- [0171] Ratledge, C., Wilkinson, S., 1988a. An overview of microbial lipids. Microbial lipids. 1, 3-22.
- [0172] Ratledge, C., Wilkinson, S. G., 1988b. Microbial lipids. Academic Press London.
- [0173] Ratledge, C., Wynn, J. P., 2002. The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. Adv Appl Microbiol. 51, 1-51.
- [0174] Raychaudhuri, S., Reddy, M. M., Rajkumar, N. R., Rajasekharan, R., 2003. Cytosolic iron superoxide dismutase is a part of the triacylglycerol biosynthetic complex in oleaginous yeast. The Biochemical journal. 372, 587-94.
- [0175] Sampaio, J. P., Gadanho, M., Bauer, R., Weiß, M., 2003. Taxonomic studies in the Microbotryomycetidae: *Leucosporidium golubevii* sp. nov., *Leucosporidiella* gen. nov. and the new orders Leucosporidiales and Sporidiobolales. Mycol Prog. 2, 53-68.
- [0176] Sorger, D., Daum, G., 2002. Synthesis of triacylglycerols by the acyl-coenzyme A:diacyl-glycerol acyltransferase Dga1p in lipid particles of the yeast *Saccharomyces cerevisiae*. Journal of bacteriology. 184, 519-24.
- [0177] Sorger, D., Daum, G., 2003. Triacylglycerol biosynthesis in yeast. Applied microbiology and biotechnology. 61, 289-99.
- [0178] Szymanski, K. M., Binns, D., Bartz, R., Grishin, N. V., Li, W.-P., Agarwal, A. K., Garg, A., Anderson, R. G. W., Goodman, J. M., 2007. The lipodystrophy protein seipin is found at endoplasmic reticulum lipid droplet junctions and is important for droplet morphology. Proceedings of the National Academy of Sciences. 104, 20890-20895.
- [0179] Tang, X., Chen, W. N., 2015. Enhanced production of fatty alcohols by engineering the TAGs synthesis pathway in *Saccharomyces cerevisiae*. Biotechnol Bioeng. 112, 386-92.
- [0180] Turchetto-Zolet, A. C., Maraschin, F. S., de Moraes, G. L., Cagliari, A., Andrade, C. M., Margis-Pinheiro, M., Margis, R., 2011. Evolutionary view of acyl-CoA diacylglycerol acyltransferase (DGAT), a key enzyme in neutral lipid biosynthesis. BMC evolutionary biology. 11, 263.
- [0181] Turcotte, G., Kosaric, N., 1988. Biosynthesis of lipids by *Rhodospiridium toruloides* ATCC 10788. J Biotechnol. 8, 221-237.
- [0182] Voelker, T. A., Davies, H. M., 1994. Alteration of the specificity and regulation of fatty acid synthesis of *Escherichia coli* by expression of a plant medium-chain acyl-acyl carrier protein thioesterase. J Bacteriol. 176, 7320-7.

- [0183] Wang, S. S., Brandriss, M. C., 1987. Proline utilization in *Saccharomyces cerevisiae*: sequence, regulation, and mitochondrial localization of the PUT1 gene product. *Mol Cell Biol.* 7, 4431-40.
- [0184] Wesley, S.V. et al. (2001). Construct design for efficient, effective and high-throughput gene silencing in plants. *Plant J* 27:581-590.
- [0185] Willis, R. M., Wahlen, B. D., Seefeldt, L. C., Barney, B. M., 2011. Characterization of a fatty acyl-CoA reductase from *Marinobacter aquaeolei* VT8: a bacterial enzyme catalyzing the reduction of fatty acyl-CoA to fatty alcohol. *Biochemistry.* 50, 10550-8.
- [0186] Yang, F., Zhang, S., Zhou, Y. J., Zhu, Z., Lin, X., Zhao, Z. K., 2012. Characterization of the mitochondrial NAD(+)-dependent isocitrate dehydrogenase of the oleaginous yeast *Rhodospiridium toruloides*. *Applied microbiology and biotechnology.*
- [0187] Yen, C. L., Stone, S. J., Koliwad, S., Harris, C., Farese, R. V., Jr., 2008. Thematic review series: glycerolipids. DGAT enzymes and triacylglycerol biosynthesis. *Journal of lipid research.* 49, 2283-301.
- [0188] Yan, P. et al. (2012). High-throughput construction of intron-containing hairpin RNA vectors for RNAi in plants. *PLOS one* 7(5):e38186.
- [0189] Yoon, K., Han, D., Li, Y., Sommerfeld, M., Hu, Q., 2012. Phospholipid:diacylglycerol acyltransferase is a multifunctional enzyme involved in membrane lipid turnover and degradation while synthesizing triacylglycerol in the unicellular green microalga *Chlamydomonas reinhardtii*. *The Plant cell.* 24, 3708-24.
- [0190] Youngquist, J. T., Schumacher, M. H., Rose, J. P., Raines, T. C., Politz, M. C., Copeland, M. F., Pfleger, B. F., 2013. Production of medium chain length fatty alcohols from glucose in *Escherichia coli*. *Metab Eng.* 20, 177-86.
- [0191] Youssar, L., Avalos, J., 2007. Genetic basis of the *ovc* phenotype of *Neurospora*: identification and analysis of a 77 kb deletion. *Current genetics.* 51, 19-30.
- [0192] Zhao, X., Wu, S., Hu, C., Wang, Q., Hua, Y., Zhao, Z. K., 2010. Lipid production from Jerusalem artichoke by *Rhodospiridium toruloides* Y4. *J Ind Microbiol Biotechnol.* 37, 581-5.
- [0193] Zheng, Y. N., Li, L. L., Liu, Q., Yang, J. M., Wang, X. W., Liu, W., Xu, X., Liu, H., Zhao, G., Xian, M., 2012. Optimization of fatty alcohol biosynthesis pathway for selectively enhanced production of C12/14 and C16/18 fatty alcohols in engineered *Escherichia coli*. *Microb Cell Fact.* 11, 65.

Claims:

1. A genetically modified fungal cell useful for producing fatty alcohols that comprises:
 - (a) a nucleic acid construct for overexpression of a heterologous fatty acyl-CoA reductase (FAR), wherein the nucleic acid construct for overexpressing the heterologous FAR comprises multiple copies of a nucleic acid encoding the heterologous FAR, wherein each copy of the nucleic acid encoding the heterologous FAR is operably linked to a different strong constitutive promoter, wherein the heterologous FAR has the amino acid sequence set forth in SEQ ID NO: 14, and wherein the overexpression of the heterologous FAR is in comparison to the corresponding non-genetically modified fungal cell;
 - (b) a deletion in an endogenous gene encoding a type 1 acyl-CoA:diacylglycerol acyltransferase (Dga1);
 - (c) a deletion in an endogenous gene encoding a type 2 phospholipid:diacylglycerol acyltransferase (Lro1);
 - (d) a deletion in an endogenous gene encoding an acyl-CoA:sterol acyltransferase (sterol ester synthase, Are1); and
 - (e) a deletion in an endogenous gene encoding a type 3 soluble acyltransferase (Dga3), wherein the fungal cell is from a *Rhodospiridium* species or a *Rhodotorula* species.
2. The genetically modified fungal cell of claim 1, further comprising a deletion in an endogenous gene encoding an acyl-CoA oxidase 1 (Pox1).
3. The genetically modified fungal cell of claim 1, wherein the fungal cell is a strain of *Rhodospiridium toruloides*.
4. The genetically modified fungal cell of claim 1, wherein FAR is encoded by a nucleic acid having the nucleotide sequence set forth in SEQ ID NO:13.
5. The genetically modified fungal cell of claim 1, wherein the nucleic acid construct comprises three copies of the nucleic acid encoding the heterologous FAR.

6. The genetically modified fungal cell of claim 5, wherein the strong constitutive promoters are a *RtPLN1_{in}* promoter having the nucleotide sequence set forth in SEQ ID NO:15, a *RtGPD1* promoter having the nucleotide sequence set forth in SEQ ID NO:16, and a *RtTEF1_{in}* promoter having the nucleotide sequence set forth in SEQ ID NO:17.
7. The genetically modified fungal cell of claim 3, wherein the strain of *Rhodospordium toruloides* is *Rhodospordium toruloides* ATCC 10657.
8. The genetically modified fungal cell of claim 2, wherein the fungal cell is a strain of *Rhodospordium toruloides*.
9. The genetically modified fungal cell of claim 8, wherein the strain of *Rhodospordium toruloides* is *Rhodospordium toruloides* ATCC 10657.
10. The genetically modified fungal cell of claim 2, wherein the nucleic acid construct comprises three copies of the nucleic acid encoding the heterologous FAR.
11. The genetically modified fungal cell of claim 10, wherein the strong constitutive promoters are a *RtPLN1_{in}* promoter having the nucleotide sequence set forth in SEQ ID NO:15, a *RtGPD1* promoter having the nucleotide sequence set forth in SEQ ID NO:16, and a *RtTEF1_{in}* promoter having the nucleotide sequence set forth in SEQ ID NO:17.
12. The genetically modified fungal cell of claim 1, wherein Dga1 has the amino acid sequence set forth in SEQ ID NO:6, Lro1 has the amino acid sequence set forth in SEQ ID NO:8, Are1 has the amino acid sequence set forth in SEQ ID NO:10, and Dga3 has the amino acid sequence set forth in SEQ ID NO:12.
13. The genetically modified fungal cell of claim 12, wherein Dga1 is encoded by a nucleic acid having the nucleotide sequence set forth in SEQ ID NO:5, Lro1 is encoded by a nucleic acid having the nucleotide sequence set forth in SEQ ID NO:7, Are1 is encoded by a nucleic acid having the nucleotide sequence set forth in SEQ ID NO:9, and Dga3 is encoded by a nucleic acid having the nucleotide sequence set forth in SEQ ID NO:11.

14. The genetically modified fungal cell of claim 2, wherein Dga1 has the amino acid sequence set forth in SEQ ID NO:6, Lro1 has the amino acid sequence set forth in SEQ ID NO:8, Are1 has the amino acid sequence set forth in SEQ ID NO:10, Dga3 has the amino acid sequence set forth in SEQ ID NO:12, and, Pox1 has the amino acid sequence set forth in SEQ ID NO:61.
15. The genetically modified fungal cell of claim 14, wherein Dga1 is encoded by a nucleic acid having the nucleotide sequence set forth in SEQ ID NO:5, Lro1 is encoded by a nucleic acid having the nucleotide sequence set forth in SEQ ID NO:7, Are1 is encoded by a nucleic acid having the nucleotide sequence set forth in SEQ ID NO:9, Dga3 is encoded by a nucleic acid having the nucleotide sequence set forth in SEQ ID NO:11; and Pox1 is encoded by a nucleic acid having the nucleotide sequence set forth in SEQ ID NO:60.
16. A method of producing fatty alcohols comprising culturing the genetically modified fungal cell of any one of claims 1-15 under conditions suitable for growth of the modified fungal cell and for production of fatty alcohols.
17. The method of claim 16, wherein the culturing is performed using a medium comprising glucose, yeast extract, K_2HPO_4 , NH_4NO_3 , $MgSO_4 \cdot 7H_2O$, and $CaCl_2 \cdot 2H_2O$.
18. The method of claim 17, wherein the culturing is performed using a medium comprising per litre: 100 g glucose, 22.5 g yeast extract, 0.75 g K_2HPO_4 , 0.7 g NH_4NO_3 , 0.4 g $MgSO_4 \cdot 7H_2O$, and 0.4 g $CaCl_2 \cdot 2H_2O$.