TAILORED OILS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. 119(e) of US Provisional Patent Application Nos.: 61/887,268, filed October 4, 2013; 61/892,399, filed October 17, 2013; 61/895,355, filed October 24, 2013; 61/923,327, filed January 3, 2014; and 62/023,109, filed July 10, 2014. Each of these applications is incorporated herein by reference in its entirety for all purposes. This application includes subject matter related to that disclosed in US Provisional Patent Application No. 62/023,112, entitled "Novel Ketoacyl ACP Synthase Genes and Uses Thereof," filed July 10, 2014, which is also hereby incorporated by reference in its entirety for all purposes. In particular, Tables 1, 7 and 8 of 62/023,112, and the corresponding sequences identified therein, are hereby incorporated by reference.

REFERENCE TO A SEQUENCE LISTING

[0002] This application includes a sequence listing appended hereto.

FIELD OF THE INVENTION

[0003] Embodiments of the present invention relate to oils/fats, fuels, foods, and oleochemicals and their production from cultures of genetically engineered cells. Specific embodiments relate to oils with a high content of triglycerides bearing fatty acyl groups upon the glycerol backbone in particular regiospecific patterns, highly stable oils, oils with high levels of oleic or mid-chain fatty acids, and products produced from such oils.

BACKGROUND OF THE INVENTION

[0004] PCT Publications WO2008/151149, WO2010/06032, WO2011/150410, WO2011/150411, WO2012/061647, and WO2012/106560 disclose oils and methods for producing those oils in microbes, including microalgae. These publications also describe the use of such oils to make oleochemicals and fuels.

[0005] Tempering is a process of converting a fat into a desired polymorphic form by manipulation of the temperature of the fat or fat-containing substance, commonly used in chocolate making.

[0006] Certain enzymes of the fatty acyl-CoA elongation pathway function to extend the length of fatty acyl-CoA molecules. Elongase-complex enzymes extend fatty acyl-CoA molecules in 2 carbon additions, for example myristoyl-CoA to palmitoyl-CoA, stearoyl-CoA to arachidyl-CoA, or oleoyl-CoA to eicosanoyl-CoA, eicosanoyl-CoA to erucyl-CoA. In addition, elongase enzymes also extend acyl chain length in 2 carbon increments. KCS enzymes condense acyl-CoA molecules with two carbons from malonyl-CoA to form beta-

ketoacyl-CoA. KCS and elongases may show specificity for condensing acyl substrates of particular carbon length, modification (such as hydroxylation), or degree of saturation. For example, the jojoba (*Simmondsia chinensis*) beta-ketoacyl-CoA synthase has been demonstrated to prefer monounsaturated and saturated C18- and C20-CoA substrates to elevate production of erucic acid in transgenic plants (Lassner et al., *Plant Cell*, 1996, Vol 8(2), pp. 281-292), whereas specific elongase enzymes of *Trypanosoma brucei* show preference for elongating short and midchain saturated CoA substrates (Lee et al., *Cell*, 2006, Vol 126(4), pp. 691-9).

[0007] The type II fatty acid biosynthetic pathway employs a series of reactions catalyzed by soluble proteins with intermediates shuttled between enzymes as thioesters of acyl carrier protein (ACP). By contrast, the type I fatty acid biosynthetic pathway uses a single, large multifunctional polypeptide.

[0008] The oleaginous, non-photosynthetic alga, *Prototheca moriformis*, stores copious amounts of triacylglyceride oil under conditions when the nutritional carbon supply is in excess, but cell division is inhibited due to limitation of other essential nutrients. Bulk biosynthesis of fatty acids with carbon chain lengths up to C18 occurs in the plastids; fatty acids are then exported to the endoplasmic reticulum where (if it occurs) elongation past C18 and incorporation into triacylglycerides (TAGs) is believed to occur. Lipids are stored in large cytoplasmic organelles called lipid bodies until environmental conditions change to favor growth, whereupon they are mobilized to provide energy and carbon molecules for anabolic metabolism.

SUMMARY OF THE INVENTION

[0009] In accordance with an embodiment, a method includes cultivating a recombinant cell, the cell

(i) expressing an exogenous KASI or KASIV gene, optionally encoding a protein having at least 60, 65, 70, 75, 80, 85, 90, or 95% amino acid sequence identity to an enzyme encoded by any of SEQ ID NOs: 46-49, and at least one FATB acyl-ACP thioesterase gene optionally encoding a protein having at least 60, 65, 70, 75, 80, 85, 90, or 95% nucleic acid sequence identity to SEQ ID NOs: 11, 87, 89, 159, 162 or 163;

(ii) expressing a gene encoding a FATA, FATB, KASI, KASII, LPAAT, SAD, or FAD2 under the control of a nitrogen-sensitive promoter having at least 60, 65, 70, 75, 80, 85, 90, or 95% sequence identity to any of SEQ ID NOs: 129 to 147; or

(iii) having a knockout or knockdown of a SAD gene, a FAD2 gene, and a FATA gene, an overexpressing an exogenous C18-preferring FATA gene, an oleoyl-preferring LPAAT gene, and a KASII gene; and

extracting oil from the cell.

[0010] In a related embodiment, the cell is of type (ii) and comprises at least a second acyl-ACP thioesterase, optionally encoding a protein having at least 60, 65, 70, 75, 80, 85, 90, or 95% nucleic acid sequence identity to any of SEQ ID NOS: : 11, 87, 89, 159, 162 or 163. The oil can have at least 30% C10:0 and at least 30% C12:0. The oil can have a viscosity of less than 30 cS and optionally of 25 cS ± 20% at 40°C as measured by ASTM D445. The C10:0 and C12:0 fatty acids can be balanced to within 20%, 10% or 5%.

[0011] In a related embodiment, the cell is of type (iii) and the cell oil comprises at least 60% stearate-oleate-stearate (SOS). Optionally,the C18-preferring FATA gene encodes a protein with at least 60, 65, 70, 75, 80, 85, 90, or 95% amino acid identity to SEQ ID NO: 156, the LPAAT gene encodes a protein with at least 60, 65, 70, 75, 80, 85, 90, or 95% amino acid identity to SEQ ID NO: 157 and/or the KASII gene encodes a protein with at least 60, 65, 70, 75, 80, 85, 90, or 95% amino acid identity to SEQ ID NO 160 or 161.

[0012] Optionally, the cell is a microalga, optionally of Trebouxiophyceae, and optionally of the genus Prototheca.

[0013] In a related embodiment, there is an oil, soap, oleochemical, foodstuff, or other oil-derived product produced according to one of the aforementioned methods.

[0014] In accordance with an embodiment of the present invention, a method comprises cultivating an oleaginous recombinant cell. The cell comprises an exogenous gene encoding a palmitate ACP-desaturase enzyme active to produce an oil having a fatty acid profile characterized by a ratio of palmitoleic acid to palmitic acid of at least 0.1 and/or palmitoleic acid levels of 0.5 % or more, as determined by FAME GC/FID analysis. Optionally, the cell is of an oleaginous recombinant eukaryotic microalga.

[0015] In related embodiments, the exogenous gene encodes a palmitoyl-ACP desaturase (PAD) having desaturating activity toward ACP-palmitate. Optionally, the exogenous PAD gene encodes a stearoyl-ACP desaturase variant having increased activity toward ACP-palmitate. The variant can be a L118W mutant. The gene can be in operable linkage with a promoter, plastid-targeting transit peptide, and 5'UTR active to express the gene product in a eukaryotic oleaginous microalga. The microalga can be of Trebouxiophyceae, and optionally of the genus Chlorella or Prototheca. Alternately, the microalga has 23S rRNA with at least 65, 70, 75, 80, 85, 90 or 95% nucleotide sequence identity to SEQ ID NO: 76.

[0016] Optionally, the fatty acid profile is further characterized by less than 3.5% saturated fatty acids. Optionally, the cell is cultivated to at least 40% oil by dry cell weight. Optionally, the microalga further comprises a knockout or knockdown of an endogenous acyl-ACP thioesterase and/or an exogenous KASII gene. This may reduce the levels of saturated fatty acids in the oil. For example, the exogenous KASII gene can be inserted into the coding region of the endogenous acyl-ACP thioesterase. Optionally, the inserted KASII gene is inverted in orientation relative to the endogenous acyl-ACP thioesterase.

[0017] In any of these embodiments, the oil can be produced by heterotrophically cultivating the microalga on sucrose and the microalga comprises an exogenous invertase gene that allows it to metabolize the sucrose.

[0018] The oil may be recovered. The recovered oil may be used for frying or as an ingredient in a prepared food. The oil may have a microalgal sterol profile. In a specific embodiment, the microalgal sterol profile is characterized by an excess of ergosterol over β-sitosterol and/or the presence of 22, 23-dihydrobrassicasterol, poriferasterol or clionasterol.

[0019] In another embodiment, a method comprises cultivating an oleaginous cell, optionally a microalga, so that the cell produces an oil with less than 10% palmitic acid, greater than Optionally the cell is a microalga with FAD and FATA knockouts and expresses an exogenous KASII gene.

[0020] In a related embodiment, a method comprises cultivating an oleaginous cell, optionally a microalga, so that the cell produces an oil with a fatty acid profile in which: the sum of lauric and myristic acids is at least 50%; total saturated fatty acids are at least 50% and levels of capric and lauric fatty acids are balanced to within 20%; or capric acid is at least 45% and lauric acid is at least 45%. In specific related embodiments the sum of lauric and myristic acids is at least 60%, 70% or 7%%. Optionally, the cell comprises an exogenous plant FATB gene.

Optionally, the cell comprises an exogenous exogenous KASI or KASIV gene.

[0021] The oil may be recovered. The recovered oil may be used for frying or as an ingredient in a prepared food. The oil may have a microalgal sterol profile. In a specific embodiment, the microalgal sterol profile is characterized by an excess of ergosterol over β-sitosterol and/or the presence of 22, 23-dihydrobrassicasterol, poriferasterol or clionasterol. The oil can be used to make a foodstuff or chemical.

[0022] In another embodiment, a method comprises cultivating an oleaginous cell, optionally a microalga, so that the cell produces an oil with a fatty acid profile characterized by 10% or less linolenic acid and 20% or more linoleic acid. The cell can comprise an

overexpressed KASII gene and a FAD gene replacement. Optionally, the cell comprises an exogenous gene encoding an oleate-specific acyl-ACP thioesterase or a knockout of one or more FATA alleles, together with an exogenous gene encoding an oleate-specific acyl-ACP thioesterase. The overexpression of the FAD gene can be by environmental control of a regulatable promoter. The oil can be recovered and used to produce a foodstuff or chemicals. The oil may comprise a microalgal sterol profile.

[0023] In another aspect, the present invention provides a method for producing a triglyceride oil, in which the method comprises: (a) cultivating an oleaginous cell under nitrogen-replete conditions, thereby increasing the number of cells, then; (b) cultivating the cells under nitrogen-poor conditions thereby causing the cells to accumulate triglycerides to at least 20% by dry cell weight; comprising a FADc (FAD2) allele, optionally a sole allele, under control of a promoter that is active under the nitrogen replete conditions and inactive under the nitrogen-starved conditions, the promoter retaining at least half of its activity at pH 5.0 as compared to pH 7.0; and (c) obtaining the oil, wherein the oil comprises reduced linoleic acid due to the downregulation of the FADc gene under the nitrogen-starved conditions.

[0024] In some embodiments, the cell is cultivated at a pH of less than 6.5 using sucrose in the presence of invertase. In some cases, the invertase is produced by the cell. In some cases, the invertase is produced from an exogenous gene expressed by the cell.

[0025] In some embodiments, the oil obtained has a fatty acid profile with less than 3%, 2%, 1%, or .5% linoleic acid.

[0026] In some embodiments, the cell further comprises a FADc knockout so as to amplify the change in linoleic acid. In some cases, the transcript level of FADc decreases by a factor of 10 or more between the nitrogen-replete and nitrogen-starved conditions.

[0027] In another aspect, the present invention provides a method for producing a triglyceride cell oil comprising cultivating a recombinant cell comprising an exogenous FATB gene and an exogenous KASI gene, wherein the expression of the KASI gene causes the oil to have a shorter chain distribution relative to a control cell with the FATB gene but without the KASI gene.

[0028] In another aspect, the present invention provides a recombinant cell comprising a FATB acyl-ACP thioesterase gene having at least 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 88% nucleotide identity to SEQ ID NOs: 90 or 91 or equivalent sequence due to the degeneracy of the genetic code, or encoding an enzyme having at least 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 88% amino acid identity to SEQ ID NOs: 90 or 91. In some

embodiments, the cell produces triglycerides that are shifted in fatty acid profile due to expression of the FATB gene.

[0029] In an embodiment of the invention, there is a process for producing an oil. The process includes obtaining a cell oil from a genetically engineered microbe, optionally a microalga, and fractionating the cell oil to produce a stearin fraction. The stearin fraction can be characterized by a TAG profile having at least 70% SOS with no more than 4% trisaturates and an sn-2 profile characterized by least 90% oleate at the sn-2 position. Optionally, the microbe is a microalga comprising one or more of an overexpressed KASII gene, a SAD knockout or knockdown, or an exogenous C18-preferring FATA gene, an exogenous LPAAT, and a FAD2 knockout or knockdown. Optionally, the stearin fraction has a maximum heat-flow temperatures or DSC-derived SFC curve that is an essentially identical to the equivalent curve of Kokum butter. The fractionation can be a two step fractionation performed at a first temperature that removes OOS, optionally about 24°C, and a second temperature that removes trisaturates, optionally about 29°C.

[0030] In accordance with an embodiment of the invention a method produces a triglyceride oil characterized by a TAG profile. The method includes providing an oleaginous plastidic host cell overexpressing a KASII gene, an exogenous FATA gene and an exogenous LPAAT gene, cultivating the cell so as to produce the oil, and isolating the oil; the TAG profile has greater than 50% SOS an less than 10% trisaturates.

[0031] In related embodiments, the cell includes a knockdown or knockout of an endogenous SAD2 gene and/or knockdown or knockout of an endogenous FATA gene. The exogenous FATA gene can encode a functional FATA acyl-ACP thioesterase protein with at least 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity to SEQ ID NO: 92. The exogenous LPAAT gene can encode a functional Lysophosphatidic acid acyltransferase protein with at least 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity to SEQ ID NO: 93. Optionally, the host cell can be a microalga, optionally of Trebouxiophyceae, and optionally of the genus Chlorella or Prototheca, and optionally having 23S rRNA with at least 65, 70, 75, 80, 85, 90 or 95% nucleotide sequence identity to SEQ ID NO: 76.

[0032] In an embodiment, a recombinant microlagal host cell optionally of Trebouxiophyceae, and optionally of the genus Chlorella or Prototheca, and optionally having 23S rRNA with at least 65, 70, 75, 80, 85, 90 or 95% nucleotide sequence identity to SEQ ID NO: 76, expresses an exogenous FATA gene encodes a functional FATA acyl-ACP thioesterase protein with at least 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity to SEQ ID NO: 92.

[0033] In an embodiment, a recombinant microlagal host cell optionally of Trebouxiophyceae, and optionally of the genus Chlorella or Prototheca, and optionally having 23S rRNA with at least 65, 70, 75, 80, 85, 90 or 95% nucleotide sequence identity to SEQ ID NO: 76, expresses an exogenous LPAAT gene encodes a functional Lysophosphatidic acid acyltransferase protein with at least 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity to SEQ ID NO: 93.

[0034] These and other aspects and embodiments of the invention are described and/or exemplified in the accompanying drawings, a brief description of which immediately follows, the detailed description of the invention, and in the examples. Any or all of the features discussed above and throughout the application can be combined in various embodiments of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0035] Figures 1-14 show fatty acid profiles and melting curves of refined, bleached and deodorized oils from genetically engineered *Prototheca moriformis* strains, as discussed in Example 4;

[0036] Figure 15 shows the stability of different oils as a function of antioxidant concentration, as discussed in Example 5;

[0037] Figure 16 shows various properties of cell oils with very low levels of polyunsaturated fatty acids in accordance with an embodiment of the invention; and

[0038] Figure 17 shows a plot of percent solid fat content for various oils as follows: (a) P. moriformis RBD oil without lipid pathway engineering; (b) Brazilian cocoa butter +25% milk fat; (c) three replicates of P. moriformis RBD oil from a strain expressing hairpin nucleic acids that reduce levels of a SAD allele thus reducing oleic acid and increasing stearic acid in the TAG profile; (d) P. moriformis RBD oil from a strain overexpressing an endogenous OTE (oleoyl acyl-ACP thioesterase, see Example 45); (e) Malaysian cocoa butter +25% milk fat; and (f) Malaysian cocoa butter. The cocoa butter and cocoa butter milk fat values are literature values (Bailey's Industrial Oils and Fat Products, 6th ed.).

[0039] Figure 18 shows the results of thermal stability testing performed on methylated oil prepared from high-oleic (HO) and high-stability high-oleic (HSAO) triglyceride oils prepared from heterotrophically grown oleaginous microalgae, in comparison to a soya methyl ester control sample.

[0040] Figure 19 shows various properties of high-oleic and high-stability high-oleic algal oils.

[0041] Figure 20 shows TAG composition of Strain K-4, Strain AU and Strain AV oils from flask and fermenter biomass. La = laurate (C12:0), M = myristate (C14:0), P = palmitate (C16:0), Po = palmitoleate (C16:1), S = stearate (C18:0), O = oleate (C18:1), L= linoleate (C18:2), Ln = α-linolenate (C18:3), A = arachidate (C20:0), B = behenate (C22:0), Lg = lignocerate (C24:0), Hx = hexacosanoate (C26:0) S-S-S refers to the sum of TAGs in which all three fatty acids are saturated. In each block of bars, the strains are shown in the order illustrated at the bottom of the figure.

[0042] Figure 21 shows TAG composition of Strain AW Strain AX and Strain AY oils from shake flask biomass. La = laurate (C12:0), M = myristate (C14:0), P = palmitate (C16:0), Po = palmitoleate (C16:1), S = stearate (C18:0), O = oleate (C18:1), L= linoleate (C18:2), Ln = α-linolenate (C18:3), A = arachidate (C20:0), B = behenate (C22:0), Lg = lignocerate (C24:0), Hx = hexacosanoate (C26:0). S-S-S refers to the sum of TAGs in which all three fatty acids are saturated. In each block of bars, the strains are shown in the order illustrated at the bottom of the figure.

[0043] Figure 22 shows the fatty acid profile and solid fat content of a refined, bleached and deodorized myristate rich oil from a genetically engineered *Prototheca moriformis* strain as discussed in Example 52.

[0044] Figure 23 shows the pairwise alignment of heterologous FAE proteins expressed in STRAIN Z.

[0045] Figure 24 shows genetic modification of a microalgal strain to produced double knockouts of FAD2/FADc and FATA.

DETAILED DESCRIPTION OF THE INVENTION

I. DEFINITIONS

[0046] An "allele" refers to a copy of a gene where an organism has multiple similar or identical gene copies, even if on the same chromosome. An allele may encode the same or similar protein.

[0047] In connection with two fatty acids in a fatty acid profile, "balanced" shall mean that the two fatty acids are within a specified percentage of their mean area percent. Thus, for fatty acid a in x% abundance and fatty acid b in y% abundance, the fatty acids are "balanced to within z%" if $|x-((x+y)/2)|$ and $|y-((x+y)/2)|$ are $\leq 100(z)$.

[0048] A "cell oil" or "cell fat" shall mean a predominantly triglyceride oil obtained from an organism, where the oil has not undergone blending with another natural or synthetic oil, or fractionation so as to substantially alter the fatty acid profile of the triglyceride. In connection with an oil comprising triglycerides of a particular regiospecificity, the cell oil or

cell fat has not been subjected to interesterification or other synthetic process to obtain that regiospecific triglyceride profile, rather the regiospecificity is produced naturally, by a cell or population of cells. For a cell oil produced by a cell, the sterol profile of oil is generally determined by the sterols produced by the cell, not by artificial reconstitution of the oil by adding sterols in order to mimic the cell oil. In connection with a cell oil or cell fat, and as used generally throughout the present disclosure, the terms oil and fat are used interchangeably, except where otherwise noted. Thus, an "oil" or a "fat" can be liquid, solid, or partially solid at room temperature, depending on the makeup of the substance and other conditions. Here, the term "fractionation" means removing material from the oil in a way that changes its fatty acid profile relative to the profile produced by the organism, however accomplished. The terms "cell oil" and "cell fat" encompass such oils obtained from an organism, where the oil has undergone minimal processing, including refining, bleaching and/or degumming, which does not substantially change its triglyceride profile. A cell oil can also be a "noninteresterified cell oil", which means that the cell oil has not undergone a process in which fatty acids have been redistributed in their acyl linkages to glycerol and remain essentially in the same configuration as when recovered from the organism.

[0049] "Exogenous gene" shall mean a nucleic acid that codes for the expression of an RNA and/or protein that has been introduced into a cell (*e.g.* by transformation/transfection), and is also referred to as a "transgene". A cell comprising an exogenous gene may be referred to as a recombinant cell, into which additional exogenous gene(s) may be introduced. The exogenous gene may be from a different species (and so heterologous), or from the same species (and so homologous), relative to the cell being transformed. Thus, an exogenous gene can include a homologous gene that occupies a different location in the genome of the cell or is under different control, relative to the endogenous copy of the gene. An exogenous gene may be present in more than one copy in the cell. An exogenous gene may be maintained in a cell as an insertion into the genome (nuclear or plastid) or as an episomal molecule.

[0050] "FADc", also referred to as "FAD2" is a gene encoding a delta-12 fatty acid desaturase.

[0051] "Fatty acids" shall mean free fatty acids, fatty acid salts, or fatty acyl moieties in a glycerolipid. It will be understood that fatty acyl groups of glycerolipids can be described in terms of the carboxylic acid or anion of a carboxylic acid that is produced when the triglyceride is hydrolyzed or saponified.

[0052] "Fixed carbon source" is a molecule(s) containing carbon, typically an organic molecule that is present at ambient temperature and pressure in solid or liquid form in a

culture media that can be utilized by a microorganism cultured therein. Accordingly, carbon dioxide is not a fixed carbon source.

[0053] "In operable linkage" is a functional linkage between two nucleic acid sequences, such a control sequence (typically a promoter) and the linked sequence (typically a sequence that encodes a protein, also called a coding sequence). A promoter is in operable linkage with an exogenous gene if it can mediate transcription of the gene.

[0054] "Microalgae" are eukaryotic microbial organisms that contain a chloroplast or other plastid, and optionally that is capable of performing photosynthesis, or a prokaryotic microbial organism capable of performing photosynthesis. Microalgae include obligate photoautotrophs, which cannot metabolize a fixed carbon source as energy, as well as heterotrophs, which can live solely off of a fixed carbon source. Microalgae include unicellular organisms that separate from sister cells shortly after cell division, such as *Chlamydomonas*, as well as microbes such as, for example, *Volvox*, which is a simple multicellular photosynthetic microbe of two distinct cell types. Microalgae include cells such as *Chlorella*, *Dunaliella*, and *Prototheca*. Microalgae also include other microbial photosynthetic organisms that exhibit cell-cell adhesion, such as *Agmenellum, Anabaena*, and *Pyrobotrys*. Microalgae also include obligate heterotrophic microorganisms that have lost the ability to perform photosynthesis, such as certain dinoflagellate algae species and species of the genus *Prototheca*.

[0055] In connection with fatty acid length, "mid-chain" shall mean C8 to C16 fatty acids.

[0056] In connection with a recombinant cell, the term "knockdown" refers to a gene that has been partially suppressed (e.g., by about 1-95%) in terms of the production or activity of a protein encoded by the gene.

[0057] Also, in connection with a recombinant cell, the term " knockout" refers to a gene that has been completely or nearly completely (e.g., >95%) suppressed in terms of the production or activity of a protein encoded by the gene. Knockouts can be prepared by homologous recombination of a noncoding sequence into a coding sequence, gene deletion, mutation or other method.

[0058] An "oleaginous" cell is a cell capable of producing at least 20% lipid by dry cell weight, naturally or through recombinant or classical strain improvement. An "oleaginous microbe" or "oleaginous microorganism" is a microbe, including a microalga that is oleaginous (especially eukaryotic microalgae that store lipid). An oleaginous cell also encompasses a cell that has had some or all of its lipid or other content removed, and both live and dead cells.

[0059] An "ordered oil" or "ordered fat" is one that forms crystals that are primarily of a given polymorphic structure. For example, an ordered oil or ordered fat can have crystals that are greater than 50%, 60%, 70%, 80%, or 90% of the β or β' polymorphic form.

[0060] In connection with a cell oil, a "profile" is the distribution of particular species or triglycerides or fatty acyl groups within the oil. A "fatty acid profile" is the distribution of fatty acyl groups in the triglycerides of the oil without reference to attachment to a glycerol backbone. Fatty acid profiles are typically determined by conversion to a fatty acid methyl ester (FAME), followed by gas chromatography (GC) analysis with flame ionization detection (FID), as in Example 1. The fatty acid profile can be expressed as one or more percent of a fatty acid in the total fatty acid signal determined from the area under the curve for that fatty acid. FAME-GC-FID measurement approximate weight percentages of the fatty acids. A "sn-2 profile" is the distribution of fatty acids found at the sn-2 position of the triacylglycerides in the oil. A "regiospecific profile" is the distribution of triglycerides with reference to the positioning of acyl group attachment to the glycerol backbone without reference to stereospecificity. In other words, a regiospecific profile describes acyl group attachment at sn-1/3 vs. sn-2. Thus, in a regiospecific profile, POS (palmitate-oleate-stearate) and SOP (stearate-oleate-palmitate) are treated identically. A "stereospecific profile" describes the attachment of acyl groups at sn-1, sn-2 and sn-3. Unless otherwise indicated, triglycerides such as SOP and POS are to be considered equivalent. A "TAG profile" is the distribution of fatty acids found in the triglycerides with reference to connection to the glycerol backbone, but without reference to the regiospecific nature of the connections. Thus, in a TAG profile, the percent of SSO in the oil is the sum of SSO and SOS, while in a regiospecific profile, the percent of SSO is calculated without inclusion of SOS species in the oil. In contrast to the weight percentages of the FAME-GC-FID analysis, triglyceride percentages are typically given as mole percentages; that is the percent of a given TAG molecule in a TAG mixture.

[0061] The term "percent sequence identity," in the context of two or more amino acid or nucleic acid sequences, refers to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same, when compared and aligned for maximum correspondence, as measured using a sequence comparison algorithm or by visual inspection. For sequence comparison to determine percent nucleotide or amino acid identity, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if

necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters. Optimal alignment of sequences for comparison can be conducted using the NCBI BLAST software (ncbi.nlm.nih.gov/BLAST/) set to default parameters. For example, to compare two nucleic acid sequences, one may use blastn with the "BLAST 2 Sequences" tool Version 2.0.12 (Apr. 21, 2000) set at the following default parameters: Matrix: BLOSUM62; Reward for match: 1; Penalty for mismatch: -2; Open Gap: 5 and Extension Gap: 2 penalties; Gap x drop-off: 50; Expect: 10; Word Size: 11; Filter: on. For a pairwise comparison of two amino acid sequences, one may use the "BLAST 2 Sequences" tool Version 2.0.12 (Apr. 21, 2000) with blastp set, for example, at the following default parameters: Matrix: BLOSUM62; Open Gap: 11 and Extension Gap: 1 penalties; Gap x drop-off 50; Expect: 10; Word Size: 3; Filter: on.

[0062] "Recombinant" is a cell, nucleic acid, protein or vector that has been modified due to the introduction of an exogenous nucleic acid or the alteration of a native nucleic acid. Thus, e.g., recombinant cells can express genes that are not found within the native (non-recombinant) form of the cell or express native genes differently than those genes are expressed by a non-recombinant cell. Recombinant cells can, without limitation, include recombinant nucleic acids that encode for a gene product or for suppression elements such as mutations, knockouts, antisense, interfering RNA (RNAi) or dsRNA that reduce the levels of active gene product in a cell. A "recombinant nucleic acid" is a nucleic acid originally formed in vitro, in general, by the manipulation of nucleic acid, e.g., using polymerases, ligases, exonucleases, and endonucleases, using chemical synthesis, or otherwise is in a form not normally found in nature. Recombinant nucleic acids may be produced, for example, to place two or more nucleic acids in operable linkage. Thus, an isolated nucleic acid or an expression vector formed in vitro by ligating DNA molecules that are not normally joined in nature, are both considered recombinant for the purposes of this invention. Once a recombinant nucleic acid is made and introduced into a host cell or organism, it may replicate using the in vivo cellular machinery of the host cell; however, such nucleic acids, once produced recombinantly, although subsequently replicated intracellularly, are still considered recombinant for purposes of this invention. Similarly, a "recombinant protein" is a protein made using recombinant techniques, *i.e.*, through the expression of a recombinant nucleic acid.

[0063] The terms "triglyceride", "triacylglyceride" and "TAG" are used interchangeably as is known in the art.

II. GENERAL

[0064] Illustrative embodiments of the present invention feature oleaginous cells that produce altered fatty acid profiles and/or altered regiospecific distribution of fatty acids in glycerolipids, and products produced from the cells. Examples of oleaginous cells include microbial cells having a type II fatty acid biosynthetic pathway, including plastidic oleaginous cells such as those of oleaginous algae and, where applicable, oil producing cells of higher plants including but not limited to commercial oilseed crops such as soy, corn, rapeseed/canola, cotton, flax, sunflower, safflower and peanut. Other specific examples of cells include heterotrophic or obligate heterotrophic microalgae of the phylum Chlorophtya, the class Trebouxiophytae, the order Chlorellales, or the family Chlorellacae. Examples of oleaginous microalgae and method of cultivation are also provided in Published PCT Patent Applications WO2008/151149, WO2010/06032, WO2011/150410, and WO2011/150411, including species of Chlorella and Prototheca, a genus comprising obligate heterotrophs. The oleaginous cells can be, for example, capable of producing 25, 30, 40, 50, 60, 70, 80, 85, or about 90% oil by cell weight, ±5%. Optionally, the oils produced can be low in highly unsaturated fatty acids such as DHA or EPA fatty acids. For example, the oils can comprise less than 5%, 2 %, or 1% DHA and/or EPA. The above-mentioned publications also disclose methods for cultivating such cells and extracting oil, especially from microalgal cells; such methods are applicable to the cells disclosed herein and incorporated by reference for these teachings. When microalgal cells are used they can be cultivated autotrophically (unless an obligate heterotroph) or in the dark using a sugar (e.g., glucose, fructose and/or sucrose) In any of the embodiments described herein, the cells can be heterotrophic cells comprising an exogenous invertase gene so as to allow the cells to produce oil from a sucrose feedstock. Alternately, or in addition, the cells can metabolize xylose from cellulosic feedstocks. For example, the cells can be genetically engineered to express one or more xylose metabolism genes such as those encoding an active xylose transporter, a xylulose-5-phosphate transporter, a xylose isomerase, a xylulokinase, a xylitol dehydrogenase and a xylose reductase. See WO2012/154626, "GENETICALLY ENGINEERED MICROORGANISMS THAT METABOLIZE XYLOSE", published Nov 15, 2012, including disclosure of genetically engineered Prototheca strains that utilize xylose.

[0065] The oleaginous cells may, optionally, be cultivated in a bioreactor/fermenter. For example, heterotrophic oleaginous microalgal cells can be cultivated on a sugar-containing

nutrient broth. Optionally, cultivation can proceed in two stages: a seed stage and a lipid-production stage. In the seed stage, the number of cells is increased from s starter culture. Thus, the seeds stage typically includes a nutrient rich, nitrogen replete, media designed to encourage rapid cell division. After the seeds stage, the cells may be fed sugar under nutrient-limiting (e.g. nitrogen sparse) conditions so that the sugar will be converted into triglycerides. For example, the rate of cell division in the lipid-production stage can be decreased by 50%, 80% or more relative to the seed stage. Additionally, variation in the media between the seed stage and the lipid-production stage can induce the recombinant cell to express different lipid-synthesis genes and thereby alter the triglycerides being produced. For example, as discussed below, nitrogen and/or pH sensitive promoters can be placed in front of endogenous or exogenous genes. This is especially useful when an oil is to be produced in the lipid-production phase that does not support optimal growth of the cells in the seed stage. In an example below, a cell has a fatty acid desaturase with a pH sensitive promoter so than an oil that is low in linoleic acid is produced in the lipid production stage while an oil that has adequate linoleic acid for cell division is produced during the seed stage. The resulting low linoleic oil has exceptional oxidative stability.

[0066] The oleaginous cells express one or more exogenous genes encoding fatty acid biosynthesis enzymes. As a result, some embodiments feature cell oils that were not obtainable from a non-plant or non-seed oil, or not obtainable at all.

[0067] The oleaginous cells (optionally microalgal cells) can be improved via classical strain improvement techniques such as UV and/or chemical mutagenesis followed by screening or selection under environmental conditions, including selection on a chemical or biochemical toxin. For example the cells can be selected on a fatty acid synthesis inhibitor, a sugar metabolism inhibitor, or an herbicide. As a result of the selection, strains can be obtained with increased yield on sugar, increased oil production (e.g., as a percent of cell volume, dry weight, or liter of cell culture), or improved fatty acid or TAG profile.

[0068] For example, the cells can be selected on one or more of 1,2-Cyclohexanedione; 19-Norethindone acetate; 2,2-dichloropropionic acid; 2,4,5-trichlorophenoxyacetic acid; 2,4,5-trichlorophenoxyacetic acid, methyl ester; 2,4-dichlorophenoxyacetic acid; 2,4-dichlorophenoxyacetic acid, butyl ester; 2,4-dichlorophenoxyacetic acid, isooctyl ester; 2,4-dichlorophenoxyacetic acid, methyl ester; 2,4-dichlorophenoxybutyric acid; 2,4-dichlorophenoxybutyric acid, methyl ester; 2,6-dichlorobenzonitrile; 2-deoxyglucose; 5-Tetradecyloxy-w-furoic acid; A-922500; acetochlor; alachlor; ametryn; amphotericin; atrazine; benfluralin; bensulide; bentazon; bromacil; bromoxynil; Cafenstrole; carbonyl

cyanide m-chlorophenyl hydrazone (CCCP); carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP); cerulenin; chlorpropham; chlorsulfuron; clofibric acid; clopyralid; colchicine; cycloate; cyclohexamide; C75; DACTHAL (dimethyl tetrachloroterephthalate); dicamba; dichloroprop ((R)-2-(2,4-dichlorophenoxy)propanoic acid); Diflufenican; dihyrojasmonic acid, methyl ester; diquat; diuron; dimethylsulfoxide; Epigallocatechin gallate (EGCG); endothall; ethalfluralin; ethanol; ethofumesate; Fenoxaprop-p-ethyl; Fluazifop-p-Butyl; fluometuron; fomasefen; foramsulfuron; gibberellic acid; glufosinate ammonium; glyphosate; haloxyfop; hexazinone; imazaquin; isoxaben; Lipase inhibitor THL ((-)-Tetrahydrolipstatin); malonic acid; MCPA (2-methyl-4-chlorophenoxyacetic acid); MCPB (4-(4-chloro-o-tolyloxy)butyric acid); mesotrione; methyl dihydrojasmonate; metolachlor; metribuzin; Mildronate; molinate; naptalam; norharman; orlistat; oxadiazon; oxyfluorfen; paraquat; pendimethalin; pentachlorophenol; PF-04620110; phenethyl alcohol; phenmedipham; picloram; Platencin; Platensimycin; prometon; prometryn; pronamide; propachlor; propanil; propazine; pyrazon; Quizalofop-p-ethyl; s-ethyl dipropylthiocarbamate (EPTC); s,s,s-tributylphosphorotrithioate; salicylhydroxamic acid; sesamol; siduron; sodium methane arsenate; simazine; T-863 (DGAT inhibitor) ; tebuthiuron; terbacil; thiobencarb; tralkoxydim; triallate; triclopyr; triclosan; trifluralin; and vulpinic acid.

[0069] The oleaginous cells produce a storage oil, which is primarily triacylglyceride and may be stored in storage bodies of the cell. A raw oil may be obtained from the cells by disrupting the cells and isolating the oil. The raw oil may comprise sterols produced by the cells. WO2008/151149, WO2010/06032, WO2011/150410, and WO2011/1504 disclose heterotrophic cultivation and oil isolation techniques for oleaginous microalgae. For example, oil may be obtained by providing or cultivating, drying and pressing the cells. The oils produced may be refined, bleached and deodorized (RBD) as known in the art or as described in WO2010/120939. The raw or RBD oils may be used in a variety of food, chemical, and industrial products or processes. Even after such processing, the oil may retain a sterol profile characteristic of the source. Microalgal sterol profiles are disclosed below. See especially Section XII of this patent application. After recovery of the oil, a valuable residual biomass remains. Uses for the residual biomass include the production of paper, plastics, absorbents, adsorbents, drilling fluids, as animal feed, for human nutrition, or for fertilizer.

[0070] Where a fatty acid profile of a triglyceride (also referred to as a "triacylglyceride" or "TAG") cell oil is given here, it will be understood that this refers to a nonfractionated sample of the storage oil extracted from the cell analyzed under conditions in which

phospholipids have been removed or with an analysis method that is substantially insensitive to the fatty acids of the phospholipids (e.g. using chromatography and mass spectrometry). The oil may be subjected to an RBD process to remove phospholipids, free fatty acids and odors yet have only minor or negligible changes to the fatty acid profile of the triglycerides in the oil. Because the cells are oleaginous, in some cases the storage oil will constitute the bulk of all the TAGs in the cell. Examples 1, 2, and 8 below give analytical methods for determining TAG fatty acid composition and regiospecific structure.

[0071] Broadly categorized, certain embodiments of the invention include (i) auxotrophs of particular fatty acids; (ii) cells that produce oils having low concentrations of polyunsaturated fatty acids, including cells that are auxotrophic for unsaturated fatty acids; (iii) cells producing oils having high concentrations of particular fatty acids due to expression of one or more exogenous genes encoding enzymes that transfer fatty acids to glycerol or a glycerol ester; (iv) cells producing regiospecific oils, (v) genetic constructs or cells encoding a newly discovered gene encoding an LPAAT enzyme from *Cuphea* PSR23 (see Example 43), (vi) cells producing low levels of saturated fatty acids and/or high levels of palmitoleic acid, (vii) cells producing erucic acid, and (viii) other inventions related to producing cell oils with altered profiles. The embodiments also encompass the oils made by such cells, the residual biomass from such cells after oil extraction, oleochemicals, fuels and food products made from the oils and methods of cultivating the cells.

[0072] In any of the embodiments below, the cells used are optionally cells having a type II fatty acid biosynthetic pathway such as microalgal cells including heterotrophic or obligate heterotrophic microalgal cells, including cells classified as Chlorophyta, Trebouxiophyceae , Chlorellales, Chlorellaceae, or Chlorophyceae, or cells engineered to have a type II fatty acid biosynthetic pathway using the tools of synthetic biology (i.e., transplanting the genetic machinery for a type II fatty acid biosynthesis into an organism lacking such a pathway). Use of a host cell with a type II pathway avoids the potential for non-interaction between an exogenous acyl-ACP thioesterase or other ACP-binding enzyme and the multienzyme complex of type I cellular machinery. In specific embodiments, the cell is of the species *Prototheca moriformis, Prototheca krugani, Prototheca stagnora* or *Prototheca zopfii* or has a 23S rRNA sequence with at least 65, 70, 75, 80, 85, 90 or 95% nucleotide identity SEQ ID NO: 76. By cultivating in the dark or using an obligate heterotroph, the cell oil produced can be low in chlorophyll or other colorants. For example, the cell oil can have less than 100, 50, 10, 5, 1, 0.0.5 ppm of chlorophyll without substantial purification.

[0073] The stable carbon isotope value δ13C is an expression of the ratio of $^{13}C/^{12}C$ relative to a standard (e.g. PDB, carbonite of fossil skeleton of *Belemnite americana* from Peedee formation of South Carolina). The stable carbon isotope value δ13C ($^{0}/_{00}$) of the oils can be related to the δ13C value of the feedstock used. In some embodiments the oils are derived from oleaginous organisms heterotrophically grown on sugar derived from a C4 plant such as corn or sugarcane. In some embodiments the δ13C ($^{0}/_{00}$) of the oil is from -10 to -17 $^{0}/_{00}$ or from -13 to -16 $^{0}/_{00}$.

[0074] In specific embodiments and examples discussed below, one or more fatty acid synthesis genes (e.g., encoding an acyl-ACP thioesterase, a keto-acyl ACP synthase, an LPAAT, a stearoyl ACP desaturase, or others described herein) is incorporated into a microalga. It has been found that for certain microalga, a plant fatty acid synthesis gene product is functional in the absence of the corresponding plant acyl carrier protein (ACP), even when the gene product is an enzyme, such as an acyl-ACP thioesterase, that requires binding of ACP to function. Thus, optionally, the microalgal cells can utilize such genes to make a desired oil without co-expression of the plant ACP gene.

[0075] For the various embodiments of recombinant cells comprising exogenous genes or combinations of genes, it is contemplated that substitution of those genes with genes having 60, 70, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% nucleic acid sequence identity can give similar results, as can substitution of genes encoding proteins having 60, 70, 80, 85, 90, 91, 92, 93, 94, 95, 95.5, 96, 96.5, 97, 97.5, 98, 98.5, 99 or 99.5% amino acid sequence identity. Likewise, for novel regulatory elements, it is contemplated that substitution of those nucleic acids with nucleic acids having 60, 70, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% nucleic acid can be efficacious. In the various embodiments, it will be understood that sequences that are not necessary for function (e.g. FLAG® tags or inserted restriction sites) can often be omitted in use or ignored in comparing genes, proteins and variants.

[0076] Although discovered using or exemplified with microalgae, the novel genes and gene combinations reported here can be used in higher plants using techniques that are well known in the art. For example, the use of exogenous lipid metabolism genes in higher plants is described in U.S. Patents 6028247, 5850022, 5639790, 5455167, 5,512,482,and 5,298,421 disclose higher plants with exogenous acyl-ACP thioesterases. WO2009129582 and WO1995027791 disclose cloning of LPAAT in plants. FAD2 suppression in higher plants is taught in WO 2013112578 , and WO 2008006171.

[0077] As described in Example 63, transcript profiling was used to discover promoters that modulate expression in response to low nitrogen conditions. The promoters are useful to

selectively express various genes and to alter the fatty acid composition of microbial oils. In accordance with an embodiment, there are non-natural constructs comprising a heterologous promoter and a gene, wherein the promoter comprises at least 60, 65, 70, 75, 80, 85, 90, or 95% sequence identity to any of the promoters of Example 63 (e.g., SEQ ID NOs: 130-147) and the gene is differentially expressed under low vs. high nitrogen conditions. Optionally, the expression is less pH sensitive than for the AMT03 promoter. For example, the promoters can be placed in front of a FAD2 gene in a linoleic acid auxotroph to produce an oil with less than 5, 4, 3, 2, or 1% linoleic acid after culturing under high, then low nitrogen conditions.

III. FATTY ACID AUXOTROPHS / REDUCING FATTY ACID LEVELS TO GROWTH INHIBITORY CONDITIONS DURING AN OIL PRODUCTION PHASE

[0078] In an embodiment, the cell is genetically engineered so that all alleles of a lipid pathway gene are knocked out. Alternately, the amount or activity of the gene products of the alleles is knocked down so as to require supplementation with fatty acids. A first transformation construct can be generated bearing donor sequences homologous to one or more of the alleles of the gene. This first transformation construct may be introduced and selection methods followed to obtain an isolated strain characterized by one or more allelic disruptions. Alternatively, a first strain may be created that is engineered to express a selectable marker from an insertion into a first allele, thereby inactivating the first allele. This strain may be used as the host for still further genetic engineering to knockout or knockdown the remaining allele(s) of the lipid pathway gene (e.g., using a second selectable marker to disrupt a second allele). Complementation of the endogenous gene can be achieved through engineered expression of an additional transformation construct bearing the endogenous gene whose activity was originally ablated, or through the expression of a suitable heterologous gene. The expression of the complementing gene can either be regulated constitutively or through regulatable control, thereby allowing for tuning of expression to the desired level so as to permit growth or create an auxotrophic condition at will. In an embodiment, a population of the fatty acid auxotroph cells are used to screen or select for complementing genes; e.g., by transformation with particular gene candidates for exogenous fatty acid synthesis enzymes, or a nucleic acid library believed to contain such candidates.

[0079] Knockout of all alleles of the desired gene and complementation of the knocked-out gene need not be carried out sequentially. The disruption of an endogenous gene of interest and its complementation either by constitutive or inducible expression of a suitable complementing gene can be carried out in several ways. In one method, this can be achieved

by co-transformation of suitable constructs, one disrupting the gene of interest and the second providing complementation at a suitable, alternative locus. In another method, ablation of the target gene can be effected through the direct replacement of the target gene by a suitable gene under control of an inducible promoter ("promoter hijacking"). In this way, expression of the targeted gene is now put under the control of a regulatable promoter. An additional approach is to replace the endogenous regulatory elements of a gene with an exogenous, inducible gene expression system. Under such a regime, the gene of interest can now be turned on or off depending upon the particular needs. A still further method is to create a first strain to express an exogenous gene capable of complementing the gene of interest, then to knockout out or knockdown all alleles of the gene of interest in this first strain. The approach of multiple allelic knockdown or knockout and complementation with exogenous genes may be used to alter the fatty acid profile, regiospecific profile, sn-2 profile, or the TAG profile of the engineered cell.

[0080] Where a regulatable promoter is used, the promoter can be pH-sensitive (e.g., amt03), nitrogen and pH sensitive (e.g., amt03), or nitrogen sensitive but pH-insensitive (e.g., newly discovered promoters of Example 63) or variants therof comprising at least 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98 or 99% sequence identity to any of the aforementioned promoters. In connection with a promoter, pH-inensitive means that the promoter is less sensitive than the amt03 promoter when environmental conditions are shifter from pH 6.8 to 5.0 (e.g., at least 5, 10, 15, or 20% less relative change in activity upon the pH-shift as compared to an equivalent cell with amt03 as the promoter).

[0081] In a specific embodiment, the recombinant cell comprises nucleic acids operable to reduce the activity of an endogenous acyl-ACP thioesterase; for example a FatA or FatB acyl-ACP thioesterase having a preference for hydrolyzing fatty acyl-ACP chains of length C18 (e.g., stearate (C18:0) or oleate (C18:1), or C8:0-C16:0 fatty acids. The activity of an endogenous acyl-ACP thioesterase may be reduced by knockout or knockdown approaches. Knockdown may be achieved, for example, through the use of one or more RNA hairpin constructs, by promoter hijacking (substitution of a lower activity or inducible promoter for the native promoter of an endogenous gene), or by a gene knockout combined with introduction of a similar or identical gene under the control of an inducible promoter. Example 34 describes the engineering of a *Prototheca* strain in which two alleles of the endogenous fatty acyl-ACP thioesterase (FATA1) have been knocked out. The activity of the Prototheca moriformis FATA1 was complemented by the expression of an exogenous FatA or FatB acyl-ACP thioesterase. Example 36 details the use of RNA hairpin constructs to

reduce the expression of FATA in *Prototheca*, which resulted in an altered fatty acid profile having less palmitic acid and more oleic acid.

[0082] Accordingly, oleaginous cells, including those of organisms with a type II fatty acid biosynthetic pathway can have knockouts or knockdowns of acyl-ACP thioesterase-encoding alleles to such a degree as to eliminate or severely limit viability of the cells in the absence of fatty acid supplementation or genetic complementations. These strains can be used to select for transformants expressing acyl-ACP-thioesterase transgenes. Alternately, or in addition, the strains can be used to completely transplant exogenous acyl-ACP-thioesterases to give dramatically different fatty acid profiles of cell oils produced by such cells. For example, FATA expression can be completely or nearly completely eliminated and replaced with FATB genes that produce mid-chain fatty acids. Alternately, an organism with an endogenous FatA gene having specificity for palmitic acid (C16) relative to stearic or oleic acid (C18) can be replaced with an exogenous FatA gene having a greater relative specificity for stearic acid (C18:0) or replaced with an exogenous FatA gene having a greater relative specificity for oleic acid (C18:1). In certain specific embodiments, these transformants with double knockouts of an endogenous acyl-ACP thioesterase produce cell oils with more than 50, 60, 70, 80, or 90% caprylic, capric, lauric, myristic, or palmitic acid, or total fatty acids of chain length less than 18 carbons. Such cells may require supplementation with longer chain fatty acids such as stearic or oleic acid or switching of environmental conditions between growth permissive and restrictive states in the case of an inducible promoter regulating a FatA gene.

[0083] In an embodiment the oleaginous cells are cultured (e.g., in a bioreactor). The cells are fully auxotrophic or partially auxotrophic (i.e., lethality or synthetic sickness) with respect to one or more types of fatty acid. The cells are cultured with supplementation of the fatty acid(s) so as to increase the cell number, then allowing the cells to accumulate oil (e.g. to at least 40% by dry cell weight). Alternatively, the cells comprise a regulatable fatty acid synthesis gene that can be switched in activity based on environmental conditions and the environmental conditions during a first, cell division, phase favor production of the fatty acid and the environmental conditions during a second, oil accumulation, phase disfavor production of the fatty acid. In the case of an inducible gene, the regulation of the inducible gene can be mediated, without limitation, via environmental pH (for example, by using the AMT3 promoter as described in the Examples).

[0084] As a result of applying either of these supplementation or regulation methods, a cell oil may be obtained from the cell that has low amounts of one or more fatty acids essential

for optimal cell propagation. Specific examples of oils that can be obtained include those low in stearic, linoleic and/or linolenic acids.

[0085] These cells and methods are illustrated in connection with low polyunsaturated oils in the section immediately below and in Example 6 (fatty acid desaturase auxotroph) in connection with oils low in polyunsaturated fatty acids and in Example 34 (acyl-ACP thioesterase auxotroph).

[0086] Likewise, fatty acid auxotrophs can be made in other fatty acid synthesis genes including those encoding a SAD, FAD, KASIII, KASI, KASII, KCS, elongase, GPAT, LPAAT, DGAT or AGPAT or PAP. These auxotrophs can also be used to select for complement genes or to eliminate native expression of these genes in favor of desired exogenous genes in order to alter the fatty acid profile, regiospecific profile, or TAG profile of cell oils produced by oleaginous cells.

[0087] Accordingly, in an embodiment of the invention, there is a method for producing an oil/fat. The method comprises cultivating a recombinant oleaginous cell in a growth phase under a first set of conditions that is permissive to cell division so as to increase the number of cells due to the presence of a fatty acid, cultivating the cell in an oil production phase under a second set of conditions that is restrictive to cell division but permissive to production of an oil that is depleted in the fatty acid, and extracting the oil from the cell, wherein the cell has a mutation or exogenous nucleic acids operable to suppress the activity of a fatty acid synthesis enzyme, the enzyme optionally being a stearoyl-ACP desaturase, delta 12 fatty acid desaturase, or a ketoacyl-ACP synthase. The oil produced by the cell can be depleted in the fatty acid by at least 50, 60, 70, 80, or 90%. The cell can be cultivated heterotrophically. The cell can be a microalgal cell cultivated heterotrophically or autotrophically and may produce at least 40, 50, 60, 70, 80, or 90% oil by dry cell weight.

IV. (A) LOW POLYUNSATURATED CELL OILS

[0088] In an embodiment of the present invention, the cell oil produced by the cell has very low levels of polyunsaturated fatty acids. As a result, the cell oil can have improved stability, including oxidative stability. The cell oil can be a liquid or solid at room temperature, or a blend of liquid and solid oils, including the regiospecific or stereospecific oils, high stearate oils, or high mid-chain oils described *infra*. Oxidative stability can be measured by the Rancimat method using the AOCS Cd 12b-92 standard test at a defined temperature. For example, the OSI (oxidative stability index) test may be run at temperatures between 110°C and 140°C. The oil is produced by cultivating cells (e.g., any of the plastidic microbial cells mentioned above or elsewhere herein) that are genetically engineered to reduce the activity of

one or more fatty acid desaturase. For example, the cells may be genetically engineered to reduce the activity of one or more fatty acyl Δ12 desaturase(s) responsible for converting oleic acid (18:1) into linoleic acid (18:2) and/or one or more fatty acyl Δ15 desaturase(s) responsible for converting linoleic acid (18:2) into linolenic acid (18:3). Various methods may be used to inhibit the desaturase including knockout or mutation of one or more alleles of the gene encoding the desaturase in the coding or regulatory regions, inhibition of RNA transcription, or translation of the enzyme, including RNAi, siRNA, miRNA, dsRNA, antisense, and hairpin RNA techniques. Other techniques known in the art can also be used including introducing an exogenous gene that produces an inhibitory protein or other substance that is specific for the desaturase. In specific examples, a knockout of one fatty acyl Δ12 desaturase allele is combined with RNA-level inhibition of a second allele.

[0089] In a specific embodiment, fatty acid desaturase (e.g., Δ12 fatty acid desaturase) activity in the cell is reduced to such a degree that the cell is unable to be cultivated or is difficult to cultivate (e.g., the cell division rate is decreased more than 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, 97 or 99%). Achieving such conditions may involve knockout, or effective suppression of the activity of multiple gene copies (e.g. 2, 3, 4 or more) of the desaturase or their gene products. A specific embodiment includes the cultivation in cell culture of a full or partial fatty acid auxotroph with supplementation of the fatty acid or a mixture of fatty acids so as to increase the cell number, then allowing the cells to accumulate oil (e.g. to at least 40% by cell weight). Alternatively, the cells comprise a regulatable fatty acid synthesis gene that can be switched in activity. For example, the regulation can be based on environmental conditions and the environmental conditions during a first, cell division, phase favor production of the fatty acid and the environmental conditions during a second, oil accumulation, phase disfavor production of the oil. For example, culture media pH and/or nitrogen levels can be used as an environmental control to switch expression of a lipid pathway gene to produce a state of high or low synthetic enzyme activity. Examples of such cells are described in Example 7.

[0090] In a specific embodiment, a cell is cultivated using a modulation of linoleic acid levels within the cell. In particular, the cell oil is produced by cultivating the cells under a first condition that is permissive to an increase in cell number due to the presence of linoleic acid and then cultivating the cells under a second condition that is characterized by linoleic acid starvation and thus is inhibitory to cell division, yet permissive of oil accumulation. For example, a seed culture of the cells may be produced in the presence of linoleic acid added to the culture medium. For example, the addition of linoleic acid to 0.25 g/L in the seed culture

of a *Prototheca* strain deficient in linoleic acid production due to ablation of two alleles of a fatty acyl Δ12 desaturase (i.e., a linoleic auxotroph) was sufficient to support cell division to a level comparable to that of wild type cells. Optionally, the linoleic acid can then be consumed by the cells, or otherwise removed or diluted. The cells are then switched into an oil producing phase (e.g., supplying sugar under nitrogen limiting conditions such as described in WO2010/063032). Surprisingly, oil production has been found to occur even in the absence of linoleic acid production or supplementation, as demonstrated in the obligate heterotroph oleaginous microalgae *Prototheca* but generally applicable to other oleaginous microalgae, microorganisms, or even multicellular organisms (e.g., cultured plant cells). Under these conditions, the oil content of the cell can increase to about 10, 20, 30, 40, 50, 60, 70, 80, 90%, or more by dry cell weight, while the oil produced can have polyunsaturated fatty acid (e.g.; linoleic + linolenic) profile with 5%, 4%, 3%, 2%, 1%, 0.5%, 0.3%, 0.2%, 0.1%, 0.05% or less, as a percent of total triacylglycerol fatty acids in the oil. For example, the oil content of the cell can be 50% or more by dry cell weight and the triglyceride of the oil produced less than 3% polyunsaturated fatty acids.

[0091] These oils can also be produced without the need (or reduced need) to supplement the culture with linoleic acid by using cell machinery to produce the linoleic acid during the cell division phase, but less or no linoleic acid in the lipid production phase. The linoleic-producing cell machinery may be regulatable so as to produce substantially less linoleic acid during the oil producing phase. The regulation may be via modulation of transcription of the desaturase gene(s) or modulation or modulation of production of an inhibitor substance (e.g., regulated production of hairpin RNA/ RNAi). For example, the majority, and preferably all, of the fatty acid Δ12 desaturase activity can be placed under a regulatable promoter regulated to express the desaturase in the cell division phase, but to be reduced or turned off during the oil accumulation phase. The regulation can be linked to a cell culture condition such as pH, and/or nitrogen level, as described in the examples herein, or other environmental condition. In practice, the condition may be manipulated by adding or removing a substance (e.g., protons via addition of acid or base) or by allowing the cells to consume a substance (e.g., nitrogen-supplying nutrients) to effect the desired switch in regulation of the desaturase activity.

[0092] Other genetic or non-genetic methods for regulating the desaturase activity can also be used. For example, an inhibitor of the desaturase can be added to the culture medium in a manner that is effective to inhibit polyunsaturated fatty acids from being produced during the oil production phase.

[0093] Accordingly, in a specific embodiment of the invention, there is a method comprising providing a recombinant cell having a regulatable delta 12 fatty acid desaturase gene, under control of a recombinant regulatory element via an environmental condition. The cell is cultivated under conditions that favor cell multiplication. Upon reaching a given cell density, the cell media is altered to switch the cells to lipid production mode by nutrient limitation (e.g. reduction of available nitrogen). During the lipid production phase, the environmental condition is such that the activity of the delta 12 fatty acid desaturase is downregulated. The cells are then harvested and, optionally, the oil extracted. Due to the low level of delta 12 fatty acid desaturase during the lipid production phase, the oil has less polyunsaturated fatty acids and has improved oxidative stability. Optionally the cells are cultivated heterotrophically and optionally microalgal cells.

[0094] Using one or more of these desaturase regulation methods, it is possible to obtain a cell oil that it is believed has been previously unobtainable, especially in large scale cultivation in a bioreactor (e.g., more than 1000L). The oil can have polyunsaturated fatty acid levels that are 5%, 4%, 3%, 2%, 1%, 0.5%, 0.3%, 0.2%, or less, as an area percent of total triacylglycerol fatty acids in the oil.

[0095] One consequence of having such low levels of polyunsaturates is that oils are exceptionally stable to oxidation. Indeed, in some cases the oils may be more stable than any previously known cell cell oil. In specific embodiments, the oil is stable, without added antioxidants, at 110°C so that the inflection point in conductance is not yet reached by 10 hours, 15 hours, 20 hours, 30 hours, 40, hours, 50 hours, 60 hours, or 70 hours under conditions of the AOCS Cd 12b-92. Rancimat test, noting that for very stable oils, replenishment of water may be required in such a test due to evaporation that occurs with such long testing periods (see Example 5). For example the oil can have and OSI value of 40-50 hours or 41-46 hours at 110°C without added antioxidants. When antioxidants (suitable for foods or otherwise) are added, the OSI value measured may be further increased. For example, with added tocopherol (100ppm) and ascorbyl palmitate (500 ppm) or PANA and ascorbyl palmitate, such an oil can have an oxidative stability index (OSI value) at 110°C in excess 100 or 200 hours, as measured by the Rancimat test. In another example, 1050 ppm of mixed tocopherols and 500 pm of ascorbyl palmitate are added to an oil comprising less than 1% linoleic acid or less than 1% linoleic + linolenic acids; as a result, the oil is stable at 110°C for 1, 2, 3, 4, 5, 6, 7, 8, or 9, 10, 11, 12, 13, 14, 15, or 16, 20, 30, 40 or 50 days, 5 to 15 days, 6 to 14 days, 7 to 13 days, 8 to 12 days, 9 to 11 days, about 10 days, or about 20 days. The oil can also be stable at 130°C for 1, 2, 3, 4, 5, 6, 7, 8, or 9, 10, 11, 12, 13, 14, 15, or 16,

20, 30, 40 or 50 days, 5 to 15 days, 6 to 14 days, 7 to 13 days, 8 to 12 days, 9 to 11 days, about 10 days, or about 20 days. In a specific example, such an oil was found to be stable for greater than 100 hours (about 128 hours as observed). In a further embodiment, the OSI value of the cell oil without added antioxidants at 120°C is greater than 15 hours or 20 hours or is in the range of 10-15, 15-20, 20-25, or 25-50 hours, or 50-100 hours.

[0096] In an example, using these methods, the oil content of a microalgal cell is between 40 and about 85% by dry cell weight and the polyunsaturated fatty acids in the fatty acid profile of the oil is between 0.001% and 3% in the fatty acid profile of the oil and optionally yields a cell oil having an OSI induction time of at least 20 hours at 110°C without the addition of antioxidants. In yet another example, there is a cell oil produced by RBD treatment of a cell oil from an oleaginous cell, the oil comprises between 0.001% and 2% polyunsaturated fatty acids and has an OSI induction time exceeding 30 hours at 110C without the addition of antioxidants. In yet another example, there is a cell oil produced by RBD treatment of a cell oil from an oleaginous cell, the oil comprises between 0.001% and 1% polyunsaturated fatty acids and has an OSI induction time exceeding 30 hours at 110C without the addition of antioxidants.

[0097] In another specific embodiment there is an oil with reduced polyunsaturate levels produced by the above-described methods. The oil is combined with antioxidants such as PANA and ascorbyl palmitate. For example, it was found that when such an oil was combined with 0.5% PANA and 500ppm of ascorbyl palmitate the oil had an OSI value of about 5 days at 130°C or 21 days at 110°C. These remarkable results suggest that not only is the oil exceptionally stable, but these two antioxidants are exceptionally potent stabilizers of triglyceride oils and the combination of these antioxidants may have general applicability including in producing stable biodegradable lubricants (e.g., jet engine lubricants). In specific embodiments, the genetic manipulation of fatty acyl Δ12 desaturase results in a 2 to 30, or 5 to 25, or 10 to 20 fold increase in OSI (e.g., at 110°C) relative to a strain without the manipulation. The oil can be produced by suppressing desaturase activity in a cell, including as described above.

[0098] Antioxidants suitable for use with the oils of the present invention include alpha, delta, and gamma tocopherol (vitamin E), tocotrienol, ascorbic acid (vitamin C), glutathione, lipoic acid, uric acid, β-carotene, lycopene, lutein, retinol (vitamin A), ubiquinol (coenzyme Q), melatonin, resveratrol, flavonoids, rosemary extract, propyl gallate (PG), tertiary butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT), N,N'-di-2-butyl-1,4-phenylenediamine,2,6-di-tert-butyl-4-methylphenol, 2,4-

dimethyl-6-tert-butylphenol, 2,4-dimethyl-6-tert-butylphenol, 2,4-dimethyl-6-tert-butylphenol, 2,6-di-tert-butyl-4-methylphenol, 2,6-di-tert-butylphenol, and phenyl-alpha-naphthylamine (PANA).

[0099] In addition to the desaturase modifications, in a related embodiment other genetic modifications may be made to further tailor the properties of the oil, as described throughout, including introduction or substitution of acyl-ACP thioesterases having altered chain length specificity and/or overexpression of an endogenous or exogenous gene encoding a KAS, SAD, LPAAT, or DGAT gene. For example, a strain that produces elevated oleic levels may also produce low levels of polyunsaturates. Such genetic modifications can include increasing the activity of stearoyl-ACP desaturase (SAD) by introducing an exogenous SAD gene, increasing elongase activity by introducing an exogenous KASII gene, and/or knocking down or knocking out a FATA gene.

[0100] In a specific embodiment, a high oleic cell oil with low polyunsaturates may be produced. For example, the oil may have a fatty acid profile with greater than 60, 70, 80, 90, or 95% oleic acid and less than 5, 4, 3, 2, or 1% polyunsaturates. In related embodiments, a cell oil is produced by a cell having recombinant nucleic acids operable to decrease fatty acid Δ12 desaturase activity and optionally fatty acid Δ15 desaturase so as to produce an oil having less than or equal to 3% polyunsaturated fatty acids with greater than 60% oleic acid, less than 2% polyunsaturated fatty acids and greater than 70% oleic acid, less than 1% polyunsaturated fatty acids and greater than 80% oleic acid, or less than 0.5% polyunsaturated fatty acids and greater than 90% oleic acid. It has been found that one way to increase oleic acid is to use recombinant nucleic acids operable to decrease expression of a FATA acyl-ACP thioesterase and optionally overexpress a KAS II gene; such a cell can produce an oil with greater than or equal to 75% oleic acid. Alternately, overexpression of KASII can be used without the FATA knockout or knockdown. Oleic acid levels can be further increased by reduction of delta 12 fatty acid desaturase activity using the methods above, thereby decreasing the amount of oleic acid the is converted into the unsaturates linoleic acid and linolenic acid. Thus, the oil produced can have a fatty acid profile with at least 75% oleic and at most 3%, 2%, 1%, or 0.5% linoleic acid. In a related example, the oil has between 80 to 95% oleic acid and about 0.001 to 2% linoleic acid, 0.01 to 2% linoleic acid, or 0.1 to 2% linoleic acid. In another related embodiment, an oil is produced by cultivating an oleaginous cell (e.g., a microalga) so that the microbe produces a cell oil with less than 10% palmitic acid, greater than 85% oleic acid, 1% or less polyunsaturated fatty acids, and less than 7% saturated fatty acids. See Example 58 in which such an oil is

produced in a microalga with FAD and FATA knockouts plus expression of an exogenous KASII gene. Such oils will have a low freezing point, with excellent stability and are useful in foods, for frying, fuels, or in chemical applications. Further, these oils may exhibit a reduced propensity to change color over time. In an illustrative chemical application, the high oleic oil is used to produce a chemical. The oleic acid double bonds of the oleic acid groups of the triglycerides in the oil can be epoxidized or hydroxylated to make a polyol. The epoxidized or hydroxylated oil can be used in a variety of applications. One such application is the production of polyurethane (including polyurethane foam) via condensation of the hydroxylated triglyceride with an isocyanate, as has been practiced with hydroxylated soybean oil or castor oil. See, e.g. US2005/0239915, US2009/0176904, US2005/0176839, US2009/0270520, and US Patent No. 4,264,743 and Zlatanic, et al, Biomacromolecules 2002, 3, 1048-1056 (2002) for examples of hydroxylation and polyurethane condensation chemistries. Suitable hydroxyl forming reactions include epoxidation of one or more double bonds of a fatty acid followed by acid catalyzed epoxide ring opening with water (to form a diol), alcohol (to form a hydroxyl ether), or an acid (to form a hydroxyl ester). There are multiple advantages of using the high-oleic/low polyunsaturated oil in producing a bio-based polyurethane: (1) the shelf-life, color or odor, of polyurethane foams may be improved; (2) the reproducibility of the product may be improved due to lack of unwanted side reactions resulting from polyunsaturates; (3) a greater degree of hydroxylation reaction may occur due to lack of polyunsaturates and the structural characteristics of the polyurethane product can be improved accordingly.

[0101] The low-polyunsaturated or high-oleic/low-polyunsaturated oils described here may be advantageously used in chemical applications where yellowing is undesirable. For example, yellowing can be undesirable in paints or coatings made from the triglycerides fatty acids derived from the triglycerides. Yellowing may be caused by reactions involving polyunsaturated fatty acids and tocotrienols and/or tocopherols. Thus, producing the high-stability oil in an oleaginous microbe with low levels of tocotrienols can be advantageous in elevating high color stability a chemical composition made using the oil. In contrast to commonly used plant oils, through appropriate choice of oleaginous microbe, the cell oils of these embodiments can have tocopherols and tocotrienols levels of 1 g/L or less. In a specific embodiment, a cell oil has a fatty acid profile with less than 2% with polyunsaturated fatty acids and less than 1 g/L for tocopherols, tocotrienols or the sum of tocopherols and tocotrienols. In another specific embodiment, the cell oil has a fatty acid profile with less

than 1% with polyunsaturated fatty acids and less than 0.5 g/L for tocopherols, tocotrienols or the sum of tocopherols and tocotrienols

[0102] Any of the high-stability (low-polyunsaturate) cell oils or derivatives thereof can be used to formulate foods, drugs, vitamins, nutraceuticals, personal care or other products, and are especially useful for oxidatively sensitive products. For example, the high-stability cell oil (e.g., less than or equal to 3%, 2% or 1% polyunsaturates) can be used to formulate a sunscreen (e.g. a composition having one or more of avobenzone, homosalate, octisalate, octocrylene or oxybenzone) or retinoid face cream with an increased shelf life due to the absence of free-radical reactions associated with polyunsaturated fatty acids. For example, the shelf-life can be increased in terms of color, odor, organoleptic properties or %active compound remaining after accelerated degradation for 4 weeks at 54°C. The high stability oil can also be used as a lubricant with excellent high-temperature stability. In addition to stability, the oils can be biodegradable, which is a rare combination of properties.

[0103] In another related embodiment, the fatty acid profile of a cell oil is elevated in C8 to C16 fatty acids through additional genetic modification, e.g. through overexpression of a short-chain to mid chain preferring acyl-ACP thioesterase or other modifications described here. A low polyunsaturated oil in accordance with these embodiments can be used for various industrial, food, or consumer products, including those requiring improved oxidative stability. In food applications, the oils may be used for frying with extended life at high temperature, or extended shelf life.

[0104] Where the oil is used for frying, the high stability of the oil may allow for frying without the addition of antioxidant and/or defoamers (e.g. silicone). As a result of omitting defoamers, fried foods may absorb less oil. Where used in fuel applications, either as a triglyceride or processed into biodiesel or renewable diesel (see, e.g., WO2008/151149 WO2010/063032, and WO2011/150410), the high stability can promote storage for long periods, or allow use at elevated temperatures. For example, the fuel made from the high stability oil can be stored for use in a backup generator for more than a year or more than 5 years. The frying oil can have a smoke point of greater than 200°C, and free fatty acids of less than 0.1% (either as a cell oil or after refining).

[0105] The low polyunsaturated oils may be blended with food oils, including structuring fats such as those that form beta or beta prime crystals, including those produced as described below. These oils can also be blended with liquid oils. If mixed with an oil having linoleic acid, such as corn oil, the linoleic acid level of the blend may approximate that of high oleic plant oils such as high oleic sunflower oils (e.g., about 80% oleic and 8% linoleic).

[0106] Blends of the low polyunsaturated cell oil can be interesterified with other oils. For example, the oil can be chemically or enzymatically interesterified. In a specific embodiment, a low polyunsaturated oil according to an embodiment of the invention has at least 10% oleic acid in its fatty acid profile and less than 5% polyunsaturates and is enzymatically interesterified with a high saturate oil (e.g. hydrogenated soybean oil or other oil with high stearate levels) using an enzyme that is specific for sn-1 and sn-2 triacylglycerol positions. The result is an oil that includes a stearate-oleate-stearate (SOS). Methods for interesterification are known in the art; see for example, "Enzymes in Lipid Modification," Uwe T. Bornschuer, ed., Wiley_VCH, 2000, ISBN 3-527-30176-3.

[0107] High stability oils can be used as spray oils. For example, dried fruits such as raisins can be sprayed with a high stability oil having less than 5, 4, 3, 2, or 1% polyunsaturates. As a result, the spray nozzle used will become clogged less frequently due to polymerization or oxidation product buildup in the nozzle that might otherwise result from the presence of polyunsaturates.

[0108] In a further embodiment, an oil that is high is SOS, such as those described below can be improved in stability by knockdown or regulation of delta 12 fatty acid desaturase.

[0109] Optionally, where the FADc promoter is regulated, it can be regulated with a promoter that is operable at low pH (e.g., one for which the level of transcription of FADc is reduced by less than half upon switching from cultivation at pH 7.0 to cultivation at pH 5.0). The promoter can be sensitive to cultivation under low nitrogen conditions such that the promoter is active under nitrogen replete conditions and inactive under nitrogen starved conditions. For example, the promoter may cause a reduction in FADc transcript levels of 5, 10, 15-fold or more upon nitrogen starvation. Because the promoter is operable at pH 5.0, more optimal invertase activity can be obtained. For example, the cell can be cultivated in the presence of invertase at a pH of less than 6.5, 6.0 or 5.5. The cell may have a FADc knockout to increase the relative gene-dosage of the regulated FADc. Optionally, the invertase is produced by the cell (natively or due to an exogenous invertase gene). Because the promoter is less active under nitrogen starved conditions, fatty acid production can proceed during the lipid production phase that would not allow for optimal cell proliferation in the cell proliferation stage. In particular, a low linoleic oil may be produced. The cell can be cultivated to an oil content of at least 20% lipid by dry cell weight. The oil may have a fatty acid profile having less than 5, 4, 3, 2, 1, or 0.5, 0.2, or 0.1% linoleic acid. Example 62 describes the discovery of such promoters.

IV. (B) HIGH 18:2/LOW 18:3 OILS OBTAINED USING FAD GENE REPLACEMENT

[0110] Surprisingly, while researching the production of low polyunsaturate oils as described above, an oil with high polyunsaturates but having a unique fatty acid profile was discovered. The discovery of this oil is described in Example 59. Thus, it is possible to use an oleaginous plastidic cell (e.g., microalgal) culture to produce an oil with a fatty acid profile characterized by 10% or less linolenic acid (C18:3) and 20% or more linoleic acid (C18:2). Such oils can be produced in an oleaginous microalga or other oleaginous plastidic cell by overexpression of a (endogenous or exogenous) KASII and gene replacement of FADc (also referred to as FAD2) and, if necessary based on the host cell, replacing native acyl-ACP thioesterase activity. In Example 58-59, an endogenous KASII was overexpressed and an endogenous FADc gene was placed under control of a pH-inducible promoter, although constitutive expression would also work. Interestingly, the oils were much higher in linoleic acid when the FADc was overexpressed in a linoleic acid auxotroph (e.g., a FADc double knockout). It is believed that this is due to the presence of a previously unrecognized gene-level regulatory system in microalgae that must be disabled in order to efficiently accumulate linoleic acid. In addition, two copies of the endogenous acyl-ACP thioesterase were knocked out and replaced with an oleate-specific plant acyl-ACP thioesterase. Under permissive pH conditions, an oil with 10% or less linolenic acid (C18:3) and 20% or more linoleic acid (C18:2). The oil can be extracted and used for various uses included in foodstuffs or chemicals. If the host cell is a microalga, the oil can comprise microalgal sterols. As with other embodiments, the host cell can be a microalga transformed to express an exogenous invertase, thus enable conversion of sucrose into the oil under conditions of heterotrophic cultivation.

[0111] In a specific embodiment, a host cell comprises a FADc knockdown, knockout, or FADc with a down-regulatable promoter combined with an exogenous KASII gene that expresses a protein having at least 80,85,90,91, 92, 93, 94, 95, 96, 97, 98 or 99% amino acid identity to the protein encoded by the Prototheca moriformis KASII gene disclose in Example 58, and optionally expresses an acyl-ACP thioesterase gene producing an oleate-specific acyl-ACP thioesterase enzyme. Optionally, the cell can be an a plant cell, a microbial cell, or a microalgal cell.

V. CELLS WITH EXOGENOUS ACYLTRANSFERASES

[0112] In various embodiments of the present invention, one or more genes encoding an acyltransferase (an enzyme responsible for the condensation of a fatty acid with glycerol or a

glycerol derivative to form an acylglyceride) can be introduced into an oleaginous cell (e.g., a plastidic microalgal cell) so as to alter the fatty acid composition of a cell oil produced by the cell. The genes may encode one or more of a glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT), also known as 1-acylglycerol-3-phosphate acyltransferase (AGPAT), phosphatidic acid phosphatase (PAP), or diacylglycerol acyltransferase (DGAT) that transfers an acyl group to the sn-3 position of DAG, thereby producing a TAG.

[0113] Recombinant nucleic acids may be integrated into a plasmid or chromosome of the cell. Alternately, the gene encodes an enzyme of a lipid pathway that generates TAG precursor molecules through fatty acyl-CoA-independent routes separate from that above. Acyl-ACPs may be substrates for plastidial GPAT and LPAAT enzymes and/or mitochondrial GPAT and LPAAT enzymes. Among further enzymes capable of incorporating acyl groups (e.g., from membrane phospholipids) to produce TAGs is phospholipid diacylglycerol acyltransferase (PDAT). Still further acyltransferases, including lysophosphosphatidylcholine acyltransferase (LPCAT), lysophosphosphatidylserine acyltransferase (LPSAT), lysophosphosphatidylethanolamine acyltransferase (LPEAT), and lysophosphosphatidylinositol acyltransferase (LPIAT), are involved in phospholipid synthesis and remodeling that may impact triglyceride composition.

[0114] The exogenous gene can encode an acyltransferase enzyme having preferential specificity for transferring an acyl substrate comprising a specific number of carbon atoms and/or a specific degree of saturation is introduced into a oleaginous cell so as to produce an oil enriched in a given regiospecific triglyceride. For example, the coconut (*Cocos nucifera*) lysophosphatidic acid acyltransferase has been demonstrated to prefer C12:0-CoA substrates over other acyl-CoA substrates (Knutzon et al., *Plant Physiology*, Vol. 120, 1999, pp. 739-746), whereas the 1-acyl-sn-3-glycerol-3-phosphate acyltransferase of maturing safflower seeds shows preference for linoleoyl-CoA and oleoyl-CoA substrates over other acyl-CoA substrates, including stearoyl-CoA (Ichihara et al., *European Journal of Biochemistry*, Vol. 167, 1989, pp. 339-347). Furthermore, acyltransferase proteins may demonstrate preferential specificity for one or more short-chain, medium-chain, or long-chain acyl-CoA or acyl-ACP substrates, but the preference may only be encountered where a particular, e.g. medium-chain, acyl group is present in the *sn*-1 or *sn*-3 position of the lysophosphatidic acid donor substrate. As a result of the exogenous gene, a TAG oil can be produced by the cell in which a particular fatty acid is found at the sn-2 position in greater than 20, 30, 40, 50, 60, 70, 90, or 90% of the TAG molecules.

[0115] In some embodiments of the invention, the cell makes an oil rich in saturated-unsaturated-saturated (sat-unsat-sat) TAGs. Sat-unsat-sat TAGS include 1,3-dihexadecanoyl-2-(9Z-octadecenoyl)-glycerol (referred to as 1-palmitoyl-2-oleyl-glycero-3-palmitoyl), 1,3-dioctadecanoyl-2-(9Z-octadecenoyl)-glycerol (referred to as 1- stearoyl -2-oleyl-glycero-3-stearoyl), and 1-hexadecanoyl-2-(9Z-octadecenoyl)-3-octadecanoy-glycerol (referred to as 1-palmitoyl-2-oleyl-glycero-3-stearoyl). These molecules are more commonly referred to as POP, SOS, and POS, respectively, where 'P' represents palmitic acid, 'S' represents stearic acid, and 'O' represents oleic acid. Further examples of saturated-unsaturated-saturated TAGs include MOM, LOL, MOL, COC and COL, where 'M' represents myristic acid, 'L' represents lauric acid, and 'C' represents capric acid (C8:0). Trisaturates, triglycerides with three saturated fatty acyl groups, are commonly sought for use in food applications for their greater rate of crystallization than other types of triglycerides. Examples of trisaturates include PPM, PPP, LLL, SSS, CCC, PPS, PPL, PPM, LLP, and LLS. In addition, the regiospecific distribution of fatty acids in a TAG is an important determinant of the metabolic fate of dietary fat during digestion and absorption.

[0116] According to certain embodiments of the present invention, oleaginous cells are transformed with recombinant nucleic acids so as to produce cell oils that comprise an elevated amount of a specified regiospecific triglyceride, for example 1-acyl-2-oleyl-glycero-3-acyl, or 1-acyl-2-lauric-glycero-3-acyl where oleic or lauric acid respectively is at the *sn*-2 position, as a result of introduced recombinant nucleic acids. Alternately, caprylic, capric, myristic, or palmitic acid may be at the sn-2 position. The amount of the specified regiospecific triglyceride present in the cell oil may be increased by greater than 5%, greater than 10%, greater than 15%, greater than 20%, greater than 25%, greater than 30%, greater than 35%, greater than 40%, greater than 50%, greater than 60%, greater than 70%, greater than 80%, greater than 90%, greater than 100-500%, or greater than 500% than in the cell oil produced by the microorganism without the recombinant nucleic acids. As a result, the sn-2 profile of the cell triglyceride may have greater than 10, 20, 30, 40, 50, 60, 70, 80, or 90% of the particular fatty acid.

[0117] The identity of the acyl chains located at the distinct stereospecific or regiospecific positions in a glycerolipid can be evaluated through one or more analytical methods known in the art (see Luddy *et al.*, *J. Am. Oil Chem. Soc.*, **41**, 693-696 (1964), Brockerhoff, *J. Lipid Res.*, **6**, 10-15 (1965), Angers and Aryl, *J. Am. Oil Chem. Soc.*,Vol. 76:4, (1999), Buchgraber et al., *Eur. J. Lipid Sci. Technol.*, **106**, 621-648 (2004)), or in accordance with Examples 1, 2, and 8 given below.

[0118] The positional distribution of fatty acids in a triglyceride molecule can be influenced by the substrate specificity of acyltransferases and by the concentration and type of available acyl moieties substrate pool. Nonlimiting examples of enzymes suitable for altering the regiospecificity of a triglyceride produced in a recombinant microorganism are listed in Tables 1-4. One of skill in the art may identify additional suitable proteins.

[0119] Table 1. Glycerol-3-phosphate acyltransferases and GenBank accession numbers.

glycerol-3-phosphate acyltransferase	*Arabidopsis thaliana*	BAA00575
glycerol-3-phosphate acyltransferase	*Chlamydomonas reinhardtii*	EDP02129
glycerol-3-phosphate acyltransferase	*Chlamydomonas reinhardtii*	Q886Q7
acyl-(acyl-carrier-protein): glycerol-3-phosphate acyltransferase	*Cucurbita moschata*	BAB39688
glycerol-3-phosphate acyltransferase	*Elaeis guineensis*	AAF64066
glycerol-3-phosphate acyltransferase	*Garcina mangostana*	ABS86942
glycerol-3-phosphate acyltransferase	*Gossypium hirsutum*	ADK23938
glycerol-3-phosphate acyltransferase	*Jatropha curcas*	ADV77219
plastid glycerol-3-phosphate acyltransferase	*Jatropha curcas*	ACR61638
plastidial glycerol-phosphate acyltransferase	*Ricinus communis*	EEF43526
glycerol-3-phosphate acyltransferase	*Vica faba*	AAD05164
glycerol-3-phosphate acyltransferase	*Zea mays*	ACG45812

[0120] Lysophosphatidic acid acyltransferases suitable for use with the microbes and methods of the invention include, without limitation, those listed in Table 2.

[0121] Table 2. Lysophosphatidic acid acyltransferases and GenBank accession numbers.

1-acyl-sn-glycerol-3-phosphate acyltransferase	*Arabidopsis thaliana*	AEE85783
1-acyl-sn-glycerol-3-phosphate acyltransferase	*Brassica juncea*	ABQ42862
1-acyl-sn-glycerol-3-phosphate acyltransferase	*Brassica juncea*	ABM92334
1-acyl-sn-glycerol-3-phosphate acyltransferase	*Brassica napus*	CAB09138
lysophosphatidic acid acyltransferase	*Chlamydomonas reinhardtii*	EDP02300

lysophosphatidic acid acyltransferase	*Limnanthes alba*	AAC49185
1-acyl-sn-glycerol-3-phosphate acyltransferase (putative)	*Limnanthes douglasii*	CAA88620
acyl-CoA:sn-1-acylglycerol-3-phosphate acyltransferase	*Limnanthes douglasii*	ABD62751
1-acylglycerol-3-phosphate O-acyltransferase	*Limnanthes douglasii*	CAA58239
1-acyl-sn-glycerol-3-phosphate acyltransferase	*Ricinus communis*	EEF39377

[0122] Diacylglycerol acyltransferases suitable for use with the microbes and methods of the invention include, without limitation, those listed in Table 3.

[0123] Table 3. Diacylglycerol acyltransferases and GenBank accession numbers.

diacylglycerol acyltransferase	*Arabidopsis thaliana*	CAB45373
diacylglycerol acyltransferase	*Brassica juncea*	AAY40784
putative diacylglycerol acyltransferase	*Elaeis guineensis*	AEQ94187
putative diacylglycerol acyltransferase	*Elaeis guineensis*	AEQ94186
acyl CoA:diacylglycerol acyltransferase	*Glycine max*	AAT73629
diacylglycerol acyltransferase	*Helianthus annus*	ABX61081
acyl-CoA:diacylglycerol acyltransferase 1	*Olea europaea*	AAS01606
diacylglycerol acyltransferase	*Ricinus communis*	AAR11479

[0124] Phospholipid diacylglycerol acyltransferases suitable for use with the microbes and methods of the invention include, without limitation, those listed in Table 4.

[0125] Table 4. Phospholipid diacylglycerol acyltransferases and GenBank accession numbers.

phospholipid:diacylglycerol acyltransferase	*Arabidopsis thaliana*	AED91921
Putative phospholipid:diacylglycerol acyltransferase	*Elaeis guineensis*	AEQ94116
phospholipid:diacylglycerol acyltransferase 1-like	*Glycine max*	XP_003541296
phospholipid:diacylglycerol acyltransferase	*Jatropha curcas*	AEZ56255
phospholipid:diacylglycerol acyltransferase	*Ricinus*	ADK92410

	communis	
phospholipid:diacylglycerol acyltransferase	*Ricinus communis*	AEW99982

[0126] In an embodiment of the invention, known or novel LPAAT genes are transformed into the oleaginous cells so as to alter the fatty acid profile of triglycerides produced by those cells, most notably by altering the sn-2 profile of the triglycerides. For example, by virtue of expressing an exogenous active LPAAT in an oleaginous cell, the percent of unsaturated fatty acid at the sn-2 position is increased by 10, 20, 30, 40, 50, 60, 70, 80, 90% or more. For example, a cell may produce triglycerides with 30% unsaturates (which may be primarily 18:1 and 18:2 and 18:3 fatty acids) at the sn-2 position. In this example, introduction of the LPAAT activity increases the unsaturates at the sn-2 position by 20% so that 36% of the triglycerides comprise unsaturates at the sn-2 position. Alternately, an exogenous LPAAT can be used to increase mid-chain fatty acids including saturated mid-chains such as C8:0, C10:0, C12:0, C14:0 or C16:0 moieties at the sn-2 position. As a result, mid-chain levels in the overall fatty acid profile may be increased. Examples 43 and 44 describe altering the sn-2 and fatty acid profiles in an oleaginous microbe. As can be seen from those examples, the choice of LPAAT gene is important in that different LPAATs can cause a shift in the sn-2 and fatty acid profiles toward different acyl group chain-lengths or saturation levels. For example, the LPAAT of Example 43 increases C10-C14 fatty acids and the LPAAT of Example 44 causes an increase in C16 and C18 fatty acids. As in these examples, introduction of an exogenous LPAAT can be combined with introduction of exogenous acyl-ACP thioesterase. Combining a mid-chain preferring LPAAT and a mid-chain preferring FatB was found to give an additive effect; the fatty acid profile was shifted more toward the mid-chain fatty acids when both an exogenous LPAAT and FatB gene was present than when only an exogenous FatB gene was present. In a specific embodiment, the oil produced by a cell comprising an exogenous mid-chain specific LPAAT and (optionally) an exogenous FatB acyl-ACP thioesterase gene can have a fatty acid profile with 40, 50, 60, 70, 80% or more of C8:0, C10:0, C12:0, C14:0, or C16:0 fatty acids (separately or in sum).

[0127] Specific embodiments of the invention are a nucleic acid construct, a cell comprising the nucleic acid construct, a method of cultivating the cell to produce a triglyceride, and the triglyceride oil produced where the nucleic acid construct has a promoter operably linked to a novel LPAAT coding sequence. The coding sequence can have an

initiation codon upstream and a termination codon downstream followed by a 3 UTR sequence. In a specific embodiment, the LPAAT gene has LPAAT activity and a coding sequence have at least 75, 80, 85, 90, 95, 96, 97, 98, or 99% sequence identity to any of the cDNAs of SEQ ID NOs: 80 to 85 or a functional fragment thereof including equivalent sequences by virtue of degeneracy of the genetic code. Introns can be inserted into the sequence as well. Alternately, the LPAAT gene codes for the amino acid sequence of SEQ ID NOs 77-79 or functional fragments thereof, or a protein having at least 75, 80, 85, 90, 95, 96, 97, 98, or 99% amino acid sequence identity . In addition to microalgae and other oleaginous cells, plants expressing the novel LPAAT as transgenes are expressly included in the embodiments and can be produced using known genetic engineering techniques.

VI. CELLS WITH EXOGENOUS ELONGASES OR ELONGASE COMPLEX ENZYMES

[0128] In various embodiments of the present invention, one or more genes encoding elongases or components of the fatty acyl-CoA elongation complex can be introduced into an oleaginous cell (e.g., a plastidic microalgal cell) so as to alter the fatty acid composition of the cell or of a cell oil produced by the cell. The genes may encode a beta-ketoacyl-CoA synthase (also referred to as 3-ketoacyl synthase, beta-ketoacyl synthase or KCS), a ketoacyl-CoA reductase, a hydroxyacyl-CoA dehydratase, enoyl-CoA reductase, or elongase. The enzymes encoded by these genes are active in the elongation of acyl-coA molecules liberated by acyl-ACP thioesterases. Recombinant nucleic acids may be integrated into a plasmid or chromosome of the cell. In a specific embodiment, the cell is of Chlorophyta, including heterotrophic cells such as those of the genus *Prototheca*.

[0129] Beta-Ketoacyl-CoA synthase and elongase enzymes suitable for use with the microbes and methods of the invention include, without limitation, those listed in Table 5.

[0130] Table 5. Beta-Ketoacyl-CoA synthases and elongases listed with GenBank accession numbers.

Trypanosoma brucei elongase 3 (GenBank Accession No. AAX70673), *Marchanita polymorpha* (GenBank Accession No. AAP74370), *Trypanosoma cruzi* fatty acid elongase, putative (GenBank Accession No. EFZ33366), *Nannochloropsis oculata* fatty acid elongase (GenBank Accession No. ACV21066.1), *Leishmania donovani* fatty acid elongase, putative (GenBank Accession No. CBZ32733.1), Glycine max 3-ketoacyl-CoA synthase 11-like (GenBank Accession No. XP_003524525.1), *Medicago truncatula* beta-ketoacyl-CoA synthase (GenBank Accession No. XP_003609222), Zea mays fatty acid elongase (GenBank

Accession No. ACG36525), Gossypium hirsutum beta-ketoacyl-CoA synthase (GenBank Accession No. ABV60087), *Helianthus annuus* beta-ketoacyl-CoA synthase (GenBank Accession No. ACC60973.1), *Saccharomyces cerevisiae* ELO1 (GenBank Accession No. P39540), *Simmondsia chinensis* beta-ketoacyl-CoA synthase (GenBank Accession No. AAC49186) ,*Tropaeolum majus* putative fatty acid elongase (GenBank Accession No. AAL99199, Brassica napus fatty acid elongase (GenBank Accession No. AAA96054)

[0131] In an embodiment of the invention, an exogenous gene encoding a beta-ketoacyl-CoA synthase or elongase enzyme having preferential specificity for elongating an acyl substrate comprising a specific number of carbon atoms and/or a specific degree of acyl chain saturation is introduced into a oleaginous cell so as to produce a cell or an oil enriched in fatty acids of specified chain length and/or saturation. Example 40 describes engineering of *Prototheca* strains in which exogenous fatty acid elongases with preferences for extending midchain fatty acyl-CoAs have been overexpressed to increase the concentration of stearate. Examples 42 and 54 describe engineering of *Prototheca* in which exogenous elongases or beta-ketoacyl-CoA synthases with preferences for extending monounsaturated and saturated C18- and C20-CoA substrates are overexpressed to increase the concentration of erucic acid.

[0132] In specific embodiments, the oleaginous cell produces an oil comprising greater than 0.5, 1, 2, 5, 10, 20, 30, 40, 50, 60 70, or 80% erucic and/or eicosenoic acid. Alternately, the cell produces an oil comprising 0.5-5, 5-10, 10-15, 15-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, or 90-99% erucic or eicosenoic acid. The cell may comprise recombinant acids described above in connection with high-oleic oils with a further introduction of an exogenous beta-ketoacyl-CoA synthase that is active in elongating oleoyl-CoA. As a result of the expression of the exogenous beta-ketoacyl-CoA synthase, the natural production of erucic or eicosenoic acid by the cell can be increased by more than 2, 3, 4, 5, 10, 20, 30, 40, 50, 70, 100, 130, 170 or 200 fold. The high erucic and/or eicosenoic oil can also be a high stability oil; e.g., one comprising less than 5, 4, 3, 2, or 1% polyunsaturates and/or having the OSI values described in Section IV or this application and accompanying Examples. In a specific embodiment, the cell is a microalgal cell, optionally cultivated heterotrophically. As in the other embodiments, the oil/fat can be produced by genetic engineering of a plastidic cell, including heterotrophic microalgae of the phylum Chlorophyta, the class Trebouxiophytae, the order Chlorellales, or the family Chlorellacae. Preferably, the cell is oleaginous and capable of accumulating at least 40% oil by dry cell weight. The cell can be

an obligate heterotroph, such as a species of Prototheca, including Prototheca moriformis or Prototheca zopfii.

[0133] In specific embodiments, an oleaginous microbial cell, optionally an oleaginous microalgal cell, optionally of the phylum Chlorophyta, the class Trebouxiophytae, the order Chlorellales, or the family Chlorellacae expresses an enzyme having 80, 85, 90, 95, 96, 97, 98, or 99% amino acid sequence identity to an enzyme of Table 5.

VII. REGIOSPECIFIC AND STEREOSPECIFIC OILS/FATS

[0134] In an embodiment, a recombinant cell produces a cell fat or oil having a given regiospecific makeup. As a result, the cell can produce triglyceride fats having a tendency to form crystals of a given polymorphic form; e.g., when heated to above melting temperature and then cooled to below melting temperature of the fat. For example, the fat may tend to form crystal polymorphs of the β or β' form (e.g., as determined by X-ray diffraction analysis), either with or without tempering. The fats may be ordered fats. In specific embodiments, the fat may directly from either β or β' crystals upon cooling; alternatively, the fat can proceed through a β form to a β' form. Such fats can be used as structuring, laminating or coating fats for food applications. The cell fats can be incorporated into candy, dark or white chocolate, chocolate flavored confections, ice cream, margarines or other spreads, cream fillings, pastries, or other food products. Optionally, the fats can be semi-solid (at room temperature) yet free of artificially produced trans-fatty acids. Such fats can also be useful in skin care and other consumer or industrial products.

[0135] As in the other embodiments, the fat can be produced by genetic engineering of a plastidic cell, including heterotrophic eukaryotic microalgae of the phylum Chlorophyta, the class Trebouxiophytae, the order Chlorellales, or the family Chlorellacae. Preferably, the cell is oleaginous and capable of accumulating at least 40% oil by dry cell weight. The cell can be an obligate heterotroph, such as a species of *Prototheca*, including *Prototheca moriformis* or *Prototheca zopfii*. The fats can also be produced in autotrophic algae or plants. Optionally, the cell is capable of using sucrose to produce oil and a recombinant invertase gene may be introduced to allow metabolism of sucrose, as described in PCT Publications WO2008/151149, WO2010/06032, WO2011/150410, WO2011/150411, and international patent application PCT/US12/23696. The invertase may be codon optimized and integrated into a chromosome of the cell, as may all of the genes mentioned here. It has been found that cultivated recombinant microalgae can produce hardstock fats at temperatures below the melting point of the hardstock fat. For example, *Prototheca moriformis* can be altered to

heterotrophically produce triglyceride oil with greater than 50% stearic acid at temperatures in the range of 15 to 30°C, wherein the oil freezes when held at 30°C.

[0136] In an embodiment, the cell fat has at least 30, 40, 50, 60, 70, 80, or 90% fat of the general structure [saturated fatty acid (sn-1)-unsaturated fatty acid (sn-2)-saturated fatty acid (sn-3)]. This is denoted below as Sat-Unsat-Sat fat. In a specific embodiment, the saturated fatty acid in this structure is preferably stearate or palmitate and the unsaturated fatty acid is preferably oleate. As a result, the fat can form primarily β or β' polymorphic crystals, or a mixture of these, and have corresponding physical properties, including those desirable for use in foods or personal care products. For example, the fat can melt at mouth temperature for a food product or skin temperature for a cream, lotion or other personal care product (e.g., a melting temperature of 30 to 40, or 32 to 35°C). Optionally, the fats can have a 2L or 3L lamellar structure (e.g., as determined by X-ray diffraction analysis). Optionally, the fat can form this polymorphic form without tempering.

[0137] In a specific related embodiment, a cell fat triglyceride has a high concentration of SOS (i.e. triglyceride with stearate at the terminal sn-1 and sn-3 positions, with oleate at the sn-2 position of the glycerol backbone). For example, the fat can have triglycerides comprising at least 50, 60, 70, 80 or 90% SOS. In an embodiment, the fat has triglyceride of at least 80% SOS. Optionally, at least 50, 60, 70, 80 or 90% of the sn-2 linked fatty acids are unsaturated fatty acids. In a specific embodiment, at least 95% of the sn-2 linked fatty acids are unsaturated fatty acids. In addition, the SSS (tri-stearate) level can be less than 20, 10 or 5% and/or the C20:0 fatty acid (arachidic acid) level may be less than 6%, and optionally greater than 1% (e.g., from 1 to 5%). For example, in a specific embodiment, a cell fat produced by a recombinant cell has at least 70% SOS triglyceride with at least 80% sn-2 unsaturated fatty acyl moieties. In another specific embodiment, a cell fat produced by a recombinant cell has TAGs with at least 80% SOS triglyceride and with at least 95% sn-2 unsaturated fatty acyl moieties. In yet another specific embodiment, a cell fat produced by a recombinant cell has TAGs with at least 80% SOS, with at least 95% sn-2 unsaturated fatty acyl moieties, and between 1 to 6% C20 fatty acids.

[0138] In yet another specific embodiment, the sum of the percent stearate and palmitate in the fatty acid profile of the cell fat is twice the percentage of oleate, ± 10, 20, 30 or 40% [e.g., (%P+%S)/%O=2.0 ± 20%]. Optionally, the sn-2 profile of this fat is at least 40%, and preferably at least 50, 60, 70, or 80% oleate (at the sn-2 position). Also optionally, this fat may be at least 40, 50, 60, 70, 80, or 90% SOS. Optionally, the fat comprises between 1 to 6% C20 fatty acids.

[0139] In any of these embodiments, the high SatUnsatSat fat may tend to form β' polymorphic crystals. Unlike previously available plant fats like cocoa butter, the SatUnsatSat fat produced by the cell may form β' polymorphic crystals without tempering. In an embodiment, the polymorph forms upon heating to above melting temperature and cooling to less that the melting temperature for 3, 2, 1, or 0.5 hours. In a related embodiment, the polymorph forms upon heating to above 60°C and cooling to 10°C for 3, 2, 1, or 0.5 hours.

[0140] In various embodiments the fat forms polymorphs of the β form, β' form, or both, when heated above melting temperature and the cooled to below melting temperature, and optionally proceeding to at least 50% of polymorphic equilibrium within 5, 4, 3, 2, 1, 0.5 hours or less when heated to above melting temperature and then cooled at 10°C. The fat may form β' crystals at a rate faster than that of cocoa butter.

[0141] Optionally, any of these fats can have less than 2 mole % diacylglycerol, or less than 2 mole% mono and diacylglycerols, in sum.

[0142] In an embodiment, the fat may have a melting temperature of between 30-60°C, 30-40°C, 32 to 37°C, 40 to 60°C or 45 to 55°C. In another embodiment, the fat can have a solid fat content (SFC) of 40 to 50%, 15 to 25%, or less than 15% at 20°C and/or have an SFC of less than 15% at 35°C.

[0143] The cell used to make the fat may include recombinant nucleic acids operable to modify the saturate to unsaturate ratio of the fatty acids in the cell triglyceride in order to favor the formation of SatUnsatSat fat. For example, a knock-out or knock-down of stearoyl-ACP desaturase (SAD) gene can be used to favor the formation of stearate over oleate or expression of an exogenous mid-chain-preferring acyl-ACP thioesterase gene can increase the levels mid-chain saturates. Alternately a gene encoding a SAD enzyme can be overexpressed to increase unsaturates.

[0144] In a specific embodiment, the cell has recombinant nucleic acids operable to elevate the level of stearate in the cell. As a result, the concentration of SOS may be increased. Example 9 demonstrates that the regiospecific profile of the recombinant microbe is enriched for the SatUnsatSat triglycerides POP, POS, and SOS as a result of overexpressing a Brassica napus C18:0-preferring thioesterase. An additional way to increase the stearate of a cell is to decrease oleate levels. For cells having high oleate levels (e.g., in excess of one half the stearate levels) one can also employ recombinant nucleic acids or classical genetic mutations operable to decrease oleate levels. For example, the cell can have a knockout, knockdown, or mutation in one or more FATA alleles, which encode an oleate liberating acyl-ACP

thioesterase, and/or one or more alleles encoding a stearoyl ACP desaturase (SAD). Example 35 describes the inhibition of SAD2 gene product expression using hairpin RNA to produce a fatty acid profile of 37% stearate in *Prototheca moriformis* (UTEX 1435), whereas the wildtype strain produced less than 4% stearate, a more than 9-fold improvement. Moreover, while the strains of Example 35 are engineered to reduce SAD activity, sufficient SAD activity remains to produce enough oleate to make SOS, POP, and POS. See the TAG profiles of Example 47. In specific examples, one of multiple SAD encoding alleles may be knocked out and/or one or more alleles are downregulated using inhibition techniques such as antisense, RNAi, or siRNA, hairpin RNA or a combination thereof. In various embodiments, the cell can produce TAGs that have 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, or 90 to about 100% stearate. In other embodiments, the cells can produce TAGs that are 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, or 90 to about 100% SOS. Optionally, or in addition to genetic modification, stearoyl ACP desaturase can be inhibited chemically; e.g., by addition of sterculic acid to the cell culture during oil production.

[0145] Surprisingly, knockout of a single FATA allele has been found to increase the presence of C18 fatty acids produced in microalgae. By knocking out one allele, or otherwise suppressing the activity of the FATA gene product (e.g., using hairpin RNA), while also suppressing the activity of stearoyl-ACP desaturase (using techniques disclosed herein), stearate levels in the cell can be increased.

[0146] Another genetic modification to increase stearate levels includes increasing a ketoacyl ACP synthase (KAS) activity in the cell so as to increase the rate of stearate production. It has been found that in microalgae, increasing KASII activity is effective in increasing C18 synthesis and particularly effective in elevating stearate levels in cell triglyceride in combination with recombinant DNA effective in decreasing SAD activity. Recombinant nucleic acids operable to increase KASII (e.g., an exogenous KasII gene) can be also be combined with a knockout or knockdown of a FatA gene, or with knockouts or knockdowns of both a FatA gene and a SAD gene). Optionally, the KASII gene encodes a protein having at least 75, 80, 85, 90, 95, 96, 97, 98, or 99% amino acid identity to the KASII Prototheca moriformis (mature protein given in SEQ ID NO: 161), or any plant KASII gene disclosed herein (e.g., in Example 60) or known in the art including a microalgal KASII.

[0147] Optionally, the cell can include an exogenous stearate-liberating acyl-ACP thioesterase, either as a sole modification or in combination with one or more other stearate-increasing genetic modifications. For example the cell may be engineered to overexpress an acyl-ACP thioesterase with preference for cleaving C18:0-ACPs. Example 9 describes the

expression of exogenous C18:0-preferring acyl-ACP thioesterases to increase stearate in the fatty acid profile of the microalgae, *Prototheca moriformis* (UTEX 1435), from about 3.7% to about 30.4% (over 8-fold). Example 41 provides additional examples of C18:0-preferring acyl-ACP thioesterases function to elevate C18:0 levels in *Prototheca*. Optionally, the stearate-preferring acyl-ACP thioesterase gene encodes an enzyme having at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 9% amino acid identity to the gene products of Example 9 or 41 (Seq ID NOS. 28, 65, 67, 69, 71, 73, or 75 omitting FLAG tags when present). Introduction of the acyl-ACP -thioesterase can be combined with a knockout or knockdown of one or more endogenous acyl-ACP thioesterase alleles. Introduction of the thioesterase can also be combined with overexpression of an elongase (KCS) or beta-ketoacyl-CoA synthase. In addition, one or more exogenous genes (e.g., encoding SAD or KASII) can be regulated via an environmental condition (e.g., by placement in operable linkage with a regulatable promoter). In a specific example, pH and/or nitrogen level is used to regulate an amt03 promoter. The environmental condition may then be modulated to tune the cell to produce the desired amount of stearate appearing in cell triglycerides (e.g., to twice the oleate concentration). As a result of these manipulations, the cell may exhibit an increase in stearate of at least 5, 10, 15, or 20 fold.

[0148] As a further modification, alone or in combination with the other stearate increasing modifications, the cell can comprise recombinant nucleic acids operable to express an elongase or a beta-ketoacyl-CoA synthase. For example, overexpression of a C18:0-preferring acyl-ACP thioesterases may be combined with overexpression of a midchain-extending elongase or KCS to increase the production of stearate in the recombinant cell. One or more of the exogenous genes (e.g., encoding a thioesterase, elongase, or KCS) can be regulated via an environmental condition (e.g., by placement in operable linkage with a regulatable promoter). In a specific example, pH and/or nitrogen level is used to regulate an amt03 promoter or any of the promoters of example 63 including those that are less pH-sensitive than amt03. The environmental condition may then be modulated to tune the cell to produce the desired amount of stearate appearing in cell triglycerides (e.g., to twice the oleate concentration). As a result of these manipulations, the cell may exhibit an increase in stearate of at least 5, 10, 15, or 20 fold. In addition to stearate, arachidic, behenic, lignoceric, and cerotic acids may also be produced.

[0149] In specific embodiments, due to the genetic manipulations of the cell to increase stearate levels, the ratio of stearate to oleate in the oil produced by the cell is 2:1 ± 30% (i.e., in the range of 1.4:1 to 2.6:1), 2:1 ± 20% or 2:1 ± 10%.

[0150] Alternately, the cell can be engineered to favor formation of SatUnsatSat where Sat is palmitate or a mixture of palmitate and stearate. In this case introduction of an exogenous palmitate liberating acyl-ACP thioesterase can promote palmitate formation. In this embodiment, the cell can produce triglycerides, that are at least 30, 40, 50, 60, 70, or 80% POP, or triglycerides in which the sum of POP, SOS, and POS is at least 30, 40, 50, 60, 70, 80, or 90% of cell triglycerides. In other related embodiments, the POS level is at least 30, 40, 50, 60, 70, 80, or 90% of the triglycerides produced by the cell.

[0151] In a specific embodiment, the melting temperature of the oil is similar to that of cocoa butter (about 30-32°C). The POP, POS and SOS levels can approximate cocoa butter at about 16, 38, and 23% respectively. For example, POP can be 16% ±20%, POS can be 38%±20%, and SOS can be 23% ± 20%. Or, POP can be 16% ±15%, POS can be 38%±15%, an SOS can be 23%±15%. Or, POP can be 16% ±10%, POS can be 38%±10%, an SOS can be 23%±10%.

[0152] As a result of the recombinant nucleic acids that increase stearate, a proportion of the fatty acid profile may be arachidic acid. For example, the fatty acid profile can be 0.01% to 5%, 0.1 to 4%, or 1 to 3% arachidic acid. Furthermore, the regiospecific profile may have 0.01% to 4%, 0.05% to 3%, or 0.07% to 2% AOS, or may have 0.01% to 4%, 0.05% to 3%, or 0.07% to 2% AOA. It is believed that AOS and AOA may reduce blooming and fat migration in confection comprising the fats of the present invention, among other potential benefits.

[0153] In addition to the manipulations designed to increase stearate and/or palmitate, and to modify the SatUnsatSat levels, the levels of polyunsaturates may be suppressed, including as described above by reducing delta 12 fatty acid desaturase activity (e.g., as encoded by a Fad gene) and optionally supplementing the growth medium or regulating FAD expression. It has been discovered that, in microalgae (as evidenced by work in *Prototheca* strains), polyunsaturates are preferentially added to the sn-2 position. Thus, to elevate the percent of triglycerides with oleate at the sn-2 position, production of linoleic acid by the cell may be suppressed. The techniques described herein, in connection with highly oxidatively stable oils, for inhibiting or ablating fatty acid desaturase (FAD) genes or gene products may be applied with good effect toward producing SatUnsatSat oils by reducing polyunsaturates at the sn-2 position. As an added benefit, such oils can have improved oxidatively stability. As also described herein, the fats may be produced in two stages with polyunsaturates supplied or produced by the cell in the first stage with a deficit of polyunsaturates during the fat producing stage. The fat produced may have a fatty acid profile having less than or equal to

15,10,7, 5, 4, 3, 2, 1, or 0.5% polyunsaturates. In a specific embodiment, the oil/fat produced by the cell has greater than 50% SatUnsatSat, and optionally greater than 50% SOS, yet has less than 3% polyunsaturates. Optionally, polyunsaturates can be approximated by the sum of linoleic and linolenic acid area% in the fatty acid profile.

[0154] In an embodiment, the cell fat is a Shea stearin substitute having 65% to 95% SOS and optionally 0.001 to 5% SSS. In a related embodiment, the fat has 65% to 95% SOS, 0.001 to 5% SSS, and optionally 0.1 to 8% arachidic acid containing triglycerides. In another related embodiment, the fat has 65% to 95% SOS and the sum of SSS and SSO is less than 10% or less than 5%.

[0155] The cell's regiospecific preference can be learned using the analytical method described below (Examples 1-2, 8). Despite balancing the saturates and unsaturates as describe above, it is possible that the cell enzymes do not place the unsaturated fatty acid at the sn-2 position. In this case, genetic manipulations can confer the desired regiospecificity by (i) reducing the activity of endogenous sn-2 specific acyl transferases (e.g., LPAAT) and/or (ii) introducing an exogenous LPAAT with the desired specificity (i.e., introduction of oleate at sn-2). Where an exogenous LPAAT is introduced, preferably the gene encoding the LPAAT is integrated into a host chromosome and is targeted to the endoplasmic reticulum. In some cases, the host cell may have both specific and non-specific LPAAT alleles and suppressing the activity of one of these alleles (e.g., with a gene knockout) will confer the desired specificity. For example, genes encoding the LPAATs of SEQ ID NO: 78 and SEQ ID NO: 79 or an LPAAT comprising at least 90, 95, 98, or 99% amino acid identity to either of these sequences, or a functional fragment thereof, can be used to add oleate to the sn-2 position in order to boost the levels of SatUnsatSat TAGs. The genes can have at least 80, 85, 90, 95, 96, 97, 98, or 99% nucleotide identity to any of SEQ ID NOs: 80 to 85 or equivalent sequences by virtue of the degeneracy of the genetic code. Alternatively, the proteins encoded by the genes can have at least 80, 85, 90, 95, 96, 97, 98, or 99% nucleotide identity to the gene products of any of SEQ ID NOs: 80 to 85. These genes can be manifest as recombinant nucleic acid constructs, vectors, chromosomes or host cells comprising these sequences or functional fragments thereof, which can be found by systematic deletion of nucleic acid from the sequences using known techniques. As a result of expression of the genes, the amount of sat-unsat-sat TAGs such as SOS, POS, POP, or triglycerides with C8 to C16 fatty acids at the sn-2 position can be increased in a host cell.

[0156] Among other discoveries, the above discussion and Examples below highlight certain pathways to obtain high Sat-Unsat-Sat oils in general and SOS oils in particular in

microorganisms or in plants. Thus, it is possible that the use of genetic engineering techniques, optionally combined with classical mutagenesis and breeding, a microalga or higher plant can be produced with an increase in the amount of SatUnsatSat or SOS produced of at least 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, or more relative to the starting strain. In another aspect, the SatUnsatSat or SOS concentration of a species for which the wild-type produces less than 20%, 30%, 40% or 50% SatUnsatSat or SOS can be increased so that the SatUnsatSat or SOS is increased to at least 30%, 40%, 50% or 60%, respectively. The key changes, relative to the starting or wild-type organism, are to increase the amount of stearate (e.g., by reducing the amount of oleate formed from stearate, e.g., by reducing SAD activity, and/or increasing the amount of palmitate that is converted to stearate by reducing the activity of FATA and/or increasing the activity of KASII) and by decreasing the amount of linoleate by reducing FAD2/FADc activity.

[0157] Optionally, the starting organism can have triacylglycerol (TAG) biosynthetic machineries which are predisposed toward the synthesis of TAG species in which oleate or unsaturated fatty acids, predominate at the sn-2 position. Many oilseed crops have this characteristic. It has been demonstrated that lysophosphatidic acyltransferases (LPAATs) play a critical role in determining the species of fatty acids which will ultimately be inserted at the sn-2 position. Indeed, manipulation, through heterologous gene expression, of LPAATs in higher plant seeds, can alter the species of fatty acid occupying the sn-2 position.

[0158] One approach to generating oils with significant levels of so-called structuring fats (typically comprised of the species SOS-stearate-oleate-stearate, POS-palmitate-oleate-stearate, or POP-palmitate-oleate-palmitate) in agriculturally important oilseeds and in algae, is through the manipulation of endogenous as well as heterologous LPAAT expression. Expression of LPAATs from seeds containing high levels of structuring fats, for example, would be one strategy to increase the level of structuring fats in an oil seed or oleaginous algae that normally contains only limited quantities of such fats.

[0159] An alternative or supplementary strategey, however, is to take advantage of the innate propensity of LPAATs in agricultuarlly important oilseeds (eg, safflower-*Carthamus sp.*, sunflower-*Helianthus sp.*, canola-*Brassica sp.*, peanut-*Arachis sp.* , soybean-*Glycine sp.*, corn-*Zea sp.*, olive-*Olea sp.*, flax-*Linum sp.*, palm-*Elaeis sp.* and cotton-*Gossypium sp.*,see representative profiles in Table 5a below) and through either genetic engineering alone or a combination of genetic engineering and classical strain improvemment (i.e. mutagenesis) selectively manipulate the *species* of fatty acids present in order to increase the levels of

structuring fats. In the case of SOS, these manipulations are comprised of a series of discrete steps, which can be carried out independently. These include:

[0160] Increasing the level of stearate. This can be achieved, as we have demonstarted in microalgae here and others have shown in higher plants, through the expression of stearate specific FATA activities or down regulation of the endogenous SAD activity; e.g., through direct gene knockout, RNA silencing, or mutation, including classical strain improvement. Simply elevating stearate levels alone, by the above approaches,however, will not be optimal. For example, in the case of palm oil, the already high levels of palmitate, coupled with increased stearate levels, will likely overwhelm the existing LPAAT activity, leading to significant amounts of stearate and palmitate incorporation into tri-saturated fatty acids (SSS, PPP, SSP, PPS ect). Hence, steps must be taken to control palmitate levels as well.

[0161] Palmitate levels must be minimized in order to create high SOS containing fats because palmitate, even with a high-functioning LPAAT, will occupy sn-1 or sn-3 positions that could be taken up by stearate, and, as outlined above, too many saturates will result in significant levels of tri-saturated TAG species. Palmitate levels can be lowered. for example, through down-regulation of endogenous FATA activity through mutation/classical strain improvement, gene knockouts or RNAi-mediated strategies, in instances wherein the endogenous FATA activity has significant palmitate activty. Alternatively, or in concert with the above, palmitate levels can be lowered through over expression of endogenous KASII activity or classical strain improvement efforts which manifest in the same effect, such that elongation from palmitate to stearate is enhanced. Simply lowering palmitate levels via the above methods may not be sufficient, however. Take again the example of palm oil. Reduction of palmitate and elevation of stearate via the previous methods would still leave significant levels of linoleic acid. The endogenous LPAAT activity in most higher plants species while they will preferentially insert oleate in the sn-2 position, will insert linoleic as the next most preferred species. As oleate levels decrease, linoleic will come to occupy the sn-2 position with increased frequency. TAG species with linoleic at the sn-2 position have poor structuring properties as the TAGs will tend to display much higher melting temperatures than what is desired in a structuring fat. Hence, increases in stearate and reductions in palmitate must in turn be balanced by reductions in levels of linoleic fatty acids.

[0162] In turn, levels of linoleic fatty acids must be minimized in order to create high SOS-containing fats because linoleate, even with a high functioning LPAAT will occupy sn-2 positions to the exclusion of oleate, creating liquid oils as opposed to the desired solid fat (at room temperature). Linoleate levels can be lowered, as we have demonstrated in microalgae

and others have shown in plant oilseeds, through down regulation of endogenous FAD2 desaturases; e.g., through mutation/classical strain improvement, FAD2 knockouts or RNAi mediated down regulation of endogenous FAD2 activity. Accordingly, the linoleic acid level in the fatty acid profile can be reduced by at least 10, 20, 30, 40, 50, 100, 200, or 300%. For example, an RNAi construct with at least 70, 75, 80, 85, 90, 95, 96, 97, 98, or 99% identity to those disclosed herein can be used to downregulate FAD2.

[0163] Although one can choose a starting strain with such an sn-2 preference one can also introduce an exogenous LPAAT gene having a greater oleate preference, to further boost oleate at the sn-2 position and to further boost Sat-Unsat-Sat in the TAG profile. Optionally, one can replace one or more endogenous LPAAT alleles with the exogenous, more specific LPAAT.

[0164] The cell oils resulting from the SatUnsatSat/SOS producing organisms can be distinguished from conventional sources of SOS/POP/POS in that the sterol profile will be indicative of the host organism as distinguishable from the conventional source. Conventional sources of SOS/POP/POS include cocoa, shea, mango, sal, illipe, kokum, and allanblackia. See section XII of this disclosure for a discussion of microalgal sterols.

[0165] Table 5a: The fatty acid profiles of some commercial oilseed strains.

Common Food Oils*	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Corn oil (*Zea mays*)		<1.0	8.0-19.0	<0.5	0.5-4.0	19-50	38-65	<2.0
Cottonseed oil (*Gossypium barbadense*)	<0.1	0.5-2.0	17-29	<1.5	1.0-4.0	13-44	40-63	0.1-2.1
Canola (*Brassica rapa, B. napus, B. juncea*)	<0.1	<0.2	<6.0	<1.0	<2.5	>50	<40	<14
Olive (*Olea europea*)		<0.1	6.5-20.0	≤3.5	0.5-5.0	56-85	3.5-20.0	≤1.2
Peanut (*Arachis hypogaea*)	<0.1	<0.2	7.0-16.0	<1.0	1.3-6.5	35-72	13.0-43	<0.6
Palm (*Elaeis guineensis*)		0.5-5.9	32.0-47.0		2.0-8.0	34-44	7.2-12.0	
Safflower (*Carthamus tinctorus*)	<0.1	<1.0	2.0-10.0	<0.5	1.0-10.0	7.0-16.0	72-81	<1.5
Sunflower (*Helianthus annus*)	<0.1	<0.5	3.0-10.0	<1.0	1.0-10.0	14-65	20-75	<0.5
Soybean (*Glycine max*)	<0.1	<0.5	7.0-12.0	<0.5	2.0-5.5	19-30	48-65	5.0-10.0
Solin-Flax (*Linum usitatissimum*)	<0.1	<0.5	2.0-9.0	<0.5	2.0-5.0	8.0-60	40-80	<5.0

*Unless otherwise indicated, data taken from the U.S. Pharacopeia's Food and Chemicals Codex, 7th Ed. 2010-2011**

[0166] Accordingly, in an embodiment of the present invention, there is a method for increasing the amount of SOS in an oil (i.e. oil or fat) produced by a cell. The method comprises providing a cell and using classical and/or genetic engineering techniques (e.g., mutation, selection, strain-improvement, introduction of an exogenous gene and/or regulator element, or RNA-level modulation such as RNAi) to (i) increase the stearate in the oil, (ii) decrease the linoleate in the oil, and optionally (iii) increase the stereospecificity of the addition of oleate in the sn-2 position. The step of increasing the stearate can comprise decreasing desaturation by SAD (e.g., knockout, knockdown or use of regulatory elements) and increasing the conversion of palmitate to stearate (including overexpression of an endogenous or exogenous KASII and/or knockout or knockdown of FATA). Optionally, an exogenous FATA with greater stearate specificity then an endogenous FATA is expressed in the cell to increase stearate levels. Here, stearate-specificity of a FATA gene is a measure of the gene product's rate of cleavage of stearate over palmitate. The stearate-specific FATA gene insertion can be combined with a knockdown or knockout of the less-specific endogenous FATA gene. In this way, the ratio of stearate to palmitate can be increased, by 10%, 20%, 30%, 40%, 50%, 100% or more. The step of decreasing the linoleate can be via reduction of FADc/FAD2 activity including knockout and/or knockdown . The step of increasing the oleate at the sn-2 position can comprise expressing an exogenous oleate-preferring LPAAT such as an LPAAT having at least 75, 80, 85, 90, 85, 96, 97, 98, or 99% amino acid identity to an LPAAT disclosed herein.

[0167] In a specific embodiment, the cell (e.g, an oleaginous microalgal or other plastidic cell) produces an oil enriched in SOS (e.g., at least 50% SOS and in some cases 60% SOS). The cell is modified in at least four genes: (i) a β-ketoacyl-ACP synthase II (KASII) is overexpressed, (ii) activity of an endogenous FATA acyl-ACP thioesterase is reduced (iii) a stearate-specific FATA acyl-ACP thioesterase is overexpressed, (iii) endogenous SAD activity is decreased, and (iv) endogenous FAD activity is decreased. Example 65 demonstrates this embodiment in a Prototheca moriformis microalga by disrupting the coding region of endogenous FATA and SAD2 through homologous recombination, overexpressing a β-ketoacyl-ACP synthase II (KASII) gene, and activating FAD2 RNAi to decrease polyunsaturates.

[0168] In another specific embodiment, the cell (e.g, an oleaginous microalgal or other plastidic cell) produces an oil enriched in SOS (e.g., at least 50% SOS and in some cases 60% SOS). The cell is modified in at least four genes: (i) a β-ketoacyl-ACP synthase II (KASII) is overexpressed, (ii) activity of an endogenous FATA acyl-ACP thioesterase is reduced (iii) a

stearate-specific FATA acyl-ACP thioesterase is overexpressed, (iv) endogenous SAD activity is decreased, (v) endogenous FAD activity is decreased and (vi) an exogenous oleate-preferring LPAAT is expressed. See Examples 65 and 66. Optionally, these genes or regulatory elements have at least 75, 80, 85, 90, 85, 96, 97, 98, or 99% nucleic acid or amino acid identity to a gene or gene-product or regulatory element disclosed herein. Optionally, one or more of these genes is under control of a pH-sensitive or nitrogen-sensitive (pH-sensitive or pH-insensitive) promoter such as one having at least 75, 80, 85, 90, 85, 96, 97, 98, or 99% nucleic acid identity to one of those disclosed herein. Optionally, the cell oil is fractionated (see Example 64).

[0169] In an embodiment, fats produced by cells according to the invention are used to produce a confection, candy coating, or other food product. As a result, a food product like a chocolate or candy bar may have the "snap" (e.g., when broken) of a similar product produced using cocoa butter. The fat used may be in a beta polymorphic form or tend to a beta polymorphic form. In an embodiment, a method includes adding such a fat to a confection. Optionally, the fat can be a cocoa butter equivalent per EEC regulations, having greater than 65% SOS, less than 45% unsaturated fatty acid, less than 5% polyunsaturated fatty acids, less than 1% lauric acid, and less than 2% trans fatty acid. The fats can also be used as cocoa butter extenders, improvers, replacers, or anti-blooming agents, or as Shea butter replacers, including in food and personal care products. High SOS fats produced using the cells and methods disclosed here can be used in any application or formulation that calls for Shea butter or Shea fraction. However, unlike Shea butter, fats produced by the embodiments of the invention can have low amounts of unsaponifiables; e.g. less than 7, 5, 3, or 2% unsaponifiables. In addition, Shea butter tends to degrade quickly due to the presence of diacylglycerides whereas fats produced by the embodiments of the invention can have low amounts of diacylglycerides; e.g., less than 5, 4, 3, 2, 1, or 0.5% diacylglycerides.

[0170] In an embodiment of the invention there is a cell fat suitable as a shortening, and in particular, as a roll-in shortening. Thus, the shortening may be used to make pastries or other multi-laminate foods. The shortening can be produced using methods disclosed herein for producing engineered organisms and especially heterotrophic microalgae. In an embodiment, the shortening has a melting temperature of between 40 to 60°C and preferably between 45-55°C and can have a triglyceride profile with 15 to 20% medium chain fatty acids (C8 to C14), 45-50% long chain saturated fatty acids (C16 and higher), and 30-35% unsaturated fatty acids (preferably with more oleic than linoleic). The shortening may form β' polymorphic crystals, optionally without passing through the β polymorphic form. The

shortening may be thixotropic. The shortening may have a solid fat content of less than 15% at 35°C. In a specific embodiment, there is a cell oil suitable as a roll-in shortening produced by a recombinant microalga, where the oil has a yield stress between 400 and 700 or 500 and 600 Pa and a storage modulus of greater than $1x10^5$ Pa or $1x10^6$ Pa. (see Example 46)

[0171] A structured solid-liquid fat system can be produced using the structuring oils by blending them with an oil that is a liquid at room temperature (e.g., an oil high in tristearin or triolein). The blended system may be suitable for use in a food spread, mayonnaise, dressing, shortening; i.e. by forming an oil-water-oil emulsion. The structuring fats according to the embodiments described here, and especially those high in SOS, can be blended with other oils/fats to make a cocoa butter equivalent, replacer, or extender. For example, a cell fat having greater than 65% SOS can be blended with palm mid-fraction to make a cocoa butter equivalent.

[0172] In general, such high Sat-Unsat-Sat fats or fat systems can be used in a variety of other products including whipped toppings, margarines, spreads, salad dressings, baked goods (e.g. breads, cookies, crackers muffins, and pastries), cheeses, cream cheese, mayonnaise, etc.

[0173] In a specific embodiment, a Sat-Unsat-Sat fat described above is used to produce a margarine, spread, or the like. For example, a margarine can be made from the fat using any of the recipes or methods found in US Patent Nos. 7118773, 6171636, 4447462, 5690985, 5888575, 5972412, 6171636, or international patent publications WO9108677A1.

[0174] In an embodiment, a fat comprises a cell (e.g., from microalgal cells) fat optionally blended with another fat and is useful for producing a spread or margarine or other food product is produced by the genetically engineered cell and has glycerides derived from fatty acids which comprises:

(a) at least 10 weight % of C18 to C24 saturated fatty acids,

(b) which comprise stearic and/or arachidic and/or behenic and/or lignoceric acid and

(c) oleic and/or linoleic acid, while

(d) the ratio of saturated C18 acid/saturated (C20+C22+C24)-acids ≥1, preferably ≥5, more preferably ≥10,

which glycerides contain:

(e) ≤5 weight % of linolenic acid calculated on total fatty acid weight

(f) ≤5 weight % of trans fatty acids calculated on total fatty acid weight

(g) ≤75 weight %, preferably ≤60 weight % of oleic acid at the sn-2 position: which glycerides contain calculated on total glycerides weight

(h) ≥8 weight % HOH+HHO triglycerides

(i) ≤5 weight % of trisaturated triglycerides, and optionally one or more of the following properties:

(j) a solid fat content of >10% at 10°C

(k) a solid fat content ≤15% at 35°C,

(l) a solid fat content of >15% at 10°C and a solid fat content ≤25% at 35°C,

(m) the ratio of (HOH+HHO) and (HLH+HHL) triglycerides is >1, and preferably >2,

where H stands for C18-C24 saturated fatty acid, O for oleic acid, and L for linoleic acid.

[0175] Optionally, the solid content of the fat (%SFC) is 11 to 30 at 10°C, 4 to 15 at 20°C, 0.5 to 8 at 30°C, and 0 to 4 at 35°C. Alternately, the %SFC of the fat is 20 to 45 at 10°C, 14 to 25 at 20°C, 2 to 12 at 30°C, and 0 to 5 at 35°C. In related embodiment, the %SFC of the fat is 30 to 60 at 10°C, 20 to 55 at 20°C, 5 to 35 at 30°C, and 0 to 15 at 35°C. The C12-C16 fatty acid content can be ≤15 weight %. The fat can have ≤5 weight % disaturated diglycerides.

[0176] In related embodiments there is a spread, margarine or other food product made with the cell oil or cell oil blend. For example, the cell fat can be used to make an edible W/O (water/oil) emulsion spread comprising 70-20 wt. % of an aqueous phase dispersed in 30-80 wt. % of a fat phase which fat phase is a mixture of 50-99 wt. % of a vegetable triglyceride oil A and 1-50 wt. % of a structuring triglyceride fat B, which fat consists of 5-100 wt. % of a hardstock fat C and up to 95 wt. % of a fat D, where at least 45 wt. % of the hardstock fat C triglycerides consist of SatOSat triglycerides and where Sat denotes a fatty acid residue with a saturated C18-C24 carbon chain and O denotes an oleic acid residue and with the proviso that any hardstock fat C which has been obtained by fractionation, hydrogenation, esterification or interesterification of the fat is excluded. The hardstock fat can be a cell fat produced by a cell according to the methods disclosed herein. Accordingly, the hardstock fat can be a fat having a regiospecific profile having at least 50, 60, 70, 80, or 90% SOS. The W/O emulsion can be prepared to methods known in the art including in US Patent No. 7,118,773.

[0177] In related embodiment, the cell also expresses an endogenous hydrolyase enzyme that produces ricinoleic acid. As a result, the oil (e.g., a liquid oil or structured fat) produced

may be more easily emulsified into a margarine, spread, or other food product or non-food product. For example, the oil produced may be emulsified using no added emulsifiers or using lower amounts of such emulsifiers. The U.S. Patent Application No. 13/365,253 discloses methods for expressing such hydroxylases in microalgae and other cells. In specific embodiments, a cell oil comprises at least 1, 2, or 5% SRS, where S is stearate and R is ricinoleic acid.

[0178] In an alternate embodiment, a cell oil that is a cocoa butter mimetic as described above (or other high sat-unsat-sat oil such as a Shea or Kolum mimetic) can be fractionated to remove trisaturates (e.g., tristearin and tripalmitin, SSP, and PPS). For example, it has been found that microalgae engineered to decrease SAD activity to increase SOS concentration make an oil that can be fractionated to remove trisaturated. See Example 47 and example 64. In specific embodiments, the melting temperature of the fractionated cell oil is similar to that of cocoa butter (about 30-32°C). The POP, POS and SOS levels can approximate cocoa butter at about 16, 38, and 23% respectively. For example, POP can be 16% ±20%, POS can be 38%±20%, an SOS can be 23% ± 20%. Or, POP can be 16% ±15%, POS can be 38%±15%, an SOS can be 23%±15%. Or, POP can be 16% ±10%, POS can be 38%±10%, an SOS can be 23%±10%. In addition, the tristearin levels can be less than 5% of the triacylglycerides.

[0179] In an embodiment, a method comprises obtaining a cell oil obtained from a genetically engineered (e.g., microalga or other microbe) cell that produces a starting oil with a TAG profile having at least 40, 50, or 60% SOS. Optionally, the cell comprises one or more of an overexpressed KASII gene, a SAD knockout or knockdown, or an exogenous C18-preferring FATA gene, an exogenous LPAAT, and a FAD2 knockout or knockdown. The oil is fractionated by dry fractionation or solvent fractionation to give an enriched oil (stearin fraction) that is increased in SOS and decreased in trisaturates relative to the starting oil. The enriched oil can have at least 60%, 70% or 80% SOS with no more than 5%, 4%, 3%, 2% or 1% trisaturates. The enriched oil can have a sn-2 profile having 85, 90, 95% or more oleate at the sn-2 position. For example, thefractionated oil can comprise at least 60% SOS, no more than 5% trisaturates and at least 85% oleate at the sn-2 position. Alternatively, the oil can comprise at least 70% SOS, no more than 4% trisaturates and at least 90% oleate at the sn-2 position or 80% SOS, no more than 4% trisaturates and at least 95% oleate at the sn-2 position. Optionally, the oil has essentially identical maximum heat-flow temperatures and/or the DSC-derived SFC curves to Kokum butter. The stearin fraction can be obtained by dry fractionation, solvent fractionation, or a combination of these. Optionally, the process

includes a 2-step dry fractionation at a first temperature and a second temperature. The first termperature can be higher or lower than the second temperature. In a specific embodiment, the first temperature is effective at removing OOS and the second temperature is effective in removing trisaturates. Optionally, the stearin fraction is washed with a solvent (e.g. acetone) to remove the OOS after treatment at the first temperature. Optionally, the first temperature is about 24°C and the second temperature is about 29°C.

VIII. HIGH MID-CHAIN OILS

[0180] In an embodiment of the present invention, the cell has recombinant nucleic acids operable to elevate the level of midchain fatty acids (e.g., C8:0, C10:0, C12:0, C14:0, or C16:0 fatty acids) in the cell or in the oil of the cell. One way to increase the levels of midchain fatty acids in the cell or in the oil of the cell is to engineer a cell to express an exogenous acyl-ACP thioesterase that has activity towards midchain fatty acyl-ACP substrates (e.g., one encoded by a FatB gene), either as a sole modification or in combination with one or more other genetic modifications. An additional genetic modification to increase the level of midchain fatty acids in the cell or oil of the cell is the expression of an exogenous lysophosphatidic acid acyltransferase gene encoding an active lysophosphatidic acid acyltransferase (LPAAT) that catalyzes the transfer of a mid-chain fatty-acyl group to the sn-2 position of a substituted acylglyceroester. For example, the LPAAT gene can have 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% amino acid sequence identity or have 75, 80, 85 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% nucleic acid sequence identity (or equivalent sequence to degeneracy of the genetic code) to the mid-chain preferring LPAATs disclosed in Examples 43-44 (SEQ ID NOs 77, 78, 79, 81,82, 84, and 85). In a specific related embodiment, both an exogenous acyl-ACP thioesterase and LPAAT are stably expressed in the cell. In an embodiment, recombinant nucleic acids are introduced into an oleaginous cell (and especially into a plastidic microbial cell) that cause expression of an exogenous mid-chain-specific thioesterase and an exogenous LPAAT that catalyzes the transfer of a mid-chain fatty-acyl group to the sn-2 position of a substituted acylglyceroester. As a result, the cell can be made to increase the percent of a midchain fatty acid in the TAGs that it produces by 10, 20 30, 40, 50, 60, 70, 80, 90-fold, or more. Introduction of the exogenous LPAAT can increase midchain fatty acids at the sn-2 position by 1.2, 1.5, 1.7, 2, 3, 4 fold or more compared to introducing an exogenous mid-chain preferring acyl-ACP thioesterase alone. In an embodiment, the mid-chain fatty acid is greater than 30, 40, 50 60, 70, 80, or 90% of the TAG fatty acids produced by the cell. In various embodiments, the mid-chain fatty acid is lauric, myristic, or palmitic. Examples 3, 43, and 44 describe expression of plant LPAATs in

microalgal cells with resulting alterations in fatty acid profiles. As in the examples, the cells can also express an exogenous acyl-ACP thioesterase (which can also be from a plant) with a preference for a given fatty acyl-ACP chain length. For example, a microalgal cell can comprise exogenous genes encoding a LPAAT and an acyl-ACP thioesterase that preferentially cleave C8, C10, C12, C14, C8-C12, or C8-C10 fatty acids. In a specific embodiment, such a cell is capable of producing a cell oil with a fatty acid profile comprising 10-20, 20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, or 90-99%, >20%, >30%, >40%, >50%, >60%, >70%, >80% or >90% C8, C10, C12, C14, C8-C12, or C8-C10 fatty acids. Other LPAATs can preferentially cleave C16 or C18 fatty acids (see Example 44). Further genetic manipulation of the fatty acid desaturase pathway (e.g., as described *infra*) can increase the stability of the oils.

[0181] Any of these cell oils can be interesterified. Interesterification can, for example, be used to lower the melting temperature or pour-point of the oil. In a specific embodiment, the cell oil comprises at least 50% of the sum of caprylic and capric acids and may be interesterified to reduce the pour point and/or kinematic viscosity. Such an oil (cell or interesterified) can optionally be a high stability oil comprising, for example, less than 2% polyunsaturated fatty acids.

[0182] Alternately, or in addition to expression of an exogenous LPAAT, the cell may comprise recombinant nucleic acids that are operable to express an exogenous KASI or KASIV enzyme and optionally to decrease or eliminate the activity of a KASII, which is particularly advantageous when a mid-chain-preferring acyl-ACP thioesterase is expressed. Example 37 describes the engineering of *Prototheca* cells to overexpress KASI or KASIV enzymes in conjunction with a mid-chain preferring acyl-ACP thioesterase to generate strains in which production of C10-C12 fatty acids is about 59% of total fatty acids. Mid-chain production can also be increased by suppressing the activity of KASI and/or KASII (e.g., using a knockout or knockdown). Example 38 details the chromosomal knockout of different alleles of *Prototheca moriformis* (UTEX 1435) KASI in conjunction with overexpression of a mid-chain preferring acyl-ACP thioesterase to achieve fatty acid profiles that are about 76% or 84% C10-C14 fatty acids. Example 39 provides recombinant cells and oils characterized by elevated midchain fatty acids as a result of expression of KASI RNA hairpin polynucleotides. In addition to any of these modifications, unsaturated or polyunsaturated fatty acid production can be suppressed (e.g., by knockout or knockdown) of a SAD or FAD enzyme.

[0183] In a particular embodiment, a recombinant cell produces TAG having 40% lauric acid or more. In another related embodiment, a recombinant cell produces TAG having a fatty acid profile of 40% or more of myristic, caprylic, capric, or palmitic acid. For example, an oleaginous recombinant clorophyte cell can produce 40% lauric or myristic acid in an oil that makes up 40, 50, or 60% or more of the cell's dry weight.

[0184] In a specific embodiment, a recombinant cell comprises nucleic acids operable to express a product of an exogenous gene encoding a lysophosphatidic acid acyltransferase that catalyzes the transfer of a mid-chain fatty-acyl group to the sn-2 position of a substituted acylglyceroester and nucleic acids operable to express a product of an acyl-ACP thioesterase exogenous gene encoding an active acyl-ACP thioesterase that catalyzes the cleavage of mid-chain fatty acids from ACP. As a result, in one embodiment, the oil produced can be characterized by a fatty acid profile elevated in C10 and C12 fatty acids and reduced in C16, C18, and C18:1 fatty acids as a result of the recombinant nucleic acids. See Example 3, in which overexpression of a *Cuphea wrightii* acyl-ACP thioesterase and a *Cocos nucifera* LPAAT gene increased the percentage of C12 fatty acids from about 0.04% in the untransformed cells to about 46% and increased the percentage of C10 fatty acids from about 0.01% in the untransformed cells to about 11%. For example, the FATB gene can have 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% amino acid sequence identity or have 75, 80, 85 90, 91, 92, 93, 94, 95, 96, 97, 98 or 99% nucleic acid sequence identity (or equivalent sequence to degeneracy of the genetic code) to SEQ ID NOs 10 or 11. In related embodiments, the increase in midchain fatty acid production is greater than 70%, from 75-85%, from 70-90%, from 90-200%, from 200-300%, from 300-400%, from 400-500%, or greater than 500%.

[0185] Average chain length can also be reduced by overexpression of a C18-specific acyl-ACP thioesterase. Recombinant nucleic acids operable to overexpress a C18 or other acyl-ACP thioesterase may be used alone or in combination with the other constructs described here to further reduce average chain length. Among other uses, the oils produced can be used as cocoa-butter/milk fat substitute. See Example 45 and the discussion of Fig. 17. In an embodiment, one of the above described high mid-chain producing cells is further engineered to produce a low polyunsaturated oil by knocking out or knocking down one or more fatty acyl desaturases, as described above in section IV. Accordingly, the oil produced can have the high stability characteristic mentioned in that section or in corresponding Examples. In a specific embodiment, the cell produces an oil comprising greater than 30% midchain fatty acids and 5% or less polyunsaturates. In a related embodiment, the cell produces an oil

comprising greater than 40% midchain fatty acids and 4% or less polyunsaturates. In a further related embodiment, the cell produces an oil comprising greater than 50% midchain fatty acids and 3% or less polyunsaturates.

[0186] In a specific embodiment, the cell produces an oil characterized by a fatty acid profile in which the sum of lauric and myristic acids is at least 50%, 60% , 70%, or 75%. This can be accomplished using the techniques of Examples 37-39, 43-44, 52, and 60-61. For example, Example 52 describes a method for producing an oil that has a fatty acid profile in which the sum of lauric and myristic acids is about 79% using a recombinant cell with an exogenous plant FATB acyl-ACP thioesterase.

[0187] In another specific embodiment, the cell produces a cell oil characterized by a fatty acid profile in which capric acid (C10:0) is at least 30% and lauric acid (C12:0) is at least 30%. For example, the absolute level of capric acid and lauric acid in the cell oil can be balanced to within 5, 10, 15, 20 or 30%. This can be accomplished using the techniques of Examples 37-39, 43-44, 52, and 60-61. As in Example 60, exogenous plant FATB and KASI (or KASIV) genes can be combined to give balanced levels of capric and lauric. Optionally, an endogenous KASI gene can be knocked out and replaced with an exogenous KASI. In addition, two or more exogenous FATB genes can be used do reach a desired fatty acid profile. In a specific embodiment, a microalgal cell expresses at least one and optionally at least two exogenous FATB genes and an exogenous KASI/KASIV gene and produces an extractable cell oil with at least 30% C10 and at least 30% C12 fatty acids. For example, the cell can express a FATB acyl-ACP thioesterase having at least 70, 75, 80, 85, 90 or 95% amino acid sequence identity to the Cuphea hookeriana FATB2 (SEQ ID NO: 158) and a beta-ketoacyl ACP synthase having at least 70, 75, 80, 85, 90 or 95% amino acid sequence identity to the Cuphea wrightii KASA1 (SEQ ID NO: 159, with alternate transit peptide). Further, a second exogenous FATB gene/enzyme can be expressed. The second FATB can have at least 70, 75, 80, 85, 90 or 95% amino acid sequence identity to the Cuphea wrightii FATB2 acyl-ACP thioesterase (SEQ ID NO: 11.) For these purposes, plastid targeted peptides can be aligned with or with out the plastid targeting transit peptides, which are less conserved and more easily replaceable than the remaining enzyme domain sequence.

[0188] In an embodiment, the cell produces an oil comprising greater than 75% saturated fatty acids. Optionally, the cell produces an oil comprising greater than 75% saturated fatty acids with less than 25% capric acid, less than 50% lauric acid, and less than 5% palmitic acid. In related embodiments, the oil comprises at least 80%, 95% or 90% saturated fatty acids. Example 60 describes the production of such oil by microalgae comprising multiple

exogenous FATB genes and replacement of an endogenous KASI gene with exogenous KASI or KASIV genes from plants.

[0189] Examples 60 and 62 also shows that selection of FATB and KAS genes can give rise to an oil with at least 50% total saturates with capric and lauric acids balanced to within 20% (or even to within 15%, or 10%).

[0190] High-mid chain oils in general, and those produced by strains similar to those of Example 60 and 62 can possess low kinematic viscosity. For example, the oil can have a kinematic viscosity as measured usng ASTM D445 at 40°C of 25 cS ± 20%, 25 cS ± 10%, or 25 cS ± 5%. Likewise, the oil can have a kinematic viscosity according to ASTM D445 at 100°C of 5.4 cS ± 20%, 5.4 cS ± 10%, or 5.4 cS ± 5%. The oil can have a viscosity index as measured using ASTM 2280 of 160 ± 20%, 160 ± 10%, or 160 ± 5%.

[0191] In a specific example, an oil prepared using a strain similar to those reported in Example 60, produced an oil with greater than 30% C10:0 and greater than 30% C12:0 fatty acids. The oil had a kinematic viscocity by ASTM 445 of 24.61 cSt at 40°C and 5.36 cSt at 100°C with a viscosity index (ASTM 2270) of 159. To make this oil, a Cuphea hookeriana FATB2 acyl-ACP thioesterase was expressed with a Cuphea wrightii KASA1 gene (with a P. moriformis SAD transit peptide) in Prototheca moriformis under control of the UAPA1 and AMT03 promoters, respectively. Neomycin resitance was used at the selection marker and the construct with incorporated in the KAS1-1 site. Accordingly, in an embodiment, a host cell comprises an exogenous gene that expresses a protein having at least 70, 75, 80, 85, 90, or 95% amino acid sequence identity to SEQ ID NO: 158 and also expresses a protein having at least 70, 75, 80, 85, 90, or 95% amino acid sequence identity to SEQ ID NO: 159. The cell produces an oil comprising at least 30% C10:0 and/or at least 30% C12:0 fatty acids. Optionally, a cell oil can be extracted from the cell that has a kinematic viscosity as measured usng ASTM D445 at 40°C of less than 30 cSt.

[0192] The high mid-chain oils or fatty acids derived from hydrolysis of these oils may be particularly useful in food, fuel and oleochemical applications including the production of lubricants and surfactants. For example, fatty acids derived from the cells can be esterified, cracked, reduced to an aldehyde or alcohol, aminated, sulfated, sulfonated, or subjected to other chemical process known in the art.

[0193] In some embodiments, the cell oil is interesterified and the kinematic viscosity of the interesterified cell oil is less than 30, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 centiStokes at 40°C. In some embodiments, the kinematic viscosity is less than 3 centiStokes at 40°C. In some embodiments, the pour point of an interesterified cell oil is less than, 5 °C , 0 °C , -10

°C, -12 °C, -15°C, -20 °C, -25°C, -30°C, -35°C , -40°C, -45°C, or -50°C. In some embodiments, the pour point is less than -10 °C. In some embodiments, the pour point is less than -20 °C.

[0194] Example 53 describes the use of a plant FatB gene in algae to produce oils in microalgae with greater than 60% myristate. In an embodiment, a gene encoding a protein having at least 90, 95, 96, 97, 98, or 99% amino acid identity to SEQ ID NO:87 or SEQ ID NO:89 is used, optionally in combination with a mid-chain preferred LPAAT as described above.

[0195] As described in Example 62, we surprisingly discovered that the combination of a KASI gene with a FATB gene can shift the fatty acid profile of an oil produced by the cell in ways that neither gene can do on its own. Specifically, recombinant cells with exogenous plant myristate-preferring acyl-ACP thioesterases were discovered to shift their fatty acid profile to a greater percentage of laurate when a KASI gene was co-expressed. This is unexpected because KASI has an elongase activity yet the fatty acid profile was shifted to shorter chains. In other words, a cell expressing both the exogenous FATB and KASI gene produced an oil having a fatty acid profile that is shifted toward shorter fatty acid chains than a control cell with the FATB gene but without the KASI gene. Accordingly, an embodiment of the invention comprises constructing a recombinant cell or using the cell to make an oil, where the cell comprises an exogenous FATB with a given chain-length preference and a KASI gene, wherein the cell makes an oil with a shift in distribution toward shorter chains than is obtained without the KASI gene. Optionally, the FATB gene has a nucleic acid sequence that is at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% identical (or an equivalent sequence by virtue of degenerecy of the genetic code) or has an amino acid sequence that is least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% identical to the CcFATB2-UcFATB2 FATB of Example 62 (SEQ ID NO: 162), the Cuphea wrightii FATB2 (SEQ ID NO: 11), Cuphea palustris FATB2 (SEQ ID NO: 87; SEQ ID NO: 89) , Cuphea hyssopifolia FATB1 (SEQ ID NO: 163), Cuphea hyssopifolia FATB3 (SEQ ID NO: 164), or Cuphea hookeriana FATB2 (SEQ ID NO: 158). Optionally, the KASI or KASIV gene has a nucleic acid sequence that is at least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% identical (or an equivalent sequence by virtue of degenerecy of the genetic code) or has an amino acid sequence that is least 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% identical to the *Cuphea wrightii* KASAI of Example 62 (SEQ ID NO: 159), the Cuphea hookeriana KASIV encoded by the sequence of SEQ ID NO:49, or the Cuphea pulch. KASIV encoded by SEQ ID NO: 48.

IX. HIGH OLEIC/PALMITIC OIL

[0196] In another embodiment, there is a high oleic oil with about 60% oleic acid, 25% palmitic acid and optionally 5% polyunsaturates or less. The high oleic oil can be produced using the methods disclosed in U.S. Patent Application No. 13/365,253, which is incorporated by reference in relevant part. For example, the cell can have nucleic acids operable to suppress an acyl-ACP thioesterase (e.g., knockout or knockdown of a gene encoding FATA) while also expressing a gene that increases KASII activity. The cell can have further modifications to inhibit expression of delta 12 fatty acid desaturase, including regulation of gene expression as described above. As a result, the polyunsaturates can be less than or equal to 5, 4, 3, 2, or 1 area%.

X. LOW SATURATE OIL

[0197] In an embodiment, a cell oil is produced from a recombinant cell. The oil produced has a fatty acid profile that has less that 4%, 3%, 2%, or 1% (area %), saturated fatty acids. In a specific embodiment, the oil has 0.1 to 3.5% saturated fatty acids. Certain of such oils can be used to produce a food with negligible amounts of saturated fatty acids. Optionally, these oils can have fatty acid profiles comprising at least 90% oleic acid or at least 90% oleic acid with at least 3% polyunsaturated fatty acids. In an embodiment, a cell oil produced by a recombinant cell comprises at least 90% oleic acid, at least 3% of the sum of linoleic and linolenic acid and has less than 3.5% saturated fatty acids. In a related embodiment, a cell oil produced by a recombinant cell comprises at least 90% oleic acid, at least 3% of the sum of linoleic and linolenic acid and has less than 3.5% saturated fatty acids, the majority of the saturated fatty acids being comprised of chain length 10 to 16. These oils may be produced by recombinant oleaginous cells including but not limited to those described here and in U.S. Patent Application No. 13/365,253. For example, overexpression of a KASII enzyme in a cell with a highly active SAD can produce a high oleic oil with less than or equal to 3.5% saturates. Optionally, an oleate-specific acyl-ACP thioesterase is also overexpressed and/or an endogenous thioesterase having a propensity to hydrolyze acyl chains of less than C18 knocked out or suppressed. The oleate-specific acyl-ACP thioesterase may be a transgene with low activity toward ACP-palmitate and ACP-stearate so that the ratio of oleic acid relative to the sum of palmitic acid and stearic acid in the fatty acid profile of the oil produced is greater than 3, 5, 7, or 10. Alternately, or in addition, a FATA gene may be knocked out or knocked down, as in Example 36 below. A FATA gene may be knocked out or knocked down and an exogenous KASII overexpressed. Another optional modification is to increase KASI and/or KASIII activity, which can further suppress the formation of shorter

chain saturates. Optionally, one or more acyltransferases (e.g., an LPAAT) having specificity for transferring unsaturated fatty acyl moieties to a substituted glycerol is also overexpressed and/or an endogenous acyltransferase is knocked out or attenuated. An additional optional modification is to increase the activity of KCS enzymes having specificity for elongating unsaturated fatty acids and/or an endogenous KCS having specificity for elongating saturated fatty acids is knocked out or attenuated. Optionally, oleate is increased at the expense of linoleate production by knockout or knockdown of a delta 12 fatty acid desaturase; e.g., using the techniques of Section IV of this patent application. Optionally, the exogenous genes used can be plant genes; e.g., obtained from cDNA derived from mRNA found in oil seeds.

[0198] As described in Example 51, levels of saturated fats may also be reduced by introduction of an exogenous gene (e.g., a plant gene) that desaturates palmitic acid to palmitoleic acid. Examples of suitable genes for use in the oleaginous cells are found in the plants, including Macfadyena unguis (Cat's claw), Macadamia integrifolia (Macadamia nut) and Hippophae rhamnoides (sea buckthorn). Variant exogenous or endogenous SADs that desaturate palmitoyl-ACP can also be used and are further discussed in Example 51. Optionally, the PAD or SAD gene has at least 95% amino acid sequence identity to the gene product described in Example 51. This modification can be used alone, or in combination with oleate-increasing modifications such as those described immediately above, in section IX and in the Examples, including knockout or knockdown of one or more endogenous FATA alleles and/or overexpression of KASII. In one embodiment, an oleaginous cell such as an oleaginous microalgae has a combination of (i) a FATA knockout or knockdown with (ii) expression of an exogenous PAD gene (this could also be a variant SAD with PAD activity such as a L118W mutant or equivalent, see Examples 55-56) and/or a mutation in an endogenous SAD gene to give PAD activity. Such as cell may further comprise an overexpressed endogenous or exogenous KASII gene. In accordance with any of these embodiments of the invention, the oleaginous cell produces an oil having a fatty acid profile with 1-2, 2-3, 3-4, 5-6, 7-8, 9-10, 10-15, 15-20, 20-30, 30-40, 40-60, 60-70, 70-80, 80-90, or 90-100 area percent palmitoleic acid. In a specific embodiment, the cell produces greater than 50% oleic acid, greater than 1% palmitoleic acid, and 3.5 area% or less of saturated fatty acids. In another specific embodiment, a eukaryotic microalgal cell comprises an exogenous gene that desaturates palmitic acid to palmitoleic acid in operable linkage with regulatory elements operable in the microalgal cell. Due to expression and activity of the exogenous gene product, the cell produces a cell oil having a fatty acid profile in which the ratio of palmitoleic acid (C16:1) to palmitic acid (C16:0) is at least 0.05, 0.1 or 0.15, or 0.18. See

Example 55 for examples of cells that produce such oils. Optionally, palmitoleic acid comprises 0.5% or more of the profile. Optionally, the cell oil comprises less than 3.5% saturated fatty acids.

[0199] In addition to the above genetic modifications, the low saturate oil can be a high-stability oil by virtue of low amounts of polyunsaturated fatty acids. Methods and characterizations of high-stability, low-polyunsaturated oils are described in the section above entitled Low Polyunsaturated Oils, including method to reduce the activity of endogenous Δ12 fatty acid desaturase. In a specific embodiment, an oil is produced by a oleaginous microbial cell having a type II fatty acid synthetic pathway and has no more than 3.5% saturated fatty acids and also has no more than 3% polyunsaturated fatty acids. In another specific embodiment, the oil has no more than 3% saturated fatty acids and also has no more than 2% polyunsaturated fatty acids. In another specific embodiment, the oil has no more than 3% saturated fatty acids and also has no more than 1% polyunsaturated fatty acids. In another specific embodiment, a eukaryotic microalgal cell comprises an exogenous gene that desaturates palmitic acid to palmitoleic acid in operable linkage with regulatory elements operable in the microalgal cell. The cell further comprises a knockout or knockdown of a FAD gene. Due to the genetic modifications, the cell produces a cell oil having a fatty acid profile in which the ratio of palmitoleic acid (C16:1) to palmitic acid (C16:0) is greater than 0.1, with no more than 3% polyunsaturated fatty acids. Optionally, palmitoleic acid comprises 0.5% or more of the profile. Optionally, the cell oil comprises less than 3.5% saturated fatty acids.

[0200] The low saturate and low saturate/high stability oil can be blended with less expensive oils to reach a targeted saturated fatty acid level at less expense. For example, an oil with 1% saturated fat can be blended with an oil having 7% saturated fat (e.g. high-oleic sunflower oil) to give an oil having 3.5% or less saturated fat.

[0201] Oils produced according to embodiments of the present invention can be used in the transportation fuel, oleochemical, and/or food and cosmetic industries, among other applications. For example, transesterification of lipids can yield long-chain fatty acid esters useful as biodiesel. Other enzymatic and chemical processes can be tailored to yield fatty acids, aldehydes, alcohols, alkanes, and alkenes. In some applications, renewable diesel, jet fuel, or other hydrocarbon compounds are produced. The present disclosure also provides methods of cultivating microalgae for increased productivity and increased lipid yield, and/or for more cost-effective production of the compositions described herein. The methods

described here allow for the production of oils from plastidic cell cultures at large scale; e.g., 1000, 10,000, 100,000 liters or more.

[0202] In an embodiment, an oil extracted from the cell has 3.5%, 3%, 2.5%, or 2% saturated fat or less and is incorporated into a food product. The finished food product has 3.5, 3, 2.5, or 2% saturated fat or less. For example, oils recovered from such recombinant microalgae can be used for frying oils or as an ingredient in a prepared food that is low in saturated fats. The oils can be used neat or blended with other oils so that the food has less than 0.5g of saturated fat per serving, thus allowing a label stating zero saturated fat (per US regulation). In a specific embodiment, the oil has a fatty acid profile with at least 90% oleic acid, less than 3% saturated fat, and more oleic acid than linoleic acid.

[0203] As with the other oils disclosed in this patent application, the low-saturate oils described in this section, including those with increased levels palmitoleic acid, can have a microalgal sterol profile as described in Section XII of this application. For example, via expression of an exogenous PAD gene, an oil can be produced with a fatty acid profile characterized by a ratio of palmitoleic acid to palmitic acid of at least 0.1 and/or palmitoleic acid levels of 0.5 % or more, as determined by FAME GC/FID analysis and a sterol profile characterized by an excess of ergosterol over β-sitosterol and/or the presence of 22, 23-dihydrobrassicasterol, poriferasterol or clionasterol.

XI. COCOA BUTTER/MILK-FAT BLEND MIMETICS

[0204] In certain embodiments, the cell produces a cell oil that has a temperature-dependent solid fat content ("SFC-curve") that approximates a blend of cocoa butter and milk fat. Such oils may be used where the cocoa butter/milk fat blend could be used; for example, in chocolates other confections, ice cream or other frozen desserts, pastries, or dough, including for quickbreads, or other baked goods. The oils may inhibit blooming, enhance flavor, enhance texture, or reduce costs. In a specific example, the cell oil approximates. Accordingly, an embodiment of the invention is using a cell oil from a recombinant microalgal cell to replace a cocoa butter/milk fat blend in a recipe. In a related embodiment,

[0205] Figure 17 shows a plot of %solid fat content for various oils as follows (a) P. moriformis RBD oil without lipid pathway engineering, (b) Brazilian cocoa butter +25% milk fat, (c) three replicates of P. moriformis RBD oil from a strain expressing hairpin nucleic acids that reduce levels of a SAD allele thus reducing oleic acid and increasing stearic acid in the TAG profile, (d) P. moriformis RBD oil from a strain overexpressing an endogenous OTE (oleoyl acyl-ACP thioesterase, see Example 45), (e) Malaysian cocoa butter +25% milk fat,

and (f) Malaysian cocoa butter. The cocoa butter and cocoa butter milk fat values are literature values (Bailey's Industrial Oils and Fat Products, 6th ed.)

[0206] In an embodiment of the present invention, a cell oil that is similar in thermal properties to a 75% cocoa butter/25% milk fat blend is produced by a microalgal or other cell described above. The cell comprises recombinant nucleic acids operable to alter the fatty acid profile of triglycerides produced by the cell so as that the oil has a solid fat content (e.g., as determined by NMR) of 38%±30% at 20°C, 32%±30% at 25°C, 17%±30% at 30°C, and less than 5%±30% at 35°C. For the sake of clarity, ±10% refers to percent of the percent SFC (e.g., 30% of 5% SFC is 1.5%SFC so the range is 3.5 to 6.5% SFC at 35°C). In related embodiments, the oil has a solid fat content (e.g., as determined by NMR) of 38%±20% at 20°C, 32%±20% at 25°C, 17%±20% at 30°C, and less than 5%±20% at 35°C or the oil has a solid fat content (e.g., as determined by NMR) of 38%±10% at 20°C, 32%±10% at 25°C, 17%±10% at 30°C, and less than 5%±10% at 35°C.

[0207] In a another embodiment a cell high oleic oil produced according to the methods of section IX or corresponding Examples, is converted into a structuring fat such as a cocoa butter equivalent, substitute, extender by enzymatic interesterification or transesterification with a source of saturated fatty acids (e.g. a hardstock fat or saturated fatty acid esters). For example, a 1,3-specific lipase can be used to add stearate, palmitate or both to a high oleic oil having greater than 80% oleic acid.

XII. MINOR OIL COMPONENTS

[0208] The oils produced according to the above methods in some cases are made using a microalgal host cell. As described above, the microalga can be, without limitation, fall in the classification of Chlorophyta, Trebouxiophyceae , Chlorellales, Chlorellaceae, or Chlorophyceae. It has been found that microalgae of Trebouxiophyceae can be distinguished from vegetable oils based on their sterol profiles. Oil produced by *Chlorella protothecoides* was found to produce sterols that appeared to be brassicasterol, ergosterol, campesterol, stigmasterol, and β-sitosterol, when detected by GC-MS. However, it is believed that all sterols produced by *Chlorella* have C24β stereochemistry. Thus, it is believed that the molecules detected as campesterol, stigmasterol, and β-sitosterol, are actually 22,23-dihydrobrassicasterol, poriferasterol and clionasterol, respectively. Thus, the oils produced by the microalgae described above can be distinguished from plant oils by the presence of sterols with C24β stereochemistry and the absence of C24α stereochemistry in the sterols present. For example, the oils produced may contain 22, 23-dihydrobrassicasterol while

lacking campesterol; contain clionasterol, while lacking in β-sitosterol, and/or contain poriferasterol while lacking stigmasterol. Alternately, or in addition, the oils may contain significant amounts of Δ^7-poriferasterol.

[0209] In one embodiment, the oils provided herein are not vegetable oils. Vegetable oils are oils extracted from plants and plant seeds. Vegetable oils can be distinguished from the non-plant oils provided herein on the basis of their oil content. A variety of methods for analyzing the oil content can be employed to determine the source of the oil or whether adulteration of an oil provided herein with an oil of a different (e.g. plant) origin has occurred. The determination can be made on the basis of one or a combination of the analytical methods. These tests include but are not limited to analysis of one or more of free fatty acids, fatty acid profile, total triacylglycerol content, diacylglycerol content, peroxide values, spectroscopic properties (e.g. UV absorption), sterol profile, sterol degradation products, antioxidants (e.g. tocopherols), pigments (e.g. chlorophyll), d13C values and sensory analysis (e.g. taste, odor, and mouth feel). Many such tests have been standardized for commercial oils such as the Codex Alimentarius standards for edible fats and oils.

[0210] Sterol profile analysis is a particularly well-known method for determining the biological source of organic matter. Campesterol, b-sitosterol, and stigmasterol are common plant sterols, with b-sitosterol being a principle plant sterol. For example, b-sitosterol was found to be in greatest abundance in an analysis of certain seed oils, approximately 64% in corn, 29% in rapeseed, 64% in sunflower, 74% in cottonseed, 26% in soybean, and 79% in olive oil (Gul et al. J. Cell and Molecular Biology 5:71-79, 2006).

[0211] Oil isolated from Prototheca moriformis strain UTEX1435 were separately clarified (CL), refined and bleached (RB), or refined, bleached and deodorized (RBD) and were tested for sterol content according to the procedure described in JAOCS vol. 60, no.8, August 1983. Results of the analysis are shown below (units in mg/100g) in Table 5b.

[0212] Table 5b. Sterol profiles of oils from UTEX 1435.

	Sterol	**Crude**	**Clarified**	**Refined & bleached**	**Refined, bleached, & deodorized**
1	Ergosterol	384 (56%)	398 (55%)	293 (50%)	302 (50%)
2	5,22-cholestadien-24-methyl-3-ol (Brassicasterol)	14.6 (2.1%)	18.8 (2.6%)	14 (2.4%)	15.2 (2.5%)

3	24-methylcholest-5-en-3-ol (Campesterol or 22,23-dihydrobrassicasterol)	10.7 (1.6%)	11.9 (1.6%)	10.9 (1.8%)	10.8 (1.8%)
4	5,22-cholestadien-24-ethyl-3-ol (Stigmasterol or poriferasterol)	57.7 (8.4%)	59.2 (8.2%)	46.8 (7.9%)	49.9 (8.3%)
5	24-ethylcholest-5-en-3-ol (β-Sitosterol or clionasterol)	9.64 (1.4%)	9.92 (1.4%)	9.26 (1.6%)	10.2 (1.7%)
6	Other sterols	209	221	216	213
	Total sterols	685.64	718.82	589.96	601.1

[0213] These results show three striking features. First, ergosterol was found to be the most abundant of all the sterols, accounting for about 50% or more of the total sterols. The amount of ergosterol is greater than that of campesterol, β-sitosterol, and stigmasterol combined. Ergosterol is steroid commonly found in fungus and not commonly found in plants, and its presence particularly in significant amounts serves as a useful marker for non-plant oils. Secondly, the oil was found to contain brassicasterol. With the exception of rapeseed oil, brassicasterol is not commonly found in plant based oils. Thirdly, less than 2% β-sitosterol was found to be present. β-sitosterol is a prominent plant sterol not commonly found in microalgae, and its presence particularly in significant amounts serves as a useful marker for oils of plant origin. In summary, *Prototheca moriformis* strain UTEX1435 has been found to contain both significant amounts of ergosterol and only trace amounts of β-sitosterol as a percentage of total sterol content. Accordingly, the ratio of ergosterol : β-sitosterol or in combination with the presence of brassicasterol can be used to distinguish this oil from plant oils.

[0214] In some embodiments, the oil content of an oil provided herein contains, as a percentage of total sterols, less than 20%, 15%, 10%, 5%, 4%, 3%, 2%, or 1% β-sitosterol. In other embodiments the oil is free from β-sitosterol. For any of the oils or cell-oils disclosed in this application, the oil can have the sterol profile of any column of Table 5b, above, with a sterol-by-sterol variation of 30%, 20%, 10% or less.

[0215] In some embodiments, the oil is free from one or more of β-sitosterol, campesterol, or stigmasterol. In some embodiments the oil is free from β-sitosterol, campesterol, and

stigmasterol. In some embodiments the oil is free from campesterol. In some embodiments the oil is free from stigmasterol.

[0216] In some embodiments, the oil content of an oil provided herein comprises, as a percentage of total sterols, less than 20%, 15%, 10%, 5%, 4%, 3%, 2%, or 1% 24-ethylcholest-5-en-3-ol. In some embodiments, the 24-ethylcholest-5-en-3-ol is clionasterol. In some embodiments, the oil content of an oil provided herein comprises, as a percentage of total sterols, at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% clionasterol.

[0217] In some embodiments, the oil content of an oil provided herein contains, as a percentage of total sterols, less than 20%, 15%, 10%, 5%, 4%, 3%, 2%, or 1% 24-methylcholest-5-en-3-ol. In some embodiments, the 24-methylcholest-5-en-3-ol is 22, 23-dihydrobrassicasterol. In some embodiments, the oil content of an oil provided herein comprises, as a percentage of total sterols, at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% 22,23-dihydrobrassicasterol.

[0218] In some embodiments, the oil content of an oil provided herein contains, as a percentage of total sterols, less than 20%, 15%, 10%, 5%, 4%, 3%, 2%, or 1% 5,22-cholestadien-24-ethyl-3-ol. In some embodiments, the 5, 22-cholestadien-24-ethyl-3-ol is poriferasterol. In some embodiments, the oil content of an oil provided herein comprises, as a percentage of total sterols, at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% poriferasterol.

[0219] In some embodiments, the oil content of an oil provided herein contains ergosterol or brassicasterol or a combination of the two. In some embodiments, the oil content contains, as a percentage of total sterols, at least 5%, 10%, 20%, 25%, 35%, 40%, 45%, 50%, 55%, 60%, or 65% ergosterol. In some embodiments, the oil content contains, as a percentage of total sterols, at least 25% ergosterol. In some embodiments, the oil content contains, as a percentage of total sterols, at least 40% ergosterol. In some embodiments, the oil content contains, as a percentage of total sterols, at least 5%, 10%, 20%, 25%, 35%, 40%, 45%, 50%, 55%, 60%, or 65% of a combination of ergosterol and brassicasterol.

[0220] In some embodiments, the oil content contains, as a percentage of total sterols, at least 1%, 2%, 3%, 4% or 5% brassicasterol. In some embodiments, the oil content contains, as a percentage of total sterols less than 10%, 9%, 8%, 7%, 6%, or 5% brassicasterol.

[0221] In some embodiments the ratio of ergosterol to brassicasterol is at least 5:1, 10:1, 15:1, or 20:1.

[0222] In some embodiments, the oil content contains, as a percentage of total sterols, at least 5%, 10%, 20%, 25%, 35%, 40%, 45%, 50%, 55%, 60%, or 65% ergosterol and less than

20%, 15%, 10%, 5%, 4%, 3%, 2%, or 1% β-sitosterol. In some embodiments, the oil content contains, as a percentage of total sterols, at least 25% ergosterol and less than 5% β-sitosterol. In some embodiments, the oil content further comprises brassicasterol.

[0223] Sterols contain from 27 to 29 carbon atoms (C27 to C29) and are found in all eukaryotes. Animals exclusively make C27 sterols as they lack the ability to further modify the C27 sterols to produce C28 and C29 sterols. Plants however are able to synthesize C28 and C29 sterols, and C28/C29 plant sterols are often referred to as phytosterols. The sterol profile of a given plant is high in C29 sterols, and the primary sterols in plants are typically the C29 sterols b-sitosterol and stigmasterol. In contrast, the sterol profile of non-plant organisms contain greater percentages of C27 and C28 sterols. For example the sterols in fungi and in many microalgae are principally C28 sterols. The sterol profile and particularly the striking predominance of C29 sterols over C28 sterols in plants has been exploited for determining the proportion of plant and marine matter in soil samples (Huang, Wen-Yen, Meinschein W. G., "Sterols as ecological indicators"; Geochimica et Cosmochimia Acta. Vol 43. pp 739-745).

[0224] In some embodiments the primary sterols in the microalgal oils provided herein are sterols other than b-sitosterol and stigmasterol. In some embodiments of the microalgal oils, C29 sterols make up less than 50%, 40%, 30%, 20%, 10%, or 5% by weight of the total sterol content.

[0225] In some embodiments the microalgal oils provided herein contain C28 sterols in excess of C29 sterols. In some embodiments of the microalgal oils, C28 sterols make up greater than 50%, 60%, 70%, 80%, 90%, or 95% by weight of the total sterol content. In some embodiments the C28 sterol is ergosterol. In some embodiments the C28 sterol is brassicasterol.

XIII. FUELS AND CHEMICALS

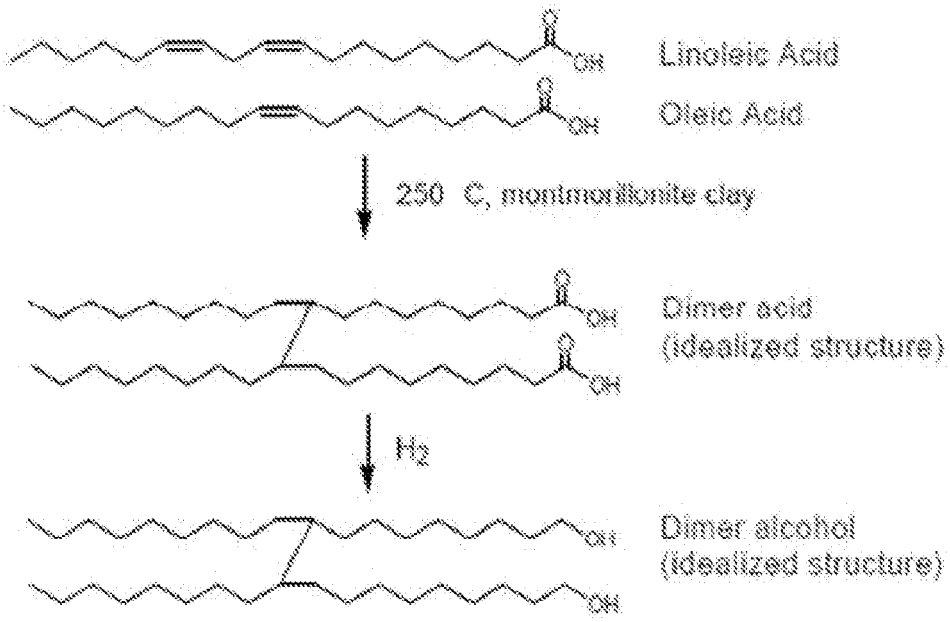
[0226] The oils discussed above alone or in combination are useful in the production of foods, fuels and chemicals (including plastics, foams, films, etc.). The oils, triglycerides, fatty acids from the oils may be subjected to C-H activation, hydroamino methylation, methoxy-carbonation, ozonolysis, enzymatic transformations, epoxidation, methylation, dimerization, thiolation, metathesis, hydro-alkylation, lactonization, or other chemical processes.

[0227] The oils can be converted to alkanes (e.g., renewable diesel) or esters (e.g., methyl or ethyl esters for biodisesel produced by transesterification). The alkanes or esters may be

used as fuel, as solvents or lubricants, or as a chemical feedstock. Methods for production of renewable diesel and biodiesel are well established in the art. See, for example, WO2011/150411.

[0228] In a specific embodiment of the present invention, a high-oleic or high-oleic-high stability oil described above is esterified. For example, the oils can be transesterified with methanol to an oil that is rich in methyl oleate. As described in Example 49, such formulations have been found to compare favorably with methyl oleate from soybean oil.

[0229] In another specific example, the oil is converted to C36 diacids or products of C36 diacids. Fatty acids produced from the oil can be polymerized to give a composition rich in C36 dimer acids. In a specific example, high-oleic oil is split to give a high-oleic fatty acid material which is polymerized to give a composition rich in C36-dimer acids. Optionally , the oil is high oleic high stability oil (e.g., greater than 60% oleic acid with less than 3% polyunsaturates, greater than 70% oleic acid with less than 2% polyunsaturates, or greater than 80% oleic acid with less than 1% polyunsaturates). It is believed that using a high oleic, high stability, starting material will give lower amounts of cyclic products, which may be desirable in some cases. After hydrolyzing the oil, one obtains a high concentration of oleic acid. In the process of making dimer acids, a high oleic acid stream will convert to a "cleaner" C36 dimer acid and not produce trimers acids (C54) and other more complex cyclic by-products which are obtained due to presence of C18:2 and C18:3 acids. For example, the oil can be hydrolyzed to fatty acids and the fatty acids purified and dimerized at 250°C in the presence of montmorillonite clay. See SRI Natural Fatty Acid, March 2009. A product rich in C36 dimers of oleic acid is recovered.



[0230] Further, the C36 dimer acids can be esterified and hydrogenated to give diols. The diols can be polymerized by catalytic dehydration. Polymers can also be produced by transesterification of dimerdiols with dimethyl carbonate.

[0231] For the production of fuel in accordance with the methods of the invention lipids produced by cells of the invention are harvested, or otherwise collected, by any convenient means. Lipids can be isolated by whole cell extraction. The cells are first disrupted, and then intracellular and cell membrane/cell wall-associated lipids as well as extracellular hydrocarbons can be separated from the cell mass, such as by use of centrifugation. Intracellular lipids produced in oleaginous cells are, in some embodiments, extracted after lysing the cells. Once extracted, the lipids are further refined to produce oils, fuels, or oleochemicals.

[0232] Various methods are available for separating lipids from cellular lysates. For example, lipids and lipid derivatives such as fatty aldehydes, fatty alcohols, and hydrocarbons such as alkanes can be extracted with a hydrophobic solvent such as hexane (see Frenz et al. 1989, Enzyme Microb. Technol., 11:717). Lipids and lipid derivatives can also be extracted using liquefaction (see for example Sawayama et al. 1999, Biomass and Bioenergy 17:33-39 and Inoue et al. 1993, Biomass Bioenergy 6(4):269-274); oil liquefaction (see for example Minowa et al. 1995, Fuel 74(12):1735-1738); and supercritical CO_2 extraction (see for example Mendes et al. 2003, Inorganica Chimica Acta 356:328-334). Miao and Wu describe a protocol of the recovery of microalgal lipid from a culture of *Chlorella protothecoides* in which the cells were harvested by centrifugation, washed with distilled water and dried by

freeze drying. The resulting cell powder was pulverized in a mortar and then extracted with *n*-hexane. Miao and Wu, Biosource Technology (2006) 97:841-846.

[0233] Lipids and lipid derivatives can be recovered by extraction with an organic solvent. In some cases, the preferred organic solvent is hexane. Typically, the organic solvent is added directly to the lysate without prior separation of the lysate components. In one embodiment, the lysate generated by one or more of the methods described above is contacted with an organic solvent for a period of time sufficient to allow the lipid and/or hydrocarbon components to form a solution with the organic solvent. In some cases, the solution can then be further refined to recover specific desired lipid or hydrocarbon components. Hexane extraction methods are well known in the art.

[0234] Lipids produced by cells *in vivo*, or enzymatically modified *in vitro*, as described herein can be optionally further processed by conventional means. The processing can include "cracking" to reduce the size, and thus increase the hydrogen:carbon ratio, of hydrocarbon molecules. Catalytic and thermal cracking methods are routinely used in hydrocarbon and triglyceride oil processing. Catalytic methods involve the use of a catalyst, such as a solid acid catalyst. The catalyst can be silica-alumina or a zeolite, which result in the heterolytic, or asymmetric, breakage of a carbon-carbon bond to result in a carbocation and a hydride anion. These reactive intermediates then undergo either rearrangement or hydride transfer with another hydrocarbon. The reactions can thus regenerate the intermediates to result in a self-propagating chain mechanism. Hydrocarbons can also be processed to reduce, optionally to zero, the number of carbon-carbon double, or triple, bonds therein. Hydrocarbons can also be processed to remove or eliminate a ring or cyclic structure therein. Hydrocarbons can also be processed to increase the hydrogen:carbon ratio. This can include the addition of hydrogen ("hydrogenation") and/or the "cracking" of hydrocarbons into smaller hydrocarbons.

[0235] Thermal methods involve the use of elevated temperature and pressure to reduce hydrocarbon size. An elevated temperature of about 800°C and pressure of about 700kPa can be used. These conditions generate "light," a term that is sometimes used to refer to hydrogen-rich hydrocarbon molecules (as distinguished from photon flux), while also generating, by condensation, heavier hydrocarbon molecules which are relatively depleted of hydrogen. The methodology provides homolytic, or symmetrical, breakage and produces alkenes, which may be optionally enzymatically saturated as described above.

[0236] Catalytic and thermal methods are standard in plants for hydrocarbon processing and oil refining. Thus hydrocarbons produced by cells as described herein can be collected

and processed or refined via conventional means. See Hillen et al. (Biotechnology and Bioengineering, Vol. XXIV:193-205 (1982)) for a report on hydrocracking of microalgae-produced hydrocarbons. In alternative embodiments, the fraction is treated with another catalyst, such as an organic compound, heat, and/or an inorganic compound. For processing of lipids into biodiesel, a transesterification process is used as described below in this Section.

[0237] Hydrocarbons produced via methods of the present invention are useful in a variety of industrial applications. For example, the production of linear alkylbenzene sulfonate (LAS), an anionic surfactant used in nearly all types of detergents and cleaning preparations, utilizes hydrocarbons generally comprising a chain of 10-14 carbon atoms. See, for example, US Patent Nos.: 6,946,430; 5,506,201; 6,692,730; 6,268,517; 6,020,509; 6,140,302; 5,080,848; and 5,567,359. Surfactants, such as LAS, can be used in the manufacture of personal care compositions and detergents, such as those described in US Patent Nos.: 5,942,479; 6,086,903; 5,833,999; 6,468,955; and 6,407,044.

[0238] Increasing interest is directed to the use of hydrocarbon components of biological origin in fuels, such as biodiesel, renewable diesel, and jet fuel, since renewable biological starting materials that may replace starting materials derived from fossil fuels are available, and the use thereof is desirable. There is an urgent need for methods for producing hydrocarbon components from biological materials. The present invention fulfills this need by providing methods for production of biodiesel, renewable diesel, and jet fuel using the lipids generated by the methods described herein as a biological material to produce biodiesel, renewable diesel, and jet fuel.

[0239] Traditional diesel fuels are petroleum distillates rich in paraffinic hydrocarbons. They have boiling ranges as broad as 370° to 780°F, which are suitable for combustion in a compression ignition engine, such as a diesel engine vehicle. The American Society of Testing and Materials (ASTM) establishes the grade of diesel according to the boiling range, along with allowable ranges of other fuel properties, such as cetane number, cloud point, flash point, viscosity, aniline point, sulfur content, water content, ash content, copper strip corrosion, and carbon residue. Technically, any hydrocarbon distillate material derived from biomass or otherwise that meets the appropriate ASTM specification can be defined as diesel fuel (ASTM D975), jet fuel (ASTM D1655), or as biodiesel if it is a fatty acid methyl ester (ASTM D6751).

[0240] After extraction, lipid and/or hydrocarbon components recovered from the microbial biomass described herein can be subjected to chemical treatment to manufacture a fuel for use in diesel vehicles and jet engines.

[0241] Biodiesel is a liquid which varies in color - between golden and dark brown - depending on the production feedstock. It is practically immiscible with water, has a high boiling point and low vapor pressure. Biodiesel refers to a diesel-equivalent processed fuel for use in diesel-engine vehicles. Biodiesel is biodegradable and non-toxic. An additional benefit of biodiesel over conventional diesel fuel is lower engine wear. Typically, biodiesel comprises C14-C18 alkyl esters. Various processes convert biomass or a lipid produced and isolated as described herein to diesel fuels. A preferred method to produce biodiesel is by transesterification of a lipid as described herein. A preferred alkyl ester for use as biodiesel is a methyl ester or ethyl ester.

[0242] Biodiesel produced by a method described herein can be used alone or blended with conventional diesel fuel at any concentration in most modern diesel-engine vehicles. When blended with conventional diesel fuel (petroleum diesel), biodiesel may be present from about 0.1% to about 99.9%. Much of the world uses a system known as the "B" factor to state the amount of biodiesel in any fuel mix. For example, fuel containing 20% biodiesel is labeled B20. Pure biodiesel is referred to as B100.

[0243] Biodiesel can be produced by transesterification of triglycerides contained in oil-rich biomass. Thus, in another aspect of the present invention a method for producing biodiesel is provided. In a preferred embodiment, the method for producing biodiesel comprises the steps of (a) cultivating a lipid-containing microorganism using methods disclosed herein (b) lysing a lipid-containing microorganism to produce a lysate, (c) isolating lipid from the lysed microorganism, and (d) transesterifying the lipid composition, whereby biodiesel is produced. Methods for growth of a microorganism, lysing a microorganism to produce a lysate, treating the lysate in a medium comprising an organic solvent to form a heterogeneous mixture and separating the treated lysate into a lipid composition have been described above and can also be used in the method of producing biodiesel. The lipid profile of the biodiesel is usually highly similar to the lipid profile of the feedstock oil.

[0244] Lipid compositions can be subjected to transesterification to yield long-chain fatty acid esters useful as biodiesel. Preferred transesterification reactions are outlined below and include base catalyzed transesterification and transesterification using recombinant lipases. In a base-catalyzed transesterification process, the triacylglycerides are reacted with an alcohol,

such as methanol or ethanol, in the presence of an alkaline catalyst, typically potassium hydroxide. This reaction forms methyl or ethyl esters and glycerin (glycerol) as a byproduct.

[0245] Transesterification has also been carried out, as discussed above, using an enzyme, such as a lipase instead of a base. Lipase-catalyzed transesterification can be carried out, for example, at a temperature between the room temperature and 80° C, and a mole ratio of the TAG to the lower alcohol of greater than 1:1, preferably about 3:1. Lipases suitable for use in transesterification include, but are not limited to, those listed in Table 9. Other examples of lipases useful for transesterification are found in, e.g., U.S. Patent Nos. 4,798,793; 4,940,845 5,156,963; 5,342,768; 5,776,741 and WO89/01032. Such lipases include, but are not limited to, lipases produced by microorganisms of Rhizopus, Aspergillus, Candida, Mucor, Pseudomonas, Rhizomucor, Candida, and Humicola and pancreas lipase.

[0246] Subsequent processes may also be used if the biodiesel will be used in particularly cold temperatures. Such processes include winterization and fractionation. Both processes are designed to improve the cold flow and winter performance of the fuel by lowering the cloud point (the temperature at which the biodiesel starts to crystallize). There are several approaches to winterizing biodiesel. One approach is to blend the biodiesel with petroleum diesel. Another approach is to use additives that can lower the cloud point of biodiesel. Another approach is to remove saturated methyl esters indiscriminately by mixing in additives and allowing for the crystallization of saturates and then filtering out the crystals. Fractionation selectively separates methyl esters into individual components or fractions, allowing for the removal or inclusion of specific methyl esters. Fractionation methods include urea fractionation, solvent fractionation and thermal distillation.

[0247] Another valuable fuel provided by the methods of the present invention is renewable diesel, which comprises alkanes, such as C10:0, C12:0, C14:0, C16:0 and C18:0 and thus, are distinguishable from biodiesel. High quality renewable diesel conforms to the ASTM D975 standard. The lipids produced by the methods of the present invention can serve as feedstock to produce renewable diesel. Thus, in another aspect of the present invention, a method for producing renewable diesel is provided. Renewable diesel can be produced by at least three processes: hydrothermal processing (hydrotreating); hydroprocessing; and indirect liquefaction. These processes yield non-ester distillates. During these processes, triacylglycerides produced and isolated as described herein, are converted to alkanes.

[0248] In one embodiment, the method for producing renewable diesel comprises (a) cultivating a lipid-containing microorganism using methods disclosed herein (b) lysing the

microorganism to produce a lysate, (c) isolating lipid from the lysed microorganism, and (d) deoxygenating and hydrotreating the lipid to produce an alkane, whereby renewable diesel is produced. Lipids suitable for manufacturing renewable diesel can be obtained via extraction from microbial biomass using an organic solvent such as hexane, or via other methods, such as those described in US Patent 5,928,696. Some suitable methods may include mechanical pressing and centrifuging.

[0249] In some methods, the microbial lipid is first cracked in conjunction with hydrotreating to reduce carbon chain length and saturate double bonds, respectively. The material is then isomerized, also in conjunction with hydrotreating. The naptha fraction can then be removed through distillation, followed by additional distillation to vaporize and distill components desired in the diesel fuel to meet an ASTM D975 standard while leaving components that are heavier than desired for meeting the D975 standard. Hydrotreating, hydrocracking, deoxygenation and isomerization methods of chemically modifying oils, including triglyceride oils, are well known in the art. See for example European patent applications EP1741768 (A1); EP1741767 (A1); EP1682466 (A1); EP1640437 (A1); EP1681337 (A1); EP1795576 (A1); and U.S. Patents 7,238,277; 6,630,066; 6,596,155; 6,977,322; 7,041,866; 6,217,746; 5,885,440; 6,881,873.

[0250] In one embodiment of the method for producing renewable diesel, treating the lipid to produce an alkane is performed by hydrotreating of the lipid composition. In hydrothermal processing, typically, biomass is reacted in water at an elevated temperature and pressure to form oils and residual solids. Conversion temperatures are typically 300° to 660°F, with pressure sufficient to keep the water primarily as a liquid, 100 to 170 standard atmosphere (atm). Reaction times are on the order of 15 to 30 minutes. After the reaction is completed, the organics are separated from the water. Thereby a distillate suitable for diesel is produced.

[0251] In some methods of making renewable diesel, the first step of treating a triglyceride is hydroprocessing to saturate double bonds, followed by deoxygenation at elevated temperature in the presence of hydrogen and a catalyst. In some methods, hydrogenation and deoxygenation occur in the same reaction. In other methods deoxygenation occurs before hydrogenation. Isomerization is then optionally performed, also in the presence of hydrogen and a catalyst. Naphtha components are preferably removed through distillation. For examples, see U.S. Patents 5,475,160 (hydrogenation of triglycerides); 5,091,116 (deoxygenation, hydrogenation and gas removal); 6,391,815 (hydrogenation); and 5,888,947 (isomerization).

[0252] One suitable method for the hydrogenation of triglycerides includes preparing an aqueous solution of copper, zinc, magnesium and lanthanum salts and another solution of alkali metal or preferably, ammonium carbonate. The two solutions may be heated to a temperature of about 20°C to about 85°C and metered together into a precipitation container at rates such that the pH in the precipitation container is maintained between 5.5 and 7.5 in order to form a catalyst. Additional water may be used either initially in the precipitation container or added concurrently with the salt solution and precipitation solution. The resulting precipitate may then be thoroughly washed, dried, calcined at about 300°C and activated in hydrogen at temperatures ranging from about 100°C to about 400°C. One or more triglycerides may then be contacted and reacted with hydrogen in the presence of the above-described catalyst in a reactor. The reactor may be a trickle bed reactor, fixed bed gas-solid reactor, packed bubble column reactor, continuously stirred tank reactor, a slurry phase reactor, or any other suitable reactor type known in the art. The process may be carried out either batchwise or in continuous fashion. Reaction temperatures are typically in the range of from about 170°C to about 250°C while reaction pressures are typically in the range of from about 300 psig to about 2000 psig. Moreover, the molar ratio of hydrogen to triglyceride in the process of the present invention is typically in the range of from about 20:1 to about 700:1. The process is typically carried out at a weight hourly space velocity (WHSV) in the range of from about 0.1 hr^{-1} to about 5 hr^{-1}. One skilled in the art will recognize that the time period required for reaction will vary according to the temperature used, the molar ratio of hydrogen to triglyceride, and the partial pressure of hydrogen. The products produced by the such hydrogenation processes include fatty alcohols, glycerol, traces of paraffins and unreacted triglycerides. These products are typically separated by conventional means such as, for example, distillation, extraction, filtration, crystallization, and the like.

[0253] Petroleum refiners use hydroprocessing to remove impurities by treating feeds with hydrogen. Hydroprocessing conversion temperatures are typically 300° to 700°F. Pressures are typically 40 to 100 atm. The reaction times are typically on the order of 10 to 60 minutes. Solid catalysts are employed to increase certain reaction rates, improve selectivity for certain products, and optimize hydrogen consumption.

[0254] Suitable methods for the deoxygenation of an oil includes heating an oil to a temperature in the range of from about 350°F to about 550°F and continuously contacting the heated oil with nitrogen under at least pressure ranging from about atmospheric to above for at least about 5 minutes.

[0255] Suitable methods for isomerization include using alkali isomerization and other oil isomerization known in the art.

[0256] Hydrotreating and hydroprocessing ultimately lead to a reduction in the molecular weight of the triglyceride feed. The triglyceride molecule is reduced to four hydrocarbon molecules under hydroprocessing conditions: a propane molecule and three heavier hydrocarbon molecules, typically in the C8 to C18 range.

[0257] Thus, in one embodiment, the product of one or more chemical reaction(s) performed on lipid compositions of the invention is an alkane mixture that comprises ASTM D975 renewable diesel. Production of hydrocarbons by microorganisms is reviewed by Metzger et al. Appl Microbiol Biotechnol (2005) 66: 486–496 and A Look Back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae, NREL/TP-580-24190, John Sheehan, Terri Dunahay, John Benemann and Paul Roessler (1998).

[0258] The distillation properties of a diesel fuel is described in terms of T10-T90 (temperature at 10% and 90%, respectively, volume distilled). The T10-T90 of the material produced in Example 13 was 57.9°C. Methods of hydrotreating, isomerization, and other covalent modification of oils disclosed herein, as well as methods of distillation and fractionation (such as cold filtration) disclosed herein, can be employed to generate renewable diesel compositions with other T10-T90 ranges, such as 20, 25, 30, 35, 40, 45, 50, 60 and 65°C using triglyceride oils produced according to the methods disclosed herein.

[0259] Methods of hydrotreating, isomerization, and other covalent modification of oils disclosed herein, as well as methods of distillation and fractionation (such as cold filtration) disclosed herein, can be employed to generate renewable diesel compositions with other T10 values, such as T10 between 180 and 295, between 190 and 270, between 210 and 250, between 225 and 245, and at least 290.

[0260] Methods of hydrotreating, isomerization, and other covalent modification of oils disclosed herein, as well as methods of distillation and fractionation (such as cold filtration) disclosed herein can be employed to generate renewable diesel compositions with certain T90 values, such as T90 between 280 and 380, between 290 and 360, between 300 and 350, between 310 and 340, and at least 290.

[0261] The FBP of the material produced in Example 13 was 300°C. Methods of hydrotreating, isomerization, and other covalent modification of oils disclosed herein, as well as methods of distillation and fractionation (such as cold filtration) disclosed herein, can be employed to generate renewable diesel compositions with other FBP values, such as FBP

between 290 and 400, between 300 and 385, between 310 and 370, between 315 and 360, and at least 300.

[0262] Other oils provided by the methods and compositions of the invention can be subjected to combinations of hydrotreating, isomerization, and other covalent modification including oils with lipid profiles including (a) at least 1%-5%, preferably at least 4%, C8-C14; (b) at least 0.25%-1%, preferably at least 0.3%, C8; (c) at least 1%-5%, preferably at least 2%, C10; (d) at least 1%-5%, preferably at least 2%, C12; and (3) at least 20%-40%, preferably at least 30% C8-C14.

[0263] A traditional ultra-low sulfur diesel can be produced from any form of biomass by a two-step process. First, the biomass is converted to a syngas, a gaseous mixture rich in hydrogen and carbon monoxide. Then, the syngas is catalytically converted to liquids. Typically, the production of liquids is accomplished using Fischer-Tropsch (FT) synthesis. This technology applies to coal, natural gas, and heavy oils. Thus, in yet another preferred embodiment of the method for producing renewable diesel, treating the lipid composition to produce an alkane is performed by indirect liquefaction of the lipid composition.

[0264] The present invention also provides methods to produce jet fuel. Jet fuel is clear to straw colored. The most common fuel is an unleaded/paraffin oil-based fuel classified as Aeroplane A-1, which is produced to an internationally standardized set of specifications. Jet fuel is a mixture of a large number of different hydrocarbons, possibly as many as a thousand or more. The range of their sizes (molecular weights or carbon numbers) is restricted by the requirements for the product, for example, freezing point or smoke point. Kerosene-type Aeroplane fuel (including Jet A and Jet A-1) has a carbon number distribution between about 8 and 16 carbon numbers. Wide-cut or naphtha-type Aeroplane fuel (including Jet B) typically has a carbon number distribution between about 5 and 15 carbons.

[0265] In one embodiment of the invention, a jet fuel is produced by blending algal fuels with existing jet fuel. The lipids produced by the methods of the present invention can serve as feedstock to produce jet fuel. Thus, in another aspect of the present invention, a method for producing jet fuel is provided. Herewith two methods for producing jet fuel from the lipids produced by the methods of the present invention are provided: fluid catalytic cracking (FCC); and hydrodeoxygenation (HDO).

[0266] Fluid Catalytic Cracking (FCC) is one method which is used to produce olefins, especially propylene from heavy crude fractions. The lipids produced by the method of the present invention can be converted to olefins. The process involves flowing the lipids produced through an FCC zone and collecting a product stream comprised of olefins, which

is useful as a jet fuel. The lipids produced are contacted with a cracking catalyst at cracking conditions to provide a product stream comprising olefins and hydrocarbons useful as jet fuel.

[0267] In one embodiment, the method for producing jet fuel comprises (a) cultivating a lipid-containing microorganism using methods disclosed herein, (b) lysing the lipid-containing microorganism to produce a lysate, (c) isolating lipid from the lysate, and (d) treating the lipid composition, whereby jet fuel is produced. In one embodiment of the method for producing a jet fuel, the lipid composition can be flowed through a fluid catalytic cracking zone, which, in one embodiment, may comprise contacting the lipid composition with a cracking catalyst at cracking conditions to provide a product stream comprising C_2-C_5 olefins.

[0268] In certain embodiments of this method, it may be desirable to remove any contaminants that may be present in the lipid composition. Thus, prior to flowing the lipid composition through a fluid catalytic cracking zone, the lipid composition is pretreated. Pretreatment may involve contacting the lipid composition with an ion-exchange resin. The ion exchange resin is an acidic ion exchange resin, such as Amberlyst™-15 and can be used as a bed in a reactor through which the lipid composition is flowed, either upflow or downflow. Other pretreatments may include mild acid washes by contacting the lipid composition with an acid, such as sulfuric, acetic, nitric, or hydrochloric acid. Contacting is done with a dilute acid solution usually at ambient temperature and atmospheric pressure.

[0269] The lipid composition, optionally pretreated, is flowed to an FCC zone where the hydrocarbonaceous components are cracked to olefins. Catalytic cracking is accomplished by contacting the lipid composition in a reaction zone with a catalyst composed of finely divided particulate material. The reaction is catalytic cracking, as opposed to hydrocracking, and is carried out in the absence of added hydrogen or the consumption of hydrogen. As the cracking reaction proceeds, substantial amounts of coke are deposited on the catalyst. The catalyst is regenerated at high temperatures by burning coke from the catalyst in a regeneration zone. Coke-containing catalyst, referred to herein as "coked catalyst", is continually transported from the reaction zone to the regeneration zone to be regenerated and replaced by essentially coke-free regenerated catalyst from the regeneration zone. Fluidization of the catalyst particles by various gaseous streams allows the transport of catalyst between the reaction zone and regeneration zone. Methods for cracking hydrocarbons, such as those of the lipid composition described herein, in a fluidized stream of catalyst, transporting catalyst between reaction and regeneration zones, and combusting coke in the regenerator are well known by those skilled in the art of FCC processes.

Exemplary FCC applications and catalysts useful for cracking the lipid composition to produce C_2-C_5 olefins are described in U.S. Pat. Nos. 6,538,169, 7,288,685, which are incorporated in their entirety by reference.

[0270] Suitable FCC catalysts generally comprise at least two components that may or may not be on the same matrix. In some embodiments, both two components may be circulated throughout the entire reaction vessel. The first component generally includes any of the well-known catalysts that are used in the art of fluidized catalytic cracking, such as an active amorphous clay-type catalyst and/or a high activity, crystalline molecular sieve. Molecular sieve catalysts may be preferred over amorphous catalysts because of their much-improved selectivity to desired products. In some preferred embodiments, zeolites may be used as the molecular sieve in the FCC processes. Preferably, the first catalyst component comprises a large pore zeolite, such as a Y-type zeolite, an active alumina material, a binder material, comprising either silica or alumina and an inert filler such as kaolin.

[0271] In one embodiment, cracking the lipid composition of the present invention, takes place in the riser section or, alternatively, the lift section, of the FCC zone. The lipid composition is introduced into the riser by a nozzle resulting in the rapid vaporization of the lipid composition. Before contacting the catalyst, the lipid composition will ordinarily have a temperature of about 149°C to about 316°C (300°F to 600°F). The catalyst is flowed from a blending vessel to the riser where it contacts the lipid composition for a time of abort 2 seconds or less.

[0272] The blended catalyst and reacted lipid composition vapors are then discharged from the top of the riser through an outlet and separated into a cracked product vapor stream including olefins and a collection of catalyst particles covered with substantial quantities of coke and generally referred to as "coked catalyst." In an effort to minimize the contact time of the lipid composition and the catalyst which may promote further conversion of desired products to undesirable other products, any arrangement of separators such as a swirl arm arrangement can be used to remove coked catalyst from the product stream quickly. The separator, e.g. swirl arm separator, is located in an upper portion of a chamber with a stripping zone situated in the lower portion of the chamber. Catalyst separated by the swirl arm arrangement drops down into the stripping zone. The cracked product vapor stream comprising cracked hydrocarbons including light olefins and some catalyst exit the chamber via a conduit which is in communication with cyclones. The cyclones remove remaining catalyst particles from the product vapor stream to reduce particle concentrations to very low levels. The product vapor stream then exits the top of the separating vessel. Catalyst

separated by the cyclones is returned to the separating vessel and then to the stripping zone. The stripping zone removes adsorbed hydrocarbons from the surface of the catalyst by counter-current contact with steam.

[0273] Low hydrocarbon partial pressure operates to favor the production of light olefins. Accordingly, the riser pressure is set at about 172 to 241 kPa (25 to 35 psia) with a hydrocarbon partial pressure of about 35 to 172 kPa (5 to 25 psia), with a preferred hydrocarbon partial pressure of about 69 to 138 kPa (10 to 20 psia). This relatively low partial pressure for hydrocarbon is achieved by using steam as a diluent to the extent that the diluent is 10 to 55 wt-% of lipid composition and preferably about 15 wt-% of lipid composition. Other diluents such as dry gas can be used to reach equivalent hydrocarbon partial pressures.

[0274] The temperature of the cracked stream at the riser outlet will be about 510°C to 621°C (950°F to 1150°F). However, riser outlet temperatures above 566°C (1050°F) make more dry gas and more olefins. Whereas, riser outlet temperatures below 566°C (1050°F) make less ethylene and propylene. Accordingly, it is preferred to run the FCC process at a preferred temperature of about 566°C to about 630°C, preferred pressure of about 138 kPa to about 240 kPa (20 to 35 psia). Another condition for the process is the catalyst to lipid composition ratio which can vary from about 5 to about 20 and preferably from about 10 to about 15.

[0275] In one embodiment of the method for producing a jet fuel, the lipid composition is introduced into the lift section of an FCC reactor. The temperature in the lift section will be very hot and range from about 700°C (1292°F) to about 760°C (1400°F) with a catalyst to lipid composition ratio of about 100 to about 150. It is anticipated that introducing the lipid composition into the lift section will produce considerable amounts of propylene and ethylene.

[0276] In another embodiment of the method for producing a jet fuel using the lipid composition or the lipids produced as described herein, the structure of the lipid composition or the lipids is broken by a process referred to as hydrodeoxygenation (HDO). HDO means removal of oxygen by means of hydrogen, that is, oxygen is removed while breaking the structure of the material. Olefinic double bonds are hydrogenated and any sulfur and nitrogen compounds are removed. Sulfur removal is called hydrodesulphurization (HDS). Pretreatment and purity of the raw materials (lipid composition or the lipids) contribute to the service life of the catalyst.

[0277] Generally in the HDO/HDS step, hydrogen is mixed with the feed stock (lipid composition or the lipids) and then the mixture is passed through a catalyst bed as a co-current flow, either as a single phase or a two phase feed stock. After the HDO/MDS step, the product fraction is separated and passed to a separate isomerization reactor. An isomerization reactor for biological starting material is described in the literature (FI 100 248) as a co-current reactor.

[0278] The process for producing a fuel by hydrogenating a hydrocarbon feed, e.g., the lipid composition or the lipids herein, can also be performed by passing the lipid composition or the lipids as a co-current flow with hydrogen gas through a first hydrogenation zone, and thereafter the hydrocarbon effluent is further hydrogenated in a second hydrogenation zone by passing hydrogen gas to the second hydrogenation zone as a counter-current flow relative to the hydrocarbon effluent. Exemplary HDO applications and catalysts useful for cracking the lipid composition to produce C_2-C_5 olefins are described in U.S. Pat. No. 7,232,935, which is incorporated in its entirety by reference.

[0279] Typically, in the hydrodeoxygenation step, the structure of the biological component, such as the lipid composition or lipids herein, is decomposed, oxygen, nitrogen, phosphorus and sulfur compounds, and light hydrocarbons as gas are removed, and the olefinic bonds are hydrogenated. In the second step of the process, i.e. in the so-called isomerization step, isomerization is carried out for branching the hydrocarbon chain and improving the performance of the paraffin at low temperatures.

[0280] In the first step, i.e. HDO step, of the cracking process, hydrogen gas and the lipid composition or lipids herein which are to be hydrogenated are passed to a HDO catalyst bed system either as co-current or counter-current flows, said catalyst bed system comprising one or more catalyst bed(s), preferably 1-3 catalyst beds. The HDO step is typically operated in a co-current manner. In case of a HDO catalyst bed system comprising two or more catalyst beds, one or more of the beds may be operated using the counter-current flow principle. In the HDO step, the pressure varies between 20 and 150 bar, preferably between 50 and 100 bar, and the temperature varies between 200 and 500°C, preferably in the range of 300-400°C. In the HDO step, known hydrogenation catalysts containing metals from Group VII and/or VIB of the Periodic System may be used. Preferably, the hydrogenation catalysts are supported Pd, Pt, Ni, NiMo or a CoMo catalysts, the support being alumina and/or silica. Typically, $NiMo/Al_2O_3$ and $CoMo/Al_2O_3$ catalysts are used.

[0281] Prior to the HDO step, the lipid composition or lipids herein may optionally be treated by prehydrogenation under milder conditions thus avoiding side reactions of the

double bonds. Such prehydrogenation is carried out in the presence of a prehydrogenation catalyst at temperatures of 50-400°C and at hydrogen pressures of 1-200 bar, preferably at a temperature between 150 and 250°C and at a hydrogen pressure between 10 and 100 bar. The catalyst may contain metals from Group VIII and/or VIB of the Periodic System. Preferably, the prehydrogenation catalyst is a supported Pd, Pt, Ni, NiMo or a CoMo catalyst, the support being alumina and/or silica.

[0282] A gaseous stream from the HDO step containing hydrogen is cooled and then carbon monoxide, carbon dioxide, nitrogen, phosphorus and sulfur compounds, gaseous light hydrocarbons and other impurities are removed therefrom. After compressing, the purified hydrogen or recycled hydrogen is returned back to the first catalyst bed and/or between the catalyst beds to make up for the withdrawn gas stream. Water is removed from the condensed liquid. The liquid is passed to the first catalyst bed or between the catalyst beds.

[0283] After the HDO step, the product is subjected to an isomerization step. It is substantial for the process that the impurities are removed as completely as possible before the hydrocarbons are contacted with the isomerization catalyst. The isomerization step comprises an optional stripping step, wherein the reaction product from the HDO step may be purified by stripping with water vapor or a suitable gas such as light hydrocarbon, nitrogen or hydrogen. The optional stripping step is carried out in counter-current manner in a unit upstream of the isomerization catalyst, wherein the gas and liquid are contacted with each other, or before the actual isomerization reactor in a separate stripping unit utilizing counter-current principle.

[0284] After the stripping step the hydrogen gas and the hydrogenated lipid composition or lipids herein, and optionally an n-paraffin mixture, are passed to a reactive isomerization unit comprising one or several catalyst bed(s). The catalyst beds of the isomerization step may operate either in co-current or counter-current manner.

[0285] It is important for the process that the counter-current flow principle is applied in the isomerization step. In the isomerization step this is done by carrying out either the optional stripping step or the isomerization reaction step or both in counter-current manner. In the isomerization step, the pressure varies in the range of 20-150 bar, preferably in the range of 20-100 bar, the temperature being between 200 and 500°C, preferably between 300 and 400°C. In the isomerization step, isomerization catalysts known in the art may be used. Suitable isomerization catalysts contain molecular sieve and/or a metal from Group VII and/or a carrier. Preferably, the isomerization catalyst contains SAPO-11 or SAPO41 or ZSM-22 or ZSM-23 or ferrierite and Pt, Pd or Ni and Al_2O_3 or SiO_2. Typical isomerization

catalysts are, for example, Pt/SAPO-11/Al_2O_3, Pt/ZSM-22/Al_2O_3, Pt/ZSM-23/Al_2O_3 and Pt/SAPO-11/SiO_2. The isomerization step and the HDO step may be carried out in the same pressure vessel or in separate pressure vessels. Optional prehydrogenation may be carried out in a separate pressure vessel or in the same pressure vessel as the HDO and isomerization steps.

[0286] Thus, in one embodiment, the product of one or more chemical reactions is an alkane mixture that comprises HRJ-5. In another embodiment, the product of the one or more chemical reactions is an alkane mixture that comprises ASTM D1655 jet fuel. In some embodiments, the composition conforming to the specification of ASTM 1655 jet fuel has a sulfur content that is less than 10 ppm. In other embodiments, the composition conforming to the specification of ASTM 1655 jet fuel has a T10 value of the distillation curve of less than 205° C. In another embodiment, the composition conforming to the specification of ASTM 1655 jet fuel has a final boiling point (FBP) of less than 300° C. In another embodiment, the composition conforming to the specification of ASTM 1655 jet fuel has a flash point of at least 38° C. In another embodiment, the composition conforming to the specification of ASTM 1655 jet fuel has a density between 775K/M^3 and 840K/M^3. In yet another embodiment, the composition conforming to the specification of ASTM 1655 jet fuel has a freezing point that is below -47° C. In another embodiment, the composition conforming to the specification of ASTM 1655 jet fuel has a net Heat of Combustion that is at least 42.8 MJ/K. In another embodiment, the composition conforming to the specification of ASTM 1655 jet fuel has a hydrogen content that is at least 13.4 mass %. In another embodiment, the composition conforming to the specification of ASTM 1655 jet fuel has a thermal stability, as tested by quantitative gravimetric JFTOT at 260° C, which is below 3mm of Hg. In another embodiment, the composition conforming to the specification of ASTM 1655 jet fuel has an existent gum that is below 7 mg/dl.

[0287] Thus, the present invention discloses a variety of methods in which chemical modification of microalgal lipid is undertaken to yield products useful in a variety of industrial and other applications. Examples of processes for modifying oil produced by the methods disclosed herein include, but are not limited to, hydrolysis of the oil, hydroprocessing of the oil, and esterification of the oil. Other chemical modification of microalgal lipid include, without limitation, epoxidation, oxidation, hydrolysis, sulfations, sulfonation, ethoxylation, propoxylation, amidation, and saponification. The modification of the microalgal oil produces basic oleochemicals that can be further modified into selected derivative oleochemicals for a desired function. In a manner similar to that described above

with reference to fuel producing processes, these chemical modifications can also be performed on oils generated from the microbial cultures described herein. Examples of basic oleochemicals include, but are not limited to, soaps, fatty acids, fatty esters, fatty alcohols, fatty nitrogen compounds including fatty amides, fatty acid methyl esters, and glycerol. Examples of derivative oleochemicals include, but are not limited to, fatty nitriles, esters, dimer acids, quats (including betaines), surfactants, fatty alkanolamides, fatty alcohol sulfates, resins, emulsifiers, fatty alcohols, olefins, drilling muds, polyols, polyurethanes, polyacrylates, rubber, candles, cosmetics, metallic soaps, soaps, alpha-sulphonated methyl esters, fatty alcohol sulfates, fatty alcohol ethoxylates, fatty alcohol ether sulfates, imidazolines, surfactants, detergents, esters, quats (including betaines), ozonolysis products, fatty amines, fatty alkanolamides, ethoxysulfates, monoglycerides, diglycerides, triglycerides (including medium chain triglycerides), lubricants, hydraulic fluids, greases, dielectric fluids, mold release agents, metal working fluids, heat transfer fluids, other functional fluids, industrial chemicals (e.g., cleaners, textile processing aids, plasticizers, stabilizers, additives), surface coatings, paints and lacquers, electrical wiring insulation, and higher alkanes. Other derivatives include fatty amidoamines, amidoamine carboxylates, amidoamine oxides, amidoamine oxide carboxylates, amidoamine esters, ethanolamine amides, sulfonates, amidoamine sulfonates, diamidoamine dioxides, sulfonated alkyl ester alkoxylates, betaines, quarternized diamidoamine betaines, and sulfobetaines.

[0288] Hydrolysis of the fatty acid constituents from the glycerolipids produced by the methods of the invention yields free fatty acids that can be derivatized to produce other useful chemicals. Hydrolysis occurs in the presence of water and a catalyst which may be either an acid or a base. The liberated free fatty acids can be derivatized to yield a variety of products, as reported in the following: US Patent Nos. 5,304,664 (Highly sulfated fatty acids); 7,262,158 (Cleansing compositions); 7,115,173 (Fabric softener compositions); 6,342,208 (Emulsions for treating skin); 7,264,886 (Water repellant compositions); 6,924,333 (Paint additives); 6,596,768 (Lipid-enriched ruminant feedstock); and 6,380,410 (Surfactants for detergents and cleaners).

[0289] In some methods, the first step of chemical modification may be hydroprocessing to saturate double bonds, followed by deoxygenation at elevated temperature in the presence of hydrogen and a catalyst. In other methods, hydrogenation and deoxygenation may occur in the same reaction. In still other methods deoxygenation occurs before hydrogenation. Isomerization may then be optionally performed, also in the presence of hydrogen and a catalyst. Finally, gases and naphtha components can be removed if desired. For example, see

U.S. Patents 5,475,160 (hydrogenation of triglycerides); 5,091,116 (deoxygenation, hydrogenation and gas removal); 6,391,815 (hydrogenation); and 5,888,947 (isomerization).

[0290] In some embodiments of the invention, the triglyceride oils are partially or completely deoxygenated. The deoxygenation reactions form desired products, including, but not limited to, fatty acids, fatty alcohols, polyols, ketones, and aldehydes. In general, without being limited by any particular theory, the deoxygenation reactions involve a combination of various different reaction pathways, including without limitation: hydrogenolysis, hydrogenation, consecutive hydrogenation-hydrogenolysis, consecutive hydrogenolysis-hydrogenation, and combined hydrogenation-hydrogenolysis reactions, resulting in at least the partial removal of oxygen from the fatty acid or fatty acid ester to produce reaction products, such as fatty alcohols, that can be easily converted to the desired chemicals by further processing. For example, in one embodiment, a fatty alcohol may be converted to olefins through FCC reaction or to higher alkanes through a condensation reaction.

[0291] One such chemical modification is hydrogenation, which is the addition of hydrogen to double bonds in the fatty acid constituents of glycerolipids or of free fatty acids. The hydrogenation process permits the transformation of liquid oils into semi-solid or solid fats, which may be more suitable for specific applications.

[0292] Hydrogenation of oil produced by the methods described herein can be performed in conjunction with one or more of the methods and/or materials provided herein, as reported in the following: US Patent Nos. 7,288,278 (Food additives or medicaments); 5,346,724 (Lubrication products); 5,475,160 (Fatty alcohols); 5,091,116 (Edible oils); 6,808,737 (Structural fats for margarine and spreads); 5,298,637 (Reduced-calorie fat substitutes); 6,391,815 (Hydrogenation catalyst and sulfur adsorbent); 5,233,099 and 5,233,100 (Fatty alcohols); 4,584,139 (Hydrogenation catalysts); 6,057,375 (Foam suppressing agents); and 7,118,773 (Edible emulsion spreads).

[0293] One skilled in the art will recognize that various processes may be used to hydrogenate carbohydrates. One suitable method includes contacting the carbohydrate with hydrogen or hydrogen mixed with a suitable gas and a catalyst under conditions sufficient in a hydrogenation reactor to form a hydrogenated product. The hydrogenation catalyst generally can include Cu, Re, Ni, Fe, Co, Ru, Pd, Rh, Pt, Os, Ir, and alloys or any combination thereof, either alone or with promoters such as W, Mo, Au, Ag, Cr, Zn, Mn, Sn, B, P, Bi, and alloys or any combination thereof. Other effective hydrogenation catalyst materials include either supported nickel or ruthenium modified with rhenium. In an

embodiment, the hydrogenation catalyst also includes any one of the supports, depending on the desired functionality of the catalyst. The hydrogenation catalysts may be prepared by methods known to those of ordinary skill in the art.

[0294] In some embodiments the hydrogenation catalyst includes a supported Group VIII metal catalyst and a metal sponge material (e.g., a sponge nickel catalyst). Raney nickel provides an example of an activated sponge nickel catalyst suitable for use in this invention. In other embodiment, the hydrogenation reaction in the invention is performed using a catalyst comprising a nickel-rhenium catalyst or a tungsten-modified nickel catalyst. One example of a suitable catalyst for the hydrogenation reaction of the invention is a carbon-supported nickel-rhenium catalyst.

[0295] In an embodiment, a suitable Raney nickel catalyst may be prepared by treating an alloy of approximately equal amounts by weight of nickel and aluminum with an aqueous alkali solution, e.g., containing about 25 weight % of sodium hydroxide. The aluminum is selectively dissolved by the aqueous alkali solution resulting in a sponge shaped material comprising mostly nickel with minor amounts of aluminum. The initial alloy includes promoter metals (i.e., molybdenum or chromium) in the amount such that about 1 to 2 weight % remains in the formed sponge nickel catalyst. In another embodiment, the hydrogenation catalyst is prepared using a solution of ruthenium (III) nitrosylnitrate, ruthenium (III) chloride in water to impregnate a suitable support material. The solution is then dried to form a solid having a water content of less than about 1% by weight. The solid may then be reduced at atmospheric pressure in a hydrogen stream at 300°C (uncalcined) or 400°C (calcined) in a rotary ball furnace for 4 hours. After cooling and rendering the catalyst inert with nitrogen, 5% by volume of oxygen in nitrogen is passed over the catalyst for 2 hours.

[0296] In certain embodiments, the catalyst described includes a catalyst support. The catalyst support stabilizes and supports the catalyst. The type of catalyst support used depends on the chosen catalyst and the reaction conditions. Suitable supports for the invention include, but are not limited to, carbon, silica, silica-alumina, zirconia, titania, ceria, vanadia, nitride, boron nitride, heteropolyacids, hydroxyapatite, zinc oxide, chromia, zeolites, carbon nanotubes, carbon fullerene and any combination thereof.

[0297] The catalysts used in this invention can be prepared using conventional methods known to those in the art. Suitable methods may include, but are not limited to, incipient wetting, evaporative impregnation, chemical vapor deposition, wash-coating, magnetron sputtering techniques, and the like.

[0298] The conditions for which to carry out the hydrogenation reaction will vary based on the type of starting material and the desired products. One of ordinary skill in the art, with the benefit of this disclosure, will recognize the appropriate reaction conditions. In general, the hydrogenation reaction is conducted at temperatures of 80°C to 250°C, and preferably at 90°C to 200°C, and most preferably at 100°C to 150°C. In some embodiments, the hydrogenation reaction is conducted at pressures from 500 KPa to 14000 KPa.

[0299] The hydrogen used in the hydrogenolysis reaction of the current invention may include external hydrogen, recycled hydrogen, in situ generated hydrogen, and any combination thereof. As used herein, the term "external hydrogen" refers to hydrogen that does not originate from the biomass reaction itself, but rather is added to the system from another source.

[0300] In some embodiments of the invention, it is desirable to convert the starting carbohydrate to a smaller molecule that will be more readily converted to desired higher hydrocarbons. One suitable method for this conversion is through a hydrogenolysis reaction. Various processes are known for performing hydrogenolysis of carbohydrates. One suitable method includes contacting a carbohydrate with hydrogen or hydrogen mixed with a suitable gas and a hydrogenolysis catalyst in a hydrogenolysis reactor under conditions sufficient to form a reaction product comprising smaller molecules or polyols. As used herein, the term "smaller molecules or polyols" includes any molecule that has a smaller molecular weight, which can include a smaller number of carbon atoms or oxygen atoms than the starting carbohydrate. In an embodiment, the reaction products include smaller molecules that include polyols and alcohols. Someone of ordinary skill in the art would be able to choose the appropriate method by which to carry out the hydrogenolysis reaction.

[0301] In some embodiments, a 5 and/or 6 carbon sugar or sugar alcohol may be converted to propylene glycol, ethylene glycol, and glycerol using a hydrogenolysis catalyst. The hydrogenolysis catalyst may include Cr, Mo, W, Re, Mn, Cu, Cd, Fe, Co, Ni, Pt, Pd, Rh, Ru, Ir, Os, and alloys or any combination thereof, either alone or with promoters such as Au, Ag, Cr, Zn, Mn, Sn, Bi, B, O, and alloys or any combination thereof. The hydrogenolysis catalyst may also include a carbonaceous pyropolymer catalyst containing transition metals (e.g., chromium, molybdenum, tungsten, rhenium, manganese, copper, cadmium) or Group VIII metals (e.g., iron, cobalt, nickel, platinum, palladium, rhodium, ruthenium, iridium, and osmium). In certain embodiments, the hydrogenolysis catalyst may include any of the above metals combined with an alkaline earth metal oxide or adhered to a catalytically active

support. In certain embodiments, the catalyst described in the hydrogenolysis reaction may include a catalyst support as described above for the hydrogenation reaction.

[0302] The conditions for which to carry out the hydrogenolysis reaction will vary based on the type of starting material and the desired products. One of ordinary skill in the art, with the benefit of this disclosure, will recognize the appropriate conditions to use to carry out the reaction. In general, they hydrogenolysis reaction is conducted at temperatures of 110°C to 300°C, and preferably at 170°C to 220°C, and most preferably at 200°C to 225°C. In some embodiments, the hydrogenolysis reaction is conducted under basic conditions, preferably at a pH of 8 to 13, and even more preferably at a pH of 10 to 12. In some embodiments, the hydrogenolysis reaction is conducted at pressures in a range between 60 KPa and 16500 KPa, and preferably in a range between 1700 KPa and 14000 KPa, and even more preferably between 4800 KPa and 11000 KPa.

[0303] The hydrogen used in the hydrogenolysis reaction of the current invention can include external hydrogen, recycled hydrogen, in situ generated hydrogen, and any combination thereof.

[0304] In some embodiments, the reaction products discussed above may be converted into higher hydrocarbons through a condensation reaction in a condensation reactor. In such embodiments, condensation of the reaction products occurs in the presence of a catalyst capable of forming higher hydrocarbons. While not intending to be limited by theory, it is believed that the production of higher hydrocarbons proceeds through a stepwise addition reaction including the formation of carbon-carbon, or carbon-oxygen bond. The resulting reaction products include any number of compounds containing these moieties, as described in more detail below.

[0305] In certain embodiments, suitable condensation catalysts include an acid catalyst, a base catalyst, or an acid/base catalyst. As used herein, the term "acid/base catalyst" refers to a catalyst that has both an acid and a base functionality. In some embodiments the condensation catalyst can include, without limitation, zeolites, carbides, nitrides, zirconia, alumina, silica, aluminosilicates, phosphates, titanium oxides, zinc oxides, vanadium oxides, lanthanum oxides, yttrium oxides, scandium oxides, magnesium oxides, cerium oxides, barium oxides, calcium oxides, hydroxides, heteropolyacids, inorganic acids, acid modified resins, base modified resins, and any combination thereof. In some embodiments, the condensation catalyst can also include a modifier. Suitable modifiers include La, Y, Sc, P, B, Bi, Li, Na, K, Rb, Cs, Mg, Ca, Sr, Ba, and any combination thereof. In some embodiments, the condensation catalyst can also include a metal. Suitable metals include Cu, Ag, Au, Pt,

Ni, Fe, Co, Ru, Zn, Cd, Ga, In, Rh, Pd, Ir, Re, Mn, Cr, Mo, W, Sn, Os, alloys, and any combination thereof.

[0306] In certain embodiments, the catalyst described in the condensation reaction may include a catalyst support as described above for the hydrogenation reaction. In certain embodiments, the condensation catalyst is self-supporting. As used herein, the term "self-supporting" means that the catalyst does not need another material to serve as support. In other embodiments, the condensation catalyst in used in conjunction with a separate support suitable for suspending the catalyst. In an embodiment, the condensation catalyst support is silica.

[0307] The conditions under which the condensation reaction occurs will vary based on the type of starting material and the desired products. One of ordinary skill in the art, with the benefit of this disclosure, will recognize the appropriate conditions to use to carry out the reaction. In some embodiments, the condensation reaction is carried out at a temperature at which the thermodynamics for the proposed reaction are favorable. The temperature for the condensation reaction will vary depending on the specific starting polyol or alcohol. In some embodiments, the temperature for the condensation reaction is in a range from 80°C to 500°C, and preferably from 125°C to 450°C, and most preferably from 125°C to 250°C. In some embodiments, the condensation reaction is conducted at pressures in a range between 0 Kpa to 9000 KPa, and preferably in a range between 0 KPa and 7000 KPa, and even more preferably between 0 KPa and 5000 KPa.

[0308] The higher alkanes formed by the invention include, but are not limited to, branched or straight chain alkanes that have from 4 to 30 carbon atoms, branched or straight chain alkenes that have from 4 to 30 carbon atoms, cycloalkanes that have from 5 to 30 carbon atoms, cycloalkenes that have from 5 to 30 carbon atoms, aryls, fused aryls, alcohols, and ketones. Suitable alkanes include, but are not limited to, butane, pentane, pentene, 2-methylbutane, hexane, hexene, 2-methylpentane, 3-methylpentane, 2,2,-dimethylbutane, 2,3-dimethylbutane, heptane, heptene, octane, octene, 2,2,4-trimethylpentane, 2,3-dimethyl hexane, 2,3,4-trimethylpentane, 2,3-dimethylpentane, nonane, nonene, decane, decene, undecane, undecene, dodecane, dodecene, tridecane, tridecene, tetradecane, tetradecene, pentadecane, pentadecene, nonyldecane, nonyldecene, eicosane, eicosene, uneicosane, uneicosene, doeicosane, doeicosene, trieicosane, trieicosene, tetraeicosane, tetraeicosene, and isomers thereof. Some of these products may be suitable for use as fuels.

[0309] In some embodiments, the cycloalkanes and the cycloalkenes are unsubstituted. In other embodiments, the cycloalkanes and cycloalkenes are mono-substituted. In still other

embodiments, the cycloalkanes and cycloalkenes are multi-substituted. In the embodiments comprising the substituted cycloalkanes and cycloalkenes, the substituted group includes, without limitation, a branched or straight chain alkyl having 1 to 12 carbon atoms, a branched or straight chain alkylene having 1 to 12 carbon atoms, a phenyl, and any combination thereof. Suitable cycloalkanes and cycloalkenes include, but are not limited to, cyclopentane, cyclopentene, cyclohexane, cyclohexene, methyl-cyclopentane, methyl-cyclopentene, ethyl-cyclopentane, ethyl-cyclopentene, ethyl-cyclohexane, ethyl-cyclohexene, isomers and any combination thereof.

[0310] In some embodiments, the aryls formed are unsubstituted. In another embodiment, the aryls formed are mono-substituted. In the embodiments comprising the substituted aryls, the substituted group includes, without limitation, a branched or straight chain alkyl having 1 to 12 carbon atoms, a branched or straight chain alkylene having 1 to 12 carbon atoms, a phenyl, and any combination thereof. Suitable aryls for the invention include, but are not limited to, benzene, toluene, xylene, ethyl benzene, para xylene, meta xylene, and any combination thereof.

[0311] The alcohols produced in the invention have from 4 to 30 carbon atoms. In some embodiments, the alcohols are cyclic. In other embodiments, the alcohols are branched. In another embodiment, the alcohols are straight chained. Suitable alcohols for the invention include, but are not limited to, butanol, pentanol, hexanol, heptanol, octanol, nonanol, decanol, undecanol, dodecanol, tridecanol, tetradecanol, pentadecanol, hexadecanol, heptyldecanol, octyldecanol, nonyldecanol, eicosanol, uneicosanol, doeicosanol, trieicosanol, tetraeicosanol, and isomers thereof.

[0312] The ketones produced in the invention have from 4 to 30 carbon atoms. In an embodiment, the ketones are cyclic. In another embodiment, the ketones are branched. In another embodiment, the ketones are straight chained. Suitable ketones for the invention include, but are not limited to, butanone, pentanone, hexanone, heptanone, octanone, nonanone, decanone, undecanone, dodecanone, tridecanone, tetradecanone, pentadecanone, hexadecanone, heptyldecanone, octyldecanone, nonyldecanone, eicosanone, uneicosanone, doeicosanone, trieicosanone, tetraeicosanone, and isomers thereof.

[0313] Another such chemical modification is interesterification. Naturally produced glycerolipids do not have a uniform distribution of fatty acid constituents. In the context of oils, interesterification refers to the exchange of acyl radicals between two esters of different glycerolipids. The interesterification process provides a mechanism by which the fatty acid constituents of a mixture of glycerolipids can be rearranged to modify the distribution pattern.

Interesterification is a well-known chemical process, and generally comprises heating (to about 200°C) a mixture of oils for a period (e.g., 30 minutes) in the presence of a catalyst, such as an alkali metal or alkali metal alkylate (e.g., sodium methoxide). This process can be used to randomize the distribution pattern of the fatty acid constituents of an oil mixture, or can be directed to produce a desired distribution pattern. This method of chemical modification of lipids can be performed on materials provided herein, such as microbial biomass with a percentage of dry cell weight as lipid at least 20%.

[0314] Directed interesterification, in which a specific distribution pattern of fatty acids is sought, can be performed by maintaining the oil mixture at a temperature below the melting point of some TAGs which might occur. This results in selective crystallization of these TAGs, which effectively removes them from the reaction mixture as they crystallize. The process can be continued until most of the fatty acids in the oil have precipitated, for example. A directed interesterification process can be used, for example, to produce a product with a lower calorie content via the substitution of longer-chain fatty acids with shorter-chain counterparts. Directed interesterification can also be used to produce a product with a mixture of fats that can provide desired melting characteristics and structural features sought in food additives or products (e.g., margarine) without resorting to hydrogenation, which can produce unwanted trans isomers.

[0315] Interesterification of oils produced by the methods described herein can be performed in conjunction with one or more of the methods and/or materials, or to produce products, as reported in the following: US Patent Nos. 6,080,853 (Nondigestible fat substitutes); 4,288,378 (Peanut butter stabilizer); 5,391,383 (Edible spray oil); 6,022,577 (Edible fats for food products); 5,434,278 (Edible fats for food products); 5,268,192 (Low calorie nut products); 5,258,197 (Reduce calorie edible compositions); 4,335,156 (Edible fat product); 7,288,278 (Food additives or medicaments); 7,115,760 (Fractionation process); 6,808,737 (Structural fats); 5,888,947 (Engine lubricants); 5,686,131 (Edible oil mixtures); and 4,603,188 (Curable urethane compositions).

[0316] In one embodiment in accordance with the invention, transesterification of the oil, as described above, is followed by reaction of the transesterified product with polyol, as reported in US Patent No. 6,465,642, to produce polyol fatty acid polyesters. Such an esterification and separation process may comprise the steps as follows: reacting a lower alkyl ester with polyol in the presence of soap; removing residual soap from the product mixture; water-washing and drying the product mixture to remove impurities; bleaching the product mixture for refinement; separating at least a portion of the unreacted lower alkyl ester

from the polyol fatty acid polyester in the product mixture; and recycling the separated unreacted lower alkyl ester.

[0317] Transesterification can also be performed on microbial biomass with short chain fatty acid esters, as reported in U.S. Patent 6,278,006. In general, transesterification may be performed by adding a short chain fatty acid ester to an oil in the presence of a suitable catalyst and heating the mixture. In some embodiments, the oil comprises about 5% to about 90% of the reaction mixture by weight. In some embodiments, the short chain fatty acid esters can be about 10% to about 50% of the reaction mixture by weight. Non-limiting examples of catalysts include base catalysts, sodium methoxide, acid catalysts including inorganic acids such as sulfuric acid and acidified clays, organic acids such as methane sulfonic acid, benzenesulfonic acid, and toluenesulfonic acid, and acidic resins such as Amberlyst 15. Metals such as sodium and magnesium, and metal hydrides also are useful catalysts.

[0318] Another such chemical modification is hydroxylation, which involves the addition of water to a double bond resulting in saturation and the incorporation of a hydroxyl moiety. The hydroxylation process provides a mechanism for converting one or more fatty acid constituents of a glycerolipid to a hydroxy fatty acid. Hydroxylation can be performed, for example, via the method reported in US Patent No. 5,576,027. Hydroxylated fatty acids, including castor oil and its derivatives, are useful as components in several industrial applications, including food additives, surfactants, pigment wetting agents, defoaming agents, water proofing additives, plasticizing agents, cosmetic emulsifying and/or deodorant agents, as well as in electronics, pharmaceuticals, paints, inks, adhesives, and lubricants. One example of how the hydroxylation of a glyceride may be performed is as follows: fat may be heated, preferably to about 30-50°C combined with heptane and maintained at temperature for thirty minutes or more; acetic acid may then be added to the mixture followed by an aqueous solution of sulfuric acid followed by an aqueous hydrogen peroxide solution which is added in small increments to the mixture over one hour; after the aqueous hydrogen peroxide, the temperature may then be increased to at least about 60°C and stirred for at least six hours; after the stirring, the mixture is allowed to settle and a lower aqueous layer formed by the reaction may be removed while the upper heptane layer formed by the reaction may be washed with hot water having a temperature of about 60°C; the washed heptane layer may then be neutralized with an aqueous potassium hydroxide solution to a pH of about 5 to 7 and then removed by distillation under vacuum; the reaction product may then be dried under

vacuum at 100°C and the dried product steam-deodorized under vacuum conditions and filtered at about 50° to 60°C using diatomaceous earth.

[0319] Hydroxylation of microbial oils produced by the methods described herein can be performed in conjunction with one or more of the methods and/or materials, or to produce products, as reported in the following: US Patent Nos. 6,590,113 (Oil-based coatings and ink); 4,049,724 (Hydroxylation process); 6,113,971 (Olive oil butter); 4,992,189 (Lubricants and lube additives); 5,576,027 (Hydroxylated milk); and 6,869,597 (Cosmetics).

[0320] Hydroxylated glycerolipids can be converted to estolides. Estolides consist of a glycerolipid in which a hydroxylated fatty acid constituent has been esterified to another fatty acid molecule. Conversion of hydroxylated glycerolipids to estolides can be carried out by warming a mixture of glycerolipids and fatty acids and contacting the mixture with a mineral acid, as described by Isbell et al., *JAOCS* 71(2):169-174 (1994). Estolides are useful in a variety of applications, including without limitation those reported in the following: US Patent Nos. 7,196,124 (Elastomeric materials and floor coverings); 5,458,795 (Thickened oils for high-temperature applications); 5,451,332 (Fluids for industrial applications); 5,427,704 (Fuel additives); and 5,380,894 (Lubricants, greases, plasticizers, and printing inks).

[0321] Another such chemical modification is olefin metathesis. In olefin metathesis, a catalyst severs the alkylidene carbons in an alkene (olefin) and forms new alkenes by pairing each of them with different alkylidine carbons. The olefin metathesis reaction provides a mechanism for processes such as truncating unsaturated fatty acid alkyl chains at alkenes by ethenolysis, cross-linking fatty acids through alkene linkages by self-metathesis, and incorporating new functional groups on fatty acids by cross-metathesis with derivatized alkenes.

[0322] In conjunction with other reactions, such as transesterification and hydrogenation, olefin metathesis can transform unsaturated glycerolipids into diverse end products. These products include glycerolipid oligomers for waxes; short-chain glycerolipids for lubricants; homo- and hetero-bifunctional alkyl chains for chemicals and polymers; short-chain esters for biofuel; and short-chain hydrocarbons for jet fuel. Olefin metathesis can be performed on triacylglycerols and fatty acid derivatives, for example, using the catalysts and methods reported in U.S. Patent No. 7,119,216, US Patent Pub. No. 2010/0160506, and U.S. Patent Pub. No. 2010/0145086.

[0323] Olefin metathesis of bio-oils generally comprises adding a solution of Ru catalyst at a loading of about 10 to 250 ppm under inert conditions to unsaturated fatty acid esters in the presence (cross-metathesis) or absence (self-metathesis) of other alkenes. The reactions are

typically allowed to proceed from hours to days and ultimately yield a distribution of alkene products. One example of how olefin metathesis may be performed on a fatty acid derivative is as follows: A solution of the first generation Grubbs Catalyst (dichloro[2(1-methylethoxy-α-O)phenyl]methylene-α-C] (tricyclohexyl-phosphine) in toluene at a catalyst loading of 222 ppm may be added to a vessel containing degassed and dried methyl oleate. Then the vessel may be pressurized with about 60 psig of ethylene gas and maintained at or below about 30°C for 3 hours, whereby approximately a 50% yield of methyl 9-decenoate may be produced.

[0324] Olefin metathesis of oils produced by the methods described herein can be performed in conjunction with one or more of the methods and/or materials, or to produce products, as reported in the following: Patent App. PCT/US07/081427 (α-olefin fatty acids) and U.S. Patent App. Nos. 12/281,938 (petroleum creams), 12/281,931 (paintball gun capsules), 12/653,742 (plasticizers and lubricants), 12/422,096 (bifunctional organic compounds), and 11/795,052 (candle wax).

[0325] Other chemical reactions that can be performed on microbial oils include reacting triacylglycerols with a cyclopropanating agent to enhance fluidity and/or oxidative stability, as reported in U.S. Patent 6,051,539; manufacturing of waxes from triacylglycerols, as reported in U.S. Patent 6,770,104; and epoxidation of triacylglycerols, as reported in "The effect of fatty acid composition on the acrylation kinetics of epoxidized triacylglycerols", Journal of the American Oil Chemists' Society, 79:1, 59-63, (2001) and Free Radical Biology and Medicine, 37:1, 104-114 (2004).

[0326] The generation of oil-bearing microbial biomass for fuel and chemical products as described above results in the production of delipidated biomass meal. Delipidated meal is a byproduct of preparing algal oil and is useful as animal feed for farm animals, e.g., ruminants, poultry, swine and aquaculture. The resulting meal, although of reduced oil content, still contains high quality proteins, carbohydrates, fiber, ash, residual oil and other nutrients appropriate for an animal feed. Because the cells are predominantly lysed by the oil separation process, the delipidated meal is easily digestible by such animals. Delipidated meal can optionally be combined with other ingredients, such as grain, in an animal feed. Because delipidated meal has a powdery consistency, it can be pressed into pellets using an extruder or expander or another type of machine, which are commercially available.

[0327] The invention, having been described in detail above, is exemplified in the following examples, which are offered to illustrate, but not to limit, the claimed invention.

XIV. EXAMPLES

EXAMPLE 1: FATTY ACID ANALYSIS BY FATTY ACID METHYL ESTER DETECTION

[0328] Lipid samples were prepared from dried biomass. 20-40 mg of dried biomass was resuspended in 2 mL of 5% H_2SO_4 in MeOH, and 200 ul of toluene containing an appropriate amount of a suitable internal standard (C19:0) was added. The mixture was sonicated briefly to disperse the biomass, then heated at 70 -75°C for 3.5 hours. 2 mL of heptane was added to extract the fatty acid methyl esters, followed by addition of 2 mL of 6% K_2CO_3 (aq) to neutralize the acid. The mixture was agitated vigorously, and a portion of the upper layer was transferred to a vial containing Na_2SO_4 (anhydrous) for gas chromatography analysis using standard FAME GC/FID (fatty acid methyl ester gas chromatography flame ionization detection) methods. Fatty acid profiles reported below were determined by this method.

EXAMPLE 2: TRIACYLGLYCERIDE PURIFICATION FROM OIL AND METHODS FOR TRIACYLGLYCERIDE LIPASE DIGESTION

[0329] The triacylglyceride (TAG) fraction of each oil sample was isolated by dissolving ~10 mg of oil in dichloromethane and loading it onto a Bond-Elut aminopropyl solid-phase extraction cartridge (500 mg) preconditioned with heptane. TAGs were eluted with dicholoromethane-MeOH (1:1) into a collection tube, while polar lipids were retained on the column. The solvent was removed with a stream of nitrogen gas. Tris buffer and 2 mg porcine pancreatic lipase (Type II, Sigma, 100-400 units/mg) were added to the TAG fraction, followed by addition of bile salt and calcium chloride solutions. The porcine pancreatic lipase cleaves sn-1 and sn-3 fatty acids, thereby generating 2-monoacylglycerides and free fatty acids. This mixture was heated with agitation at 40 ℃ for three minutes, cooled briefly, then quenched with 6 N HCl. The mixture was then extracted with diethyl ether and the ether layer was washed with water then dried over sodium sulfate. The solvent was removed with a stream of nitrogen. To isolate the monoacylglyceride (MAG) fraction, the residue was dissolved in heptane and loaded onto a second aminopropyl solid phase extraction cartridge pretreated with heptane. Residual TAGs were eluted with diethyl ether-dichloromethane-heptane (1:9:40), diacylglycerides (DAGs) were eluted with ethyl acetate-heptane (1:4), and MAGs were eluted from the cartridge with dichloromethane-methanol (2:1). The resulting MAG, DAG, and TAG fractions were then concentrated to dryness with a stream of nitrogen and subjected to routine direct transesterification method of GC/FID analysis as described in Example 1.

EXAMPLE 3: ENGINEERING MICROORGANISMS FOR FATTY ACID AND SN-2 PROFILES INCREASED IN LAURIC ACID THROUGH EXOGENOUS LPAAT EXPRESSION

[0330] This example describes the use of recombinant polynucleotides that encode a *C. nucifera* 1-acyl-sn-glycerol-3-phosphate acyltransferase (*Cn* LPAAT) enzyme to engineer a microorganism in which the fatty acid profile and the sn-2 profile of the transformed microorganism has been enriched in lauric acid.

[0331] A classically mutagenized strain of *Prototheca moriformis* (UTEX 1435), Strain A, was initially transformed with the plasmid construct pSZ1283 according to biolistic transformation methods as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. pSZ1283, described in PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696 hereby incorporated by reference, comprised the coding sequence of the *Cuphea wrightii* FATB2 (*Cw*TE2) thioesterase (SEQ ID NO: 10), 5' (SEQ ID NO: 1) and 3' (SEQ ID NO: 2) homologous recombination targeting sequences (flanking the construct) to the 6S genomic region for integration into the nuclear genome, and a *S. cerevisiae suc2* sucrose invertase coding region (SEQ ID NO: 4), to express the protein sequence given in SEQ ID NO: 3, under the control of *C. reinhardtii* β-tubulin promoter/5'UTR (SEQ ID NO: 5) and *Chlorella vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6). This *S. cerevisiae suc2* expression cassette is listed as SEQ ID NO: 7 and served as a selectable marker. The *Cw*TE2 protein coding sequence to express the protein sequence given in SEQ ID NO: 11, was under the control of the *P. moriformis* Amt03 promoter/5'UTR (SEQ ID NO: 8) and *C. vulgaris* nitrate reductase 3'UTR. The protein coding regions of *CwTE2* and *suc2* were codon optimized to reflect the codon bias inherent in *P. moriformis* UTEX 1435 nuclear genes as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696.

[0332] Upon transformation of pSZ1283 into Strain A, positive clones were selected on agar plates with sucrose as the sole carbon source. Primary transformants were then clonally purified and a single transformant, Strain B, was selected for further genetic modification. This genetically engineered strain was transformed with plasmid construct pSZ2046 to interrupt the pLoop genomic locus of Strain B. Construct pSZ2046 comprised the coding sequence of the *C. nucifera* 1-acyl-sn-glycerol-3-phosphate acyltransferase (*Cn* LPAAT) enzyme (SEQ ID NO: 12), 5' (SEQ ID NO: 13) and 3' (SEQ ID NO: 14) homologous recombination targeting sequences (flanking the construct) to the pLoop genomic region for

integration into the nuclear genome, and a neomycin resistance protein-coding sequence under the control of *C. reinhardtii* β-tubulin promoter/5'UTR (SEQ ID NO: 5), and *Chlorella vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6). This NeoR expression cassette is listed as SEQ ID NO: 15 and served as a selectable marker. The *Cn* LPAAT protein coding sequence was under the control of the *P. moriformis* Amt03 promoter/5'UTR (SEQ ID NO: 8) and *C. vulgaris* nitrate reductase 3'UTR. The protein coding regions of *Cn* LPAAT and NeoR were codon optimized to reflect the codon bias inherent in *P. moriformis* UTEX 1435 nuclear genes as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. The amino acid sequence of *Cn LPAAT* is provided as SEQ ID NO: 16.

[0333] Upon transformation of pSZ2046 into Strain B, thereby generating Strain C, positive clones were selected on agar plates comprising G418 (Geneticin). Individual transformants were clonally purified and grown at pH 7.0 under conditions suitable for lipid production as detailed in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. Lipid samples were prepared from dried biomass from each transformant and fatty acid profiles from these samples were analyzed using standard fatty acid methyl ester gas chromatography flame ionization (FAME GC/FID) detection methods as described in Example 1.The fatty acid profiles (expressed as Area % of total fatty acids) of *P. moriformis* UTEX 1435 (U1) grown on glucose as a sole carbon source, untransformed Strain B and five pSZ2046 positive transformants (Strain C, 1-5) are presented in Table 6.

[0334] Table 6. Effect of LPAAT expression on fatty acid profiles of transformed Prototheca moriformis (UTEX 1435) comprising a mid-chain preferring thioesterase.

Area % Fatty acid	U1	Strain B	Strain C-1	Strain C-2	Strain C-3	Strain C-4	Strain C-5
C10:0	0.01	5.53	11.37	11.47	10.84	11.13	11.12
C12:0	0.04	31.04	46.63	46.47	45.84	45.80	45.67
C14:0	1.27	15.99	15.14	15.12	15.20	15.19	15.07
C16:0	27.20	12.49	7.05	7.03	7.30	7.20	7.19
C18:0	3.85	1.30	0.71	0.72	0.74	0.74	0.74
C18:1	58.70	24.39	10.26	10.41	10.95	11.31	11.45

C18:2	7.18	7.79	7.05	6.93	7.30	6.88	7.01
C10-C12	0.50	36.57	58.00	57.94	56.68	56.93	56.79

[0335] As shown in Table 6, the fatty acid profile of Strain B expressing *Cw*TE2 showed increased composition of C10:0, C12:0, and C14:0 fatty acids and a decrease in C16:0, C18:0, and C18:1 fatty acids relative to the fatty acid profile of the untransformed UTEX 1435 strain. The impact of additional genetic modification on the fatty acid profile of the transformed strains, namely the expression of CnLPAAT in Strain B, is a still further increase in the composition of C10:0 and C12:0 fatty acids, a still further decrease in C16:0, C18:0, and C18:1 fatty acids, but no significant effect on the C14:0 fatty acid composition. These data indicate that the CnLPAAT shows substrate preference in the context of a microbial host organism.

[0336] The untransformed *P. moriformis* Strain Ais characterized by a fatty acid profile comprising less than 0.5% C12 fatty acids and less than 1% C10-C12 fatty acids. In contrast, the fatty acid profile of Strain B expressing a *C. wrightii* thioesterase comprised 31% C12:0 fatty acids, with C10-C12 fatty acids comprising greater than 36% of the total fatty acids. Further, fatty acid profiles of Strain C, expressing a higher plant thioesterase and a *Cn*LPAAT enzyme, comprised between 45.67% and 46.63% C12:0 fatty acids, with C10-C12% fatty acids comprising between 71 and 73% of total fatty acids. The result of expressing an exogenous thioesterase was a 62-fold increase in the percentage of C12 fatty acid present in the engineered microbe. The result of expressing an exogenous thioesterase and exogenous LPAAT was a 92-fold increase in the percentage of C12 fatty acids present in the engineered microbe.

[0337] The TAG fraction of oil samples extracted from Strains A, B, and C were analyzed for the sn-2 profile of their triacylglycerides. The TAGs were extracted and processed as described in Example 2 and analyzed as in Examples 1 and 2. The fatty acid composition and the sn-2 profiles of the TAG fraction of oil extracted from Strains A, B, and C (expressed as Area % of total fatty acids) are presented in Table 7. Values not reported are indicated as "n.r."

[0338] Table 7. Effect of LPAAT expression on the fatty acid composition and the sn-2 profile of TAGs produced from transformed Prototheca moriformis (UTEX 1435) comprising a mid-chain preferring thioesterase.

Strain	Strain A (untransformed)		Strain B (pSZ1500)		Strain C (pSZ1500 + pSZ2046)	
Area % fatty acid	FA	sn-2 profile	FA	sn-2 profile	FA	sn-2 profile
C10:0	n.r.	n.r.	11.9	14.2	12.4	7.1
C12:0	n.r.	n.r.	42.4	25	47.9	52.8
C14:0	1.0	0.6	12	10.4	13.9	9.1
C16:0	23.9	1.6	7.2	1.3	6.1	0.9
C18:0	3.7	0.3	n.r	n.r.	0.8	0.3
C18:1	64.3	90.5	18.3	36.6	9.9	17.5
C18:2	4.5	5.8	5.8	10.8	6.5	10
C18:3	n.r.	n.r.	n.r.	n.r.	1.1	1.6

[0339] As shown in Table 7, the fatty acid composition of triglycerides (TAGs) isolated from Strain B expressing *Cw*TE2 was increased for C10:0, C12:0, and C14:0 fatty acids and decrease in C16:0 and C18:1 fatty acids relative to the fatty acid profile of TAGs isolated from untransformed Strain A. The impact of additional genetic modification on the fatty acid profile of the transformed strains, namely the expression of CnLPAAT, was a still further increase in the composition of C10:0 and C12:0 fatty acids, a still further decrease in C16:0, C18:0, and C18:1 fatty acids, but no significant effect on the C14:0 fatty acid composition. These data indicate that expression of the exogenous CnLPAAT improves the midchain fatty acid profile of transformed microbes.

[0340] The untransformed *P. moriformis* Strain A is characterized by an sn-2 profile of about 0.6% C14, about 1.6% C16:0, about 0.3% C18:0, about 90% C18:1, and about 5.8% C18:2. In contrast to Strain A, Strain B, expressing a *C. wrightii* thioesterase is characterized by an sn-2 profile that is higher in midchain fatty acids and lower in long chain fatty acids. C12 fatty acids comprised 25% of the sn-2 profile of Strain B. The impact of additional genetic modification on the sn-2 profile of the transformed strains, namely the expression of CnLPAAT, was still a further increase in C12 fatty acids (from 25% to 52.8%), a decrease in C18:1 fatty acids (from 36.6% to 17.5%), and a decrease in C10:0 fatty acids. (The sn-2 profile composition of C14:0 and C16:0 fatty acids was relatively similar for Strains B and C.)

[0341] These data demonstrate the utility and effectiveness of polynucleotides permitting exogenous LPAAT expression to alter the fatty acid profile of engineered microorganisms, and in particular in increasing the concentration of C10:0 and C12:0 fatty acids in microbial cells. These data further demonstrate the utility and effectiveness of polynucleotides permitting exogenous thioesterase and exogenous LPAAT expression to alter the sn-2 profile of TAGs produced by microbial cells, in particular in increasing the C12 composition of sn-2 profiles and decreasing the C18:1 composition of sn-2 profiles.

EXAMPLE 4: THERMAL BEHAVIOR OF OILS PRODUCED FROM RECOMBINANT MICROALGAE.

[0342] Figures 1-14 include fatty acid profiles and melting curves of refined, bleached and deodorized oils from genetically engineered *Prototheca moriformis* strains. In some cases, modifications of the melting curves are obtained via genetic engineering. For example, some of the oils produced have shallower or sharper melting transitions relative to control microalgal oils (i.e., those produced from strains lacking a given genetic modification) or relative to widely available plant oils. In addition, Figure 12 shows scanning calorimetry for a high palmitic oil when tempered by holding at room temperature for several days (lower trace) and for the same oil after performing the first scan (upper trace). The scans ranged from -60°C to +50°C with a heating rate of 10°C/minute. The differences between the two traces suggests that tempering of the oil caused a change in crystal structure within the oil.

[0343] Also of note, Figures 10 and 11 show stability testing of RBD-5 and RBD 6. Remarkably, RBD-6, an oil with less than 0.1% 18:2 and 18:3 fatty acids was substantially stable as measured by the oxidative stability index (AOCS Method Cd 12b-92) even after 36 hours of heating at 110°C.

[0344] Table 8, below, gives details of the genetic engineering of the strains identified in Figures 1-13.

[0345] Table 8. Genetically engineered strains.

RB Z	Ulmus Americana thioesterase
RBD-1	Cuphea wrightii FATB2 thioesterase driven by amt03
RBD-2	Ulmus americana thioesterase
RBD-3	Native C. hookeriana C16:0-specific thioesterase with amt03 promoter
RBD Y	Ulmus Americana thioesterase with Btub promoter

RBD X	SAD2B knockout with native C wrightii FAT2B thioesterase, amt03 promoter
RBD W	SAD2B KO with Native C. wrightii FATB2 driven by amt03 at insertion site
RBD-4	control strain
RBD-5	FATA-1 knockout with Carthamus oleate sp. TE driven by amt03 promoter at insertion site
RBD-6	FADc knockout with *Carthamus tinctorius* oleoyl thioesterase

EXAMPLE 5: CHARACTERISTICS OF PROCESSED OIL PRODUCED FROM ENGINEERED MICROORGANISMS

[0346] Methods and effects of transforming *Prototheca moriformis* (UTEX 1435) with transformation vector pSZ1500 (SEQ ID NO: 17) have been previously described in PCT Application Nos. PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696.

[0347] A classically mutagenized (for higher oil production) derivative of *Prototheca moriformis* (UTEX 1435), Strain A, was transformed with pSZ1500 according to biolistic transformation methods as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. pSZ1500 comprised nucleotide sequence of the *Carthamus tinctorius* oleyl-thioesterase (CtOTE) gene, codon-optimized for expression in *P. moriformis* UTEX 1435. The pSZ1500 expression construct included 5' (SEQ ID NO: 18) and 3' (SEQ ID NO: 19) homologous recombination targeting sequences (flanking the construct) to the FADc genomic region for integration into the nuclear genome and a *S. cerevisiae* suc2 sucrose invertase coding region under the control of *C. reinhardtii* β-tubulin promoter/5'UTR (SEQ ID NO: 5) and *Chlorella vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6). This *S. cerevisiae* suc2 expression cassette is listed as SEQ ID NO: 7 and served as a selection marker. The CtOTE coding region was under the control of the *P. moriformis* Amt03 promoter/5'UTR (SEQ ID NO: 8) and *C. vulgaris* nitrate reductase 3'UTR, and the native transit peptide was replaced with the *C. protothecoides* stearoyl-ACP desaturase transit peptide (SEQ ID NO: 9). The protein coding regions of *Ct*OTE and *suc2* were codon optimized to reflect the codon bias inherent in *P. moriformis* UTEX 1435 nuclear genes as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696.

[0348] Primary pSZ1500 transformants of Strain A were selected on agar plates containing sucrose as a sole carbon source, clonally purified, and a single engineered line, Strain D was selected for analysis. Strain D was grown as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. Hexane extraction of the oil from the generated biomass was then performed using standard methods, and the resulting triglyceride oil was determined to be free of residual hexane. Other methods of extraction of oil from microalgae using an expeller press are described in PCT Application No. PCT/US2010/031108 and are hereby incorporated by reference.

[0349] Different lots of oil extracted from biomass of Strain D were refined, bleached, and deodorized using standard vegetable oil processing methods. These procedures generated oil samples RBD437, RBD469, RBD501, RBD 502, RBD503, and RBD529, which were subjected to analytical testing protocols according to methods defined through the American Oil Chemists' Society, the American Society for Testing and Materials, and the International Organization for Standardization. The results of these analyses are summarized below in Tables 9-14.

[0350] Table 9. Analytical results for oil sample RBD469.

Method Number	Test Description	Results	Units
AOCS Ca 3a-46	Insoluble impurities	<0.01	%
AOCS Ca 5a-40	Free Fatty Acids (Oleic)	0.02	%
AOCS Ca 5a-40	Acid Value	0.04	mg KOH/g
AOCS CA 9f-57	Neutral oil	98.9	%
D97	Cloud Point	-15	deg C
D97	Pour Point	-18	deg C
	Karl Fischer Moisture	0.01	%
AOCS Cc 13d-55 (modified)	Chlorophyll	<0.01	ppm
	Iodine Value	78.3	g I_2/100g
AOCS Cd 8b-90	Peroxide Value	0.31	meq/kg

ISO 6885	p-Anisidine Value	0.65	
AOCS Cc 18-80	Dropping Melting point (Mettler)	6.2	deg C
AOCS Cd 11d-96	Triacylglycerides	98.6	%
AOCS Cd 11d-96	Monoglyceride	<0.01	%
AOCS Cd 11d-96	Diglycerides	0.68	%
AOCS Cd 20-91	Total Polar Compounds	2.62	%
IUPAC, 2.507 and 2.508	Oxidized & Polymerized Triacylglycerides	17.62	%
AOCS Cc 9b-55	Flash Point	244	deg C
AOCS Cc 9a-48	Smoke Point	232	deg C
AOCS Cd 12b-92	Oxidative Stability Index Rancimat (110°C)	31.6	hours
AOCS Ca 6a-40	Unsaponified Matter	2.28	%

[0351] RBD469 oil was analyzed for trace element content, solid fat content, and Lovibond color according to AOCS methods. Results of these analyses are presented below in Table 10, Table 10, and Table 11.

[0352] Table 10. ICP Elemental Analysis of RBD469 oil.

Method Number	Test Description	Results in ppm
AOCS Ca 20-99 and AOCS Ca 17-01 (modified)	Phosphorus	1.09
	Calcium	0.1
	Magnesium	0.04
	Iron	<0.02
	Sulfur	28.8
	Copper	<0.05
	Potassium	<0.50
	Sodium	<0.50
	Silicon	0.51
	Boron	0.06
	Aluminum	<0.20
	Lead	<0.20

	Lithium	<0.02
	Nickel	<0.20
	Vanadium	<0.05
	Zinc	<0.02
	Arsenic	<0.20
	Mercury	<0.20
	Cadmium	<0.03
	Chromium	<0.02
	Manganese	<0.05
	Silver	<0.05
	Titanium	<0.05
	Selenium	<0.50
UOP779	Chloride organic	<1
UOP779	Chloride inorganic	7.24
AOCS Ba 4e-93	Nitrogen	6.7

[0353] Table 11. Solid Fat Content of RBD469 Oil

Method Number	Solid Fat Content	Result
AOCS Cd 12b-93	Solid Fat Content 10℃	0.13%
AOCS Cd 12b-93	Solid Fat Content 15℃	0.13%
AOCS Cd 12b-93	Solid Fat Content 20℃	0.28%
AOCS Cd 12b-93	Solid Fat Content 25℃	0.14%
AOCS Cd 12b-93	Solid Fat Content 30℃	0.08%
AOCS Cd 12b-93	Solid Fat Content 35℃	0.25%

[0354] Table 12. Lovibond Color of RBD469 Oil

Method Number	Color	Result	Unit

AOCS Cc 13j-97	red	2	Unit
AOCS Cc 13j-97	yellow	27	Unit

[0355] RBD469 oil was subjected to transesterification to produce fatty acid methyl esters (FAMEs). The resulting FAME profile of RBD469 is shown in Table 12.

[0356] Table 13. FAME Profile of RBD469 Oil

Fatty Acid	Area %
C10	0.01
C12:0	0.04
C14:0	0.64
C15:0	0.08
C16:0	8.17
C16:1 iso	0.39
C16:1	0.77
C17:0	0.08
C18:0	1.93
C18:1	85.88
C18:1 iso	0.05
C18:2	0.05
C20:0	0.3
C20:1	0.06
C20:1	0.44
C22:0	0.11
C23:0	0.03
C24:0	0.1
Total FAMEs Identified	99.13

[0357] The oil stability indexes (OSI) of 6 RBD oil samples without supplemented antioxidants and 3 RBD oil samples supplemented with antioxidants were analyzed according to the Oil Stability Index AOCS Method Cd 12b-92. Shown in Table 14 are the results of OSI AOCS Cd 12b-92 tests, conducted at 110°C, performed using a Metrohm 873 Biodiesel

Rancimat. Results, except where indicated with an asterisks (*), are the average of multiple OSI runs. Those samples not analyzed are indicated (NA).

[0358] Table 14. Oil Stability Index at 110°C of RBD oil samples with and without antioxidants.

		OSI (hours) for each RBD Sample					
		RBD 437	RBD 469	RBD 502	RBD 501	RBD 503	RBD 529
Antioxidant added	Antioxidant Concentration						
None	0	65.41	38.33	72.10	50.32	63.04	26.68
Tocopherol & Ascorbyl Palmitate	35 ppm/16.7 ppm	77.72	48.60	82.67	NA	NA	NA
Tocopherol & Ascorbyl Palmitate	140 ppm/66.7 ppm	130.27	81.54*	211.49*	NA	NA	NA
Tocopherol & Ascorbyl Palmitate	1050 ppm/500 ppm	>157*	>144	242.5*	NA	NA	NA
Tocopherol	50 ppm	NA	46.97	NA	NA	NA	NA
TBHQ	20 ppm	63.37	37.4	NA	NA	NA	NA

[0359] The untransformed *P. moriformis* (UTEX 1435) acid profile comprises less than 60% C18:1 fatty acids and greater than 7% C18:2 fatty acids. In contrast, Strain D (comprising pSZ1500) exhibited fatty acid profiles with an increased composition of C18:1 fatty acids (to above 85%) and a decrease in C18:2 fatty acids (to less than 0.06%). Upon refining, bleaching, and degumming, RBD oils samples prepared from the oil made from strain E exhibited OSI values > 26 hrs. With addition of antioxidants, the OSI of RBD oils prepared from oils of Strain D increased from 48.60 hours to greater than 242 hours. In other experiments, OSI values of over 400 hours were achieved. Additional properties of a low polyunsaturated oil according to embodiments of the invention are given in Fig. 16.

EXAMPLE 6: IMPROVING THE LEVELS OF OLEIC ACID OF ENGINEERED MICROBES THROUGH ALLELIC DISRUPTION OF A FATTY ACID DESATURASE AND AN ACYL-ACP THIOESTERASE

[0360] This example describes the use of a transformation vector to disrupt a FATA locus of a *Prototheca moriformis* strain previously engineered for high oleic acid and low linoleic acid production. The transformation cassette used in this example comprised a selectable marker and nucleotide sequences encoding a *P. moriformis* KASII enzyme to engineer microorganisms in which the fatty acid profile of the transformed microorganism has been altered for further increased oleic acid and lowered palmitic acid levels.

[0361] Strain D, described in Example 5 and in PCT/US2012/023696, is a classically mutagenized (for higher oil production) derivative of *P. moriformis* (UTEX 1435) subsequently transformed with the transformation construct pSZ1500 (SEQ ID NO: 17) according to biolistic transformation methods as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. This strain was used as the host for transformation with construct pSZ2276 to increase expression of a KASII enzyme while concomitantly ablating an endogenous acyl-ACP thioesterase genetic locus to generate Strain E. The pSZ2276 transformation construct included 5' (SEQ ID NO: 20) and 3' (SEQ ID NO: 21) homologous recombination targeting sequences (flanking the construct) to the FATA1 genomic region for integration into the *P. moriformis* nuclear genome, an *A. thaliana* THIC protein coding region under the control of the *C. prototheccoides* actin promoter/5'UTR (SEQ ID NO: 22) and *C. vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6). This *At*THIC expression cassette is listed as SEQ ID NO: 23 and served as a selection marker. The *P. moriformis* KASII protein coding region was under the control of the *P. moriformis* Amt03 promoter/5'UTR (SEQ ID NO: 8) and *C. vulgaris* nitrate reductase 3'UTR, and the native transit peptide of the KASII enzyme was replaced with the *C. prototheccoides* stearoyl-ACP desaturase transit peptide (SEQ ID NO: 9). The codon-optimized sequence of PmKASII (Prototheca moriformis KASII) comprising a *C. prototheccoides* S106 stearoyl-ACP desaturase transit peptide is provided the sequence listings as SEQ ID NO: 24. SEQ ID NO: 25 provides the protein translation of SEQ ID NO: 24. The protein coding regions of PmKASII and *suc2* were codon optimized to reflect the codon bias inherent in *P. moriformis* UTEX 1435 nuclear genes as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696.

[0362] Primary pSZ2276 transformants of Strain D were selected on agar plates lacking thiamine, clonally purified, and a single engineered line, strain E was selected for analysis. Strain E was cultivated under heterotrophic lipid production conditions at pH5.0 and pH7.0 as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. Lipid samples were prepared from dried biomass from each transformant and fatty acid profiles from these samples were analyzed using standard fatty acid methyl ester gas chromatography flame ionization (FAME GC/FID) detection methods as described in Example 1. The fatty acid profiles (expressed as Area % of total fatty acids) from the transgenic line arising from transformation with pSZ2276 into Strain D are shown in Table 15.

[0363] Table 15. Fatty acid profiles of *Prototheca moriformis* (UTEX 1435) Strains A, D, and E engineered for increased oleic acid and lowered linoleic acid levels.

			Area % Fatty Acid				
Strain	Transformation Construct(s)	pH	C16:0	C18:0	C18:1	C18:2	C20:1
Strain A	None	pH 5	26.6	3.3	60.5	6.7	0.07
Strain A	None	pH 7	28.3	4.1	58	6.5	0.06
Strain D	pSZ1500	pH 5	17	3.6	77.1	0.01	0.14
Strain D	pSZ1500	pH 7	19.5	5.3	72.6	0.01	0.09
Strain E	pSZ1500 + pSZ2276	pH 5	4.1	2.36	88.5	0.04	3.1
Strain E	pSZ1500+ pSZ2276	pH 7	2.1	7.8	87.9	0.01	0.5

[0364] As shown in Table 15, targeted interruption of FADc alleles with a CtOTE expression cassette impacted the fatty acid profiles of transformed microorganisms. Fatty acid profiles of Strain D (comprising the pSZ1500 transformation vector) showed increased composition of C18:1 fatty acids with a concomitant decrease in C16:0 and C18:2 fatty acids relative to Strain A. Subsequent transformation of Strain D with pSZ2276 to overexpress a *P. moriformis* (UTEX 1435) KASII protein while concomitantly ablating a FATA genetic locus (thereby generating Strain E) resulted in still further impact on the fatty acid profiles of the transformed microorganisms. Fatty acid profiles of Strain E showed increased composition of C18:1 fatty acids, with a further decrease in C16:0 fatty acids relative to Strains A and D.

Propagation of Strain E in culture conditions at pH 7, to induce expression from the Amt03 promoter, resulted in a fatty acid profile that was higher in C18:0 and C18:1 fatty acids and lower in C16:0 fatty acids, relative to the same strain cultured at pH 5.

[0365] These data demonstrate the utility of multiple genetic modifications to impact the fatty acid profile of a host organism for increased levels of oleic acid with concomitant decreased levels of linoleic acid and palmitic acid. Further, this example illustrates the use of recombinant polynucleotides to target gene interruption of an endogenous FATA allele with a cassette comprising a pH-regulatable promoter to control expression of an exogenous KASII protein-coding region in order to alter the fatty acid profile of a host microbe.

EXAMPLE 7: Conditional Expression of a Fatty Acid Desaturase

[0366] This example describes the use of a transformation vector to conditionally express a delta 12 fatty acid desaturase (FADs) in a *Prototheca moriformis* strain previously engineered for high oleic acid and very low linoleic acid production in both seed and lipid productivity stages of propagation. Very low linoleic acid levels in cell oils are sought for use in certain applications. However, absence of linoleic acid during cell division phase ("seed stage") of a host microbe is disadvantageous. Linoleic acid may be supplemented to the seed medium to hasten cell division and not added during lipid production, but this addition imposes unwanted costs. To overcome this challenge, a transformation cassette was constructed for regulated expression of a FAD2 enzyme such that levels of linoleic acids sufficient for cell division could be achieved and oil with very low levels of linoleic acids could be produced during the oil production phase of culture of a microorganism. The transformation cassette used in this example comprised a selectable marker, a pH-regulatable promoter, and nucleotide sequences encoding a *P. moriformis* FAD2 enzyme to engineer microorganisms in which the fatty acid profile of the transformed microorganism has been altered for increased oleic acid production and regulatable linoleic acid production.

[0367] Strain D, described in Examples 5, 6, and in PCT/US2012/023696, is a classically mutagenized (for higher oil production) derivative of *P. moriformis* (UTEX 1435) subsequently transformed with the transformation construct pSZ1500 (SEQ ID NO: 17) according to biolistic transformation methods as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. This strain was used as the host for transformation with construct pSZ2413 to introduce a pH-driven promoter for regulation of a *P. moriformis* FAD2 enzyme. The pSZ2413 transformation construct included 5' (SEQ ID NO: 1) and 3' (SEQ ID NO: 2)

homologous recombination targeting sequences (flanking the construct) to the 6S genomic region for integration into the *P. moriformis* nuclear genome, an *A. thaliana* THIC protein coding region under the control of the *C. protothecoides* actin promoter/5'UTR (SEQ ID NO: 22) and *C. vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6). This *At*THIC expression cassette is listed as SEQ ID NO: 23 and served as a selection marker. The *P. moriformis* FAD2 protein coding region was under the control of the *P. moriformis* Amt03 promoter/5'UTR (SEQ ID NO: 8) and *C. vulgaris* nitrate reductase 3'UTR. The codon-optimized sequence of *Pm*FAD2 is provided the sequence listings as SEQ ID NO: 26. SEQ ID NO: 27 provides the protein translation of SEQ ID NO: 26. The protein coding regions of PmFAD2 and *suc2* were codon optimized to reflect the codon bias inherent in *P. moriformis* UTEX 1435 nuclear genes as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696.

[0368] Primary pSZ2413 transformants of Strain D were selected on agar plates lacking thiamine, clonally purified, and isolates of the engineered line, Strain F were selected for analysis. These isolates were cultivated under heterotrophic lipid production conditions at pH7.0 (to activate expression of FAD2 from the *Pm*Amt03 promoter) and at pH5.0, as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. Lipid samples were prepared from dried biomass from each transformant and fatty acid profiles from these samples were analyzed using standard fatty acid methyl ester gas chromatography flame ionization (FAME GC/FID) detection methods as described in Example 1. The resulting profile of C18:2 fatty acids (expressed in Area %) from nine representative isolates of transgenic Strain F (F-1 through F-9) arising from transformation with pSZ2413 into Strain D are shown in Table 16.

[0369] Table 16. C18:2 fatty acid profiles of *Prototheca moriformis* (UTEX 1435) Strains A, D, and F.

Strain	**Transformation Construct (s)**	**Area % C18:2**	
		pH 5.0	**pH 7.0**
A	**None**	6.07	7.26
D	**pSZ1500**	0.01	0.01
F-1	**pSZ1500 +** pSZ2413	0.37	**5.29**
F-2	**pSZ1500 +**	0.45	**6.87**

	pSZ2413		
F-3	**pSZ1500 +** pSZ2413	0.50	**6.79**
F-4	**pSZ1500 +** pSZ2413	0.57	**5.06**
F-5	**pSZ1500 +** pSZ2413	0.57	**7.58**
F-6	**pSZ1500 +** pSZ2413	0.60	**6.88**
F-7	**pSZ1500 +** pSZ2413	0.62	**6.52**
F-8	**pSZ1500 +** pSZ2413	0.63	**5.79**
F-9	**pSZ1500 +** pSZ2413	0.77	**4.53**

[0370] As shown in Table 16 the impact of regulated expression of the *Pm*FAD2 enzyme, effected though strain culture at different pH levels, is a clear increase in the composition of C18:2 fatty acids in the transformed microorganism. Linoleic acid comprises about 6% to about 7.3% of fatty acids of Strain A. In contrast, Strain D (comprising the pSZ1500 transformation vector to ablate both FAD2 alleles) is characterized by a fatty acid profile of 0.01% linoleic acid. Transformation of Strain D with pSZ2413 to generate Strain F results in a recombinant microbe in which the production of linoleic acid is regulated by the Amt03 promoter. Propagation of Strain F isolates in culture conditions at pH 7, to induce FAD2 expression from the Amt03 promoter, resulted in a fatty acid profile characterized by about 4.5% to about 7.5% linoleic acid. In contrast, propagation of Strain F isolates in culture conditions at pH 5 resulted in a fatty acid profile characterized by about 0.33 to about 0.77% linoleic acid.

[0371] These data demonstrate the utility of and effectiveness of recombinant polynucleotides permitting conditional expression of a FAD2 enzyme to alter the fatty acid profile of engineered microorganisms, and in particular in regulating the production of C18:2 fatty acids in microbial cells.

EXAMPLE 8: Analysis of Regiospecific Profile

[0372] LC/MS TAG distribution analyses were carried out using a Shimadzu Nexera ultra high performance liquid chromatography system that included a SIL-30AC autosampler, two LC-30AD pumps, a DGU-20A5 in-line degasser, and a CTO-20A column oven, coupled to a Shimadzu LCMS 8030 triple quadrupole mass spectrometer equipped with an APCI source. Data was acquired using a Q3 scan of *m/z* 350-1050 at a scan speed of 1428 u/sec in positive ion mode with the CID gas (argon) pressure set to 230 kPa. The APCI, desolvation line, and heat block temperatures were set to 300, 250, and 200°C, respectively, the flow rates of the nebulizing and drying gases were 3.0 L/min and 5.0 L/min, respectively, and the interface voltage was 4500 V. Oil samples were dissolved in dichloromethane-methanol (1:1) to a concentration of 5 mg/mL, and 0.8 µL of sample was injected onto Shimadzu Shim-pack XR-ODS III (2.2 µm, 2.0 x 200 mm) maintained at 30°C. A linear gradient from 30% dichloromethane-2-propanol (1:1)/acetonitrile to 51% dichloromethane-2-propanol (1:1)/acetonitrile over 27 minutes at 0.48 mL/min was used for chromatographic separations.

EXAMPLE 9: Engineering Microbes for Increased Production of SOS, POP, And POS Triacylglycerides

[0373] This example describes the use of recombinant polynucleotides that encode a C18:0-preferring *Brassica napus* thioesterase (*Bn*OTE) enzyme to engineer a microorganism in which the triacylglyceride distribution of the transformed microorganism has been enriched in SOS, POS, and POP triacylglycerides.

[0374] A classically mutagenized strain of *Prototheca moriformis* (UTEX 1435), Strain A, was initially transformed with the plasmid construct pSZ1358 according to biolistic transformation methods as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. pSZ1358, described in PCT/US2012/023696, hereby incorporated by reference, comprised the coding sequence of the *Brassica napus* thioesterase (*Bn*OTE) thioesterase (SEQ ID NO: 28), 5' (SEQ ID NO: 1) and 3' (SEQ ID NO: 2) homologous recombination targeting sequences (flanking the construct) to the 6S genomic region for integration into the nuclear genome, and a *S. cerevisiae suc2* sucrose invertase coding region (SEQ ID NO: 4), to express the protein sequence given in SEQ ID NO: 3, under the control of *C. reinhardtii* β-tubulin promoter/5'UTR (SEQ ID NO: 5) and *Chlorella vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6). This *S. cerevisiae suc2* expression cassette is listed as SEQ ID NO: 7 and served as a selectable marker. The *Bn*OTE protein coding sequence to express the protein sequence given

in SEQ ID NO: 29, was under the control of the *P. moriformis* Amt03 promoter/5'UTR (SEQ ID NO: 8) and *C. vulgaris* nitrate reductase 3'UTR. The protein coding regions of *Bn*OTE and *suc2* were codon optimized to reflect the codon bias inherent in *P. moriformis* UTEX 1435 nuclear genes as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696.

[0375] Primary pSZ1358 transformants of Strain A were selected on agar plates containing sucrose as a sole carbon source, clonally purified, and single engineered line, Strain G was selected for analysis. Strain G was cultivated under heterotrophic lipid production conditions at pH7.0 (to activate expression of *Bn*OTE from the *Pm*Amt03 promoter) as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. Oil samples obtained from Strain A and Strain G were analyzed for fatty acid composition using methods described in Examples 1 and 2, and, using the methods described in Example 8, for the regiospecificity of triacylglycerides in the oil. Fatty acid profiles of TAGs isolated from Strain A and G are shown in Table 17. Table 18 presents the regiospecificity profile of POP, POS, and SOS TAGs present in oil samples from Strain A and G.

[0376] Table 17. Effect of *Bn*OTE expression on the fatty acid composition and the sn-2 profile of TAGs produced from transformed *Prototheca moriformis*.

	Strain A	Strain G (pSZ1358)
Area % Fatty acid	FA profile	FA profile
C10:0	n.r.	0.5
C12:0	n.r.	0.5
C14:0	1.0	1.3
C16:0	23.9	25.8
C18:0	3.7	30.4
C18:1	64.3	30.2
C18:2	4.5	8.8
C18:3 α	n.r.	0.4

[0377] Table 18. Effect of *Bn*OTE expression on the regiospecific profile of POP, POS, and SOS TAGs produced from transformed *Prototheca moriformis*.

	Strain A (untransformed)		Strain G (pSZ1358)		Cocoa Butter	
TAG	Area %	Normalized Area %	Area %	Normalized Area %	Area %	Normalized Area %
POP	13.09	76.8	10.6	23.5	17.9	22.1
POS	3.51	20.5	21.0	46.6	39.2	48.4
SOS	0.45	2.6	13.5	29.9	23.9	29.5
total	17.05	100	45.0	100	81.1	100

[0378] As shown in Table 17, the fatty acid composition of TAGs isolated from Strain G expressing *Bn*OTE was markedly increased for C18:0 fatty acids (from 3.7% to 30.4%) and decreased in C18:1 fatty acids (from 64.3% to 30.2%) relative to the fatty acid profile of TAGs isolated from untransformed Strain A. The fatty acid composition of TAGs isolated from Strain A was characterized by about 23.9% palmitic acid, 3.7% stearic acid, and 64.3% oleic acid, a ratio for P:S:O of about 6.5:1:17.4. In contrast, the fatty acid composition of TAGs isolated from Strain G was characterized by about 25.8% palmitic acid, 30.4% stearic acid, and 30.2 % oleic acid, a ratio for P:O:S of about 1:1.18:1.17.

[0379] The impact of expression of a C18:0 preferring thioesterase on the regiospecific profile of POP, POS, and SOS TAGs of oils produced from the transformed microorganism was an increase in all three TAGs as a proportion of the total TAGs present in the oil. As shown in Table 18, the sum of POP + POS + SOS TAGs accounted for 45% of the TAGs produced by Strain G, whereas POP, POS, and SOS TAGs summed to only about 17% of TAGs produced in Strain A. The percentages of POP, POS and SOS of strain G are compared to Cocoa butter in Table 18. As can be seen, ratios of POP, POS and SOS of Strain G are very similar to the ratios observed in cocoa butter.

[0380] These data demonstrate the utility and effectiveness of polynucleotides permitting exogenous thioesterase expression to alter the fatty acid and regiospecific profiles of TAGs of engineered microorganisms, in particular to increase the distribution of POP, POS, and SOS TAGs.

EXAMPLES 10-33: ENGINEERING OF MICROORGANISMS

[0381] Examples 10-33 below describe the engineering of various microorganisms in accordance with the present invention. To alter the fatty acid profile of a microorganism, microorganisms can be genetically modified wherein endogenous or exogenous lipid biosynthesis pathway enzymes are expressed, overexpressed, or attenuated. Steps to genetically engineer a microbe to alter its fatty acid profile as to the degree of fatty acid unsaturation and to decrease or increase fatty acid chain length comprise the design and construction of a transformation vector (*e.g.*, a plasmid), transformation of the microbe with one or more vectors, selection of transformed microbes (transformants), growth of the transformed microbe, and analysis of the fatty acid profile of the lipids produced by the engineered microbe.

[0382] Transgenes that alter the fatty acid profiles of host organisms can be expressed in numerous eukaryotic microbes. Examples of expression of transgenes in eukaryotic microbes including *Chlamydomonas reinhardtii*, *Chlorella ellipsoidea*, *Chlorella saccarophila, Chlorella vulgaris*, *Chlorella kessleri*, *Chlorella sorokiniana*, *Haematococcus pluvialis*, *Gonium pectorale, Volvox carteri*, *Dunaliella tertiolecta, Dunaliella viridis*, *Dunaliella salina*, *Closterium peracerosum–strigosum–littorale complex, Nannochloropsis sp.*, *Thalassiosira pseudonana, Phaeodactylum tricornutum*, *Navicula saprophila*, *Cylindrotheca fusiformis*, *Cyclotella cryptica*, *Symbiodinium microadriacticum, Amphidinium sp., Chaetoceros sp., Mortierella alpina, and Yarrowia lipolytica* can be found in the scientific literature. These expression techniques can be combined with the teachings of the present invention to produce engineered microorganisms with altered fatty acid profiles.

[0383] Transgenes that alter the fatty acid profiles of host organisms or alter the regiospecific distribution of glycerolipids produced by host organisms can also be expressed in numerous prokaryotic microbes. Examples of expression of transgenes in oleaginous microbes including *Rhodococcus opacus* can be found in the literature. These expression techniques can be combined with the teachings of the present invention to produce engineered microorganisms with altered fatty acid profiles.

[0384] Tables 19A-D. Codon preference listing.

Amino Acid	Codon	*Chlorella sorokiniana*	*Chlorella vulgaris*	*Chlorella ellipsoidea*	*Chlorella kessleri*	*Dunaliella tertiolecta*	*Volvox carteri*	*Haematococcus pluvialis*
Ala	GCG	0.20	0.25	0.15	0.14	0.09	0.25	0.21

Ala	GCA	0.05	0.24	0.32	0.10	0.17	0.13	0.27
Ala	GCT	0.12	0.16	0.26	0.18	0.31	0.26	0.17
Ala	GCC	0.63	0.35	0.27	0.58	0.43	0.36	0.35
Arg	AGG	0.03	0.09	0.10	0.09	0.26	0.08	0.14
Arg	AGA	0.04	0.05	0.14	0.01	0.09	0.03	0.05
Arg	CGG	0.06	0.19	0.09	0.06	0.06	0.17	0.15
Arg	CGA	0.00	0.10	0.08	0.00	0.08	0.08	0.10
Arg	CGT	0.06	0.09	0.37	0.14	0.12	0.22	0.13
Arg	CGC	0.81	0.48	0.22	0.71	0.40	0.43	0.42
Asn	AAT	0.04	0.16	0.43	0.06	0.27	0.23	0.21
Asn	AAC	0.96	0.84	0.57	0.94	0.73	0.77	0.79
Asp	GAT	0.13	0.25	0.47	0.12	0.40	0.35	0.27
Asp	GAC	0.87	0.75	0.53	0.88	0.60	0.65	0.73
Cys	TGT	0.06	0.13	0.43	0.09	0.20	0.17	0.27
Cys	TGC	0.94	0.87	0.57	0.91	0.80	0.83	0.64
End	TGA	0.00	0.72	0.14	0.14	0.36	0.24	0.70
End	TAG	0.33	0.11	0.29	0.00	0.00	0.18	0.22
End	TAA	0.67	0.17	4.00	0.86	0.64	0.59	0.09
Gln	CAG	0.42	0.40	0.15	0.40	0.27	0.29	0.33
Gln	CAA	0.04	0.04	0.21	0.40	0.27	0.07	0.10
Glu	GAG	0.53	0.50	0.33	0.40	0.27	0.53	0.49
Glu	GAA	0.02	0.06	0.31	0.40	0.27	0.11	0.07
Gly	GGG	0.04	0.16	0.19	0.08	0.10	0.12	0.22
Gly	GGA	0.02	0.11	0.13	0.07	0.13	0.12	0.11
Gly	GGT	0.03	0.12	0.39	0.24	0.25	0.23	0.15
Gly	GGC	0.91	0.61	0.29	0.96	0.51	0.53	0.52
His	CAT	0.14	0.16	0.30	0.08	0.25	0.35	0.27
His	CAC	0.86	0.84	0.70	0.93	0.75	0.65	0.73
Ile	ATA	0.00	0.04	0.07	0.01	0.04	0.08	0.09
Ile	ATT	0.15	0.30	0.63	0.29	0.31	0.35	0.29
Ile	ATC	0.85	0.66	0.65	0.69	0.65	0.57	0.62
Leu	TTG	0.03	0.07	0.03	0.05	0.14	0.14	0.16
Leu	TTA	0.00	0.01	0.32	0.00	0.02	0.03	0.02
Leu	CTG	0.72	0.61	0.34	0.61	0.60	0.45	0.53
Leu	CTA	0.01	0.03	0.03	0.04	0.04	0.07	0.07
Leu	CTT	0.04	0.08	0.16	0.06	0.06	0.14	0.09
Leu	CTC	0.20	0.20	0.12	0.24	0.14	0.17	0.13
Lys	AAG	0.98	0.94	0.54	0.98	0.90	0.90	0.84
Lys	AAA	0.02	0.06	0.46	0.02	0.10	0.10	0.16
Met	ATG	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Phe	TTT	0.28	0.32	0.42	0.31	0.24	0.27	0.35
Phe	TTC	0.72	0.68	0.58	0.69	0.76	0.73	0.65
Pro	CCG	0.18	0.31	0.09	0.07	0.04	0.34	0.15
Pro	CCA	0.06	0.17	0.36	0.07	0.04	0.20	0.24
Pro	CCT	0.10	0.14	0.25	0.17	0.04	0.19	0.29

Pro	CCC	0.66	0.38	0.29	0.69	0.04	0.27	0.32
Ser	AGT	0.03	0.04	0.14	0.02	0.08	0.08	0.07
Ser	AGC	0.27	0.38	0.18	0.18	0.31	0.27	0.31
Ser	TCG	0.12	0.14	0.08	0.10	0.02	0.19	0.10
Ser	TCA	0.03	0.08	0.14	0.08	0.09	0.09	0.14
Ser	TCT	0.09	0.11	0.26	0.18	0.19	0.14	0.13
Ser	TCC	0.47	0.24	0.20	0.44	0.30	0.24	0.24
Thr	ACG	0.11	0.20	0.13	0.05	0.12	0.27	0.19
Thr	ACA	0.01	0.20	0.32	0.07	0.20	0.12	0.23
Thr	ACT	0.12	0.13	0.29	0.12	0.24	0.20	0.18
Thr	ACC	0.76	0.47	0.26	0.76	0.44	0.41	0.40
Trp	TGG	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Tyr	TAT	0.07	0.15	0.43	0.27	0.28	0.24	0.19
Tyr	TAC	0.93	0.85	0.57	0.73	0.72	0.76	0.81
Val	GTG	0.71	0.54	0.37	0.60	0.54	0.46	0.62
Val	GTA	0.00	0.05	0.25	0.03	0.09	0.07	0.09
Val	GTT	0.11	0.14	0.24	0.09	0.14	0.17	0.09
Val	GTC	0.18	0.27	0.14	0.28	0.23	0.30	0.21

Amino Acid	Codon	*Closterium peracerosum-strigosum-littorale complex*	*Dunaliella viridis*	*Dunaliella salina*	*Gonium pectorale*	*Phaeodactylum tricornutum*	*Chaetoceros compressum*
Ala	GCG	0.48	0.13	0.15	0.43	0.15	0.08
Ala	GCA	0.10	0.27	0.20	0.09	0.10	0.37
Ala	GCT	0.15	0.25	0.27	0.08	0.23	0.36
Ala	GCC	0.26	0.35	0.39	0.41	0.52	0.18
Arg	AGG	0.04	0.25	0.22	0.13	0.02	0.14
Arg	AGA	0.00	0.06	0.05	0.00	0.04	0.29
Arg	CGG	0.18	0.08	0.12	0.40	0.10	0.00
Arg	CGA	0.00	0.06	0.06	0.05	0.12	0.19
Arg	CGT	0.13	0.15	0.13	0.08	0.41	0.38
Arg	CGC	0.64	0.39	0.43	0.35	0.31	0.00
Asn	AAT	0.04	0.17	0.23	0.07	0.30	0.58
Asn	AAC	0.96	0.83	0.77	0.93	0.65	0.42
Asp	GAT	0.30	0.38	0.40	0.11	0.41	0.53
Asp	GAC	0.70	0.62	0.60	0.89	0.59	0.47
Cys	TGT	0.06	0.24	0.17	0.20	0.39	0.44
Cys	TGC	0.94	0.76	0.83	0.90	0.61	0.56
End	TGA	0.75	0.31	0.37	0.50	0.06	0.50
End	TAG	0.00	0.15	0.14	0.00	0.13	0.00
End	TAA	0.25	0.54	0.49	0.50	0.81	0.50
Gln	CAG	0.53	0.36	0.32	0.31	0.23	0.16

Gln	CAA	0.09	0.12	0.08	0.07	0.14	0.19
Glu	GAG	0.31	0.44	0.51	0.56	0.21	0.28
Glu	GAA	0.06	0.09	0.09	0.07	0.42	0.37
Gly	GGG	0.31	0.14	0.10	0.18	0.08	0.12
Gly	GGA	0.06	0.11	0.12	0.09	0.34	0.33
Gly	GGT	0.09	0.22	0.22	0.07	0.30	0.39
Gly	GGC	0.53	0.54	0.56	0.65	0.28	0.16
His	CAT	0.33	0.25	0.25	0.43	0.28	0.84
His	CAC	0.67	0.75	0.75	0.57	0.72	0.16
Ile	ATA	0.03	0.03	0.03	0.07	0.03	0.12
Ile	ATT	0.23	0.25	0.31	0.33	0.51	0.65
Ile	ATC	0.74	0.72	0.66	0.59	0.46	0.23
Leu	TTG	0.04	0.11	0.12	0.04	0.26	0.11
Leu	TTA	0.00	0.01	0.01	0.00	0.02	0.14
Leu	CTG	0.31	0.60	0.61	0.64	0.15	0.05
Leu	CTA	0.01	0.05	0.04	0.01	0.05	0.08
Leu	CTT	0.04	0.07	0.08	0.05	0.18	0.51
Leu	CTC	0.60	0.16	0.14	0.26	0.34	0.11
Lys	AAG	0.86	0.87	0.89	0.93	0.75	0.52
Lys	AAA	0.14	0.13	0.11	0.07	0.25	0.48
Met	ATG	1.00	1.00	1.00	1.00	1.00	1.00
Phe	TTT	0.09	0.25	0.29	0.10	0.44	0.65
Phe	TTC	0.91	0.75	0.71	0.90	0.56	0.35
Pro	CCG	0.28	0.10	0.08	0.53	0.29	0.05
Pro	CCA	0.15	0.10	0.17	0.09	0.12	0.45
Pro	CCT	0.12	0.10	0.30	0.04	0.20	0.33
Pro	CCC	0.44	0.10	0.45	0.34	0.40	0.17
Ser	AGT	0.04	0.09	0.06	0.02	0.12	0.14
Ser	AGC	0.05	0.31	0.32	0.20	0.12	0.07
Ser	TCG	0.22	0.04	0.06	0.42	0.19	0.08
Ser	TCA	0.16	0.08	0.10	0.09	0.06	0.31
Ser	TCT	0.05	0.17	0.15	0.07	0.15	0.23
Ser	TCC	0.47	0.31	0.30	0.20	0.35	0.18
Thr	ACG	0.30	0.16	0.13	0.42	0.23	0.10
Thr	ACA	0.06	0.21	0.18	0.03	0.13	0.38
Thr	ACT	0.22	0.18	0.23	0.08	0.19	0.27
Thr	ACC	0.42	0.46	0.46	0.47	0.45	0.25
Trp	TGG	1.00	1.00	1.00	1.00	1.00	1.00
Tyr	TAT	0.07	0.16	0.21	0.12	0.18	0.67
Tyr	TAC	0.93	0.84	0.79	0.88	0.82	0.33
Val	GTG	0.50	0.64	0.62	0.57	0.22	0.30
Val	GTA	0.02	0.03	0.05	0.04	0.09	0.27
Val	GTT	0.06	0.11	0.11	0.04	0.22	0.10
Val	GTC	0.42	0.22	0.23	0.35	0.47	0.33

Amino Acid	Codon	*Cylindro-theca fusiformis*	*Amphi-dinium carterae*	*Symbiodinium micro-adriacticum*	*Nanno-chloropsis sp*	*Cyclotella cryptica*	*Navicula pelliculosa*	*Thalassiosira pseudonana*	*C. reinhardtii*
Ala	GCG	0.07	0.17	0.22	0.24	0.11	0.00	0.11	0.35
Ala	GCA	0.14	0.33	0.26	0.10	0.16	0.13	0.25	0.08
Ala	GCT	0.35	0.29	0.20	0.17	0.45	0.44	0.33	0.13
Ala	GCC	0.43	0.20	0.32	0.48	0.27	0.44	0.30	0.43
Arg	AGG	0.09	0.15	0.27	0.00	0.09	0.05	0.18	0.05
Arg	AGA	0.14	0.03	0.27	0.00	0.05	0.10	0.17	0.01
Arg	CGG	0.06	0.08	0.09	0.00	0.04	0.05	0.06	0.20
Arg	CGA	0.16	0.18	0.09	0.29	0.08	0.35	0.11	0.04
Arg	CGT	0.34	0.18	0.09	0.14	0.47	0.20	0.34	0.09
Arg	CGC	0.22	0.40	0.18	0.57	0.28	0.25	0.15	0.62
Asn	AAT	0.42	0.37	0.21	0.00	0.25	0.47	0.43	0.09
Asn	AAC	0.58	0.63	0.79	1.00	0.75	0.53	0.57	0.91
Asp	GAT	0.54	0.54	0.50	0.20	0.52	0.20	0.56	0.14
Asp	GAC	0.46	0.46	0.50	0.80	0.48	0.80	0.44	0.86
Cys	TGT	0.44	0.75	0.50	0.00	0.29	0.10	0.54	0.10
Cys	TGC	0.56	0.25	0.50	1.00	0.71	0.90	0.46	0.90
End	TGA	0.13	0.50	1.00	0.00	0.10	0.00	0.31	0.27
End	TAG	0.10	0.00	0.00	0.00	0.00	0.00	0.38	0.22
End	TAA	0.77	0.50	0.00	1.00	0.90	1.00	0.31	0.52
Gln	CAG	0.12	0.33	0.28	0.41	0.19	0.21	0.16	0.38
Gln	CAA	0.25	0.15	0.17	0.00	0.17	0.28	0.19	0.04
Glu	GAG	0.23	0.41	0.50	0.59	0.38	0.17	0.40	0.55
Glu	GAA	0.39	0.10	0.06	0.00	0.26	0.34	0.26	0.03
Gly	GGG	0.06	0.19	0.32	0.10	0.10	0.03	0.12	0.11
Gly	GGA	0.47	0.10	0.12	0.05	0.45	0.28	0.51	0.06
Gly	GGT	0.35	0.34	0.16	0.25	0.22	0.13	0.23	0.11
Gly	GGC	0.12	0.37	0.40	0.60	0.24	0.56	0.14	0.72
His	CAT	0.39	0.12	0.40	0.00	0.42	1.00	0.50	0.11
His	CAC	0.61	0.88	0.60	1.00	0.58	0.00	0.50	0.89
Ile	ATA	0.06	0.05	0.00	0.00	0.04	0.00	0.08	0.03
Ile	ATT	0.42	0.53	0.38	0.14	0.53	0.73	0.38	0.22
Ile	ATC	0.52	0.42	0.63	0.86	0.42	0.27	0.54	0.75
Leu	TTG	0.26	0.35	0.39	0.22	0.20	0.16	0.29	0.04
Leu	TTA	0.09	0.01	0.00	0.00	0.03	0.00	0.05	0.01
Leu	CTG	0.09	0.22	0.39	0.09	0.06	0.12	0.08	0.73
Leu	CTA	0.05	0.00	0.04	0.00	0.03	0.04	0.06	0.03
Leu	CTT	0.37	0.31	0.13	0.04	0.39	0.36	0.20	0.05
Leu	CTC	0.13	0.12	0.04	0.65	0.29	0.32	0.32	0.15
Lys	AAG	0.60	0.93	0.85	1.00	0.70	0.83	0.76	0.95
Lys	AAA	0.40	0.07	0.15	0.00	0.30	0.17	0.24	0.05

Met	ATG	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Phe	TTT	0.37	0.21	0.25	0.20	0.31	0.78	0.38	0.16
Phe	TTC	0.63	0.79	0.75	0.80	0.69	0.22	0.62	0.84
Pro	CCG	0.11	0.14	0.18	0.08	0.10	0.21	0.16	0.33
Pro	CCA	0.33	0.42	0.09	0.08	0.16	0.29	0.31	0.08
Pro	CCT	0.32	0.22	0.41	0.25	0.35	0.21	0.31	0.13
Pro	CCC	0.24	0.22	0.32	0.58	0.39	0.29	0.23	0.47
Ser	AGT	0.12	0.13	0.09	0.00	0.09	0.13	0.18	0.04
Ser	AGC	0.09	0.24	0.14	0.13	0.08	0.28	0.11	0.35
Ser	TCG	0.13	0.03	0.05	0.00	0.15	0.25	0.17	0.25
Ser	TCA	0.12	0.25	0.05	0.00	0.12	0.08	0.12	0.05
Ser	TCT	0.30	0.16	0.23	0.13	0.39	0.25	0.23	0.07
Ser	TCC	0.24	0.19	0.45	0.75	0.18	0.03	0.19	0.25
Thr	ACG	0.09	0.14	0.10	0.28	0.10	0.18	0.21	0.30
Thr	ACA	0.15	0.28	0.10	0.00	0.15	0.09	0.19	0.08
Thr	ACT	0.39	0.12	0.10	0.17	0.33	0.41	0.28	0.10
Thr	ACC	0.37	0.47	0.70	0.56	0.43	0.32	0.32	0.52
Trp	TGG	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Tyr	TAT	0.38	0.32	0.20	0.00	0.38	0.20	0.39	0.10
Tyr	TAC	0.62	0.68	0.80	1.00	0.62	0.80	0.61	0.90
Val	GTG	0.11	0.65	0.67	0.31	0.16	0.18	0.29	0.67
Val	GTA	0.06	0.05	0.00	0.00	0.09	0.09	0.16	0.03
Val	GTT	0.38	0.08	0.11	0.15	0.42	0.09	0.28	0.07
Val	GTC	0.46	0.21	0.22	0.54	0.33	0.64	0.27	0.22

Amino Acid	Codon	*Yarrowia lipolytica*	*Mortierella alpina*	*Rhodococcus opacus*
Ala	GCG	0.08	0.14	0.35
Ala	GCA	0.11	0.12	0.14
Ala	GCT	0.35	0.29	0.09
Ala	GCC	0.46	0.45	0.43
Arg	AGG	0.05	0.05	0.05
Arg	AGA	0.13	0.06	0.02
Arg	CGG	0.12	0.06	0.26
Arg	CGA	0.52	0.09	0.12
Arg	CGT	0.11	0.32	0.11
Arg	CGC	0.07	0.42	0.44
Asn	AAT	0.17	0.15	0.21
Asn	AAC	0.83	0.85	0.79
Asp	GAT	0.35	0.42	0.24
Asp	GAC	0.65	0.58	0.76
Cys	TGT	0.46	0.13	0.26
Cys	TGC	0.54	0.87	0.74

End	TGA	0.16	0.05	0.72
End	TAG	0.38	0.25	0.17
End	TAA	0.46	0.70	0.11
Gln	CAG	0.33	0.36	0.28
Gln	CAA	0.08	0.06	0.06
Glu	GAG	0.44	0.49	0.45
Glu	GAA	0.14	0.09	0.22
Gly	GGG	0.05	0.03	0.18
Gly	GGA	0.28	0.29	0.15
Gly	GGT	0.32	0.32	0.20
Gly	GGC	0.34	0.36	0.48
His	CAT	0.34	0.27	0.20
His	CAC	0.66	0.73	0.80
Ile	ATA	0.03	0.01	0.05
Ile	ATT	0.44	0.33	0.14
Ile	ATC	0.53	0.66	0.81
Leu	TTG	0.09	0.27	0.09
Leu	TTA	0.02	0.00	0.01
Leu	CTG	0.37	0.26	0.41
Leu	CTA	0.05	0.02	0.03
Leu	CTT	0.18	0.12	0.06
Leu	CTC	0.29	0.32	0.40
Lys	AAG	0.84	0.91	0.80
Lys	AAA	0.16	0.09	0.20
Met	ATG	1.00	1.00	1.00
Phe	TTT	0.38	0.39	0.09
Phe	TTC	0.62	0.61	0.91
Pro	CCG	0.10	0.07	0.52
Pro	CCA	0.10	0.08	0.09
Pro	CCT	0.32	0.36	0.07
Pro	CCC	0.47	0.49	0.32
Ser	AGT	0.07	0.05	0.08
Ser	AGC	0.11	0.14	0.23
Ser	TCG	0.16	0.32	0.33
Ser	TCA	0.08	0.08	0.07
Ser	TCT	0.28	0.12	0.05
Ser	TCC	0.30	0.29	0.24
Thr	ACG	0.11	0.17	0.28
Thr	ACA	0.14	0.10	0.11
Thr	ACT	0.26	0.23	0.07
Thr	ACC	0.49	0.49	0.53
Trp	TGG	1.00	1.00	1.00
Tyr	TAT	0.18	0.20	0.18
Tyr	TAC	0.82	0.80	0.82
Val	GTG	0.33	0.22	0.37

Val	GTA	0.05	0.02	0.05
Val	GTT	0.26	0.27	0.10
Val	GTC	0.36	0.49	0.49

[0385] Table 20. Lipid biosynthesis pathway proteins.

3-Ketoacyl ACP synthase

Cuphea hookeriana 3-ketoacyl-ACP synthase (GenBank Acc. No. AAC68861.1), *Cuphea wrightii beta*-ketoacyl-ACP synthase II (GenBank Acc. No. AAB37271.1), *Cuphea lanceolata beta*-ketoacyl-ACP synthase IV (GenBank Acc. No. CAC59946.1), *Cuphea wrightii beta-ketoacyl-ACP synthase II* (GenBank Acc. No. AAB37270.1), *Ricinus communis ketoacyl-ACP synthase* (GenBank Acc. No. XP_002516228), Gossypium hirsutum *ketoacyl-ACP synthase* (GenBank Acc. No. ADK23940.1), *Glycine max plastid 3-keto-acyl-ACP synthase II-A* (GenBank Acc No. AAW88763.1), *Elaeis guineensis* beta-ketoacyl-ACP synthase II (GenBank Acc. No. AAF26738.2), *Helianthus annuus* plastid 3-keto-acyl-ACP synthase I (GenBank Acc. No. ABM53471.1), Glycine max3-keto-acyl-ACP synthase I (GenkBank Acc. No. NP_001238610.1), *Helianthus annuus* plastid 3-keto-acyl-ACP synthase II (GenBank Acc ABI18155.1), *Brassica napus beta-ketoacyl-ACP synthetase 2* (GenBank Acc. No. *AAF61739.1), Perilla frutescens* beta-ketoacyl-ACP synthase II (GenBank Acc. No. AAC04692.1), *Helianthus annus* beta-ketoacyl-ACP synthase II (GenBank Accession No. ABI18155*), Ricinus communis* beta-ketoacyl-ACP synthase II (GenBank Accession No. AAA33872), *Haematococcus pluvialis* beta-ketoacyl acyl carrier protein synthase (GenBank Accession No. HM560033.1), *Jatropha curcasbeta* ketoacyl-ACP synthase I (GenBank Accession No. ABJ90468.1), *Populus trichocarpa* beta-ketoacyl-ACP synthase I (GenBank Accession No. XP_002303661.1), *Coriandrum sativum* beta-ketoacyl-ACP synthetase I (GenBank Accession No. AAK58535.1), *Arabidopsis thaliana* 3-oxoacyl-[acyl-carrier-protein] synthase I (GenBank Accession No. NP_001190479.1), *Vitis vinifera* 3-oxoacyl-[acyl-carrier-protein] synthase I (GenBank Accession No. XP_002272874.2)

Fatty acyl-ACP Thioesterases

Umbellularia californica fatty acyl-ACP thioesterase (GenBank Acc. No. AAC49001), *Cinnamomum camphora* fatty acyl-ACP thioesterase (GenBank Acc. No. Q39473), *Umbellularia californica* fatty acyl-ACP thioesterase (GenBank Acc. No. Q41635), *Myristica fragrans* fatty acyl-ACP thioesterase (GenBank Acc. No. AAB71729), *Myristica fragrans* fatty acyl-ACP thioesterase (GenBank Acc. No. AAB71730), *Elaeis guineensis* fatty acyl-

ACP thioesterase (GenBank Acc. No. ABD83939), *Elaeis guineensis* fatty acyl-ACP thioesterase (GenBank Acc. No. AAD42220), *Populus tomentosa* fatty acyl-ACP thioesterase (GenBank Acc. No. ABC47311), *Arabidopsis thaliana* fatty acyl-ACP thioesterase (GenBank Acc. No. NP_172327), *Arabidopsis thaliana* fatty acyl-ACP thioesterase (GenBank Acc. No. CAA85387), *Arabidopsis thaliana* fatty acyl-ACP thioesterase (GenBank Acc. No. CAA85388), *Gossypium hirsutum* fatty acyl-ACP thioesterase (GenBank Acc. No. Q9SQI3), *Cuphea lanceolata* fatty acyl-ACP thioesterase (GenBank Acc. No. CAA54060), *Cuphea hookeriana* fatty acyl-ACP thioesterase (GenBank Acc. No. AAC72882), *Cuphea calophylla subsp. mesostemon* fatty acyl-ACP thioesterase (GenBank Acc. No. ABB71581), *Cuphea lanceolata* fatty acyl-ACP thioesterase (GenBank Acc. No. CAC19933), *Elaeis guineensis* fatty acyl-ACP thioesterase (GenBank Acc. No. AAL15645), *Cuphea hookeriana* fatty acyl-ACP thioesterase (GenBank Acc. No. Q39513), *Gossypium hirsutum* fatty acyl-ACP thioesterase (GenBank Acc. No. AAD01982), *Vitis vinifera* fatty acyl-ACP thioesterase (GenBank Acc. No. CAN81819), *Garcinia mangostana* fatty acyl-ACP thioesterase (GenBank Acc. No. AAB51525), *Brassica juncea* fatty acyl-ACP thioesterase (GenBank Acc. No. ABI18986), *Madhuca longifolia* fatty acyl-ACP thioesterase (GenBank Acc. No. AAX51637), *Brassica napus* fatty acyl-ACP thioesterase (GenBank Acc. No. ABH11710),*Brassica napus* fatty acyl-ACP thioesterase (GenBank Acc. No. CAA52070.1), *Oryza sativa* (indica cultivar-group) fatty acyl-ACP thioesterase (GenBank Acc. No. EAY86877), *Oryza sativa* (japonica cultivar-group) fatty acyl-ACP thioesterase (GenBank Acc. No. NP_001068400), *Oryza sativa* (indica cultivar-group) fatty acyl-ACP thioesterase (GenBank Acc. No. EAY99617), *Cuphea hookeriana* fatty acyl-ACP thioesterase (GenBank Acc. No. AAC49269), *Ulmus Americana* fatty acyl-ACP thioesterase (GenBank Acc. No. AAB71731), *Cuphea lanceolata* fatty acyl-ACP thioesterase (GenBank Acc. No. CAB60830), *Cuphea palustris* fatty acyl-ACP thioesterase (GenBank Acc. No. AAC49180), *Iris germanica* fatty acyl-ACP thioesterase (GenBank Acc. No. AAG43858, *Iris germanica* fatty acyl-ACP thioesterase (GenBank Acc. No. AAG43858.1), *Cuphea palustris* fatty acyl-ACP thioesterase (GenBank Acc. No. AAC49179), *Myristica fragrans* fatty acyl-ACP thioesterase (GenBank Acc. No. AAB71729), *Myristica fragrans* fatty acyl-ACP thioesterase (GenBank Acc. No. AAB717291.1), *Cuphea hookeriana* fatty acyl-ACP thioesterase GenBank Acc. No. U39834), *Umbelluaria californica* fatty acyl-ACP thioesterase (GenBank Acc. No. M94159), *Cinnamomum camphora* fatty acyl-ACP thioesterase (GenBank Acc. No. U31813), *Ricinus communis* fatty acyl-ACP thioesterase (GenBank Acc. No. ABS30422.1), *Helianthus annuus* acyl-ACP thioesterase (GenBank Accession No. AAL79361.1), *Jatropha curcas* acyl-ACP

thioesterase (GenBank Accession No. ABX82799.3), *Zea mays* oleoyl-acyl carrier protein thioesterase, (GenBank Accession No. ACG40089.1), *Haematococcus pluvialis* fatty acyl-ACP thioesterase (GenBank Accession No. HM560034.1)

Desaturase Enzymes

Linum usitatissimum fatty acid desaturase 3C, (GenBank Acc. No. ADV92272.1), *Ricinus communis* omega-3 fatty acid desaturase, endoplasmic reticulum, putative, (GenBank Acc. No. EEF36775.1), *Vernicia fordii* omega-3 fatty acid desaturase, (GenBank Acc. No. AAF12821), *Glycine max* chloroplast omega 3 fatty acid desaturase isoform 2, (GenBank Acc. No. ACF19424.1), *Prototheca moriformis* FAD-D omega 3 desaturase (SEQ ID NO: 35), *Prototheca moriformis* linoleate desaturase (SEQ ID NO: 36), *Carthamus tinctorius* delta 12 desaturase, (GenBank Accession No. ADM48790.1), *Gossypium hirsutum* omega-6 desaturase, (GenBank Accession No. CAA71199.1), *Glycine max* microsomal desaturase (GenBank Accession No. BAD89862.1), *Zea mays* fatty acid desaturase (GenBank Accession No. ABF50053.1), *Brassica napa* linoleic acid desaturase (GenBank Accession No. AAA32994.1), *Camelina sativa* omega-3 desaturase (SEQ ID NO: 37), *Prototheca moriformis* delta 12 desaturase allele 2 (SEQ ID NO: 38, *Camelina sativa* omega-3 FAD7-1 (SEQ ID NO: 39), *Helianthus annuus* stearoyl-ACP desaturase, (GenBank Accession No. AAB65145.1), *Ricinus communis* stearoyl-ACP desaturase, (GenBank Accession No. AACG59946.1), *Brassica juncea* plastidic delta-9-stearoyl-ACP desaturase (GenBank Accession No. AAD40245.1), *Glycine max* stearoyl-ACP desaturase (GenBank Accession No. ACJ39209.1), *Olea europaea* stearoyl-ACP desaturase (GenBank Accession No. AAB67840.1), *Vernicia fordii* stearoyl-acyl-carrier protein desaturase, (GenBank Accession No. ADC32803.1), *Descurainia sophia* delta-12 fatty acid desaturase (GenBank Accession No. ABS86964.2), *Euphorbia lagascae* delta12-oleic acid desaturase (GenBank Acc. No. AAS57577.1), *Chlorella vulgaris* delta 12 fatty acid desaturase (GenBank Accession No. ACF98528), *Chlorella vulgaris* omega-3 fatty acid desaturase (GenBank Accession No. BAB78717), *Haematococcus pluvialis* omega-3 fatty acid desaturase (GenBank Accession No. HM560035.1), *Haematococcus pluvialis* stearoyl-ACP-desaturase GenBank Accession No. EF586860.1, *Haematococcus pluvialis* stearoyl-ACP-desaturase GenBank Accession No. EF523479.1

Oleate 12-hydroxylase Enzymes

Ricinus communis oleate 12-hydroxylase (GenBank Acc. No. AAC49010.1), *Physaria lindheimeri* oleate 12-hydroxylase (GenBank Acc. No. ABQ01458.1), *Physaria lindheimeri* mutant bifunctional oleate 12-hydroxylase:desaturase (GenBank Acc. No. ACF17571.1), *Physaria lindheimeri* bifunctional oleate 12-hydroxylase:desaturase (GenBank Accession No. ACQ42234.1), *Physaria lindheimeri* bifunctional oleate 12-hydroxylase:desaturase (GenBank Acc. No. AAC32755.1), *Arabidopsis lyrata subsp. Lyrata* (GenBank Acc. No. XP_002884883.1)

Glycerol-3-phosphate Enzymes

Arabidopsis thaliana glycerol-3-phosphate acyltransferase BAA00575, *Chlamydomonas reinhardtii* glycerol-3-phosphate acyltransferase (GenBank Acc. No. EDP02129), *Chlamydomonas reinhardtii* glycerol-3-phosphate acyltransferase (GenBank Acc. No. Q886Q7), *Cucurbita moschata* acyl-(acyl-carrier-protein):glycerol-3-phosphate acyltransferase (GenBank Acc. No. BAB39688), *Elaeis guineensis* glycerol-3-phosphate acyltransferase, ((GenBank Acc. No. AAF64066), *Garcina mangostana* glycerol-3-phosphate acyltransferase (GenBank Acc. No. ABS86942), *Gossypium hirsutum* glycerol-3-phosphate acyltransferase (GenBank Acc. No. ADK23938), *Jatropha curcas* glycerol-3-phosphate acyltransferase (GenBank Acc. No. ADV77219), *Jatropha curcas* plastid glycerol-3-phosphate acyltransferase (GenBank Acc. No. ACR61638), *Ricinus communis* plastidial glycerol-phosphate acyltransferase (GenBank Acc. No. EEF43526), *Vica faba* glycerol-3-phosphate acyltransferase (GenBank Accession No. AAD05164), *Zea mays* glycerol-3-phosphate acyltransferase (GenBank Acc. No. ACG45812)

Lysophosphatidic acid acyltransferase Enzymes

Arabidopsis thaliana 1-acyl-sn-glycerol-3-phosphate acyltransferase (GenBank Accession No. AEE85783), *Brassica juncea* 1-acyl-sn-glycerol-3-phosphate acyltransferase (GenBank Accession No. ABQ42862), *Brassica juncea* 1-acyl-sn-glycerol-3-phosphate acyltransferase (GenBank Accession No. ABM92334), *Brassica napus* 1-acyl-sn-glycerol-3-phosphate acyltransferase (GenBank Accession No. CAB09138), *Chlamydomonas reinhardtii* lysophosphatidic acid acyltransferase (GenBank Accession No. EDP02300), *Cocos nucifera* lysophosphatidic acid acyltransferase (GenBank Acc. No. AAC49119), *Limnanthes alba* lysophosphatidic acid acyltransferase (GenBank Accession No. EDP02300), *Limnanthes douglasii* 1-acyl-sn-glycerol-3-phosphate acyltransferase (putative) (GenBank Accession No.

CAA88620), *Limnanthes douglasii* acyl-CoA:sn-1-acylglycerol-3-phosphate acyltransferase (GenBank Accession No. ABD62751), *Limnanthes douglasii* 1-acylglycerol-3-phosphate O-acyltransferase (GenBank Accession No. CAA58239), *Ricinus communis* 1-acyl-sn-glycerol-3-phosphate acyltransferase (GenBank Accession No. EEF39377)

Diacylglycerol acyltransferase Enzymes

Arabidopsis thaliana diacylglycerol acyltransferase (GenBank Acc. No. CAB45373), *Brassica juncea* diacylglycerol acyltransferase (GenBank Acc. No. AAY40784), *Elaeis guineensis*
putative diacylglycerol acyltransferase (GenBank Acc. No. AEQ94187), *Elaeis guineensis* putative diacylglycerol acyltransferase (GenBank Acc. No. AEQ94186), *Glycine max* acyl CoA:diacylglycerol acyltransferase (GenBank Acc. No. AAT73629), *Helianthus annus* diacylglycerol acyltransferase (GenBank Acc. No. ABX61081), *Olea europaea* acyl-CoA:diacylglycerol acyltransferase 1 (GenBank Acc. No. AAS01606), *Ricinus communis* diacylglycerol acyltransferase (GenBank Acc. No. AAR11479)

Phospholipid diacylglycerol acyltransferase Enzymes

Arabidopsis thaliana phospholipid:diacylglycerol acyltransferase (GenBank Acc. No. AED91921), *Elaeis guineensis* putative phospholipid:diacylglycerol acyltransferase (GenBank Acc. No. AEQ94116), *Glycine max* phospholipid:diacylglycerol acyltransferase 1-like (GenBank Acc. No. XP_003541296), *Jatropha curcas* phospholipid:diacylglycerol acyltransferase (GenBank Acc. No. AEZ56255), *Ricinus communis* phospholipid:diacylglycerol acyltransferase (GenBank Acc. No. ADK92410), *Ricinus communis* phospholipid:diacylglycerol acyltransferase (GenBank Acc. No. AEW99982)

EXAMPLE 10: Engineering *Chlorella sorokiniana*

[0386] Expression of recombinant genes in accordance with the present invention in *Chlorella sorokiniana* can be accomplished by modifying the methods and vectors taught by Dawson *et al.* as discussed herein. Briefly, Dawson *et al., Current Microbiology* Vol. 35 (1997) pp. 356–362, reported the stable nuclear transformation of *Chlorella sorokiniana* with plasmid DNA. Using the transformation method of microprojectile bombardment, Dawson introduced the plasmid pSV72-NRg, encoding the full *Chlorella vulgaris* nitrate reductase gene (NR, GenBank Accession No. U39931), into mutant *Chlorella sorokiniana* (NR-

mutants). The NR-mutants are incapable of growth without the use of nitrate as a source of nitrogen. Nitrate reductase catalyzes the conversion of nitrate to nitrite. Prior to transformation, *Chlorella sorokiniana* NR-mutants were unable to grow beyond the microcolony stage on culture medium comprising nitrate (NO_3^-) as the sole nitrogen source. The expression of the *Chlorella vulgaris* NR gene product in NR-mutant *Chlorella sorokiniana* was used as a selectable marker to rescue the nitrate metabolism deficiency. Upon transformation with the pSV72-NRg plasmid, NR-mutant *Chlorella sorokiniana* stably expressing the *Chlorella vulgaris* NR gene product were obtained that were able to grow beyond the microcolony stage on agar plates comprising nitrate as the sole carbon source. Evaluation of the DNA of the stable transformants was performed by Southern analysis and evaluation of the RNA of the stable transformants was performed by RNase protection. Selection and maintenance of the transformed *Chlorella sorokiniana* (NR mutant) was performed on agar plates (pH 7.4) comprising 0.2 g/L $MgSO_4$, 0.67 g/L KH_2PO_4, 3.5 g/L K_2HPO_4, 1.0 g/L $Na_3C_6H_5O_7 \cdot H_2O$ and 16.0 g/L agar, an appropriate nitrogen source (e.g., NO_3^-), micronutrients, and a carbon source. Dawson also reported the propagation of *Chlorella sorokiniana* and *Chlorella sorokiniana* NR mutants in liquid culture medium. Dawson reported that the plasmid pSV72-NRg and the promoter and 3' UTR/terminator of the *Chlorella vulgaris* nitrate reductase gene were suitable to enable heterologous gene expression in *Chlorella sorokiniana* NR-mutants. Dawson also reported that expression of the *Chlorella vulgaris* nitrate reductase gene product was suitable for use as a selectable marker in *Chlorella sorokiniana* NR-mutants.

[0387] In an embodiment of the present invention, vector pSV72-NRg, comprising nucleotide sequence encoding the *Chlorella vulgaris* nitrate reductase (*Cv*NR) gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected from Table 20, each protein-coding sequence codon-optimized for expression in *Chlorella sorokiniana to* reflect the codon bias inherent in nuclear genes of *Chlorella sorokiniana* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *Cv*NR promoter upstream of the protein-coding sequence and operably linked to the *Cv*NR 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Chlorella sorokiniana* genome for targeted genomic integration of the transformation vector.

Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. Stable transformation of *Chlorella sorokiniana* with the transformation vector is achieved through well-known transformation techniques including microprojectile bombardment or other known methods. Activity of the *Cv*NR gene product can be used as a selectable marker to rescue the nitrogen assimilation deficiency of *Chlorella sorokiniana* NR mutant strains and to select for *Chlorella sorokiniana* NR-mutants stably expressing the transformation vector. Growth media suitable for *Chlorella sorokiniana* lipid production include, but are not limited to 0.5 g/L KH_2PO_4, 0.5g/L K_2HPO_4, 0.25 g/L $MgSO_4$-7H2O, with supplemental micronutrients and the appropriate nitrogen and carbon sources (Patterson, *Lipids* Vol.5:7 (1970), pp.597-600). Evaluation of fatty acid profiles of *Chlorella sorokiniana lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 11: Engineering *Chlorella vulgaris*

[0388] Expression of recombinant genes in accordance with the present invention in *Chlorella vulgaris* can be accomplished by modifying the methods and vectors taught by Chow and Tung *et al.* as discussed herein. Briefly, Chow and Tung *et al., Plant Cell Reports*, Volume 18 (1999), pp. 778-780, reported the stable nuclear transformation of *Chlorella vulgaris* with plasmid DNA. Using the transformation method of electroporation, Chow and Tung introduced the plasmid pIG121-Hm (GenBank Accession No. AB489142) into *Chlorella vulgaris.* The nucleotide sequence of pIG121-Hm comprised sequence encoding a beta-glucuronidase (GUS) reporter gene product operably-linked to a CaMV 35S promoter upstream of the GUS protein-coding sequence and further operably linked to the 3' UTR/terminator of the nopaline synthase (*nos*) gene downstream of the GUS protein-coding sequence. The sequence of plasmid pIG121-Hm further comprised a hygromycin B antibiotic resistance cassette. This hygromycin B antibiotic resistance cassette comprised a CaMV 35S promoter operably linked to sequence encoding the hygromycin phosphotransferase (*hpt*, GenBank Accession No. BAH24259) gene product. Prior to transformation, *Chlorella vulgaris* was unable to be propagated in culture medium comprising 50 ug/ml hygromycin B. Upon transformation with the pIG121-Hm plasmid, transformants of *Chlorella vulgaris* were obtained that were propagated in culture medium comprising 50 ug/ml hygromycin B. The expression of the *hpt* gene product in *Chlorella vulgaris* enabled propagation of transformed *Chlorella vulgaris* in the presence of 50 ug/mL hygromycin B, thereby establishing the utility of the a hygromycin B resistance cassette as a

selectable marker for use in *Chlorella vulgaris*. Detectable activity of the GUS reporter gene indicated that CaMV 35S promoter and nos 3'UTR are suitable for enabling heterologous gene expression in *Chlorella vulgaris*. Evaluation of the genomic DNA of the stable transformants was performed by Southern analysis. Selection and maintenance of transformed *Chlorella vulgaris* was performed on agar plates comprising YA medium (agar and 4 g/L yeast extract). The propagation of *Chlorella vulgaris* in liquid culture medium was conducted as discussed by Chow and Tung. Propagation of *Chlorella vulgaris* in media other than YA medium has been described (for examples, see Chader et al., *Revue des Energies Renouvelabes*, Volume 14 (2011), pp. 21-26 and Illman et al., *Enzyme and Microbial Technology*, Vol. 27 (2000), pp. 631–635). Chow and Tung reported that the plasmid pIG121-Hm, the CaMV 35S promoter, and the *Agrobacterium tumefaciens* nopaline synthase gene 3'UTR/terminator are suitable to enable heterologous gene expression in *Chlorella vulgaris*. In addition, Chow and Tung reported the hygromycin B resistance cassette was suitable for use as a selectable marker in *Chlorella vulgaris*. Additional plasmids, promoters, 3'UTR/terminators, and selectable markers suitable for enabling heterologous gene expression in *Chlorella vulgaris* have been discussed in Chader *et al.*, *Revue des Energies Renouvelabes*, Volume 14 (2011), pp. 21-26.

[0389] In an embodiment of the present invention, pIG121-Hm, comprising the nucleotide sequence encoding the hygromycin B gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected from Table 20, each protein-coding sequence codon-optimized for expression in *Chlorella vulgaris to* reflect the codon bias inherent in nuclear genes of *Chlorella vulgaris* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the CaMV 35S promoter upstream of the protein-coding sequence and operably linked to the *Agrobacterium tumefaciens* nopaline synthase gene 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Chlorella vulgaris* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. Stable transformation of *Chlorella vulgaris* with the transformation vector is achieved through well-known transformation techniques including electroporation or other known methods. Activity of the hygromycin B resistance gene

product can be used as a marker to select for *Chlorella vulgaris* transformed with the transformation vector on, but not limited to, agar medium comprising hygromycin. Growth media suitable for *Chlorella vulgaris* lipid production include, but are not limited to BG11 medium (0.04 g/L KH_2PO_4, 0.075 g/L $CaCl_2$, 0.036 g/L citric acid, 0.006 g/L Ammonium Ferric Citrate, 1mg/L EDTA, and 0.02 g/L Na_2CO_3) supplemented with trace metals, and optionally 1.5 g/L NaNO3. Additional media suitable for culturing *Chlorella vulgaris* for lipid production include, for example, Watanabe medium (comprising 1.5 g/L KNO_3, 1.25 g/L KH_2PO_4, 1.25 g l^{-1} $MgSO_4 \cdot 7H_2O$, 20 mg l^{-1} $FeSO_4 \cdot 7H_2O$ with micronutrients and low-nitrogen medium (comprising 203 mg/l $(NH_4)_2HPO_4$, 2.236 g/l KCl, 2.465 g/l $MgSO_4$, 1.361 g/l KH_2PO_4 and 10 mg/l $FeSO_4$) as reported by Illman et al., *Enzyme and Microbial Technology*, Vol. 27 (2000), pp. 631–635. Evaluation of fatty acid profiles of *Chlorella vulgaris lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 12: Engineering *Chlorella ellipsoidea*

[0390] Expression of recombinant genes in accordance with the present invention in *Chlorella ellipsoidea* can be accomplished by modifying the methods and vectors taught by Chen *et al.* as discussed herein. Briefly, Chen *et al.*, *Current Genetics*, Vol. 39:5 (2001), pp. 365-370, reported the stable transformation of *Chlorella ellipsoidea* with plasmid DNA. Using the transformation method of electroporation, Chen introduced the plasmid pBinUΩNP-1 into *Chlorella ellipsoidea.* The nucleotide sequence of pBinUΩNP-1 comprised sequence encoding the neutrophil peptide-1 (NP-1) rabbit gene product operably linked to a *Zea mays* Ubiquitin (ubi1) gene promoter upstream of the NP-1 protein-coding region and operably linked to the 3' UTR/terminator of the nopaline synthase (*nos*) gene downstream of the NP-1 protein-coding region. The sequence of plasmid pBinUΩNP-1 further comprised a G418 antibiotic resistance cassette. This G418 antibiotic resistance cassette comprised sequence encoding the aminoglycoside 3'-phosphotransferase (*aph 3'*) gene product. The *aph 3'* gene product confers resistance to the antibiotic G418. Prior to transformation, *Chlorella ellipsoidea* was unable to be propagated in culture medium comprising 30 ug/mL G418. Upon transformation with the pBinUΩNP-1 plasmid, transformants of *Chlorella ellipsoidea* were obtained that were propagated in selective culture medium comprising 30 ug/mL G418. The expression of the *aph 3'* gene product in *Chlorella ellipsoidea* enabled propagation of transformed *Chlorella ellipsoidea* in the presence of 30 ug/mL G418, thereby establishing the utility of the G418 antibiotic resistance

cassette as selectable marker for use in *Chlorella ellipsoidea*. Detectable activity of the NP-1 gene product indicated that the ubi1 promoter and *nos* 3' UTR are suitable for enabling heterologous gene expression in *Chlorella ellipsoidea*. Evaluation of the genomic DNA of the stable transformants was performed by Southern analysis. Selection and maintenance of the transformed *Chlorella ellipsoidea* was performed on Knop medium (comprising 0.2 g/L K_2HPO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.12 g/L KCl, and 10 mg/L FeCl3, pH 6.0-8.0 supplemented with 0.1% yeast extract and 0.2% glucose) with 15 ug/mL G418 (for liquid cultures) or with 30 ug/mL G418 (for solid cultures comprising 1.8% agar). Propagation of *Chlorella ellipsoidea* in media other than Knop medium has been reported (see Cho *et al.*, Fisheries Science, Vol. 73:5 (2007), pp. 1050-1056, Jarvis and Brown, *Current Genetics*, Vol. 19 (1991), pp.317-321 and Kim *et al.*, *Marine Biotechnology*, Vol. 4 (2002), pp.63-73). Additional plasmids, promoters, 3'UTR/terminators, and selectable markers suitable for enabling heterologous gene expression in *Chlorella ellipsoidea* have been reported (see Jarvis and Brown and Kim *et al., Marine Biotechnology*, Vol. 4 (2002), pp.63-73). Chen reported that the plasmid pBinUΩNP-1, the ubi1 promoter, and the *Agrobacterium tumefaciens* nopaline synthase gene 3'UTR/terminator are suitable to enable exogenous gene expression in *Chlorella ellipsoidea*. In addition, Chen reported that the G418 resistance cassette encoded on pBinUΩNP-1 was suitable for use as a selectable marker in *Chlorella ellipsoidea*.

[0391] In an embodiment of the present invention, vector pBinUΩNP-1, comprising the nucleotide sequence encoding the *aph 3'* gene product, conferring resistance to G418, for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected from Table 20, each protein-coding sequence codon-optimized for expression in *Chlorella ellipsoidea to* reflect the codon bias inherent in nuclear genes of *Chlorella ellipsoidea* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *Zea mays* ubi1 promoter upstream of the protein-coding sequence and operably linked to the *Agrobacterium tumefaciens* nopaline synthase gene 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Chlorella ellipsoidea* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. Stable

transformation of *Chlorella ellipsoidea* with the transformation vector is achieved through well-known transformation techniques including electroporation or other known methods. Activity of the *aph 3'* gene product can be used as a marker to select for *Chlorella ellipsoidea* transformed with the transformation vector on, but not limited to, Knop agar medium comprising G418. Growth media suitable for *Chlorella ellipsoidea* lipid production include, but are not limited to, Knop medium and those culture medium reported by Jarvis and Brown and Kim *et al.* Evaluation of fatty acid profiles of *Chlorella ellipsoidea lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 13: Engineering *Chlorella kessleri*

[0392] Expression of recombinant genes in accordance with the present invention in *Chlorella kessleri* can be accomplished by modifying the methods and vectors taught by El-Sheekh *et al.* as discussed herein. Briefly, El-Sheekh *et al.*, *Biologia Plantarium*, Vol. 42:2 (1999), pp. 209-216, reported the stable transformation of *Chlorella kessleri* with plasmid DNA. Using the transformation method of microprojectile bombardment, El-Sheekh introduced the plasmid pBI121 (GenBank Accession No. AF485783) into *Chlorella kessleri.* Plasmid pBI121 comprised a kanamycin/neomycin antibiotic resistance cassette. This kanamycin/neomycin antibiotic resistance cassette comprised the *Agrobacterium tumefaciens* nopaline synthase (*nos*) gene promoter, sequence encoding the neomycin phosphotransferase II (*npt*II) gene product (GenBank Accession No. AAL92039) for resistance to kanamycin and G418, and the 3' UTR/terminator of the *Agrobacterium tumefaciens* nopaline synthase (*nos*) gene. pBI121 further comprised sequence encoding a beta-glucuronidase (GUS) reporter gene product operably linked to a CaMV 35S promoter and operably linked to a 3' UTR/terminator of the *nos* gene. Prior to transformation, *Chlorella kessleri* was unable to be propagated in culture medium comprising 15 ug/L kanamycin. Upon transformation with the pBI121plasmid, transformants of *Chlorella kessleri* were obtained that were propagated in selective culture medium comprising 15 mg/L kanamycin. The express ion of the *npt*II gene product in *Chlorella kessleri* enabled propagation in the presence of 15 mg/L kanamycin, thereby establishing the utility of the kanamycin/neomycin antibiotic resistance cassette as selectable marker for use in *Chlorella kessleri.* Detectable activity of the GUS gene product indicated that the CaMV 35S promoter and nos 3' UTR are suitable for enabling heterologous gene expression in *Chlorella kessleri.* Evaluation of the genomic DNA of the stable transformants was performed by Southern analysis. As reported by El-Sheekh, selection and maintenance of transformed *Chlorella kessleri* was conducted on semisolid agar plates

comprising YEG medium (1% yeast extract, 1% glucose) and 15 mg/L kanamycin. El-Sheekh also reported the propagation of *Chlorella kessleri* in YEG liquid culture media. Additional media suitable for culturing *Chlorella kessleri* for lipid production are disclosed in Sato *et al.*, *BBA Molecular and Cell Biology of Lipids*, Vol. 1633 (2003), pp. 27-34). El-Sheekh reported that the plasmid pBI121, the CaMV promoter, and the nopaline synthase gene 3'UTR/terminator are suitable to enable heterologous gene expression in *Chlorella kessleri*. In addition, El-Sheekh reported that the kanamycin/neomycin resistance cassette encoded on pBI121 was suitable for use as a selectable marker in *Chlorella kessleri*.

[0393] In an embodiment of the present invention, vector pBI121, comprising the nucleotide sequence encoding the kanamycin/neomycin resistance gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected from Table 20, each protein-coding sequence codon-optimized for expression in *Chlorella kessleri to* reflect the codon bias inherent in nuclear genes of *Chlorella kessleri* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the CaMV 35S promoter upstream of the protein-coding sequence and operably linked to the *Agrobacterium tumefaciens* nopaline synthase gene 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Chlorella kessleri* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. Stable transformation of *Chlorella kessleri* with the transformation vector is achieved through well-known transformation techniques including microprojectile bombardment or other known methods. Activity of the *nptII* gene product can be used as a marker to select for *Chlorella kessleri* transformed with the transformation vector on, but not limited to, YEG agar medium comprising kanamycin or neomycin. Growth media suitable for *Chlorella kessleri* lipid production include, but are not limited to, YEG medium, and those culture media reported by Sato *et al.* Evaluation of fatty acid profiles of *Chlorella kessleri lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 14: Engineering *Dunaliella tertiolecta*

[0394] Expression of recombinant genes in accordance with the present invention in *Dunaliella tertiolecta* can be accomplished by modifying the methods and vectors taught by Walker *et al.* as discussed herein. Briefly, Walker *et al., Journal of Applied Phycology*, Vol. 17 (2005), pp. 363-368, reported stable nuclear transformation of *Dunaliella tertiolecta* with plasmid DNA. Using the transformation method of electroporation, Walker introduced the plasmid pDbleFLAG1.2 into *Dunaliella tertiolecta*. pDbleFLAG1.2 comprised sequence encoding a bleomycin antibiotic resistance cassette, comprising sequence encoding the *Streptoalloteichus hindustanus* Bleomycin binding protein (*ble*), for resistance to the antibiotic phleomycin, operably linked to the promoter and 3' UTR of the *Dunaliella tertiolecta* ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit gene (*rbcS1*, GenBank Accession No. AY530155). Prior to transformation, *Dunaliella tertiolecta* was unable to be propagated in culture medium comprising 1 mg/L phleomycin. Upon transformation with the pDbleFLAG1.2 plasmid, transformants of *Dunaliella tertiolecta* were obtained that were propagated in selective culture medium comprising 1 mg/L phleomycin. The expression of the *ble* gene product in *Dunaliella tertiolecta* enabled propagation in the presence of 1 mg/L phleomycin, thereby establishing the utility of the bleomycin antibiotic resistance cassette as selectable marker for use in *Dunaliella tertiolecta*. Evaluation of the genomic DNA of the stable transformants was performed by Southern analysis. As reported by Walker, selection and maintenance of transformed *Dunaliella tertiolecta* was conducted in Dunaliella medium (DM, as described by Provasoli et al., *Archiv fur Mikrobiologie*, Vol. 25 (1957), pp. 392-428) further comprising 4.5 g/L NaCl and 1 mg/L pheomycin. Additional media suitable for culturing *Dunaliella tertiolecta* for lipid production are discussed in Takagi *et al., Journal of Bioscience and Bioengineering*, Vol. 101:3 (2006), pp. 223-226 and in Massart and Hanston, Proceedings Venice 2010, *Third International Symposium on Energy from Biomass and Waste*. Walker reported that the plasmid pDbleFLAG1.2 and the promoter and 3' UTR of the *Dunaliella tertiolecta* ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit gene are suitable to enable heterologous expression in *Dunaliella tertiolecta*. In addition, Walker reported that the bleomycin resistance cassette encoded on pDbleFLAG1.2 was suitable for use as a selectable marker in *Dunaliella tertiolecta*.

[0395] In an embodiment of the present invention, vector pDbleFLAG1.2, comprising the nucleotide sequence encoding the *ble* gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway

expression cassette encodes one or more lipid biosynthesis pathway proteins selected from Table 20, each protein-coding sequence codon-optimized for expression in *Dunaliella tertiolecta to* reflect the codon bias inherent in nuclear genes of *Dunaliella tertiolecta* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *rbcS*1 promoter upstream of the protein-coding sequence and operably linked to the *rbcS*1 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Dunaliella tertiolecta* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. Stable transformation of *Dunaliella tertiolecta* with the transformation vector is achieved through well-known transformation techniques including electroporation or other known methods. Activity of the *ble* gene product can be used as a marker to select for *Dunaliella tertiolecta* transformed with the transformation vector on, but not limited to, DM medium comprising pheomycin. Growth medium suitable for *Dunaliella tertiolecta* lipid production include, but are not limited to DM medium and those culture media described by Takagi *et al.* and Massart and Hanston. Evaluation of fatty acid profiles of *Dunaliella tertiolecta lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 15: Engineering *Volvox carteri*

[0396] Expression of recombinant genes in accordance with the present invention in *Volvox carteri* can be accomplished by modifying the methods and vectors taught by Hallman and Rappel *et al.* as discussed herein. Briefly, Hallman and Rappel *et al.*, *The Plant Journal*, Volume 17 (1999), pp. 99-109, reported the stable nuclear transformation of *Volvox carteri* with plasmid DNA. Using the transformation method of microprojectile bombardment, Hallman and Rappel introduced the pzeoE plasmid into *Volvox carteri.* The pzeoE plasmid comprised sequence encoding a bleomycin antibiotic resistance cassette, comprising sequence encoding the *Streptoalloteichus hindustanus* Bleomycin binding protein (*ble*), for resistance to the antibiotic zeocin, operably linked to and the promoter and 3' UTR of the *Volvox carteri* beta-tubulin gene (GenBank Accession No. L24547). Prior to transformation, *Volvox carteri* was unable to be propagated in culture medium comprising 1.5 ug/ml zeocin. Upon transformation with the pzeoE plasmid, transformants of *Volvox carteri* were obtained that were propagated in selective culture medium comprising greater than 20 ug/ml zeocin.

The expression of the *ble* gene product in *Volvox carteri* enabled propagation in the presence of 20 ug/ml zeocin, thereby establishing the utility of the bleomycin antibiotic resistance cassette as selectable marker for use in *Volvox carteri*. Evaluation of the genomic DNA of the stable transformants was performed by Southern analysis. As reported by Hallman and Rappel, selection and maintenance of transformed *Volvox carteri* was conducted in Volvox medium (VM, as described by Provasoli and Pintner, The Ecology of Algae, Special Publication No. 2 (1959), Tyron, C.A. and Hartman, R.T., eds., Pittsburgh: University of Pittsburgh, pp. 88-96) with 1 mg/L pheomycin. Media suitable for culturing *Volvox carteri* for lipid production are also discussed by Starr in *Starr* R,C,. *Dev Biol* Suppl., Vol. 4 (1970), pp.59-100). Hallman and Rappel reported that the plasmid pzeoE and the promoter and 3' UTR of the *Volvox carteri* beta-tubulin gene are suitable to enable heterologous expression in *Volvox carteri*. In addition, Hallman and Rappel reported that the bleomycin resistance cassette encoded on pzeoE was suitable for use as a selectable marker in *Volvox carteri*. Additional plasmids, promoters, 3'UTR/terminators, and selectable markers suitable for enabling heterologous gene expression in *Volvox carteri* and suitable for use as selective markers *Volvox carteri* in have been reported (for instance see Hallamann and Sumper, *Proceedings of the National Academy of Sciences*, Vol. 91 (1994), pp 11562-11566 and Hallman and Wodniok, *Plant Cell Reports*, Volume 25 (2006), pp. 582-581).

[0397] In an embodiment of the present invention, vector pzeoE, comprising the nucleotide sequence encoding the *ble* gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected from Table 19, each protein-coding sequence codon-optimized for expression in *Volvox carteri to* reflect the codon bias inherent in nuclear genes of *Volvox carteri* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *Volvox carteri* beta-tubulin promoter upstream of the protein-coding sequence and operably linked to the *Volvox carteri* beta-tubulin 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Volvox carteri* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. One skilled in the art can identify such homology regions within the sequence of the *Volvox carteri* genome (referenced in the publication by Prochnik et al.,

Science, Vol. 329:5988 (2010), pp223-226). Stable transformation of *Volvox carteri* with the transformation vector is achieved through well-known transformation techniques including microprojectile bombardment or other known methods. Activity of the *ble* gene product can be used as a marker to select for *Volvox carteri* transformed with the transformation vector on, but not limited to, VM medium comprising zeocin. Growth medium suitable for *Volvox carteri* lipid production include, but are not limited to VM medium and those culture media discussed by Starr. Evaluation of fatty acid profiles of *Volvox carteri lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 16: Engineering *Haematococcus pluvialis*

[0398] Expression of recombinant genes in accordance with the present invention in *Haematococcus pluvialis* can be accomplished by modifying the methods and vectors taught by Steinbrenner and Sandmann *et al.* as discussed herein. Briefly, Steinbrenner and Sandmann *et al.*, *Applied and Environmental Microbiology*, Vol. 72:12 (2006), pp.7477-7484, reported the stable nuclear transformation of *Haematococcus pluvialis* with plasmid DNA. Using the transformation method of microprojectile bombardment, Steinbrenner introduced the plasmid pPlat-pds-L504R into *Haematococcus pluvialis*. The plasmid pPlat-pds-L504R comprised a norflurazon resistance cassette, which comprised the promoter, protein-coding sequence, and 3'UTR of the *Haematococcus pluvialis* phytoene desaturase gene (Pds, GenBank Accession No. AY781170), wherein the protein-coding sequence of Pds was modified at position 504 (thereby changing a leucine to an arginine) to encode a gene product (Pds-L504R) that confers resistance to the herbicide norflurazon. Prior to transformation with pPlat-pds-L504R, *Haematococcus pluvialis* was unable to propagate on medium comprising 5 uM norflurazon. Upon transformation with the pPlat-pds-L504R plasmid, transformants of *Haematococcus pluvialis* were obtained that were propagated in selective culture medium comprising 5 uM norflurazon. The expression of the Pds-L504R gene product in *Haematococcus pluvialis* enabled propagation in the presence of 5 uM norflurazon, thereby establishing the utility of the norflurazon herbicide resistance cassette as selectable marker for use in *Haematococcus pluvialis*. Evaluation of the genomic DNA of the stable transformants was performed by Southern analysis. As reported by Steinbrenner, selection and maintenance of transformed *Haematococcus pluvialis* was conducted on agar plates comprising OHA medium (OHM (0.41 g/L KNO_3, 0.03 g/L Na_2HPO_4, 0.246 g/L $MgSO_4 \cdot 7H_2O$, 0.11 g/L $CaCl_2 \cdot 2H_2O$, 2.62 mg/L $Fe_{(III)}$citrate x H_2O, 0.011 mg/L $CoCl_2 \cdot 6H_2O$, 0.012 mg/L $CuSO_4 \cdot 5H_2O$, 0.075 mg/L Cr_2O_3, 0.98 mg/L $MnCl_2 \cdot 4H_2O$, 0.12

mg/L Na_2MoO_4 x $2H_20$, 0.005 mg/L SeO_2 and 25 mg/L biotin, 17.5 mg/L thiamine, and 15 mg/L vitamin B12), supplemented with 2.42 g/L Tris-acetate, and 5mM norflurazon. Propagation of *Haematococcus pluvialis* in liquid culture was performed by Steinbrenner and Sandmann using basal medium (basal medium as described by Kobayashi *et al., Applied and Environmental Microbiology*, Vol. 59 (1993), pp.867-873). Steinbrenner and Sandmann reported that the pPlat-pds-L504R plasmid and promoter and 3' UTR of the *Haematococcus pluvialis* phytoene desaturase gene are suitable to enable heterologous expression in *Haematococcus pluvialis*. In addition, Steinbrenner and Sandmann reported that the norflurazon resistance cassette encoded on pPlat-pds-L504R was suitable for use as a selectable marker in *Haematococcus pluvialis*. Additional plasmids, promoters, 3'UTR/terminators, and selectable markers suitable for enabling heterologous gene expression in *Haematococcus pluvialis* have been reported (see Kathiresan *et al., Journal of Phycology*, Vol. 45 (2009), pp 642-649).

[0399] In an embodiment of the present invention, vector pPlat-pds-L504R, comprising the nucleotide sequence encoding the Pds-L504R gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected from Table 20, each protein-coding sequence codon-optimized for expression in *Haematococcus pluvialis to* reflect the codon bias inherent in nuclear genes of *Haematococcus pluvialis* in accordance with Tables 19 A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *Haematococcus pluvialis pds* gene promoter upstream of the protein-coding sequence and operably linked to the *Haematococcus pluvialis pds* gene 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Haematococcus pluvialis* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. Stable transformation of *Haematococcus pluvialis* with the transformation vector is achieved through well-known transformation techniques including microprojectile bombardment or other known methods. Activity of the Pds-L504R gene product can be used as a marker to select for *Haematococcus pluvialis* transformed with the transformation vector on, but not limited to, OHA medium comprising norflurazon. Growth media suitable for *Haematococcus pluvialis* lipid production include, but are not limited to basal medium and those culture media described by

Kobayashi *et al.,* Kathiresan et al, and Gong and Chen, *Journal of Applied Phycology*, Vol. 9:5 (1997), pp. 437-444). Evaluation of fatty acid profiles of *Haematococcus pluvialis lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 17: Engineering *Closterium peracerosum–strigosum–littorale complex*

[0400] Expression of recombinant genes in accordance with the present invention in *Closterium peracerosum–strigosum–littorale complex* can be accomplished by modifying the methods and vectors taught by Abe *et al.* as discussed herein. Briefly, Abe *et al.*, *Plant Cell Physiology*, Vol. 52:9 (2011), pp. 1676-1685, reported the stable nuclear transformation of *Closterium peracerosum–strigosum–littorale complex* with plasmid DNA. Using the transformation methods of microprojectile bombardment, Abe introduced the plasmid pSA106 into *Closterium peracerosum–strigosum–littorale complex.* Plasmid pSA106 comprised a bleomycin resistance cassette, comprising sequence encoding the *Streptoalloteichus hindustanus* Bleomycin binding protein gene (ble, GenBank Accession No. CAA37050) operably linked to the promoter and 3' UTR of the *Closterium peracerosum–strigosum–littorale complex Chlorophyll a/b-binding protein gene* (CAB, GenBank Accession No. AB363403). Prior to transformation with pSA106, *Closterium peracerosum–strigosum–littorale complex* was unable to propagate on medium comprising 3 ug/ml phleomycin. Upon transformation with pSA106, transformants of *Closterium peracerosum–strigosum–littorale complex* were obtained that were propagated in selective culture medium comprising 3 ug/ml phleomycin. The expression of the ble gene product in *Closterium peracerosum–strigosum–littorale complex* enabled propagation in the presence of 3 ug/ml phleomycin, thereby establishing the utility of the bleomycin antibiotic resistance cassette as selectable marker for use in *Closterium peracerosum–strigosum–littorale complex*. Evaluation of the genomic DNA of the stable transformants was performed by Southern analysis. As reported by Abe, selection and maintenance of transformed *Closterium peracerosum–strigosum–littorale complex* was conducted first in top agar with C medium (0.1 g/L KNO_3, 0.015 g/L $Ca(NO_3)_2 \cdot 4H2O$, 0.05 g/L glycerophosphate-Na2, 0.04 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L Tris (hydroxylmethyl) aminomethane, trace minerals, biotin, vitamins B_1 and B_{12}) and then subsequently isolated to agar plates comprising C medium supplemented with phleomycin. As reported by Abe, propagation of *Closterium peracerosum–strigosum–littorale complex* in liquid culture was performed in C medium. Additional liquid culture medium suitable for propagation of *Closterium peracerosum–strigosum–littorale complex* are discussed by Sekimoto *et al., DNA Research*, 10:4 (2003),

pp. 147-153. Abe reported that the pSA106 plasmid and promoter and 3' UTR of the *Closterium peracerosum–strigosum–littorale complex* CAB gene are suitable to enable heterologous gene expression in *Closterium peracerosum–strigosum–littorale complex*. In addition, Abe reported that the bleomycin resistance cassette encoded on pSA106 was suitable for use as a selectable marker in *Closterium peracerosum–strigosum–littorale complex*. Additional plasmids, promoters, 3'UTR/terminators, and selectable markers suitable for enabling heterologous gene expression in *Closterium peracerosum–strigosum–littorale complex* have been reported (see Abe *et al., Plant Cell Physiology*, Vol. 49 (2008), pp. 625-632).

[0401] In an embodiment of the present invention, vector pSA106, comprising the nucleotide sequence encoding the *ble* gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected from Table 20, each protein-coding sequence codon-optimized for expression in *Closterium peracerosum–strigosum–littorale complex to* reflect the codon bias inherent in nuclear genes of *Closterium peracerosum–strigosum–littorale complex* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *Closterium peracerosum–strigosum–littorale complex* CAB gene promoter upstream of the protein-coding sequence and operably linked to the *Closterium peracerosum–strigosum–littorale complex* CAB gene 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Closterium peracerosum–strigosum–littorale complex* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. Stable transformation of *Closterium peracerosum–strigosum–littorale complex* with the transformation vector is achieved through well-known transformation techniques including microprojectile bombardment or other known methods. Activity of the *ble* gene product can be used as a marker to select for *Closterium peracerosum–strigosum–littorale complex* transformed with the transformation vector on, but not limited to, C medium comprising phleomycin. Growth media suitable for *Closterium peracerosum–strigosum–littorale complex* lipid production include, but are not limited to C medium and those culture media reported by Abe *et al.* and Sekimoto *et al.* Evaluation of fatty acid profiles of *Closterium peracerosum–strigosum–littorale complex lipids* can be

assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 18: Engineering *Dunaliella viridis*

[0402] Expression of recombinant genes in accordance with the present invention in *Dunaliella viridis* can be accomplished by modifying the methods and vectors taught by Sun *et al.* as discussed herein. Briefly, Sun *et al.*, *Gene*, Vol. 377 (2006), pp.140-149, reported the stable transformation of *Dunaliella viridis* with plasmid DNA. Using the transformation method of electroporation, Sun introduced the plasmid pDVNR, encoding the full *Dunaliella viridis* nitrate reductase gene into mutant *Dunaliella viridis* (*Dunaliella viridis* NR-mutants.) The NR-mutants are incapable of growth without the use of nitrate as a source of nitrogen. Nitrate reductase catalyzes the conversion of nitrate to nitrite. Prior to transformation, *Dunaliella viridis* NR-mutants were unable to propagate in culture medium comprising nitrate (NO_3^-) as the sole nitrogen source. The expression of the *Dunaliella viridis* NR gene product in NR-mutant *Dunaliella viridis* was used as a selectable marker to rescue the nitrate metabolism deficiency. Upon transformation with the pDVNR plasmid, NR-mutant *Dunaliella viridis* stably expressing the *Dunaliella viridis* NR gene product were obtained that were able to grow on agar plates comprising nitrate as the sole carbon source. Evaluation of the DNA of the stable transformants was performed by Southern analysis. Selection and maintenance of the transformed *Dunaliella viridis* (NR mutant) was performed on agar plates comprising 5 mM KNO_3. Sun also reported the propagation of *Dunaliella viridis* and *Dunaliella viridis* NR mutants in liquid culture medium. Additional media suitable for propagation of *Dunaliella viridis* are reported by Gordillo *et al.*, *Journal of Applied Phycology*, Vol. 10:2 (1998), pp. 135-144 and by Moulton and Burford, *Hydrobiologia*, Vols. 204-205:1 (1990), pp. 401-408. Sun reported that the plasmid pDVNR and the promoter and 3' UTR/terminator of the *Dunaliella viridis* nitrate reductase gene were suitable to enable heterologous expression in *Dunaliella viridis* NR-mutants. Sun also reported that expression of the *Dunaliella viridis* nitrate reductase gene product was suitable for use as a selectable marker in *Dunaliella viridis* NR-mutants.

[0403] In an embodiment of the present invention, vector pDVNR, comprising the nucleotide sequence encoding the *Dunaliella viridis* nitrate reductase (*Dv*NR) gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected Table 20, each protein-coding sequence codon-optimized for

expression in *Dunaliella viridis to* reflect the codon bias inherent in nuclear genes of *Dunaliella viridis* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *DvNR* promoter upstream of the protein-coding sequence and operably linked to the *DvNR* 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Dunaliella viridis* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. Stable transformation of *Dunaliella viridis NR mutants* with the transformation vector is achieved through well-known transformation techniques including electroporation or other known methods. Activity of the *DvNR* gene product can be used as a selectable marker to rescue the nitrogen assimilation deficiency of *Dunaliella viridis* NR mutant strains and to select for *Dunaliella viridis* NR-mutants stably expressing the transformation vector. Growth media suitable for *Dunaliella viridis* lipid production include, but are not limited to those discussed by Sun *et al.*, Moulton and Burford, and Gordillo *et al.* Evaluation of fatty acid profiles of *Dunaliella viridis lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 19: Engineering *Dunaliella salina*

[0404] Expression of recombinant genes in accordance with the present invention in *Dunaliella salina* can be accomplished by modifying the methods and vectors taught by Geng *et al.* as discussed herein. Briefly, Geng *et al., Journal of Applied Phycology*, Vol. 15 (2003), pp. 451-456, reported the stable transformation of *Dunaliella salina* with plasmid DNA. Using the transformation method of electroporation, Geng introduced the pUΩHBsAg-CAT plasmid into *Dunaliella salina.* pUΩHBsAg-CAT comprises a hepatitis B surface antigen (HBsAG) expression cassette comprising sequence encoding the hepatitis B surface antigen operably linked to a *Zea mays* ubi1 promoter upstream of the HBsAG protein-coding region and operably linked to the *3'UTR/terminator* of the *Agrobacterium tumefaciens* nopaline synthase gene (*nos*) downstream of the HBsAG protein-coding region. pUΩHBsAg-CAT further comprised a chloramphenicol resistance cassette, comprising sequence encoding the chloramphenicol *acetyltransferase* (CAT) gene product, conferring resistance to the antibiotic chloramphenicol, operably linked to the simian virus 40 promoter and enhancer. Prior to transformation with pUΩHBsAg-CAT, *Dunaliella salina* was unable to propagate on medium comprising 60 mg/L chloramphenicol. Upon transformation with the pUΩHBsAg-

CAT plasmid, transformants of *Dunaliella salina* were obtained that were propagated in selective culture medium comprising 60 mg/L chloramphenicol. The expression of the CAT gene product in *Dunaliella salina* enabled propagation in the presence of 60 mg/L chloramphenicol, thereby establishing the utility of the chloramphenicol resistance cassette as selectable marker for use in *Dunaliella salina*. Detectable activity of the HBsAg gene product indicated that ubi1 promoter and nos 3'UTR/terminator are suitable for enabling gene expression in *Dunaliella salina*. Evaluation of the genomic DNA of the stable transformants was performed by Southern analysis. Geng reported that selection and maintenance of the transformed *Dunaliella salina* was performed on agar plates comprising Johnson's medium (J1, described by Borowitzka and Borowitzka (eds), Micro-algal Biotechnology. Cambridge University Press, Cambridge, pp. 460-461) with 60 mg/L chloramphenicol. Liquid propagation of *Dunaliella salina* was performed by Geng in J1 medium with 60 mg/L chloramphenicol. Propagation of *Dunaliella salina* in media other than J1 medium has been discussed (see Feng *et al., Mol. Bio. Reports*, Vol. 36 (2009), pp.1433-1439 and Borowitzka *et al.*, *Hydrobiologia*, Vols. 116-117:1 (1984), pp. 115-121). Additional plasmids, promoters, 3'UTR/terminators, and selectable markers suitable for enabling heterologous gene expression in *Dunaliella salina* have been reported by Feng *et al.* Geng reported that the plasmid pUΩHBsAg-CAT, the ubi1 promoter, and the *Agrobacterium tumefaciens* nopaline synthase gene 3'UTR/terminator are suitable to enable exogenous gene expression in *Dunaliella salina*. In addition, Geng reported that the CAT resistance cassette encoded on pUΩHBsAg-CAT was suitable for use as a selectable marker in *Dunaliella salina*.

[0405] In an embodiment of the present invention, vector pUΩHBsAg-CAT, comprising the nucleotide sequence encoding the CAT gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected Table 20, each protein-coding sequence codon-optimized for expression in *Dunaliella salina to* reflect the codon bias inherent in nuclear genes of *Dunaliella salina* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *ubi1* promoter upstream of the protein-coding sequence and operably linked to the *Agrobacterium tumefaciens* nopaline synthase gene 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the

Dunaliella salina genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. Stable transformation of *Dunaliella salina* with the transformation vector is achieved through well-known transformation techniques including electroporation or other known methods. Activity of the CAT gene product can be used as a selectable marker to select for *Dunaliella salina* transformed with the transformation vector in, but not limited to, J1 medium comprising chloramphenicol. Growth medium suitable for *Dunaliella salina* lipid production include, but are not limited to J1 medium and those culture media described by Feng *et al.* and Borowitzka *et al.* Evaluation of fatty acid profiles of *Dunaliella salina lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 20: Engineering *Gonium pectoral*

[0406] Expression of recombinant genes in accordance with the present invention in *Gonium pectoral* can be accomplished by modifying the methods and vectors taught by Lerche and Hallman *et al.* as discussed herein. Briefly, Lerche and Hallman *et al.*, *BMC Biotechnology*, Volume 9:64, 2009, reported the stable nuclear transformation of *Gonium pectorale* with plasmid DNA. Using the transformation method of microprojectile bombardment, Lerche introduced the plasmid pPmr3 into *Gonium pectorale.* Plasmid pPmr3 comprised a paromomycin resistance cassette, comprising a sequence encoding the aminoglycoside 3'-phosphotransferase (*aphVIII*) gene product (GenBank Accession No. AAB03856) of *Streptomyces rimosus* for resistance to the antibiotic paromomycin, operably linked to the *Volvox carteri* hsp70A-rbcS3 hybrid promoter upstream of the *aphVIII* protein-coding region and operably linked to the 3' UTR/terminator of the *Volvox carteri* rbcS3 gene downstream of the *aphVIII* protein-coding region. Prior to transformation with pPmr3, *Gonium pectorale* was unable to propagate on medium comprising 0.06 ug/ml paromomycin. Upon transformation with pPmr3, transformants of *Gonium pectorale* were obtained that were propagated in selective culture medium comprising 0.75 and greater ug/ml paromomycin. The expression of the *aphVIII* gene product in *Gonium pectorale* enabled propagation in the presence of 0.75 and greater ug/ml paromomycin, thereby establishing the utility of the paromomycin antibiotic resistance cassette as selectable marker for use in *Gonium pectorale*. Evaluation of the genomic DNA of the stable transformants was performed by Southern analysis. Lerche and Hallman reported that selection and maintenance of the transformed *Gonium pectorale* was performed in liquid Jaworski's

medium (20 mg/L $Ca(NO_3)_2 \cdot 4H_2O$, 12.4 mg/L KH_2PO_4, 50 mg/L $MgSO_4 \cdot 7H_2O$, 15.9 mg/L $NaHCO_3$, 2.25 mg/L EDTA-FeNa, 2.25 mg/L EDTA Na_2, 2.48 g/L H_3BO_3, 1.39 g/L $MnCl_2.4H_2O$, 1 mg/L $(NH_4)_6MO_7O_24.4H_2O$, 0.04 mg/L vitamin B12, 0.04 mg/L Thiamine-HCl, 0.04 mg/L biotin, 80 mg/L $NaNO_3$, 36 mg/L $Na_4HPO_4.12H_2O$) with 1.0 ug/ml paromomycin. Additional plasmids, promoters, 3'UTR/terminators, and selectable markers suitable for enabling heterologous gene expression in *Gonium pectorale* are further discussed by Lerche and Hallman. Lerche and Hallman reported that the plasmid pPmr3, *Volvox carteri* hsp70A-rbcS3 hybrid promoter, and the 3' UTR/terminator of the *Volvox carteri* rbcS3 gene are suitable to enable exogenous gene expression in *Gonium pectorale*. In addition, Lerche and Hallman reported that the paromomycin resistance cassette encoded pPmr3 was suitable for use as a selectable marker in *Gonium pectorale*.

[0407] In an embodiment of the present invention, vector pPmr3, comprising the nucleotide sequence encoding the *aphVIII* gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected Table 20, each protein-coding sequence codon-optimized for expression in *Gonium pectorale to* reflect the codon bias inherent in nuclear genes of *Gonium pectorale* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *Volvox carteri* hsp70A-rbcS3 hybrid promoter upstream of the protein-coding sequence and operably linked to the *Volvox carteri* rbcS3 gene 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Gonium pectorale* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. Stable transformation of *Gonium pectorale* with the transformation vector can be achieved through well-known transformation techniques including microprojectile bombardment or other known methods. Activity of the *aphVIII* gene product can be used as a selectable marker to select for *Gonium pectorale* transformed with the transformation vector in, but not limited to, Jaworski's medium comprising paromomycin. Growth media suitable for *Gonium pectorale* lipid production include Jaworski's medium and media reported by Stein, American Journal of Botany, Vol. 45:9 (1958), pp. 664-672. Evaluation of fatty acid profiles of *Gonium pectorale lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 21: Engineering *Phaeodactylum tricornutum*

[0408] Expression of recombinant genes in accordance with the present invention in *Phaeodactylum tricornutum* can be accomplished by modifying the methods and vectors taught by Apt *et al.* as discussed herein. Briefly, Apt *et al., Molecular and General Genetics*, Vol. 252 (1996), pp. 572-579, reported the stable nuclear transformation of *Phaeodactylum tricornutum* with vector DNA. Using the transformation technique of microprojectile bombardment, Apt introduced the plasmid pfcpA into *Phaeodactylum tricornutum.* Plasmid pfcpA comprised a bleomycin resistance cassette, comprising sequence encoding the *Streptoalloteichus hindustanus* Bleomycin binding protein (*ble*), for resistance to the antibiotics phleomycin and zeocin, operably linked to the promoter of the *Phaeodactylum tricornutum* fucoxanthin chlorophyll a binding protein gene (fcpA) upstream of the *ble* protein-coding region and operably linked to the 3' UTR/terminator of the *Phaeodactylum tricornutum* fcpA gene at the 3' region, or downstream of the *ble* protein-coding region. Prior to transformation with pfcpA, *Phaeodactylum tricornutum* was unable to propagate on medium comprising 50 ug/ml zeocin. Upon transformation with pfcpA, transformants of *Phaeodactylum tricornutum* were obtained that were propagated in selective culture medium comprising 50 ug/ml zeocin. The expression of the *ble* gene product in *Phaeodactylum tricornutum* enabled propagation in the presence of 50 ug/ml zeocin, thereby establishing the utility of the bleomycin antibiotic resistance cassette as selectable marker for use in *Phaeodactylum tricornutum.* Evaluation of the genomic DNA of the stable transformants was performed by Southern analysis. Apt reported that selection and maintenance of the transformed *Phaeodactylum tricornutum* was performed on agar plates comprising LDM medium (as reported by Starr and Zeikus, *Journal of Phycology*, Vol. 29, Supplement, (1993)) with 50 mg/L zeocin. Apt reported liquid propagation of *Phaeodactylum tricornutum* transformants in LDM medium with 50 mg/L zeocin. Propagation of *Phaeodactylum tricornutum* in medium other than LDM medium has been discussed (by Zaslavskaia *et al., Science*, Vol. 292 (2001), pp. 2073-2075, and by Radokovits *et al., Metabolic Engineering*, Vol. 13 (2011), pp. 89-95). Additional plasmids, promoters, 3'UTR/terminators, and selectable markers suitable for enabling heterologous gene expression in *Phaeodactylum tricornutum* have been reported in the same report by Apt *et al.,* by Zaslavskaia *et al.,* and by Radokovits *et al.*). Apt reported that the plasmid pfcpA, and the *Phaeodactylum tricornutum* fcpA promoter and 3' UTR/terminator are suitable to enable exogenous gene expression in *Phaeodactylum tricornutum.* In addition, Apt reported that the bleomycin resistance cassette encoded on pfcpA was suitable for use as a selectable marker in *Phaeodactylum tricornutum.*

[0409] In an embodiment of the present invention, vector pfcpA, comprising the nucleotide sequence encoding the *ble* gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected Table 20, each protein-coding sequence codon-optimized for expression in *Phaeodactylum tricornutum to* reflect the codon bias inherent in nuclear genes of *Phaeodactylum tricornutum* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *Phaeodactylum tricornutum* fcpA gene promoter upstream of the protein-coding sequence and operably linked to the *Phaeodactylum tricornutum* fcpA gene 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Phaeodactylum tricornutum* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. One skilled in the art can identify such homology regions within the sequence of the *Phaeodactylum tricornutum* genome (referenced in the publication by Bowler et al., *Nature*, Vol. 456 (2008), pp. 239-244). Stable transformation of *Phaeodactylum tricornutum* with the transformation vector is achieved through well-known transformation techniques including microprojectile bombardment or other known methods. Activity of the *ble* gene product can be used as a marker to select for *Phaeodactylum tricornutum* transformed with the transformation vector in, but not limited to, LDM medium comprising paromomycin. Growth medium suitable for *Phaeodactylum tricornutum* lipid production include, but are not limited to f/2 medium as reported by Radokovits *et al.* Evaluation of fatty acid profiles of *Phaeodactylum tricornutum lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 22: Engineering *Chaetoceros sp.*

[0410] Expression of recombinant genes in accordance with the present invention in *Chaetoceros sp.* can be accomplished by modifying the methods and vectors taught by Yamaguchi *et al.* as discussed herein. Briefly, Yamaguchi *et al.*, Phycological Research, Vol. 59:2 (2011), pp.113-119, reported the stable nuclear transformation of *Chaetoceros sp.* with plasmid DNA. Using the transformation method of microprojectile bombardment, Yamaguchi introduced the plasmid pTpfcp/nat into *Chaetoceros sp.* pTpfcp/nat comprised a

nourseothricin resistance cassette, comprising sequence encoding the nourseothricin acetyltransferase (*nat*) gene product (GenBank Accession No. AAC60439) operably linked to the *Thalassiosira pseudonana* fucoxanthin chlorophyll a/c binding protein gene (fcp) promoter upstream of the *nat* protein-coding region and operably linked to the *Thalassiosira pseudonana* fcp gene 3' UTR/ terminator at the 3' region (downstream of the *nat* protein coding-sequence). The *nat* gene product confers resistance to the antibiotic nourseothricin. Prior to transformation with pTpfcp/nat, *Chaetoceros sp.* was unable to propagate on medium comprising 500 ug/ml nourseothricin. Upon transformation with pTpfcp/nat, transformants of *Chaetoceros sp.* were obtained that were propagated in selective culture medium comprising 500 ug/ml nourseothricin. The expression of the *nat* gene product in *Chaetoceros sp.* enabled propagation in the presence of 500 ug/ml nourseothricin, thereby establishing the utility of the nourseothricin antibiotic resistance cassette as selectable marker for use in *Chaetoceros sp.* Evaluation of the genomic DNA of the stable transformants was performed by Southern analysis. Yamaguchi reported that selection and maintenance of the transformed *Chaetoceros sp.* was performed on agar plates comprising f/2 medium (as reported by Guilard, R.R., Culture of Phytoplankton for feeding marine invertebrates, *In* Culture of Marine Invertebrate Animals, Smith and Chanley (eds) 1975, Plenum Press, New York, pp. 26-60) with 500 ug/ml nourseothricin. Liquid propagation of *Chaetoceros* sp. transformants, as performed by Yamaguchi, was carried out in f/2 medium with 500 mg/L nourseothricin. Propagation of *Chaetoceros sp.* in additional culture medium has been reported (for example in Napolitano *et al.*, *Journal of the World Aquaculture Society*, Vol. 21:2 (1990), pp. 122-130, and by Volkman *et al.*, *Journal of Experimental Marine Biology and Ecology*, Vol. 128:3 (1989), pp. 219-240). Additional plasmids, promoters, 3'UTR/terminators, and selectable markers suitable for enabling heterologous gene expression in *Chaetoceros sp.* have been reported in the same report by Yamaguchi *et al.* Yamaguchi reported that the plasmid pTpfcp/nat, and the *Thalassiosira pseudonana* fcp promoter and 3' UTR/terminator are suitable to enable exogenous gene expression in *Chaetoceros sp.* In addition, Yamaguchi reported that the nourseothricin resistance cassette encoded on pTpfcp/nat was suitable for use as a selectable marker in *Chaetoceros sp.*

[0411] In an embodiment of the present invention, vector pTpfcp/nat, comprising the nucleotide sequence encoding the *nat* gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected from

Table 20, each protein-coding sequence codon-optimized for expression in the closely-related *Chaetoceros compressum to* reflect the codon bias inherent in nuclear genes of *Chaetoceros compressum* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *Thalassiosira pseudonana* fcp gene promoter upstream of the protein-coding sequence and operably linked to the *Thalassiosira pseudonana* fcp gene 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Chaetoceros sp.* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. Stable transformation of *Chaetoceros sp.* with the transformation vector is achieved through well-known transformation including microprojectile bombardment or other known methods. Activity of the *nat* gene product can be used as a selectable marker to select for *Chaetoceros sp.* transformed with the transformation vector in, but not limited to, f/2 agar medium comprising nourseothricin. Growth medium suitable for *Chaetoceros sp.* lipid production include, but are not limited to, f/2 medium, and those culture media discussed by Napolitano *et al.* and Volkman *et al.* Evaluation of fatty acid profiles of *Chaetoceros sp lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 23: Engineering *Cylindrotheca fusiformis*

[0412] Expression of recombinant genes in accordance with the present invention in *Cylindrotheca fusiformis* can be accomplished by modifying the methods and vectors taught by Poulsen and Kroger *et al.* as discussed herein. Briefly, Poulsen and Kroger *et al.*, *FEBS Journal*, Vol. 272 (2005), pp.3413-3423, reported the transformation of *Cylindrotheca fusiformis* with plasmid DNA. Using the transformation method of microprojectile bombardment, Poulsen and Kroger introduced the pCF-ble plasmid into *Cylindrotheca fusiformis.* Plasmid pCF-ble comprised a bleomycin resistance cassette, comprising sequence encoding the *Streptoalloteichus hindustanus* Bleomycin binding protein (*ble*), for resistance to the antibiotics zeocin and phleomycin, operably linked to the *Cylindrotheca fusiformis* fucozanthin chlorophyll a/c binding protein gene (fcpA, GenBank Accession No. AY125580) promoter upstream of the *ble* protein-coding region and operably linked to the *Cylindrotheca fusiformis* fcpA gene 3'UTR/terminator at the 3' region (down-stream of the *ble* protein-coding region). Prior to transformation with pCF-ble, *Cylindrotheca fusiformis* was unable to propagate on medium comprising 1 mg/ml zeocin. Upon transformation with pCF-ble,

transformants of *Cylindrotheca fusiformis* were obtained that were propagated in selective culture medium comprising 1 mg/ml zeocin. The expression of the *ble* gene product in *Cylindrotheca fusiformis* enabled propagation in the presence of 1 mg/ml zeocin, thereby establishing the utility of the bleomycin antibiotic resistance cassette as selectable marker for use in *Cylindrotheca fusiformis*. Poulsen and Kroger reported that selection and maintenance of the transformed *Cylindrotheca fusiformis* was performed on agar plates comprising artificial seawater medium with 1 mg/ml zeocin. Poulsen and Kroger reported liquid propagation of *Cylindrotheca fusiformis* transformants in artificial seawater medium with 1 mg/ml zeocin. Propagation of *Cylindrotheca fusiformis* in additional culture medium has been discussed (for example in Liang *et al.*, *Journal of Applied Phycology*, Vol. 17:1 (2005), pp. 61-65, and by Orcutt and Patterson, *Lipids*, Vol. 9:12 (1974), pp. 1000-1003). Additional plasmids, promoters, and 3'UTR/terminators for enabling heterologous gene expression in *Chaetoceros sp.* have been reported in the same report by Poulsen and Kroger. Poulsen and Kroger reported that the plasmid pCF-ble and the *Cylindrotheca fusiformis* fcp promoter and 3' UTR/terminator are suitable to enable exogenous gene expression in *Cylindrotheca fusiformis*. In addition, Poulsen and Kroger reported that the bleomycin resistance cassette encoded on pCF-ble was suitable for use as a selectable marker in *Cylindrotheca fusiformis*.

[0413] In an embodiment of the present invention, vector pCF-ble, comprising the nucleotide sequence encoding the *ble* gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected Table 20, each protein-coding sequence codon-optimized for expression in *Cylindrotheca fusiformis to* reflect the codon bias inherent in nuclear genes of *Cylindrotheca fusiformis* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *Cylindrotheca fusiformis* fcp gene promoter upstream of the protein-coding sequence and operably linked to the *Cylindrotheca fusiformis* fcp gene 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Cylindrotheca fusiformis* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. Stable transformation of *Cylindrotheca fusiformis* with the transformation vector is achieved through well-known transformation techniques including microprojectile bombardment or other known methods.

Activity of the *ble* gene product can be used as a selectable marker to select for *Cylindrotheca fusiformis* transformed with the transformation vector in, but not limited to, artificial seawater agar medium comprising zeocin. Growth media suitable for *Cylindrotheca fusiformis* lipid production include, but are not limited to, artificial seawater and those media reported by Liang et al. and Orcutt and Patterson. Evaluation of fatty acid profiles of *Cylindrotheca fusiformis lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 24: Engineering *Amphidinium sp.*

[0414] Expression of recombinant genes in accordance with the present invention in *Amphidinium sp.* can be accomplished by modifying the methods and vectors taught by ten Lohuis and Miller *et al.* as discussed herein. Briefly, ten Lohuis and Miller *et al.*, *The Plant Journal*, Vol. 13:3 (1998), pp. 427-435, reported the stable transformation of *Amphidinium sp.* with plasmid DNA. Using the transformation technique of agitation in the presence of silicon carbide whiskers, ten Lohuis introduced the plasmid pMT NPT/GUS into *Amphidinium sp.* pMT NPT/GUS comprised a neomycin resistance cassette, comprising sequence encoding the neomycin phosphotransferase II (*nptII*) gene product (GenBank Accession No. AAL92039) operably linked to the *Agrobacterium tumefaciens* nopaline synthase (*nos*) gene promoter upstream, or 5' of the *nptII* protein-coding region and operably linked to the 3' UTR/terminator of the *nos* gene at the 3' region (down-stream of the *nptII* protein-coding region). The *nptII* gene product confers resistance to the antibiotic G418. The pMT NPT/GUS plasmid further comprised sequence encoding a beta-glucuronidase (GUS) reporter gene product operably-linked to a CaMV 35S promoter and further operably linked to the CaMV 35S 3' UTR/terminator. Prior to transformation with pMT NPT/GUS, *Amphidinium sp.* was unable to be propagated on medium comprising 3 mg/ml G418. Upon transformation with pMT NPT/GUS, transformants of *Amphidinium sp.* were obtained that were propagated in selective culture medium comprising 3 mg/ml G418. The expression of the *nptII* gene product in *Amphidinium sp.* enabled propagation in the presence of 3 mg/ml G418, thereby establishing the utility of the neomycin antibiotic resistance cassette as selectable marker for use in *Amphidinium sp.* Detectable activity of the GUS reporter gene indicated that CaMV 35S promoter and 3'UTR are suitable for enabling gene expression in *Amphidinium sp.* Evaluation of the genomic DNA of the stable transformants was performed by Southern analysis. ten Lohuis and Miller reported liquid propagation of *Amphidinium sp* transformants in medium comprising seawater supplemented with F/2 enrichment solution

(provided by the supplier Sigma) and 3 mg/ml G418 as well as selection and maintenance of *Amphidinium sp.* transformants on agar medium comprising seawater supplemented with F/2 enrichment solution and 3 mg/ml G418. Propagation of *Amphidinium sp.* in additional culture medium has been reported (for example in Mansour *et al.*, *Journal of Applied Phycology*, Vol. 17:4 (2005) pp. 287-v300). An additional plasmid, comprising additional promoters, 3'UTR/terminators, and a selectable marker for enabling heterologous gene expression in *Amphidinium sp.* have been reported in the same report by ten Lohuis and Miller. ten Lohuis and Miller reported that the plasmid pMT NPT/GUS and the promoter and 3' UTR/terminator of the *nos* and CaMV 35S genes are suitable to enable exogenous gene expression in *Amphidinium sp.* In addition, ten Lohuis and Miller reported that the neomycin resistance cassette encoded on pMT NPT/GUS was suitable for use as a selectable marker in *Amphidinium sp.*

[0415] In an embodiment of the present invention, vector pMT NPT/GUS, comprising the nucleotide sequence encoding the *nptII* gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected from Table 20, each protein-coding sequence codon-optimized for expression in *Amphidinium sp. to* reflect the codon bias inherent in nuclear genes of the closely-related species, *Amphidinium carterae* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *Agrobacterium tumefaciens* nopaline synthase (*nos*) gene promoter upstream of the protein-coding sequence and operably linked to the *nos* 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Amphidinium sp.* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. Stable transformation of *Amphidinium sp.* with the transformation vector is achieved through well-known transformation techniques including silicon fibre-mediated microinjection or other known methods. Activity of the *nptII* gene product can be used as a selectable marker to select for *Amphidinium sp.* transformed with the transformation vector in, but not limited to, seawater agar medium comprising G418. Growth media suitable for *Amphidinium sp.* lipid production include, but are not limited to, artificial seawater and those media reported by Mansour *et al.* and ten Lohuis and Miller. Evaluation of fatty acid profiles of *Amphidinium sp. lipids* can be

assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 25: Engineering *Symbiodinium microadriacticum*

[0416] Expression of recombinant genes in accordance with the present invention in *Symbiodinium microadriacticum* can be accomplished by modifying the methods and vectors taught by ten Lohuis and Miller *et al.* as discussed herein. Briefly, ten Lohuis and Miller *et al.*, *The Plant Journal*, Vol. 13:3 (1998), pp. 427-435, reported the stable transformation of *Symbiodinium microadriacticum* with plasmid DNA. Using the transformation technique of silicon fibre-mediated microinjection, ten Lohuis introduced the plasmid pMT NPT/GUS into *Symbiodinium microadriacticum.* pMT NPT/GUS comprised a neomycin resistance cassette, comprising sequence encoding the neomycin phosphotransferase II (*nptII*) gene product (GenBank Accession No. AAL92039) operably linked to the *Agrobacterium tumefaciens* nopaline synthase (*nos*) gene promoter upstream, or 5' of the *nptII* protein-coding region and operably linked to the 3' UTR/terminator of the *nos* gene at the 3' region (down-stream of the *nptII* protein-coding region). The *nptII* gene product confers resistance to the antibiotic G418. The pMT NPT/GUS plasmid further comprised sequence encoding a beta-glucuronidase (GUS) reporter gene product operably-linked to a CaMV 35S promoter and further operably linked to the CaMV 35S 3' UTR/terminator. Prior to transformation with pMT NPT/GUS, *Symbiodinium microadriacticum* was unable to be propagated on medium comprising 3 mg/ml G418. Upon transformation with pMT NPT/GUS, transformants of *Symbiodinium microadriacticum* were obtained that were propagated in selective culture medium comprising 3 mg/ml G418. The expression of the *nptII* gene product in *Symbiodinium microadriacticum* enabled propagation in the presence of 3 mg/ml G418, thereby establishing the utility of the neomycin antibiotic resistance cassette as selectable marker for use in *Symbiodinium microadriacticum.* Detectable activity of the GUS reporter gene indicated that CaMV 35S promoter and 3'UTR are suitable for enabling gene expression in *Symbiodinium microadriacticum.* Evaluation of the genomic DNA of the stable transformants was performed by Southern analysis. ten Lohuis and Miller reported liquid propagation of *Symbiodinium microadriacticum* transformants in medium comprising seawater supplemented with F/2 enrichment solution (provided by the supplier Sigma) and 3 mg/ml G418 as well as selection and maintenance of *Symbiodinium microadriacticum* transformants on agar medium comprising seawater supplemented with F/2 enrichment solution and 3 mg/ml G418. Propagation of *Symbiodinium microadriacticum* in additional culture medium has been discussed (for example in Iglesias-Prieto *et al.*, *Proceedings of the*

National Academy of Sciences, Vol. 89:21 (1992) pp. 10302-10305). An additional plasmid, comprising additional promoters, 3'UTR/terminators, and a selectable marker for enabling heterologous gene expression in *Symbiodinium microadriacticum* have been discussed in the same report by ten Lohuis and Miller. ten Lohuis and Miller reported that the plasmid pMT NPT/GUS and the promoter and 3' UTR/terminator of the *nos* and CaMV 35S genes are suitable to enable exogenous gene expression in *Symbiodinium microadriacticum.* In addition, ten Lohuis and Miller reported that the neomycin resistance cassette encoded on pMT NPT/GUS was suitable for use as a selectable marker in *Symbiodinium microadriacticum.*

[0417] In an embodiment of the present invention, vector pMT NPT/GUS, comprising the nucleotide sequence encoding the *nptII* gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected Table 20, each protein-coding sequence codon-optimized for expression in *Symbiodinium microadriacticum to* reflect the codon bias inherent in nuclear genes of *Symbiodinium microadriacticum* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *Agrobacterium tumefaciens* nopaline synthase (*nos*) gene promoter upstream of the protein-coding sequence and operably linked to the *nos* 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Symbiodinium microadriacticum* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. Stable transformation of *Symbiodinium microadriacticum* with the transformation vector is achieved through well-known transformation techniques including silicon fibre-mediated microinjection or other known methods. Activity of the *nptII* gene product can be used as a selectable marker to select for *Symbiodinium microadriacticum* transformed with the transformation vector in, but not limited to, seawater agar medium comprising G418. Growth media suitable for *Symbiodinium microadriacticum* lipid production include, but are not limited to, artificial seawater and those media reported by Iglesias-Prieto *et al.* and ten Lohuis and Miller. Evaluation of fatty acid profiles of *Symbiodinium microadriacticum lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 26: Engineering *Nannochloropsis sp.*

[0418] Expression of recombinant genes in accordance with the present invention in *Nannochloropsis sp.* W2J3B can be accomplished by modifying the methods and vectors taught by Kilian *et al.* as discussed herein. Briefly, Kilian *et al.*, *Proceedings of the National Academy of Sciences*, Vol. 108:52 (2011) pp.21265-21269, reported the stable nuclear transformation of *Nannochloropsis* with a transformation construct. Using the transformation method of electroporation, Kilian introduced the transformation construct C2 into *Nannochloropsis sp.* W2J3B. The C2 transformation construct comprised a bleomycin resistance cassette, comprising the coding sequence for the *Streptoalloteichus hindustanus* Bleomycin binding protein (*ble*), for resistance to the antibiotics phleomycin and zeocin, operably linked to and the promoter of the *Nannochloropsis sp.* W2J3B violaxanthin/chlorophyll a-binding protein gene VCP2 upstream of the ble protein-coding region and operably linked to the 3'UTR/terminator of the *Nannochloropsis sp.* W2J3B violaxanthin/chlorophyll a-binding gene VCP1 downstream of the ble protein-coding region. Prior to transformation with C2, *Nannochloropsis sp.* W2J3B was unable to propagate on medium comprising 2 ug/ml zeocin. Upon transformation with C2, transformants of *Nannochloropsis sp.* W2J3B were obtained that were propagated in selective culture medium comprising 2 ug/ml zeocin. The expression of the ble gene product in *Nannochloropsis sp.* W2J3B enabled propagation in the presence of 2 ug/ml zeocin, thereby establishing the utility of the bleomycin antibiotic resistance cassette as selectable marker for use in *Nannochloropsis*. Evaluation of the genomic DNA of the stable transformants was performed by PCR. Kilian reported liquid propagation of *Nannochloropsis sp.* W2J3B transformants in F/2 medium (reported by Guilard and Ryther, *Canadian Journal of Microbiology*, Vol. 8 (1962), pp. 229-239) comprising fivefold levels of trace metals, vitamins, and phosphate solution, and further comprising 2 ug/ml zeocin. Kilian also reported selection and maintenance of *Nannochloropsis sp.* W2J3B transformants on agar F/2 medium comprising artificial seawater 2 mg/ml zeocin. Propagation of *Nannochloropsis* in additional culture medium has been discussed (for example in Chiu *et al.*, *Bioresour Technol., Vol. 100:2 (2009), pp. 833-838* and Pal *et al.*, *Applied Microbiology and Biotechnology*, Vol. 90:4 (2011), pp. 1429-1441.). Additional transformation constructs, comprising additional promoters and 3'UTR/terminators for enabling heterologous gene expression in *Nannochloropsis sp.* W2J3B and selectable markers for selection of transformants have been described in the same report by Kilian. Kilian reported that the transformation construct C2 and the promoter of the *Nannochloropsis sp.* W2J3B

violaxanthin/chlorophyll a-binding protein gene VCP2 and 3' UTR/terminator of the *Nannochloropsis sp.* W2J3B violaxanthin/chlorophyll a-binding protein gene VCP1 are suitable to enable exogenous gene expression in *Nannochloropsis sp.* W2J3B. In addition, Kilian reported that the bleomycin resistance cassette encoded on C2 was suitable for use as a selectable marker in *Nannochloropsis sp.* W2J3B.

[0419] In an embodiment of the present invention, transformation construct C2, comprising the nucleotide sequence encoding the *ble* gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected from Table 20, each protein-coding sequence codon-optimized for expression in *Nannochloropsis sp.* W2J3B *to* reflect the codon bias inherent in nuclear genes of *Nannochloropsis sp.* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *Nannochloropsis sp.* W2J3B VCP2 gene promoter upstream of the protein-coding sequence and operably linked to the *Nannochloropsis sp.* W2J3B VCP1 gene 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Nannochloropsis sp.* W2J3B genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. Stable transformation of *Nannochloropsis sp.* W2J3B with the transformation vector is achieved through well-known transformation techniques including electroporation or other known methods. Activity of the *ble* gene product can be used as a selectable marker to select for *Nannochloropsis sp.* W2J3B transformed with the transformation vector in, but not limited to, F/2 medium comprising zeocin. Growth media suitable for *Nannochloropsis sp.* W2J3B lipid production include, but are not limited to, F/2 medium and those media reported by Chiu et al. and Pal et al. Evaluation of fatty acid profiles of *Nannochloropsis sp.* W2J3B *lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 27: Engineering *Cyclotella cryptica*

[0420] Expression of recombinant genes in accordance with the present invention in *Cyclotella cryptica* can be accomplished by modifying the methods and vectors taught by Dunahay *et al.* as discussed herein. Briefly, Dunahay *et al., Journal of Phycology*, Vol. 31

(1995), pp. 1004-1012, reported the stable transformation of *Cyclotella cryptica* with plasmid DNA. Using the transformation method of microprojectile bombardment, Dunahay introduced the plasmid pACCNPT5.1 into *Cyclotella cryptica.* Plasmid pACCNPT5.1 comprised a neomycin resistance cassette, comprising the coding sequence of the neomycin phosphotransferase II (*nptII*) gene product operably linked to the promoter of the *Cyclotella cryptica* acetyl-CoA carboxylase (ACCase) gene (GenBank Accession No. L20784) upstream of the *nptII* coding-region and operably linked to the 3'UTR/terminator of the *Cyclotella cryptica* ACCase gene at the 3' region (downstream of the *nptII* coding-region). The *nptII* gene product confers resistance to the antibiotic G418. Prior to transformation with pACCNPT5.1, *Cyclotella cryptica* was unable to propagate on 50% artificial seawater medium comprising 100 ug/ml G418. Upon transformation with pACCNPT5.1, transformants of *Cyclotella cryptica* were obtained that were propagated in selective 50% artificial seawater medium comprising 100 ug/ml G418. The expression of the *nptII* gene product in *Cyclotella cryptica* enabled propagation in the presence of 100 ug/ml G418, thereby establishing the utility of the neomycin antibiotic resistance cassette as selectable marker for use in *Cyclotella cryptica.* Evaluation of the genomic DNA of the stable transformants was performed by Southern analysis. Dunahay reported liquid propagation of *Cyclotella cryptica* in artificial seawater medium (ASW, as discussed by Brown, L., *Phycologia*, Vol. 21 (1982), pp. 408-410) supplemented with 1.07 mM sodium silicate and with 100 ug/ml G418. Dunahay also reported selection and maintenance of *Cyclotella cryptica* transformants on agar plates comprising ASW medium with 100 ug/ml G418. Propagation of *Cyclotella cryptica* in additional culture medium has been discussed (for example in Sriharan *et al.*, *Applied Biochemistry and Biotechnology,* Vol. 28-29:1 (1991), *pp.* 317-326 and Pahl *et al.*, *Journal of Bioscience and Bioengineering*, Vol. 109:3 (2010), pp. 235–239). Dunahay reported that the plasmid pACCNPT5.1 and the promoter of the *Cyclotella cryptica* acetyl-CoA carboxylase (ACCase) gene are suitable to enable exogenous gene expression in *Cyclotella cryptica.* In addition, Dunahay reported that the neomycin resistance cassette encoded on pACCNPT5.1 was suitable for use as a selectable marker in *Cyclotella cryptica.*

[0421] In an embodiment of the present invention, vector pACCNPT5.1, comprising the nucleotide sequence encoding the *nptII* gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected from

Table 20, each protein-coding sequence codon-optimized for expression in *Cyclotella cryptica to* reflect the codon bias inherent in nuclear genes of *Cyclotella cryptica* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *Cyclotella cryptica* ACCase promoter upstream of the protein-coding sequence and operably linked to the *Cyclotella cryptica* ACCase 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Cyclotella cryptica* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. Stable transformation of *Cyclotella cryptica* with the transformation vector is achieved through well-known transformation techniques including microprojectile bombardment or other known methods. Activity of the *nptII* gene product can be used as a marker to select for *Cyclotella cryptica* transformed with the transformation vector in, but not limited to, agar ASW medium comprising G418. Growth media suitable for *Cyclotella cryptica* lipid production include, but are not limited to, ASW medium and those media reported by Sriharan *et al.*, 1991 and Pahl *et al.* Evaluation of fatty acid profiles of *Cyclotella cryptica lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 28: Engineering *Navicula saprophila*

[0422] Expression of recombinant genes in accordance with the present invention in *Navicula saprophila* can be accomplished by modifying the methods and vectors taught by Dunahay *et al.* as discussed herein. Briefly, Dunahay *et al.*, *Journal of Phycology*, Vol. 31 (1995), pp. 1004-1012, reported the stable transformation of *Navicula saprophila* with plasmid DNA. Using the transformation method of microprojectile bombardment, Dunahay introduced the plasmid pACCNPT5.1 into *Navicula saprophila.* Plasmid pACCNPT5.1 comprised a neomycin resistance cassette, comprising the coding sequence of the neomycin phosphotransferase II (*nptII*) gene product operably linked to the promoter of the *Cyclotella cryptica* acetyl-CoA carboxylase (ACCase) gene (GenBank Accession No. L20784) upstream of the *nptII* coding-region and operably linked to the 3'UTR/terminator of the *Cyclotella cryptica* ACCase gene at the 3' region (downstream of the *nptII* coding-region). The *nptII* gene product confers resistance to the antibiotic G418. Prior to transformation with pACCNPT5.1, *Navicula saprophila* was unable to propagate on artificial seawater medium comprising 100 ug/ml G418. Upon transformation with pACCNPT5.1, transformants of

Navicula saprophila were obtained that were propagated in selective artificial seawater medium comprising 100 ug/ml G418. The expression of the *nptII* gene product in *Navicula saprophila* enabled propagation in the presence of G418, thereby establishing the utility of the neomycin antibiotic resistance cassette as selectable marker for use in *Navicula saprophila*. Evaluation of the genomic DNA of the stable transformants was performed by Southern analysis. Dunahay reported liquid propagation of *Navicula saprophila* in artificial seawater medium (ASW, as discussed by Brown, L., *Phycologia*, Vol. 21 (1982), pp. 408-410) supplemented with 1.07 mM sodium silicate and with 100 ug/ml G418. Dunahay also reported selection and maintenance of *Navicula saprophila* transformants on agar plates comprising ASW medium with 100 ug/ml G418. Propagation of *Navicula saprophila* in additional culture medium has been discussed (for example in Tadros and Johansen, *Journal of Phycology*, Vol. 24:4 (1988), pp. 445-452 and Sriharan *et al.*, *Applied Biochemistry and Biotechnology,* Vol. 20-21:1 (1989), *pp.* 281-291). Dunahay reported that the plasmid pACCNPT5.1 and the promoter of the *Cyclotella cryptica* acetyl-CoA carboxylase (ACCase) gene are suitable to enable exogenous gene expression in *Navicula saprophila*. In addition, Dunahay reported that the neomycin resistance cassette encoded on pACCNPT5.1 was suitable for use as a selectable marker in *Navicula saprophila*.

[0423] In an embodiment of the present invention, vector pACCNPT5.1, comprising the nucleotide sequence encoding the *nptII* gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected from Table 20, each protein-coding sequence codon-optimized for expression in *Navicula saprophila to* reflect the codon bias inherent in nuclear genes of *the closely-related Navicula pelliculosa* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *Cyclotella cryptica* ACCase gene promoter upstream of the protein-coding sequence and operably linked to the *Cyclotella cryptica* ACCase gene 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Navicula saprophila* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. Stable transformation of *Navicula saprophila* with the transformation vector is achieved through well-known transformation techniques including microprojectile bombardment or other known methods.

Activity of the *nptII* gene product can be used as a selectable marker to select for *Navicula saprophila* transformed with the transformation vector in, but not limited to, agar ASW medium comprising G418. Growth media suitable for *Navicula saprophila* lipid production include, but are not limited to, ASW medium and those media reported by Sriharan *et al.* 1989 and Tadros and Johansen. Evaluation of fatty acid profiles of *Navicula saprophila lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 29: Engineering *Thalassiosira pseudonana*

[0424] Expression of recombinant genes in accordance with the present invention in *Thalassiosira pseudonana* can be accomplished by modifying the methods and vectors taught by Poulsen *et al.* as discussed herein. Briefly, Poulsen *et al.*, *Journal of Phycology*, Vol. 42 (2006), pp. 1059-1065, reported the stable transformation of *Thalassiosira pseudonana* with plasmid DNA. Using the transformation method of microprojectile bombardment, Poulsen introduced the plasmid pTpfcp/nat in to *Thalassiosira pseudonana*. pTpfcp/nat comprised a nourseothricin resistance cassette, comprising sequence encoding the nourseothricin acetyltransferase (*nat*) gene product (GenBank Accession No. AAC60439) operably linked to the *Thalassiosira pseudonana* fucoxanthin chlorophyll a/c binding protein gene (fcp) promoter upstream of the *nat* protein-coding region and operably linked to the *Thalassiosira pseudonana* fcp gene 3' UTR/ terminator at the 3' region (downstream of the *nat* protein coding-sequence). The *nat* gene product confers resistance to the antibiotic nourseothricin. Prior to transformation with pTpfcp/nat, *Thalassiosira pseudonana* was unable to propagate on medium comprising 10 ug/ml nourseothricin. Upon transformation with pTpfcp/nat, transformants of *Thalassiosira pseudonana* were obtained that were propagated in selective culture medium comprising 100 ug/ml nourseothricin. The expression of the *nat* gene product in *Thalassiosira pseudonana* enabled propagation in the presence of 100 ug/ml nourseothricin, thereby establishing the utility of the nourseothricin antibiotic resistance cassette as selectable marker for use in *Thalassiosira pseudonana*. Evaluation of the genomic DNA of the stable transformants was performed by Southern analysis. Poulsen reported that selection and maintenance of the transformed *Thalassiosira pseudonana* was performed in liquid culture comprising modified ESAW medium (as discussed by Harrison *et al.*, *Journal of Phycology*, Vol. 16 (1980), pp. 28-35) with 100 ug/ml nourseothricin. Propagation of *Thalassiosira pseudonana* in additional culture medium has been discussed (for example in Volkman *et al.*, *Journal of Experimental Marine Biology and Ecology*, Vol. 128:3 (1989), pp.

219-240). An additional plasmid, comprising additional selectable markers suitable for use in *Thalassiosira pseudonana* has been discussed in the same report by Poulsen. Poulsen reported that the plasmid pTpfcp/nat, and the *Thalassiosira pseudonana* fcp promoter and 3' UTR/terminator are suitable to enable exogenous gene expression in *Thalassiosira pseudonana*. In addition, Poulsen reported that the nourseothricin resistance cassette encoded on pTpfcp/nat was suitable for use as a selectable marker in *Thalassiosira pseudonana*.

[0425] In an embodiment of the present invention, vector pTpfcp/nat, comprising the nucleotide sequence encoding the *nat* gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected from Table 20, each protein-coding sequence codon-optimized for expression in *Thalassiosira pseudonana to* reflect the codon bias inherent in nuclear genes of *Thalassiosira pseudonana* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *Thalassiosira pseudonana* fcp gene promoter upstream of the protein-coding sequence and operably linked to the *Thalassiosira pseudonana* fcp gene 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Thalassiosira pseudonana* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. One skilled in the art can identify such homology regions within the sequence of the *Thalassiosira pseudonana* genome (referenced in the publication by Armbrust *et al.*, *Science*, Vol. 306: 5693 (2004): pp. 79-86). Stable transformation of *Thalassiosira pseudonana* with the transformation vector is achieved through well-known transformation techniques including microprojectile bombardment or other known methods. Activity of the *nat* gene product can be used as a marker to select for *Thalassiosira pseudonana* transformed with the transformation vector in but not limited to, ESAW agar medium comprising nourseothricin. Growth media suitable for *Thalassiosira pseudonana* lipid production include, but are not limited to, ESAW medium, and those culture media discussed by Volkman *et al.* and Harrison *et al.* Evaluation of fatty acid profiles of *Thalassiosira pseudonana lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 30: Engineering *Chlamydomonas reinhardtii*

[0426] Expression of recombinant genes in accordance with the present invention in *Chlamydomonas reinhardtii* can be accomplished by modifying the methods and vectors taught by Cerutti *et al.* as discussed herein. Briefly, Cerutti *et al.*, *Genetics,* Vol. 145:1 (1997), pp. 97–110, reported the stable nuclear transformation of *Chlamydomonas reinhardtii* with a transformation vector. Using the transformation method of microprojectile bombardment, Cerutti introduced transformation construct P[1030] into *Chlamydomonas reinhardtii*. Construct P[1030] comprised a spectinomycin resistance cassette, comprising sequence encoding the aminoglucoside 3''-adenyltransferase (*aadA*) gene product operably linked to the *Chlamydomonas reinhardtii* ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit gene (*RbcS2*, GenBank Accession No. X04472) promoter upstream of the *aadA* protein-coding region and operably linked to the *Chlamydomonas reinhardtii RbcS2* gene 3' UTR/ terminator at the 3' region (downstream of the *aadA* protein coding-sequence). The *aadA* gene product confers resistance to the antibiotic spectinomycin. Prior to transformation with P[1030], *Chlamydomonas reinhardtii* was unable to propagate on medium comprising 90 ug/ml spectinomycin. Upon transformation with P[1030], transformants of *Chlamydomonas reinhardtii* were obtained that were propagated in selective culture medium comprising 90 ug/ml spectinomycin, thereby establishing the utility of the spectinomycin antibiotic resistance cassette as a selectable marker for use in *Chlamydomonas reinhardtii*. Evaluation of the genomic DNA of the stable transformants was performed by Southern analysis. Cerutti reported that selection and maintenance of the transformed *Chlamydomonas reinhardtii* was performed on agar plates comprising Tris-acetate-phosphate medium (TAP, as described by Harris, *The Chlamydomonas Sourcebook*, Academic Press, San Diego, 1989) with 90 ug/ml spectinomycin. Cerutti additionally reported propagation of *Chlamydomonas reinhardtii* in TAP liquid culture with 90 ug/ml spectinomycin. Propagation of *Chlamydomonas reinhardtii* in alternative culture medium has been discussed (for example in Dent *et al.*, *African Journal of Microbiology Research*, Vol. 5:3 (2011), pp. 260-270 and Yantao et al., *Biotechnology and Bioengineering*, Vol. 107:2 (2010), pp. 258-268). Additional constructs, comprising additional selectable markers suitable for use in *Chlamydomonas reinhardtii as well as numerous regulatory sequences, including promoters and 3' UTRs suitable for promoting heterologous gene expression in Chlamydomonas reinhardtii* are known in the art and have been discussed (for a review, see Radakovits *et al., Eukaryotic Cell*, Vol. 9:4 (2010), pp. 486-501). Cerutti reported that the transformation vector P[1030] and the *Chlamydomonas reinhardtii* promoter and 3' UTR/terminator are suitable to enable exogenous gene expression in *Chlamydomonas reinhardtii*. In addition,

Cerutti reported that the spectinomycin resistance cassette encoded on P[1030] was suitable for use as a selectable marker in *Chlamydomonas reinhardtii*.

[0427] In an embodiment of the present invention, vector P[1030], comprising the nucleotide sequence encoding the *aadA* gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected from Table 20, each protein-coding sequence codon-optimized for expression in *Chlamydomonas reinhardtii to* reflect the codon bias inherent in nuclear genes of *Chlamydomonas reinhardtii* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *Chlamydomonas reinhardtii RbcS*2 promoter upstream of the protein-coding sequence and operably linked to the *Chlamydomonas reinhardtii RbcS*2 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Chlamydomonas reinhardtii* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic site of an endogenous lipid biosynthesis pathway gene. One skilled in the art can identify such homology regions within the sequence of the *Chlamydomonas reinhardtii* genome (referenced in the publication by Merchant et al., *Science*, Vol. 318:5848 (2007), pp. *245-250*). Stable transformation of *Chlamydomonas reinhardtii* with the transformation vector is achieved through well-known transformation techniques including microprojectile bombardment or other known methods. Activity of the *aadA* gene product can be used as a marker to select for *Chlamydomonas reinhardtii* transformed with the transformation vector on, but not limited to, TAP agar medium comprising spectinomycin. Growth media suitable for *Chlamydomonas reinhardtii* lipid production include, but are not limited to, ESAW medium, and those culture media discussed by Yantao *et al.* and Dent *et al.* Evaluation of fatty acid profiles of *Chlamydomonas reinhardtii lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 31: Engineering *Yarrowia lipolytica*

[0428] Expression of recombinant genes in accordance with the present invention in *Yarrowia lipolytica* can be accomplished by modifying the methods and vectors taught by Fickers et al. as discussed herein. Briefly, Fickers *et al., Journal of Microbiological*

Methods, Vol. 55 (2003), pp. 727-737, reported the stable nuclear transformation of *Yarrowia lipolytica* with plasmid DNA. Using a lithium acetate transformation method, Fickers introduced the plasmid JMP123 into *Yarrowia lipolytica*. Plasmid JMP123 comprised a hygromycin B resistance cassette, comprising sequence encoding the hygromycin B phosphotransferase gene product (*hph*), operably-linked to the *Yarrowia lipolytica LIP2 gene promoter (GenBank Accession No. AJ012632) upstream of the hph protein-coding region and operably linked to the Yarrowia lipolytica LIP2 gene* 3'UTR/terminator downstream of the *hph protein-coding region. Prior to transformation with JMP123, Yarrowia lipolytica were unable to propagate on medium comprising 100 ug/ml hygromycin. Upon transformation with JMP123, transformed Yarrowia lipolytica were obtained that were able to propagate on medium comprising 100 ug/ml hygromycin, thereby establishing the* hygromycin B antibiotic resistance cassette as a selectable marker for use in *Yarrowia lipolytica. The nucleotide sequence provided on JMP123 of the promoter and 3'UTR/terminator of the Yarrowia lipolytica LIP2 gene served as donor sequences for homologous recombination of the hph coding sequence into the LIP2 locus.* Evaluation of the genomic DNA of the stable transformants was performed by Southern. Fickers reported that selection and maintenance of the transformed *Yarrowia lipolytica was performed on agar plates comprising standard YPD medium (*Yeast Extract Peptone Dextrose) with 100 ug/ml hygromycin. Liquid culturing of transformed *Yarrowia lipolytica was performed in YPD medium with hygromycin.* Other media and techniques used for culturing *Yarrowia lipolytica have been reported* and numerous other plasmids, promoters, 3' UTRs, and selectable markers for use in *Yarrowia lipolytica have been reported (for example see* Pignede *et al., Applied and Environmental Biology*, Vol. 66:8 (2000), pp. 3283-3289, Chuang *et al., New Biotechnology*, Vol. 27:4 (2010), pp. 277-282, and Barth and Gaillardin, (1996), In: K,W. (Ed.), Nonconventional Yeasts in Biotechnology. Sprinter-Verlag, Berlin-Heidelber, pp. 313-388). Fickers reported that the transformation vector JMP123 and the *Yarrowia lipolytica LIP2 gene* promoter and 3' UTR/terminator are suitable to enable heterologous gene expression in *Yarrowia lipolytica*. In addition, Fickers reported that the hygromycin resistance cassette encoded on JMP123 was suitable for use as a selectable marker in *Yarrowia lipolytica.*

[0429] In an embodiment of the present invention, vector JMP123, comprising the nucleotide sequence encoding the *hph* gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected from

Table 20, each protein-coding sequence codon-optimized for expression in *Yarrowia lipolytica to* reflect the codon bias inherent in nuclear genes of *Yarrowia lipolytica* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *Yarrowia lipolytica LIP2 gene* promoter upstream of the protein-coding sequence and operably linked to the *Yarrowia lipolytica LIP2 gene* 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Yarrowia lipolytica* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. One skilled in the art can identify such homology regions within the sequence of the *Yarrowia lipolytica* genome (referenced in the publication by Dujun *et al.*, *Nature*, Vol. 430 (2004), pp. *35-44*). Stable transformation of *Yarrowia lipolytica* with the transformation vector is achieved through well-known transformation techniques including lithium acetate transformation or other known methods. Activity of the *hph* gene product can be used as a marker to select for *Yarrowia lipolytica* transformed with the transformation vector on, but not limited to, *YPD medium* comprising hygromycin. Growth media suitable for *Yarrowia lipolytica* lipid production include, but are not limited to, *YPD* medium, and those culture media described by Chuang *et al.* Evaluation of fatty acid profiles of *Yarrowia lipolytica lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 32: Engineering *Mortierella alpine*

[0430] Expression of recombinant genes in accordance with the present invention in *Mortierella alpine* can be accomplished by modifying the methods and vectors taught by Mackenzie *et al.* as discussed herein. Briefly, Mackenzie *et al.*, *Applied and Environmental Microbiology*, Vol. 66 (2000), pp. 4655-4661, reported the stable nuclear transformation of *Mortierella alpina* with plasmid DNA. Using a protoplast transformation method, MacKenzie introduced the plasmid pD4 into *Mortierella alpina*. Plasmid pD4 comprised a hygromycin B resistance cassette, comprising sequence encoding the hygromycin B phosphotransferase gene product (*hpt*), operably-linked to the *Mortierella alpina histone H4.1 gene promoter (GenBank Accession No. AJ249812) upstream of the hpt protein-coding region and operably linked to the Aspergillus nidulans N-(5'-phophoribosyl)anthranilate isomerase (trpC) gene* 3'UTR/terminator downstream of the *hpt protein-coding region. Prior to transformation with pD4, Mortierella alpina were unable to propagate on medium*

comprising 300 ug/ml hygromycin. Upon transformation with pD4, transformed Mortierella alpina were obtained that were propagated on medium comprising 300 ug/ml hygromycin, thereby establishing the hygromycin B antibiotic resistance cassette as a selectable marker for use in *Mortierella alpina.* Evaluation of the genomic DNA of the stable transformants was performed by Southern. Mackenzie reported that selection and maintenance of the transformed *Mortierella alpina was performed on PDA (potato dextrose agar) medium comprising hygromycin.* Liquid culturing of transformed *Mortierella alpina by Mackenzie was performed in PDA medium or in S2GYE medium (comprising* 5% glucose, 0.5% yeast extract, 0.18% NH_4SO_4, 0.02% $MgSO_4$-$7H_2O$, 0.0001% $FeCl_3$- $6H_2O$, 0.1%, trace elements, 10 mM K_2HPO_4–NaH_2PO_4), *with hygromycin.* Other media and techniques used for culturing *Mortierella alpina have been reported* and other plasmids, promoters, 3' UTRs, and selectable markers for use in *Mortierella alpina have been reported (for example see* Ando *et al., Applied and Environmental Biology*, Vol. 75:17 (2009) pp. 5529-35 and Lu *et al., Applied Biochemistry and Biotechnology*, Vol. 164:7 (2001), pp. 979-90). Mackenzie reported that the transformation vector pD4 and the *Mortierella alpina histone H4.1* promoter and *A. nidulans trpC gene* 3' UTR/terminator are suitable to enable heterologous gene expression in *Mortierella alpina.* In addition, Mackenzie reported that the hygromycin resistance cassette encoded on pD4 was suitable for use as a selectable marker in *Mortierella alpina.*

[0431] In an embodiment of the present invention, vector pD4, comprising the nucleotide sequence encoding the *hpt* gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected from Table 20, each protein-coding sequence codon-optimized for expression in *Mortierella alpina to* reflect the codon bias inherent in nuclear genes of *Mortierella alpina* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the *Mortierella alpina histone H4.1 gene promoter* upstream of the protein-coding sequence and operably linked to the *A. nidulans trpC* 3'UTR/terminator at the 3' region, or downstream, of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Mortierella alpina* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. One skilled in the art can identify such homology regions

within the sequence of the *Mortierella alpina* genome (referenced in the publication by Wang et al., *PLOS One*, Vol. 6:12 (2011)). Stable transformation of *Mortierella alpina* with the transformation vector is achieved through well-known transformation techniques including protoplast transformation or other known methods. Activity of the *hpt* gene product can be used as a marker to select for *Mortierella alpina* transformed with the transformation vector on, but not limited to, PDA medium comprising hygromycin. Growth media suitable for *Mortierella alpina* lipid production include, but are not limited to, *S2GYE* medium, and those culture media described by Lu *et al.* and Ando *et al.* Evaluation of fatty acid profiles of *Mortierella alpina lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 33: Engineering *Rhodococcus opacus PD630*

[0432] Expression of recombinant genes in accordance with the present invention in *Rhodococcus opacus PD630* can be accomplished by modifying the methods and vectors taught by Kalscheuer *et al.* as discussed herein. Briefly, Kalscheuer *et al., Applied and Environmental Microbiology*, Vol. 52 (1999), pp. 508-515, reported the stable transformation of *Rhodococcus opacus* with plasmid DNA. Using the transformation method of electroporation, Kalscheuer introduced the plasmid pNC9501 into *Rhodococcus opacus PD630*. Plasmid pNC9501 comprised a thiostrepton resistance (thio^r) cassette, comprising the full nucleotide sequence of the *Streptomyces azureus* 23S rRNA A1067 methyltransferase gene, including the gene's promoter and 3' terminator sequence. *Prior to transformation with p*NC9501, *Rhodococcus opacus was unable to propagate on medium comprising 1 mg/ml* thiostrepton. Upon transformation of *Rhodococcus opacus PD630* with pNC9501, transformants were obtained that propagated on culture medium comprising 1 mg/ml thiostrepton, thereby establishing the use of the thiostrepton resistance cassette as a selectable marker in *Rhodococcus opacus PD630*. A second plasmid described by Kalscheuer, pAK68, comprised the resistance thio^r cassette as well as the gene sequences of the *Ralstonia eutropha* beta-ketothiolase (phaB), acetoacetyl-CoA reductase (phaA), and poly3-hydroxyalkanoic acid synthase (phaC) genes for *polyhydroxyalkanoate biosynthesis, driven by the lacZ promoter*. Upon pAK68 transformation of a *Rhodococcus opacus PD630 strain deficient in polyhydroxyalkanoate biosynthesis,* transformed *Rhodococcus opacus PD630 were obtained that produced* higher amounts of *polyhydroxyalkanoates* than the untransformed strain. Detectable activity of the introduced *Ralstonia eutropha* phaB, phaA, and phaC enzymes indicted that the regulatory elements encoded on the pAK68 plasmid were

suitable for heterologous gene expression in *Rhodococcus opacus PD630.* Kalscheuer reported that selection and maintenance of the transformed *Rhodococcus opacus PD630 was performed* on standard Luria Broth (LB) medium, nutrient broth (NB), or mineral salts medium (MSM) *comprising* thiostrepton. Other media and techniques used for culturing *Rhodococcus opacus PD630 have been described (for example see* Kurosawa *et al., Journal of Biotechnology*, Vol. 147:3-4 (2010), pp. 212-218 and Alverez *et al., Applied Microbial and Biotechnology*, Vol. 54:2 (2000), pp.218-223). Kalscheuer reported that the transformation vectors pNC9501 and pAK68, the promoters of the *Streptomyces azureus* 23S rRNA A1067 methyltransferase gene and lacZ gene are suitable to enable heterologous gene expression in *Rhodococcus opacus PD630.* In addition, Kalscheuer reported that the thio^r cassette encoded on pNC9501 and pAK68 was suitable for use as a selectable marker in *Rhodococcus opacus PD630.*

[0433] In an embodiment of the present invention, vector pNC9501, comprising the nucleotide sequence encoding the thio^r gene product for use as a selectable marker, is constructed and modified to further comprise a lipid biosynthesis pathway expression cassette sequence, thereby creating a transformation vector. The lipid biosynthesis pathway expression cassette encodes one or more lipid biosynthesis pathway proteins selected from Table 20, each protein-coding sequence codon-optimized for expression in *Rhodococcus opacus PD630 to* reflect the codon bias inherent in nuclear genes of *Rhodococcus opacus* in accordance with Tables 19A-D. For each lipid biosynthesis pathway protein of Table 20, the codon-optimized gene sequence can individually be operably linked to the lacZ *gene promoter* upstream of the protein-coding sequence. The transformation construct may additionally comprise homology regions to the *Rhodococcus opacus PD630* genome for targeted genomic integration of the transformation vector. Homology regions may be selected to disrupt one or more genomic sites of endogenous lipid biosynthesis pathway genes. One skilled in the art can identify such homology regions within the sequence of the *Rhodococcus opacus PD630* genome (referenced in the publication by Holder *et al., PLOS Genetics*, Vol. 7:9 (2011). Transformation of *Rhodococcus opacus PD630* with the transformation vector is achieved through well-known transformation techniques including electroporation or other known methods. Activity of the *Streptomyces azureus* 23S rRNA A1067 methyltransferase gene product can be used as a marker to select for *Rhodococcus opacus PD630* transformed with the transformation vector on, but not limited to, LB medium comprising thiostrepton. Growth media suitable *Rhodococcus opacus PD630* lipid production include, but are not limited to those culture media discussed by Kurosawa *et al.*

and Alvarez *et al*. Evaluation of fatty acid profiles of *Rhodococcus opacus PD630 lipids* can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 34: ENGINEERING MICROALGAE FOR FATTY ACID AUXOTROPHY

[0434] Strain B of Example 3, *Prototheca moriformis* (UTEX 1435) engineered to express a *Cuphea wrightii* thioesterase (*Cw*TE2), was used as the host organism for further genetic modification to knockout both endogenous thioesterase alleles, FATA1-1 and FATA1-2. Here, a first transformation construct was generated to integrate a neomycin expression cassette into Strain B at the FATA1-1 locus. This construct, pSZ2226, included 5' (SEQ ID NO: 30) and 3' (SEQ ID NO: 31) homologous recombination targeting sequences (flanking the construct) to the FATA1-1 locus of the nuclear genome and a neomycin resistance protein-coding sequence under the control of the *C. reinhardtii* β-tubulin promoter/5'UTR (SEQ ID NO: 5) and the *Chlorella vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6). This NeoR expression cassette is listed as SEQ ID NO: 15 and served as a selectable marker.

[0435] Upon transformation of pSZ2226 into Strain B, individual transformants were selected on agar plates comprising sucrose and G418. A single isolate, Strain H, was selected for further genetic modification. A second transformation construct, pSZ2236, was generated to integrate polynucleotides enabling expression of a thiamine selectable marker into Strain H at the FATA1-2 locus. pSZ2236 included 5' (SEQ ID NO: 32) and 3' (SEQ ID NO: 33) homologous recombination targeting sequences (flanking the construct) to the FATA1-2 genomic region for integration into the *P. moriformis* (UTEX 1435) nuclear genome and an *A. thaliana* THIC protein coding region under the control of the *C. prototheco*ides actin promoter/5'UTR (SEQ ID NO: 22) and *C. vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6). This *At*THIC expression cassette is listed as SEQ ID NO: 23 and served as a selectable marker. Upon transformation of Strain H with pSZ2236 to generate Strain I, individual transformants, were selected on agar plates comprising free fatty acids. Strain I was able to propagate on agar plates and in medium lacking thiamine and supplemented with free fatty acids.

EXAMPLE 35: ENGINEERING MICROORGANISMS FOR INCREASED PRODUCTION OF STEARIC ACID

[0436] A classically mutagenized strain of *Prototheca moriformis* (UTEX 1435), Strain J, was transformed with the plasmid construct pSZ2281 according to biolistic transformation methods as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463,

PCT/US2011/038464, and PCT/US2012/023696. pSZ2281 included polynucleotides encoding RNA hairpins (SAD2hpC, SEQ ID NO: 34) to down-regulate the expression of stearoyl-ACP desaturase, 5' (SEQ ID NO: 1) and 3' (SEQ ID NO: 2) homologous recombination targeting sequences (flanking the construct) to the 6S genomic region for integration into the nuclear genome, and a *S. cerevisiae suc2* sucrose invertase coding region (SEQ ID NO: 4), to express the protein sequence given in SEQ ID NO: 3, under the control of *C. reinhardtii* β-tubulin promoter/5'UTR (SEQ ID NO: 5) and *Chlorella vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6). This *S. cerevisiae suc2* expression cassette is listed as SEQ ID NO: 7 and served as a selectable marker. The polynucleotide sequence encoding the SAD2hpC RNA hairpin was under the control of the *C. protothecoides* actin promoter/5'UTR (SEQ ID NO: 22) and *C. vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6).

[0437] Upon transformation of Strain J with construct pSZ2281, thereby generating Strain K, positive clones were selected on agar plates containing sucrose as a sole carbon source. Individual transformants were clonally purified and propagated under heterotrophic conditions suitable for lipid production as those detailed in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. Lipid samples were prepared from dried biomass and analyzed using standard fatty acid methyl ester gas chromatography flame ionization detection methods as described in Example 1 (also see PCT/US2012/023696). The fatty acid profiles (expressed as Area % of total fatty acids) of *P. moriformis* UTEX Strain J propagated on glucose as a sole carbon source and three representative isolates of Strain K, propagated on sucrose as a sole carbon source, are presented in Table 21.

[0438] Table 21. Fatty acid profiles of *Prototheca moriformis* (UTEX 1435) cells engineered to express a hairpin RNA construct targeting stearoyl ACP desaturase gene/gene products.

Area % Fatty acid	Strain J	Strain K-1	Strain K-2	Strain K-3	Strain K-4
C8:0					0.02
C10:0	0.01	0.00	0.02	0.02	0.04
C12:0	0.03	0.05	0.05	0.05	0.08

C14:0	1.22	0.89	0.87	0.77	1.2
C16:0	26.75	29.23	28.96	27.55	28.06
C18:0	3.06	37.39	36.76	36.41	40.82
C18:1	59.62	23.90	24.76	26.92	22.02
C18:2	7.33	5.44	5.54	5.54	4.53
C18:3					0.14
C20:0					1.43

[0439] The data presented in Table 21 show a clear impact of the expression of SAD2 hairpin RNA construct on the C18:0 and C18:1 fatty acid profiles of the transformed organism. The fatty acid profiles of Strain K transformants comprising a SAD2 hairpin RNA construct demonstrated an increase in the percentage of saturated C18:0 fatty acids with a concomitant diminution of unsaturated C18:1 fatty acids. Fatty acid profiles of the untransformed strain comprise about 3% C18:0. Fatty acid profiles of the transformed strains comprise about 37% C18:0. These data illustrate the successful expression and use of polynucleotides enabling expression of a SAD RNA hairpin construct in *Prototheca moriformis* to alter the percentage of saturated fatty acids in the engineered host microbes, and in particular in increasing the concentration of C18:0 fatty acids and decreasing C18:1 fatty acids in microbial cells.

[0440] Also shown in Table 21, strain K-4 had a yet further elevated level of stearate. Strain K4 was created by inserting the construct of strains K1-K3 into the SAD2B locus. Thus, by knocking out one copy of the SAD gene and inhibiting the remaining copies at the RNA level, a further reduction in oleic acid and corresponding increase in stearate was obtained. Triglyceride analysis of RBD oil obtained from strain K4 showed about 12% POP, 27%POS and 18%SOS.

EXAMPLE 36: ENGINEERING MICROORGANISMS FOR INCREASED PRODUCTION OF OLEIC ACID THROUGH KNOCKDOWN OF AN ENDOGENOUS ACYL-ACP THIOESTERASE

[0441] A classically mutagenized strain of *Prototheca moriformis* (UTEX 1435), Strain J, was transformed independently with each of the constructs pSZ2402-pSZ2407 according to biolistic transformation methods as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. Each of the constructs pSZ2402-pSZ2407 included different polynucleotides encoding a hairpin RNA

targeted against *Prototheca moriformis* FATA1 mRNA transcripts to down-regulate the expression of fatty acyl-ACP thioesterase, 5' (SEQ ID NO: 1) and 3' (SEQ ID NO: 2) homologous recombination targeting sequences (flanking the construct) to the 6S genomic region for integration into the nuclear genome, and a *S. cerevisiae suc2* sucrose invertase coding region (SEQ ID NO: 4) to express the protein sequence given in SEQ ID NO: 3 under the control of *C. reinhardtii* β-tubulin promoter/5'UTR (SEQ ID NO: 5) and *Chlorella vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6). This *S. cerevisiae suc2* expression cassette is listed as SEQ ID NO: 7 and served as a selectable marker. Sequence listing identities for the polynucleotides corresponding to each hairpin are listed in Table 22. The polynucleotide sequence encoding each RNA hairpin was under the control of the *C. reinhardtii* β-tubulin promoter/5'UTR (SEQ ID NO: 5) and *C. vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6).

[0442] Table 22. Plasmid constructs used to transform *Prototheca moriformis* (UTEX 1435) Strain J.

Plasmid construct	Hairpin designation	SEQ ID NO:
pSZ2402	PmFATA-hpB	SEQ ID NO: 40
pSZ2403	PmFATA-hpC	SEQ ID NO: 41
pSZ2404	PmFATA-hpD	SEQ ID NO: 42
pSZ2405	PmFATA-hpE	SEQ ID NO: 43
pSZ2406	PmFATA-hpF	SEQ ID NO: 44
pSZ2407	PmFATA-hpG	SEQ ID NO: 45

[0443] Upon independent transformation of Strain J with each of the constructs listed in Table 22, positive clones were selected on agar plates containing sucrose as a sole carbon source. Individual transformants were clonally purified and propagated under heterotrophic conditions suitable for lipid production as those detailed in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. Lipid samples were prepared from dried biomass and analyzed using standard fatty acid methyl ester gas chromatography flame ionization detection methods as described in Example 1 (also see PCT/US2012/023696). The fatty acid profiles (expressed as Area % of total fatty acids) of *P. moriformis* (UTEX 1435) Strain J propagated on glucose as a sole carbon source and representative isolates of each transformation of Strain J, propagated on sucrose as a sole carbon source, are presented in Table 23.

[0444] Table 23. Fatty acid profiles of *Prototheca moriformis* (UTEX 1435) cells engineered to express hairpin RNA constructs targeting fatty acyl-ACP thioesterase gene/gene products.

	Area % Fatty Acid						
Construct	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2
Strain J untransformed	0	0.05	1.32	26.66	3.1	59.07	7.39
PmFATA-hpB	0.04	0.07	1.36	24.88	2.24	61.92	6.84
	0	0.08	1.33	25.34	2.39	61.72	6.5
	0	0.07	1.29	25.44	2.26	61.7	6.69
	0	0.06	1.33	25.1	2.37	61.56	6.87
PmFATA-hpC	0	0.08	1.18	22.03	1.71	63.8	8.63
	0	0.07	1.21	24.5	2.23	62.32	7.19
	0	0.08	1.29	24.93	2.24	62.02	7.01
	0.05	0.06	1.29	25.45	2.26	61.81	6.76
PmFATA-hpD	0	0.02	0.68	15.8	1.88	72.64	6.96
	0	0.03	0.78	17.56	1.7	71.8	6.03
	0	0.03	0.92	19.04	2.03	68.82	7.05
	0	0.04	1.27	23.14	2.25	65.27	6.07
PmFATA-hpE	0	0.03	0.79	18.55	2.13	69.66	6.77
	0	0.04	1.11	21.01	1.74	65.18	8.55
	0	0.03	1.08	21.11	1.54	64.76	8.87
	0	0.03	1.17	21.93	1.71	63.89	8.77
PmFATA-hpF	0.03	0.04	0.34	8.6	1.69	78.08	8.87
	0	0.03	0.49	10.2	1.52	76.97	8.78
	0	0.03	1	20.47	2.22	66.34	7.45
	0	0.03	1.03	21.61	1.88	65.39	7.76
PmFATA-hpG	0	0.03	1.03	20.57	2.36	64.73	8.75
	0	0.03	1.2	24.39	2.47	61.9	7.49
	0	0.04	1.29	24.14	2.29	61.41	8.22

[0445] The data presented in Table 23 show a clear impact of the expression of FATA hairpin RNA constructs on the C18:0 and C18:1 fatty acid profiles of the transformed

organism. The fatty acid profiles of Strain J transformants comprising a FATA hairpin RNA construct demonstrated an increase in the percentage of C18:1 fatty acids with a concomitant diminution of C16:0 and C18:0 fatty acids. Fatty acid profiles of the untransformed Strain J are about 26.66% C16:0, 3% C18:0, and about 59% C18:1 fatty acids. In contrast, the fatty acid profiles of the transformed strains comprise as low as 8.6% C16:0 and 1.54% C18:0 and greater than 78% C18:1 fatty acids.

[0446] These data illustrate the utility and successful use of polynucleotide FATA RNA hairpin constructs in *Prototheca moriformis* to alter the fatty acids profile of engineered microbes, and in particular in increasing the concentration of C18:1 fatty acids and decreasing C18:0 and C16:0 fatty acids in microbial cells.

EXAMPLE 37: ENGINEERING MICROORGANISMS FOR INCREASED PRODUCTION OF MID-CHAIN FATTY ACIDS THROUGH KASI OR KASIV OVEREXPRESSION

[0447] This example describes the use of recombinant polynucleotides that encode KASI or KASIV enzymes to engineer microorganisms in which the fatty acid profiles of the transformed microorganisms have been enriched in lauric acid, C10:0, and total saturated fatty acids.

[0448] Each of the constructs pSZD1132, pSZD1133, pSZD1134, or pSZD1201 was used independently to transform Strain B of Example 3, *Prototheca moriformis* (UTEX 1435) engineered to express a *Cuphea wrightii* thioesterase (*Cw*TE2), according to biolistic transformation methods as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. Each of the above constructs included different polynucleotides encoding a KASI or KASIV enzyme, 5' (SEQ ID NO: 13) and 3' (SEQ ID NO: 14) homologous recombination targeting sequences (flanking the construct) to the pLoop genomic region for integration into the nuclear genome, and a neomycin resistance protein-coding sequence under the control of the *C. reinhardtii* β-tubulin promoter/5'UTR (SEQ ID NO: 5) and the *Chlorella vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6). This NeoR expression cassette is listed as SEQ ID NO: 15 and served as a selectable marker. Sequence listing identities for the polynucleotides corresponding to each construct are listed in Table 20. The polynucleotide sequence encoding each KAS enzyme was under the control of the *P. moriformis* UTEX 1435 Amt03 promoter/5'UTR (SEQ ID NO: 8) and *C. vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6). The protein coding regions of the KAS enzymes and neomycin resistance gene were codon optimized to

reflect the codon bias inherent in *P. moriformis* UTEX 1435 nuclear genes as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696.

[0449] Upon transformation of individual plasmids into Strain B, positive clones were selected on agar plates comprising G418. Individual transformants were clonally purified and grown on sucrose as a sole carbon source at pH 7.0 under conditions suitable for lipid production as detailed in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. Lipid samples were prepared from dried biomass from each transformant and fatty acid profiles from these samples were analyzed using standard fatty acid methyl ester gas chromatography flame ionization (FAME GC/FID) detection methods as described in Example 1. The fatty acid profiles (expressed as Area % of total fatty acids) of Strain B and four positive transformants of each of pSZ2046 (Strains M-P, 1-4) are presented in Table 24.

[0450] Table 24. Plasmid constructs used to transform *Prototheca moriformis* (UTEX 1435) Strain B.

Plasmid construct	KASI / KASIV source	Transit peptide	SEQ ID NO:
pSZD1134	*Cuphea wrightii* GenBank Accession No. U67317	Native	SEQ ID NO: 46
pSZD1201	*Cuphea wrightii*	PmSAD	SEQ ID NO: 47
pSZD1132	*Cuphea pulcherrima* GenBank Accession No. AAC68860	Native	SEQ ID NO: 48
pSZD1133	*Cuphea hookeriana*	Native	SEQ ID NO: 49

[0451] Table 25. Fatty acid profiles of *Prototheca moriformis* (UTEX 1435) Strain B engineered for increased C10, lauric acid, and total saturated fatty acids.

		Fatty Acid (Area%)								
Plasmid construct(s)	No	C10	C12	C14	C16	C18:0	C18:1	C18:2	C10-C12	%Saturates/Total
pSZ1283		7.89	35.49	16.58	11.5	1.09	19.64	6.49	43.38	72.55

pSZ1283, pSZD1134	1	14.94	43.97	12.19	7.56	0.72	14.11	5.31	58.91	79.38
pSZ1283, pSZD1134	2	10.27	39.61	15.35	9.61	0.94	17.1	5.88	49.88	75.78
pSZ1283, pSZD1134	3	11.69	41.83	15.21	8.77	0.83	15.04	5.40	53.52	78.33
D1134-20	4	10.76	40.77	15.32	9.19	0.88	16.06	5.76	51.53	76.92
pSZ1283, pSZD1132	1	10.77	40.31	15.21	9.43	0.88	16.18	5.97	51.08	76.6
pSZ1283, pSZD1132	2	9.19	37.03	15.02	10.52	1.00	19.63	6.29	46.22	72.76
pSZ1283, pSZD1132	3	8.97	36.09	15.01	10.77	1.05	20.38	6.39	45.06	71.89
pSZ1283, pSZD1132	4	9.51	38.12	14.96	9.96	0.94	18.93	6.32	47.63	73.49
pSZ1283, pSZD1201	1	13.06	46.21	9.84	7.12	0.75	16.7	5.22	59.27	76.98
pSZ1283, pSZD1201	2	11.02	43.91	13.01	7.78	0.86	16.53	5.77	54.93	76.58
pSZ1283, pSZD1201	3	11.59	45.14	12.41	7.61	0.82	15.72	5.65	56.73	77.57
pSZ1283, pSZD1201	4	10.66	41.32	13.74	8.75	0.68	18.64	5.21	51.98	75.15
pSZ1283, pSZD1133	1	6.90	36.08	15.15	11.02	1.00	21.74	6.77	42.98	70.15
pSZ1283, pSZD1133	2	7.01	35.88	15.01	10.75	1.07	22.02	6.93	42.89	69.72
pSZ1283, pSZD1133	3	10.65	41.94	12.38	8.48	0.85	18.28	6.15	52.59	74.3
pSZ1283, pSZD1133	4	10.23	41.88	12.58	8.52	0.82	18.48	6.22	52.11	74.03

[0452] The data presented in Table 25 show a clear impact of the exogenous expression of KASI and KASIV enzymes on the C10:0 and C12 fatty acid profiles of the transformed organism. The fatty acid profiles of Strain B, expressing the *Cuphea wrightii* thioesterase alone, comprised about 8% C10:0 and about 35.5% C12:0, with saturated fatty acids accounting for 72.55% of total fatty acids. In contrast, transformants of Strain B engineered to additionally express a *Cuphea wrightii* KASI with a *P. moriformis* stearoyl ACP desaturase transit peptide were characterized by a fatty acid profile of about 13% C10:0 and about 46% C12:0. Saturated fatty acids accounted for as high as 77% in transformants of Strain B co-expressing the *C. wrightii* KASI fusion protein. Similarly, transformants of Strain B engineered to express the *C. wrightii* KASI with the enzyme's native transit peptide were characterized by a fatty acid profile of about 15% C10, about 44% C12, and about 79% saturated fatty acids. The fatty acid profiles or many transformants of Strain B expressing either *Cuphea pulcherrima* KASIV or *Cuphea hookeriana* KASIV also displayed elevated C10% and C12% levels, compared to the fatty acid profile of Strain B itself.

[0453] These data demonstrate the utility and effectiveness of polynucleotides enabling expression of KASI and KASIV constructs in *Prototheca moriformis* (UTEX 1435) to alter the percentage of saturated fatty acids in the engineered host microbes, and in particular in increasing the concentration of C10:0 and C12:0 fatty acids in microbial cells.

EXAMPLE 38: ENGINEERING MICROORGANISMS FOR INCREASED PRODUCTION OF MID-CHAIN FATTY ACIDS THROUGH KASI KNOCKOUT

[0454] This example describes the use of recombinant polynucleotides that disrupt different KASI alleles to engineer microorganisms in which the fatty acid profiles of the transformed microorganisms have been enriched in C10:0 and midchain fatty acids.

[0455] Constructs pSZ2302 and pSZ2304 were used to independently transform Strain B of Example 3, *Prototheca moriformis* (UTEX 1435) engineered to express a *Cuphea wrightii* thioesterase (*Cw*TE2), according to biolistic transformation methods as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. pSZ2302 included 5' (SEQ ID NO: 50) and 3' (SEQ ID NO: 51) homologous recombination targeting sequences (flanking the construct) to the KAS1 allele 1 genomic region for integration into the *P. moriformis* nuclear genome, an *A. thaliana* THIC protein coding region under the control of the *C. prototheciodes* actin promoter/5'UTR (SEQ ID NO: 22) and *C. vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6). pSZ2304 included 5' (SEQ ID NO: 52) and 3' (SEQ ID NO: 53) homologous recombination targeting sequences

(flanking the construct) to the KAS1 allele 2 genomic region for integration into the *P. moriformis* nuclear genome, an *A. thaliana* THIC protein coding region under the control of the *C. protothecoides* actin promoter/5'UTR (SEQ ID NO: 22) and *C. vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6).This *At*THIC expression cassette is listed as SEQ ID NO: 23 and served as a selection marker. The protein coding region of *At*THIC was codon optimized to reflect the codon bias inherent in *P. moriformis* UTEX 1435 nuclear genes as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696.

[0456] Upon independent transformation pSZ2302 and pSZ2304 into Strain B, thereby generating Strain Q and R, positive clones were selected on agar plates comprising thiamine. Individual transformants were clonally purified and cultivated on sucrose as a sole carbon source at pH 5.0 or pH 7.0 under heterotrophic conditions suitable for lipid production as detailed in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. Lipid samples were prepared from dried biomass from each transformant and fatty acid profiles from these samples were analyzed using fatty acid methyl ester gas chromatography flame ionization (FAME GC/FID) detection methods as described in Example 1. The fatty acid profiles (expressed as Area % of total fatty acids) of Strain B and positive pSZ2302 (Strain Q, 1-5) and pSZ2304 (Strain R, 1-5) transformants are presented in Tables 26 and 27.

[0457] Table 26. Fatty acid profiles of *Prototheca moriformis* (UTEX 1435) Strains B, Q, and R engineered for increased midchain fatty acids, cultured at pH 5.0.

		Fatty Acid (Area%)							
Strain	Transformation plasmid(s)	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C10-C14
UTEX 1435	None	0.00	0.04	1.28	26.67	3.05	59.96	7.19	1.32
Strain B	pSZ1283	0.01	0.09	1.09	21.60	2.21	65.15	7.94	1.19
Strain Q-1	pSZ1283, pSZ2302	0.08	1.21	7.52	38.71	1.38	38.32	8.75	8.81
Strain Q-2	pSZ1283, pSZ2302	0.15	1.36	7.51	38.23	1.33	38.27	8.94	9.02
Strain Q-3	pSZ1283,	0.16	1.43	7.49	38.88	1.30	37.58	8.73	9.08

	pSZ2302								
Strain Q-4	pSZ1283, pSZ2302	0.00	1.71	7.42	37.67	1.43	37.26	10.38	9.13
Strain Q-5	pSZ1283, pSZ2302	0.13	1.21	7.36	38.81	1.31	38.07	8.71	8.7
Strain R-1	pSZ1283, pSZ2304	0.19	1.78	8.47	40.11	1.34	33.46	9.98	10.44
Strain R-2	pSZ1283, pSZ2304	0.90	8.00	7.78	28.96	1.15	30.26	17.14	16.68
Strain R-3	pSZ1283, pSZ2304	0.26	3.58	7.77	34.98	1.56	32.86	14.60	11.61
Strain R-4	pSZ1283, pSZ2304	1.64	13.50	7.61	21.38	0.90	36.13	14.73	22.75
Strain R-5	pSZ1283, pSZ2304	1.03	9.63	7.56	25.61	1.00	31.70	18.23	18.22

[0458] Table 27. Fatty acid profiles of *Prototheca moriformis* (UTEX 1435), Strains B, Q, and R engineered for increased midchain fatty acids, cultured at pH 7.0.

		Fatty Acid (Area%)							
Strain	Transformation plasmid(s)	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C10-C14
UTEX 1435	None	0.01	0.04	1.34	27.94	3.24	57.46	7.88	1.39
Strain B	pSZ1283	4.72	29.57	15.56	12.63	1.20	27.65	7.39	49.85
Strain Q-1	pSZ1283, pSZ2302	16.00	50.61	9.52	5.33	0.54	11.79	5.28	76.13
Strain Q-2	pSZ1283, pSZ2302	16.32	49.79	9.82	5.52	0.54	12.28	4.87	75.93
Strain Q-3	pSZ1283, pSZ2302	15.08	47.58	10.23	5.93	0.56	15.12	4.50	72.89
Strain Q-4	pSZ1283, pSZ2302	14.27	47.30	10.44	6.17	0.56	15.50	4.59	72.01
Strain Q-	pSZ1283,	14.75	47.28	10.32	6.04	0.59	15.50	4.65	72.35

5	pSZ2302								
Strain R-1	pSZ1283, pSZ2304	21.25	55.42	7.97	3.65	0.00	5.46	5.66	84.64
Strain R-2	pSZ1283, pSZ2304	13.00	55.05	10.88	5.78	0.28	7.90	6.29	78.93
Strain R-3	pSZ1283, pSZ2304	12.89	53.15	11.11	6.13	0.00	9.87	6.13	77.15
Strain R-4	pSZ1283, pSZ2304	12.80	51.64	13.86	6.69	0.00	7.51	6.70	78.3
Strain R-5	pSZ1283, pSZ2304	16.61	51.42	9.84	5.27	0.33	11.15	4.79	77.87

[0459] The data presented in Tables 26 and 27 show a clear impact of disruption of different KASI alleles on the fatty acid profiles of the transformed organisms. When cultivated at pH 5.0, the fatty acid profiles of *Prototheca moriformis* (UTEX 1435) and *Prototheca moriformis* (UTEX 1435) Strain B, expressing a *Cuphea wrightii* FATB2 thioesterase under control of a pH regulatable promoter were very similar. These profiles were characterized by about 1% C14:0, about 21-26% C16:0, about 2-3% C18:0, about 60-65% C18:1, about 7% C18:2, with C10-C14 fatty acids comprising about 1.19-1.3% of total fatty acids. In contrast, when cultivated at pH 5.0, Strain B further engineered to disrupt KASI allele 1 (Strain Q) or KASI allele 2 (Strain R) demonstrated altered fatty acid profiles that were characterized by increased levels of C12, increased levels of C14, decreased levels of C18, and decreased levels of C18:1 fatty acids compared to Strain B or UTEX 1435. The fatty acid profiles of isolates of Strains Q and R differed in that Strain R (allele 2 knockout) isolates had generally greater C12s and lower C16s and C18:1s than Strain Q (allele 1 knockout).

[0460] When cultivated at pH 7.0, the fatty acid profile of *Prototheca moriformis* (UTEX 1435) is distinct from that *Prototheca moriformis* (UTEX 1435) Strain B expressing a *Cuphea wrightii* FATB2 thioesterase under control of a pH regulatable promoter. When cultured at pH 7.0, Strain B was characterized by a fatty acid profile elevated in C10, C12, and C14 fatty acids (these comprised about 50% of the total fatty acids). When cultured at pH 7.0, Strain Q and Strain R demonstrated fatty acid profiles with still further increases in C10, C12, and C14 fatty acids and still further decreases in C18:0 and C18:1 fatty acids relative to that of Strain B. Again, differences in fatty acid profiles between Strain Q and R were

observed with the profile of Strain R comprising greater percentage levels of C12 and lower levels of C18:1 than that of Strain Q.

[0461] These data illustrate the successful expression and use of polynucleotides enabling expression of KASI and KASIV constructs in *Prototheca moriformis* to alter the percentage of saturated fatty acids in the engineered host microbes, and in particular in increasing the concentration of C10:0 and C12:0 fatty acids and decreasing the concentration of C18:0 and C18:1 fatty acids in microbial cells. In addition, the data here indicate the different KASI alleles can be disrupted to result in altered fatty acid profiles of the transformed organisms.

EXAMPLE 39: ENGINEERING MICROORGANISMS FOR INCREASED PRODUCTION OF MID-CHAIN FATTY ACIDS THROUGH KASI KNOCKDOWN

[0462] This example describes the use of recombinant polynucleotides that encode RNA hairpins to attenuate a KASI enzyme to engineer a microorganism in which the fatty acid profile of the transformed microorganism has been enriched in midchain fatty acids.

[0463] A classically mutagenized strain of *Prototheca moriformis* (UTEX 1435), Strain S, was transformed independently with each of the constructs pSZ2482-pSZ2485 according to biolistic transformation methods as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. Each of the constructs pSZ2482-pSZ2485 included different polynucleotides encoding hairpin RNAs targeted against *Prototheca moriformis* (UTEX 1435) KASI mRNA transcripts to down-regulate the expression of fatty acyl-ACP thioesterase, 5' (SEQ ID NO: 1) and 3' (SEQ ID NO: 2) homologous recombination targeting sequences (flanking the construct) to the 6S genomic region for integration into the nuclear genome, and a *S. cerevisiae suc2* sucrose invertase coding region (SEQ ID NO: 4) to express the protein sequence given in SEQ ID NO: 3 under the control of *C. reinhardtii* β-tubulin promoter/5'UTR (SEQ ID NO: 5) and *Chlorella vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6). This *S. cerevisiae suc2* expression cassette is listed as SEQ ID NO: 7 and served as a selectable marker. Sequence listing identities for the polynucleotides corresponding to each KASI hairpin are listed in Table 28. The polynucleotide sequence encoding each RNA hairpin was under the control of the *P. moriformis* Amt03 promoter/5'UTR (SEQ ID NO: 8) and *C. vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6). The protein coding region of the *suc2* expression cassette was codon optimized to reflect the codon bias inherent in *P. moriformis* UTEX 1435 nuclear genes as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696.

[0464] Table 28. Plasmid constructs used to transform *Prototheca moriformis* (UTEX 1435) Strain S.

Transformation construct	Hairpin	SEQ ID NO:
pSZ2482	KASI hairpin B	SEQ ID NO: 54
pSZ2483	KASI hairpin C	SEQ ID NO: 55
pSZ2484	KASI hairpin D	SEQ ID NO: 56
pSZ2485	KASI hairpin E	SEQ ID NO: 57

[0465] Upon independent transformation of Strain S with each of the constructs listed in Table 28, positive clones were selected on agar plates containing sucrose as a sole carbon source. Individual transformants were clonally purified and propagated under heterotrophic conditions suitable for lipid production as those detailed in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. Lipid samples were prepared from dried biomass and analyzed using fatty acid methyl ester gas chromatography flame ionization detection methods as described in Example 1 (also see PCT/US2012/023696). The fatty acid profiles (expressed as Area % of total fatty acids) of *P. moriformis* UTEX 1435 propagated on glucose as a sole carbon source and four representative isolates of each transformation of Strain S, propagated on sucrose as a sole carbon source, are presented in Table 29.

[0466] Table 29. Fatty acid profiles of *Prototheca moriformis* (UTEX 1435) cells engineered to express hairpin RNA constructs targeting KASI gene/gene products.

			Fatty Acid (Area%)							
Strain	Plasmid	No.	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3
UTEX 1435	none	1	0.00	0.04	1.45	27.97	3.18	58.35	6.78	0.60
Strain S	pSZ2482	1	0.19	0.74	8.47	38.30	2.15	36.24	9.45	1.42
		2	0.07	0.25	4.16	32.46	2.62	49.57	7.73	0.82
		3	0.03	0.10	2.68	27.48	2.65	56.40	8.14	0.55
		4	0.03	0.10	2.60	27.44	2.01	55.54	9.15	0.78
	pSZ2483	1	0.00	0.06	1.94	30.58	1.55	53.26	9.31	0.76
		2	0.20	0.05	1.76	28.01	2.31	56.61	8.70	0.60
		3	0.00	0.06	1.60	24.38	2.65	58.25	9.93	1.15

		4	0.00	0.04	1.56	26.65	2.96	60.06	6.92	0.52
	pSZ2484	1	0.72	3.71	19.15	38.03	1.68	14.22	15.00	4.21
		2	0.66	2.76	16.34	38.19	1.78	18.52	14.91	3.38
		3	0.69	2.96	16.20	37.28	1.77	19.05	15.26	3.48
		4	0.18	0.70	8.61	36.80	2.35	36.22	10.89	1.10
	pSZ2485	1	0.00	0.04	1.41	25.34	3.16	60.12	7.78	0.48
		2	0.03	0.04	1.41	23.85	2.19	61.23	8.75	0.67
		3	0.00	0.04	1.41	24.41	2.23	60.64	8.69	0.67
		4	0.00	0.04	1.41	24.51	2.16	60.85	8.91	0.66

[0467] The data presented in Table 29 show a clear impact of the expression of KAS hairpin RNA constructs on the fatty acid profiles of the transformed organisms. The fatty acid profiles of Strain S transformants comprising either pSZ2482 or pSZ2484 KASI hairpin RNA construct demonstrated an increase in the percentage of C10, C12, C14, and C16 fatty acids with a concomitant diminution of C18:0 and C18:1 fatty acids relative to the fatty acid profile of UTEX 1435.

[0468] These data illustrate the utility and successful use of polynucleotide KASI RNA hairpin constructs in *Prototheca moriformis* (UTEX 1435) to alter the fatty acids profile of engineered microbes, and in particular in increasing the concentration of midchain fatty acids and decreasing C18:0 and C18:1 fatty acids in microbial cells.

EXAMPLE 40: ENGINEERING MICROORGANISMS FOR INCREASED PRODUCTION OF STEARIC ACID THROUGH FATTY ACID ELONGASE OVEREXPRESSION

[0469] This example describes the use of recombinant polynucleotides that encode fatty acid elongases to engineer a microorganism in which the fatty acid profile of the transformed microorganism has been enriched in stearic acid, arachidic acid, and docosadienoic acid.

[0470] A classically mutagenized strain of *Prototheca moriformis* (UTEX 1435), Strain J, was transformed independently with each of the constructs pSZ2323, pSZ2324, or pSZ2328 according to biolistic transformation methods as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. Each of the constructs included a protein coding region to overexpress an elongase, 5' (SEQ ID NO: 1) and 3' (SEQ ID NO: 2) homologous recombination targeting

sequences (flanking the construct) to the 6S genomic region for integration into the nuclear genome, and a *S. cerevisiae suc2* sucrose invertase coding region (SEQ ID NO: 4) to express the protein sequence given in SEQ ID NO: 3 under the control of *C. reinhardtii* β-tubulin promoter/5'UTR (SEQ ID NO: 5) and *Chlorella vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6). This *S. cerevisiae suc2* expression cassette is listed as SEQ ID NO: 7 and served as a selectable marker. Sequence listing identities for the polynucleotides corresponding to each elongase are listed in Table 30. The polynucleotide sequence encoding each elongase was under control of the *P. moriformis* Amt03 promoter/5'UTR (SEQ ID NO: 8) and *C. vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6). The protein coding regions of the exogenous elongases and the *suc2* expression cassette were codon optimized to reflect the codon bias inherent in *P. moriformis* UTEX 1435 nuclear genes as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696.

[0471] Table 30. Plasmid constructs used to transform *Prototheca moriformis* (UTEX 1435) Strain J.

Plasmid construct	Elongase source	GenBank Accession No.	SEQ ID NO:
pSZ2328	*Marchantia polymorpha*	AP74370	58, 59
pSZ2324	*Trypanosoma brucei*	AX70673	60, 61
pSZ2323	*Saccharomyces cerevisiae*	39540	62, 63

[0472] Upon independent transformation of Strain J with the constructs listed in Table 30, positive clones were selected on agar plates containing sucrose as a sole carbon source. Individual transformants were clonally purified and propagated under heterotrophic conditions suitable for lipid production as those detailed in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. Lipid samples were prepared from dried biomass and analyzed using fatty acid methyl ester gas chromatography flame ionization detection methods as described

in Example 1 (also see PCT/US2012/023696). The fatty acid profiles (expressed as Area % of total fatty acids) of *P. moriformis* UTEX 1435 Strain J propagated on glucose as a sole carbon source and three representative isolates of each transformation of Strain J, propagated on sucrose as a sole carbon source are presented in Table 31.

[0473] Table 31. Fatty acid profiles of *Prototheca moriformis* (UTEX 1435) Strain J cells engineered to overexpress elongases.

Plasmid construct	No.	Fatty Acid Area %								
		C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3α	C20:0	C22:2n6
None	1	1.39	27.42	0.77	3.33	57.46	8.05	0.61	0.30	0.03
pSZ2328	1	1.25	19.23	0.85	8.26	57.54	9.34	0.79	0.73	0.94
pSZ2328	2	1.22	17.76	0.69	7.86	60.56	9.38	0.59	0.6	0.47
pSZ2328	3	1.26	18.37	0.92	7.83	58.77	10.01	0.72	0.64	0.52
pSZ2324	1	1.51	22.97	1.09	8.71	53.01	9.63	0.65	0.68	0.55
pSZ2324	2	1.29	20.6	0.92	7.53	56.97	9.92	0.73	0.64	0.43
pSZ2324	3	1.28	20.59	0.93	7.33	57.52	9.68	0.65	0.58	0.42
pSZ2323	1	1.65	27.27	0.67	3.56	56.68	8.72	0.33	0.36	0.00
pSZ2323	2	1.56	28.44	0.74	3.36	55.22	9.07	0.46	0.39	0.03
pSZ2323	3	1.64	28.7	0.75	3.34	55.29	8.59	0.49	0.36	0.02

[0474] The data presented in Table 31 show a clear impact of the expression of *Marchantia polymorpha* and *Trypanosoma brucei* enzymes on the C14, C16, C18:0, C20:0, and C22:2n6 fatty acid profiles of the transformed organisms. The fatty acid profile of untransformed Strain J was about 27.42% C16:0, about 3% C18:0, about 57.5% C18:1, about 0.3% C20:0 and about 0.03% C22:2n6 fatty acids. In contrast to that of Strain J, the fatty acid profiles of Strain J transformed with different plasmid constructs to express elongases comprised lower percentage levels of C16 and higher percentage levels of C18:0, C20:0, and C22:2n6 fatty acids. The result of overexpression of *Marchantia polymorpha* elongase was about a 2.5 fold increase in percentage levels of C18:0 fatty acids, a 2 fold increase in percentage levels of C20:0 fatty acids, and about a 15 to 30 fold increase in percentage levels of C22:2n6 fatty acids relative to the fatty acid profile of Strain J.

[0475] These data illustrate the successful use of polynucleotides encoding elongases for expression in *Prototheca moriformis* (UTEX 1435) to alter the fatty acid profile of engineered microbes, and in particular in increasing the concentration of C18:0, C20:0, and C22:2n6 fatty acids and decreasing C16:0 fatty acids in recombinant microbial cells.

EXAMPLE 41: ENGINEERING MICROORGANISMS FOR INCREASED PRODUCTION OF STEARIC ACID THROUGH ACYL-ACP THIOESTERASE OVEREXPRESSION

[0476] This example describes the use of recombinant polynucleotides that encode different C18:0-preferring acyl-ACP thioesterases to engineer microorganisms in which the fatty acid profiles of the transformed microorganisms have been enriched in stearic acid.

[0477] Classically mutagenized strains of *Prototheca moriformis* (UTEX 1435), Strain J or Strain A, were transformed independently with the constructs listed in Table 32 according to biolistic transformation methods as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. Each of the constructs included a protein coding region to overexpress a fatty acyl-ACP thioesterase with a C-terminal 3X FLAG® epitope tag, 5' (SEQ ID NO: 1) and 3' (SEQ ID NO: 2) homologous recombination targeting sequences (flanking the construct) to the 6S genomic region for integration into the nuclear genome, and a *S. cerevisiae suc2* sucrose invertase coding region (SEQ ID NO: 4) to express the protein sequence given in SEQ ID NO: 3 under the control of *C. reinhardtii* β-tubulin promoter/5'UTR (SEQ ID NO: 5) and *Chlorella vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6). This *S. cerevisiae suc2* expression cassette is listed as SEQ ID NO: 7 and served as a selectable marker. Sequence listing identities for the polynucleotides corresponding to each thioesterase are listed in Table 32. The polynucleotide sequence encoding each thioesterase was under control of the *P. moriformis* Amt03 promoter/5'UTR (SEQ ID NO: 8) and *C. vulgaris* nitrate reductase 3' UTR (SEQ ID NO: 6). The protein coding regions of the exogenous thioesterases and the *suc2* expression cassette were codon optimized to reflect the codon bias inherent in *P. moriformis* UTEX 1435 nuclear genes as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696.

[0478] Table 32. Plasmid constructs used to transform *Prototheca moriformis* (UTEX 1435) Strain A or Strain J.

Plasmid construct	Acyl-ACP Thioesterase, GenBank Accession No.	Acyl-ACP Thioesterase source	Transit Peptide source	SEQ ID NO:
	FATA, CAA52070	*Brassica napus*	native	64, 65

SZD581				
SZD643	FATA, CAA52070	*Brassica napus*	UTEX 250 SAD	66, 67
SZD645	FATA, AAA33019	*C. tinctorius*	UTEX 250 SAD	68, 69
SZD644	FATA, ABS30422	*Ricinis communis*	native	70, 71
SZD1323	FATA, AAB51523	*G. mangostana*	native	72, 73
SZD1320	FATA	*Theobroma cacao*	native	74, 75

[0479] Upon independent transformation of Strain A or J with the constructs listed in Table 32, positive clones were selected on agar plates containing sucrose as a sole carbon source. Individual transformants were clonally purified and propagated under heterotrophic conditions suitable for lipid production as those detailed in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. Lipid samples were prepared from dried biomass and analyzed using fatty acid methyl ester gas chromatography flame ionization detection methods as described in Example 1 (also see PCT/US2012/023696). The fatty acid profiles (expressed as Area % of total fatty acids) of *P. moriformis* UTEX 1435 Strain J propagated on glucose as a sole carbon source and representative isolates of each transformation of Strain J, propagated on sucrose as a sole carbon source are presented in Table 33.

[0480] Table 33. Fatty acid profiles of *Prototheca moriformis* (UTEX 1435) Strain J cells engineered to overexpress exogenous acyl-ACP thioesterase enzymes.

			Fatty Acid Area %					
Strain	Plasmid construct	No.	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3α
A	None	1	1.08	25.48	3.23	59.70	8.25	0.70
J	None	1	1.41	27.33	3.38	57.07	8.15	0.64
A	pSZD581	1	1.02	26.60	14.47	44.80	10.05	0.65
		2	1.08	28.24	13.57	43.89	10.07	0.68
		3	0.97	24.70	9.13	50.85	11.27	0.82

A	pSZD643	1	1.39	26.97	16.21	44.10	8.43	0.83
		2	1.37	27.91	11.15	48.31	8.40	0.78
A	pSZD645	1	0.90	23.39	8.35	50.69	13.34	0.96
A	pSZD644	1	1.67	19.70	4.40	59.15	12.32	1.01
J	pSZD1323	1	1.33	23.26	9.28	53.42	10.35	0.69
		2	1.47	26.84	7.36	52.78	9.29	0.64
		3	1.43	26.31	6.05	54.45	9.37	0.66
J	pSZD1320	1	1.30	24.76	3.84	60.90	6.96	0.55
		2	1.36	26.30	3.27	58.19	8.66	0.48
		3	1.39	25.51	3.18	58.78	8.85	0.45

[0481] The data presented in Table 33 show a clear impact of the expression of exogenous acyl-ACP enzymes on the fatty acid profiles of the transformed microorganisms. The fatty acid profiles of untransformed Strain A and J were about 25% C16:0, about 3.3% C18:0, about 57 to 60% C18:1. In contrast, the fatty acid profiles of Strain A transformed with different plasmid constructs to express acyl-ACP enzymes comprised greater percentage levels of C18:0 and lower percentage levels of C18:1 fatty acids than that of Strain A. Expression of *FATA* enzymes from *B. napus*, *C. tinctorius*, *R. communis* and *G. mangostana* in Strain A or J enabled the accumulation of stearate levels in the transformed organisms. The result of overexpression of a *Brassica napus* acyl-ACP thioesterase was about a 2 to 5 fold increase in the percentage levels of C18:0 fatty acids of the fatty acid profile of the transformed organisms relative to the fatty acid profile of Strain A. Fatty acid profiles of cells engineered to overexpress a *G. mangostana* acyl-ACP FATA thioesterase with a *C. protothecoides* SAD1 transit peptide were characterized by about a 2 to 3 fold increase in the percentage levels of C18:0 fatty acids of the fatty acid profile of the transformed organism relative to the fatty acid profile of Strain J.

[0482] These data illustrate the utility and effective use of polynucleotides encoding fatty acyl-ACP thioesterases for expression in *Prototheca moriformis* (UTEX 1435) to alter the fatty acid profile of engineered microbes, and in particular in increasing the concentration of C18:0 and decreasing C18:1 fatty acids in recombinant microbial cells.

EXAMPLE 42: ENGINEERING MICROORGANISMS FOR INCREASED PRODUCTION OF ERUCIC ACID THROUGH ELONGASE OR BETA-KETOACYL-COA SYNTHASE OVEREXPRESSION

[0483] In an embodiment of the present invention, a recombinant polynucleotide transformation vector operable to express an exogenous elongase or beta-ketoacyl-CoA synthase in an optionally plastidic oleaginous microbe is constructed and employed to transform *Prototheca moriformis* (UTEX 1435) according to the biolistic transformation methods as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696 to obtain a cell increased for production of erucic acid. The transformation vector includes a protein coding region to overexpress an elongase or beta-ketoacyl-CoA synthase such as those listed in Table 5, promoter and 3'UTR control sequences to regulate expression of the exogenous gene, 5' and 3' homologous recombination targeting sequences targeting the recombinant polynucleotides for integration into the *P. moriformis* (UTEX 1435) nuclear genome, and nucleotides operable to express a selectable marker. The protein-coding sequences of the transformation vector are codon-optimized for expression in *P. moriformis* (UTEX 1435) as described in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. Recombinant polynucleotides encoding promoters, 3' UTRs, and selectable markers operable for expression in *P. moriformis* (UTEX 1435) are disclosed herein and in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696.

[0484] Upon transformation of the transformation vector into *P. moriformis* (UTEX 1435) or a classically-mutagenized strain of *P. moriformis* (UTEX 1435), positive clones are selected on agar plates. Individual transformants are clonally purified and cultivated under heterotrophic conditions suitable for lipid production as detailed in PCT/US2009/066141, PCT/US2009/066142, PCT/US2011/038463, PCT/US2011/038464, and PCT/US2012/023696. Lipid samples are prepared from dried biomass from each transformant and fatty acid profiles from these samples are analyzed using fatty acid methyl ester gas chromatography flame ionization (FAME GC/FID) detection methods as described in Example 1. As a result of these manipulations, the cell may exhibit an increase in erucic acid of at least 5, 10, 15, or 20 fold.

EXAMPLE 43: GENERATION OF CAPRIC, LAURIC, AND MYRISTIC ACID RICH OILS IN STRAIN UTEX1435 BY THE EXPRESSION OF CUPHEA PSR23 LPAATS

[0485] We tested the effect of expression of two 1-acyl-sn-glycerol–3-phosphate acyltransferases (*LPAATs*) in a previously described *P. moriformis* (UTEX 1435) transgenic strain, expressing the acyl ACP thioesterase (FATB2) from *Cuphea wrightii*. The *LPAAT2* and *LPAAT3* genes from *Cuphea* PSR23 (*Cu*PSR23) were identified by analysis of a combination of *Cu*PSR23 genomic sequences and transcriptomic sequences derived from seed RNAs. The two LPAATs have not been previously described. The genes were codon optimized to reflect UTEX 1435 codon usage. Transformations, cell culture, lipid production and fatty acid analysis were all carried out as previously described.

[0486] Increased capric, lauric, and myristic accumulation in strain B by the expression of the *Cuphea* PSR23 1-acyl-sn-glycerol–3-phosphate acyltransferases (*LPAAT2 and LPAAT3*) [pSZ2299 and pSZ2300, respectively]: In this example, transgenic strains were generated via transformation of strain B with the constructs pSZ2299 or pSZ2300, encoding *Cu*PSR23 LPAAT2 and LPAAT3, respectively. The transgenic strains were selected for resistance to the antibiotic G418. Construct pSZ2299 can be written as pLOOP5'::CrTUB2:NeoR:CvNR::PmAMT3:CuPSR23LPAAT2-1:CvNR::pLOOP3'. Construct pSZ2300 can be written as pLOOP5'::CrTUB2:NeoR:CvNR::PmAMT3:CuPSR23LPAAT3-1:CvNR::pLOOP3'. The sequence of the transforming DNA (pSZ2299 and pSZ2300) is provided below. The relevant restriction sites in the construct from 5'-3', BspQI, KpnI, XbaI, Mfe I, BamHI, EcoRI, SpeI, XhoI, SacI, BspQI, respectively, are indicated in lowercase, bold, and underlined. BspQI sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences at the 5' and 3' end of the construct represent genomic DNA from UTEX 1435 that target integration to the *pLoop* locus via homologous recombination. Proceeding in the 5' to 3' direction, the selection cassette has the *C. reinhardtii β-tubulin* promoter driving expression of the *NeoR* gene (conferring resistance to G418) and the *Chlorella vulgaris Nitrate Reductase* (*NR*) gene 3' UTR. The promoter is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for *NeoR* are indicated by uppercase italics, while the coding region is indicated with lowercase italics. The 3' UTR is indicated by lowercase underlined text. The spacer region between the two cassettes is indicated by upper case text. The second cassette containing the codon optimized *LPAAT2* gene (pSZ2299) or *LPAAT3* gene (pSZ2300) from *Cuphea* PSR23 is driven by the *Prototheca moriformis* endogenous

AMT3 promoter, and has the same *Chlorella vulgaris Nitrate Reductase* (*NR*) gene 3' UTR. In this cassette, the *AMT3* promoter in indicated by lowercase, boxed text. The initiator ATG and terminator TGA for the *Cu*PSR23 *LPAAT2* and *LPAAT3* genes are indicated in uppercase italics, while the coding regions are indicated by lowercase italics. The 3' UTR is indicated by lowercase underlined text. The final constructs were sequenced to ensure correct reading frames and targeting sequences.

[0487] pSZ2299 Transforming Construct:

gctcttccgctaacggaggtctgtcaccaaatggaccccgtctattgcgggaaaccacggcgatggcacgtttcaaaacttgat
gaaatacaatattcagtatgtcgcgggcggcgacggcggggagctgatgtcgcgctgggtattgcttaatcgccagcttcgcc
cccgtcttggcgcgaggcgtgaacaagccgaccgatgtgcacgagcaaatcctgacactagaagggctgactcgcccggca
cggctgaattacacaggcttgcaaaaataccagaatttgcacgcaccgtattcgcggtattttgttggacagtgaatagcgatg
cggcaatggcttgtggcgttagaaggtgcgacgaaggtggtgccaccactgtgccagccagtcctggcggctcccagggccc
cgatcaagagccaggacatccaaactacccacagcatcaacgccccggcctatactcgaaccccacttgcactctgcaatggt
atgggaaccacggggcagtcttgtgtgggtcgcgcctatcgcggtcggcgaagaccgggaa**ggtacc**ctttcttgcgctatgac
acttccagcaaaaggtagggcgggctgcgagacggcttcccggcgctgcatgcaacaccgatgatgcttcgacccccccgaagctcc
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gctaccaaagccatattcaaacacctagatcactaccacttctacacaggccactcgagcttgtgatcgcactccgctaaggggcgc
ctcttcctcttcgtttcagtcacaacccgcaaac**tctaga**atatca*ATGatcgagcaggacggcctccacgccggctcccccgccg*
cctgggtggagcgcctgttcggctacgactgggcccagcagaccatcggctgctccgacgccgccgtgttccgcctgtccgccca
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*A***caattg**gcagcagcagctcggatagtatcgacacactctggacgctggtcgtgtgatggactgttgccgccacacttgctgccttga
cctgtgaatatccctgccgcttttatcaaacagcctcagtgtgtttgatcttgtgtgtacgcgcttttgcgagttgctagctgcttgtgctatttt
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caatgctgatgcacgggaagtagtgggatgggaacacaaatgga**ggatcc**CGCGTCTCGAACAGAGCGCGCA
GAGGAACGCTGAAGGTCTCGCCTCTGTCGCACCTCAGCGCGGCATACACCACAA

TAACCACCTGACGAATGCGCTTGGTTCTTCGTCCATTAGCGAAGCGTCCGGTTCA
CACACGTGCCACGTTGGCGAGGTGGCAGGTGACAATGATCGGTGGAGCTGATGG
TCGAAACGTTCACAGCCTAGGGATATC**gaattc***ggccgacaggacgcgcgtcaaaggtgctggtcgtgt*
atgccctggccggcaggtcgttgctgctggttagtgattccgcaaccctgattttggcgtcttattttggcgtggcaaacgctggc
gcccgcgagccgggccggcggcgatgcggtgccccacggctgccggaatccaagggaggcaagagcgcccgggtcagttga
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cctcaccagcggacaaagcaccggtgtatcaggtccgtgtcatccactctaaagagctcgactacgacctactgatggccctaga
ttcttcatcaaaaacgcctgagacacttgcccaggattgaaactccctgaagggaccaccaggggccctgagttgttccttccccc
cgtggcgagctgccagccaggctgtacctgtgatcgaggctggcgggaaaataggcttcgtgtgctcaggtcatgggaggtgca
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ctagt*ATGgcgatcgcggccgcggcggtgatcttcctgttcggcctgatcttcttcgcctccggcctgatcatcaacctgttccagg*
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gaacacaaatggaaagctt**gagctcagcggcgacggtcctgctaccgtacgacgttgggcacgcccatgaaagtttgtataccg agcttgttgagcgaactgcaagcgcggctcaaggatacttgaactcctggattgatatcggtccaataatggatggaaaatcc gaacctcgtgcaagaactgagcaaacctcgttacatggatgcacagtcgccagtccaatgaacattgaagtgagcgaactgtt cgcttcggtggcagtactactcaaagaatgagctgctgttaaaaatgcactctcgttctctcaagtgagtggcagatgagtgctc acgccttgcacttcgctgcccgtgtcatgccctgcgccccaaaatttgaaaaaagggatgagattattgggcaatggacgacgt cgtcgctccgggagtcaggaccggcggaaaataagaggcaacacactccgcttcttagctcttcg** (SEQ ID NO:90)

[0488] pSZ2300 Transforming Construct:

gctcttccgctaacggaggtctgtcaccaaatggaccccgtctattgcgggaaaccacggcgatggcacgtttcaaaacttgat gaaatacaatattcagtatgtcgcgggcggcgacggcggggagctgatgtcgcgctgggtattgcttaatcgccagcttcgcc cccgtcttggcgcgaggcgtgaacaagccgaccgatgtgcacgagcaaatcctgacactagaagggctgactcgcccggca cggctgaattacacaggcttgcaaaaataccagaatttgcacgcaccgtattcgcggtattttgttggacagtgaatagcgatg cggcaatggcttgtggcgttagaaggtgcgacgaaggtggtgccaccactgtgccagccagtcctggcggctcccagggccc cgatcaagagccaggacatccaaactacccacagcatcaacgccccggcctatactcgaaccccacttgcactctgcaatggt atgggaaccacggggcagtcttgtgtgggtcgcgcctatcgcggtcggcgaagaccgggaaggtaccctttcttgcgctatgac acttccagcaaaaggtagggcgggctgcgagacggcttcccggcgctgcatgcaacaccgatgatgcttcgacccccccgaagctcc ttcggggctgcatgggcgctccgatgccgctccagggcgagcgctgtttaaatagccaggcccccgattgcaaagacattatagcga gctaccaaagccatattcaaacacctagatcactaccacttctacacaggccactcgagcttgtgatcgcactccgctaaggggcgc ctcttcctcttcgtttcagtcacaacccgcaaac**tctaga**atatca*ATGatcgagcaggacggcctccacgccggctcccccgccg cctgggtggagcgcctgttcggctacgactgggcccagcagaccatcggctgctccgacgccgccgtgttccgcctgtccgccca gggccgccccgtgctgttcgtgaagaccgacctgtccggcgccctgaacgagctgcaggacgaggccgcccgcctgtcctggct ggccaccaccggcgtgccctgcgccgccgtgctggacgtggtgaccgaggccggccgcgactggctgctgctgggcgaggtgc ccggccaggacctgctgtcctcccacctggcccccgccgagaaggtgtccatcatggccgacgccatgcgccgcctgcacaccc tggaccccgccacctgcccctttcgaccaccaggccaagcaccgcatcgagcgcgcccgcacccgcatggaggccggcctggtg gaccaggacgacctggacgaggagcaccagggcctggcccccgccgagctgttcgcccgcctgaaggcccgcatgcccgacg gcgaggacctggtggtgacccacggcgacgcctgcctgcccaacatcatggtggagaacggccgcttctccggcttcatcgactg cggccgcctgggcgtggccgaccgctaccaggacatcgccctggccacccgcgacatcgccgaggagctgggcggcgagtgg gccgaccgcttcctggtgctgtacggcatcgccgcccccgactcccagcgcatcgccttctaccgcctgctggacgagttcttc*TG A**caattg**gcagcagcagctcggatagtatcgacacactctggacgctggtcgtgtgatggactgttgccgccacacttgctgccttga cctgtgaatatccctgccgcttttatcaaacagcctcagtgtgtttgatcttgtgtgtacgcgcttttgcgagttgctagctgcttgtgctattt gcgaataccacccccagcatccccttccctcgtttcatatcgcttgcatcccaaccgcaacttatctacgctgtcctgctatccctcagcg ctgctcctgctcctgctcactgcccctcgcacagccttggtttgggctccgcctgtattctcctggtactgcaacctgtaaaccagcactg caatgctgatgcacgggaagtagtgggatgggaacacaaatgga**ggatcc**CGCGTCTCGAACAGAGCGCGCA GAGGAACGCTGAAGGTCTCGCCTCTGTCGCACCTCAGCGCGGCATACACCACAA

TAACCACCTGACGAATGCGCTTGGTTCTTCGTCCATTAGCGAAGCGTCCGGTTCA
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TCGAAACGTTCACAGCCTAGGGATATC**gaattc***ggccgacaggacgcgcgtcaaaggtgctggtcgtgt*
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gagggccgagggtttgggacgggccgtcccgaaatgcagttgcacccggatgcgtggcaccttttttgcgataatttatgcaatgg
actgctctgcaaaattctggctctgtcgccaaccctaggatcagcggcgtaggatttcgtaatcattcgtcctgatggggagctacc
gactaccctaatatcagcccgactgcctgacgccagcgtccacttttgtgcacacattccattcgtgcccaagacatttcattgtggt
*gcgaagcgtccccagttacgctcacctgtttcccgacctccttactgttctgtcgacagagcgggcccacaggccggtcgcagcc***a**
ctagt*ATGgccatcgcggcggccgcggtgatcgtgcccctgtccctgctgttcttcgtgtccggcctgatcgtcaacctggtgcag*
gccgtctgcttcgtcctgatccgccccctgtccaagaacacgtaccgccgcatcaaccgcgtggtcgcggagctgctgtggctgga
gctggtgtggctgatcgactggtgggcgggcgtgaagatcaaggtcttcacggaccacgagacgttccacctgatgggcaagga
gcacgccctggtcatctgcaaccacaagtccgacatcgactggctggtcggctgggtcctgggccagcgctccggctgcctgggc
tccaccctggcggtcatgaagaagtcctccaagttcctgcccgtcctgggctggtccatgtggttctccgagtacctgttcctggagc
gctcctgggccaaggacgagatcacgctgaagtccggcctgaaccgcctgaaggactacccccctgcccttctggctggcgctgtt
cgtggagggcacgcgcttcacccgcgcgaagctgctggcggcgcagcagtacgccgcgtcctccggcctgcccgtgccccgca
acgtgctgatcccccgcacgaagggcttcgtgtcctccgtgtcccacatgcgctccttcgtgcccgcgatctacgacgtcaccgtgg
ccatccccaagacgtccccccccccccacgctgatccgcatgttcaagggccagtcctccgtgctgcacgtgcacctgaagcgcca
cctgatgaaggacctgcccgagtccgacgacgccgtcgcgcagtggtgccgcgacatcttcgtggagaaggacgcgctgctgg
acaagcacaacgccgaggacaccttctccggccaggagctgcaggagaccggccgccccatcaagtccctgctggtcgtcatct
cctgggccgtcctggaggtgttcggcgccgtcaagttcctgcagtggtcctccctgctgtcctcctggaagggcctggcgttctccgg
catcggcctgggcgtgatcaccctgctgatgcacatcctgatcctgttctcccagtccgagcgctccacccccgccaaggtggccc
*ccgcgaagcccaagaacgagggcgagtcctccaagaccgagatggagaaggagaagTGAttaattaa***ctcgag**gcagcag
cagctcggatagtatcgacacactctggacgctggtcgtgtgatggactgttgccgccacacttgctgccttgacctgtgaatatccctg
ccgcttttatcaaacagcctcagtgtgtttgatcttgtgtgtacgcgcttttgcgagttgctagctgcttgtgctatttgcgaataccacccccc
agcatccccttccctcgtttcatatcgcttgcatcccaaccgcaacttatctacgctgtcctgctatccctcagcgctgctcctgctcctgct
cactgcccctcgcacagccttggtttgggctccgcctgtattctcctggtactgcaacctgtaaaccagcactgcaatgctgatgcacgg

gaagtagtgggatgggaacacaaatggaaagcttgagctcagcggcgacggtcctgctaccgtacgacgttgggcacgcccatg aaagtttgtataccgagcttgttgagcgaactgcaagcgcggctcaaggatacttgaactcctggattgatatcggtccaataa tggatggaaaatccgaacctcgtgcaagaactgagcaaacctcgttacatggatgcacagtcgccagtccaatgaacattga agtgagcgaactgttcgcttcggtggcagtactactcaaagaatgagctgctgttaaaaatgcactctcgttctctcaagtgagt ggcagatgagtgctcacgcctgcacttcgctgcccgtgtcatgccctgcgccccaaaatttgaaaaaagggatgagattattgg gcaatggacgacgtcgtcgctccgggagtcaggaccggcggaaaataagaggcaacacactccgcttcttagctcttcg (SEQ ID NO:91)

[0489] To determine the impact of the *Cu*PSR23 *LPAAT2* and *LPAAT3* genes on mid-chain fatty acid accumulation, the above constructs containing the codon optimized *Cu*PSR23 *LPAAT2* or *LPAAT3* genes driven by the UTEX 1453 *AMT3* promoter were transformed into strain B.

[0490] Primary transformants were clonally purified and grown under standard lipid production conditions at pH7.0 (all the strains require growth at pH 7.0 to allow for maximal expression of the *Cu*PSR23 *LPAAT2* or *LPAAT3* gene driven by the pH-regulated *AMT3* promoter). The resulting profiles from a set of representative clones arising from these transformations are shown in Table 34, below. D1520 represents clones of Strain B with *Cu*PSR23 *LPAAT2* and D1521 represents clones of Strain B with *Cu*PSR23 *LPAAT3*.

[0491] Table 34. Fatty acid profiles of Strain B and representative transgenic lines transformed with pSZ2299 and pSZ2300 DNA.

Sample ID	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2
Strain B	4.83	28.54	15.64	12.64	1.3	27.99	7.75
Sample ID	**C10:0**	**C12:0**	**C14:0**	**C16:0**	**C18:0**	**C18:1**	**C18:2**
D1520-A	8.59	35.09	16.55	11.96	1.69	19.49	5.59
D1520-B	8.13	33.93	16.46	12.44	1.57	20.66	5.96
D1520-C	7.6	33.1	16.21	12.65	1.5	21.41	6.48
D1520-D	7.35	32.54	16.03	12.79	1.67	22.16	6.41
D1520-E	7.28	32.21	16.2	12.99	1.73	22.39	6.28
Sample ID	**C10:0**	**C12:0**	**C14:0**	**C16:0**	**C18:0**	**C18:1**	**C18:2**
D1521-A	6.14	31.5	15.98	12.96	1.96	22.52	8
D1521-B	6.17	31.38	15.98	12.87	2.08	22.54	7.92
D1521-C	5.99	31.31	15.75	12.79	2.23	22.45	8.36
D1521-D	5.95	31.05	15.71	12.84	2.48	22.69	8.32
D1521-E	5.91	30.58	15.85	13.22	1.97	23.55	7.84

[0492] The transgenic *Cu*PSR23 *LPAAT2* strains (D1520A-E) show a significant increase in the accumulation of C10:0, C12:0, and C14:0 fatty acids with a concomitant decrease in C18:1 and C18:2. The transgenic *Cu*PSR23 *LPAAT3* strains (D1521A-E) show a significant increase in the accumulation of C10:0, C12:0, and C14:0 fatty acids with a concomitant

decrease in C18:1. The expression of the *Cu*PSR23 *LPAAT* in these transgenic lines appears to be directly responsible for the increased accumulation of mid-chain fatty acids in general, and especially laurates. While the transgenic lines show a shift from longer chain fatty acids (C16:0 and above) to mid-chain fatty acids, the shift is targeted predominantly to C10:0 and C12:0 fatty acids with a slight effect on C14:0 fatty acids. The data presented also show that co-expression of the *LPAAT2* and *LPAAT3* genes from *Cuphea* PSR23 and the *FATB2* from *C. wrightii* (expressed in the strain Strain B) have an additive effect on the accumulation of C12:0 fatty acids.

[0493] Our results suggest that the LPAAT enzymes from *Cuphea* PSR23 are active in the algal strains derived from UTEX 1435. These results also demonstrate that the enzyme functions in conjunction with the heterologous FatB2 acyl-ACP thioesterase enzyme expressed in Strain B, which is derived from *Cuphea wrightii.*

EXAMPLE 44: ALTERATION OF FATTY ACID LEVELS IN STRAIN UTEX1435 BY THE EXPRESSION OF *CUPHEA* PSR23 *LPAATX* IN COMBINATION WITH *CUPHEA WRIGHTII FATB2*

[0494] Here we demonstrate the effect of expression of a 1-acyl-sn-glycerol–3-phosphate acyltransferase (*LPAAT*) in a previously described *P. moriformis* (UTEX 1435) transgenic strain, Strain B. As described above, Strain B is a transgenic strain expressing the acyl ACP thioesterase (*FATB2*) from *Cuphea wrightii,* which accumulates C12:0 fatty acids between 40 to 49%. Further to Example 43, a third *Cu*PSR23 LPAAT, *LPAATx*, was identified by analysis of a combination of *Cu*PSR23 genomic sequences and transcriptomic sequences derived from seed RNAs. Expression of a mid-chain specific LPAAT should thus increase the percentage of TAGs that have a capric acid (C10:0 fatty acid), lauric acid (C12:0 fatty acid), or myristic acid (C14:0 fatty acid) at the *sn*-2 position, and should consequently elevate the overall levels of these fatty acids. In Example 43, *LPAAT2* and *LPAAT3* were shown to increase caprate, laurate, and myristate accumulation in strain B. *LPAATx* was introduced into strain B to determine its effect on fatty acid levels in this strain. The *LPAATx* gene was codon optimized to reflect UTEX 1435 codon usage. Transformations, cell culture, lipid production and fatty acid analysis were all carried out as previously described.

[0495] Decreased caprate, laurate, and myristate accumulation and increased palmitate and stearate accumulation in strain Strain B by the expression of the *Cuphea* PSR23 1-acyl-sn-glycerol–3-phosphate acyltransferase (*LPAATx*) [pSZ2575]: In this example, transgenic strains were generated via transformation of strain B with the construct

pSZ2575 encoding *Cu*PSR23 LPAATx. The transgenic strains were selected for resistance to the antibiotic G418. Construct pSZ2575 can be written as pLOOP5'::CrTUB2:NeoR:CvNR::PmAMT3:CuPSR23LPAATx:CvNR ::pLOOP3'. The sequence of the transforming DNA is provided below (pSZ2575). The relevant restriction sites in the construct from 5'-3', BspQ1, KpnI, XbaI, MfeI, BamHI, EcoRI, SpeI, XhoI, SacI, BspQ1, respectively, are indicated in lowercase, bold, and underlined. BspQ1 sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences at the 5' and 3' end of the construct represent genomic DNA from UTEX 1435 that target integration to the *pLoop* locus via homologous recombination. Proceeding in the 5' to 3' direction, the selection cassette has the *C. reinhardtii β-tubulin* promoter driving expression of the *NeoR* gene (conferring resistance to G418) and the *Chlorella vulgaris Nitrate Reductase* (*NR*) gene 3' UTR. The promoter is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for *NeoR* are indicated by uppercase italics, while the coding region is indicated with lowercase italics. The 3' UTR is indicated by lowercase underlined text. The spacer region between the two cassettes is indicated by upper case text. The second cassette containing the codon optimized *LPAATx* gene (pSZ2575) from *Cuphea* PSR23 is driven by the *Prototheca moriformis* endogenous *AMT3* promoter, and has the same *Chlorella vulgaris Nitrate Reductase* (*NR*) gene 3' UTR. In this cassette, the *AMT3* promoter is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for the *Cu*PSR23 *LPAATx* genes are indicated in uppercase italics, while the coding region is indicated by lowercase italics. The 3' UTR is indicated by lowercase underlined text. The final construct was sequenced to ensure correct reading frame and targeting sequences.

[0496] pSZ2575 Transforming Construct

gctcttccgctaacggaggtctgtcaccaaatggaccccgtctattgcgggaaaccacggcgatggcacgtttcaaaacttgat gaaatacaatattcagtatgtcgcgggcggcgacggcggggagctgatgtcgcgctgggtattgcttaatcgccagcttcgcc cccgtcttggcgcgaggcgtgaacaagccgaccgatgtgcacgagcaaatcctgacactagaagggctgactcgcccggca cggctgaattacacaggcttgcaaaaataccagaatttgcacgcaccgtattcgcggtattttgttggacagtgaatagcgatg cggcaatggcttgtggcgttagaaggtgcgacgaaggtggtgccaccactgtgccagccagtcctggcggctcccagggccc cgatcaagagccaggacatccaaactacccacagcatcaacgccccggcctatactcgaaccccacttgcactctgcaatggt atgggaaccacggggcagtcttgtgtgggtcgcgcctatcgcggtcggcgaagaccgggaaggtaccctttcttgcgctatgac acttccagcaaaaggtagggcgggctgcgagacggcttcccggcgctgcatgcaacaccgatgatgcttcgacccccccgaagctcc ttcggggctgcatgggcgctccgatgccgctccagggcgagcgctgtttaaatagccaggcccccgattgcaaagacattatagcga gctaccaaagccatattcaaacacctagatcactaccacttctacacaggccactcgagcttgtgatcgcactccgctaaggggcgc

ctcttcctcttcgtttcagtcacaacccgcaaac**tctaga**atatca*ATGatcgagcaggacggcctccacgccggctcccccgccg*
cctgggtggagcgcctgttcggctacgactgggcccagcagaccatcggctgctccgacgccgccgtgttccgcctgtccgccca
gggccgccccgtgctgttcgtgaagaccgacctgtccggcgccctgaacgagctgcaggacgaggccgcccgcctgtcctggct
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ccggccaggacctgctgtcctcccacctggcccccgccgagaaggtgtccatcatggccgacgccatgcgccgcctgcacaccc
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gccgaccgcttcctggtgctgtacggcatcgccgcccccgactcccagcgcatcgccttctaccgcctgctggacgagttcttcTG
*A***caattg**gcagcagcagctcggatagtatcgacacactctggacgctggtcgtgtgatggactgttgccgccacacttgctgccttga
cctgtgaatatccctgccgcttttatcaaacagcctcagtgtgtttgatcttgtgtgtacgcgcttttgcgagttgctagctgcttgtgctattt
gcgaataccacccccagcatccccttccctcgtttcatatcgcttgcatcccaaccgcaacttatctacgctgtcctgctatccctcagcg
ctgctcctgctcctgctcactgcccctcgcacagccttggtttgggctccgcctgtattctcctggtactgcaacctgtaaaccagcactg
caatgctgatgcacgggaagtagtgggatgggaacacaaatgga**ggatcc**CGCGTCTCGAACAGAGCGCGCA
GAGGAACGCTGAAGGTCTCGCCTCTGTCGCACCTCAGCGCGGCATACACCACAA
TAACCACCTGACGAATGCGCTTGGTTCTTCGTCCATTAGCGAAGCGTCCGGTTCA
CACACGTGCCACGTTGGCGAGGTGGCAGGTGACAATGATCGGTGGAGCTGATGG
TCGAAACGTTCACAGCCTAGGGATATC**gaattc***ggccgacaggacgcgcgtcaaaggtgctggtcgtgt*
atgccctggccggcaggtcgttgctgctgctggttagtgattccgcaaccctgattttggcgtcttattttggcgtggcaaacgctggc
gcccgcgagccgggccggcggcgatgcggtgccccacggctgccggaatccaagggaggcaagagcgcccgggtcagttga
agggctttacgcgcaaggtacagccgctcctgcaaggctgcgtggtggaattggacgtgcaggtcctgctgaagttcctccaccg
cctcaccagcggacaaagcaccggtgtatcaggtccgtgtcatccactctaaagagctcgactacgacctactgatggccctaga
ttcttcatcaaaaacgcctgagacacttgcccaggattgaaactccctgaagggaccaccaggggccctgagttgttccttccccc
cgtggcgagctgccagccaggctgtacctgtgatcgaggctggcgggaaaataggcttcgtgtgctcaggtcatgggaggtgca
ggacagctcatgaaacgccaacaatcgcacaattcatgtcaagctaatcagctatttcctcttcacgagctgtaattgtcccaaaat
tctggtctaccgggggtgatccttcgtgtacgggcccttccctcaaccctaggtatgcgcgcatgcggtcgccgcgcaactcgcgc
gagggccgagggtttgggacgggccgtcccgaaatgcagttgcacccggatgcgtggcacctttttgcgataatttatgcaatgg
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gactaccctaatatcagcccgactgcctgacgccagcgtccacttttgtgcacacattccattcgtgcccaagacatttcattgtggt
*gcgaagcgtccccagttacgctcacctgtttcccgacctccttactgttctgtcgacagagcgggcccacaggccggtcgcagcc***a**
ctagt*ATGgagatccccccccactgcctgtgctccccctccccgccccctcccagctgtactacaagaagaagaagcacgcc*
atcctgcagacccagaccccctaccgctaccgcgtgtccccccacctgcttcgccccccccccgcctgcgcaagcagcacccctacc

*ccctgcccgtgctgtgctaccccaagctgctgcacttctcccagccccgctacccccctggtgcgctcccacctggccgaggccggc gtggcctaccgccccggctacgagctgctgggcaagatccgcggcgtgtgcttctacgccgtgaccgccgccgtggccctgctgct gttccagtgcatgctgctgctgcaccccttegtgctgctgttcgacccctteccccgcaaggcccaccacaccatcgccaagctgtg gtccatctgctccgtgtccctgttctacaagatccacatcaagggcctggagaacctgccccccccccactcccccgccgtgtacgt gtccaaccaccagtccttcctggacatctacaccctgctgaccctgggccgcaccttcaagttcatctccaagaccgagatcttcctg taccccatcatcggctgggccatgtacatgctgggcaccatcccccctgaagcgcctggactcccgctcccagctggacaccctga agcgctgcatggacctgatcaagaagggcgcctccgtgttcttcttccccgagggcacccgctccaaggacggcaagctgggcg ccttcaagaagggcgccttctccatcgccgccaagtccaaggtgcccgtggtgcccatcaccctgatcggcaccggcaagatcat gccccccggctccgagctgaccgtgaaccccggcaccgtgcaggtgatcatccacaagcccatcgagggctccgacgccgagg ccatgtgcaacgaggcccgcgccaccatctcccactccctggacgacTGAttaattaa***<u>**ctcgag**</u>***gcagcagcagctcggatagt atcgacacactctggacgctggtcgtgtgatggactgttgccgccacacttgctgccttgacctgtgaatatccctgccgcttttatcaaa cagcctcagtgtgtttgatcttgtgtgtacgcgcttttgcgagttgctagctgcttgtgctatttgcgaataccacccccagcatccccttcc ctcgtttcatatcgcttgcatcccaaccgcaacttatctacgctgtcctgctatccctcagcgctgctcctgctcctgctcactgcccctcgc acagccttggtttgggctccgcctgtattctcctggtactgcaacctgtaaaccagcactgcaatgctgatgcacgggaagtagtgggat gggaacacaaatggaaagctt**<u>**gagctc**</u>**agcggcgacggtcctgctaccgtacgacgttgggcacgcccatgaaagtttgtatac cgagcttgttgagcgaactgcaagcgcggctcaaggatacttgaactcctggattgatatcggtccaataatggatggaaaat ccgaacctcgtgcaagaactgagcaaacctcgttacatggatgcacagtcgccagtccaatgaacattgaagtgagcgaact gttcgcttcggtggcagtactactcaaagaatgagctgctgttaaaaatgcactctcgttctctcaagtgagtggcagatgagtg ctcacgccttgcacttcgctgcccgtgtcatgccctgcgccccaaaatttgaaaaaagggatgagattattgggcaatggacga cgtcgtcgctccgggagtcaggaccggcggaaaataagaggcaacacactccgcttcttag<u>**ctcttcg**</u>*** (SEQ ID NO:92)

[0497] To determine the impact of the *Cu*PSR23 *LPAATx* gene on fatty acid accumulation, the above construct containing the codon optimized *Cu*PSR23 *LPAATx* gene driven by the UTEX 1453 *AMT*3 promoter was transformed into strain B.

[0498] Primary transformants were clonally purified and grown under low nitrogen conditions at pH7.0; the strains require growth at pH 7.0 to allow for maximal expression of the *Cu*PSR23 *LPAATx* and *CwFATB2* genes driven by the pH-regulated *AMT3* promoter. The resulting profiles from a set of representative clones arising from these transformations are shown in Table 35, below. D1542 represents clones of Strain B with *Cu*PSR23 *LPAATx*.

[0499] Table 35. Fatty acid profiles of Strain B and representative transgenic lines transformed with pSZ2575.

Sample ID	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2

Strain B	4.77	28.63	15.48	12.65	1.28	28.20	7.57
Sample ID	**C10:0**	**C12:0**	**C14:0**	**C16:0**	**C18:0**	**C18:1**	**C18:2**
D1542-A	1.19	13.25	10.48	21.34	4.49	32.07	14.78
D1542-B	1.15	14.01	10.62	20.61	3.99	32.12	15.24
D1542-C	1.21	13.69	10.83	20.40	3.59	33.54	15.05
D1542-D	1.56	16.83	11.51	18.44	2.94	33.97	12.74
D1542-E	2.15	18.58	11.94	18.22	3.17	32.63	11.62

[0500] The transgenic *Cu*PSR23 *LPAATx* strains (D1542A-E) show a significant decrease in the accumulation of C10:0, C12:0, and C14:0 fatty acids relative to the parent, Strain B, with a concomitant increase in C16:0, C18:0, C18:1 and C18:2. The expression of the *Cu*PSR23 *LPAATx* gene in these transgenic lines appears to be directly responsible for the decreased accumulation of mid-chain fatty acids (C10-C14) and the increased accumulation of C16:0 and C18 fatty acids, with the most pronounced increase observed in palmitates (C16:0). The data presented also show that despite the expression of the midchain specific *FATB2* from *C. wrightii* (present in Strain B), the expression of *Cu*PSR23 *LPAATx* appears to favor incorporation of longer chain fatty acids into TAGs.

[0501] Our results suggest that the *LPAATx* enzyme from *Cuphea* PSR23 is active in the algal strains derived from UTEX 1435. Contrary to *Cuphea* PSR23 *LPAAT2* and *LPAAT3*, which increase mid-chain fatty acid levels, *Cu*PSR23 *LPAATx* leads to increased C16:0 and C18:0 levels. These results demonstrate that the different LPAATs derived from CuPSR23 (*LPAAT2*, *LPAAT3*, and *LPAATx*) exhibit different fatty acid specificities in Strain B as judged by their effects on overall fatty acid levels.

EXAMPLE 45: REDUCTION IN CHAIN LENGTH OF FATTY ACID PROFILE AS A RESULT OF OVEREXPRESSING AN ENDOGENOUS MICROALGAL FATA ACYL-ACP THIOESTERASE

[0502] Here, we demonstrate that over expression of the Prototheca moriformis endogenous thioesterases FATA1 in UTEX1435 results in a clear diminution of cell triglyceride C18:0 and C18:1 acyl chains with an increase in C16:0, C14:0.

[0503] **Constructs used for the over expression of the *P. moriformis* FATA1 gene (pSZ2422, pSZ2421):** To over express the PmFATA1 in P. moriformis STRAIN J, a codon optimized PmFATA1 gene was been transformed into STRAIN J. The Saccharomyces cerevisiae invertase gene was utilized as the selectable marker to confer the ability of growing on sucrose media. The construct pSZ2422 that have been expressed in STRAIN J

can be written as: 6SA:: CrTUB2-ScSUC2-CvNR3':PmAMT3-Pm FATA1 (opt)-CvNR3'::6SB, and the construct pSZ2421 can be written as 6SA:: CrTUB2-ScSUC2-CvNR3':PmAMT3-S106SAD TP-Pm FATA1 (opt)-CvNR3'::6SB.

[0504] The sequence of the transforming DNA is provided below. Relevant restriction sites in the construct pSZ2422 are indicated in lowercase, bold and underlining and are 5'-3' BspQ 1, Kpn I, Xba I, Mfe I, BamH I, EcoR I, Spe I, Asc I, Cla I, Sac I, BspQ I, respectively. BspQI sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent genomic DNA from STRAIN J that permit targeted integration at 6s locus via homologous recombination. Proceeding in the 5' to 3' direction, the C. reinhardtii β-tubulin promoter driving the expression of the yeast sucrose invertase gene (conferring the ability of STRAIN J to metabolize sucrose) is indicated by boxed text. The initiator ATG and terminator TGA for invertase are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics. The Chlorella vulgaris nitrate reductase 3' UTR is indicated by lowercase underlined text followed by an endogenous amt03 promoter of P. moriformis, indicated by boxed italics text. The Initiator ATG and terminator TGA codons of the PmFATA1 are indicated by uppercase, bold italics, while the remainder of the gene is indicated by bold italics. The C. vulgaris nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the STRAIN J 6S genomic region indicated by bold, lowercase text.

[0505] Relevant restriction sites in the construct pSZ2421 are the same as pSZ2422. In pSZ2421, the PmFATA1 is fused to the Chlorella protothecoides S106 stearoyl-ACP desaturase transit peptide and the transit peptide is located between initiator ATG of PmFATA1 and the Asc I site.

[0506] Nucleotide sequence of transforming DNA contained in pSZ2422

gctcttcgccgccgccactcctgctcgagcgcgcccgcgcgtgcgccgccagcgccttggccttttcgccgcgctcgtgcgcgtc
gctgatgtccatcaccaggtccatgaggtctgccttgcgccggctgagccactgcttcgtccgggcggccaagaggagcatga
gggaggactcctggtccagggtcctgacgtggtcgcggctctgggagcgggccagcatcatctggctctgccgcaccgaggc
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*cgctcacctgtttcccgacctccttactgttctgtcgacagagcgggcccacaggccggtcgcagcc***actagt***ATGgccccac***

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tcaaaccgcgtacctctgctttcgcgcaatctgccctgttgaaatcgccaccacattcatattgtgacgcttgagcagtctgtaat

tgcctcagaatgtggaatcatctgccccctgtgcgagcccatgccaggcatgtcgcgggcgaggacacccgccactcgtacag

cagaccattatgctacctcacaatagttcataacagtgaccatatttctcgaagctccccaacgagcacctccatgctctgagtg

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[0507] To determine the impact on fatty acid profiles when the endogenous FATA1 gene have been over expressed in STRAIN J, both the P. moriformis FATA1 with native transit peptide and PmFATA1 fused to a Chlorella protothecoides SAD transit peptide were driven by the amt03 promoter and the resulting plasmids were transformed independently into STRAIN J.

[0508] Primary transformants were clonally purified and grown under low-nitrogen lipid production conditions at pH7.0 (all the plasmids require growth at pH 7.0 to allow for maximal PmFATA1 gene expression when driven by the pH regulated amt03 promoter). The resulting profiles from representative clones arising from transformations with pSZ2422 and pSZ2421 into STRAIN J are shown in the tables below.

[0509] In Table 36, below, the impact of over expressing native PmFATA1 is a clear diminution of C18:1 chain lengths with an increase in C16:0, C14:0, and possibly in C18:0. Considering the protein localization of processing, we also tried the PmFATA1 fused to a Chlorella protothecoides stearoyl-ACP desaturase transit peptide. Similar to the results we observed in the amt03-native PmFATA1 construct, the C16:0 and C14:0 levels are significantly higher than the parental strain J.

[0510] Table 36. Fatty acid profiles in Strain J and derivative transgenic lines transformed with pSZ2422 DNA.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2
pH 7; Strain J; T374; D1377-7 96well	7.69	55.00	4.92	24.94	5.19
pH 7; Strain J; T374; D1377-13 96well	6.39	54.11	5.85	25.91	5.76
pH 7; Strain J; T374; D1377-14 96well	6.57	53.55	4.68	27.18	5.74
pH 7; Strain J; T374; D1377-16 96well	5.29	49.93	4.24	30.76	7.27
pH 7; Strain J; T374; D1377-9 96well	4.76	49.10	4.75	32.36	6.77
pH 7; Strain J; T374; D1377-19 96well	4.28	46.06	5.14	35.87	6.69

Ctrl-pH7; Strain J	1.42	27.63	3.31	57.20	8.00

[0511] Table 37. Fatty acid profiles in STRAIN J and derivative transgenic lines transformed with pSZ2421 DNA.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2
pH 7; STRAIN J; T374; D1376-21 96well	6.76	57.06	4.12	23.66	6.07
pH 7; STRAIN J; T374; D1376-22 96well	6.56	54.62	5.44	25.69	5.64
pH 7; STRAIN J; T374; D1376-23 96well	4.54	48.38	4.27	33.23	7.24
pH 7; STRAIN J; T374; D1376-19 96well	4.48	47.66	4.60	34.28	6.91
pH 7; STRAIN J; T374; D1376-20 96well	4.53	47.30	4.67	34.51	6.80
pH 7; STRAIN J; T374; D1376-17 96well	3.56	42.70	4.03	39.85	7.52
Ctrl-pH7; STRAIN J	1.42	27.63	3.31	57.20	8.00

[0512] Thus, we conclude that percent myristic and lauric acid levels in the fatty acid profile of a microalgal cell can be increased by overexpression of a C18-preferring acyl-ACP thioesterase.

EXAMPLE 46: CELL OILS SUITABLE FOR USE AS ROLL-IN SHORTENINGS

[0513] The nutritional and functional properties of edible fats have been traditionally associated with specific chemical compositions and crystallization conditions. Switching from one oil source to another is usually a difficult task since both the melting behavior and structure of the fat changes dramatically, leading to adverse changes in functionality. In recent history, we can recall the painful period when partially hydrogenated fats were replaced with palm oil and palm oil fractions. We examined how the yield stress, elastic modulus, polymorphism, microstructure and melting profile of two fats with vastly different chemical compositions can be matched. Oil A was produced from Prototheca moriformis cells expressing an exogenous invertase and an *Ulmus americana* acyl-ACP thioesterase with a Chlorella protothecoides plastid targeting sequence. Oil B was produced from Prototheca moriformis cells expressing an exogenous invertase and a *Cuphea hookeriana* acyl-ACP thioesterase. Oil A contained greater than 62% (w/w) medium chain fatty acids, or MCT (C8:0-C14:0), 23% (C16:0+C18:0) and 9% C18:1, while Oil B contained less than 2% C8:0-

C14:0, 54% (C16:0+C18:0) and 29% C18:1. Oil A was thus a medium chain triglyceride rich fat, while Oil B resembled palm oil. Both oils had a solid fat content of ~45% at 20°C, and very similar SFC versus temperature profiles. DSC (dynamic scanning calorimetry) melting profiles showed two major peaks centered around ~12-13°C and ~28-35°C. Both fats were in the beta-prime polymorphic form (as determined by X-ray diffraction) and displayed asymmetric, elongated crystallite morphology with characteristic features. The yield stresses and storage moduli (G') of Oil A and Oil B were 520-550 Pa, and $7x10^6$Pa-$1.8x10^7$Pa, respectively. A yield stress in this region suggests a satisfactory plasticity, which combined with a high storage modulus makes for an ideal roll-in shortening. Thus, it is possible to alter the chemical composition of a food oil while retaining its lamination functionality.

[0514] Other suitable enzymes for use with the cells and methods of any of the above embodiments of the invention include those that have at least 70% amino acid identity with one of the proteins listed in the description above and that exhibit the corresponding desired enzymatic activity. In additional embodiments, the enzymatic activity is present in a sequence that has at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% identity with one of the above described nucleic acid sequences, all of which are hereby incorporated by reference as if fully set forth.

EXAMPLE 47: FRACTIONATION TO REMOVE TRISATURATES FROM A TAILORED MICROBIAL OIL THAT IS A COCOA BUTTER MIMETIC

[0515] A refined bleached and deodorized oil was obtained from Strain K4 (see Example 35). The oil was heated to 70°C and cooled at 0.5°C per min to 36°C and held at 36°C for 1 hour. An approximately 2.5 ml sample was then centrifuged at 36°C for 1 hour at 4300. A liquid supernatant was recovered and analyzed using lipase and mass spectrometry. The sample was found to be depleted in tristearin (SSS), SSP, and PPS. The triacylglycerols of the sample were found to be very similar to that of cocoa butter and the liquid supernatant was even closer to that of cocoa butter in terms of low amounts of trisaturates. Further fractionation experiments are described in Example 64.

[0516] Table 38. TAG profile of oil from the K4 strain before and after fractionation as compared to cocoa butter.

TAG	K4 oil	fractionation upper layer (liquid)	cocoa butter
OOL (+?)	0.12	0.12	0.00

POL	0.23	0.31	0.33
PLP	2.41	3.38	1.58
MOP	0.93	1.25	0.00
PPM (+ MMS)	0.42	0.29	0.00
OOO	0.23	0.34	0.00
SOL	0.36	0.47	0.32
OOP	0.95	1.42	2.44
PLS	5.66	7.90	2.90
POP (+ MSO)	11.80	15.20	17.93
PPP + MPS	2.22	1.07	0.36
OOS	1.19	1.68	3.02
SLS (+ PLA)	3.96	5.11	1.77
POS	27.22	32.80	40.25
PPS (+ SSM)	6.47	1.52	0.49
MaOO	0.00	0.00	0.36
SLA	0.31	0.34	0.00
SOS (+ POA)	17.84	22.50	24.93
SSP (+ PPA)	9.24	0.96	0.63
SOA (+ POB)	1.39	1.68	1.51
SSS (+ PSA)	5.25	0.23	0.33
SOB + LgOP	0.38	0.44	0.27
SSA	0.41	0.00	0.00
SOLg	0.41	0.00	0.00

PSLg + ASB	0.26	0.00	0.00
SOHx	0.12	0.51	0.00
SSLg	0.21	0.14	0.15
SUM area %	100.00	99.67	99.57

EXAMPLE 48: PRODUCTION OF HIGH-STEARATE TRIGLYCERIDE OIL IN AN OLEAGINOUS CELL BY OVEREXPRESSION OF KASII, KNOCKOUT OF ONE SAD ALLELE AND REPRESSION OF A SECOND SAD ALLELE

[0517] The oleaginous, non-photosynthetic alga, *Prototheca moriformis*, stores copious amounts of triacylglyceride oil under conditions where the nutritional carbon supply is in excess, but cell division is inhibited due to limitation of other essential nutrients. Bulk biosynthesis of fatty acids with carbon chain lengths up to C18 occurs in the plastids; fatty acids are then exported to the endoplasmic reticulum where elongation past C18 and incorporation into triacylglycerides (TAGs) is believed to occur. Lipids are stored in large cytoplasmic organelles called lipid bodies until environmental conditions change to favor growth, whereupon they are rapidly mobilized to provide energy and carbon molecules for anabolic metabolism. Wild-type *P. moriformis* storage lipid is mainly comprised of ~60% oleic (C18:1), ~25-30% palmitic (C16:0), and ~5-8% linoleic (C18:2) acids, with minor amounts of stearic (C18:0), myristic (C14:0), α-linolenic (C18:3 α), and palmitoleic (C16:1) acids. This fatty acid profile results from the relative activities and substrate affinities of the enzymes of the endogenous fatty acid biosynthetic pathway. *P. moriformis* is amenable to manipulation of fatty acid and lipid biosynthesis using molecular genetic tools, enabling the production of oils with fatty acid profiles that are very different to the wild-type composition. Herein we describe strains where we have modified the expression of *stearoyl-ACP desaturase* (*SAD*) and *β-ketoacyl-ACP synthase II* (*KASII*) genes in order to generate strains with up to 57% stearate and as little as 7% palmitate. We identify additional strains with up to 55% stearate and as low as 2.4% linoleate when we perform similar modifications in conjunction with down-regulating the expression of the *FATA* thioesterase and the *FAD2* fatty acid desaturase genes.

[0518] Soluble SADs are plastid-localized, di-iron enzymes which catalyze the desaturation of acyl carrier protein (ACP)-bound stearate to oleate (C18:1 cis-Δ^9). Previously, we have established that hairpin constructs targeting the *SAD1* or *SAD2* transcripts activate the cellular

RNA interference (RNAi) machinery, down-regulating SAD activity and resulting in elevated levels of C18:0 in the storage lipid. SAD activity is also reduced in strains where we disrupt one of the two alleles of *SAD2*, encoding the major SADs that are expressed during storage lipid biosynthesis. The *Fatty Acid Desaturase 2* (*FAD2*) gene encodes an endoplasmic reticulum membrane-associated desaturase that converts oleate to linoleate (C18:2 cis-Δ^9, cis-Δ^{12}). Hairpin RNAi constructs targeting *FAD2* reduce linoleate levels to 1-2%. KASII is a fatty acid synthase which specifically catalyzes the condensation of malonyl-ACP with palmitoyl (C16:0)-ACP to form β-keto-stearoyl-ACP. We have shown that overexpression of KASII in *P. moriformis* causes C16:0 levels to decrease with a concomitant increase in C18:1 abundance. In the examples below we demonstrate that by down-regulating *SAD* gene expression using RNAi, disrupting an allele of the *SAD2* gene, and overexpressing the KASII fatty acid synthase, we generate strains capable of accumulating stearate in excess of 50% of the total fatty acids, and with SOS as the major TAG species. SOS levels increase up to 47% in strains which combine *SAD2* and *FAD2* down-regulation with *KASII* overexpression.

[0519] Constructs used for *SAD2* knockout/RNAi in S1920: A DNA construct, pSZ2282, was made to simultaneously disrupt the *SAD2-1* allele and express a *SAD2* hairpin construct in Strain J. A Saccharomyces cerevisiae *SUC2* gene, encoding sucrose invertase, which was codon-optimized for expression in *P. moriformis*, was utilized as a selectable marker for transformation. The sequence of the transforming DNA is provided immediately below. Relevant restriction sites are indicated in lowercase, bold, and are from 5'-3' BspQI, KpnI, AscI, MfeI, BamHI, AvrII, EcoRV, EcoRI, SpeI, BamHI, HinDIII, and SacI, respectively. BspQI sites delimit the 5' and 3' ends of the transforming DNA. Underlined sequences at the 5' and 3' flanks of the construct represent genomic DNA from *P. moriformis* that enable targeted integration of the transforming DNA via homologous recombination at the *SAD2-1* locus. Proceeding in the 5' to 3' direction, the *Chlamydomonas reinhardtii TUB2* promoter driving the expression of the *Saccharomyces cerevisiae SUC2* gene (encoding sucrose hydrolyzing activity, thereby permitting the strain to grow on sucrose) is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for *SUC2* are indicated by uppercase italics, while the coding region is indicated with lowercase italics. The 3' UTR of the *Chlorella vulgaris nitrate reductase* (*NR*) gene is indicated by small capitals, followed by a spacer region indicated by lowercase text. A second *C. reinhardtii TUB2* promoter sequence, indicated by lowercase boxed text, drives expression of the *SAD2* hairpin C sequence. The sense and antisense strands are indicated with uppercase, bold italics, and are separated by the *P. moriformis* Δ^{12}-fatty acid desaturase (*FAD2*) intron and the first 10 bases

of the *FAD2* second exon (uppercase italics). A second *C. vulgaris NR* 3' UTR is indicated by small capitals.

[0520] Nucleotide sequence of the transforming DNA from pSZ2282:

gctcttcgggtcgccgcgctgcctcgcgtcccctggtggtgcgcgcggtcgccagcgaggccccgctgggcgttccgccctcggtgca
gcgcccctcccccgtggtctactccaagctggacaagcagcaccgcctgacgcccgagcgcctggagctggtgcagagcatggggc
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*tctacatcgacaagttccaggtgcgcgaggtcaagTGA***caattg**GCAGCAGCAGCTCGGATAGTATCGACACACTCTGGAC

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[0521] **Identification and analysis of *SAD2* knockout/knockdown strains**: Construct D1283, derived from pSZ2282, was transformed into Strain J. Primary transformants were clonally purified and grown under standard lipid production conditions at pH 5. The resulting fatty acid profiles from representative clones arising from transformation with pSZ2282 into Strain J are summarized in Table 39, below. D1283 transformants accumulated up to ~42% C18:0 at the expense of C18:1, indicating that SAD activity was significantly reduced in these strains.

[0522] Table 39. Fatty acid profiles of D1283 [pSZ2282] primary transformants, compared to the wild-type parental strain, Strain J.

Strain		J	D1283-4	D1283-7	D1283-19	D1283-27	D1283-40	D1283-24
Fatty Acid Area %	C12:0	0.04	0.05	0.06	0.07	0.06	0.04	0.05
	C14:0	1.31	0.92	1.07	1.01	1.08	1.03	0.96
	C16:0	26.68	28.23	29.21	27.24	27.67	27.02	27.07
	C16:1	0.78	0.05	0.06	0.08	0.33	0.14	0.12
	C17:0	0.11	0.12	0.15	0.10	0.10	0.12	0.13
	C18:0	3.15	**41.98**	**40.94**	**34.20**	**26.26**	**23.18**	**22.82**
	C18:1	59.30	19.37	18.17	26.87	34.77	38.74	39.38
	C18:2	7.47	6.22	7.43	7.42	7.31	7.25	7.38
	C18:3α	0.57	0.93	1.03	0.75	0.71	0.72	0.51
	C20:0	0.32	1.81	1.67	1.75	1.35	1.36	1.23
	C20:1	0.00	0.10	0.00	0.12	0.00	0.12	0.11
	C22:0	0.05	0.17	0.13	0.20	0.16	0.16	0.15
	C24:0	0.00	0.00	0.00	0.10	0.00	0.00	0.00
sum C18		70.49	68.5	67.57	69.24	69.05	69.89	70.09
saturates		31.66	73.28	73.23	64.67	56.68	52.91	52.41
unsaturates		68.12	26.67	26.69	35.24	43.12	46.97	47.50

[0523] In Table 39, Stearate (C18:0) levels greater than the wild-type level are highlighted with bold text.

[0524] The fatty acid profiles of transformants D1283-4 and -7 were determined to be stable after more than 30 generations of growth in the absence of selection (growth on sucrose). The performance of selected strains in shake flask assays was then evaluated, and the fatty acid profiles and lipid titers are presented in Table 40, below. Strain X had the

highest level of C18:0 (~44%) and the best lipid titer (~26%) relative to the Strain J parent, and so was selected for further fermentation development.

[0525] Table 40. Fatty acid profiles and lipid titers of SAD2 knockout/knock-down strains derived from D1283 primary transformants, compared to the wild-type parental strain, Strain J.

Primary			T342;D1283-4			T342;D1283-7		
Strain		J	S	T	U	V	W	X
Fatty Acid Area %	C14:0	1.59	1.61	1.58	1.55	1.81	1.84	1.34
	C16:0	30.47	29.41	28.58	29.24	28.77	29.09	28.47
	C16:1	0.82	0.05	0.07	0.05	0.07	0.05	0.06
	C17:0	0.10	0.30	0.29	0.28	0.46	0.37	0.19
	C18:0	3.58	**42.85**	**41.86**	**43.38**	**39.99**	**41.41**	**44.42**
	C18:1	56.96	13.52	15.55	13.49	13.57	12.98	15.64
	C18:2	5.50	8.01	7.85	7.65	10.37	9.47	5.72
	C18:3α	0.37	0.78	0.73	0.82	0.95	0.91	0.64
	C20:0	0.22	2.06	2.11	2.11	1.98	1.98	2.32
	C22:0	0.05	0.32	0.34	0.33	0.33	0.32	0.35
	C24:0	0.03	0.43	0.42	0.44	0.49	0.49	0.37
lipid titer (% parent)		100	12.3	12.6	13.6	6.2	8.2	25.9

[0526] In Table 40, Stearate (C18:0) levels greater than the wild-type level are highlighted with bold text.

[0527] We optimized the performance of Strain X in 7-L fermentations, and found that we could match the ~44% C18:0 level obtained in shake flasks, with lipid productivities that were ~45% of the wild-type parent. The fatty acid profiles and lipid titers of representative strain K-4 fermentations are summarized in Table 41, below. Fermentation of Strain X under optimal conditions yielded nearly 44% C18:0, which was similar to the stearate level that accumulated in shake flask assays. Strain X produced high C18:0 levels at both flask and 7-L scale and had acceptable lipid productivity in 7-L fermentations; consequently this strain was selected as a base strain for additional modifications aimed at increasing C18:0 accumulation.

[0528] Table 41. Fatty acid profiles and lipid titers of Strain X, compared to a control transgenic Strain Y.

Strain		Y	K-4	K-4	K-4
Fermentation		110088F14	120489F5	120531F8	120580F1
Fatty Acid Area %	C14:0	1.47	1.18	1.15	1.27
	C16:0	25.66	28.68	28.38	28.35
	C16:1	0.71	0.11	0.09	0.06

	C18:0	3.16	**41.63**	**42.40**	**43.67**
	C18:1	62.24	20.78	19.38	17.63
	C18:2	5.90	5.06	5.38	5.58
	C18:3α	0.16	0.24	0.25	0.25
	C20:0	0.24	1.36	1.99	2.11
	C22:0	0.05	0.19	0.28	0.31
	C24:0	0.05	0.34	0.29	0.31
sum C18		71.46	67.71	67.41	67.13
saturates		30.63	73.38	74.49	76.02
unsaturates		69.01	26.19	25.10	23.52
total lipid (g/L)		930	383	539	475

[0529] In Table 41, Stearate (C18:0) levels greater than the control are highlighted with bold text. Strain Y contains S. cerevisiae SUC2, encoding sucrose invertase, integrated at the 6S locus, and has a fatty acid profile that is indistinguishable from the Strain J wild-type parent.

[0530] **Constructs used for *KASII* overexpression in Strain K-4:** DNA construct pSZ2734 was made to overexpress a codon-optimized *P. moriformis KASII* gene in Strain X. The *neoR* gene from transposon *Tn5*, conferring resistance to aminoglycoside antibiotics, was used as a selectable marker for transformation. The sequence of the transforming DNA is provided immediately below. Relevant restriction sites are indicated in lowercase, bold, and are from 5'-3' BspQI, KpnI, XbaI, MfeI, BamHI, AvrII, EcoRV, SpeI, AscI, ClaI, BglII, AflII, HinDIII and SacI, respectively. BspQI sites delimit the 5' and 3' ends of the transforming DNA. Underlined sequences at the 5' and 3' flanks of the construct represent genomic DNA from *P. moriformis* that enable targeted integration of the transforming DNA via homologous recombination at the *6S* locus. Proceeding in the 5' to 3' direction, the *C. reinhardtii TUB2* promoter driving the expression of *neoR* (encoding aminoglycoside phosphotransferase activity, thereby permitting the strain to grow on G418) is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for *neoR* are indicated by uppercase italics, while the coding region is indicated with lowercase italics. The 3' UTR of the *C. vulgaris NR* gene is indicated by small capitals, followed by a spacer region indicated by lowercase text. The *P. moriformis SAD2-2* promoter sequence, indicated by boxed text, drives expression of the codon-optimized *P. moriformis KASII* gene. The region encoding the KASII plastid targeting sequence is indicated by uppercase italics. The sequence that encodes the mature *P. moriformis* KASII polypeptide is indicated with bold, uppercase italics, while a

3xFLAG epitope encoding sequence is in bold, underlined, uppercase italics. A second *C. vulgaris NR* 3' UTR is indicated by small capitals.

[0531] Nucleotide sequence of the transforming DNA from pSZ2734:

gctcttcgccgccgccactcctgctcgagcgcgcccgcgcgtgcgccgccagcgccttggccttttcgccgcgctcgtgcgcgtcgct
gatgtccatcaccaggtccatgaggtctgccttgcgccggctgagccactgcttcgtccgggcggccaagaggagcatgagggagg
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GCTCGCGTGCATGCCTCGCCCCGTCGGGTGTCATGACCGGGACTGGAATCCCCCCTCGCGACCCTCC

TGCTAACGCTCCCGACTCTCCCGCCCGCGCGCAGGATAGACTCTAGTTCAACCAATCGACA**actagt***AT*

GCAGACCGCCCACCAGCGCCCCCCCACCGAGGGCCACTGCTTCGGCGCCCGCCTGCCCACCGCCTCCC

*GCCGCGCCGTGCGCCGCGCCTGGTCCCGCATCGCCCGCG***ggcgcgcc*** ***GCCGCCGCCGCCGACGCCAAC***

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gagctt**gaagagc** (SEQ ID NO:95)

[0532] **Overexpression of *KASII* in Strain X:** Construct D1643 derived from pSZ2734 was transformed into Strain X as described previously. Primary transformants were clonally purified and grown under standard lipid production conditions at pH 5. The resulting fatty acid profiles from representative clones arising from transformation of Strain X with D1643 are summarized in Table 42, below. Overexpression of *KASII* in the *SAD2* knockout/knock-down Strain K-4 background resulted in multiple strains accumulating over 50% C18:0 and with substantially reduced levels of C16:0. We also observed that *KASII* over-expressing lines had lower overall ratios of saturated to unsaturated fatty acids compared to Strain X.

[0533] Table 42. Fatty acid profiles of D1653 [pSZ2734] primary transformants, compared to the Strain X base strain and the wild-type parental strain, Strain J.

Strain		J	K-4	D1653-89	D1653-10A	D1653-2B	D1653-5B	D1653-7A	D1653-75	D1653-90	D1653-9B	D1653-72	D1653-6B	D1653-82	D1653-66
Fatty Acid Area %	C12:0	0.04	0.06	0.27	0.13	0.20	0.19	0.24	0.13	0.12	0.27	0.16	0.18	0.25	0.22
	C14:0	1.44	1.06	1.55	1.65	1.79	1.67	1.70	1.53	1.50	1.74	1.57	1.64	1.48	1.56
	C16:0	29.23	29.83	**8.16**	**11.45**	**10.68**	**10.11**	**9.27**	**11.14**	**11.08**	**9.40**	**9.78**	**9.95**	**8.12**	**8.65**
	C16:1	0.88	0.10	0.04	0.00	0.00	0.00	0.00	0.04	0.04	0.00	0.04	0.00	0.05	0.06
	C18:0	2.97	**40.17**	**54.25**	**53.87**	**53.61**	**53.46**	**53.32**	**53.32**	**53.15**	**52.43**	**52.20**	**51.23**	**50.52**	**50.02**
	C18:1	58.07	20.15	23.52	22.12	22.20	23.48	24.02	22.73	23.45	23.94	25.21	26.07	28.00	28.29
	C18:2	6.25	5.25	6.75	6.05	6.42	6.25	6.56	6.19	5.96	6.88	6.28	6.31	6.59	6.31
	C18:3α	0.50	0.68	0.79	0.88	0.78	0.79	0.79	0.85	0.82	0.86	0.78	0.78	0.78	0.83
	C20:0	0.22	1.88	3.21	2.81	3.01	2.91	3.02	2.86	2.77	3.21	2.74	2.80	2.87	2.80
	C20:1	0.02	0.07	0.19	0.21	0.34	0.27	0.28	0.12	0.11	0.41	0.14	0.30	0.28	0.26
	C22:0	0.05	0.26	0.41	0.34	0.40	0.37	0.37	0.36	0.35	0.42	0.36	0.37	0.36	0.37
	C24:0	0.04	0.27	0.49	0.38	0.42	0.41	0.45	0.38	0.36	0.46	0.39	0.37	0.41	0.41
sum C18		67.78	66.24	85.31	82.92	83.01	83.98	84.69	83.09	83.38	84.11	84.47	84.39	85.89	85.45
saturates		33.97	73.52	68.34	70.63	70.11	69.12	68.37	69.72	69.33	67.93	67.20	***66.54***	***64.01***	***64.03***
unsatur		65.	26.	31.	29.	29.	30.	31.	29.	30.	32.	32.	***33.***	***35.***	***35.***

ates	71	23	29	26	74	79	65	93	38	09	45	***46***	***70***	***75***

[0534] In Table 42, Stearate (C18:0) levels greater than the wild-type level are highlighted with bold text. Palmitate (C16:0) levels lower than Strain X or J are highlighted with bold. For three strains the ratio of saturated to unsaturated fatty acids is ≤ 2:1; these are highlighted with bold, italicized text.

[0535] Stable lines were isolated from the primary transformants shown in Table 42. The fatty acid profiles and lipid titers of shake flask cultures are presented in Table 43, below. The strains accumulated up to 55% C18:0, with as low as 7% C16:0, with comparable lipid titers to the Strain X parent. The saturates:unsaturates ratios were substantially reduced compared to Strain X. Strains AU and AV were selected for evaluation in 3-L high-density fermentations.

[0536] Table 43. Shake flask assays of strains derived from D1653, expressing KASII, driven by the PmSAD2-2 promoter, targeted to the 6S locus.

Primary				**1653-6B**		**1653-9B**	**1653-10A**		**1653-72**		**D1653-89**		
Strain			**K-4**	**5664**	**AU**	**BM**	**BN**	**BO**	**BP**	**BQ**	**BR**	**AV**	**BS**
Fatty Acid Area%	**10:0**	.02	.04	.08	.09	.12	.06	.06	.08	.09	.12	.12	.12
	12:0	.04	.09	.28	.29	.35	.20	.20	.23	.26	.32	.32	.33
	14:0	.42	.12	.81	.66	.73	.75	.72	.50	.61	.38	.43	.38
	16:0	5.59	8.56	**.39**	**.61**	**.44**	**.98**	**0.11**	**.26**	**.95**	**.81**	**.21**	**.63**
	16:1	.03	.10	.06	.05	.06	.06	.06	.04	.04	.03	.03	.03
	18:0	.60	**0.13**	**7.60**	**2.47**	**5.12**	**0.25**	**9.73**	**4.56**	**4.01**	**2.96**	**3.68**	**2.12**
	18:1	2.08	0.74	7.78	3.93	1.31	5.37	5.70	2.86	2.87	4.37	3.99	5.17
	18:2	.16	.83	.98	.52	.72	.55	.64	.20	.24	.11	.83	.04
	18:3α	.40	.89	.21	.22	.49	.17	.07	.20	.29	.28	.24	.31
	20:0	.18	.82	.62	.93	.75	.65	.66	.97	.72	.43	.10	.59
	20:1	.04	.13	.37	.36	.39	.34	.34	.35	.34	.48	.41	.47

	20:1	.07	.00	.00	.00	.00	.00	.00	.00	.00	.00	.00	.00
	20:1	.15	.08	.11	.09	.11	.10	.10	.09	.10	.12	.10	.12
	22:0	.02	.20	.28	.30	.24	.29	.28	.30	.27	.32	.29	.35
	24:0	.00	.03	.16	.29	.00	.03	.15	.16	.02	.05	.04	.07
Sum C18		1.23	7.58	4.57	5.13	5.63	4.34	4.13	5.81	5.40	6.71	6.73	6.63
Saturates		9.86	1.97	***2.22***	***6.63***	8.74	***5.20***	***4.90***	8.05	7.90	***5.37***	***6.17***	***4.57***
Unsaturates		9.91	7.76	***7.50***	***3.15***	1.07	***4.57***	***4.89***	1.73	1.87	***4.37***	***3.59***	***5.13***

[0537] In Table 43, Strain X is the parent strain; Strain J is the wild-type base strain. Stearate (C18:0) levels at least two-fold higher than in the wild-type strain are highlighted in bold. Palmitate levels that are less than in Strain J and Strain K-4 are highlighted bold. Bold italics indicate that the saturates:unsaturates ratio is ≤2:1.

[0538] The fatty acid profiles and performance metrics of strains AU and AV are detailed in Table 44, below. The fatty acid profile of the parent strain X, grown under the same fermentation conditions, is presented for comparison. The strains that over-express *KASII* accumulate about 11% more C18:0 than the strain K-4 parent. C16:0 is reduced to 7-9%, and levels of unsaturated fatty acids increase by 4-5%. The lipid titers of Strain AU and AV were comparable to K-4, indicating that *KASII* over-expression did not have deleterious effects on lipid production.

[0539] Table 44. End point fatty acid profiles of biomass from Strain X, AU and AV fermentations.

Strain	**K-4**	**AU**	**AV**
Fermentation	**120580F1**	**130097F3**	**130098F4**
pH	**5**	**5**	**5**
C14:0	1.27	1.50	1.35
C16:0	28.35	**8.88**	**7.33**
C16:1	0.06	0.02	0.03
C18:0	**43.67**	**56.88**	**57.24**
C18:1	17.63	21.57	21.66
C18:2	5.58	6.06	6.94
C18:3α	0.25	0.29	0.22
C20:0	2.11	3.28	3.46
C22:0	0.31	0.40	0.40
C24:0	0.31	0.37	0.40

sum C18	67.13	84.80	86.06
saturates	76.02	71.31	70.18
unsaturates	23.52	27.94	28.85
total lipid (g/L)	475	529	418

[0540] The fermentations were cultured for 6 days using a fed-batch process. The Strain X fatty acid profile from fermentation 120580F1 was presented in Table 41, and is shown again in Table 44 for comparison with Strains AU and AV. All fermentations were carried out at 32°C, pH 5, 30% dissolved oxygen (DO), 300 mM nitrogen [N], and 557.5 μM iron. The sugar source was 70% sucrose (S70). Stearate (C18:0) levels higher than in the wild-type strain are indicated with bold. Palmitate (C16:0) levels that are less than in the wild-type are highlighted bold.

[0541] Lab scale oils were prepared from biomass derived from the shake flasks and fermentations described above. The TAG compositions of these oils were determined by LC/MS. SOS is the major TAG species in both Strain AU and AV, ranging from 33-35% in the biomass from shake flasks, and reaching 37% in the high-density fermentation biomass. The major palmitate-containing TAGs are substantially reduced, and trisaturate levels are less than half of those observed in Strain X oils. These results demonstrate that *KASII* over-expression in a high-stearate background significantly improves SOS accumulation, and reduces the accumulation of trisaturated TAGs.

[0542] **Constructs used for *FATA-1* disruption, *KASII* over-expression and *FAD2* RNAi in Strain J:** A DNA construct, pSZ2419, was made to simultaneously disrupt the *FATA-1* allele, over-express *P. moriformis KASII* and express a *FAD2* hairpin construct in Strain J. A version of the *S. cerevisiae SUC2* gene, encoding sucrose invertase, which was codon-optimized for expression in *P. moriformis*, was utilized as a selectable marker for transformation. The sequence of the transforming DNA is provided immediately below. Relevant restriction sites are indicated in lowercase, bold, and are from 5'-3' BspQI, KpnI, AscI, MfeI, BamHI, AvrII, EcoRV, EcoRI, SpeI, AscI, ClaI, BglII, AflII, HinDIII, SacI, SpeI, and XhoI, respectively. BspQI sites delimit the 5' and 3' ends of the transforming DNA. Underlined sequences at the 5' and 3' flanks of the construct represent genomic DNA from *P. moriformis* that enable targeted integration of the transforming DNA via homologous recombination at the *FATA-1* locus. Proceeding in the 5' to 3' direction, the *C. reinhardtii TUB2* promoter driving the expression of the *S. cerevisiae SUC2* gene (encoding sucrose hydrolyzing activity, thereby permitting the strain to grow on sucrose) is indicated by

lowercase, boxed text. The initiator ATG and terminator TGA for *SUC2* are indicated by uppercase italics, while the coding region is indicated with lowercase italics. The 3' UTR of the *C. vulgaris* nitrate reductase (*NR*) gene is indicated by small capitals, followed by a spacer region indicated by lowercase text. The *P. moriformis AMT3* promoter, indicated by lowercase boxed text, drives expression of the *P. moriformis KASII* gene. The region encoding the plastid targeting peptide from *Chlorella protothecoides* SAD1 is indicated by uppercase italics. The sequence that encodes the mature *P. moriformis* KASII polypeptide is indicated with bold, uppercase italics, while a 3xFLAG epitope encoding sequence is in bold, underlined, uppercase italics. A second *C. vulgaris NR* 3' UTR is indicated by small capitals. A second *C. reinhardtii TUB2* promoter sequence, indicated by lowercase boxed text, drives expression of the *P. moriformis FAD2* hairpin A sequence. The sense and antisense strands are indicated with uppercase, bold italics, and are separated by the *FAD2* intron and the first 10 bases of the *FAD2* second exon (uppercase italics). A third *C. vulgaris NR* 3' UTR is indicated by small capitals, followed by a second spacer region that is indicated by lowercase text.

[0543] Nucleotide sequence of the transforming DNA from pSZ2419:

gctcttcggagtcactgtgccactgagttcgactggtagctgaatggagtcgctgctccactaaacgaattgtcagcaccgccagcc
ggccgaggacccgagtcatagcgagggtagtagcgcgccatggcaccgaccagcctgcttgccagtactggcgtctcttccgcttct
ctgtggtcctctgcgcgctccagcgcgtgcgcttttccggtggatcatgcggtccgtggcgcaccgcagcggccgctgcccatgcagc
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atgcgttccttcacctctcatttctcatttctgaatccctccctgctcactctttctcctcctccttcccgttcacgcagcattcgg**ggtacc**
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GCGCCTCCCGCCCCTGGGACGCCGACCGCGACGGCTTCGTGATGGGCGAGGGCGCCGGCGTGCTG

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ACCACCCCCAGCATCCCCTTCCCTCGTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTC

CTGCTATCCCTCAGCGCTGCTCCTGCTCCTGCTCACTGCCCCTCGCACAGCCTTGGTTTGGGCTCCGC

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GTGGGTCAAGTACGGCATCATGTGGCCGCTCTACTGGTTCTTCCAG*GTGTGTTTGAGGGTTTTGGTT*

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*ACCCTCCCGGCACCTTCCAGGGCGCGTACG****GGAAGAACCAGTAGAGCGGCCACATGATGCCGTACT***

TGACCCACGTAGGCACCGGTGCAGGGTCGATGTACGTCGACGCGACGTAGAGCAGGGACATGACC

GCGATGTCAAAGGCCAGGTACATGCTGCTACGAAGCGCCGAGCGCTCGAAACAGTGCGCGGGGA

TGGCCTTGCGCAGCGTCCCGATCGTGAACGGAGGCTTCTCCACAGGCTGCCTGTTCGTCTTGATAGC

***CAT*ctcgag**GCAGCAGCAGCTCGGATAGTATCGACACACTCTGGACGCTGGTCGTGTGATGGACTGTT

GCCGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCCGCTTTTATCAAACAGCCTCAGTGTGTTT

GATCTTGTGTGTACGCGCTTTTGCGAGTTGCTAGCTGCTTGTGCTATTTGCGAATACCACCCCCAGCA

TCCCCTTCCCTCGTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCTATCCCTCAG

CGCTGCTCCTGCTCCTGCTCACTGCCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCTCCTGGT

ACTGCAACCTGTAAACCAGCACTGCAATGCTGATGCACGGGAAGTAGTGGGATGGGAACACAAATG

GAaagctgtattgttttccagaaggagttgctccttgagcctttcattctcagcctcgataacctccaaagccgctctaattgtggagg

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agtcactcctgcacaggcccttgtgcgcagtgctcctgtaccctttaccgctccttccattctgcgaggcccctattgaatgtattcg

ttgcctgtgtggccaagcgggctgctgggcgcgccgccgtcgggcagtgctcggcgactttggcggaagccgattgttcttctgtaag

ccacgcgcttgctgctttgggaagagaaggggggggtactgaatggatgaggaggagaaggaggggtattggtattatctgagtt

gggt**gaagagc** (SEQ ID NO:96)

[0544] Identification and analysis of *FATA*-1 knockout, KASII over-expression and *FAD2* RNAi strains: Construct D1358, derived from pSZ2419, was transformed into Strain J as described previously. Primary transformants were clonally purified and grown under

standard lipid production conditions at pH 5. The resulting fatty acid profiles from representative clones arising from transformation of Strain J with D1358 are summarized in Table 45, below. The *P. moriformis AMT3* promoter is repressed at pH 5 so the observed phenotypes did not reflect over-expression of P. moriformis *KASII*. Nevertheless, we observed that multiple strains had substantially reduced levels of C16:0 and 10-15% increases in C18:1, suggesting that the construct had disrupted the *FATA-1* target gene, increasing the amount of palmitoyl-ACP available for extension by endogenous KASII. One line, D1358-13, was selected for further analysis. D1358-13 accumulated ~17% C16:0, ~75% C18:1 and less than 2% C18:2, indicating that we had successfully integrated at *FATA-1* and down-regulated activity of the FAD2 Δ^{12}-desaturase in this strain.

[0545] Table 45. Fatty acid profiles of D1358 [pSZ2419] primary transformants, compared to the wild-type parental strain, Strain J.

Strain		J	D1358-13	D1358-19	D1358-11	D1358-9	D1358-30	D1358-28	D1358-6	D1358-8	D1358-10	D1358-3
Fatty Acid Area %	C12:0	0.05	0.08	0.06	0.08	0.06	0.07	0.07	0.09	0.07	0.08	0.10
	C14:0	1.32	0.79	0.83	0.85	0.87	0.84	0.91	0.86	0.89	0.92	0.60
	C16:0	26.66	**17.43**	**18.84**	**20.03**	**16.27**	**18.4**	**19.1**	**18.18**	**15.6**	**16.42**	**11.24**
	C16:1	0.84	0.74	0.79	0.97	0.60	0.77	1.17	0.75	0.56	0.61	0.57
	C18:0	3.10	2.87	2.97	2.36	3.20	2.67	2.10	2.82	3.22	3.19	2.30
	C18:1	59.07	**74.78**	**69.54**	**68.78**	**71.48**	**69.55**	**69.02**	**68.93**	**70.44**	**69.64**	**75.27**
	C18:2	7.39	1.97	5.47	5.61	6.22	6.31	6.42	6.8	7.68	7.78	8.51
	C18:3α	0.55	0.23	0.59	0.51	0.26	0.39	0.46	0.38	0.24	0.27	0.24
	C20:0	0.24	0.22	0.20	0.13	0.32	0.20	0.03	0.20	0.33	0.31	0.22
	C20:1	0.11	0.40	0.29	0.37	0.23	0.33	0.33	0.39	0.36	0.27	0.40
	C22:0	0.11	0.09	0.08	0.07	0.09	0.08	0.08	0.08	0.09	0.11	0.11
sum C18		70.11	79.85	78.57	77.26	81.16	78.92	78.00	78.93	81.58	80.88	86.32
saturates		31.48	21.48	22.98	23.52	20.81	22.26	22.29	22.23	20.20	21.03	14.57
unsatura		67.9	78.1	76.6	76.2	78.7	77.3	77.4	77.2	79.2	78.5	84.9

tes	6	2	8	4	9	5		5	8	7	9

[0546] In Table 45, Oleate (C18:1) levels greater than the wild-type level are highlighted with bold text. Palmitate (C16:0) levels less than the wild-type are highlighted with bold text. Levels of linoleate (C18:2) reduced by 1% or more compared to the Strain J parent are highlighted with bold text.

[0547] The fatty acid profiles of strains derived from transformant D1358-13 were determined to be stable after more than 60 generations of growth in the absence of selection (growth on sucrose). The performance of selected strains in shake flask assays was then evaluated, and the fatty acid profiles and lipid titers are presented in Table 46, below. Flask experiments were performed at pH 7, enabling activation of the *PmAMT3* promoter driving expression of the KASII transgene. The combination of *KASII* over-expression and *FATA-1* knockout leads to further reductions in palmitate levels and enhanced oleate accumulation compared to the phenotypes observed at pH 5 (Table 45). With more than 82% C18:1, less than 11% C16:0, less than 2% C18:2 and ~83% of the wild-type lipid titer, Strain AA was determined to be the most appropriate strain from this set to serve as a host strain for subsequent modifications to elevate stearate levels. DNA blot analysis showed that S5003 has a simple insertion of construct D1358 [pSZ2419] at the *FATA-1* locus.

[0548] Table 46. Fatty acid profiles and lipid titers of FATA-1 knockout, KASII over-expressing, FAD2 RNAi lines derived from D1358-13 primary transformants, compared to the wild-type parental strain, Strain J.

Primary			T389;D1358-13											
Strain		J	AA	AB	AC	AD	AE	AF	AG	AH	AI	AJ	AK	AL	AM
Fatty Acid Area %	C12:0	0.05	0.08	0.09	0.11	0.19	0.11	0.14	0.10	0.12	0.08	0.11	0.09	0.20	0.20
	C14:0	1.34	0.96	0.98	1.03	1.04	0.96	1.02	0.98	1.03	0.98	1.01	1.00	1.03	1.02
	C16:0	29.69	**10.72**	**10.47**	**8.90**	**6.99**	**9.53**	**9.27**	**10.13**	**8.99**	**10.76**	**9.58**	**10.00**	**6.64**	**6.38**
	C16:1	0.88	0.42	0.39	0.31	0.29	0.39	0.37	0.41	0.32	0.40	0.35	0.35	0.27	0.27
	C18:0	2.78	2.92	3.00	3.16	2.71	2.88	2.85	2.91	3.21	3.03	3.10	3.20	2.77	2.71
	C18:1	58.45	**82.08**	**82.24**	**83.66**	**85.49**	**83.28**	**83.38**	**82.57**	**83.51**	**82.12**	**83.10**	**82.63**	**85.88**	**86.13**
	C18:2	5.83	1.89	1.88	1.80	2.01	1.83	1.89	1.89	1.77	1.73	1.75	1.76	1.94	1.96

	C18:3α	0.42	0.23	0.23	0.25	0.35	0.27	0.29	0.27	0.25	0.22	0.24	0.23	0.34	0.36
	C20:0	0.17	0.15	0.16	0.17	0.15	0.15	0.16	0.16	0.17	0.14	0.16	0.16	0.15	0.15
	C20:1	0.05	0.23	0.24	0.27	0.36	0.28	0.29	0.26	0.27	0.21	0.25	0.24	0.38	0.39
sum C18		67.48	87.12	87.35	88.87	90.56	88.26	88.41	87.64	88.74	87.10	88.19	87.82	90.93	91.16
saturates		34.03	14.83	14.70	13.37	11.08	13.63	13.44	14.28	13.52	14.99	13.96	14.45	10.79	10.46
unsaturates		65.63	84.85	84.98	86.29	88.50	86.05	86.22	85.40	86.12	84.68	85.69	85.21	88.81	89.11
lipid titer (% parent)		100.0	82.8	81.1	72.8	54.4	68.3	63.7	70.6	72.2	106.9	76.5	77.5	56.7	54.6

[0549] In Table 46, Stearate (C18:1) levels greater than the wild-type level are highlighted with bold text. Palmitate (C16:0) levels lower than the wild-type are highlighted with bold text. Linoleate (C18:2) levels that are lower than the wild-type are indicated with bold text.

[0550] **Constructs used for *SAD2* knockout/RNAi in S5003:** Two DNA constructs, pSZ2283 and pSZ2697, were made to simultaneously disrupt the *SAD2-1* allele and express a *SAD2* hairpin construct in Strain AA. In each construct, the *neoR* gene from transposon *Tn5*, conferring resistance to aminoglycoside antibiotics, was used as a selectable marker for transformation. The sequence of the transforming DNA derived from pSZ2283 is provided immediately below. Relevant restriction sites are indicated in lowercase, bold, and are from 5'-3' BspQI, KpnI, XbaI, MfeI, BamHI, AvrII, EcoRV, EcoRI, SpeI, BamHI, HinDIII, and SacI, respectively. BspQI sites delimit the 5' and 3' ends of the transforming DNA. Underlined sequences at the 5' and 3' flanks of the construct represent genomic DNA from *P. moriformis* that enable targeted integration of the transforming DNA via homologous recombination at the *SAD2-1* locus. Proceeding in the 5' to 3' direction, the *Chlamydomonas reinhardtii TUB2* promoter driving the expression of *neoR* (encoding aminoglycoside phosphotransferase activity, thereby permitting the strain to grow on G418) is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for neoR are indicated by uppercase italics, while the coding region is indicated with lowercase italics. The 3' UTR of the C. vulgaris NR gene is indicated by small capitals, followed by a spacer region indicated by lowercase text. A second *C. reinhardtii TUB2* promoter sequence, indicated by lowercase boxed text, drives expression of the *SAD2* hairpin C sequence. The sense and antisense strands are indicated with uppercase, bold italics, and are separated by the *P. moriformis*

FAD2 intron and the first 10 bases of the *FAD2* second exon (uppercase italics). A second *C. vulgaris NR* 3' UTR is indicated by small capitals.

[0551] Nucleotide sequence of the transforming DNA from pSZ2283:

gctcttcgggtcgccgcgctgcctcgcgtcccctggtggtgcgcgcggtcgccagcgaggccccgctgggcgttccgccctcggtgca
gcgcccctcccccgtggtctactccaagctggacaagcagcaccgcctgacgcccgagcgcctggagctggtgcagagcatggggc
agtttgcggaggagagggtgctgcccgtgctgcaccccgtggacaagctgtggcagccgcaggacttttgcccgaccccgagtcgc
ccgacttcgaggatcaggtggcggagctgcgcgcgcgcgccaaggacctgcccgacgagtactttgtggtgctggtgggggacatg
atcacggaggaggcgctgccgacctacatggccatgctcaacacgctggacggcgtgcgcgacgacagggcgcggccgaccacc
cgtgggcgcgctggacgcggcagtgggtggccgaggagaaccggcacggcgacctgctgaacaagtactgctggctgacggggc
gcgtcaacatgcgggccgtggaggtgaccatcaacaacctgatcaagagcggcatgaacccgcagacggacaacaacccttattt
ggggttcgtctacacctccttccaggagcgcgccaccaagta**ggtacc**ctttcttgcgctatgacacttccagcaaaaggtagggcg
ggctgcgagacggcttcccggcgctgcatgcaacaccgatgatgcttcgacccccgaagctccttcggggctgcatgggcgctccg
atgccgctccagggcgagcgctgtttaaatagccaggcccccgattgcaaagacattatagcgagctaccaaagccatattcaaac
acctagatcactaccacttctacacaggccactcgagcttgtgatcgcactccgctaaggggcgcctcttcctcttcgtttcagtcac
aacccgcaaac**tctaga**atatca*ATGatcgagcaggacggcctccacgccggctcccccgccgcctgggtggagcgcctgttc*
ggctacgactgggcccagcagaccatcggctgctccgacgccgccgtgttccgcctgtccgcccagggccgccccgtgctgttc
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cctgcgccgccgtgctggacgtggtgaccgaggccggccgcgactggctgctgctgggcgaggtgcccggccaggacctgct
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ccccttcgaccaccaggccaagcaccgcatcgagcgcgcccgcacccgcatggaggccggcctggtggaccaggacgacctg
gacgaggagcaccagggcctggcccccgccgagctgttcgcccgcctgaaggcccgcatgcccgacggcgaggacctggtg
gtgacccacggcgacgcctgcctgcccaacatcatggtggagaacggccgcttctccggcttcatcgactgcggccgcctgggc
gtggccgaccgctaccaggacatcgccctggccacccgcgacatcgccgaggagctgggcggcgagtgggccgaccgcttcc
*tggtgctgtacggcatcgccgcccccgactcccagcgcatcgccttctaccgcctgctggacgagttcttc*TGA**caattg**GCAG
CAGCAGCTCGGATAGTATCGACACACTCTGGACGCTGGTCGTGTGATGGACTGTTGCCGCCACACTT
GCTGCCTTGACCTGTGAATATCCCTGCCGCTTTTATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGT
ACGCGCTTTTGCGAGTTGCTAGCTGCTTGTGCTATTTGCGAATACCACCCCCAGCATCCCCTTCCCTC
GTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCTATCCCTCAGCGCTGCTCCTG
CTCCTGCTCACTGCCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCTCCTGGTACTGCAACCT
GTAAACCAGCACTGCAATGCTGATGCACGGGAAGTAGTGGGATGGGAACACAAATGGA**ggatcc**cgc
gtctcgaacagagcgcgcagaggaacgctgaaggtctcgcctctgtcgcacctcagcgcggcatacaccacaataaccacctgacg

aatgcgcttggttcttcgtccattagcgaagcgtccggttcacacacgtgccacgttggcgaggtggcaggtgacaatgatcggtgg
agctgatggtcgaaacgttcacag**cctagggatatcgaattc**ctttcttgcgctatgacacttccagcaaaaggtagggcgggctgc
gagacggcttcccggcgctgcatgcaacaccgatgatgcttcgacccccgaagctccttcggggctgcatgggcgctccgatgccg
ctccagggcgagcgctgtttaaatagccaggccccgattgcaaagacattatagcgagctaccaaagccatattcaaacacctag
atcactaccacttctacacaggccactcgagcttgtgatcgcactccgctaaggggcgcctcttcctcttcgtttcagtcacaacccg
caaac**actagt*GCGCTGGACGCGGCAGTGGGTGGCCGAGGAGAACCGGCACGGCGACCTGCTGAAC***
AAGTACTGTTGGCTGACGGGGCGCGTCAACATGCGGGCCGTGGAGGTGACCATCAACAACCTGAT
CAAGAGCGGCATGAACCCGCAGACGGACAACAACCCTTACTTGGGCTTCGTCTACACCTCCTTCCAG
GAGCGCGCGACCAAGTACAGCCACGGCAACACCGCGCGCCTTGCGGCCGAGCA*GTGTGTTTGAGG*
GTTTTGGTTGCCCGTATCGAGGTCCTGGTGGCGCGCATGGGGGAGAAGGCGCCTGTCCCGCTGACC
*CCCCCGGCTACCCTCCCGGCACCTTCCAGGGCGCGTACG***ggatcc*TGCTCGGCCGCAAGGCGCGCGGT***
GTTGCCGTGGCTGTACTTGGTCGCGCGCTCCTGGAAGGAGGTGTAGACGAAGCCCAAGTAAGGGT
TGTTGTCCGTCTGCGGGTTCATGCCGCTCTTGATCAGGTTGTTGATGGTCACCTCCACGGCCCGCAT
GTTGACGCGCCCCGTCAGCCAACAGTACTTGTTCAGCAGGTCGCCGTGCCGGTTCTCCTCGGCCACC
***CACTGCCGCGTCCAGCGC*aagctt**GCAGCAGCAGCTCGGATAGTATCGACACACTCTGGACGCTGGTC
GTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCCGCTTTTATCAAAC
AGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGCGAGTTGCTAGCTGCTTGTGCTATTTGCGA
ATACCACCCCCAGCATCCCCTTCCCTCGTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTG
TCCTGCTATCCCTCAGCGCTGCTCCTGCTCCTGCTCACTGCCCCTCGCACAGCCTTGGTTTGGGCTCCG
CCTGTATTCTCCTGGTACTGCAACCTGTAAACCAGCACTGCAATGCTGATGCACGGGAAGTAGTGGG
ATGGGAACACAAATGGAaagctg**gagctc**cagccacggcaacaccgcgcgccttgcggccgagcacggcgacaagaacc
tgagcaagatctgcgggctgatcgccagcgacgagggccggcacgagatcgcctacacgcgcatcgtggacgagttcttccgcctc
gaccccgagggcgccgtcgccgcctacgccaacatgatgcgcaagcagatcaccatgcccgcgcacctcatggacgacatgggcc
acggcgaggccaacccgggccgcaacctcttcgccgacttctccgcggtcgccgagaagatcgacgtctacgacgccgaggactac
tgccgcatcctggagcacctcaacgcgcgctggaaggtggacgagcgccaggtcagcggccaggccgccgcggaccaggagtac
gtcctgggcctgccccagcgcttccggaaactcgccgagaagaccgccgccaagcgcaagcgcgtcgcgcgcaggcccgtcgcctt
ctcctggatctccgggcgcgagatcatggtctagggagcgacgagtgtgcgtgcggggctggcgggagtgggacgccctcctcgct
cctctctgttctgaacggaacaatcggccaccccgcgctacgcgccacgcatcgagcaacgaagaaaaccccccgatgataggttg
cggtggctgccgggatatagatccggccgcacatcaaagggcccctccgccagagaagaagctcctttcccagcagactcct**gaag**
agc (SEQ ID NO:97)

[0552] The sequence of the transforming DNA derived from pSZ2697 is provided immediately below. Relevant restriction sites are indicated in lowercase, bold, and are from 5'-3' NsiI, SpeI, BamHI, HinDIII, SacII, EcoRV, KpnI, XbaI, MfeI, BamHI, AvrII, EcoRV, EcoRI and XbaI, respectively. Underlined sequences at the 5' and 3' flanks of the construct represent genomic DNA from *P. moriformis* that enable targeted integration of the transforming DNA via homologous recombination at the *SAD2-1* locus. Proceeding in the 5' to 3' direction, the *SAD2* hairpin C sense and antisense strands are indicated with uppercase, bold italics, and are separated by the *P. moriformis FAD2* intron and the first 10 bases of the *FAD2* second exon (uppercase italics). The 3' UTR of the *C. vulgaris NR* gene is indicated by small capitals. The *Chlorella sorokiniana Glutamate Dehydrogenase* (*GDH*) promoter, driving the expression of *neoR* (encoding aminoglycoside phosphotransferase activity, thereby permitting the strain to grow on G418) is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for *neoR* are indicated by uppercase italics, while the coding region is indicated with lowercase italics. A second *C. vulgaris NR* 3' UTR is indicated by small capitals, followed by a spacer region indicated by lowercase text.

[0553] Nucleotide sequence of the transforming DNA from pSZ2697:

atgcatgccggtcaccacccgcatgctcgtactacagcgcacgcaccgcttcgtgatccaccgggtgaacgtagtcctcgacggaa
acatctggttcgggcctcctgcttgcactcccgcccatgccgacaacctttctgctgttaccacgacccacaatgcaacgcgacacga
ccgtgtgggactgatcggttcactgcacctgcatgcaattgtcacaagcgcttactccaattgtattcgtttgttttctgggagcagttg
ctcgaccgcccgcgtcccgcaggcagcgatgacgtgtgcgtggcctgggtgtttcgtcgaaaggccagcaaccctaaatcgcaggc
gatccggagattgggatctgatccgagtttggaccagatccgccccgatgcggcacgggaactgcatcgactcggcgcggaaccca
gctttcgtaaatgccagattggtgtccgatacctggatttgccatcagcgaaacaagacttcagcagcgagcgtatttggcgggcgt
gctaccagggttgcatacattgcccatttctgtctggaccgctttactggcgcagagggtgagttgatggggttggcaggcatcgaaa
cgcgcgtgcatggtgtgcgtgtctgttttcggctgcacgaattcaatagtcggatgggcgacggtagaattgggtgtggcgctcgcgt
gcatgcctcgccccgtcgggtgtcatgaccgggactggaatccccctcgcgaccatcttgctaacgctcccgactctcccg**actagt**
GCGCTGGACGCGGCAGTGGGTGGCCGAGGAGAACCGGCACGGCGACCTGCTGAACAAGTACTGT
TGGCTGACGGGGCGCGTCAACATGCGGGCCGTGGAGGTGACCATCAACAACCTGATCAAGAGCG
GCATGAACCCGCAGACGGACAACAACCCTTACTTGGGCTTCGTCTACACCTCCTTCCAGGAGCGCGC
GACCAAGTACAGCCACGGCAACACCGCGCGCCTTGCGGCCGAGCA*GTGTGTTTGAGGGTTTTGGTT*
GCCCGTATCGAGGTCCTGGTGGCGCGCATGGGGGAGAAGGCGCCTGTCCCGCTGACCCCCCCGGCT
*ACCCTCCCGGCACCTTCCAGGGCGCGTACG***ggatcc*****TGCTCGGCCGCAAGGCGCGCGGTGTTGCCGTG***
GCTGTACTTGGTCGCGCGCTCCTGGAAGGAGGTGTAGACGAAGCCCAAGTAAGGGTTGTTGTCCG
TCTGCGGGTTCATGCCGCTCTTGATCAGGTTGTTGATGGTCACCTCCACGGCCCGCATGTTGACGCG

CCCCGTCAGCCAACAGTACTTGTTCAGCAGGTCGCCGTGCCGGTTCTCCTCGGCCACCCACTGCCGC

***GTCCAGCGC*aagctt**GCAGCAGCAGCTCGGATAGTATCGACACACTCTGGACGCTGGTCGTGTGATG

GACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCCGCTTTTATCAAACAGCCTCAG

TGTGTTTGATCTTGTGTGTACGCGCTTTTGCGAGTTGCTAGCTGCTTGTGCTATTTGCGAATACCACC

CCCAGCATCCCCTTCCCTCGTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCTA

TCCCTCAGCGCTGCTCCTGCTCCTGCTCACTGCCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATT

CTCCTGGTACTGCAACCTGTAAACCAGCACTGCAATGCTGATGCACGGGAAGTAGTGGGATGGGAA

CACAAATGGAAAGCTG**gagctc**aaa**gatatc**aacttaattaaccaa**ggtacc**cgcctgcaacgcaagggcagccacagcc

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gatgaaatcgtgtgcacgccgatcagctgattgcccggctcgcgaagtaggcgccctcctttctgctcgccctctctccgtcccgcc**tc**

tagaatatca*ATGatcgagcaggacggcctccacgccggctcccccgccgcctgggtggagcgcctgttcggctacgactggg*

cccagcagaccatcggctgctccgacgccgccgtgttccgcctgtccgcccagggccgccccgtgctgttcgtgaagaccgacct

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gccaagcaccgcatcgagcgcgcccgcacccgcatggaggccggcctggtggaccaggacgacctggacgaggagcacca

gggcctggcccccgccgagctgttcgcccgcctgaaggcccgcatgcccgacggcgaggacctggtggtgacccacggcgac

*gcctgcctgcccaacatcatggtggagaacggccgcttctccggcttcatcgactgcggccgcctgggcgtggccgaccgctac caggacatcgccctggccacccgcgacatcgccgaggagctgggcggcgagtgggccgaccgcttcctggtgctgtacggca tcgccgcccccgactcccagcgcatcgccttctaccgcctgctggacgagttcttcTGA***caattg***GCAGCAGCAGCTCGGA* TAGTATCGACACACTCTGGACGCTGGTCGTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACC TGTGAATATCCCTGCCGCTTTTATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGC GAGTTGCTAGCTGCTTGTGCTATTTGCGAATACCACCCCCAGCATCCCCTTCCCTCGTTTCATATCGCT TGCATCCCAACCGCAACTTATCTACGCTGTCCTGCTATCCCTCAGCGCTGCTCCTGCTCCTGCTCACTG CCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCTCCTGGTACTGCAACCTGTAAACCAGCACT GCAATGCTGATGCACGGGAAGTAGTGGGATGGGAACACAAATGGA***ggatcc***cgcgtctcgaacagagcgcg cagaggaacgctgaaggtctcgcctctgtcgcacctcagcgcggcatacaccacaataaccacctgacgaatgcgcttggttcttcg tccattagcgaagcgtccggttcacacacgtgccacgttggcgaggtggcaggtgacaatgatcggtggagctgatggtcgaaacg ttcacag***cctagggatatcgaattc***cgggtcgccgcgctgcctcgcgtcccctggtggtgcgcgcggtcgccagcgaggccccgctg ggcgttccgccctcggtgcagcgcccctcccccgtggtctactccaagctggacaagcagcaccgcctgacgcccgagcgcctgga gctggtgcagagcatggggcagtttgcggaggagagggtgctgcccgtgctgcaccccgtggacaagctgtggcagccgcaggac tttttgcccgaccccgagtcgcccgacttcgaggatcaggtggcggagctgcgcgcgcgcgccaaggacctgcccgacgagtacttt gtggtgctggtgggggacatgatcacggaggaggcgctgccgacctacatggccatgctcaacacgctggacggcgtgcgcgacg acacgggcgcggccgaccacccgtgggcgcgctggacgcggcagtgggtggccgaggagaaccggcacggcgacctgctgaaca agtactgctggctgacggggcgcgtcaacatgcgggccgtggaggtgaccatcaacaacctgatcaagagcggcatgaacccgca gacggacaacaaccccttatttggggttcgtctacacctccttccaggagcgcgccaccaagta***tctaga*** (SEQ ID NO:98)

[0554] Identification and analysis of *SAD2* knockout/knockdown strains in the S5003 background: Constructs D1639, derived from pSZ2697, and D1682, derived from pSZ2283, were transformed into Strain AA as described previously. Primary transformants were clonally purified and grown under standard lipid production conditions at pH 7. The resulting fatty acid profiles from representative clones arising from transformation are summarized in Table 47, below. D1639 transformants accumulated up to 56% C18:0, and D1682 transformants accumulated a maximum of about 35% C18:0. Most of the increases in stearate came at the expense of C18:1, indicating that SAD activity was significantly reduced by the *SAD2* knockout/RNAi constructs in these strains. C16:0 levels varied from 6% to 14%; C18:2 ranged from 2-5%. Most strains maintained the low C16:0 and C18:2 phenotypes of the Strain AA parent. These fatty acid profiles demonstrate that down-regulating *SAD2* expression using knockout/RNAi constructs, in a background with disrupted *FATA-1*, *KASII* over-expression and *FAD2* RNAi, produces strains with high C18:0, low

C16:0 and low C18:2 phenotypes. These strains will be useful for production of high stability, high stearate, high oleic oils, and oils which have high SOS content.

[0555] Table 47. Fatty acid profiles of D1639 [pSZ2697] and D1682 [pSZ2283] primary transformants, compared to the wild-type strain, Strain J, and the Strain AA parental base strain.

Strain		J	AA	D1682-4	D1682-17	D1682-7	D1682-6	D1639-2	D1639-5	D1639-10	D1639-19
Fatty Acid Area %	**C12:0**	0.04	0.11	0.14	0.10	0.32	0.31	0.00	0.19	0.17	0.00
	C14:0	1.29	0.98	1.03	0.94	1.11	1.15	1.64	1.39	1.61	1.02
	C16:0	27.50	**7.75**	**8.68**	**10.41**	**5.70**	**5.96**	**7.54**	**9.90**	**14.39**	**12.02**
	C16:1	0.71	0.30	0.06	0.07	0.07	0.10	0.00	0.00	0.00	0.00
	C18:0	3.28	3.60	**35.46**	**29.92**	**24.66**	**22.30**	**55.96**	**53.38**	**43.46**	**37.30**
	C18:1	57.80	**84.14**	48.39	52.49	61.04	63.60	23.70	26.79	32.93	42.81
	C18:2	7.90	2.09	**2.37**	**2.36**	**3.03**	**2.88**	**5.09**	**3.50**	**3.22**	**2.79**
	C18:3α	0.57	0.32	0.50	0.65	0.66	0.58	1.59	0.98	1.01	0.85
	C20:0	0.28	0.23	2.07	1.87	1.75	1.51	3.04	2.73	2.29	2.22
	C20:1	0.18	0.35	0.54	0.49	0.78	0.83	0.37	0.33	0.30	0.40
	C22:0	0.06	0.02	0.27	0.27	0.23	0.20	0.43	0.36	0.29	0.29
	C24:0	0.09	0.02	0.33	0.26	0.34	0.26	0.64	0.45	0.32	0.31
sum C18		69.55	90.14	86.72	85.42	89.39	89.36	86.34	84.65	80.62	83.75
saturates		32.54	12.70	47.98	43.77	34.11	31.69	69.25	68.40	62.53	53.16
unsaturates		67.16	87.21	51.86	56.06	65.58	67.99	30.75	31.60	37.46	46.85

[0556] In Table 47, Stearate (C18:0) levels greater than the wild-type level are highlighted with bold text. Oleate (C18:1) levels that are higher than in the wild-type are indicated with bold text. Palmitate (C16:0) levels less than the wild-type level are highlighted with bold. Reduced levels of linoleate (C18:2) compared to the wild-type are highlighted with bold text.

[0557] Stable lines were isolated from a number of D1639 and D1682 transformants. Shake flask assays were carried out to evaluate the performance of four lines derived from D1639-5. Fatty acid profiles and relative lipid titers from the biomass are shown in Table 48, below.

[0558] Table 48. Shake flask assays of strains derived from D1639-5, expressing *SAD2hpC*, driven by the *CrTUB2* promoter, targeted to the *SAD2-1* locus.

Primary				T530;D1639-5			
Strain		J	AA	AW	AX	AY	BL
Fatty Acid Area %	C10:0	0.01	0.00	0.07	0.08	0.05	0.04
	C12:0	0.02	0.11	0.19	0.22	0.25	0.23
	C14:0	1.52	1.10	1.35	1.32	1.30	1.43
	C16:0	31.61	**9.59**	**9.28**	**8.44**	**7.74**	**9.46**
	C16:1	1.04	0.34	0.03	0.02	0.01	0.01
	C17:0	0.10	0.11	0.10	0.10	0.10	0.09
	C18:0	2.98	4.36	**53.01**	**53.52**	**55.32**	**52.09**
	C18:1	54.81	**80.84**	27.26	27.52	27.42	28.06
	C18:2	6.88	**2.42**	**3.55**	**3.52**	**2.38**	**3.45**
	C18:3α	0.53	0.33	0.97	1.03	0.82	1.06
	C20:0	0.26	0.31	2.88	2.94	3.15	2.72
	C20:1	0.05	0.34	0.38	0.38	0.40	0.37
	C22:0	0.03	0.06	0.36	0.37	0.39	0.35
	C24:0	0.07	0.08	0.53	0.54	0.53	0.60
sum C18		65.19	87.95	84.79	85.58	85.94	84.66
saturates		36.59	15.70	67.76	67.52	68.82	66.99
unsaturates		63.30	84.26	32.19	32.46	31.02	32.95
% wild-type lipid titer		100.0	70.3	34.8	33.7	31.4	35.3

[0559] In Table 48, Strain AA is the parent strain; Strain J is the wild-type base strain. Stearate (C18:0) levels higher than in the wild-type strain are indicated with bold. Bold text indicates the increased level of oleate (C18:1) in Strain AA compared to the wild-type. Palmitate (C16:0) levels that are less than in the wild-type are highlighted bold. Linoleate (C18:2) levels that are less than in the wild-type are indicated with bold.

[0560] Lab scale oils were prepared from biomass collected from the Strain AW, AX and AY shake flasks. The TAG compositions of these oils were determined by LC/MS, and are shown in Figure 21. SOS accumulation ranged from 42-47% in these strains. POS was the next most abundant TAG, at 16-17%. Linoleate-containing TAGs were reduced by more than 50% compared to the Strain AU and AV oils, described above. Strain AW,AX, and AY oils contained 12-13% trisaturated TAGs (S-S-S), similar to the amounts that accumulated in

the Strain AU and AX oils. Modulation of SAD activity during oil production to prevent overproduction of saturated fatty acids may help to reduce accumulation of trisaturates.

EXAMPLE 49: PROPERTIES OF METHYL OLEATE FROM HIGH OLEIC MICROALGAL OILS.

[0561] Esterified oils high in methyl oleate are useful in a variety of applications such as cleaning and lubrication of machinery. For some of these applications, high thermal stability is desired. Thermal stability testing was performed on methylated oil prepared from high-oleic and high-stability-high oleic triglyceride oils prepared from heterotrophically grown oleaginous microalgae as described above. The oils were bleached and deodorized prior to methylation. Commercially available soya methyl ester was used as a control.

[0562] High Oleic (HO) oil was prepared from a high oil-yielding strain of *Prototheca moriformis* transformed with a plasmid that can be described as FatA1_Btub:inv:nr::amt03-CwTE2:nr_FatA1. This plasmid was designed to homologously recombine in the FATA1 chromosomal site, thus ablating a FATA acyl-ACP thioesterase chromosomal allele, while expressing an exogenous acyl-ACP thioesterase from Cuphea. wrightii (CwTE2, SEQ ID NO: 11) under control of the pH-regulatable amt3 promoter. The CwTE2 gene can be downregulated by cultivation at pH 5 during oil production to further elevate oleate production. Sucrose invertase was also expressed as a selection marker and to allow for cultivation of the strain on sucrose as a sole carbon source. The 3' UTR sequences are from the *Chlorella vulgaris* nitrate reductase gene. The resulting HO strain is denoted Stain Q. The fatty acid profile of the oil produced by Strain Q is listed below in Table 49.

[0563] Table 49. Fatty acid profile of high oleic oil from Strain Q.

Fatty Acid	Area %
C10	0.01
C12:0	0.03
C14:0	0.43
C15:0	0.03
C16:0	7.27
C16:1 iso	0.81
C16:1	0.689
C17:0	0.06
C18:0	1.198

C18:1	80.15
C18:1 iso	0.08
C18:2	8.38
C18:3 ALPHA	0.25
C20:0	0.02
C20:1	0.38
C22:0	0.04
C24:0	0.03

[0564] A high-stability-high-oleic oil (HSAO) was also prepared from a high oil-yielding strain of *Prototheca moriformis* transformed with a plasmid that can be described as FADc5'_Btub:inv:nr::btub-CpSAD_CtOTE:nr_FADc3' . The resulting strain (Strain R) expresses sucrose invertase as a selectable marker and to allow for cultivation on sucrose as a sole carbon source. In addition, a FAD allele (encoding fatty acid desaturase responsible for the conversion of oleate to linoleate) is disrupted and an oleate-specific acyl-ACP thioesterase (*Carthamus tinctorius* OTE, see example 5) fused to the transit peptide from the SAD gene of *Chlorella protothecoides* is expressed under control of the beta tubulin promoter. The 3' UTR sequences are from the *Chlorella vulgaris* nitrate reductase gene. The fatty acid profile of the oil produced by Strain R after heterotrophic cultivation is listed below in Table 50. The fatty acid profile has greater than 85% oleate yet almost none of the major polyunsaturates, linoleic and linolenic acids.

[0565] Table 50. Fatty acid profile of high oleic oil from Strain R.

Fatty Acid	Area %
C10	0.02
C12:0	0.07
C14:0	0.09
C15:0	0.05
C16:0	7.28
C16:1	0.70
C17:0	0.08
C18:0	2.15
C18:1	86.32

C20:0	0.30
C20:1	0.46
C22:0	0.08
C23:0	0.01
C24:0	0.06

[0566] The HO and HSAO oils were methylated by known biodiesel production techniques to make methyl-HO and methyl-HSAO esters. These methyl esters where then subjection to thermal testing according to the following procedure:

1. Prepare equipment as shown in Figure 1.
2. Add 1 litre of water to test vessel and bring to an active boil on the hotplate.
3. To each test product add 50ppm Cobalt (0.083g of 6% Cobalt Napthenate in 100.0 gram sample) and mix thoroughly.
4. Weigh out, in a watch glass, 7.0g of 100% cotton gauze, (#50 Cheese Cloth).
5. Evenly distribute 14.0g of test product, as prepared in step 3, onto the gauze.
6. Place thermocouple (thermometer) through the center of #15 stopper. Wrap cotton around the thermocouple.
7. Place wrapped cotton into 24 mesh wire frame cylinder so that it occupies the upper 4 ½ inches.
8. Position cylinder with wrapped gauze into the 1L tall form beaker. Secure the beaker in the boiling water and begin recording the temperature increase with time.
9. Continue monitoring the temperature for 2 hours or until a 10 degree temperature drop in observed.
10. Plot temperature vs time on a graph.
11. Any sample which shows a temperature exceeding 100 degrees C in 1 hour or 200 degrees C in 2 hours should be regarded as a dangerous oxidation risk or one that is likely to spontaneously combust.

[0567] Results: The HO and HSAO methyl ester did not exhibit auto-oxidation as evidenced by a temperature rise. The control soya methyl ester sample did exhibit the potential for auto-oxidation. The time-temperature profiles are shown in Figure 18.

[0568] In addition, methylated fatty acid from oil produced by Strain Q was found to have the following characteristics:

- Flash Point (ASTM D93) of 182°C

- Non-VOC
- Kauri Butanol value (ASTM D1133) of 53.5
- Viscosity at 40°C (ASTM D445) of 4.57 mm2/s
- Acid Number (ASTM D664) of 0.17 mg KOH/g
- Boiling range distribution (ASTM D2887) 325-362°C.

EXAMPLE 50: FURTHER PROPERTIES OF HIGH OLEIC (HO) AND HIGH-STABILITY-HIGH-OLEIC (HSAO) MICROALGAL OILS.

[0569] The high oleic oil and the high-stability high-oleic algal oils can have the properties shown in Figure 19 or these values ±20% for the measured parameters.

[0570] In one experiment, HSAO microalgal oil showed 512 hour stability measured by OSI at 110°C (estimated using 130°C data) with antioxidants of 0.5% phenyl-alpha-naphthylamine (PANA) and 500 ppm ascorbyl palmitate (AP).

EXAMPLE 51: PRODUCTION OF LOW SATURATE OIL BY CONVERSION OF PALMITIC TO PALMITOLEATE.

[0571] As described in the examples above, genetic manipulation of microalgae can decrease saturated fat levels, especially by increasing the production of oleic acid. However, in some cases, the acyl-ACP thioesterases expressed in the oleaginous cell liberate more than desirable amounts of palmitate. Here, we describe methods for converting palmitate (16:0) to palmitoleate (16:1) by overexpressing a palmitoyl-ACP desaturase (PAD) gene. The PAD gene can be obtained from natural sources such as Macfadyena unguis (Cat's claw), Macadamia integrifolia (Macadamia nut), Hippophae rhamnoides (sea buckthorn), or by creating a PAD via mutation of a stearoyl-ACP desaturase to have 16:1 activity. The *Macfadyena unguis* desaturase is denoted (MuPAD).

[0572] A high-oil-producing strain of *Prototheca moriformis* (Strain Z) is biolistically transformed with plasmid DNA constructs with a PAD gene. For example, one of the high oleic strains described in the Examples 6, 36, or 49 can further comprise an exogenous *PAD* gene. The constructs comprises sucrose invertase as a selectable marker and either the *MuPAD* or a *SAD* gene (e.g.., *Olea europaea* stearoyl-ACP desaturase, GenBank Accession No. AAB67840.1) having the L118W mutation to shift substrate-specificity toward palmitate. See Cahoon, et al., Plant Physoil (1998) 117:593-598. Both the amt3 and beta tubulin (Btub) promoters are used. In addition, the native transit peptide of a plant PAD gene can be swapped with one known to be effective in microalgae (e.g., the transit peptide from the *Chlorella vularis* SAD gene).

[0573] The *PAD* gene can be expressed in a variety of strains including those with a FATA knockout or knockdown and/or a KASII knockin to produce high-oleic oil. Optionally, these strains can also produce high-stability (low polyunsaturate) oil by virtue of a FAD (delta 12 fatty acid desaturase) knockout, knockdown, or by placing FAD expression under control of a regulatable promoter and producing oil under conditions that downregulate FAD. In addition, useful base strains for the introduction of *PAD* gene activities might also include strains possessing *KASII* knockouts, and *FATA* Knockins, whereby levels of C16:0 palmitate are elevated.

[0574] As a result, lower levels of palmitic acid are found in the fatty acid profile of the microalgal oil as this is converted into cis-palmitoleic and cis-vaccenic acids. In some cases the total area percent of saturated fatty acids is less than equal to 3.5%, 3% or 2.5%.

[0575] Constructs for over expression of *Macfadyena unguis* C16:0 desaturase (MuPAD) follow:

[0576] **1) pSZ3142: 6S::CrTUB2:ScSUC2:CvNR::PmAMT3:CpSADtp :MuPAD:CvNR::6S**

Relevant restriction sites in the construct pSZ3142 6S::CrTUB2:ScSUC2:CvNR::PmAMT3:CpSADtp :MuPAD:CvNR::6S are indicated in lowercase, bold and underlining and are 5'-3' *BspQ* 1, *Kpn* I, Xba I, *Mfe* I, *BamH* I, *EcoR* I, *Spe* I, Asc I, *Cla* I, *Sac* I, *BspQ* I, respectively. *BspQI* sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent genomic DNA from that permit targeted integration at 6s locus via homologous recombination. Proceeding in the 5' to 3' direction, the *C. reinhardtii* β-tubulin promoter driving the expression of the yeast sucrose invertase gene (conferring the ability of Strain Z to metabolize sucrose) is indicated by boxed text. The initiator ATG and terminator TGA for invertase are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics. The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase underlined text followed by an endogenous amt03 promoter of *Prototheca moriformis,* indicated by boxed italics text. The Initiator ATG and terminator TGA codons of the MuPAD are indicated by uppercase, bold italics, while the remainder of the coding region is indicated by bold italics. The *Chlorella protothecoides* S106 stearoyl-ACP desaturase transit peptide is located between initiator ATG and the *Asc* I site. The *C. vulgaris* nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the 6S genomic region indicated by bold, lowercase text.

[0577] Nucleotide sequence of transforming DNA contained in pSZ3142:

gctcttcgccgccgccactcctgctcgagcgcgcccgcgcgtgcgccgccagcgccttggccttttcgccgcgctcgtgcgcgtcgct
gatgtccatcaccaggtccatgaggtctgccttgcgccggctgagccactgcttcgtccgggcggccaagaggagcatgagggag
gactcctggtccagggtcctgacgtggtcgcggctctgggagcgggccagcatcatctggctctgccgcaccgaggccgcctccaa
ctggtcctccagcagccgcagtcgccgccgaccctggcagaggaagacaggtgagggggtatgaattgtacagaacaaccacg
agccttgtctaggcagaatccctaccagtcatggctttacctggatgacggcctgcgaacagctgtccagcgaccctcgctgccgcc
gcttctcccgcacgcttctttccagcaccgtgatggcgcgagccagcgccgcacgctggcgctgcgcttcgccgatctgaggacagt
cggggaactctgatcagtctaaaccccccttgcgcgttagtgttgccatcctttgcagaccggtgagagccgacttgttgtgcgccac
cccccacaccacctcctcccagaccaattctgtcacctttttggcgaaggcatcggcctcggcctgcagagaggacagcagtgccc
agccgctgggggttggcggatgcacgctcaggtaccctttcttgcgctatgacacttccagcaaaaggtagggcgggctgcgagac
ggcttcccggcgctgcatgcaacaccgatgatgcttcgaccccccgaagctccttcggggctgcatgggcgctccgatgccgctcca
gggcgagcgctgtttaaatagccaggccccgattgcaaagacattatagcgagctaccaaagccatattcaaacacctagatcac
taccacttctacacaggccactcgagcttgtgatcgcactccgctaagggggcgcctcttcctcttcgtttcagtcacaacccgcaaa
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gacgccaagtggcacctgtacttccagtacaacccgaacgacaccgtctgggggacgcccttgttctggggccacgccacgtc
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tggtggactacaacaacacctccggcttcttcaacgacaccatcgacccgcgccagcgctgcgtggccatctggacctacaaca
ccccggagtccgaggagcagtacatctcctacagcctggacggcggctacaccttcaccgagtaccagaagaaccccgtgctg
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ccaggactacaagatcgagatctactcctccgacgacctgaagtcctggaagctggagtccgcgttcgccaacgagggcttcc
tcggctaccagtacgagtgccccggcctgatcgaggtccccaccgagcaggaccccagcaagtcctactgggtgatgttcatct
ccatcaaccccggcgccccggccggcggctccttcaaccagtacttcgtcggcagcttcaacggcacccacttcgaggccttcga
caaccagtcccgcgtggtggacttcggcaaggactactacgccctgcagaccttcttcaacaccgacccgacctacgggagcg
ccctgggcatcgcgtgggcctccaactgggagtactccgccttcgtgcccaccaacccctggcgctcctccatgtccctcgtgcgc
aagttctccctcaacaccgagtaccaggccaacccggagacggagctgatcaacctgaaggccgagccgatcctgaacatca
gcaacgccggcccctggagccggttcgccaccaacaccacgttgacgaaggccaacagctacaacgtcgacctgtccaacag
caccggcaccctggagttcgagctggtgtacgccgtcaacaccacccagacgatctccaagtccgtgttcgcggacctctccctc
tggttcaagggcctggaggaccccgaggagtacctccgcatgggcttcgaggtgtccgcgtcctccttcttcctggaccgcggg
aacagcaaggtgaagttcgtgaaggagaacccctacttcaccaaccgcatgagcgtgaacaaccagcccttcaagagcgag
aacgacctgtcctactacaaggtgtacggcttgctggaccagaacatcctggagctgtacttcaacgacggcgacgtcgtgtcc
accaacacctacttcatgaccaccgggaacgccctgggctccgtgaacatgacgacgggggtggacaacctgttctacatcga

caagttccaggtgcgcgaggtcaagTGAcaattggcagcagcagctcggatagtatcgacacactctggacgctggtcgtgtga
tggactgttgccgccacacttgctgccttgacctgtgaatatccctgccgcttttatcaaacagcctcagtgtgtttgatcttgtgtgtac
gcgcttttgcgagttgctagctgcttgtgctatttgcgaataccacccccagcatccccttccctcgtttcatatcgcttgcatcccaacc
gcaacttatctacgctgtcctgctatccctcagcgctgctcctgctcctgctcactgcccctcgcacagccttggtttgggctccgcctgt
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gcgtctcgaacagagcgcgcagaggaacgctgaaggtctcgcctctgtcgcacctcagcgcggcatacaccacaataaccacctga
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ggagctgatggtcgaaacgttcacagcctagggatatcgaattcggccgacaggacgcgcgtcaaaggtgctggtcgtgtatgc
cctggccggcaggtcgttgctgctgctggttagtgattccgcaaccctgattttggcgtcttattttggcgtggcaaacgctggcg
cccgcgagccgggccggcggcgatgcggtgccccacggctgccggaatccaagggaggcaagagcgcccgggtcagttga
agggctttacgcgcaaggtacagccgctcctgcaaggctgcgtggtggaattggacgtgcaggtcctgctgaagttcctccac
cgcctcaccagcggacaaagcaccggtgtatcaggtccgtgtcatccactctaaagaactcgactacgacctactgatggccct
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[0578] 2) pSZ3145: 6S::CrTUB2:ScSUC2:CvNR::PmAMT3:MuPAD:CvNR::6S

Relevant restriction sites in the construct pSZ3145 6S::CrTUB2:ScSUC2:CvNR::PmAMT3: MuPAD:CvNR::6S are indicated in lowercase, bold and underlining and are 5'-3' *BspQ* 1, *Kpn* I, Xba I, *Mfe* I, *BamH* I, *EcoR* I, *Spe* I, *Cla* I, *Sac* I, *BspQ* I, respectively. *BspQI* sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent genomic DNA from that permit targeted integration at 6s locus via homologous recombination. Proceeding in the 5' to 3' direction, the *C. reinhardtii* β-tubulin promoter driving the expression of the yeast sucrose invertase gene (conferring the ability of Strain Z to metabolize sucrose) is indicated by boxed text. The initiator ATG and terminator TGA for invertase are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics. The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase underlined text followed by an endogenous amt03 promoter of *Prototheca moriformis,* indicated by boxed italics text. The Initiator ATG and terminator TGA codons of the

MuPAD are indicated by uppercase, bold italics, while the remainder of the coding region is indicated by bold italics. The *C. vulgaris* nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the 6S genomic region indicated by bold, lowercase text.

[0579] Nucleotide sequence of transforming DNA contained in pSZ3145:

gctcttcgccgccgccactcctgctcgagcgcgcccgcgcgtgcgccgccagcgccttggccttttcgccgcgctcgtgcgcgtcgct
gatgtccatcaccaggtccatgaggtctgccttgcgccggctgagccactgcttcgtccgggcggccaagaggagcatgagggag
gactcctggtccagggtcctgacgtggtcgcggctctgggagcgggccagcatcatctggctctgccgcaccgaggccgcctccaa
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[0580] 3) pSZ3137: 6S::CrTUB2:ScSUC2:CvNR::CrTUB2:CpSADtp :MuPAD:CvNR::6S

Relevant restriction sites in the construct pSZ3137 6S::CrTUB2:ScSUC2:CvNR::CrTUB2:CpSADtp :MuPAD:CvNR::6S are indicated in lowercase, bold and underlining and are 5'-3' *BspQ* 1, *Kpn* I, Xba I, *Mfe* I, *BamH* I, *EcoR* I, *Spe* I, Asc I, *Cla* I, *Sac* I, *BspQ* I, respectively. *BspQI* sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent genomic DNA from that permit targeted integration at 6s locus via homologous recombination. Proceeding in the 5' to 3' direction, the *C. reinhardtii* β-tubulin promoter driving the expression of the yeast sucrose

invertase gene (conferring the ability of Strain Z to metabolize sucrose) is indicated by boxed text. The initiator ATG and terminator TGA for invertase are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics. The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase underlined text followed by *C. reinhardtii* β-tubulin promoter, indicated by boxed italics text. The Initiator ATG and terminator TGA codons of the MuPAD are indicated by uppercase, bold italics, while the remainder of the coding region is indicated by bold italics. The *Chlorella protothecoides* S106 stearoyl-ACP desaturase transit peptide is located between initiator ATG and the *Asc* I site. The *C. vulgaris* nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the 6S genomic region indicated by bold, lowercase text.

[0581] Nucleotide sequence of transforming DNA contained in pSZ3137:

gctcttcgccgccgccactcctgctcgagcgcgcccgcgcgtgcgccgccagcgccttggccttttcgccgcgctcgtgcgcgtcgct
gatgtccatcaccaggtccatgaggtctgccttgcgccggctgagccactgcttcgtccgggcggccaagaggagcatgagggag
gactcctggtccagggtcctgacgtggtcgcggctctgggagcgggccagcatcatctggctctgccgcaccgaggccgcctccaa
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agccttgtctaggcagaatccctaccagtcatggctttacctggatgacggcctgcgaacagctgtccagcgaccctcgctgccgcc
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agccgctgggggttggcggatgcacgctcaggtaccctttcttgcgctatgacacttccagcaaaaggtagggcgggctgcgagac
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aaacacctagatcactaccacttctacacaggccactcgagcttgtgatcgcactccgctaagggggcgcctcttcctcttcgttt

*cagtcacaacccgcaaac***actagt*ATGgccaccgcatccacttttctcggcgttcaatgcccgctgcggcgacctgcgtcgctc***

ggcgggctccgggccccggcgcccagcgaggcccctccccgtgcgcgggcgcgccgccaccctgcgctccggcctgcgcgac

gtggagaccgtgaagaagaccttctcccccgcccgcgaggtgcacgtgcaggtgacccactccatggccccccagaagatc

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EXAMPLE 52: MYRISTATE RICH OIL PRODUCED BY OVEREXPRESSING A *CUPHEA PALUSTRIS* THIOESTERASE

[0582] Here, we demonstrate that over expression of a *Cuphea palustris* thioesterase (*Cpal* FATB2, accession AAC49180) in UTEX1435 results in a large increase in C14:0 production (over 60% of the fatty acid profile).

[0583] Constructs used for the overexpression of the *Cpal* FATB2 gene were codon optimized for expression in *P. moriformis* as described herein. *Cuphea palustris* FATB2 is a C14 preferring thioesterase. Two constructs, both encoding the *Cpal* FATB2 gene, were prepared. The first construct, pSZ2479, can be written as 6SA::CrTUB2-ScSUC2-CvNR:PmAMT3-CpSAD1tpExt- CpalFATB2ExtA-CvNR::6SB. The FatB2 coding sequence is given as SEQ ID NO: 86 and the amino acid sequence is given as SEQ ID NO: 87. The second construct, pSZ2480 can be written as 6SA::CrTUB2-ScSUC2-

CvNR:PmAMT3-CpSAD1tpExt_CpalFATB2FLAG_ExtA-CvNR::6SB. The nucleic acid sequence and amino acid sequence are given as SEQ ID NO: 88 and SEQ ID NO: 89.

[0584] *P. moriformis* transformed with pSZ2480 produced high levels of myristic acid. The myristate content was 65.70 percent. This is a very large increase when compared to the myristate content of the wild-type oil produced by the base strain, which has a myristate content of approximately 1%.

[0585] The fatty acid profile of the high myristate strain is shown in the Table 51 below.

[0586] Table 51. Fatty acid profile of high myristate strain.

Fatty Acid	%
C10:0	0.04
C12:0	1.19
C14:0	65.7
C16:0	13.55
C18:0	0.57
C18:1	12.2
C18:2	5.13
C20:0	0.05
C22:0	0.01
C24:0	0.01

EXAMPLE 53: PRODUCTION OF EICOSENOIC AND ERUCIC FATTY ACIDS

[0587] In this example we demonstrate that expression of heterologous fatty acid elongase (FAE), also known as 3-ketoacyl-CoA synthase (KCS), genes from *Cramble abyssinica* (*CaFAE*, Accession No: AY793549), *Lunaria annua* (*LaFAE*, ACJ61777), and *Cardamine graeca* (*CgFAE*, ACJ61778) leads to production of very long chain monounsaturated fatty acids such as eicosenoic ($20 : 1^{\Delta 11}$) and erucic ($22 : 1^{\Delta 13}$) acids in classically mutagenized derivative of UTEX 1435, Strain Z. On the other hand a putative FAE gene from *Tropaeolum majus* (*TmFAE*, ABD77097) and two FAE genes from *Brassica napus* (*BnFAE1*, AAA96054 and *BnFAE2*, AAT65206), while resulting in modest increase in eicosenoic ($20 : 1^{\Delta 11}$), produced no detectable erucic acid in STRAIN Z. Interestingly the unsaturated fatty acid profile obtained with heterologous expression of *BnFAE1* in STRAIN Z resulted in noticeable increase in Docosadienoic acid (22:2n6). All the genes were codon optimized to reflect UTEX 1435 codon usage. These results suggest that *CaFAE*, *LaFAE* or *CgFAE* genes encode condensing enzymes involved in the biosynthesis of very long-chain utilizing monounsaturated and saturated acyl substrates, with specific capability for improving the eicosenoic and erucic acid content.

[0588] Construct used for the expression of the *Cramble abyssinica* fatty acid elongase (CaFAE) in *P. moriformis* (UTEX 1435 strain Z) - [pSZ3070]: In this example STRAIN Z strains, transformed with the construct pSZ3070, were generated, which express sucrose invertase (allowing for their selection and growth on medium containing sucrose) and *C. abyssinica* FAE gene. Construct pSZ3070 introduced for expression in STRAIN Z can be written as 6S::CrTUB2-ScSUC2-Cvnr:PmAmt03-CaFAE-Cvnr::6S.

[0589] The sequence of the transforming DNA is provided below. Relevant restriction sites in the construct are indicated in lowercase, bold, and are from 5'-3' *BspQ*I, *Kpn*I, *Xba*I, *Mfe*I, *BamH*I, *EcoR*I, *Spe*I, *Afl*II, *Sac*I, *BspQ*I, respectively. *BspQ*I sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent genomic DNA from STRAIN Z that permit targeted integration at the 6S locus via homologous recombination. Proceeding in the 5' to 3' direction, the *C. reinhardtii* β-tubulin promoter driving the expression of the *Saccharomyces cerevisiae SUC2* gene (encoding sucrose hydrolyzing activity, thereby permitting the strain to grow on sucrose) is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for *SUC2* are indicated by uppercase italics, while the coding region is indicated with lowercase italics. The *Chlorella vulgaris nitrate reductase* (*NR*) gene 3' UTR is indicated by lowercase underlined text followed by an endogenous AMT3 promoter of *P. moriformis,* indicated by boxed italicized text. The Initiator ATG and terminator TGA codons of the *CaFAE* are indicated by uppercase, bold italics, while the remainder of the gene is indicated by bold italics. The *C. vulgaris* nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the STRAIN Z 6S genomic region indicated by bold, lowercase text. The final construct was sequenced to ensure correct reading frames and targeting sequences.

[0590] Nucleotide sequence of transforming DNA contained in plasmid pSZ3070:

gctcttcgccgccgccactcctgctcgagcgcgcccgcgcgtgcgccgccagcgccttggccttttcgccgcgctcgtgcgcgtcgctgatgt
ccatcaccaggtccatgaggtctgccttgcgccggctgagccactgcttcgtccgggcggccaagaggagcatgagggaggactcctggt
ccagggtcctgacgtggtcgcggctctgggagcgggccagcatcatctggctctgccgcaccgaggccgcctccaactggtcctccagca
gccgcagtcgccgccgaccctggcagaggaagacaggtgaggggggtatgaattgtacagaacaaccacgagccttgtctaggcagaa
tccctaccagtcatggctttacctggatgacggcctgcgaacagctgtccagcgaccctcgctgccgccgcttctcccgcacgcttctttcca
gcaccgtgatggcgcgagccagcgccgcacgctggcgctgcgcttcgccgatctgaggacagtcggggaactctgatcagtctaaacccc
cttgcgcgttagtgttgccatcctttgcagaccggtgagagccgacttgttgtgcgccacccccccacaccacctcctcccagaccaattctgt
cacctttttggcgaaggcatcggcctcggcctgcagagaggacagcagtgcccagccgctgggggttggcggatgcacgctcaggtacccc

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aagctccttcggggctgcatgggcgctccgatgccgctccagggcgagcgctgtttaaatagccaggcccccgattgcaaagacattatagcgag

ctaccaaagccatattcaaacacctagatcactaccacttctacacaggccactcgagcttgtgatcgcactccgctaaggggcgcctcttcctctt

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acctgaccaactgggaggaccagcccatcgccatcgccccgaagcgcaacgactccggcgccttctccggctccatggtggtggactacaa

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aacatgacgacgggggtggacaacctgttctacatcgacaagttccaggtgcgcgaggtcaag***TGA***<u>**caattg**gcagcagcagctcggata</u>

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<u>agtgtgtttgatcttgtgtgtacgcgcttttgcgagttgctagctgcttgtgctatttgcgaataccacccccagcatccccttccctcgtttcatatcgctt</u>

<u>gcatcccaaccgcaacttatctacgctgtcctgctatccctcagcgctgctcctgctcctgctcactgcccctcgcacagccttggtttgggctccgcc</u>

<u>tgtattctcctggtactgcaacctgtaaaccagcactgcaatgctgatgcacgggaagtagtgggatgggaacacaaatgga**ggatcc**</u>cgcgtctc

gaacagagcgcgcagaggaacgctgaaggtctcgcctctgtcgcacctcagcgcggcatacaccacaataaccacctgacgaatgcgcttggtt

cttcgtccattagcgaagcgtccggttcacacacgtgccacgttggcgaggtggcaggtgacaatgatcggtggagctgatggtcgaaacgttcac

agcctagggatatc**gaattc***ggccgacaggacgcgcgtcaaaggtgctggtcgtgtatgccctggccggcaggtcgttgctgctgctggttagt*

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tgcaacctgtaaaccagcactgcaatgctgatgcacgggaagtagtgggatgggaacacaaatggaaagcttaattaa**gagctcttgttttccaga**

aggagttgctccttgagcctttcattctcagcctcgataacctccaaagccgctctaattgtggagggggttcgaatttaaaagcttggaatg

ttggttcgtgcgtctggaacaagcccagacttgttgctcactgggaaaaggaccatcagctccaaaaaacttgccgctcaaaccgcgtacc

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gtgcttctgtccgaagcaggggttgctagggatcgctccgagtccgcaaacccttgtcgcgtggcggggcttgttcgagcttgaagagc

(SEQ ID NO:102)

[0591] Constructs used for the expression of the FAE genes from higher plants in STRAIN Z: In addition to the *CaFAE* gene (pSZ3070), *LaFAE* (pSZ3071) from *Lunaria*

annua, CgFAE (pSZ3072) from *Cardamine graeca*, *TmFAE* (pSZ3067) *Tropaeolum majus* and *BnFAE1* (pSZ3068) and *BnFAE2* (pSZ3069) genes from *Brassica napus* have been constructed for expression in STRAIN Z. These constructs can be described as:

pSZ3071 - 6S::CrTUB2-ScSUC2-Cvnr:PmAmt03-LaFAE-Cvnr::6S

pSZ3072 - 6S::CrTUB2-ScSUC2-Cvnr:PmAmt03-CgFAE-Cvnr::6S

pSZ3067 - 6S::CrTUB2-ScSUC2-Cvnr:PmAmt03-TmFAE-Cvnr::6S

pSZ3068 - 6S::CrTUB2-ScSUC2-Cvnr:PmAmt03-BnFAE1-Cvnr::6S

pSZ3069 - 6S::CrTUB2-ScSUC2-Cvnr:PmAmt03-BnFAE2-Cvnr::6S

[0592] All these constructs have the same vector backbone; selectable marker, promoters, and 3' utr as pSZ3070, differing only in the respective FAE genes. Relevant restriction sites in these constructs are also the same as in pSZ3070. The sequences of *LaFAE*, *CgFAE*, *TmFAE*, *BnFAE1* and *BnFAE2* are shown below. Relevant restriction sites as bold text including *Spe*I and *Afl*II are shown 5'-3' respectively.

[0593] Nucleotide sequence of *LaFAE* contained in pSZ3071:

actagt*ATGacctccatcaacgtgaagctgctgtaccactacgtgatcaccaacttcttcaacctgtgcttcttccccctgaccgccat cctggccggcaaggcctcccgcctgaccaccaacgacctgcaccacttctactcctacctgcagcacaacctgatcaccctgacc ctgctgttcgccttcaccgtgttcggctccgtgctgtacttcgtgacccgccccaagcccgtgtacctggtggactactcctgctacctg ccccccccagcacctgtccgccggcatctccaagaccatggagatcttctaccagatccgcaagtccgacccccctgcgcaacgtgg ccctggacgactcctcctccctggacttcctgcgcaagatccaggagcgctccggcctgggcgacgagacctacggccccgagg gcctgttcgagatcccccccccgcaagaacctggcctccgcccgcgaggagaccgagcaggtgatcaacggcgccctgaagaa cctgttcgagaacaccaaggtgaaccccaaggagatcggcatcctggtggtgaactcctccatgttcaaccccacccccctccctgt ccgccatggtggtgaacaccttcaagctgcgctccaacatcaagtccttcaacctgggcggcatgggctgctccgccggcgtgatc gccatcgacctggccaaggacctgctgcacgtgcacaagaacacctacgccctggtggtgtccaccgagaacatcacccagaa catctacaccggcgacaaccgctccatgatggtgtccaactgcctgttccgcgtgggcggcgccgccatcctgctgtccaacaagc ccggcgaccgccgccgctccaagtaccgcctggcccacaccgtgcgcacccacaccggcgccgacgacaagtccttcggctgc gtgcgccaggaggaggacgactccggcaagaccggcgtgtccctgtccaaggacatcaccggcgtggccggcatcaccgtgc agaagaacatcaccaccctgggccccctggtgctgcccctgtccgagaagatcctgttcgtggtgaccttcgtggccaagaagct gctgaaggacaagatcaagcactactacgtgcccgacttcaagctggccgtggaccacttctgcatccacgccggcggccgcgc cgtgatcgacgtgctggagaagaacctgggcctgtcccccatcgacgtggaggcctcccgctccaccctgcaccgcttcggcaac acctcctcctcctccatctggtacgagctggcctacatcgaggccaagggccgcatgaagaagggcaacaaggcctggcagatc gccgtgggctccggcttcaagtgcaactccgccgtgtgggtggccctgcgcaacgtgaaggcctccgccaactccccctgggagc actgcatccacaagtaccccgtgcagatgtactccggctcctccaagtccgagacccgcgcccagaacggccgctcc*TGA****ctta ag*** (SEQ ID NO:103)

[0594] Nucleotide sequence of *CgFAE* contained in pSZ3072:

actagtATGacctccatcaacgtgaagctgctgtaccactacgtgctgaccaacttcttcaacctgtgcctgttcccccctgaccgcctt ccccgccggcaaggcctcccagctgaccaccaacgacctgcaccacctgtactcctacctgcaccacaacctgatcaccgtgac cctgctgttcgccttcaccgtgttcggctccatcctgtacatcgtgacccgccccaagcccgtgtacctggtggactactcctgctacc tgccccccgccacctgtcctgcggcatctcccgcgtgatggagatcttctacgagatccgcaagtccgacccctcccgcgaggtg ccttcgacgacccctcctccctggagttcctgcgcaagatccaggagcgctccggcctgggcgacgagacctacggccccag ggcctggtgcacgacatgcccctgcgcatgaacttcgccgccgcccgcgaggagaccgagcaggtgatcaacggcgccctgga gaagctgttcgagaacaccaaggtgaaccccgcgagatcggcatcctggtggtgaactcctccatgttcaaccccaccccctcc ctgtccgccatggtggtgaacaccttcaagctgcgctccaacatcaagtccttctccctgggcggcatgggctgctccgccggcatc atcgccatcgacctggccaaggacctgctgcacgtgcacaagaacacctacgccctggtggtgtccaccgagaacatcacccac tccacctacaccggcgacaaccgctccatgatggtgtccaactgcctgttccgcatgggcggcgccgccatcctgctgtccaacaa ggccggcgaccgccgccgctccaagtacaagctggcccacaccgtgcgcacccacaccggcgccgacgaccagtccttccgct gcgtgcgccaggaggacgacgaccgcggcaagatcggcgtgtgcctgtccaaggacatcaccgccgtggccggcaagaccgt gaccaagaacatcgccaccctgggccccctggtgctgccccctgtccgagaagttcctgtacgtggtgtccctgatggccaagaag ctgttcaagaacaagatcaagcacacctacgtgcccgacttcaagctggccatcgaccacttctgcatccacgccggcggccgcg ccgtgatcgacgtgctggagaagaacctggccctgtcccccgtggacgtggaggcctcccgctccaccctgcaccgcttcggcaa cacctcctcctcctccatctggtacgagctggcctacatcgaggccaagggccgcatgaagaagggcaacaaggtgtggcagat cgccatcggctccggcttcaagtgcaactccgccgtgtgggtggccctgtgcaacgtgaagccctccgtgaactcccctgggag cactgcatcgaccgctaccccgtggagatcaactacggctcctccaagtccgagacccgcgcccagaacggccgctccTGActtaag (SEQ ID NO:104)

[0595] Nucleotide sequence of *TmFAE* contained in pSZ3067:

actagtATGtccggcaccaaggccacctccgtgtccgtgcccctgcccgacttcaagcagtccgtgaacctgaagtacgtgaagc tgggctaccactactccatcacccacgccatgtacctgttcctgacccccctgctgctgatcatgtccgcccagatctccaccttctcc atccaggacttccaccacctgtacaaccacctgatcctgcacaacctgtcctccctgatcctgtgcatcgccctgctgctgttcgtgct gaccctgtacttcctgacccgccccaccccgtgtacctgctgaacttctcctgctacaagcccgacgccatccacaagtgcgacc gccgccgcttcatggacaccatccgcggcatgggcacctacaccgaggagaacatcgagttccagcgcaaggtgctggagcgc tccggcatcggcgagtcctcctacctgcccccccaccgtgttcaagatccccccccgcgtgtacgacgccgaggagcgcgccgag gccgagatgctgatgttcggcgccgtggacggcctgttcgagaagatctccgtgaagcccaaccagatcggcgtgctggtggtga actgcggcctgttcaaccccatcccctccctgtcctccatgatcgtgaaccgctacaagatgcgcggcaacgtgttctcctacaacct gggcggcatgggctgctccgccggcgtgatctccatcgacctggccaaggacctgctgcaggtgcgccccaactcctacgccctg gtggtgtccctggagtgcatctccaagaacctgtacctgggcgagcagcgctccatgctggtgtccaactgcctgttccgcatgggc ggcgccgccatcctgctgtccaacaagatgtccgaccgctggcgctccaagtaccgcctggtgcacaccgtgcgcacccacaag ggcaccgaggacaactgcttctcctgcgtgacccgcaaggaggactccgacggcaagatcggcatctccctgtccaagaacctg

*atggccgtggccggcgacgccctgaagaccaacatcaccaccctgggcccccctggtgctgcccatgtccgagcagctgctgttctt cgccaccctggtgggcaagaaggtgttcaagatgaagctgcagccctacatccccgacttcaagctggccttcgagcacttctgc atccacgccggcggccgcgccgtgctggacgagctggagaagaacctgaagctgtcctcctggcacatggagccctcccgcat gtccctgtaccgcttcggcaacacctcctcctcctccctgtggtacgagctggcctactccgaggccaagggccgcatcaagaagg gcgaccgcgtgtggcagatcgccttcggctccggcttcaagtgcaactccgccgtgtggaaggccctgcgcaacgtgaaccccg ccgaggagaagaacccctggatggacgagatccacctgttccccgtggaggtgcccctgaac*TGA*cttaag* (SEQ ID NO:105)

[0596] Nucleotide sequence of *BnFAE1* contained in pSZ3068:

*actagt*ATG*acctccatcaacgtgaagctgctgtaccactacgtgatcaccaacctgttcaacctgtgcttcttcccccctgaccgcc atcgtggccggcaaggcctacctgaccatcgacgacctgcaccacctgtactactcctacctgcagcacaacctgatcaccatcg ccccccctgctggccttcaccgtgttcggctccgtgctgtacatcgccacccgccccaagcccgtgtacctggtggagtactcctgcta cctgcccccccacccactgccgctcctccatctccaaggtgatggacatcttcttccaggtgcgcaaggccgacccctcccgcaacg gcacctgcgacgactcctcctggctggacttcctgcgcaagatccaggagcgctccggcctgggcgacgagacccacggcccc gagggcctgctgcaggtgcccccccgcaagaccttcgcccgcgcccgcgaggagaccgagcaggtgatcatcggcgccctgg agaacctgttcaagaacaccaacgtgaaccccaaggacatcggcatcctggtggtgaactcctccatgttcaaccccaccccctc cctgtccgccatggtggtgaacaccttcaagctgcgctccaacgtgcgctccttcaacctgggcggcatgggctgctccgccggcg tgatcgccatcgacctggccaaggacctgctgcacgtgcacaagaacacctacgccctggtggtgtccaccgagaacatcacct acaacatctacgccggcgacaaccgctccatgatggtgtccaactgcctgttccgcgtgggcggcgccgccatcctgctgtccaac aagccccgcgaccgccgccgctccaagtacgagctggtgcacaccgtgcgcacccacaccggcgccgacgacaagtccttcc gctgcgtgcagcagggcgacgacgagaacggccagaccggcgtgtccctgtccaaggacatcaccgacgtggccggccgcac cgtgaagaagaacatcgccaccctgggcccccctgatcctgcccctgtccgagaagctgctgttcttcgtgaccttcatgggcaaga agctgttcaaggacgagatcaagcactactacgtgcccgacttcaagctggccatcgaccacttctgcatccacgccggcggcaa ggccgtgatcgacgtgctggagaagaacctgggcctggcccccatcgacgtggaggcctcccgctccaccctgcaccgcttcgg caacacctcctcctcctccatctggtacgagctggcctacatcgagcccaagggccgcatgaagaagggcaacaaggtgtggca gatcgccctgggctccggcttcaagtgcaactccgccgtgtgggtggccctgaacaacgtgaaggcctccaccaactccccctgg gagcactgcatcgaccgctaccccgtgaagatcgactccgactccggcaagtccgagacccgcgtgcccaacggccgctcc*TG A*cttaag* (SEQ ID NO:106)

[0597] Nucleotide sequence of *BnFAE2* contained in pSZ3069:

*actagt*ATG*gagcgcaccaactccatcgagatggaccaggagcgcctgaccgccgagatggccttcaaggactcctcctccgc cgtgatccgcatccgccgccgcctgcccgacttcctgacctccgtgaagctgaagtacgtgaagctgggcctgcacaactccttca acttcaccaccttcctgttcctgctgatcatcctgcccctgaccggcaccgtgctggtgcagctgaccggcctgaccttcgagaccttc tccgagctgtggtacaaccacgccgcccagctggacggcgtgacccgcctggcctgcctggtgtccctgtgcttcgtgctgatcatc tacgtgaccaaccgctccaagcccgtgtacctggtggacttctcctgctacaagcccgaggacgagcgcaagatgtccgtggact*

*ccttcctgaagatgaccgagcagaacggcgccttcaccgacgacaccgtgcagttccagcagcgcatctccaaccgcgccggc ctgggcgacgagacctacctgccccgcggcatcacctccacccccccaagctgaacatgtccgaggcccgcgccgaggccga ggccgtgatgttcggcgccctggactccctgttcgagaagaccggcatcaagcccgccgaggtgggcatcctgatcgtgtcctgct ccctgttcaaccccacccctccctgtccgccatgatcgtgaaccactacaagatgcgcgaggacatcaagtcctacaacctggg cggcatgggctgctccgccggcctgatctccatcgacctggccaacaacctgctgaaggccaaccccaactcctacgccgtggtg gtgtccaccgagaacatcaccctgaactggtacttcggcaacgaccgctccatgctgctgtgcaactgcatcttccgcatgggcgg cgccgccatcctgctgtccaaccgccgccaggaccgctccaagtccaagtacgagctggtgaacgtggtgcgcacccacaagg gctccgacgacaagaactacaactgcgtgtaccagaaggaggacgagcgcggcaccatcggcgtgtccctggcccgcgagct gatgtccgtggccggcgacgccctgaagaccaacatcaccaccctgggccccatggtgctgcccctgtccggccagctgatgttct ccgtgtccctggtgaagcgcaagctgctgaagctgaaggtgaagccctacatccccgacttcaagctggccttcgagcacttctgc atccacgccggcggccgcgccgtgctggacgaggtgcagaagaacctggacctggaggactggcacatggagccctcccgca tgaccctgcaccgcttcggcaacacctcctcctcctccctgtggtacgagatggcctacaccgaggccaagggccgcgtgaaggc cggcgaccgcctgtggcagatcgccttcggctccggcttcaagtgcaactccgccgtgtggaaggccctgcgcgtggtgtccacc gaggagctgaccggcaacgcctgggccggctccatcgagaactaccccgtgaagatcgtgcag*TGA<u>*cttaag*</u> (SEQ ID NO:107)

[0598] To determine their impact on fatty acid profiles, the above constructs containing various heterologous FAE genes, driven by the PmAMT3 promoter, were transformed independently into STRAIN Z.

[0599] Primary transformants were clonally purified and grown under low-nitrogen lipid production conditions at pH7.0 (all the plasmids require growth at pH 7.0 to allow for maximal FAE gene expression when driven by the pH regulated PmAMT03 promoter). The resulting profiles from a set of representative clones arising from transformations with pSZ3070, pSZ3071, pSZ3072, pSZ3067, pSZ3068 and pSZ3069 into STRAIN Z are shown in Tables 52-57, respectively, below.

[0600] All the transgenic STRAIN Z strains expressing heterologous FAE genes show an increased accumulation of C20:1 and C22:1 fatty acid (see Tables 52-57). The increase in eicosenoic ($20:1^{\Delta 11}$) and erucic ($22:1^{\Delta 13}$) acids levels over the wildtype is consistently higher than the wildtype levels. Additionally, the unsaturated fatty acid profile obtained with heterologous expression of BnFAE1 in STRAIN Z resulted in noticeable increase in Docosadienoic acid (C22:2n6). Protein alignment of aforementioned FAE expressed in STRAIN Z is shown in Figure 23.

[0601] Table 52. Unsaturated fatty acid profile in STRAIN Z and representative derivative transgenic lines transformed with pSZ3070 (CaFAE) DNA.

Sample ID	C18:1	C18:2	C18:3a	C20:1	C22:1	C22:2n6	C22:5
STRAIN Z; T588; D1828-20	51.49	9.13	0.65	**4.35**	**1.24**	0.11	0.00
STRAIN Z; T588; D1828-23	55.59	7.65	0.50	**3.78**	**0.85**	0.00	0.13
STRAIN Z; T588; D1828-43	54.70	7.64	0.50	**3.44**	**0.85**	0.09	0.00
STRAIN Z; T588; D1828-12	52.43	7.89	0.59	**2.72**	**0.73**	0.00	0.00
STRAIN Z; T588; D1828-19	56.02	7.12	0.52	**3.04**	**0.63**	0.10	0.11
Cntrl STRAIN Z pH 7	57.99	6.62	0.56	0.19	0.00	0.06	0.05
Cntrl STRAIN Z pH 5	57.70	7.08	0.54	0.11	0.00	0.05	0.05

[0602] Table 53. Unsaturated fatty acid profile in STRAIN Z and representative derivative transgenic lines transformed with pSZ3071 (LaFAE) DNA.

Sample ID	C18:1	C18:2	C18:3 a	C20:1	C22:1	C22:2n6	C22:5
STRAIN Z; T588; D1829-36	54.66	7.04	0.52	**1.82**	**0.84**	0.12	0.09
STRAIN Z; T588; D1829-24	56.27	6.72	0.51	**1.70**	**0.72**	0.09	0.00
STRAIN Z; T588; D1829-11	56.65	8.36	0.54	**2.04**	**0.67**	0.00	0.00
STRAIN Z; T588; D1829-35	55.57	7.71	0.53	**0.10**	**0.66**	0.00	0.00
STRAIN Z; T588; D1829-42	56.03	7.06	0.54	**1.54**	**0.51**	0.06	0.08
Cntrl STRAIN Z pH 7	57.70	7.08	0.54	0.11	0.00	0.06	0.05
Cntrl STRAIN Z pH 5	57.99	6.62	0.56	0.19	0.00	0.05	0.05

[0603] Table 54. Unsaturated fatty acid profile in STRAIN Z and representative derivative transgenic lines transformed with pSZ3072 (CgFAE) DNA.

Sample ID	C18:1	C18:2	C18:3 a	C20:1	C22:1	C22:2n6	C22:5
STRAIN Z; T588; D1830-47	57.74	7.79	0.52	**1.61**	**0.25**	0.11	0.05
STRAIN Z; T588; D1830-16	58.06	7.39	0.55	**1.64**	**0.22**	0.07	0.06
STRAIN Z; T588; D1830-12	57.77	6.86	0.51	**1.34**	**0.19**	0.09	0.00
STRAIN Z; T588; D1830-37	58.45	7.54	0.49	**1.65**	**0.19**	0.06	0.00
STRAIN Z; T588; D1830-44	57.10	7.28	0.56	**1.43**	**0.19**	0.07	0.00

Cntrl STRAIN Z pH 7	57.70	7.08	0.54	0.11	0.00	0.06	0.05
Cntrl STRAIN Z pH 5	57.99	6.62	0.56	0.19	0.00	0.05	0.05

[0604] Table 55. Unsaturated fatty acid profile in Strain AR and representative derivative transgenic lines transformed with pSZ3070 (TmFAE) DNA. No detectable Erucic (22:1) acid peaks were reported for these transgenic lines.

Sample ID	C18:1	C18:2	C18:3 a	C20:1	C22:2n6	C22:5
STRAIN Z; T588; D1825-47	59.97	7.44	0.56	**0.57**	0.00	0.00
STRAIN Z; T588; D1825-35	58.77	7.16	0.51	**0.50**	0.09	0.11
STRAIN Z; T588; D1825-27	60.40	7.82	0.47	**0.44**	0.07	0.07
STRAIN Z; T588; D1825-14	58.07	7.32	0.54	**0.41**	0.05	0.05
STRAIN Z; T588; D1825-40	58.66	7.74	0.46	**0.39**	0.08	0.00
Cntrl STRAIN Z pH 7	57.99	6.62	0.56	0.19	0.05	0.05
Cntrl STRAIN Z pH 5	57.70	7.08	0.54	0.11	0.06	0.05

[0605] Table 56. Unsaturated fatty acid profile in STRAIN Z and representative derivative transgenic lines transformed with pSZ3068 (BnFAE1) DNA. No detectable Erucic (22:1) acid peaks were reported for these transgenic lines.

Sample ID	C18:1	C18:2	C18:3 a	C20:1	C22:2n6	C22:5
STRAIN Z; T588; D1826-30	59.82	7.88	0.55	**0.32**	**0.17**	0.10
STRAIN Z; T588; D1826-23	59.32	8.02	0.58	**0.27**	**0.18**	0.07
STRAIN Z; T588; D1826-45	59.63	7.49	0.55	**0.27**	**0.19**	0.08
STRAIN Z; T588; D1826-24	59.35	7.78	0.57	**0.26**	**0.23**	0.00
STRAIN Z; T588; D1826-34	59.14	7.61	0.57	**0.25**	**0.22**	0.05
Cntrl STRAIN Z pH 7	57.81	7.15	0.59	0.19	0.04	0.06
Cntrl STRAIN Z pH 5	58.23	6.70	0.58	0.18	0.05	0.06

[0606] Table 57. Unsaturated fatty acid profile in STRAIN Z and representative derivative transgenic lines transformed with pSZ3069 (BnFAE2) DNA. No detectable Erucic (22:1) acid peaks were reported for these transgenic lines.

Sample ID	C18:1	C18:2	C18:3 a	C20:1	C22:2n6	C22:5
STRAIN Z; T588; D1827-6	60.59	8.20	0.57	**0.34**	0.00	0.07

STRAIN Z; T588; D1827-42	59.62	6.44	0.52	**0.30**	0.07	0.00
STRAIN Z; T588; D1827-48	59.71	7.99	0.59	**0.30**	0.06	0.00
STRAIN Z; T588; D1827-43	60.66	8.21	0.59	**0.29**	0.04	0.00
STRAIN Z; T588; D1827-3	60.26	7.99	0.57	**0.28**	0.04	0.00
Cntrl STRAIN Z pH 7	57.81	7.15	0.59	0.19	0.04	0.06
Cntrl STRAIN Z pH 5	58.23	6.70	0.58	0.18	0.05	0.06

EXAMPLE 54: ELEVATING TOTAL UNSATURATED FATTY ACIDS LEVEL BY EXPRESSING HETEROLOGOUS DESATURASE GENES

[0607] One of the approaches to generate a "zero SAT FAT" (e.g., total unsaturated fatty acids target at 97% or more/ less than or equal to 3% saturated fat) strain is to express desaturase genes in a high oleic strain such as Strain N, which we found to produce about 85% C18:1 with total un-saturates around 93% in multiple fermentation runs. We investigated if the total saturates will be further diminished by expressing desaturase genes in Strain N.

[0608] In the examples below, we demonstrated the ability to reduce stearic and palmitic levels in wild type strain UTEX1435 by over expression of heterologous stearoyl-ACP desaturase genes, including desaturases from *Olea europaea*, *Ricinus communis,* and *Chlorella protothecoides.*

[0609] **Construct used for the expression of the *Olea europaea* stearoyl-ACP desaturase:** To introduce the *O. europaea* stearoyl-ACP desaturase (Accession No: AAB67840.1) into UTEX1435, Strain A, the *Saccharomyces cerevisiae* invertase gene was utilized as the selectable marker to confer the ability of growing on sucrose media. The construct that has been expressed in UTEX1435, Strain A can be written as 6SA::CrTUB2:ScSUC2:CvNR::CrTUB2:CpSADtp :OeSAD:CvNR::6SB and is termed pSZ1377.

[0610] Relevant restriction sites in the construct pSZ1377 are indicated in lowercase, bold and underlining and are 5'-3' *BspQ* 1, *Kpn* I, *Xba* I, *Mfe* I, *BamH* I, *EcoR* I, *Spe* I, *Asc* I, *Cla* I, *Sac* I, *BspQ* I, respectively. *BspQI* sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent genomic DNA that permit targeted integration at 6s locus via homologous recombination. Proceeding in the 5' to 3' direction, the *C. reinhardtii* β -tubulin promoter driving the expression of the yeast sucrose invertase gene is indicated by boxed text. The initiator ATG and terminator TGA for invertase are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics. The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase underlined text

followed by the second *C. reinhardtii* β -tubulin promoter driving the expression of the OeSAD, indicated by boxed italics text. The Initiator ATG and terminator TGA codons of the OeSAD are indicated by uppercase, bold italics, while the remainder of the stearoyl-ACP desaturase coding region is indicated by bold italics. The *Chlorella protothecoides* stearoyl-ACP desaturase transit peptide is located between initiator ATG and the *Asc* I site. The *C. vulgaris* nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the 6S genomic region indicated by bold, lowercase text.

[0611] Nucleotide sequence of transforming DNA contained in pSZ 1377:

gctcttcgccgccgccactcctgctcgagcgcgcccgcgcgtgcgccgccagcgccttggccttttcgccgcgctcgtgcgcgtcgctgatgtcca
tcaccaggtccatgaggtctgccttgcgccggctgagccactgcttcgtccgggcggccaagaggagcatgagggaggactcctggtccaggg
tcctgacgtggtcgcggctctgggagcgggccagcatcatctggctctgccgcaccgaggccgcctccaactggtcctccagcagccgcagtcg
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agccagcgccgcacgctggcgctgcgcttcgccgatctgaggacagtcggggaactctgatcagtctaaaccccccttgcgcgttagtgttgcca
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[0612] **Construct used for the expression of the *Ricinus communis* stearoyl-ACP desaturase:** To introduce the *Ricinus communis* stearoyl-ACP desaturase (Accession No: AAA74692.1) into UTEX1435, Strain A, the *Saccharomyces cerevisiae* invertase gene was utilized as the selectable marker to confer the ability of growing on sucrose media. The construct that has been expressed in UTEX1435, Strain A can be written as 6SA::CrTUB2:ScSUC2:CvNR::PmAMT03:CpSADtp:RcSAD:CvNR::6SB and is termed pSZ1454.

[0613] Relevant restriction sites in the construct pSZ1454 are indicated in lowercase, bold and underlining and are 5'-3' *BspQ* 1, *Kpn* I, *Xba* I, *Mfe* I, *BamH* I, *EcoR* I, *Spe* I, *Asc* I, *Cla* I, *Sac* I, *BspQ* I, respectively. *BspQI* sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent genomic DNA that permit targeted integration at 6s nuclear chromosomal locus via homologous recombination. Proceeding in the 5' to 3' direction, the *C. reinhardtii* β -tubulin promoter driving the expression of the yeast sucrose invertase gene is indicated by boxed text. The initiator ATG and terminator TGA for invertase are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics. The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase underlined text followed by the endogenous AMT03 promoter driving the expression of the RcSAD, indicated by boxed italics text. The Initiator ATG and terminator TGA codons of the RcSAD are indicated by uppercase, bold italics, while the remainder of the stearoyl-ACP desaturase coding region is indicated by bold italics. The *Chlorella protothecoides* stearoyl-ACP desaturase transit peptide is located between initiator ATG and the *Asc* I site. The *C. vulgaris* nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the 6S genomic region indicated by bold, lowercase text.

[0614] Nucleotide sequence of transforming DNA contained in pSZ1454 :

gctcttcgccgccgccactcctgctcgagcgcgcccgcgcgtgcgccgccagcgccttggccttttcgccgcgctcgtgcgcgtc gctgatgtccatcaccaggtccatgaggtctgccttgcgccggctgagccactgcttcgtccgggcggccaagaggagcatga gggaggactcctggtccagggtcctgacgtggtcgcggctctgggagcgggccagcatcatctggctctgccgcaccgaggc cgcctccaactggtcctccagcagccgcagtcgccgccgaccctggcagaggaagacaggtgaggggggtatgaattgtaca

gaacaaccacgagccttgtctaggcagaatccctaccagtcatggctttacctggatgacggcctgcgaacagctgtccagcg
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gccactcgtacagcagaccattatgctacctcacaatagttcataacagtgaccatatttctcgaagctccccaacgagcacct
ccatgctctgagtggccacccccccggccctggtgcttgcggagggcaggtcaaccggcatggggctaccgaaatccccgacc
ggatcccaccacccccgcgatgggaagaatctctccccgggatgtgggcccaccaccagcacaacctgctggcccaggcga
gcgtcaaaccataccacacaaatatccttggcatcggccctgaattccttctgccgctctgctacccggtgcttctgtccgaagc
aggggttgctagggatcgctccgagtccgcaaacccttgtcgcgtggcggggcttgttcgagcttgaagagc (SEQ ID NO: 109)

[0615] **Construct used for the expression of the *Chlorella protothecoides* stearoyl-ACP desaturase:** To introduce the *Chlorella protothecoides* stearoyl-ACP desaturase into UTEX1435, Strain Z, the *Saccharomyces cerevisiae* invertase gene was utilized as the selectable marker to confer the ability of growing on sucrose media. The construct that has been expressed in UTEX1435, Strain Z can be written as 6SA::CrTUB2:ScSUC2:CvNR::PmAMT03:CpSAD1:CvNR::6SB and is termed pSZ3144.

[0616] Relevant restriction sites in the construct pSZ3144 are indicated in lowercase, bold and underlining and are 5'-3' *BspQ* 1, *Kpn* I, *Xba* I, *Mfe* I, *BamH* I, *EcoR* I, *Spe* I, *Cla* I, *Sac* I, *BspQ* I, respectively. *BspQI* sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent genomic DNA that permit targeted integration at 6s locus via homologous recombination. Proceeding in the 5' to 3' direction, the *C. reinhardtii* β -tubulin promoter driving the expression of the yeast sucrose invertase gene is indicated by boxed text. The initiator ATG and terminator TGA for invertase are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics. The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase underlined text followed by the endogenous AMT03 promoter driving the expression of the CpSAD1, indicated by boxed italics text. The Initiator ATG and terminator TGA codons of the CpSAD1 are indicated by uppercase, bold italics, while the remainder of the stearoyl-ACP desaturase coding region is indicated by bold italics. The *C. vulgaris* nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the 6S genomic region indicated by bold, lowercase text.

[0617] Nucleotide sequence of transforming DNA contained in pSZ3144:

gctcttcgccgccgccactcctgctcgagcgcgcccgcgcgtgcgccgccagcgccttggccttttcgccgcgctcgtgcgcgtcgct
gatgtccatcaccaggtccatgaggtctgccttgcgccggctgagccactgcttcgtccgggcggccaagaggagcatgagggag
gactcctggtccagggtcctgacgtggtcgcggctctgggagcgggccagcatcatctggctctgccgcaccgaggccgcctccaa
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[0618] Primary transformants were clonally purified and grown under low-nitrogen lipid production conditions at either pH5.0 or pH7.0, depending on the promoters that drive the expression of the desaturase genes. Transgenic lines arising from the transformations with pSZ1377 (D583) were assayed in (low-nitrogen) lipid production media at pH5.0, because of the nature of the promoters and the fact that *P. moriformis* produces more lipid at pH5.0. Transgenic lines generated from the transformation of pSZ1454 (D648) and pSZ3144 (D1923) are assayed at pH 7.0 to allow for maximal desaturase gene expression when driven by the pH regulated PmAMT3 promoter. The resulting profiles from representative clones arising from transformations with D583, D648, and D1923 are shown in Tables 58, 59 and 60, respectively, below. The result of expression of OeSAD and CpSAD1 genes is a clear diminution of C18:0 chain lengths with an increase in C18:1. Also we noticed that there is a subtle increase in the level of C16:1, indicating these stearoyl-ACP desaturases may have broad specificity. The transformants resulted from the expression of RcSAD gene also diminishes in the level of C18:0, and elevation in C16:1. Notably, C16:1 could be increased

from under 1% to over 1.5% or over 2%. However, there is also a drop in the level of C18:1 fatty acid and increase in C18:2, which may be caused by the growth defect of these transgenic lines.

[0619] Table 58. Lipid profile of representative clones arising from transformation with D583 (pSZ1377) DNA.

Sample ID	C16:0	C16:1	C18:0	C18:1	C18:2
D583-25	19.20	1.53	1.15	64.08	11.76
D583-10	21.86	0.99	1.77	61.43	11.42
D583-3	21.94	0.95	1.85	62.22	10.53
D583-33	20.76	0.95	1.85	61.76	12.17
D583-26	20.18	0.92	1.89	62.56	11.97
D583-1	21.28	0.95	1.90	62.63	10.94
S1331	25.48	0.71	3.23	59.70	8.25

[0620] Table 59. Lipid profile of representative clones arising from transformation with D648 (pSZ1454) DNA.

Sample ID	C16:0	C16:1	C18:0	C18:1	C18:2
D648-9	26.92	2.30	1.12	54.27	11.30
D648-28	26.54	2.50	1.32	52.58	12.90
D648-15	29.47	1.68	1.48	51.74	11.48
D648-12	27.39	1.41	1.66	54.45	11.58
D648-43	29.74	1.52	1.68	52.59	10.85
D648-7	26.98	1.62	1.69	54.51	11.39
S1331-pH7	25.86	0.96	2.84	58.33	9.16

[0621] Table 60. Lipid profile of representative clones arising from transformation with D1923 (pSZ3144) DNA.

Sample ID	C14:0	C14.1	C16:0	C16:1	C18:0	C18:1	C18:2
Block 2; E2; pH7; STRAIN Z; T613; D1923-2	1.46	0.11	20.74	2.54	0.86	63.99	9.03
Block 2; G12; pH7; STRAIN Z; T613; D1923-36	1.52	0.10	25.20	1.97	1.67	61.10	7.38

Block 2; E8; pH7; STRAIN Z; T613; D1923-8	1.48	0.09	26.41	1.78	1.54	60.54	7.01
Block 2; F3; pH7STRAIN Z; T613; D1923-15	1.50	0.07	25.87	1.75	1.62	61.25	6.94
Block 2; F9; pH7; STRAIN Z; T613; D1923-21	1.47	0.07	27.02	1.73	1.84	60.15	6.55
Block 2; F4; pH7; STRAIN Z; T613; D1923-16	1.44	0.07	24.30	1.71	1.41	62.79	7.29
pH7 STRAIN Z	1.47	0.00	28.25	0.82	3.16	58.27	6.72

EXAMPLE 55: GENERATION OF PALMITOLEIC ACID BY INTRODUCING MUTATED (L118W) STEAROYL-ACP DESATURASES

[0622] To generate lower total saturates (Zero SAT FAT) strains, we have introduced both putative stearoyl-ACP desaturases (SAD) and palmitoyl-ACP desaturase (PAD) genes into *Prototheca moriformis*. We found that a single amino acid substitution (L118W) in *P. moriformis* SAD2-1 and *Olea europaea* SAD resulted in an increase in desaturation of palmitate moieties in the triglycerides produced by the cell. Oils with fatty acid profiles of over 5% palmitoleic acid were produced in the resulting transgenic lines. Therefore, these mutated SADs could be very useful to elevate palmitoleic as a route to lower total saturates, or to obtain palmitoleic acid containing oils. Oils with over 2, 3, 4, and 5 area% palmitoleic were obtained.

[0623] The *Saccharomyces cerevisiae* invertase gene (Accession no: NP 012104) was utilized as the selectable marker to introduce the *Prototheca moriformis* stearoyl-ACP desaturase PmSAD2-1 (L118W) and *Olea europaea* stearoyl-ACP desaturase OeSAD (L118W) into 6S nuclear chromosomal locus of *P. moriformis* strain Z by homologous recombination using previously described biolistic transformation methods.

[0624] The constructs that have we used to transform Strain Z can be written as:

1)6SA::CrTUB2:ScSUC2:CvNR::PmUAPA1: PmSAD2-1(L118W)-CvNR::6SB (pSZ3305, D2066)

2) 6SA::CrTUB2:ScSUC2:CvNR::CrTUB2: PmSAD2-1(L118W)-CvNR::6SB (pSZ3299, D2060)

3) 6SA::CrTUB2:ScSUC2:CvNR::CrTUB2:CpSADtp-OeSAD (L118W)-CvNR::6SB (pSZ3298, D2059)

[0625] **--Construct pSZ3305: 6SA::CrTUB2:ScSUC2:CvNR::PmUAPA1: PmSAD2-1(L118W)-CvNR::6SB** The sequence of the pSZ3305 transforming DNA is provided below. Relevant restriction sites in pSZ3305 6SA::CrTUB2:ScSUC2:CvNR::PmUAPA1: PmSAD2-1(L118W)-CvNR::6SB are indicated in lowercase, bold and underlining and are 5'-3' *BspQ* 1, *Kpn* I, *Asc* I, *Mfe* I, EcoRV, SpeI, AscI, ClaI, *Sac* I, *BspQ* I, respectively. *BspQI* sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent 6SA genomic DNA that permit targeted integration at 6S locus via homologous recombination. Proceeding in the 5' to 3' direction, the *C. reinhardtii* β -tubulin promoter driving the expression of the yeast sucrose invertase gene is indicated by boxed text. The initiator ATG and terminator TGA for invertase are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics. The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase underlined text followed by the *P. moriformis* UAPA1 promoter, indicated by boxed italics text. The initiator ATG and terminator TGA codons of the PmSAD2-1 (L118W) are indicated by uppercase, bold italics, while the remainder of the coding region is indicated by bold italics. The *C. vulgaris* nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the 6SB genomic region indicated by bold, lowercase text.

[0626] Nucleotide sequence of transforming DNA contained in pSZ3305:

gctcttcgccgccgccactcctgctcgagcgcgcccgcgcgtgcgccgccagcgccttggccttttcgccgcgctcgtgcgcgtc
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ggaacacaaatggaaagcttaattaa**gagctc**ttgttttccagaaggagttgctccttgagcctttcattctcagcctcgataacctc

caaagccgctctaattgtggagggggttcgaatttaaaagcttggaatgttggttcgtgcgtctggaacaagcccagacttgtt

gctcactgggaaaaggaccatcagctccaaaaaacttgccgctcaaaccgcgtacctctgctttcgcgcaatctgccctgttga

aatcgccaccacattcatattgtgacgcttgagcagtctgtaattgcctcagaatgtggaatcatctgccccctgtgcgagccca

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atatttctcgaagctccccaacgagcacctccatgctctgagtggccacccccccggccctggtgcttgcggagggcaggtcaac

cggcatggggctaccgaaatccccgaccggatcccaccacccccgcgatgggaagaatctctccccgggatgtgggcccacc

accagcacaacctgctggcccaggcgagcgtcaaaccataccacacaaatatccttggcatcggccctgaattccttctgccg

ctctgctacccggtgcttctgtccgaagcaggggttgctagggatcgctccgagtccgcaaacccttgtcgcgtggcggggctt

gttcgagcttgaagagc (SEQ ID NO:111)

[0627] --Construct pSZ3299: 6SA::CrTUB2:ScSUC2:CvNR::CrTUB2: PmSAD2-1(L118W)-CvNR::6SB The sequence of the pSZ3299 transforming DNA is provided in

Sequence 56-2. Relevant restriction sites in pSZ3299 6SA::CrTUB2:ScSUC2:CvNR::CrTUB2:PmSAD2-1(L118W)-CvNR::6SB are indicated in lowercase, bold and underlining and are 5'-3' *BspQ* 1, *Kpn* I, *XbaI*, *Mfe* I, EcoRV, SpeI, AscI, ClaI, *Sac* I, *BspQ* I, respectively. *BspQI* sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent 6SA genomic DNA that permit targeted integration at 6S locus via homologous recombination. Proceeding in the 5' to 3' direction, the *C. reinhardtii* β -tubulin promoter driving the expression of the yeast sucrose invertase gene is indicated by boxed text. The initiator ATG and terminator TGA for invertase are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics. The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase underlined text followed by the *C. reinhardtii* β -tubulin promoter, indicated by boxed italics text. The Initiator ATG and terminator TGA codons of the PmSAD2-1 (L118W) are indicated by uppercase, bold italics, while the remainder of the coding region is indicated by bold italics. The *C. vulgaris* nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the 6SB genomic region indicated by bold, lowercase text.

[0628] Nucleotide sequence of transforming DNA contained in pSZ3299:

gctcttcgccgccgccactcctgctcgagcgcgcccgcgcgtgcgccgccagcgccttggccttttcgccgcgctcgtgcgcgtc gctgatgtccatcaccaggtccatgaggtctgccttgcgccggctgagccactgcttcgtccgggcggccaagaggagcatga gggaggactcctggtccagggtcctgacgtggtcgcggctctgggagcgggccagcatcatctggctctgccgcaccgaggc cgcctccaactggtcctccagcagccgcagtcgccgccgaccctggcagaggaagacaggtgaggggggtatgaattgtaca gaacaaccacgagccttgtctaggcagaatccctaccagtcatggctttacctggatgacggcctgcgaacagctgtccagcg accctcgctgccgccgcttctcccgcacgcttctttccagcaccgtgatggcgcgagccagcgccgcacgctggcgctgcgctt cgccgatctgaggacagtcggggaactctgatcagtctaaaccccccttgcgcgttagtgttgccatcctttgcagaccggtgag agccgacttgttgtgcgccacccccccacaccacctcctcccagaccaattctgtcacctttttggcgaaggcatcggcctcggcc tgcagagaggacagcagtgcccagccgctgggggttggcggatgcacgctcaggtaccctttcttgcgctatgacacttccagca aaaggtagggcgggctgcgagacggcttcccggcgctgcatgcaacaccgatgatgcttcgacccccccgaagctccttcggggctg catgggcgctccgatgccgctccagggcgagcgctgtttaaatagccaggcccccgattgcaaagacattatagcgagctaccaaag ccatattcaaacacctagatcactaccacttctacacaggccactcgagcttgtgatcgcactccgctaagggggcgcctcttcctcttc gtttcagtcacaacccgcaaac**tctaga**atatca***ATG****ctgctgcaggccttcctgttcctgctggccggcttcgccgccaagatcag cgcctccatgacgaacgagacgtccgaccgccccctggtgcacttcacccccaacaagggctggatgaacgaccccaacggcc tgtggtacgacgagaaggacgccaagtggcacctgtacttccagtacaacccgaacgacaccgtctgggggacgcccttgttctg gggccacgccacgtccgacgacctgaccaactgggaggaccagcccatcgccatcgccccgaagcgcaacgactccggcgc cttctccggctccatggtggtggactacaacaacacctccggcttcttcaacgacaccatcgacccgcgccagcgctgcgtggcca

tctggacctacaacaccccggagtccgaggagcagtacatctcctacagcctggacggcggctacaccttcaccgagtaccaga
agaaccccgtgctggccgccaactccacccagttccgcgacccgaaggtcttctggtacgagccctcccagaagtggatcatgac
cgcggccaagtcccaggactacaagatcgagatctactcctccgacgacctgaagtcctggaagctggagtccgcgttcgccaa
cgagggcttcctcggctaccagtacgagtgccccggcctgatcgaggtccccaccgagcaggaccccagcaagtcctactgggt
gatgttcatctccatcaaccccggcgccccggccggcggctccttcaaccagtacttcgtcggcagcttcaacggcacccacttcg
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cgggagcgccctgggcatcgcgtgggcctccaactgggagtactccgccttcgtgcccaccaaccccctggcgctcctccatgtccc
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caacagcaccggcaccctggagttcgagctggtgtacgccgtcaacaccacccagacgatctccaagtccgtgttcgcggacctc
tccctctggttcaagggcctggaggaccccgaggagtacctccgcatgggcttcgaggtgtccgcgtcctccttcttcctggaccgc
gggaacagcaaggtgaagttcgtgaaggagaaccctacttcaccaaccgcatgagcgtgaacaaccagcccttcaagagcg
agaacgacctgtcctactacaaggtgtacggcttgctggaccagaacatcctggagctgtacttcaacgacggcgacgtcgtgtcc
accaacacctacttcatgaccaccgggaacgccctgggctccgtgaacatgacgacgggggtggacaacctgttctacatcgac
*aagttccaggtgcgcgaggtcaag**TGA*****caattg**gcagcagcagctcggatagtatcgacacactctggacgctggtcgtgtgat
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cttggttcttcgtccattagcgaagcgtccggttcacacacgtgccacgttggcgaggtggcaggtgacaatgatcggtggagctgatg
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ggcgagcgctgtttaaatagccaggcccccgattgcaaagacattatagcgagctaccaaagccatattcaaacacctagatca
ctaccacttctacacaggccactcgagcttgtgatcgcactccgctaaggggcgcctcttcctcttcgtttcagtcacaacccgcaa
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cgtgctgcccgtgctgcaccccgtggacaagctgtggcagccccaggacttcctgcccgaccccgagtccccccgacttcgaggac
caggtggccgagctgcgcgcccgcgccaaggacctgcccgacgagtacttcgtggtgctggtgggcgacatgatcaccgagga
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cgctggacccgccagtgggtggccgaggagaaccgccacggcgacctgctgaacaagtactgctggctgaccggccgcgtga
acatgcgcgccgtggaggtgaccatcaacaacctgatcaagtccggcatgaaccccagaccgacaacaaccctacctgggc

ttcgtgtacacctccttccaggagcgcgccaccaagtactcccacggcaacaccgcccgcctggccgccgagcacggcgacaa gggcctgtccaagatctgcggcctgatcgcctccgacgagggccgccacgagatcgcctacacccgcatcgtggacgagttcttc cgcctggaccccgagggcgccgtggccgcctacgccaacatgatgcgcaagcagatcaccatgcccgcccacctgatggacg acatgggccacggcgaggccaaccccggccgcaacctgttcgccgacttctccgccgtggccgagaagatcgacgtgtacgac gccgaggactactgccgcatcctggagcacctgaacgcccgctggaaggtggacgagcgccaggtgtccggccaggccgccg ccgaccaggagtacgtgctgggcctgccccagcgcttccgcaagctggccgagaagaccgccgccaagcgcaagcgcgtggc ccgccgccccgtggccttctcctggatctccggccgcgagatcatggtgTGA<u>**atcgat**</u>agatctcttaaggcagcagcagctcgg atagtatcgacacactctggacgctggtcgtgtgatggactgttgccgccacacttgctgccttgacctgtgaatatccctgccgcttttat caaacagcctcagtgtgtttgatcttgtgtgtacgcgcttttgcgagttgctagctgcttgtgctatttgcgaataccacccccagcatccc cttccctcgtttcatatcgcttgcatcccaaccgcaacttatctacgctgtcctgctatccctcagcgctgctcctgctcctgctcactgccc ctcgcacagccttggtttgggctccgcctgtattctcctggtactgcaacctgtaaaccagcactgcaatgctgatgcacgggaagtagt gggatgggaacacaaatgg</u>aaagcttaattaa<u>**gagctc**</u>**ttgttttccagaaggagttgctccttgagcctttcattctcagcctcgat aacctccaaagccgctctaattgtggagggggttcgaatttaaaagcttggaatgttggttcgtgcgtctggaacaagcccaga cttgttgctcactgggaaaaggaccatcagctccaaaaaacttgccgctcaaaccgcgtacctctgctttcgcgcaatctgccct gttgaaatcgccaccacattcatattgtgacgcttgagcagtctgtaattgcctcagaatgtggaatcatctgccccctgtgcga gcccatgccaggcatgtcgcgggcgaggacacccgccactcgtacagcagaccattatgctacctcacaatagttcataacag tgaccatatttctcgaagctccccaacgagcacctccatgctctgagtggccacccccccggccctggtgcttgcggagggcagg tcaaccggcatggggctaccgaaatccccgaccggatcccaccacccccgcgatgggaagaatctctccccgggatgtgggc ccaccaccagcacaacctgctggcccaggcgagcgtcaaaccataccacacaaatatccttggcatcggccctgaattccttc tgccgctctgctacccggtgcttctgtccgaagcaggggttgctagggatcgctccgagtccgcaaacccttgtcgcgtggcgg ggcttgttcgagcttgaagagc** (SEQ ID NO:112)

[0629] **--Construct pSZ3298: 6SA::CrTUB2:ScSUC2:CvNR::CrTUB2:CpSADtp-OeSAD(L118W)-CvNR::6SB** The sequence of the pSZ3299 transforming DNA is provided below. Relevant restriction sites in the construct pSZ3298 6SA::CrTUB2:ScSUC2:CvNR::CrTUB2:CpSADtp-OeSAD(L118W)-CvNR::6SB are indicated in lowercase, bold and underlining and are 5'-3' *BspQ* 1, *Kpn* I, *XbaI*, *Mfe* I, EcoRV, SpeI, AscI, ClaI, *Sac* I, *BspQ* I, respectively. *BspQI* sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent 6SA genomic DNA that permit targeted integration at 6S locus via homologous recombination. Proceeding in the 5' to 3' direction, the *C. reinhardtii* β -tubulin promoter driving the expression of the yeast sucrose invertase gene is indicated by boxed text. The initiator ATG and terminator TGA for invertase are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics. The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase

underlined text followed by the *C. reinhardtii* β -tubulin promoter, indicated by boxed italics text. The Initiator ATG and terminator TGA codons of the OeSAD (L118W) are indicated by uppercase, bold italics, while the remainder of the coding region is indicated by bold italics. The *Chlorella protothecoides* S106 stearoyl-ACP desaturase transit peptide is located between initiator ATG and the *Asc* I site. The *C. vulgaris* nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the 6SB genomic region indicated by bold, lowercase text.

[0630] Nucleotide sequence of transforming DNA contained in pSZ3298:

gctcttcgccgccgccactcctgctcgagcgcgcccgcgcgtgcgccgccagcgccttggccttttcgccgcgctcgtgcgcgtc
gctgatgtccatcaccaggtccatgaggtctgccttgcgccggctgagccactgcttcgtccgggcggccaagaggagcatga
gggaggactcctggtccagggtcctgacgtggtcgcggctctgggagcgggccagcatcatctggctctgccgcaccgaggc
cgcctccaactggtcctccagcagccgcagtcgccgccgaccctggcagaggaagacaggtgaggggggtatgaattgtaca
gaacaaccacgagccttgtctaggcagaatccctaccagtcatggctttacctggatgacggcctgcgaacagctgtccagcg
accctcgctgccgccgcttctcccgcacgcttctttccagcaccgtgatggcgcgagccagcgccgcacgctggcgctgcgctt
cgccgatctgaggacagtcggggaactctgatcagtctaaacccccttgcgcgttagtgttgccatcctttgcagaccggtgag
agccgacttgttgtgcgccacccccccacaccacctcctcccagaccaattctgtcacctttttggcgaaggcatcggcctcggcc
tgcagagaggacagcagtgcccagccgctgggggttggcggatgcacgctcaggtaccctttcttgcgctatgacacttccagca
aaaggtagggcgggctgcgagacggcttcccggcgctgcatgcaacaccgatgatgcttcgacccccccgaagctccttcggggctg
catgggcgctccgatgccgctccagggcgagcgctgtttaaatagccaggcccccgattgcaaagacattatagcgagctaccaaag
ccatattcaaacacctagatcactaccacttctacacaggccactcgagcttgtgatcgcactccgctaaggggggcgcctcttcctcttc
gtttcagtcacaacccgcaaac**tctaga**atatca***ATG****ctgctgcaggccttcctgttcctgctggccggcttcgccgccaagatcag*
cgcctccatgacgaacgagacgtccgaccgccccctggtgcacttcacccccaacaagggctggatgaacgaccccaacggcc
tgtggtacgacgagaaggacgccaagtggcacctgtacttccagtacaacccgaacgacaccgtctgggggacgcccttgttctg
gggccacgccacgtccgacgacctgaccaactgggaggaccagcccatcgccatcgccccgaagcgcaacgactccggcgc
cttctccggctccatggtggtggactacaacaacacctccggcttcttcaacgacaccatcgacccgcgccagcgctgcgtggcca
tctggacctacaacaccccggagtccgaggagcagtacatctcctacagcctggacggcggctacaccttcaccgagtaccaga
agaaccccgtgctggccgccaactccacccagttccgcgacccgaaggtcttctggtacgagccctcccagaagtggatcatgac
cgcggccaagtcccaggactacaagatcgagatctactcctccgacgacctgaagtcctggaagctggagtccgcgttcgccaa
cgagggcttcctcggctaccagtacgagtgccccggcctgatcgaggtccccaccgagcaggaccccagcaagtcctactgggt
gatgttcatctccatcaaccccggcgccccggccggcggctccttcaaccagtacttcgtcggcagcttcaacggcacccacttcg
aggccttcgacaaccagtcccgcgtggtggacttcggcaaggactactacgccctgcagaccttcttcaacaccgacccgaccta
cgggagcgccctgggcatcgcgtgggcctccaactgggagtactccgccttcgtgcccaccaaccccctggcgctcctccatgtccc
tcgtgcgcaagttctccctcaacaccgagtaccaggccaacccggagacggagctgatcaacctgaaggccgagccgatcctg

aacatcagcaacgccggcccctggagccggttcgccaccaacaccacgttgacgaaggccaacagctacaacgtcgacctgtc
caacagcaccggcaccctggagttcgagctggtgtacgccgtcaacaccacccagacgatctccaagtccgtgttcgcggacctc
tccctctggttcaagggcctggaggaccccgaggagtacctccgcatgggcttcgaggtgtccgcgtcctccttcttcctggaccgc
gggaacagcaaggtgaagttcgtgaaggagaaccccctacttcaccaaccgcatgagcgtgaacaaccagcccttcaagagcg
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accaacacctacttcatgaccaccgggaacgccctgggctccgtgaacatgacgacgggggtggacaacctgttctacatcgac
*aagttccaggtgcgcgaggtcaag**TGA*<u>**caattg**</u>gcagcagcagctcggatagtatcgacacactctggacgctggtcgtgtgat
<u>ggactgttgccgccacacttgctgccttgacctgtgaatatccctgccgcttttatcaaacagcctcagtgtgtttgatcttgtgtgtacgcg</u>
<u>cttttgcgagttgctagctgcttgtgctatttgcgaataccacccccagcatccccttccctcgtttcatatcgcttgcatcccaaccgcaac</u>
<u>ttatctacgctgtcctgctatccctcagcgctgctcctgctcctgctcactgcccctcgcacagccttggtttgggctccgcctgtattctcc</u>
<u>tggtactgcaacctgtaaaccagcactgcaatgctgatgcacgggaagtagtgggatgggaacacaaatgga</u>**<u>ggatcc</u>**cgcgtctcg
aacagagcgcgcagaggaacgctgaaggtctcgcctctgtcgcacctcagcgcggcatacaccacaataaccacctgacgaatgcg
cttggttcttcgtccattagcgaagcgtccggttcacacacgtgccacgttggcgaggtggcaggtgacaatgatcggtggagctgatg
gtcgaaacgttcacagcctagg**gatatc**gaattc*ctttcttgcgctatgacacttccagcaaaaggtagggcgggctgcgagacgg*
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ggcgagcgctgtttaaatagccaggcccccgattgcaaagacattatagcgagctaccaaagccatattcaaacacctagatca
ctaccacttctacacaggccactcgagcttgtgatcgcactccgctaaggggcgcctcttcctcttcgtttcagtcacaacccgcaa
*ac***<u>**actagt**</u>***ATGgccaccgcatccactttctcggcgttcaatgcccgctgcggcgacctgcgtcgctcggcgggctccgggcccc***
ggcgcccagcgaggcccctccccgtgcgcgggcgcgccgaggtgcacgtgcaggtgacccactccctggcccccgagaagcg
cgagatcttcaactccctgaacaactgggcccaggagaacatcctggtgctgctgaaggacgtggacaagtgctggcagccctc
cgacttcctgcccgactccgcctccgagggcttcgacgagcaggtgatggagctgcgcaagcgctgcaaggagatccccgacg
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gcgcgacgagaccggcgcctccctgacccccctgggccatctggacccgcgcctggaccgccgaggagaaccgccacggcga
cctgctgaacaagtacctgtacctgtccggccgcgtggacatgaagcagatcgagaagaccatccagtacctgatcggctccgg
catggacccccgcaccgagaacaaccccctacctgggcttcatctacacctccttccaggagcgcgccaccttcatctcccacggc
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cacgagaccgcctacaccaagatcgtggagaagctgttcgagatcgaccccgacggcaccgtgctggccctggccgacatgat
gcgcaagaaggtgtccatgcccgcccacctgatgtacgacggccaggacgacaacctgttcgagaacttctcctccgtggccca
gcgcctgggcgtgtacaccgccaaggactacgccgacatcctggagttcctggtgggccgctgggacatcgagaagctgaccg
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gtcccgcgtgaagaaggcctccgccacccccttctcctggatcttcggccgcgagatcaacctgatggactacaaggaccacgac
***ggcgactacaaggaccacgacatcgactacaaggacgacgacgacaagTGA*<u>**atcgat**</u>**<u>agatctcttaaggcagcagcagct</u>**
<u>cggatagtatcgacacactctggacgctggtcgtgtgatggactgttgccgccacacttgctgccttgacctgtgaatatccctgccgctt</u>

ttatcaaacagcctcagtgtgtttgatcttgtgtgtacgcgcttttgcgagttgctagctgcttgtgctatttgcgaataccacccccagcatc
ccctttccctcgtttcatatcgcttgcatcccaaccgcaacttatctacgctgtcctgctatccctcagcgctgctcctgctcctgctcactgc
ccctcgcacagccttggtttgggctccgcctgtattctcctggtactgcaacctgtaaaccagcactgcaatgctgatgcacgggaagta
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ataacctccaaagccgctctaattgtggagggggttcgaatttaaaagcttggaatgttggttcgtgcgtctggaacaagccca
gacttgttgctcactgggaaaaggaccatcagctccaaaaaacttgccgctcaaaccgcgtacctctgctttcgcgcaatctgc
cctgttgaaatcgccaccacattcatattgtgacgcttgagcagtctgtaattgcctcagaatgtggaatcatctgccccctgtgc
gagcccatgccaggcatgtcgcgggcgaggacacccgccactcgtacagcagaccattatgctacctcacaatagttcataac
agtgaccatatttctcgaagctccccaacgagcacctccatgctctgagtggccacccccggccctggtgcttgcggagggca
ggtcaaccggcatggggctaccgaaatccccgaccggatcccaccacccccgcgatgggaagaatctctccccgggatgtgg
gcccaccaccagcacaacctgctggcccaggcgagcgtcaaaccataccacacaaatatccttggcatcggccctgaattcct
tctgccgctctgctacccggtgcttctgtccgaagcaggggttgctagggatcgctccgagtccgcaaacccttgtcgcgtggcg
gggcttgttcgagcttgaagagc (SEQ ID NO:113)

[0631] Primary transformants were clonally purified and grown under low-nitrogen lipid production conditions at pH5.0. The resulting profiles from representative clones arising from transformations with pSZ3305, pSZ3299 and pSZ3298 into Strain Z are shown in Tables 61-63 respectively. Thus , introductions of such mutations or genes can increase levels pf palmitoleic acid and decrease levels of saturation in the fatty acid profiles of oils produced by recombinant microalgae. Oils were obtained with C16:1/C16:0 ratios of at least 0.1, 0.15, and 0.18.

[0632] Table 61. Fatty acid profiles in Strain Z and derivative transgenic lines transformed with pSZ3305 (D2066).

Sample ID	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C16:1:C16:0 ratio
pH5; T657; D2066-29	1.27	24.73	4.55	3.63	58.62	5.84	0.18
pH5; T657; D2066-16	1.27	22.89	3.94	3.17	60.69	6.61	0.17
pH5; T657; D2066-36	1.33	25.47	3.07	3.58	59.32	5.86	0.12
pH5; T657; D2066-19	1.28	22.48	2.42	3.66	61.65	7.02	0.11
pH5;; T657; D2066-12	1.29	26.25	2.26	3.99	59.27	5.50	0.09
pH5; T657; D2066-21	1.33	24.49	2.26	3.24	61.42	6.01	0.09
pH5; Strain Z (200:1)	1.40	27.70	0.89	3.91	57.34	7.05	0.03

[0633] Table 62. Fatty acid profiles in Strain Z and derivative transgenic lines transformed with pSZ3299 (D2060).

Sample ID	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C16:1:C16:0 ratio
pH5; T655; D2060-9	1.35	24.67	2.73	3.21	60.34	6.22	0.11

pH5; T655; D2060-23	1.52	30.05	2.64	1.65	55.38	7.03	0.09
pH5; T655; D2060-21	1.29	23.54	2.43	2.94	62.25	6.18	0.10
pH5; T655; D2060-2	1.29	24.30	2.22	2.57	62.09	6.28	0.09
pH5; T655; D2060-12	1.37	27.67	1.90	2.84	59.69	5.41	0.07
pH5; T655; D2060-14	1.41	25.01	1.62	2.47	61.30	6.96	0.06
pH5 Strain Z	1.40	27.89	0.87	3.25	57.84	7.19	0.03

[0634] Table 63. Fatty acid profiles in Strain Z and derivative transgenic lines transformed with pSZ3298 (D2059).

Sample ID	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C16:1:C16:0 ratio
pH5; T655; D2059-21	1.09	25.44	5.04	1.86	54.78	10.44	0.19
pH5; T655; D2059-19	1.28	23.11	2.71	2.19	60.66	8.64	0.12
pH5; T655; D2059-4	1.68	28.19	1.61	2.54	58.39	6.37	0.06
pH5; T655; D2059-23	1.37	23.25	1.45	2.92	62.15	7.44	0.06
pH5; T655; D2059-1	1.38	23.34	1.28	2.68	62.31	7.62	0.05
pH5 Strain Z	1.40	27.89	0.87	3.25	57.84	7.19	0.03

EXAMPLE 56: DOWN REGULATION OF FATA AND OVER EXPRESSION OF THE *PROTOTHECA MORIFORMIS* KETO-ACYL-ACP SYNTHASE II (PMKASII) GENE

[0635] A transgenic *P. moriformis* line was created with downregulation of an endogenous FATA1 gene combined with overexpression of an endogenous KASII gene. The resulting strain produced a triglyceride-rich oil that was enriched in oleate.

[0636] In the example below, we have followed up on previous work demonstrating that triacylglycerols in algae can be significantly enriched in levels of oleate (C18:1) utilizing molecular genetic approaches, such as down regulating endogenous FATA1 (a single FATA allele) and over-expression of endogenous KASII activity. In this example, we focus our efforts on combining these approaches into a single transgenic line. Constructs that disrupt a single copy of the FATA1 allele while simultaneously overexpressing the *P. moriformis* KASII gene (PmKASII). were introduced into a high oleic Prototheca moriformis Strain AO. Strain AO was derived from a high 18:1 producing mutant derived from UTEX 1435 using classical mutagenesis techniques. One of the resulting strains, termed Strain AP, produced an oil with a fatty acid profile having 85% C18:1 with total un-saturates around 93% in multiple fermentation runs. The strain AP also had high lipid productivity.

[0637] The *Saccharomyces cerevisiae* invertase gene (Accession no: NP 012104) was utilized as the selectable marker to introduce the PmKASII into the FATA1 nuclear chromosomal locus of *P. moriformis* strain AO by homologous recombination using biolistic

transformation. To investigate the KASII activity when driven by different promoters, PmKASII was fused to several promoters: PmUAPA1, PmLDH1, and PmAMT3. Note that the integration constructs are all designed as reverse orientation to the FATA1 gene; this was found to give a greater likelihood of stable invertase expression. Therefore, the constructs that have been expressed in Strain AH can be written as:
1)FATA1 3'::CrTUB2:ScSUC2:CvNR::PmUAPA1:PmKASII-CvNR::FATA1 5' (pSZ2533)
2) FATA1 3'::CrTUB2:ScSUC2:CvNR::PmLDH1:PmKASII-CvNR::FATA1 5' (pSZ2532)
3) FATA1 3'::CrTUB2:ScSUC2:CvNR::PmAMT3:PmKASII-CvNR::FATA1 5' (pSZ2750)

[0638] Strain AP is one of the transformants generated from pSZ2533. Relevant restriction sites in the construct pSZ2533 FATA13'::CrTUB2:ScSUC2:CvNR::PmUAPA1:PmKASII-CvNR::FATA1 5' are indicated in lowercase, bold and underlining and are 5'-3' *BspQ* 1, *Kpn* I, *Asc* I, *Mfe* I, EcoRV, SpeI, AscI, ClaI, *Sac* I, *BspQ* I, respectively. *BspQI* sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent FATA1 3' genomic DNA that permit targeted integration at FATA1 locus via homologous recombination. Proceeding in the 5' to 3' direction, the *C. reinhardtii* β -tubulin promoter driving the expression of the yeast sucrose invertase gene is indicated by boxed text. The initiator ATG and terminator TGA for invertase are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase underlined text followed by the *P. moriformis* UAPA1 promoter, indicated by boxed italics text. The Initiator ATG and terminator TGA codons of the PmKASII are indicated by uppercase, bold italics, while the remainder of the coding region is indicated by bold italics. The *Chlorella protothecoides* S106 stearoyl-ACP desaturase transit peptide is located between initiator ATG and the *Asc* I site. The *C. vulgaris* nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the FATA1 5' genomic region indicated by bold, lowercase text.

[0639] Nucleotide sequence of transforming DNA contained in pSZ2533:

gctcttcacccaactcagataataccaataccctccttctcctcctcatccattcagtacccccccccttctcttcccaaagcagc aagcgcgtggcttacagaagaacaatcggcttccgccaaagtcgccgagcactgcccgacggcggcgcgcccagcagcccg cttggccacacaggcaacgaatacattcaataggggcctcgcagaatggaaggagcggtaaagggtacaggagcactgc gcacaaggggcctgtgcaggagtgactgactgggcgggcagacggcgcaccgcgggcgcaggcaagcagggaagattga agcggcagggaggaggatgctgattgaggggggcatcgcagtctctcttggacccgggataaggaagcaaatattcggccg gttgggttgtgtgtgtgcacgttttcttcttcagagtcgtgggtgtgcttccagggaggatataagcagcaggatcgaatcccgc gaccagcgtttccccatccagccaaccaccctgtcggtaccctttcttgcgctatgacacttccagcaaaaggtagggcgggctgc

gagacggcttcccggcgctgcatgcaacaccgatgatgcttcgaccccccgaagctccttcggggctgcatgggcgctccgatgcc
gctccagggcgagcgctgtttaaatagccaggcccccgattgcaaagacattatagcgagctaccaaagccatattcaaacacctaga
tcactaccacttctacacaggccactcgagcttgtgatcgcactccgctaaggggggcgcctcttcctcttcgtttcagtcacaacccgca
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gccaagtggcacctgtacttccagtacaacccgaacgacaccgtctgggggacgcccttgttctggggccacgccacgtccgac
gacctgaccaactgggaggaccagcccatcgccatcgccccgaagcgcaacgactccggcgccttctccggctccatggtggtg
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agtacgagtgccccggcctgatcgaggtccccaccgagcaggaccccagcaagtcctactgggtgatgttcatctccatcaaccc
cggcgccccggccggcggctccttcaaccagtacttcgtcggcagcttcaacggcacccacttcgaggccttcgacaaccagtcc
cgcgtggtggacttcggcaaggactactacgccctgcagaccttcttcaacaccgacccgacctacgggagcgccctgggcatc
gcgtgggcctccaactgggagtactccgccttcgtgcccaccaaccccctggcgctcctccatgtccctcgtgcgcaagttctccctc
aacaccgagtaccaggccaacccggagacggagctgatcaacctgaaggccgagccgatcctgaacatcagcaacgccggc
ccctggagccggttcgccaccaacaccacgttgacgaaggccaacagctacaacgtcgacctgtccaacagcaccggcaccct
ggagttcgagctggtgtacgccgtcaacaccacccagacgatctccaagtccgtgttcgcggacctctcccctctggttcaagggcct
ggaggaccccgaggagtacctccgcatgggcttcgaggtgtccgcgtcctccttcttcctggaccgcgggaacagcaaggtgaa
gttcgtgaaggagaacccctacttcaccaaccgcatgagcgtgaacaaccagcccttcaagagcgagaacgacctgtcctacta
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ccaccgggaacgccctgggctccgtgaacatgacgacgggggtggacaacctgttctacatcgacaagttccaggtgcgcgag
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gctgccttgacctgtgaatatccctgccgcttttatcaaacagcctcagtgtgtttgatcttgtgtgtacgcgcttttgcgagttgctagctgc
ttgtgctatttgcgaataccacccccagcatcccccttccctcgtttcatatcgcttgcatcccaaccgcaacttatctacgctgtcctgctatc
cctcagcgctgctcctgctcctgctcactgcccctcgcacagccttggtttgggctccgcctgtattctcctggtactgcaacctgtaaac
cagcactgcaatgctgatgcacgggaagtagtgggatgggaacacaaatggaggatcccgcgtctcgaacagagcgcgcagagga
acgctgaaggtctcgcctctgtcgcacctcagcgcggcatacaccacaataaccacctgacgaatgcgcttggttcttcgtccattagc
gaagcgtccggttcacacacgtgccacgttggcgaggtggcaggtgacaatgatcggtggagctgatggtcgaaacgttcacagcct
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gctttgcattatccacacactattcatcgctgctgcggcaaggctgcagagtgtatttttgtggcccaggagctgagtccgaagtcga
cgcgacgagcggcgcaggatccgacccctagacgagctctgtcattttccaagcacgcagctaaatgcgctgagaccgggtcta
aatcatccgaaaagtgtcaaaatggccgattgggttcgcctaggacaatgcgctgcggattcgctcgagtccgctgccggccaaa

aggcggtggtacaggaaggcgcacggggccaaccctgcgaagccgggggcccgaacgccgaccgccggccttcgatctcgg

gtgtccccctcgtcaatttcctctctcgggtgcagccacgaaagtcgtgacgcaggtcacgaaatccggttacgaaaaacgcagg

tcttcgcaaaaacgtgagggtttcgcgtctcgccctagctattcgtatcgccgggtcagacccacgtgcagaaaagcccttgaata

acccgggaccgtggttaccgcgccgcctgcaccaggggggcttatataagcccacaccacacctgtctcaccacgcatttctccaa

ctcgcgacttttcggaagaaattgttatccacctagtatagactgccacctgcaggaccttgtgtcttgcagtttgtattggtcccggcc

gtcgagctcgacagatctgggctagggttggcctggccgctcggcactccccttttagccgcgcgcatccgcgttccagaggtgcg

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gcgtggtgacctccctgggccagaccatcgagcagttctactcctccctgctggagggcgtgtccggcatctcccagatccagaag

ttcgacaccaccggctacaccaccaccatcgccggcgagatcaagtccctgcagctggacccctacgtgcccaagcgctgggcc

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cggcgtggaggccctgacccgcggcggcgtgcgcaagatgaaccccttctgcatccccttctccatctccaacatgggcggcgcc

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tccaactccttcggcttcggcggccacaactcctgcgtgatcttccgcaagtacgacgagatggactacaaggaccacgacggcg

actacaaggaccacgacatcgactacaaggacgacgacgacaagTGA **atcgat** agatctcttaaggcagcagcagctcggat

agtatcgacacactctggacgctggtcgtgtgatggactgttgccgccacacttgctgccttgacctgtgaatatccctgccgcttttatca

aacagcctcagtgtgtttgatcttgtgtgtacgcgcttttgcgagttgctagctgcttgtgctatttgcgaataccacccccagcatcccctt

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gcacagccttggtttgggctccgcctgtattctcctggtactgcaacctgtaaaccagcactgcaatgctgatgcacgggaagtagtgg

gatgggaacacaaatggaaagcttaattaa**gagctcttgttttccagaaggagttgctccttgagcctttcattctcagcctcgataa**

cctccaaagccgctctaattgtggagggggttcgaaccgaatgctgcgtgaacgggaaggaggaggagaaagagtgagca

gggagggattcagaaatgagaaatgagaggtgaaggaacgcatccctatgcccttgcaatggacagtgtttctggccaccgc

caccaagacttcgtgtcctctgatcatcatgcgattgattacgttgaatgcgacggccggtcagccccggacctccacgcaccg gtgctcctccaggaagatgcgcttgtcctccgccatcttgcagggctcaagctgctcccaaaactcttgggcgggttccggacg gacggctaccgcgggtgcggccctgaccgccactgttcggaagcagcggcgctgcatgggcagcggccgctgcggtgcgcc acggaccgcatgatccaccggaaaagcgcacgcgctggagcgcgcagaggaccacagagaagcggaagagacgccagta ctggcaagcaggctggtcggtgccatggcgcgctactaccctcgctatgactcgggtcctcggccggctggcggtgctgacaa ttcgtttagtggagcagcgactccattcagctaccagtcgaactcagtggcacagtgactccgctcttc (SEQ ID NO:114)

[0640] In addition to the construct pSZ2533, we also investigated the PmKASII activity when the KASII gene driven by other promoters, including PmLDH1, and PmAMT3. The plasmid pSZ2532 can be written as FATA1 3'::CrTUB2:ScSUC2:CvNR::PmLDH1:PmKASII-CvNR::FATA1 5', while the plasmid pSZ2750 can be written as FATA1 3'::CrTUB2:ScSUC2: CvNR::PmAMT3:PmKASII-CvNR::FATA1 5'. Since the sequences of these two plasmids are the same as pSZ2533 except for the promoter that drives the PmKASII, the following sequences only show the sequence of the PmLDH1 and PmAMT3 promoters.

[0641] Nucleotide sequence of PmLDH1 promoter that drive the expression of PmKASII in pSZ2532:

Gatatc*tccctccgtctctgcactctggcgcccctcctccgtctcgtggactgacggacgagagtctgggcgccgcttttctatccac accgccctttccgcatcgaagacaccacccatcgtgccgccaggtcttccccaatcacccgccctgtggtcctctctcccagccgtg tttggtcgctgcgtccacatttttccattcgtgccccacgatcctcgcccatcttggcgccttggataggcacccttttttcagcacgccc tggtgtgtagcacaacctgacctctctctaccgcatcgcctccctcccacacctcagttgactccctcgtcgcacgttgcacccgcaa gctccccatttcatcctattgacaatcgcacactgtacatgtatgctcattatttttgcaaaaaaacaggggtcggttcactcctggca gacgacgcggtgctgccgcgcgccgctgaggcggcgtcgcgacggcaacacccatcgcaccgcacgtcgacgagtcaaccc accctgctcaacggtgatctccccatcgcgacacccccgtgaccgtactatgtgcgtccatacgcaacatgaaaaggaccttggt ccccggaggcggcgagctcgtaatcccgaggttggccccgcttccgctggacacccatcgcatcttccggctcgcccgctgtcga gcaagcgccctcgtgcgcgcaaccttgtggtgcctgcccgcagagccgggcataaaggcgagcaccacacccgaaccagtc caatttgctttctgcattcactcaccaacttttacatccacacatcgtactaccacacctgcccagtcgggtttgatttctattgcaaagg tgcgggggggttggcgcactgcgtgggttgtgcagccggccgccgcggctgtacccagcgatcaggtagcttgggctgtatcttct caagcattaccttgtcctgggcgtaggtttgcc**actagt** (SEQ ID NO:115)

[0642] Nucleotide sequence of PmAMT3 promoter that drive the expression of PmKASII in pSZ2750:

Gatatcgaattcggccgacaggacgcgcgtcaaaggtgctggtcgtgtatgccctggccggcaggtcgttgctgctggttagtg
attccgcaaccctgattttggcgtcttattttggcgtggcaaacgctggcgcccgcgagccgggccggcggcgatgcggtgccccac
ggctgccggaatccaagggaggcaagagcgcccgggtcagttgaagggctttacgcgcaaggtacagccgctcctgcaaggctgc
gtggtggaattggacgtgcaggtcctgctgaagttcctccaccgcctcaccagcggacaaagcaccggtgtatcaggtccgtgtcatc
cactctaaagaactcgactacgacctactgatggccctagattcttcatcaaaaacgcctgagacacttgcccaggattgaaactccctg
aagggaccaccaggggccctgagttgttccttccccccgtggcgagctgccagccaggctgtacctgtgatcgaggctggcgggaa
aataggcttcgtgtgctcaggtcatgggaggtgcaggacagctcatgaaacgccaacaatcgcacaattcatgtcaagctaatcagct
atttcctcttcacgagctgtaattgtcccaaaattctggtctaccgggggtgatccttcgtgtacgggcccttccctcaaccctaggtatgc
gcgcatgcggtcgccgcgcaactcgcgcgagggccgagggtttgggacgggccgtcccgaaatgcagttgcacccggatgcgtg
gcacctttttgcgataatttatgcaatggactgctctgcaaaattctggctctgtcgccaaccctaggatcagcggcgtaggatttcgtaa
tcattcgtcctgatggggagctaccgactaccctaatatcagcccgactgcctgacgccagcgtccacttttgtgcacacattccattcgt
gcccaagacatttcattgtggtgcgaagcgtccccagttacgctcacctgtttcccgacctccttactgttctgtcgacagagcgggccc
acaggccggtcgcagcc**actagt** (SEQ ID NO:116)

[0643] Primary transformants were clonally purified and grown under low-nitrogen lipid production conditions at either pH5.0 or pH7.0, depend on the promoters that driven the expression of the PmKASII gene. Transgenic lines arising from the transformations with pSZ2533 (D1636) and pSZ2532 (D1637) were assayed in lipid production media at pH5.0, because of the nature of the promoters and the fact that *P. moriformis* produces more lipid at pH5.0. Transgenic lines generated from the transformation of pSZ2750 (D1684) were assayed at pH 7.0 to allow for maximal PmKASII gene expression when driven by the pH regulated PmAMT3 promoter. The resulting profiles from representative clones arising from transformations with D1636 (pSZ2533), D1637 (pSZ2532), and D1684 (pSZ2750) are shown in Tables 64-66, respectively.

[0644] The impact of FATA1 knock-out and simultaneously overexpressing the *P. moriformis* KASII gene is a clear diminution of C16:0 chain lengths with a significant increase in C18:1. At pH5.0, it appears that PmUAPA1 is stronger than PmLDH1, the palmitate level in D1636 transformants is close to 3%, while none of the transformants in D1637 go below 7% at the same condition.

[0645] Table 64. Lipid profile of representative clones arising from transformation with D1636 (pSZ2533) DNA.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2
pH5; T523; D1636-3	0.53	3.31	6.15	79.89	7.19
pH5; T523; D1636-4	0.48	3.54	5.34	80.78	6.92

pH5; T523; D1636-5	0.48	3.59	5.41	81.37	6.55
pH5; T523; D1636-12	0.61	3.59	3.67	80.52	8.93
pH5; T523; D1636-13	0.55	3.80	4.88	81.83	6.61
pH5; T523; D1636-21	0.54	4.18	2.82	82.26	8.17
pH5; Strain AO	0.89	17.28	2.69	70.53	6.86

[0646] Table 65. Lipid profile of representative clones arising from transformation with D1637 (pSZ2532) DNA.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2
pH5; T523; D1637-6	0.46	7.64	3.43	80.08	6.33
pH5; T523; D1637-12	0.66	8.49	1.90	77.06	9.59
pH5; T523; D1637-13	0.47	8.59	3.18	79.39	6.54
pH5;T523; D1637-15	0.60	9.60	2.51	76.41	8.85
pH5; T523; D1637-7	0.61	11.16	2.21	75.82	8.04
pH5; T523; D1637-8	0.93	11.29	3.61	74.84	6.61
pH5; Strain AO	0.89	17.28	2.69	70.53	6.86

[0647] Table 66. Lipid profile of representative clones arising from transformation with D1684 (pSZ2750) DNA.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2
pH7; T532; D1684-14	0.55	5.04	4.90	78.88	8.19
pH7; T532; D1684-23	0.58	5.80	4.98	77.51	8.69
pH7; T532; D1684-1	0.59	6.37	4.99	77.47	8.03
pH7; T532; D1684-24	0.55	6.37	4.83	77.98	7.73
pH7; T532; D1684-11	0.61	6.61	4.88	76.14	8.96
pH7; T532; D1684-16	0.57	6.61	5.01	77.74	7.83
pH7; Strain AO	0.84	20.12	3.52	66.86	6.77

EXAMPLE 57: GENERATION OF A HIGH-OLEIC HIGH-STABILITY (HOHS) OIL-PRODUCING STRAIN

[0648] Strain AP of Example 56 produces oil with about 85% oleic acid with total un-saturates around 93%. Here we show that that that the oxidative stability of the high-oleic oil can be improved by knock-down of a delta 12 fatty acid desaturase, thereby reducing linoleic acid production in the oleaginous cell.

[0649] We expressed a hairpin-RNA-producing construct in Strain AP targeting an endogenous FAD gene, PmFAD2. The resulting strains, including Strain AQ, produce >90% C18:1 and <1% C18:2 in fermenters. Most importantly, Strain AQ retains the same level of lipid productivity and sucrose hydrolyzing ability as its parental strain, Strain AP.

[0650] **Generation of high oleic high stability oil producing strain AQ:** ***Construct used for down regulating PmFAD2.*** To generate a strain that produces oil with high oxidative

stability, the hairpin PmFAD2 was introduced into AP for down regulating PmFAD2 expression. Strain AQ is a stable line generated from the transformation of pSZ3372 DNA (6SA::PmHXT1:ScarMEL1:CvNR::CrTUB2: Hairpin PmFAD2:CvNR::6SB) into Strain AP. In this construct, the *Saccharomyces carlbergensis MEL1 gene* was utilized as the selectable marker to introduce the Hairpin PmFAD2 into the 6S nuclear chromosomal locus of *P. moriformis* strain AQ by homologous recombination using previously described transformation methods (biolistics).

[0651] The sequence of the pSZ3372 transforming DNA is provided below. Relevant restriction sites in pSZ3372 are indicated in lowercase, bold and underlining and are 5'-3' *BspQ* 1, *Kpn* I, *Spe*I, *Mfe* I, BamHI, EcoRV, SpeI, XhoI, SacI, *BspQ* I, respectively. *BspQI* sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent 6SA genomic DNA that permits targeted integration at 6S locus via homologous recombination. Proceeding in the 5' to 3' direction, the *P.moriformis HXT1* promoter driving the expression of the *S.carlbergensis* MEL1 gene is indicated by boxed text. The initiator ATG and terminator TGA for ScarMEL1 are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics. The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase underlined text followed by the *C. reinhardtii* β -tubulin promoter, indicated by boxed italics text. The hairpin PmFAD2 cassette includes the *P. moriformis* FAD2 exon1 (indicated by italics underlined text), the intron of PmFAD2 (italics lowercase text), and followed by the inverted PmFAD2 exon1 (indicated by italics underlined text). The *C. vulgaris* nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the 6SB genomic region indicated by bold, lowercase text.

[0652] Nucleotide sequence of transforming DNA contained in pSZ3372:

gctcttcgccgccgccactcctgctcgagcgcgcccgcgcgtgcgccgccagcgccttggccttttcgccgcgctcgtgcgcgtc
gctgatgtccatcaccaggtccatgaggtctgccttgcgccggctgagccactgcttcgtccgggcggccaagaggagcatga
gggaggactcctggtccagggtcctgacgtggtcgcggctctgggagcgggccagcatcatctggctctgccgcaccgaggc
cgcctccaactggtcctccagcagccgcagtcgccgccgaccctggcagaggaagacaggtgaggggggtatgaattgtaca
gaacaaccacgagccttgtctaggcagaatccctaccagtcatggctttacctggatgacggcctgcgaacagctgtccagcg
accctcgctgccgccgcttctcccgcacgcttctttccagcaccgtgatggcgcgagccagcgccgcacgctggcgctgcgctt
cgccgatctgaggacagtcggggaactctgatcagtctaaacccccttgcgcgttagtgttgccatcctttgcagaccggtgag
agccgacttgttgtgcgccacccccacaccacctcctcccagaccaattctgtcacctttttggcgaaggcatcggcctcggcc
tgcagagaggacagcagtgcccagccgctgggggttggcggatgcacgctcaggtacc**gcggtgagaatcgaaaatgcatcgt
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catgggcctcccgacgcggccatcaggagcccaaacagcgtgtcagggtatgtgaaactcaagaggtccctgctgggcactccggc

cccactccgggggcgggacgccaggcattcgcggtcggtcccgcgcgacgagcgaaatgatgattcggttacgagaccaggacgt

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acgccgacgaggaccaatccccggcatcagccttcatcgacggctgcgccgcacatataaagccggacgcctaaccggtttcgtgg

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c*ctttcttgcgctatgacacttccagcaaaaggtagggcgggctgcgagacggcttcccggcgctgcatgcaacaccgatgatgc*

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*atcgcactccgctaaggggcgcctcttcctcttcgtttcagtcacaacccgcaaac***actagt*** ***ATG*** *gctatcaagacgaacagg*
cagcctgtggagaagcctccgttcacgatcgggacgctgcgcaaggccatccccgcgcactgtttcgagcgctcggcgcttcgta
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gagcggccacatgatgccgtacttgacccacgtaggcaccggtgcagggtcgatgtacgtcgacgcgacgtagagcagggaca
tgaccgcgatgtcaaaggccaggtacatgctgctacgaagcgccgagcgctcgaaacagtgcgcggggatggccttgcgcagc
*gtcccgatcgtgaacggaggcttctccacaggctgcctgttcgtcttgatagccat****ctcgag*** gcagcagcagctcggatagtatcga
cacactctggacgctggtcgtgtgatggactgttgccgccacacttgctgccttgacctgtgaatatccctgccgcttttatcaaacagcc
tcagtgtgtttgatcttgtgtgtacgcgcttttgcgagttgctagctgcttgtgctatttgcgaataccacccccagcatccccttccctcgttt
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cttggtttgggctccgcctgtattctcctggtactgcaacctgtaaaccagcactgcaatgctgatgcacgggaagtagtgggatggga
acacaaatggaaagctgta**gagctcttgttttccagaaggagttgctccttgagcctttcattctcagcctcgataacctccaaagc**
cgctctaattgtggaggggttcgaatttaaaagcttggaatgttggttcgtgcgtctggaacaagcccagacttgttgctcact
gggaaaaggaccatcagctccaaaaaacttgccgctcaaaccgcgtacctctgctttcgcgcaatctgccctgttgaaatcgcc
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acaacctgctggcccaggcgagcgtcaaaccataccacacaaatatccttggcatcggccctgaattccttctgccgctctgct
acccggtgcttctgtccgaagcaggggttgctagggatcgctccgagtccgcaaacccttgtcgcgtggcggggcttgttcgag
cttgaagagc (SEQ ID NO: 117)

[0653] We introduced the hairpin PmFAD2 construct into strain AP. Transgenic lines arising from the transformations with pSZ3372 (D2082) were assayed in lipid production media at pH5.0, the resulting profiles from representative clones are shown in Table 67. Among more than 400 transformants we had screened, the strain AQ was isolated from the transformant D2082.1, which produced <1% C18:2 during the initial profile screening. Thus, this strain can be used to produce a triglyceride oil that is both high in oleic acid and low in polyunsaturates. Due to the low polyunsaturate levels, the oil is expected to have a high oxidative stability when tested via the AOCS Cd 12b-92 method (see Section IV of this patent application and corresponding examples).

[0654] Table 67. Lipid profile of representative clones arising from transformation with D2082 (pSZ3372) DNA.

Sample ID	C16:0	C18:0	C18:1	C18:2	C18:3 α
SAP_pH5.0_glucose_day5-T658;D2082-1	4.42	3.80	89.36	0.65	0.10
StrainAP_pH5.0_glucose_day5-T658;D2082-87	3.77	4.01	88.70	1.52	0.19
StrainAP _pH5.0_glucose_day5-T658;D2082-93	5.14	3.58	87.63	1.65	0.19
StrainAP _pH5.0_glucose_day5-T658;D2082-78	3.74	2.40	89.69	1.97	0.23
StrainAP _pH5.0_glucose_day5	4.10	3.77	83.55	6.41	0.40

EXAMPLE 58: GENERATING HIGH OLEIC "ZERO" LINOLEIC STRAINS BY KNOCK-OUT PROTOTHECA MORFORMIS (PM) FAD2 AND FATA GENES AND OVER-EXPRESSION OF PMKASII GENE

[0655] Triacylglycerols in microalgae can be significantly enriched in levels of oleate (C18:1) utilizing molecular genetic approaches, such as down regulating endogenous FATA1 and FADc genes and over-expression of endogenous KASII activity. In this example, we focus our efforts on combining these approaches into a single transgenic line. Constructs that disrupt a single copy of the FATA1 allele while simultaneously overexpressing the *Prototheca moriformis* KASII gene were introduced into different Δ*fad2* lines, termed Strain R and Strain D (see genealogy in Figure 24). The resulting strains, such as Strain AS and Strain AZ produces around 90% C18:1 with <0.05% C18:2.

[0656] Strain D and Strain R are Δ*fad2* lines that produce oils comprised of 0% C18:2, and between 76% to 87% C18:1, depending upon whether they are grown in shake flasks or high cell density fermentations, respectively. To further elevate oleate levels in Strain D and Strain R, constructs that disrupt a single copy of the FATA1 allele while simultaneously overexpressing the *P. moriformis* KAS II gene were introduced in StrainD/Strain R via particle bombardment.

[0657] **Construct to knock out FATA genes and over expression of PmKASII in S2530 background.** Relevant restriction sites in the construct FATA1::CpACT-AtThic-nr:AMT03-S106SAD-PmKASII-nr::FATA1 (termed pSZ2276) are indicated in lowercase, bold and underlining and are 5'-3' *BspQ* 1, *Kpn* I, *Xba* I, *Mfe* I, *BamH* I, *EcoR* I, *Spe* I, *Asc* I, *Cla* I, *Sac* I, *BspQ* I, respectively. *BspQI* sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent genomic DNA from UTEX1435 that permit

targeted integration at FATA1 gene via homologous recombination. Proceeding in the 5' to 3' direction, the actin gene promoter from UTEX 250 driving the expression of the *Arabidopsis thaliana* THIC gene is indicated by the boxed text. The initiator ATG and terminator TGA for AtTHIC are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics. The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase underlined text followed by an endogenous AMT03 promoter of *Prototheca moriformis,* indicated by boxed italics text. The Initiator ATG and terminator TGA codons of the *P. moriformis* KASII gene are indicated by uppercase, bold italics, while the remainder of the PmKASII coding region is indicated by bold italics. The *Chlorella protothecoides* UTEX 250 stearoyl-ACP desaturase transit peptide is located between initiator ATG and the *Asc* I site. The *C. vulgaris* nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the UTEX1435 FATA1 genomic region indicated by bold, lowercase text.

[0658] Nucleotide sequence of transforming DNA contained in pSZ2276:

gctcttcggagtcactgtgccactgagttcgactggtagctgaatggagtcgctgctccactaaacgaattgtcagcaccgcca
gccggccgaggacccgagtcatagcgagggtagtagcgcgccatggcaccgaccagcctgcttgccagtactggcgtctcttc
cgcttctctgtggtcctctgcgcgctccagcgcgtgcgcttttccggtggatcatgcggtccgtggcgcaccgcagcggccgctg
cccatgcagcgccgctgcttccgaacagtggcggtcagggccgcacccgcggtagccgtccgtccggaacccgcccaagagt
ttgggagcagcttgagccctgcaagatggcggaggacaagcgcatcttcctggaggagcaccggtgcgtggaggtccgggg
ctgaccggccgtcgcattcaacgtaatcaatcgcatgatgatcagaggacacgaagtcttggtggcggtggccagaaacact
gtccattgcaagggcatagggatgcgttccttcacctctcatttctcatttctgaatccctccctgctcactctttctcctcctcttc
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gtaggatttcgtaatcattcgtcctgatggggagctaccgactaccctaatatcagcccgactgcctgacgccagcgtccacttttgt

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*gtcgacagagcgggcccacaggccggtcgcagcc***actagt***ATGcagaccgcccaccagcgcccccccaccgagggccact*

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ttggtattatctgagttgggtgaagagc (SEQ ID NO:118)

[0659] **Construct to knock out FATA genes and over expression of PmKASII in S2532 background.** Relevant restriction sites in the construct FATA1::CpACT-AtThic-nr:PmUAPA1-S106SAD-PmKASII-nr::FATA1 (termed pSZ2441) are indicated in lowercase, bold and underlining and are 5'-3' *BspQ* 1, *Kpn* I, *Xba* I, *Mfe* I, *BamH* I, *EcoR* V, *Spe* I, *Asc* I, *Cla* I, *Sac* I, *BspQ* I, respectively. *BspQI* sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent genomic DNA from UTEX1435 that permit targeted integration at FATA1 gene via homologous recombination. Proceeding in the 5' to 3' direction, the actin gene promoter from UTEX 250 driving the expression of the *A. thaliana* THIC gene is indicated by the boxed text. The initiator ATG and terminator TGA for AtTHIC are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics. The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase underlined text followed by an endogenous UAPA1 promoter of *Prototheca moriformis,* indicated by boxed italics text. The Initiator ATG and terminator TGA codons of the *P. moriformis* KASII gene are indicated by uppercase, bold italics, while the remainder of the PmKASII coding region is indicated by bold italics. The *Chlorella protothecoides* UTEX 250 stearoyl-ACP desaturase transit peptide is located between initiator ATG and the Asc I site. The C. vulgaris nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the UTEX1435 FATA1 genomic region indicated by bold, lowercase text.

[0660] Nucleotide sequence of transforming DNA contained in pSZ2441:

gctcttcggagtcactgtgccactgagttcgactggtagctgaatggagtcgctgctccactaaacgaattgtcagcaccgcca
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caaggccctgtccaagcgcaacgacgagcccgagcgcgcctcccgcccctgggacgccgaccgcgacggcttcgtgatgggc gagggcgccggcgtgctggtgctggaggagctggagcacgccaagcgccgcggcgccaccatcctggccgagctggtgggcg gcgccgccacctccgacgcccaccacatgaccgagcccgacccccagggccgcggcgtgcgcctgtgcctggagcgcgccct ggagcgcgcccgcctggcccccgagcgcgtgggctacgtgaacgcccacggcacctccacccccgccggcgacgtggccgag taccgcgccatccgcgccgtgatcccccaggactccctgcgcatcaactccaccaagtccatgatcggccacctgctgggcggcg ccggcgccgtggaggccgtggccgccatccaggccctgcgcaccggctggctgcaccccaacctgaacctggagaaccccgc ccccggcgtggaccccgtggtgctggtgggcccccgcaaggagcgcgccgaggacctggacgtggtgctgtccaactccttcgg cttcggcggccacaactcctgcgtgatcttccgcaagtacgacgagatggactacaaggaccacgacggcgactacaaggacc acgacatcgactacaaggacgacgacgacaagTGA<u>**atcgat**</u>agatctcttaaggcagcagcagctcggatagtatcgacacac tctggacgctggtcgtgtgatggactgttgccgccacacttgctgccttgacctgtgaatatccctgccgcttttatcaaacagcctcagt gtgtttgatcttgtgtgtacgcgcttttgcgagttgctagctgcttgtgctatttgcgaataccacccccagcatccccttccctcgtttcatat cgcttgcatcccaaccgcaacttatctacgctgtcctgctatccctcagcgctgctcctgctcctgctcactgcccctcgcacagccttgg tttgggctccgcctgtattctcctggtactgcaacctgtaaaccagcactgcaatgctgatgcacgggaagtagtgggatgggaacaca aatgga</u>aagcttaattaagagctcttgttttccagaaggagttgctccttgagcctttcattctcagcctcgataacctccaaagccgctcta attgtggaggggggttcgaa**gacagggtggttggctggatggggaaacgctggtcgcgggattcgatcctgctgcttatatcctcc ctggaagcacacccacgactctgaagaagaaaacgtgcacacacacaacccaaccggccgaatatttgcttccttatcccgg gtccaagagagactgcgatgcccccctcaatcagcatcctcctccctgccgcttcaatcttccctgcttgcctgcgcccgcggtg cgccgtctgcccgcccagtcagtcactcctgcacaggcccccttgtgcgcagtgctcctgtacccctttaccgctccttccattctgc gaggcccccctattgaatgtattcgttgcctgtgtggccaagcgggctgctgggcgcgccgccgtcgggcagtgctcggcgactt tggcggaagccgattgttcttctgtaagccacgcgcttgctgctttgggaagagaaggggggggtactgaatggatgaggag gagaaggaggggtattggtattatctgagttgggtgaagagc**</u> (SEQ ID NO:119)

[0661] Southern blot analysis of Strain AS and Strain AZ indicated that both are PmFATA double knock-out mutants. Since the PmFAD2 disruption cassettes contain a *Carthamus tinctorius* putative oleoyl-specific ACP-thioesterase (CtOTE), the absence of the endogenous FATA genes seems to be fully complemented by the expression of the CtOTE.

[0662] To determine the impact of FATA1 inactivation and over expression of PmKASII gene on lipid composition in Δ*fad2* lines Strain D/Strain R, the primary transformants of D1266/Strain D and D1415/Strain R were clonally purified and grown under standard lipid production conditions at both pH5.0 and pH7.0. The resulting profiles from the transgenic line arising from transformation with pSZ2276 into Strain D are shown in Table 68, and transgenic lines arising from transformation with pSZ2441 into Strain R are shown in Table 69.

[0663] As can be seen from Table 68, in Strain AZ at pH7.0, the combination of full activity of PmKASII driven by AMT03 and FATA1 knock results in very low levels of C16:0 (2%). Meanwhile, the *Carthamus tinctorius* thioesterase is also activated since it is also driven by AMT03 promoter. We observe 7.8% C18:0 when Strain AZ is cultivated at pH7. At pH5.0, decrease of the C16:0 level is largely contributed by the FATA1 inactivation, although PmKASII can be partially activated since we run the seed culture at pH6.8. The stearic level of Strain AZ is low at pH5.0 due to the low expression of the *C. tinctorius* TE. Overall, the oleic levels of Strain AZ exceed 85% (around 88%) at both pH7.0 and pH5.0.

[0664] Table 68. Fatty acid profiles in S1331, S2530 and S4266 at both pH5.0 and pH7.0

Strains	C16:0	C18:0	C18:1	C18:2	C20:1
Strain A_pH5	26.6	3.3	60.5	6.7	0.07
Strain A_pH7	28.3	4.1	58	6.5	0.06
Strain D_pH5	17	3.6	77.1	0.01	0.14
Strain D_pH7	19.5	5.3	72.6	0.01	0.09
Strain AZ_pH5	4.1	2.36	88.5	0.04	3.1
Strain AZ_pH7	2.1	7.8	87.9	0.01	0.5

[0665] In the transgenic line Strain AS, both CrTUB2 and PmUAPA1 promoters are pH unbiased, hence, as reported in Table 69, the lipid profile at pH5.0 and pH7.0 are essentially same. Relative to Strain AZ, Strain AS produces much less stearic acid. Although the palmitic level in Strain AS is bit higher than that in Strain AZ, the oleic level in Strain AS is above 90%, which is the highest level we observed in the shake flask experiment.

[0666] Table 69. Fatty acid profiles in S1331, S2532 and S5204 at both pH5.0 and pH7.0

Strains	C16:0	C18:0	C18:1	C18:2
Strain A_pH5	26.6	3.3	60.5	6.7
Strain A_pH7	28.3	4.1	58	6.5
Strain R_pH5	23.3	2.1	72.1	0.01
Strain R_pH7	23.4	2.3	71.9	0.01
Strain AS_pH5	5.5	1.4	91.5	0.01
Strain AS_pH7	5.6	1.6	91.3	0.01

EXAMPLE 59: COMPLEMENTATION OF FAD2 AND FATA KNOCKOUT AND KASII OVEREXPRESSION GENERATES A UNIQUE OIL WITH HIGH C18-2 AND LOW C18-3 LEVELS

[0667] As described in Example 58, Strain AS was generated by knocking both copies of *PmFATA1* in a *Prototheca moriformis* strain while simultaneously overexpressing *PmKASII* gene into a Δ*fad2* line (Strain R). Strain R is a FAD2 (also known as FADc) knockout strain generated by insertion of a oleate-specific *C. tinctorius* acyl-ACP thioesterase (GenBank

Accession No: AAA33019.1) into a high-lipid producing strain derived from UTEX 1435, under the control of CrTUB2 promoter at the FAD2 locus. Strain AS and its parent, Strain R, have a disrupted endogenous PmFAD2-1 gene resulting in no Δ12 specific desaturase activity manifested as 0% C18:2 (linoleic acid) levels in both nitrogen-rich seed and nitrogen-poor lipid production conditions. Lack of C18:2 in Stain AS (and its parent Strain R) resulted in growth defects which could be partially mitigated by exogenous addition of linoleic acid in the seed stage. However, for industrial applications, exogenous addition of linoleic acid is expensive. Complementation of Strain R (and a second Δ*fad2* strain) with PmFAD2-1 restored C18:2 levels back to wild type levels and also resulted in rescued growth characteristics during seed and lipid production without any linoleic supplementation.

[0668] In the present example we demonstrate that:

- *In trans* expression of fatty acid desaturase-2 gene from *Prototheca moriformis* (PmFad2-1) under the control of a pH inducible PmAMT3 promoter results in functional complementation of PmFAD2-1 with restored growth and C18:2 levels in Δ*fad2*, Δ*fata1* strain AS;
- Complementation of Strain AS is conditional/inducible and occurs at pH 7.0 when the AMT3 promoter is actively driving the expression of PmFAD2-1 as opposed to pH 5.0 when the AMT3 promoter is inactive; and
- Over expression of PmFAD2-1 at pH 7.0 results in strains with >20% C18:2 levels. ***The fatty acid profile of these high C18:2 strains mimic canola oil closely except that the new oil has 5 fold less C18:3 than the canola oil (10%).*** The elevated C18:2 levels are seen only in strains derived from Strain AS overexpressing PmFAD2-1 since overexpression of the same gene in wild-type (i.e., non-engineered) control Strain Z does not result in higher C18:2 levels.

[0669] Construct used for the expression of the *Prototheca moriformis* fatty acid desaturase 2 *(PmFAD2-1)* in Δ*fad2* strains Strain AS and Strain Z - [pSZ2721]. Δ*fad2* Δ*fata1* Strain AS and Strain Z were transformed with the construct pSZ2721. The sequence of the transforming DNA is provided below. Relevant restriction sites in the construct pSZ2721 (6S::CpACT-ScMEL1-CvNR::PmAMT3-PmFAD2-1-CvNR::6S) are indicated in lowercase, underlined and bold, and are from 5'-3' *BspQ* 1, KpnI, Xba I, *Mfe* I, *BamH* I, *EcoR* I, *Spe* I, *Cla* I, *Sac* I, *BspQ* I, respectively. *BspQI* sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent genomic DNA from UEX 1435 that permits targeted integration of PmFAD2-1 at the 6S locus via homologous recombination.

Proceeding in the 5' to 3' direction, the actin (ACT) gene promoter from UTEX 250 driving the expression of the *Saccharomyces cerevisiae* MEL1 gene is indicated by the boxed text. The initiator ATG and terminator TGA for ScMEL1 are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics. The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase underlined text followed by an endogenous AMT03 promoter of *Prototheca moriformis,* indicated by boxed italics text. The Initiator ATG and terminator TGA codons of the PmFAD2-1 are indicated by uppercase, bold italics, while the remainder of the gene is indicated by bold italics. The *C. vulgaris* nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the UTEX 1435 6S genomic region indicated by bold, lowercase text. The final construct was sequenced to ensure correct reading frames and targeting sequences.

[0670] Nucleotide sequence of transforming DNA contained in plasmid pSZ2721:

gctcttcggagtcactgtgccactgagttcgactggtagctgaatggagtcgctgctccactaaacgaattgtcagcaccgcca gccggccgaggacccgagtcatagcgagggtagtagcgcgccatggcaccgaccagcctgcttgccagtactggcgtctcttc cgcttctctgtggtcctctgcgcgctccagcgcgtgcgcttttccggtggatcatgcggtccgtggcgcaccgcagcggccgctg cccatgcagcgccgctgcttccgaacagtggcggtcagggccgcacccgcggtagccgtccgtccggaacccgcccaagagt ttgggagcagcttgagccctgcaagatggcggaggacaagcgcatcttcctggaggagcaccggtgcgtggaggtccgggg ctgaccggccgtcgcattcaacgtaatcaatcgcatgatgatcagaggacacgaagtcttggtggcggtggccagaaacact gtccattgcaagggcatagggatgcgttccttcacctctcatttctcatttctgaatccctccctgctcactctttctcctcctcttc ccgttcacgcagcattcggggtaccagtttaggtccagcgtccgtgggggggacgggctgggagcttgggccgggaagggcaa gacgatgcagtccctctggggagtcacagccgactgtgtgtgttgcactgtgcggcccgcagcactcacacgcaaaatgcctggccg acaggcaggccctgtccagtgcaacatccacggtccctctcatcaggctcaccttgctcattgacataacggaatgcgtaccgctctttc agatctgtccatccagagaggggagcaggctccccaccgacgctgtcaaacttgcttcctgcccaaccgaaaacattattgtttgagg gggggggggggggggcagattgcatggcgggatatctcgtgaggaacatcactgggacactgtggaacacagtgagtgcagtatg cagagcatgtatgctaggggtcagcgcaggaaggggggcctttcccagtctcccatgccactgcaccgtatccacgactcaccaggac cagcttcttgatcggcttccgctcccgtggacaccagtgtgtagcctctggactccaggtatgcgtgcaccgcaaaggccagccgatc gtgccgattcctggggtggaggatatgagtcagccaacttggggctcagagtgcacactggggcacgatacgaaacaacatctacac cgtgtcctccatgctgacacaccacagcttcgctccacctgaatgtgggcgcatgggcccgaatcacagccaatgtcgctgctgccat aatgtgatccagaccctctccgcccagatgccgagcggatcgtgggcgctgaatagattcctgtttcgatcactgtttgggtcctttccttt tcgtctcggatgcgcgtctcgaaacaggctgcgtcgggctttcggatccctttgctccctccgtcaccatcctgcgcgcgggcaagtt gcttgaccctgggctgtaccagggttggagggtattaccgcgtcaggccattcccagcccggattcaattcaaagtctgggccaccac cctccgccgctctgtctgatcactccacattcgtgcatacactacgttcaagtcctgatccaggcgtgtctcgggacaaggtgtgcttga gtttgaatctcaaggacccactccagcacagctgctggttgaccccgccctcgcaatctaga***ATG***ttcgcgttctacttcctgacggc

ctgcatctccctgaagggcgtgttcggcgtctccccctcctacaacggcctgggcctgacgccccagatgggctgggacaactgg
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tacaagtacatcatcctggacgactgctggtcctccggccgcgactccgacggcttcctggtcgccgacgagcagaagttcccca
acggcatgggccacgtcgccgaccacctgcacaacaactccttcctgttcggcatgtactcctccgcgggcgagtacacgtgcgc
cggctaccccggctccctgggccgcgaggaggaggacgcccagttcttcgcgaacaaccgcgtggactacctgaagtacgaca
actgctacaacaagggccagttcggcacgcccgagatctcctaccaccgctacaaggccatgtccgacgccctgaacaagacg
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actccaacggcatccccgccacgcgcgtctggcgctactacgtgtccgacacggacgagtacggccagggcgagatccagatg
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acaactccacggcgtccgccatcctgggccgcaacaagaccgccaccggcatcctgtacaacgccaccgagcagtcctacaag
gacggcctgtccaagaacgacacccgcctgttcggccagaagatcggctccctgtcccccaacgcgatcctgaacacgaccgtc
*cccgcccacggcatcgcgttctaccgcctgcgcccctcctcc**TGA**caattg*gcagcagcagctcggatagtatcgacacactctg
gacgctggtcgtgtgatggactgttgccgccacacttgctgccttgacctgtgaatatccctgccgcttttatcaaacagcctcagtgtgtt
tgatcttgtgtgtacgcgcttttgcgagttgctagctgcttgtgctatttgcgaataccacccccagcatcccctcccctcgtttcatatcgctt
gcatcccaaccgcaacttatctacgctgtcctgctatccctcagcgctgctcctgctcctgctcactgcccctcgcacagccttggtttgg
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aaccacctgacgaatgcgcttggttcttcgtccattagcgaagcgtccggttcacacacgtgccacgttggcgaggtggcaggtgaca
atgatcggtggagctgatggtcgaaacgttcacagcctagggatatc**gaattc***ggccgacaggacgcgcgtcaaaggtgctggtc*
gtgtatgccctggccggcaggtcgttgctgctgctggttagtgattccgcaaccctgattttggcgtcttattttggcgtggcaaacgct
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cgccacacttgctgccttgacctgtgaatatccctgccgcttttatcaaacagcctcagtgtgtttgatcttgtgtgtacgcgcttttgcgagt

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gtcctgctatccctcagcgctgctcctgctcctgctcactgcccctcgcacagccttggtttgggctccgcctgtattctcctggtactgca

acctgtaaaccagcactgcaatgctgatgcacgggaagtagtgggatgggaacacaaatggaaagcttaattaagagctcttgttttcc

agaaggagttgctccttgagcctttcattctcagcctcgataacctccaaagccgctctaattgtggagggggttcgaa**gacagggtgg**

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gaaaacgtgcacacacacaacccaaccggccgaatatttgcttccttatcccgggtccaagagagactgcgatgcccccctca

atcagcatcctcctccctgccgcttcaatcttccctgcttgcctgcgcccgcggtgcgccgtctgcccgcccagtcagtcactcct

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tgtggccaagcgggctgctgggcgcgccgccgtcgggcagtgctcggcgactttggcggaagccgattgttcttctgtaagcc

acgcgcttgctgctttgggaagagaaggggggggtactgaatggatgaggaggagaaggaggggtattggtattatctgag

ttgggtgaagagc (SEQ ID NO:120)

[0671] To determine its impact on growth and fatty acid profiles, the above construct was transformed independently into a Δ*fad2* Δ*fata1* Strain AS or wild type Strain Z. Primary transformants were clonally purified and grown under low-nitrogen lipid production conditions at pH7.0 (AMT3 promoter active) and pH5.0 (AMT3 promoter inactive) for Strain

AS transformants or at pH7.0 for Strain Z transformants. The resulting profiles from a set of representative clones arising from transformations are shown in Tables 70-73 respectively.

[0672] Expression of endogenous PmFad2-1 driven by AMT3 promoter at pH 7.0, in Strain AS resulted in Δ12 specific desaturase activity with complete restoration of C18:2 fatty acid levels of the base strain A (Table 70). No such Δ12 specific desaturase activity and thus no significant C18:2 restoration is detected when the lipid production is run at pH 5.0 when the AMT3 promoter is inactive (Table 71).

[0673] Interestingly, lipid production in complemented Strain AS strains at pH 7.0 results in several strains with 2 fold or more increase in C18:2 levels. The resulting strains produce an oil profile that is similar to Canola oil except that the new oil has less C18:3 levels than the commercially available canola oil (Table 72). The increase in C18:2 is not seen in wild type (Strain Z) strains transformed with the same AMT3 driven PmFAD2-1.

[0674] While we have seen other strains with high C18:2 levels, all of them were associated with growth defects in seed as well as lipid production media. Here, however, we have been able to increase the C18:2 levels in a targeted manner without any detrimental effect on the growth of resulting strains. While Δ*fad2* strain R *and* Δ*fad2* Δ*fata1* strain AS grow very poorly and hardly reach an OD750 of 10-20 in 42 hours, complemented Strain AS (D1673) lines grow very rapidly in the same time span and reach OD750 between 50-80.

[0675] Thus, it can be seen that we were able to produce cell oils with fatty acid profiles of less than 10% linolenic acid yet >20% linoleic acid (indeed we achieved <2% linolenic acid and >20% linoleic acid) . It is surprising that C18:2 levels are elevated only in Strain AS, which has almost 90% C18:1 levels as compared to Strain Z with only 57% C18:1 levels, suggests excess availability of substrate C18:1 in the ER is a key to boost C18:2 levels. Since *Prototheca* has evolved to utilize C18:1 onto TAGS very efficiently, in wild type situations most likely the substrate leaves the ER very rapidly before being further desaturated by FAD2 enzymes. This limitation may be overcome in strains like Strain AS with very high C18:1 levels that likely stays available for desaturation by PmFAD2-1.

[0676] Table 70. Fatty acid profile in representative complemented (D1673) and parent Strain AS lines at pH 7.0 transformed with pSZ2721 (PmFAD2-1) DNA.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3a
AS; T533; D1673- 16; pH 7.0	0.49	6.33	2.44	66.53	**21.36**	1.38
AS T533; D1673- 17; pH 7.0	0.44	6.02	2.25	68.97	**19.53**	1.36
AS; T533; D1673- 02; pH 7.0	0.38	5.92	2.30	71.01	**17.77**	1.30
AS; T533; D1673- 03; pH 7.0	0.38	5.83	2.31	71.31	**17.45**	1.29

AS; T533; D1673- 10; pH 7.0	0.38	5.63	2.21	71.72	**17.37**	1.23
AS; pH 7.0	0.30	5.59	1.63	90.88	0.10	0.00
AT; pH 7.0	1.34	27.99	3.54	55.48	9.07	0.79

[0677] Table 71. Fatty acid profile in same representative complemented (D1673) and parent Strain AS lines at pH 5.0 transformed with pSZ2721 (PmFAD2-1) DNA.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3a
AS; T533; D1673- 16; pH 5.0	0.47	5.16	1.76	90.94	**0.06**	0.18
AS; T533; D1673- 17; pH 5.0	0.45	4.97	1.72	91.32	**0.05**	0.00
AS; T533; D1673- 02; pH 5.0	0.46	5.20	1.75	90.94	**0.05**	0.18
AS; T533; D1673- 03; pH 5.0	0.41	4.93	1.65	89.92	**1.56**	0.16
AS; T533; D1673- 10; pH 5.0	0.45	4.97	1.69	89.96	**1.35**	0.16
AS; pH 5.0	0.39	5.67	1.36	91.13	0.00	0.00
AT; pH 5.0	1.03	24.69	3.30	63.47	5.80	0.38

[0678] Table 72. Fatty acid profile of a stable D1673 line along with base strain Z and Canola oil.

Sample ID	C16:0	C18:0	C18:1	C18:2	C18:3 α	C20:1
pH5 Strain Z	27.54	3.29	57.91	7.17	0.59	0.10
pH7 Strain Z	27.92	3.09	58.30	6.71	0.59	0.07
pH7;AS;T533;D1673.5.2-1	4.43	1.31	70.32	**20.30**	**1.72**	0.75
pH7;AS;T533;D1673.5.2-2	4.55	1.26	67.53	**22.17**	**1.82**	1.22
pH7;AS;T533;D1673.5.2-3	4.34	1.29	69.51	**20.78**	**1.65**	1.01
pH7;AS;T533;D1673.5.2-4	4.81	1.26	68.08	**21.53**	**1.77**	1.06
pH7;AS;T533;D1673.5.2-5	4.61	1.30	68.02	**21.57**	**1.74**	1.17
pH7;AS;T533;D1673.5.2-6	4.36	1.30	68.88	**21.16**	**1.68**	1.10
pH7;AS;T533;D1673.5.2-7	4.38	1.28	69.30	**21.08**	**1.70**	0.97
pH7;AS;T533;D1673.5.2-8	4.87	1.27	68.44	**20.87**	**1.83**	1.14
Canola Oil	4.00	2.00	62.00	22.00	10.00	1.00

[0679] Table 73. Fatty acid profile in Strain Z at pH 5.0 and pH 7.0 and representative derivative transgenic lines at pH 7.0 transformed with pSZ2721 (PmFAD2-1) DNA. The lines are sorted by C18:2 levels.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3a
Z; T573; D1791-23; pH 7.0	1.45	29.96	3.28	54.72	**7.99**	0.66
Z; T573; D1791-6; pH 7.0	1.73	30.25	2.48	55.01	**7.74**	0.69
Z; T573; D1791-17; pH 7.0	1.41	29.00	3.42	55.77	**7.64**	0.68
Z; T573; D1791-14; pH 7.0	1.48	29.82	3.45	55.22	**7.56**	0.67
Z; T573; D1791-8; pH 7.0	2.30	37.15	2.54	47.62	**7.44**	0.67
Z; T573; D1791-2; pH 7.0	1.38	29.29	3.45	56.10	**7.12**	0.63
Z; T573; D1791-10; pH 7.0	1.46	29.30	3.39	56.16	**7.11**	0.60
Z; T573; D1791-5; pH 7.0	1.45	29.45	3.36	56.15	**7.02**	0.61

Z; T573; D1791-11; pH 7.0	1.43	29.52	3.44	55.99	**7.01**	0.60
Z; T573; D1791-13; pH 7.0	1.41	28.96	3.46	56.47	**7.01**	0.62
Z; pH 7.0	1.41	27.76	3.45	57.71	7.17	0.58
Z; pH 5.0	1.49	28.19	3.27	58.04	6.65	0.57

EXAMPLE 60: COMBINATORIAL EXPRESSION OF MID-CHAIN THIOESTERASES AND KETOACYL SYNTHASES TO GENERATE OILS WITH HIGHLY ELEVATED AND BALANCED C10:0 AND C12:0 FATTY ACID LEVELS

[0680] In this example we describe two molecular approaches to generate oils with highly elevated and balanced C10:0 and C12:0 fatty acids in a classically mutagenized high-oil-yielding derivative of UTEX 1435, Strain BA. Resulting transgenic strains co-express two distinct mid-chain specific thioesterases, the broad specificity C10:0-C14:0 *Cuphea wrightii* FATB2 thioesterase (expressed in Stain BA), and predominantly C10:0 – specific *Cuphea hookeriana* FATB2 thioesterase (part of incoming vectors). In addition, D1550 transformants express *C.wrightii KASIV* elongase gene integrated at a neutral genomic site, Thi4b, (vector pSZ2424), while D1681 transformants – *C. wrightii* KASAI elongase as a part of an endogenous *KASI* disruption cassette (vector pSZ2746). The use of different KASI activities of plant origin in combination with the exogenous thioesterases resulted in a significant increase in overall C10-C12 levels as well as improved C10:0 specificity of the *C. hookeriana* thioesterase. The best strain synthesized about 85% total C10:0 -C12:0 fatty acids with balanced levels of about 42% C10:0 and ca. 44% C12:0 fatty acids, respectively, less than 4% C14:0, and less than 1.5% C8:0. The results show that selection of FATB and KAS genes can give rise to an oil with at least 50% total saturates with capric and lauric acids balanced to within 20% (or even to within 15%, or 10%).

[0681] Relevant restriction sites in pSZ2424 are indicated in lowercase, bold and underlining text and are 5'-3' *Pme I, Kpn I, Xba I, Mfe I, Eco RI, Spe I, Xho I, Hind III, SnaBI, Spe I, Asc I, Xho I, Eco RI, Sac I, BspQ I,* respectively. *Pme I* and *BspQI* sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent genomic DNA from UTEX1435 that permit targeted integration at Thi4b locus via homologous recombination. Proceeding in the 5' to 3' direction, the *C. reinhardtii* B-tubulin promoter driving the expression of the neomycin phosphotransferase gene (*NeoR*, conferring the ability of cells to grow on G418) is indicated by boxed text. The initiator ATG and terminator TGA for *NeoR* are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics. The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase underlined text. Next is the Amt03 promoter of *Prototheca moriformis* indicated by boxed

lowercase text driving the expression of *Cuphea hookeriana KASIV* gene (*ChKASIV*) indicated in lowercase italics. The initiator ATG and terminator TGA for *ChKASIV* are indicated by uppercase, bold italics. The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase underlined text. Next is the Amt03 promoter of *Prototheca moriformis* indicated by boxed lowercase text driving the expression of *Cuphea hookeriana FATB2* gene (*ChFATB2*) fused to plastid transit peptide sequence derived from *Prototheca moriformis FAD* gene indicated in lowercase italics. The initiator ATG and terminator TGA for *ChFATB2* are indicated by uppercase, bold italics. The *C. vulgaris* nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the UTEX1435 Thi4b flanking sequence.

[0682] >pSZ2424 [Thi4b::CrTUB2-NeoR-CvNR:PmAmt03-ChKASIV-CvNR:PmAMT03-ChTE2-CvNR::Thi4b]. Nucleotide sequence of transforming DNA contained in pSZ2424:

gtttaaaccctcaactgcgacgctgggaaccttctccgggcaggcgatgtgcgtgggtttgcctccttggcacggctctacacc ttcgagtacgccatgaggcggtgatggctgtggctgtgccccacttcgtccagggacggcaagtccatcatctgcatgcttggt gcgacgctacagcagtccctctgcagcagaggagcacgactttggccatttcacgcactcgagtgtacacaattcatttttctta aagtaaatgactgctgattgaccagatgctgtaacgctgatttcgctccagatcgcacagtcacagattgcgaccatgttgctg cgtctgaaaatctggattccgaattcgaccctggcgctccatccatgcaacagatggcgacacttgttacaattcctgtcgccca tcggcatggagcaggtccacttagatccccgatcacccacgcgcatctcgctaatagtcattcattcgtgtcttcgatcaaagtc aggtgagtatgcatggatcttggttgacgatgcggtatgggtttgcgccgctgactgcagggtctgtccaaggcaagccaaccc agctcctctcctcgacaatactctcgcagacaaagccagccacttgccatccagattgccaataaactcaatcatggcttctgtc atgccatccatgggtctgatgaatggtcacgctcgtgtcctgaccgttccccagcctctggcgtcccctgccccgcccaccagcc cacgccgcgcggcagtcgctgccaaggctgtctcggaggtaccctttcttgcgctatgacacttccagcaaaaggtagggcgggct gcgagacggcttcccggcgctgcatgcaacaccgatgatgcttcgaccccccgaagctccttcggggctgcatgggcgctccgatg ccgctccagggcgagcgctgtttaaatagccaggcccccgattgcaaagacattatagcgagctaccaaagccatattcaaacacctа gatcactaccacttctacacaggccactcgagcttgtgatcgcactccgctaagggggcgcctcttcctcttcgtttcagtcacaacccg caaac**tctaga**atatca***ATG****atcgagcaggacggcctccacgccggctcccccgccgcctgggtggagcgcctgttcggctac gactgggcccagcagaccatcggctgctccgacgccgccgtgttccgcctgtccgcccagggccgcccctgtgctgttcgtgaaga ccgacctgtccggcgccctgaacgagctgcaggacgaggccgcccgcctgtcctggctggccaccaccggcgtgccctgcgcc gccgtgctggacgtggtgaccgaggccggccgcgactggctgctgctgggcgaggtgcccggccaggacctgctgtcctcccac ctggcccccgccgagaaggtgtccatcatggccgacgccatgcgccgcctgcacaccctggaccccgccacctgcccccttcgac caccaggccaagcaccgcatcgagcgcgcccgcacccgcatggaggccggcctggtggaccaggacgacctggacgagga gcaccagggcctggcccccgccgagctgttcgcccgcctgaaggcccgcatgcccgacggcgaggacctggtggtgacccacg gcgacgcctgcctgcccaacatcatggtggagaacggccgcttctccggcttcatcgactgcggccgcctgggcgtggccgaccg

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[0683] Relevant restriction sites in pSZ2746 are indicated in lowercase, bold and underlining text and are 5'-3' *BspQ 1, Kpn I, Xba I, Mfe I, Hind III, AscI, Spe I, Xho I, Eco RI, Nde I, Sna BI, Xho I, Hind III, Sac I, BspQ I,* respectively. *BspQI* sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent genomic DNA from UTEX1435 that permit targeted integration (and knockout) at the *KASI* locus via homologous recombination. Proceeding in the 5' to 3' direction, the *C. reinhardtii* B-tubulin promoter driving the expression of the neomycin phosphotransferase gene (NeoR, conferring the ability of cells to grow on G418) is indicated by boxed text. The initiator ATG and terminator TGA for NeoR are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics. The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase underlined text. Next is the UAPA1 promoter of *Prototheca moriformis* indicated by boxed lowercase text driving the expression of *Cuphea hookeriana* FATB2 gene (ChFATB2) fused to plastid transit peptide sequence derived from *Prototheca moriformis FAD* gene indicated in lowercase italics. The initiator ATG and terminator TGA for *ChFATB2* are indicated by uppercase, bold italics. The *B.braunii cd191* 3'UTR is indicated by lowercase underlined text. Next is the Amt03 promoter of *Prototheca moriformis* indicated by boxed lowercase

text driving the expression of *Cuphea wrightii KASAI* gene indicated by lowercase italics fused to *Prototheca moriformis SAD1* plastid transit peptide sequence. The *C.wrightii KASAI* sequence is in lowercase italics and is delineated by initiator ATG and terminator TGA. The *C. vulgaris* nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the UTEX1435 *KASI* flanking sequence.

[0684] >pSZ2746 [KASI-1::CrTUB2-NeoR-CvNR:PmUAPA1-ChFATB2-Bbcd181;PmAmt03-PmSADtp-CwKASA1-CvNR::KasI-1]. Nucleotide sequence of transforming DNA contained in pSZ2746:

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[0685] Fatty acids profiles from representative shake flask cultures of stable lines derived from D1550 transformants are shown in Table 74. Two independent genetic lineages yielded strains with high and balanced levels of C10-C12:0 fatty acids.

[0686] Table 74. Fatty acid profiles in S5050 and derivative transgenic lines generated after transformation with pSZ2424 DNA.

Strain		Fatty Acid (area %)								Total Saturates
		C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	
Strain BA		0.32	14.96	43.84	16.60	10.49	0.54	9.64	2.49	86.75
D1550-29.C4.A2		4.02	32.82	40.98	7.37	5.06	0.40	5.61	2.37	90.65
D1550-29.C4.A3		4.50	33.93	40.23	7.09	4.91	0.36	5.38	2.30	91.02
D1550-29.C4.A4		3.57	34.31	41.04	6.86	4.90	0.36	4.98	2.56	91.04
D1550-29.C4.A5		4.66	34.23	39.68	6.96	4.90	0.36	5.55	2.32	90.79
D1550-29.C6.E2		3.59	35.44	40.49	6.32	4.74	0.34	4.94	2.63	90.92
D1550-29.C6.E3		3.60	35.55	40.90	6.33	4.67	0.34	4.66	2.52	91.39
D1550-29.C6.E4	BB	3.97	35.85	40.23	6.26	4.65	0.34	4.83	2.51	91.30
D1550-29.C6.E5		4.02	35.19	39.89	6.59	4.79	0.34	5.12	2.60	90.82
D1550-29-1.14		3.30	39.62	40.04	5.16	4.04	0.30	3.49	2.67	92.46
D1550-29-1.2		3.12	39.50	40.22	5.13	3.86	0.29	3.42	2.82	92.12
D1550-29-1.12		3.26	39.36	39.91	5.13	4.15	0.30	3.73	2.77	92.11
D1550-29-1.17		3.25	39.21	40.21	5.22	4.11	0.30	3.70	2.67	92.30
D1550-29-1.39		4.12	38.44	39.23	5.83	4.25	0.30	3.96	2.46	92.17
D1550-29-1.35	BC	3.60	38.06	39.79	5.89	4.35	0.29	3.98	2.58	91.98
D1550-29-1.7		3.15	39.18	40.04	5.24	4.05	0.32	3.68	2.88	91.98

D1550-29-1.1		2.87	38.29	40.76	5.20	4.20	0.31	3.79	2.86	91.63

[0687] Next, we analyzed the performance of D1681 strains that were constructed using the *KASI* replacement strategy. Interestingly, unlike D1550 transformants, the D1681 strains demonstrated greater variability in fatty acid profiles (Table 75). In addition, the D1681 derived lines had lower C8:0 levels than what we observed in the D1550 derived transgenic lines suggesting a direct role of *C. wrightii KASA1* in improving C10:0 specificity of *C. hookeriana* FATB2 thioesterase.

[0688] Table 75. Fatty acid profiles in Strain BA and derivative transgenic lines generated after transformation with pSZ2746 DNA.

Strain		Fatty Acid (area %)								Total Saturates
		C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	
Strain BA		0.89	13.17	40.52	17.53	11.60	0.59	11.38	2.95	84.30
D1681.3.7-2	**BD**	1.44	31.83	44.97	6.52	4.83	0.30	6.53	2.45	89.89
D1681.3.7-10		1.84	31.41	43.64	6.90	5.15	0.31	7.08	2.46	89.25
D1681.3.7-12	**BE**	1.85	31.64	43.50	6.76	5.08	0.31	7.16	2.49	89.14
D1681.3.4-1		1.29	31.61	45.92	6.65	4.49	0.29	6.20	2.46	90.25
D1681.3.4-6	**BF**	1.48	32.26	45.11	6.55	4.56	0.29	6.23	2.41	90.25
D1681.3.4-9		1.42	31.22	45.40	6.93	4.69	0.30	6.49	2.47	89.96
D1681.3.8-1		1.35	27.72	44.72	8.78	5.97	0.37	7.36	2.51	88.91
D1681.3.8-4		1.44	27.51	44.34	8.72	6.05	0.36	7.84	2.51	88.42
D1681-2.1-37		0.64	34.80	47.17	4.84	3.81	0.31	4.37	2.43	91.57
D1681-2.1-34		0.62	35.26	47.07	4.77	3.77	0.30	4.22	2.36	91.79
D1681-2.1-28	**BG**	0.64	35.99	46.80	4.65	3.68	0.29	4.02	2.34	92.05
D1681-2.1-12		0.67	34.78	47.21	4.94	3.79	0.34	4.30	2.35	91.73
D1681.2.4-1.3	**BH**	0.57	35.95	47.73	4.93	3.39	0.03	0.24	3.96	92.60
D1681.2.4-1.4		0.56	36.71	47.55	4.86	3.26	0.03	0.24	3.61	92.97
D1681.2.4-1.12	**BI**	1.89	34.72	44.70	6.32	4.03	0.02	0.27	5.18	91.68
D1681.2.4-1.2		1.73	36.43	44.25	5.57	4.09	0.03	0.32	4.55	92.10

[0689] Eight strains representing D1550 and D1681 families (from Tables 74-75) were subsequently evaluated in high cell density fermentations as shown in Table 76. Fermentations resulted in oils with a slightly improved mid-chain profile or the balance of C10-C12:0 fatty acid levels compared to the lab scale fermentation. Strain BE evaluated in two independent fermentations demonstrated superior profile reaching 85.2% C10-C12:0

fatty acid levels, 3.5% C14:0 levels, and ca. 1.2% C8:0 fatty acid levels, and accumulated over 92% total saturates.

[0690] Table 76. End-point fatty acid profiles in D1550 and D1681 derivative transgenic lines subjected to high cell density fermentation.

Strain		BB	BC	BG	BH	BI	BF	BE	
Run		130067	130196	130197	130291	130292	130253	130246	PF13029
Fatty Acid Profile (Area %)	C8:0	5.3	4.62	4.62	0.54	1.53	1.59	2.1	1.19
	C10:0	36.19	36.16	36.16	33.24	40	40.46	40.94	41.59
	C12:0	39.07	38.77	38.77	47.65	43.09	42.39	41.25	43.6
	C14:0	5.31	5.18	5.18	5	4.62	4.42	4.2	3.49
	C16:0	3.72	3.9	3.9	3.4	2.83	2.63	2.8	2.28
	C18:0	0.24	0.28	0.28	0.27	0.22	0.32	0.28	0.15
	C18:1	6.12	6.79	6.79	5.88	4.95	5.34	5.8	4.76
	C18:2	2.43	2.76	2.76	2.49	1.95	2.05	1.89	1.96
	C10-C14	80.57	80.11	80.11	85.89	87.71	87.27	86.39	88.68
	C10-C12	75.26	74.93	74.93	80.89	83.09	82.85	82.19	85.19
	Total Saturates	89.83	88.91	88.91	90.1	92.29	91.81	91.57	92.3

EXAMPLE 61: TAG REGIOSPECIFICITY IN UTEX1435 BY EXPRESSION OF *CUPHEA PSR23 LPAAT2* AND *LPAAT3* GENES

[0691] In Example 43, we demonstrated that the expression of 2 different 1-acyl-sn-glycerol-3-phosphate acyltransferases (LPAATs), the *LPAAT2* and *LPAAT3* genes from *Cuphea* PSR23 (*Cu*PSR23) in the UTEX1435 derivative strain S2014 resulted in elevation of C10:0, C12:0 and C14:0 fatty acids levels. In this example we provide evidence that *Cuphea PSR23* LPAAT2 exhibits high specificity towards incorporating C10:0 fatty acids at sn-2 position in TAGs. The *Cuphea PSR23* LPAAT3 specifically incorporates C18:2 fatty acids at sn-2 position in TAGs.

[0692] Composition and properties of *Prototheca moriformis* (UTEX 1435) transgenic strain B, transforming vectors pSZ2299 and pSZ2300 that express *CuPSR23 LPAAT2* and *LPAAT3* genes, respectively, and their sequences were described previously.

[0693] To determine the impact of *Cuphea PSR23 LPAAT* genes on the resulting fatty acid profiles we have taken advantage of Strain B which synthesizes both mid chain and long chain fatty acids at relatively high levels. As shown in Table 77, the expression of the *LPAAT2* gene (D1520) in Strain B resulted in increased C10-C12:0 levels (up to 12% in the

best strain, D1520.3-7) suggesting that this LPAAT is specific for mid chain fatty acids. Alternatively, expression of the *LPAAT3* gene resulted in a relatively modest increase, (up to 5% in the best strain, D1521.28-7) indicating it has little or no impact on mid-chain levels.

[0694] Table 77. Fatty acid profiles of Strain B and representative transgenic lines transformed with pSZ2299 (D1520) and pSZ2300 (D1521) DNA.

Strain	Fatty Acid (area %)									Total Saturates
	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2	C10-C12	
Strain B	0.09	4.95	29.02	15.59	12.55	1.27	27.93	7.60	33.97	63.47
D1520.8-6	0.00	6.71	31.15	15.80	13.04	1.42	24.32	6.56	37.86	68.12
D1520.13-4	0.00	6.58	30.96	16.14	13.34	1.25	24.32	6.27	37.54	68.27
D1520.19-4	0.00	7.53	32.94	16.64	12.63	1.17	21.96	6.11	40.47	70.91
D1520.3-7	0.06	9.44	36.26	16.71	11.44	1.28	18.41	5.59	45.70	75.19
D1521.13-8	0.00	6.21	33.13	16.70	12.30	1.18	20.84	8.70	39.34	69.52
D1521.18-2	0.00	5.87	31.91	16.46	12.60	1.22	22.14	8.59	37.78	68.06
D1521.24-8	0.00	5.75	31.47	16.13	12.60	1.42	23.31	8.22	37.22	67.37
D1521.28-7	0.00	6.28	32.82	16.33	12.27	1.43	21.98	7.91	39.10	69.13

[0695] To determine if expression of the *Cuphea* PSR23 *LPAAT* genes affected regiospecificity of fatty acids at the sn-2 position, we analyzed TAGs from representative D1520 and D1521 strains utilizing the porcine pancreatic lipase method. See Example 2. As demonstrated in Table 78, the *Cuphea PSR23 LPAAT2* gene shows remarkable specificity towards C10:0 fatty acids and appears to incorporate 50% more C10:0 fatty acids into the sn-2 position. The *Cuphea PSR23 LPAAT3* gene appears to act exclusively on C18:2 fatty acids, resulting in redistribution of C18:2 fatty acids onto sn-2 position. Accordingly, microbial triglyceride oils with sn-2 profiles of greater than 15% or 20% C10:0 or C18:2 fatty acids are obtainable by introduction of an exogenous LPAAT gene having corresponding specificity.

[0696] Table 78. TAG and sn-2 fatty acid profiles in oils of parental S2014 strain and the progeny strains expressing Cuphea PSR23 LPAAT2 (BJ) and LPAAT3 (BK) genes.

Strain	Strain B	Strain BI (D1520.3-7)	Strain BK (D1521.13-8)

Analysis		TAG Profile	sn-2 Profile	TAG Profile	sn-2 Profile	TAG Profile	sn-2 Profile
Fatty Acid (area %)	C8:0	0	0	0.1	0	0	0
	C10:0	12	14.2	11	24.9	6.21	6.3
	C12:0	42.8	25.1	40.5	24.3	33.13	19.5
	C14:0	12.1	10.4	16.3	10	16.7	11.8
	C16:0	7.3	1.3	10.2	1.4	12.3	3
	C18:0	0.7	0.2	0.9	0.6	1.18	0.5
	C18:1	18.5	36.8	15.4	29.2	20.84	36.3
	C18:2	5.8	10.9	4.9	8.7	8.7	20.9
	C18:3a	0.6	0.8	0.4	0.8	0.48	1.2
	C10-C14	66.9	49.7	67.8	59.2	56.0	37.6
	C10-C12	54.8	39.3	51.5	49.2	39.3	25.8

EXAMPLE 62: INTRODUCTION OF HETEROLOGOUS THIOESTERASES INTO A HETEROLOGOUS KAS-EXPRESSING PROTOTHECA MORIFORMIS STRAIN

[0697] Here we demonstrate that heterologous fatty acyl-ACP thioesterases exhibit altered thioesterase specificity when combined with a heterologous plant KASI gene, *Cuphea wrightii* β-ketoacyl-ACP synthase (KAS), CwKASA1, in *P. moriformis* (UTEX 1435) transgenic strain, S5818. S5818 is a transgenic strain expressing a thioesterase chimera from *Cinnamomum camphora* and *Umbellularia californica*, CcFATB2-UcFATB2 chimera B, at the *6S* locus and additionally expressing the *Cuphea wrightii* KAS, *CwKASA1*, at the *pLOOP* locus. The addition of the *CcFATB2-UcFATB2 chimera B* and *CwKASA1* genes leads to an S5818 fatty acid profile with 45% C12:0 and 14% C14:0. Five different constructs encoding thioesterases that were previously shown to exhibit predominantly C14:0 thioesterase activity and with less pronounced C12:0 thioesterase activity in *P. moriformis* were introduced into S5818 in an effort to increase C14:0 and C12:0 levels in this background. However, introduction of the five different C14:0 thioesterases into S5818 led to unexpected but significant increases in C12:0 fatty acid levels (>50% overall) with only modest increases in C14:0 fatty acid levels (<20% overall). This result suggests that the KASI- FATB thioesterase combination exhibits a unique activity not displayed when either gene is introduced separately. The results demonstrate that combination of heterologous KAS genes with heterologous thioesterases in oleaginous cells can be used to produce fatty acid profiles not exhibited by introduction of either gene alone. Furthermore, introduction of heterologous KASs may be an important and fruitful approach for revealing novel specificities of additional heterologous thioesterases.

[0698] Strain S5818 generation. S5818 was created by two successive transformations. The UTEX1435 base strain, S3150 (Strain Z above), was transformed with pSZ2448 (6SA::CrTUB2-ScSUC2-CvNR:PmAMT3-CpSAD1tpExt-CcFATB2-UcFATB2-chimeraB-ExtA-CvNR::6SB), encoding the CcFATB2-UcFATB2 chimera B thioesterase targeting the 6S locus, to yield strain S4954. S4954 produces ~32% C12:0 and ~16% C14:0 fatty acid levels (Table 62-1). S4954 was subsequently transformed with pSZ2229 (pLOOP::CrTUB2-NeoR-CvNR:PmAMT3-PmSADtp_CwKASAI-CvNR::pLOOP), encoding the C. wrightii KASA1 gene targeting the pLOOP locus, to yield strain S5818. S5818 produces ~45% C12:0 and ~14% C14:0 fatty acid levels (Table 79).

[0699] Table 79. Fatty acid profiles of S3150, S4954, and S5818.

Sample ID	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2
Strain Z	0	0.05	1.49	28.83	3.24	57.87	6.27
S4954	0.17	31.52	16.39	9.81	1.19	32.14	7.19
S5818	0.34	45.16	13.77	8.54	0.81	24.63	5.38

[0700] Identification of C14:0 thioesterases. In an effort to increase C14:0 fatty acid levels, and to a lesser degree C12:0 fatty acid levels, several thioesterases that were found to exhibit C14:0 and C12:0 thioesterase activity in *P. moriformis* were cloned into vectors for introduction into S5818. The *Cuphea hyssopifolia* thioesterase *ChsFATB3* was discovered by us as part of efforts to identify novel thioesterases by sequencing the mature, plant oilseeds of *C. hyssopifolia*. Although *C. hyssopifolia* seeds exhibit ~84% C12:0 and ~5% C14:0 fatty acid levels, the ChsFATB3 thioesterase we identified exhibits strong C14:0 thioesterase activity when expressed in S3150 (up to ~34% C14:0). A version of *ChsFATB3* in which we optimized the putative plastid-targeting transit peptide, named pSAD1tp_trimmed:ChsFATB3, similarly exhibited strong C14:0 thioesterase activity (~33% C14:0; Table 80).

[0701] Table 80. Fatty acid profiles of *Cuphea hyssopifolia* seeds and S3150 with introduction of ChsFATB3 or CpSAD1tp_trimmed:ChsFATB3.

Sample ID	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2
Cuphea hyssopifolia seeds	0.24	6.53	83.69	5.13	1.10	0.12	0.00	1.74
S3150	0.00	0.00	0.05	1.49	28.83	3.24	57.87	6.27
S3150 + ChsFATB3 (T537; D1701-48)	0.00	0.00	8.09	33.66	26.46	1.57	23.75	5.3
S3150 + CpSAD1tp_trimmed:ChsFATB3 (T580; D1813-8)	0.00	0.14	7.25	33.32	27.04	1.57	24.37	5.12

[0702] Similarly, we also identified the *Cuphea heterophylla* thioesterase *ChtFATB1a* as part of our efforts to identify novel thioesterases by sequencing the mature, plant oil seeds of *C. heterophylla*. Although *C. heterophylla* seeds exhibit ~44% C10:0, ~40% C12:0 fatty acid levels, and only ~4% C14:0, the transit peptide optimized version of the ChtFATB1a thioesterase we identified, CpSAD1tp_trimmed:ChtFATB1a, exhibits strong C14:0 thioesterase activity when expressed in S3150 (up to ~35% C14:0; Table 81).

[0703] Table 81. Fatty acid profiles of *Cuphea heterophylla* seeds and S3150 with introduction of CpSAD1tp_trimmed:ChtFATB1a.

Sample ID	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2
Cuphea heterophylla seeds	3.50	44.27	40.04	4.26	1.22	0.25	2.21	3.56
S3150	0.00	0.00	0.05	1.49	28.83	3.24	57.87	6.27
S3150 + CpSAD1tp_trimmed:ChtFATB1a (T580; D1811-44)	0.00	0.15	13.18	35.16	24.1	1.19	18.87	6.02

[0704] A published *Cuphea palustris* C14:0 thioesterase, CpalFATB2, was also introduced into S5818 (*vide infra*).

[0705] Introduction of C14:0 thioesterases into S5818. Five constructs were generated using C14:0 thioesterases for introduction into S5818 (Table 82).

[0706] Table 82. Constructs engineered for introduction into S5818.

D#	pSZ#	Construct
D2104	pSZ3390	DAO1b:: PmHXT1-ScarMel1 -CvNR:PmUAPA1noSacI-CpSAD1tpExt-CpalFATB2FLAGExtA-CvNR::DAO1b
D2202	pSZ3493	DAO1b5'::PmHXT1-ScarMEL1-CvNR:PmAMT3-ChsFATB3-CvNR::DAO1b3'
D2203	pSZ3494	DAO1b5'::PmHXT1-ScarMEL1-CvNR:PmAMT3-CpSAD1tp_trimmed:ChsFATB3-CvNR::DAO1b3'
D2204	pSZ3495	DAO1b5'::PmHXT1-ScarMEL1-CvNR:PmAMT3-CpSAD1tp_trimmed:ChtFATB1a-CvNR::DAO1b3'
D2235	pSZ3531	THI4A:: PmHXT1-ScarMel1 - CpEF1a:PmUAPA1noSacI-CpSAD1tpExt-CpalFATB2FLAGExtA-CvNR:: THI4A

[0707] pSZ3390 and pSZ3531 introduce the *CpalFATB2* thioesterase gene into the *DAO1b* and *THI4A* loci, respectively, under the control of the pH5-responsive *UAPA1* promoter. pSZ3493, pSZ3494, and pSZ3495 introduce *ChsFATB3*, *CpSAD1tp_trimmed:ChsFATB3*, and *CpSAD1tp_trimmed:ChtFATB1a*, respectively, into the *DAO1b* locus under the control of the pH7-responsive *AMT3* promoter. Transgenic strains were selected for the ability to grow on melibiose. Cell culture, lipid production, and fatty acid analysis were all carried out as previously described. The transforming DNA for pSZ3390, pSZ3493, pSZ3494, pSZ3495, and pSZ3531 are provided below.

[0708] **pSZ3390:** pSZ3390 can be written as DAO1b:: PmHXT1-ScarMel1 - CvNR:PmUAPA1noSacI-CpSAD1tpExt-CpalFATB2FLAGExtA-CvNR::DAO1b. The

relevant restriction sites in the construct from 5'-3', BspQI, KpnI, SpeI, SnaBI, XhoI, EcoRI, SpeI, HindIII, SacI, BspQI, respectively, are indicated in lowercase, bold, and underlined. BspQI sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences at the 5' and 3' end of the construct represent genomic DNA from UTEX 1435 that target integration to the *DAO1b* locus via homologous recombination. Proceeding in the 5' to 3' direction, the selection cassette has the *P.moriformis HXT1* promoter driving the expression of the *S.carlbergensis MEL1* gene (conferring the ability to grow on melibiose) and the *Chlorella vulgaris Nitrate Reductase* (*NR*) gene 3' UTR. The promoter is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for *ScarMEL1* are indicated by bold, uppercase italics, while the coding region is indicated with lowercase italics. The 3'UTR is indicated by lowercase, underlined text. The second cassette containing the *CpSAD1tpExt-CpalFATB2FLAGExtA* gene, fused to the heterologous *Chlorella protothecoides SAD1* plastid-targeting transit peptide, is driven by the *P. moriformis* UAPA1 pH5-responsive promoter and has the *Chlorella vulgaris Nitrate Reductase* (*NR*) gene 3' UTR. In this cassette, the *UAPA1* promoter is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for the *CpSAD1tpExt-CpalFATB2FLAGExtA* gene are indicated in bold, uppercase italics, while the coding region is indicated by lowercase italics. The 3' UTR is indicated by lowercase, underlined text.

[0709] pSZ3390 transforming construct:

gaagagcGCCCAATGTTTAAAC**agcccgcaccctcgttgatctgggagccctgcgcagccccttaaatcatctcag tcaggtttctgtgttcaactgagcctaaagggctttcgtcatgcgcacgagcacacgtatatcggccacgcagtttctcaaaagc ggtagaacagttcgcgagccctcgtaggtcgaaaacttgcgccagtactattaaattaaattaattgatcgaacgagacgcga aacttttgcagaatgccaccgagtttgcccagagaatgggagtggcgccattcaccatccgcctgtgcccggcttgattcgccg agacgatggacggcgagaccagggagcggcttgcgagccccgagccggtagcaggaacaatgatcgacaatcttcctgtcc aattactggcaaccattagaaagagccggagcgcgttgaaagtctgcaatcgagtaatttttcgatacgtcgggcctgctgaa ccctaaggctccggactttgtttaaggcgatccaagatgcacgcggccccaggcacgtatctcaagcacaaaccccagcctta gtttcgagactttgggagatagcgaccgatatctagtttggcattttgtatattaattacctcaagcaatggagcgctctgatgcg gtgcagcgtcggctgcagcacctggcagtggcgctagggtcgccctatcgctcggaacctggtcagctggctcccgcctcctgc tcagcctcttccggtacc**gcggtgagaatcgaaaatgcatcgtttctaggttcggagacggtcaattccctgctccggcgaatctgtcg gtcaagctggccagtggacaatgttgctatggcagcccgcgcacatgggcctcccgacgcggccatcaggagcccaaacagcgtgt cagggtatgtgaaactcaagaggtccctgctgggcactccggccccactccgggggcgggacgccaggcattcgcggtcggtccc gcgcgacgagcgaaatgatgattcggttacgagaccaggacgtcgtcgaggtcgagaggcagcctcggacacgtctcgctagggc aacgccccgagtccccgcgagggccgtaaacattgtttctgggtgtcggagtgggcattttgggcccgatccaatcgcctcatgccgc

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acattgtgcccactgaagagc (SEQ ID NO:123)

[0710] **pSZ3493, pSZ3494, and pSZ3495:** pSZ3493 can be written as DAO1b5'::PmHXT1-ScarMEL1-CvNR:PmAMT3-ChsFATB3-CvNR::DAO1b3'. pSZ3494 can be written as DAO1b5'::PmHXT1-ScarMEL1-CvNR:PmAMT3-CpSAD1tp_trimmed:ChsFATB3-CvNR::DAO1b3'. pSZ3495 can be written as DAO1b5'::PmHXT1-ScarMEL1-CvNR:PmAMT3-CpSAD1tp_trimmed:ChtFATB1a-CvNR::DAO1b3'. The sequences of the three constructs differ only in the sequence of the thioesterase gene. The full transforming sequence for pSZ3493 is displayed in SEQ ID NO:124. The sequences of the *CpSAD1tp_trimmed:ChsFATB3* and *CpSAD1tp_trimmed:ChtFATB1a* genes alone, which take the place of *ChsFATB3* from pSZ3493 in the pSZ3494 and pSZ3495 sequences, are displayed in SEQ ID NOs:125 and 126, respectively, along with flanking restriction sites.

[0711] The relevant restriction sites in the pSZ3493 construct from 5'-3', BspQI, KpnI, SpeI, SnaBI, XhoI, EcoRI, SpeI, XhoI, SacI, BspQI, respectively, are indicated in lowercase, bold, and underlined. BspQI sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences at the 5' and 3' end of the construct represent genomic DNA from UTEX 1435 that target integration to the *DAO1b* locus via homologous recombination. Proceeding in the 5' to 3' direction, the selection cassette has the *P.moriformis HXT1* promoter driving the expression of the *S.carlbergensis MEL1* gene (conferring the ability to grow on melibiose) and the *Chlorella vulgaris Nitrate reductase* (*NR*) gene 3' UTR. The promoter is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for *ScarMEL1* are indicated by bold, uppercase italics, while the coding region is indicated with lowercase italics. The 3'UTR is indicated by lowercase, underlined text. The second cassette is comprised of the *ChsFATB3* gene driven by the *P. moriformis AMT3* pH7-responsive promoter and with the *Chlorella vulgaris Nitrate Reductase* (*NR*) gene 3' UTR. In this cassette, the *AMT3* promoter is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for the *ChsFATB3* gene are indicated in bold, uppercase italics, while the coding region is indicated by lowercase italics. The 3' UTR is indicated by lowercase, underlined text.

[0712] pSZ3493 transforming construct:

gaagagcGCCCAATGTTTAAAC**agcccgcaccctcgttgatctgggagccctgcgcagccccttaaatcatctcag tcaggtttctgtgttcaactgagcctaaagggctttcgtcatgcgcacgagcacacgtatatcggccacgcagtttctcaaaagc ggtagaacagttcgcgagccctcgtaggtcgaaaacttgcgccagtactattaaattaaattaattgatcgaacgagacgcga aacttttgcagaatgccaccgagtttgcccagagaatgggagtggcgccattcaccatccgcctgtgcccggcttgattcgccg agacgatggacggcgagaccagggagcggcttgcgagccccgagccggtagcaggaacaatgatcgacaatcttcctgtcc**

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caccgtgttcgtggccgccgagaagcagtggaccatgctggaccgcaagtccaagcgccccgacatgctgatggacccccttcgg
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gcagcgactgcgggtgcgaaacggatgacggtttggtcgtgtatgtcacagcatgtgctggatcttgcgggctaactcccccctg
ccacggcccattgcaggtgtcatgttgactggagggtacgacctttcgtccgtcaaattcccagaggaggacccgctctgggcc
gacattgtgcccactgaagagc (SEQ ID NO:124)

[0713] CpSAD1tp_trimmed:ChsFATB3 (from pSZ3494):

actagtAACA***ATG***gccaccgcctccaccttctccgccttcaacgcccgctgcggcgacctgcgccgctccgccggctccgg
ccccgccgccccgcccgcccctgcccgtgcgcgccgccatcaacgcctccgcccgccccaaggccaacggctccgccgtgt
ccctgaagtccggctccctggacacccaggaggacacctcctcctcctcccccccccgcaccttcatcaaccagctgcccgac
tggtccatgctgctgtccgccatcaccaccgtgttcgtggccgccgagaagcagtggaccatgctggaccgcaagtccaagcgcc
ccgacatgctgatggacccttcggcgtggaccgcgtggtgcaggacggcgccgtgttccgccagtccttctccatccgctcctac
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ggcctgctgaacgacggcttcggccgcacccccgagatgtgcaagcgcgacctgatctgggtggtgaccaagatgcacgtgga
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gctccctgtaccagcacctgctgcgcctggaggacggcgccgacatcgccaagggccgcaccaagtggcgccccaagaacgc
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acaaggaccacgacatcgactacaaggacgacgacgacaag***TGA***ctcgag (SEQ ID NO:125)

[0714] CpSAD1tp_trimmed:ChtFATB1a (from pSZ3495):

actagtAACA***ATG***gccaccgcctccaccttctccgccttcaacgcccgctgcggcgacctgcgccgctccgccggctccgg
ccccgccgccccgcccgcccctgcccgtgcgcgccgccatcaacgcctccgcccaccccaaggccaacggctccgccgtga
acctgaagtccggctccctggagacccaggaggacacctcctcctcccccccccccgcaccttcatcaagcagctgcccga
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*cgcctccatcgagaccctgatgaacatcttccaggagacctccctgaaccactgcaagtccatcggcctgctgaacgacggcttc ggccgcacccccgagatgtgcaagcgcgacctgatctgggtggtgaccaagatccaggtggaggtgaaccgctaccccacctg gggcgacaccatcgaggtgaacacctgggtgtccgagtccggcaagaacggcatgggccgcgactggctgatctccgactgcc gcaccggcgagatcctgatccgcgccacctccgtgtgggccatgatgaaccgcaagacccgccgcctgtccaagttcccctacg aggtgcgccaggagatcgccccccacttcgtggactccgcccccgtgatcgaggacgacaagaagctgcacaagctggacgtg aagaccggcgactccatccgcaagggcctgacccccgctggaacgacctggacgtgaaccagcacgtgaacaacgtgaagt acatcggctggatcctgaagtccgtgcccgccgaggtgttcgagacccaggagctgtgcggcgtgaccctggagtaccgccgcg agtgcggccgcgactccgtgctggagtccgtgaccgccatggacaccgccaaggagggcgaccgctccctgtaccagcacctg ctgcgcctggaggacggcgccgacatcaccatcggccgcaccgagtggcgccccaagaacgccggcgccaacggcgccatct ccaccggcaagacctccaacgagaactccgtgtccatggactacaaggaccacgacggcgactacaaggaccacgacatcga ctacaaggacgacgacgacaag**TGA**ctcgag* (SEQ ID NO:126)

[0715] **pSZ3531:** pSZ3531 can be written as THI4A::PmHXT1-ScarMel1 - CpEF1a:PmUAPA1noSacI-CpSAD1tpExt-CpalFATB2FLAGExtA-CvNR::THI4A. The relevant restriction sites in the construct from 5'-3', BspQI, KpnI, SpeI, SnaBI, EcoRV, EcoRI, SpeI, HindIII, SacI, BspQI, respectively, are indicated in lowercase, bold, and underlined. BspQI sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences at the 5' and 3' end of the construct represent genomic DNA from UTEX 1435 that target integration to the *THI4A* locus via homologous recombination. Proceeding in the 5' to 3' direction, the selection cassette has the *P.moriformis HXT1* promoter driving the expression of the *S.carlbergensis MEL1* gene (conferring the ability to grow on melibiose) and the *Chlorella protothecoides EF1A* gene 3' UTR. The promoter is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for *ScarMEL1* are indicated by bold, uppercase italics, while the coding region is indicated with lowercase italics. The 3'UTR is indicated by lowercase, underlined text. The second cassette containing the *CpSAD1tpExt-CpalFATB2FLAGExtA* gene, fused to the heterologous *Chlorella protothecoides SAD1* plastid-targeting transit peptide, is driven by the *P. moriformis UAPA1* pH5-responsive promoter and has the *Chlorella vulgaris Nitrate Reductase* (*NR*) gene 3' UTR. In this cassette, the *UAPA1* promoter is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for the *CpSAD1tpExt-CpalFATB2FLAGExtA* gene are indicated in bold, uppercase italics, while the coding region is indicated by lowercase italics. The 3' UTR is indicated by lowercase, underlined text.

[0716] pSZ3531 transforming construct:

gaagagcGCCCAATGTTTAAACC**cctcaactgcgacgctgggaaccttctccgggcaggcgatgtgcgtgggttt gcctccttggcacggctctacaccgtcgagtacgccatgaggcggtgatggctgtgtcggttgccacttcgtccagagacggca agtcgtccatcctctgcgtgtgtggcgcgacgctgcagcagtccctctgcagcagatgagcgtgactttggccatttcacgcact cgagtgtacacaatccatttttcttaaagcaaatgactgctgattgaccagatactgtaacgctgatttcgctccagatcgcaca gatagcgaccatgttgctgcgtctgaaaatctggattccgaattcgaccctggcgctccatccatgcaacagatggcgacactt gttacaattcctgtcacccatcggcatggagcaggtccacttagattcccgatcacccacgcacatctcgctaatagtcattcgtt cgtgtcttcgatcaatctcaagtgagtgtgcatggatcttggttgacgatgcggtatgggtttgcgccgctggctgcagggtctg cccaaggcaagctaacccagctcctctccccgacaatactctcgcaggcaaagccggtcacttgccttccagattgccaataa actcaattatggcctctgtcatgccatccatgggtctgatgaatggtcacgctcgtgtcctgaccgttccccagcctctggcgtcc cctgccccgcccaccagcccacgccgcgcggcagtcgctgccaaggctgtctcggaggtacc**gcggtgagaatcgaaaatgca tcgtttctaggttcggagacggtcaattccctgctccggcgaatctgtcggtcaagctggccagtggacaatgttgctatggcagcccg cgcacatgggcctcccgacgcggccatcaggagcccaaacagcgtgtcagggtatgtgaaactcaagaggtccctgctgggcactc cggccccactccgggggcgggacgccaggcattcgcggtcggtcccgcgcgacgagcgaaatgatgattcggttacgagaccag gacgtcgtcgaggtcgagaggcagcctcggacacgtctcgctagggcaacgccccgagtccccgcgagggccgtaaacattgtttc tgggtgtcggagtgggcattttgggcccgatccaatcgcctcatgccgctctcgtctggtcctcacgttcgcgtacggcctggatcccg gaaagggcggatgcacgtggtgttgccccgccattggcgcccacgtttcaaagtccccggccagaaatgcacaggaccggcccgg ctcgcacaggccatgctgaacgcccagatttcgacagcaacaccatctagaataatcgcaaccatccgcgttttgaacgaaacgaaac ggcgctgtttagcatgtttccgacatcgtgggggccgaagcatgctccgggggggaggaaagcgtggcacagcggtagcccattctgt gccacacgccgacgaggaccaatccccggcatcagccttcatcgacggctgcgccgcacatataaagccggacgcctaaccggttt cgtggttatg**actagt*ATG***ttcgcgttctacttcctgacggcctgcatctccctgaagggcgtgttcggcgtctcccccctcctacaac *ggcctgggcctgacgccccagatgggctgggacaactggaacacgttcgcctgcgacgtctccgagcagctgctgctggacacg gccgaccgcatctccgacctgggcctgaaggacatgggctacaagtacatcatcctggacgactgctggtcctccggccgcgact ccgacggcttcctggtcgccgacgagcagaagttccccaacggcatgggccacgtcgccgaccacctgcacaacaactccttcc tgttcggcatgtactcctccgcgggcgagtacacgtgcgccggctaccccggctccctgggccgcgaggaggaggacgcccagt tcttcgcgaacaaccgcgtggactacctgaagtacgacaactgctacaacaagggccagttcggcacgcccgagatctcctacc accgctacaaggccatgtccgacgccctgaacaagacgggccgccccatcttctactccctgtgcaactggggccaggacctga ccttctactggggctccggcatcgcgaactcctggcgcatgtccggcgacgtcacggcggagttcacgcgccccgactcccgctg cccctgcgacggcgacgagtacgactgcaagtacgccggcttccactgctccatcatgaacatcctgaacaaggccgcccccat gggccagaacgcgggcgtcggcggctggaacgacctggacaacctggaggtcggcgtcggcaacctgacggacgacgagg agaaggcgcacttctccatgtgggccatggtgaagtccccccctgatcatcggcgcgaacgtgaacaacctgaaggcctcctccta ctccatctactcccaggcgtccgtcatcgccatcaaccaggactccaacggcatccccgccacgcgcgtctggcgctactacgtgt ccgacacggacgagtacggccagggcgagatccagatgtggtccggcccccctggacaacggcgaccaggtcgtggcgctgct gaacggcggctccgtgtcccgccccatgaacacgaccctggaggagatcttcttcgactccaacctgggctccaagaagctgac**

ctccacctgggacatctacgacctgtgggcgaaccgcgtcgacaactccacggcgtccgccatcctgggccgcaacaagaccg
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cgcaggtcttcgcaaaaacgtgagggtttcgcgtctcgccctagctattcgtatcgccgggtcagacccacgtgcagaaaagcccttg
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gttttcgggccaagcgagcccctctcacctcgtcgcccccccgcattccctctctcttgcagc**Cactagt**AACA***ATG****gccaccg*
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gatagtatcgacacactctggacgctggtcgtgtgatggactgttgccgccacacttgctgccttgacctgtgaatatccctgccgctttt atcaaacagcctcagtgtgtttgatcttgtgtgtacgcgcttttgcgagttgctagctgcttgtgctatttgcgaataccacccccagcatcc ccttccctcgtttcatatcgcttgcatcccaaccgcaacttatctacgctgtcctgctatccctcagcgctgctcctgctcctgctcactgcc cctcgcacagccttggtttgggctcccgcctgtattctcctggtactgcaacctgtaaaccagcactgcaatgctgatgcacgggaagta gtgggatgggaacacaaatggaaagctg**gagctccagcgccatgccacgccctttgatggcttcaagtacgattacggtgttgg attgtgtgtttgttgcgtagtgtgcatggtttagaataatacacttgatttcttgctcacggcaatctcggcttgtccgcaggttcaa ccccatttcggagtctcaggtcagccgcgcaatgaccagccgctacttcaaggacttgcacgacaacgccgaggtgagctatg tttaggacttgattggaaattgtcgtcgacgcatattcgcgctccgcgacagcacccaagcaaaatgtcaagtgcgttccgatttt gcgtccgcaggtcgatgttgtgatcgtcggcgccggatccgccggtctgtcctgcgcttacgagctgaccaagcaccctgacgt ccgggtacgcgagctgagattcgattagacataaattgaagattaaacccgtagaaaaatttgatggtcgcgaaactgtgctc gattgcaagaaattgatcgtcctccactccgcaggtcgccatcatcgagcagggcgttgctcccggcggcggcgcctggctgg ggggacagctgttctcggccatgtgtgtacgtagaaggatgaatttcagctggttttcgttgcacagctgtttgtgcatgatttgtt tcagactattgttgaatgtttttagatttcttaggatgcatgatttgtctgcatgcgactgaagagc** (SEQ ID NO:127)

[0717] Increased C12:0 levels in strain S5818 by the expression of heterologous "C14:0-specific" thioesterases. In an effort to increase C14:0 fatty acid levels in S5818, several thioesterases that had previously displayed pronounced C14:0 thioesterase activity in *P. moriformis* were transformed into the S5818 background. Contrary to our expectations, we observed marked increases in C12:0 levels with decreases or only marginal increases in C14:0 levels. For example, introduction of the *ChsFATB3* thioesterase (which leads to an increase in C14:0 levels of up to 34% in S3150) into S5818 causes C12:0 levels to rise to ~77% (Δ = +32% C12:0) and C14:0 levels to drop to ~7% (Δ = -7%). In addition, introduction of *CpalFATB2* into S5818 at the *DAO1b* locus causes C12:0 levels to rise to ~64% (Δ = +19%) and C14:0 levels to drop to ~12% (Δ = -2%). The results for the top five transformants for each of the five constructs are displayed in Table 83.

[0718] Of note, S5818 expresses the *C. wrightii KASA1* gene from the *pLOOP* locus. As *C. wrightii* produces seed oil with 62% C12:0, we believe it likely that the *CwKASA1* gene has evolved to be specific for production of C12:0 fatty acids when combined with *C. wrightii* thioesterases. Indeed, *C. wrightii FATB2* encodes a thioesterase that exhibits C12:0 activity when introduced into *P. moriformis*. Thus, it is possible that the "C14:0" thioesterase genes identified in our transcriptome sequencing, namely *ChsFATB3* and *ChtFATB1a*, exhibit C14:0 activity only when in combination with the *P. moriformis* endogenous *KASI* gene. These results further extend to *CpalFATB2*, which has been repeatedly shown to increase C14:0 levels in *P. moriformis* (data not shown). However, when *ChsFATB3*,

ChtFATB1a, and *CpalFATB2* are combined with a *KASI* gene from a *Cuphea* species that produces high C12:0 fatty acids, such as *CwKASA1* from *Cuphea wrightii*, then a C12:0 activity of these thioesterases is revealed/exhibited. It should be further noted that *C. hyssopifolia* and *C. heterophylla* produce only low levels of C14:0 in oilseeds (5% and 4%, respectively) while producing relatively high levels of C12:0 (84% and 40%, respectively). Since the ChsFATB3 and ChtFATB1a thioesterases were identified from RNAs expressed in mature oil seeds, it is possible that these thioesterases indeed exhibited C12:0 activity in *Cuphea* seeds, significantly contributing to the high levels of C12:0 found therein.

[0719] Our results indicate that the combination of thioesterase and KAS is likely to be extremely important in determining the specificity of the thioesterase-KAS machinery in generating midchain fatty acids. Furthermore, introduction of heterologous KASs may be an important and fruitful approach for revealing novel specificities of additional heterologous thioesterases.

[0720] Table 83. Fatty acid profiles for the top 5 transformants for each of the pSZ3493, pSZ3494, pSZ3495, pSZ3390, and pSZ3531 constructs upon introduction into S5818.

Sample ID									
pSZ#; construct	Strain #	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2
N/A	S5818	0.34	45.16	13.77	8.54	0.81	24.63	5.38
pSZ3493; ChsFATB3	Block 6; C6; pH7; S5818; T678; D2202-30	0.76	76.58	7.49	3.76	0.32	6.58	3.77
	Block 6; D6; pH7; S5818; T678; D2202-42	0.61	62.82	13.92	6.75	0.37	10.76	3.88
	Block 6; D11; pH7; S5818; T678; D2202-47	4.60	55.99	9.26	5.90	0.44	18.32	4.40
	Block 6; A9; pH7; S5818; T678; D2202-9	0.47	53.94	17.59	8.51	0.44	13.70	4.33
	Block 6; D3; pH7; S5818; T678; D2202-39	0.43	53.94	15.62	8.04	0.43	15.99	4.45
pSZ3494; CpSAD1tp_trimmed:ChsFATB3	Block 2; B8; pH7; S5818; T678; D2203-20	0.43	56.76	14.15	7.60	0.56	14.23	5.09
	Block 2; C1; pH7; S5818; T678; D2203-25	0.46	54.82	17.03	7.81	0.46	13.67	4.85
	Block 2; D1; pH7; S5818; T678; D2203-37	0.43	54.47	11.51	8.14	0.95	18.58	4.99
	Block 2; D7; pH7; S5818; T678; D2203-43	0.43	52.86	18.70	8.91	0.58	13.18	4.45
	Block 2; C11; pH7; S5818; T678; D2203-35	0.44	52.81	19.54	8.87	0.54	12.57	4.29
pSZ3495; CpSAD1tp_trimmed:ChtFATB1a	Block 2; G10; pH7; S5818; T678; D2204-34	0.58	55.18	19.86	7.72	0.60	10.88	4.29
	Block 2; H7; pH7; S5818; T678; D2204-43	0.68	54.79	20.14	7.78	0.56	10.99	4.18
	Block 2; H5; pH7; S5818; T678; D2204-41	0.60	54.69	20.38	7.39	0.55	11.50	4.13
	Block 2; G8; pH7; S5818; T678; D2204-32	0.66	54.26	20.39	7.69	0.55	11.45	4.26
	Block 2; F6; pH7; S5818; T678; D2204-18	0.67	54.23	20.04	7.60	0.56	11.80	4.23
pSZ3390; CpSAD1tpExt-CpalFATB2FLAGExtA (DAO1b)	Block 4; A5; pH7; S5818; T674; D2104-5	0.58	63.83	12.10	5.89	0.55	10.46	5.44
	Block 4; A12; pH7; S5818; T674; D2104-12	0.48	61.92	16.15	5.87	0.50	9.86	4.41
	Block 4; B9; pH7; S5818; T674; D2104-21	0.41	54.31	18.41	7.26	0.51	13.80	4.49
	Block 4; B5; pH7; S5818; T674; D2104-17	0.37	53.56	16.54	7.25	0.58	16.34	4.42
	Block 4; B11; pH7; S5818; T674; D2104-23	0.41	52.44	17.99	7.60	0.54	15.38	4.66
pSZ3531; CpSAD1tpExt-CpalFATB2FLAGExtA (THI4A)	Block 5B; A8; pH7; S5818; T684; D2235-8	0.52	59.36	15.70	6.93	0.45	11.41	4.63
	Block 5B; A12; pH7; S5818; T684; D2235-12	0.44	55.60	16.98	6.98	0.53	14.21	4.59
	Block 5B; B11; pH7; S5818; T684; D2235-23	0.36	49.58	17.43	8.72	0.57	17.44	4.62
	Block 5B; A4; pH7; S5818; T684; D2235-4	0.35	49.43	18.63	8.22	0.62	17.29	4.54
	Block 5B; A11; pH7; S5818; T684; D2235-11	0.36	48.92	15.93	7.84	0.68	20.38	4.96

EXAMPLE 63: A SUITE OF REGULATABLE PROMOTERS TO CONDITIONALLY CONTROL GENE EXPRESSION LEVELS IN OLEAGINOUS CELLS IN SYNCHRONY WITH LIPID PRODUCTION

[0721] S5204 was generated by knocking out both copies of *FATA1* in *Prototheca moriformis* (PmFATA1) while simultaneously overexpressing the endogenous *PmKASII* gene in a Δ*fad2* line, S2532. S2532 itself is a *FAD2* (also known as *FADc*) double knockout strain that was previously generated by insertion of *C. tinctorius* ACP thioesterase (Accession No: AAA33019.1) into S1331, under the control of *CrTUB2* promoter at the *FAD2* locus. S5204 and its parent S2532 have a disrupted endogenous *PmFAD2-1* gene resulting in no Δ12 specific desaturase activity manifested as 0% C18:2 (linoleic acid) levels in both seed and lipid production stages. Lack of any C18:2 in S5204 (and its parent S2532) results in growth defects which can be partially mitigated by exogenous addition of linoleic acid in the seed stage. For industrial applications of a zero linoleic oil however, exogenous addition of linoleic acid entails additional cost. We have previously shown that complementation of S5204 (and other Δ*fad2* strains S2530 and S2532) with pH inducible *AMT03p* driven *PmFAD2-1* restores C18:2 to wild-type levels at pH 7.0 and also results in rescued growth characteristics during seed stage without any linoleic supplementation. Additionally when the seed from pH 7.0 grown complemented lines is subsequently transferred into low-nitrogen lipid production flasks with pH adjusted to 5.0 (to control *AMT03p* driven FAD2 protein levels), the resulting final oil profile matches the parent S5204 or S2532 profile with zero linoleic levels but with rescued growth and productivity metrics. Thus in essence with *AMT03p* driven *FAD2-1* we have developed a pH regulatable strain that potentially could be used to generate oils with varying linoleic levels depending on the desired application.

[0722] *Prototheca moriformis* undergoes rapid cell division during the first 24-30 hrs in fermenters before nitrogen runs out in the media and the cells switch to storing lipids. This initial cell division and growth in fermenters is critical for the overall strain productivity and, as reported above, FAD2 protein is crucial for sustaining vigorous growth characteristic of a particular strain. However when first generation, single insertion, genetically clean, *PmFAD2-1* complemented strains (S4694 and S4695) were run in 7L fermenters at pH 5.0 (with seed grown at pH 7.0), they did not perform on par with the original parent base strain (S1331) in terms of productivity. Western data suggested that AMT03p promoter driving *PmFAD2-1* (as measured by FAD2 protein levels) is severely down regulated between 0 – 30 hrs in fermenters irrespective of fermenter pH (5.0 or 7.0). Work on fermentation conditions

(batched vs unbatched/limited initial N, pH shift from 7 to 5 at different time points during production phase) suggested that initial batching (and excess amounts) of nitrogen during early lipid production was the likely cause of *AMT03p* promoter down regulation in fermenters. Indeed, this initial repression in AMT03 can be directly seen in transcript time-course during fermentation. A significant depression of Amt03 expression was observed early in the run, which corresponds directly with NH4 levels in the fermenter.

[0723] When the fermentations were performed with limited N, we were able to partially rescue the AMT03p promoter activity and while per cell productivity of S4694/S4695 was on par with the parent S1331, the overall productivity still lagged behind. These results suggest that a suboptimal or inactive *AMT03p* promoter and thus limitation of FAD2 protein in early fermentation stages inhibits any complemented strains from attaining their full growth potential and overall productivity. Here we identify new, improved promoter that allow differential gene activity during high-nitrogen growth and low-nitrogen lipid production phases.

[0724] In particular, we observed that:

- *In trans* expression of the fatty acid desaturase-2 gene from *Prototheca moriformis* (*PmFad2-1*) under the control of *down regulated* promoter elements identified using a transcriptome based bioinformatics approach results in functional complementation of *PmFAD2-1* with restored growth in Δ*fad2*, Δ*fata1* strain S5204.
- Complementation of S5204 manifested in a robust growth phenotype only occurs in seed and early fermentation stages when the new promoter elements are actively driving the expression of *PmFAD2-1*.
- Once the cells enter the active lipid production phase (around the time when N runs out in the fermenter), the newly identified promoters are down regulated resulting in no additional FAD2 protein and the final oil profile of the complemented lines is same as the parent S5204 albeit with better growth characteristics.
- These strains should potentially mitigate the problems that were encountered with AMT03p driven FAD2 in earlier complemented strains.
- *Importantly, we have identified down-regulatable promoters of varying strengths, some of which are relatively strong in the beginning with low-to-moderate levels provided during the remainder of the run. Thus depending on phenotype these promoters can be selected for fine-tuning the desired levels of transgenes.*

[0725] Bioinformatics Methods: RNA was prepared from cells taken from 8 time points during a typical fermenter run. RNA was polyA-selected for run on an Illumina HiSeq. Illumina paired-end data (100bp reads x 2, ~600bp fragment size) was collected and processed for read quality using FastQC [www.bioinformatics.babraham.ac.uk/projects/fastqc/]. Reads were run through a custom read-processing pipeline that de-duplicates, quality-trims, and length-trims reads.

[0726] Transcripts were assembled from Illumina paired-end reads using Oases/velvet [Velvet: algorithms for de novo short read assembly using de Bruijn graphs. D.R. Zerbino and E. Birney. Genome Research 18:821-829] and assessed by N50 and other metrics. The transcripts from all 8 time points were further collapsed using CD-Hit. [Limin Fu, Beifang Niu, Zhengwei Zhu, Sitao Wu and Weizhong Li, CD-HIT: accelerated for clustering the next generation sequencing data. Bioinformatics, (2012), 28 (23): 3150-3152. doi: 10.1093/bioinformatics/bts565; Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences", Weizhong Li & Adam Godzik Bioinformatics, (2006) 22:1658-9].

[0727] These transcripts were used as the base (reference assembly) for expression-level analysis. Reads from the 8 time points were analyzed using RSEM which provides raw read counts as well as a normalized value provided in Transcripts Per Million (TPM). [Li, Bo & Dewey, Colin N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome, BioMed Central: The Open Access Publisher. Retrieved at October 10, 2012, from the website temoa : Open Educational Resources (OER) Portal at www.temoa.info/node/441614] The TPM was used to determine expression levels. Genes previously identified in screens for strong promoters were also used to gauge which levels should be considered as significantly high or low. This data was loaded into a Postgres database and visualized with Spotfire, along with integrated data that includes gene function and other characteristics such as categorization based on expression profile. This enabled rapid and targeted analysis of genes with significant changes in expression.

[0728] The promoters for genes, which we selected, were mapped onto a high-quality reference genome for S376 (our reference *Prototheca moriformis* strain). Briefly, PacBio long reads (~2kb) were error-corrected by high-quality PacBio CCS reads (~600bp) and assembled using the Allora assembler in SMRTPipe [pacbiodevnet.com]. This reference genome, in conjunction with transcriptome read mapping, was used to annotate the precise gene structures, promoter and UTR locations, and promoter elements within the region of interest, which then guided further sequencing and promoter element selection.

[0729] The criteria used for identifying new promoter elements were:

1. Reasonable expression (e.g., > 500, <100, or <50 transcripts per million [TPM]) of a downstream gene in seed and early lipid production stages (T0 - T30 hrs)
2. Severe down regulation of the gene above (e.g., > 5-fold. 10-fold, or 15-fold) when the nitrogen gets depleted in the fermenters.
3. pH neutrality of the promoter elements (e.g., less than a 2-fold change in TPM on going from pH 5.0 top 7.0 in cultivation conditions), or at least effective operation under pH5 conditions.

[0730] Using the above described criteria we identified several potentially down regulated promoter elements that were eventually used to drive *PmFAD2-1* expression in S5204. A range of promoters was chosen that included some that started as being weak promoters and went down to extremely low levels, through those that started quite high and dropped only to moderately low levels. This was done because it was unclear *a priori* how much expression would be needed for FAD2 early on to support robust growth, and how little FAD2 would be required during the lipid production phase in order to achieve the zero linoleic phenotype.

[0731] The promoter elements that were selected for screening and their allelic forms were named after their downstream gene and are as follows:

1. Carbamoyl phosphate synthase (*PmCPS1p* and *PmCPS2p*)
2. Dipthine synthase (*PmDPS1p* and *PmDPS2p*)
3. Inorganic pyrophosphatase (*PmIPP1p*)
4. Adenosylhomocysteinase (*PmAHC1p* and *PmAHC2p*)
5. Peptidyl-prolyl cis-trans isomerase (*PmPPI1p* and *PmPPI2p*)
6. GMP Synthetase (*PmGMPS1p* and *PmGMPS2p*)
7. Glutamate Synthase (*PmGSp*)
8. Citrate Synthase (*PmCS1p* and *PmCS2p*)
9. Gamma Glutamyl Hydrolase (*PmGGH1p*)
10. Acetohydroxyacid Isomerase (*PmAHI1p* and *PmAHI2p*)
11. Cysteine Endopeptidase (*PmCEP1p*)
12. Fatty acid desaturase 2 (*PmFAD2-1p* and *PmFad2-2p*) [CONTROL]

[0732] The transcript profile of two representative genes viz. *PmIPP* (Inorganic Pyrophosphatase) and *PmAHC*, (Adenosylhomocysteinase) start off very strong (4000-5000 TPM) but once the cells enter active lipid production their levels fall off very quickly. While the transcript levels of *PmIPP* drop off to nearly 0 TPM, the levels of *PmAHC* drop to around

250 TPM and then stay steady for the rest of the fermentation. All the other promoters (based on their downstream gene transcript levels) showed similar downward expression profiles.

[0733] The elements were PCR amplified and wherever possible promoters from allelic genes were identified, cloned and named accordingly e.g. the promoter elements for 2 genes of Carbamoyl phosphate synthase were named *PmCPS1p* and *PmCPS2p*. As a comparator promoter elements from *PmFAD2-1* and *PmFAD2-2* were also amplified and used to drive *PmFAD2-1* gene. While, in the present example, we used FAD2-1 expression and hence C18:2 levels to interrogate the newly identified down regulated promoters, in principle these promoter elements can be used to down regulate any gene of interest.

[0734] **Construct used for the expression of the *Prototheca moriformis* fatty acid desaturase 2 *(PmFAD2-1) under the expression of PmCPS1p* in *Δfad2* strains S5204 - [pSZ3377]:** The Δ*fad2* Δ*fata1* S5204 strain was transformed with the construct pSZ3377. The sequence of the transforming DNA is provided below. Relevant restriction sites in the construct pSZ3377 (6S::PmHXT1p-ScMEL1-CvNR::PmCPS1p-PmFAD2-1-CvNR::6S) are indicated in lowercase, underlined and bold, and are from 5'-3' *BspQ* 1, *KpnI, SpeI, SnaBI, EcoRV, SpeI, AflII, SacI, BspQ* I, respectively. *BspQI* sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent genomic DNA from UTEX 1435 that permits targeted integration of the transforming DNA at the 6S locus via homologous recombination. Proceeding in the 5' to 3' direction, the Hexose transporter (HXT1) gene promoter from UTEX 1435 driving the expression of the *Saccharomyces cerevisiae* Melibiase (ScMEL1) gene is indicated by the boxed text. The initiator ATG and terminator TGA for ScMEL1 are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics. The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase underlined text followed by an UTEX 1435 CPS1p promoter of *Prototheca moriformis,* indicated by boxed italics text. The Initiator ATG and terminator TGA codons of the PmFAD2-1 are indicated by uppercase, bold italics, while the remainder of the gene is indicated by bold italics. The *C. vulgaris* nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the UTEX 1435 6S genomic region indicated by bold, lowercase text. The final construct was sequenced to ensure correct reading frames and targeting sequences.

[0735] Nucleotide sequence of transforming DNA contained in plasmid pSZ3377:

gctcttcggagtcactgtgccactgagttcgactggtagctgaatggagtcgctgctccactaaacgaattgtcagcaccgcca gccggccgaggacccgagtcatagcgagggtagtagcgcgccatggcaccgaccagcctgcttgccagtactggcgtctcttc

cgcttctctgtggtcctctgcgcgctccagcgcgtgcgcttttccggtggatcatgcggtccgtggcgcaccgcagcggccgctg
cccatgcagcgccgctgcttccgaacagtggcggtcagggccgcacccgcggtagccgtccgtccggaacccgcccaagagt
tttgggagcagcttgagccctgcaagatggcggaggacaagcgcatcttcctggaggagcaccggtgcgtggaggtccgggg
ctgaccggccgtcgcattcaacgtaatcaatcgcatgatgatcagaggacacgaagtcttggtggcggtggccagaaacact
gtccattgcaagggcatagggatgcgttccttcacctctcatttctcatttctgaatccctccctgctcactctttctcctcctcttc
ccgttcacgcagcattcggggtaccgcggtgagaatcgaaaatgcatcgtttctaggttcggagacggtcaattccctgctccggcg
aatctgtcggtcaagctggccagtggacaatgttgctatggcagcccgcgcacatgggcctcccgacgcggccatcaggagcccaa
acagcgtgtcagggtatgtgaaactcaagaggtccctgctgggcactccggccccactccgggggcgggacgccaggcattcgcg
gtcggtcccgcgcgacgagcgaaatgatgattcggttacgagaccaggacgtcgtcgaggtcgagaggcagcctcggacacgtctc
gctagggcaacgccccgagtccccgcgagggccgtaaacattgtttctgggtgtcggagtgggcattttgggcccgatccaatcgcct
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ccacgtttcaaagtccccggccagaaatgcacaggaccggcccggctcgcacaggccatgctgaacgcccagatttcgacagcaac
accatctagaataatcgcaaccatccgcgttttgaacgaaacgaaacggcgctgtttagcatgtttccgacatcgtgggggccgaagc
atgctccgggggggaggaaagcgtggcacagcggtagcccattctgtgccacacgccgacgaggaccaatccccggcatcagcctt
catcgacggctgcgccgcacatataaagccggacgcctaaccggtttcgtggttatg**actagt***ATGttcgcgttctacttcctgacg*
gcctgcatctccctgaagggcgtgttcggcgtctccccctcctacaacggcctgggcctgacgccccagatgggctgggacaact
ggaacacgttcgcctgcgacgtctccgagcagctgctgctggacacggccgaccgcatctccgacctgggcctgaaggacatgg
gctacaagtacatcatcctggacgactgctggtcctccggccgcgactccgacggcttcctggtcgccgacgagcagaagttccc
caacggcatgggccacgtcgccgaccacctgcacaacaactccttcctgttcggcatgtactcctccgcgggcgagtacacgtgc
gccggctaccccggctccctgggccgcgaggaggaggacgcccagttcttcgcgaacaaccgcgtggactacctgaagtacga
caactgctacaacaagggccagttcggcacgcccgagatctcctaccaccgctacaaggccatgtccgacgccctgaacaaga
cgggccgccccatcttctactccctgtgcaactggggccaggacctgaccttctactggggctccggcatcgcgaactcctggcgc
atgtccggcgacgtcacggcggagttcacgcgccccgactcccgctgcccctgcgacggcgacgagtacgactgcaagtacgc
cggcttccactgctccatcatgaacatcctgaacaaggccgccccatgggccagaacgcgggcgtcggcggctggaacgacct
ggacaacctggaggtcggcgtcggcaacctgacggacgacgaggagaaggcgcacttctccatgtgggccatggtgaagtccc
ccctgatcatcggcgcgaacgtgaacaacctgaaggcctcctcctactccatctactcccaggcgtccgtcatcgccatcaaccag
gactccaacggcatccccgccacgcgcgtctggcgctactacgtgtccgacacggacgagtacggccagggcgagatccagat
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ggaggagatcttcttcgactccaacctgggctccaagaagctgacctccacctgggacatctacgacctgtgggcgaaccgcgtc
gacaactccacggcgtccgccatcctgggccgcaacaagaccgccaccggcatcctgtacaacgccaccgagcagtcctacaa
ggacggcctgtccaagaacgacacccgcctgttcggccagaagatcggctccctgtcccccaacgcgatcctgaacacgaccgt
ccccgcccacggcatcgcgttctaccgcctgcgcccctcctccTGA**tacgta**gcagcagcagctcggatagtatcgacacactct
ggacgctggtcgtgtgatggactgttgccgccacacttgctgccttgacctgtgaatatccctgccgcttttatcaaacagcctcagtgtg

tttgatcttgtgtgtacgcgcttttgcgagttgctagctgcttgtgctatttgcgaataccacccccagcatcccccttccctcgtttcatatcgc
ttgcatcccaaccgcaacttatctacgctgtcctgctatccctcagcgctgctcctgctcctgctcactgcccctcgcacagccttggtttg
ggctccgcctgtattctcctggtactgcaacctgtaaaccagcactgcaatgctgatgcacgggaagtagtgggatgggaacacaaat
gga**gatatc***gcgaggggtctgcctgggccagccgctccctctaaacacgggacgcgtggtccaattcgggcttcgggacccttg*
gcggtttgaacgccagggatggggcgcccgcgagcctggggacccccggcaacggcttccccagagcctgccttgcaatctcgc
gcgtcctctccctcagcacgtggcggttccacgtgtggtcgggcttcccggactagctcgcgtcgtgacctagcttaatgaacccag
*ccgggcctgtagcaccgcctaagaggttttgattatttcattataccaatctattcgcc***actagt*ATGgccatcaagaccaaccgc**
cagcccgtggagaagcccccccttcaccatcggcaccctgcgcaaggccatccccgcccactgcttcgagcgctccgccctgcgct
cctccatgtacctggccttcgacatcgccgtgatgtccctgctgtacgtggcctccacctacatcgaccccgcccccgtgcccacctg
ggtgaagtacggcgtgatgtggcccctgtactggttcttccagggcgccttcggcaccggcgtgtgggtgtgcgcccacgagtgcg
gccaccaggccttctcctcctcccaggccatcaacgacggcgtgggcctggtgttccactccctgctgctggtgccctactactcctg
gaagcactcccaccgccgccaccactccaacaccggctgcctggacaaggacgaggtgttcgtgccccccccaccgcgccgtgg
cccacgagggcctggagtgggaggagtggctgcccatccgcatgggcaaggtgctggtgaccctgaccctgggctggcccctgt
acctgatgttcaacgtggcctcccgcccctacccccgcttcgccaaccacttcgaccccctggtccccccatcttctccaagcgcgagc
gcatcgaggtggtgatctccgacctggccctggtggccgtgctgtccggcctgtccgtgctgggccgcaccatgggctgggcctgg
ctggtgaagacctacgtggtgccctacctgatcgtgaacatgtggctggtgctgatcaccctgctgcagcacacccaccccgccct
gccccactacttcgagaaggactgggactggctgcgcggcgccatggccaccgtggaccgctccatgggcccccccttcatgga
caacatcctgcaccacatctccgacacccacgtgctgcaccacctgttctccaccatcccccactaccacgccgaggaggcctcc
gccgccatccgccccatcctgggcaagtactaccagtccgactcccgctgggtgggccgcgccctgtgggaggactggcgcgac
tgccgctacgtggtgcccgacgcccccgaggacgactccgccctgtggttccacaagTAGatcgat**cttaag**gcagcagcagct
cggatagtatcgacacactctggacgctggtcgtgtgatggactgttgccgccacacttgctgccttgacctgtgaatatccctgccgctt
ttatcaaacagcctcagtgtgtttgatcttgtgtgtacgcgcttttgcgagttgctagctgcttgtgctatttgcgaataccacccccagcatc
ccctttcctcgtttcatatcgcttgcatcccaaccgcaacttatctacgctgtcctgctatccctcagcgctgctcctgctcctgctcactgc
ccctcgcacagccttggtttgggctccgcctgtattctcctggtactgcaacctgtaaaccagcactgcaatgctgatgcacgggaagta
gtgggatgggaacacaaatggaaagcttaattaa**gagctcttgttttccagaaggagttgctccttgagcctttcattctcagcctcg**
ataacctccaaagccgctctaattgtggagggggttcgaatttaaaagcttggaatgttggttcgtgcgtctggaacaagccca
gacttgttgctcactgggaaaaggaccatcagctccaaaaaacttgccgctcaaaccgcgtacctctgctttcgcgcaatctgc
cctgttgaaatcgccaccacattcatattgtgacgcttgagcagtctgtaattgcctcagaatgtggaatcatctgccccctgtgc
gagcccatgccaggcatgtcgcgggcgaggacacccgccactcgtacagcagaccattatgctacctcacaatagttcataac
agtgaccatatttctcgaagctccccaacgagcacctccatgctctgagtggccacccccggccctggtgcttgcggagggca
ggtcaaccggcatggggctaccgaaatccccgaccggatcccaccacccccgcgatgggaagaatctctccccgggatgtgg
gcccaccaccagcacaacctgctggcccaggcgagcgtcaaaccataccacacaaatatccttggcatcggccctgaattcct

tctgccgctctgctacccggtgcttctgtccgaagcaggggttgctagggatcgctccgagtccgcaaacccttgtcgcgtggcg gggcttgttcgagcttgaagagc (SEQ ID NO:128)

[0736] The recombination between *C. vulgaris* nitrate reductase 3' UTR's in the construct pSZ3377 results in multiple copies of PmFAD2-1 in transgenic lines which would then manifest most likely as higher C18:2 levels at the end of fermentation. Since the goal was to create a strain with 0% terminal C18:2, we took precautions to avoid this recombination. In another version of the above plasmid ScMEL1 gene was followed by *Chlorella protothecoides* (UTEX 250) elongation factor 1a (CpEF1a) 3' UTR instead of *C. vulgaris* 3' UTR. The sequence of *C. protothecoides* (UTEX 250) elongation factor 1a (CpEF1a) 3' UTR used in construct pSZ3384 and other constructs with this 3' UTR (described below) is shown below. Plasmid pSZ3384 could be written as 6S::PmHXT1p-ScMEL1-CpEF1a::PmCPS1p-PmFAD2-1-CvNR::6S.

[0737] Nucleotide sequence of *Chlorella protothecoides* (UTEX 250) elongation factor 1a (CpEF1a) 3' UTR in pSZ3384:

tacaacttat**tacgta**acggagcgtcgtgcgggagggagtgtgccgagcggggagtcccggtctgtgcgaggcccggcagctgac gctggcgagccgtacgccccgagggtccccctcccctgcaccctcttcccctccctctgacggccgcgcctgttcttgcatgttcagc gacgag**gatatc** (SEQ ID NO:129)

[0738] The *C. protothecoides* (UTEX 250) elongation factor 1a 3' UTR sequence is flanked by restriction sites *SnaBI* on 5' and *EcoRV* on 3' ends shown in lowercase bold underlined text. Note that the plasmids containing CpEF1a 3' UTR (pSZ3384 and others described below) after ScMEL1 stop codon contains 10 extra nucleotides before the 5' *SnaBI* site. These nucleotides are not present in the plasmids that contain *C. vulgaris* nitrate reductase 3' UTR after the *S.* ScMEL1 stop codon.

[0739] In addition to plasmids pSZ3377 and pSZ3384 expressing either a recombinative CvNR-Promoter-PmFAD2-1-CvNR or non-recombinative CpEF1a-Promoter-PmFAD2-1-CvNR expression unit described above, plasmids using other promoter elements mentioned above were constructed for expression in S5204. These constructs along with their transformation identifiers (D #) can be described as:

Plasmid ID	D #	Description
pSZ3378	D2090	6SA::pPmHXT1-ScarlMEL1-CvNR:PmCPS2p-PmFad2-1-CvNR::6SB

pSZ3385	D2097	6SA::pPmHXT1-ScarlMEL1-CpEF1a:PmCPS2p-PmFad2-1-CvNR::6SB
pSZ3379	D2091	6SA::pPmHXT1-ScarlMEL1-CvNR:PmDPS1p-PmFad2-1-CvNR::6SB
pSZ3386	D2098	6SA::pPmHXT1)-ScarlMEL1-CpEF1a:PmDPS1p-PmFad2-1-CvNR::6SB
pSZ3380	D2092	6SA::pPmHXT1-ScarlMEL1-CvNR:PmDPS2p-PmFad2-1-CvNR::6SB
pSZ3387	D2099	6SA::pPmHXT1-ScarlMEL1-CpEF1a:PmDPS2p-PmFad2-1-CvNR::6SB
pSZ3480	D2259	6SA::pPmHXT1-ScarlMEL1-CvNR:PmIPP1p-PmFad2-1-CvNR::6SB
pSZ3481	D2260	6SA::pPmHXT1-ScarlMEL1-CpEF1a:PmIPP1p-PmFad2-1-CvNR::6SB
pSZ3509	D2434	6SA::pPmHXT1-ScarlMEL1-CvNR:PmAHC1p-PmFad2-1-CvNR::6SB
pSZ3516	D2266	6SA::pPmHXT1-ScarlMEL1-CpEF1a:PmAHC1p-PmFad2-1-CvNR::6SB
pSZ3510	D2435	6SA::pPmHXT1-ScarlMEL1-CvNR:PmAHC2p-PmFad2-1-CvNR::6SB
pSZ3513	D2263	6SA::pPmHXT1-ScarlMEL1-CvNR:PmPPI1p-PmFad2-1-CvNR::6SB
pSZ3689	D2440	6SA::pPmHXT1-ScarlMEL1-CpEF1a:PmPPI1p-PmFad2-1-CvNR::6SB
pSZ3514	D2264	6SA::pPmHXT1-ScarlMEL1-CvNR:PmPPI2p-PmFad2-1-CvNR::6SB
pSZ3518	D2268	6SA::pPmHXT1-ScarlMEL1-CpEF1a:PmPPI2p-PmFad2-1-CvNR::6SB
pSZ3515	D2265	6SA::pPmHXT1-ScarlMEL1-CvNR:PmGMPS1p-PmFad2-1-CvNR::6SB
pSZ3519	D2269	6SA::pPmHXT1-ScarlMEL1-CpEF1a:PmGMPS1p-PmFad2-1-CvNR::6SB
pSZ3520	D2270	6SA::pPmHXT1-ScarlMEL1-CpEF1a:PmGMPS2p-PmFad2-1-CvNR::6SB
pSZ3684	D2436	6SA::pPmHXT1-ScarlMEL1-CvNR:PmCS1p-PmFad2-1-CvNR::6SB
pSZ3686	D2438	6SA::pPmHXT1-ScarlMEL1-CpEF1A:PmCS1p-PmFad2-1-CvNR::6SB
pSZ3685	D2437	6SA::pPmHXT1-ScarlMEL1-CvNR:PmCS2p-PmFad2-1-CvNR::6SB
pSZ3688	D2439	6SA::pPmHXT1-ScarlMEL1-CvNR:PmGGHp-PmFad2-1-CvNR::6SB
pSZ3511	D2261	6SA::pPmHXT1-ScarlMEL1-CvNR:PmAHI2p-PmFad2-1-CvNR::6SB
pSZ3517	D2267	6SA::pPmHXT1-ScarlMEL1-CpEF1a:PmAHI1p-PmFad2-1-CvNR::6SB
pSZ3512	D2262	6SA::pPmHXT1-ScarlMEL1-CvNR:PmCEP1p-PmFad2-1-CvNR::6SB
pSZ3375	D2087	6SA::pPmHXT1-ScarlMEL1-CvNR:PmFAD2-1p-PmFad2-1-

		CvNR::6SB
pSZ3382	D2094	6SA::pPmHXT1-ScarlMEL1-CpEF1a:PmFAD2-1p-PmFad2-1-CvNR::6SB
pSZ3376	D2088	6SA::pPmHXT1-ScarlMEL1-CvNR:PmFAD2-2p-PmFad2-1-CvNR::6SB
pSZ3383	D2095	6SA::pPmHXT1-ScarlMEL1-CpEF1a:PmFAD2-2p-PmFad2-1-CvNR::6SB

[0740] The above constructs are the same as pSZ3377 or pSZ3384 except for the promoter element that drives PmFAD2-1. The sequences of different promoter elements used in the above constructs are shown below.

[0741] Nucleotide sequence of Carbamoyl phosphate synthase allele 2 promoter contained in plasmid pSZ3378 and pSZ3385 (PmCPS2p promoter sequence):

gcgaggggtctgcctgggccagccgctccctctgaacacgggacgcgtggtccaattcgggcttcgggacccttggcggtttg aacgcctgggagagggcgcccgcgagcctggggaccccggcaacggcttccccagagcctgccttgcaatctcgcgcgtcctc tccctcagcacgtggcggttccacgtgtggtcgggcgtcccggactagctcacgtcgtgacctagcttaatgaacccagccggg cctgcagcaccaccttagaggttttgattatttgattagaccaatctattcacc (SEQ ID NO:130)

[0742] Nucleotide sequence of Dipthine synthase allele 1 promoter contained in plasmid pSZ3379 and pSZ3386 (PmDPS1p promoter sequence):

ggcgaatagattggtataatgaaataatcaaaacctcttaggcggtgctacaggcccggctgggttcattaagctaggtcacg acgcgagctagtccgggaagcccgaccacacgtggaaccgccacgtgctgagggagaggacgcgcgagattgcaaggca ggctctggggaagccgttgccggggtccccaggctcgcgggcgccccatccctggcgttcaaaccgccaaagggtcccgaag cccgaattggaccacgcgtcccgtgtttagagggagcggctggcccaggcagacccctcgc (SEQ ID NO:131)

[0743] Nucleotide sequence of Dipthine synthase allele 2 promoter contained in plasmid pSZ3380 and pSZ3387 (PmDPS2p promoter sequence):

ggtgaatagattggtctaatcaaataatcaaaacctctaaggtggtgctgcaggcccggctgggttcattaagctaggtcacg acgtgagctagtccgggacgcccgaccacacgtggaaccgccacgtgctgagggagaggacgcgcgagattgcaaggcag gctctggggaagccgttgccggggtccccaggctcgcgggcgccctctcccaggcgttcaaaccgccaaagggtcccgaagc ccgaattggaccacgcgtcccgtgttcagagggagcggctggcccaggcagacccctcgc (SEQ ID NO:132)

[0744] Nucleotide sequence of Inorganic pyrophosphatase allele 1 promoter contained in plasmid pSZ3480 and pSZ3481 (PmIPP1p promoter sequence):

gtgatgggttctttagacgatccagcccaggatcatgtgttgcccacatggagcctatccacgctggcctagaaggcaagcac atttcaaggtgaacccacgtccatggagcgatggcgccaatatctcgcctctagaccaagcggttctcaccccaactgcgtcat

ttgtatgtatggctgcaaagttgtcggtacgatagaggccgccaacctggcggcgagggcgaggagctggttgccgatctgt
gcccaagcatgtgtcggagctcggctgtctcggcagcgagctcctgtgcaaggggcttgcatcgagaatgtcaggcgataga
cactgcacgttggggacacggaggtgcccctgtggcgtgtcctggatgccctcgggtccgtcgcgagaagctctggcgaccag
cacccggccacaaccgcagcaggcgttcacccacaagaatcttccagatcgtgatgcgcatgtatcgtgacacgattggcgag
gtccgcaggacgcacacggactcgtccactcatcagaactggtcagggcacccatctgcgtccctttcaggaaccacccaccg
ctgccaggcaccttcgccagcggcggactccacacagagaatgccttgctgtgagagaccatggccggcaagtgctgtcgga
tctgcccgcatacggtcagtccccagcacaaggaagccaagagtacaggctgttggtgtcgatggaggagtggccgttccca
caagtagtgagcggcagctgctcaacggcttccccctgttcatcttggcaaagccagtgacttcctacaagtatgtgatgcaga
tcggcactgcaatctgtcggcatgcgtacagaacatcggctcgccagggcagcgttgctcgctctggatgagctgcttgggag
gaatcatcggcacacgcccgtgccgtgcccgcgccccgcgcccgtcgggaaaggcccccggttaggacactgccgcgtcagcc
agtcgtgggatcgatcggacgtggcgaatcctcgcccggacaccctcatcacaccccacatttccctgcaagcaatcttgccga
caaaatagtcaagatccattgggtttagggaacacgtgcgagactgggcagctgtatctgtccttgccccgcgtcaaattcctg
ggcgtgacgcagtcacaggagaatctattagaccctggacttgcagctcagtcatgggcgtgagtggctaaagcacctaggt
caggcgagtaccgccccttccccaggattcactcttctgcgattgacgttgagcctgcatcgggctgcttcgtcacc (SEQ ID NO:133)

[0745] Nucleotide sequence of Adenosylhomocysteinase allele 1 promoter contained in plasmid pSZ3509 and pSZ3516 (PmAHC1p promoter sequence):

tcggagctaaagcagagactggacaagacttgcgttcgcatactggtgacacagaatagctcccatctattcatacgcctttg
ggaaaaggaacgagccttgtggcctctgcattgctgcctgctttgaggccgaggacggtgcgggacgctcagatccatcagc
gatcgccccaccctcagagcacctccgatccaaggcaatactatcaggcaaagtttccaaattcaaacattccaaaatcacgc
cagggactggatcacacacgcagatcagcgccgttttgctctttgcctacgggcgactgtgccacttgtcgacccctggtgacg
ggagggaccacgcctgcggttggcatccacttcgacggacccagggacggtttctcatgccaaacctgagatttgagcaccca
gatgagcacattatgcgttttaggatgcctgagcagcgggcgtgcaggaatctggtctcgccagattcaccgaagatgcgccc
atcggagcgaggcgagggctttgtgaccacgcaaggcagtgtgaggcaaacacatagggacacctgcgtctttcaatgcac
agacatctatggtgcccatgtatataaaatgggctacttctgagtcaaaccaacgcaaactgcgctatggcaaggccggcca
aggttggaatcccggtctgtctggatttgagtttgtgggggctatcacgtgacaatccctgggattgggcggcagcagcgcac
ggcctgggtggcaatggcgcactaatactgctgaaagcacggctctgcatccctttctcttgacctgcgattggtccttttcgcaa
gcgtgatcatc (SEQ ID NO:134)

[0746] Nucleotide sequence of Adenosylhomocysteinase allele 2 promoter contained in plasmid pSZ3510 (PmAHC2p promoter sequence):

tcggagctaaagcagaaactgaacaagacttgcgttcgcatacttgtgacactgaataggttcaatctattcatacgcctttgg gaaactgaacgagccttgtggcctctgcattgctgcctgctttgaggccgaggacggcgcggaacgcacagatccatcagcg atcgccccaccctcagagtacatccgatccaaggcaatactatcaggcaaagtttccaaattcaaacattccaaaattacgtca gggactggatcacacacgcagatcagcgccgttttgctctttgcctacgggcgactgtgccacttgtcgacgcctggtgacggg agggaccacgcctgcggttggcatccacttcgacggacccagggacggtctcacatgccaaacctgagatttgagcaccaag atgagcacattatgcgtttttggatgcctgagcagcgggcgtgcaggaatctggtctcgccagattcaccgaagatgcggcca tcggagcgaggcgagggctgtgtggccacgccaggcagtgtgaggcaaacacacagggacatctgcttctttcgatgcaca gacatctatgttgcccgtgcatataaaatgggctacttctgaatcaaaccaacgcaaacttcgctatggcaaggccggccaag gttggaatcccggtctgtctggatttgagtttgtgggggctatcacgtgacaatccctgggattgggcggcagcagcgcacgg cctggatggcaatggcgcactaatactgctgaaagcacggctctgcatccctttctcttgacctgcgattggtccttttcgcaagc gtgatcatc (SEQ ID NO:135)

[0747] Nucleotide sequence of Peptidyl-prolyl cis-trans isomerase allele 1 promoter contained in plasmid pSZ3513 and pSZ3689 (PmPPI1p promoter sequence):

caccgatcactccgtcgccgcccaagagaaatcaacctcgatggagggcgaggtggatcagaggtattggttatcgttcgttc ttagtctcaatcaatcgtacaccttgcagttgcccgagtttctccacacatacagcacctcccgctcccagcccattcgagcgacc caatccgggcgatcccagcgatcgtcgtcgcttcagtgctgaccggtggaaagcaggagatctcgggcgagcaggaccacat ccagcccaggatcttcgactggctcagagctgaccctcacgcggcacagcaaaagtagcacgcacgcgttatgcaaactggtt acaacctgtccaacagtgttgcgacgttgactggctacattgtctgtctgtcgcgagtgcgcctgggcccttacggtgggacact ggaactccgccccgagtcgaacacctagggcgacgcccgcagcttggcatgacagctctccttgtgttctaaataccttgcgcg tgtgggaga (SEQ ID NO:136)

[0748] Nucleotide sequence of Peptidyl-prolyl cis-trans isomerase allele 2 promoter contained in plasmid pSZ3514 and pSZ3518 (PmPPI2p promoter sequence):

atccaccgatcactccgtcgccgcccaagagaattcaacctcgatggagggcaaggtggatcagaggtattggttatcgttcg ctattagtctcaatcaatcgtgcaccttgcagttgctcgagtttctccacacatacagcacctcccgctcccagcccattcgagcg acccaatccgggcgatcccagcgatcgtcgtcgcttcagtgctgaccggtggaaagcaggagatctcgggcgagcaggacc acatccagcacaggatcttcgactggctcagagctgaccctcacgcggcacagcaaaagtagcccgcacgcgttatgcaaac aggttacaacctgtccaacactgttgcgacgttgactggctacattgtctgtctgtcgcgagtacgcctggacccttacggtggg acactggaactccgccccgagtcgaacacctagggcgacgcccgcagcttggcatgacagctctccttgtattctaaatacctc gcgcgtgtgggagaa (SEQ ID NO:137)

[0749] Nucleotide sequence of GMP Synthetase allele 1 promoter contained in plasmid pSZ3515 and pSZ3519 (PmGMPS1p promoter sequence):

atgatgcgcgtgtacgactatcaaggaagaaagaggacttaatttcttaccttctaaccaccatattctttttgctggatgcttgc
tcgtctcgatgacaattgtgaacctcttgtgtgaccctgaccctgctgcaaggctctccgaccgcacgcaaggcgcagccggcg
cgtccggaggcgatcggatccaatccagtcgtcctcccgcagcccgggcacgtttgcccatgcaggcccttccacaccgctcaa
gagactcccgaacaccgcccactcggcactcgcttcggctgccgagtgcgcgtttgagtttgccctgccacagaagacacc

(SEQ ID NO:138)

[0750] Nucleotide sequence of GMP Synthetase allele 2 promoter contained in plasmid pSZ3520 (PmGMPS2p promoter sequence):

atgatgcgcgtgtacgactatcaaggaagaaagaggacttaatttcttaccttctaaccaccatattctttttgctggatgcttgc
tcgtctcgatgacaattgtgaacctcttgtgtgaccctgaccctgctgcaaggctctccgaccgcacgcaaggcgcagccggcg
cgtccggaggcgatcggatccaatccagtcgtcctcccgcagcccgggcacgtttgcccatgcaggcccttccacaccgctcaa
gagactcccgaacaccgcccactcggcactcgcttcggctgccgagtgcgcgtttgagtttgccctgccacaggagacatc

(SEQ ID NO:139)

[0751] Nucleotide sequence of Citrate synthase allele 1 promoter contained in plasmid pSZ3684 and pSZ3686 (PmCS1p promoter sequence):

cccgggcgagctgtacgcctacggagcgaggcctggtgtgaccgttgcgatctcgccagcagacgtcgcggagcctcgtccca
aaggccctttctgatcgagcttgtcgtccactggacgctttaagttgcgcgcgcgatgggataaccgagctgatctgcactcag
attttggtttgttttcgcgcatggtgcagcgaggggaggtactacgctggggtacgagatcctccggattcccagaccgtgttg
ccggcatttacccggtcatcgccagcgattcgggacgacaaggccttatcctgtgctgagacgctcgagcacgtttataaaatt
gtgggtaccgcggtatgcacagcgttcaacacgcgccacgccgaaattggttggtgggggagcacgtatgggactgacgtat
ggccagcagcgaacactcaccgaacaagtgccaatgtataccttgcatcaatgatgctccggcagcttcgattgactgtctcga
aaaagtgtgagcaagcagatcatgtggccgctctgtcgcgcagcacctgacgcattcgacacccacggcaatgcccaggcca
gggaatagagagtaagacaactcccattgttcagcaaaacattgcactgcagtgccttcacaactatacaatgaatgggagg
gaatatgggctctgcatgggacagcttagctgggacattcggctactgaacaagaaaaccccacgagaaccaattggcgaa
acctgccgggaggaggtgatcgtttctgtaaatggcttacgcattcccccccggcggctcacgaggggtgtggtgaaccctgcc
agctgatcaagtgcttgctgacgtcggccagggaggtgtatgtgattgggccgtggggcgtgagttatcctaccgccggaccc
gcgaagtcacatgacgaatggccgtgcgggatgacgagagcacgactcgctctttcttcgccggcccggcttcatggaggac
aataataaagggtggccaccggcaacagccctccatacctgaaccgattccagacccaaacctcttgaattttgagggatcca
gttcaccggtatagtcacg (SEQ ID NO:140)

[0752] Nucleotide sequence of Citrate synthase allele 2 promoter contained in plasmid pSZ3685 (PmCS2p promoter sequence):

atccccgggcgagctgtacgcctacggagcgaggcctggtgtgaccgttgcgatctcgccagcagacgtcgcggagcctcgtc
ccaaaggccctttctgatcgagcttgtcgtccactggacgctttaagttgcgcgcgcgatgggataaccgagctgatctgcactc
agattttggtttgttttcgcgcatggtgcagcgaggggaggtactacgctggggtacgagatcctccggattcccagaccgtgt
tgccggcatttacccggtcatcgccagcgattcgggacgacaaggccttatcctgtgctgagacgctcgagcacgtttataaaa
ttgtggtcaccgtggtacgcacagcgtccaacacgcgccacgccgaaattcgttggtgggggagcacgtatcggactgacgt
atggccagcagcgaacactcaccaaacaggtgccaatgtatagcttgcatcaatgatgctctggcagcttcgattgactgtctc
gaaaaagtgtgtgcaaacagattatgtggccgctctgtggccgcgcagcacctgacgcactcgacacccacggcaatgccca
ggccaaggaacagagagtaagacaactcccattgttcagtaaaacattgcactgcagtgccttcacaaacatacaacgaatg
ggagggaatatgggcttcgaatgggacagcttagctgggacattcggttactgaacaagaaaaccccacgagaaccaactg
gcgaaacctgccgggaggaggtgatcgtttttgtaaatggcttacgcattccccccccggcggctcacgggggtgtggtgaa
ccctgccagctgatcaagtgcttgctgacgtcggccagggaggtgtatgtgatttggccgtggggcgtgagttatcctaccgcc
ggacccgcgaagtcacatgacgaatggccgtgcgggatgacgagagcagggctcgctctttcttcgccggcccggcttcatgg
aggacaataataaagggtggccaccggcaacagccctccatacctgaaccgattccagacccaaacctcttgaattttgagg
gatccagttcaccggtatagtcacga (SEQ ID NO:141)

[0753] Nucleotide sequence of Gamma Glutamyl Hydrolase allele 1 promoter contained in plasmid pSZ3688 (PmGGH1p promoter sequence):

gcgagtggttttgctgccgggaagggagtggggagcgtcgagcgagggacgcggcgctcgaggcgcacgtcgtctgtcaac
gcgcgcggccctcgcggcccgcggccccacccagctctaatcatcgaaaactaagaggctccacacgcctgtcgtagaatgca
tgggattcgccagtagaccacgatctgcgccgaagaagctggtctacccgacgtttttttgttgctcctttattctgaatgatatga
agatagtgtgcgcagtgccacgcataggcatcaggagcaagggaggacgggtcaacttgaaagaaccaaaccatccatcc
gagaaatgcgcatcatctttgtagtaccatcaaacgccttggccaatgtcttctgcatggacaacacaacctgctcctggccac
acggtcgacttggagcgccccatgcgcccaggtcgccacgacccgcggcccagcgcgcggcgattcgcctcacgagatcccg
gcggacccggcacgcccgcgggccgacggtgcgcttggcgatgctgctcattaacccacggccgtcacccgatccacatgctct
ttttcaacacatccacattggaatagagctctaccagggtgagtactgcattctttggggctgggaggaccccactcgacacct
ggtccttcatcggccgaaagcccgaacctgagcgcttccccgccccgttcctcatccccgactttccgatggcccattgcagtttc
aaac (SEQ ID NO:142)

[0754] Nucleotide sequence of Acetohydroxyacid Isomerase allele 1 promoter contained in plasmid pSZ3517 (PmAHI1p promoter sequence):

atctgggtggaggactgggagtaagatgtaaggatattaattaaacattctagtttgttgatggcacaacagtcaatgcattt cagtcgtcttgctccttataacctatgcgtgtgccatcgccggccatgcacctgtggcgtggtaccgaccatcggggagaggcc cgagattcggaggtacctcccgccctgggcgagcccttcacgtgacggcacaagtcccttgcatcggcccgcgagcacggaat acagagccccgtgccccccacgggccctcacatcatccactccattgttcttgccacaccgatcagca (SEQ ID NO:143)

[0755] Nucleotide sequence of Acetohydroxyacid Isomerase allele 2 promoter contained in plasmid pSZ3511 (PmAHI2p promoter sequence):

tgggtggaggactgggaagaagatgtaaggatatcaatttaacattctagtttgttgatggcacaacagtcactgaataccg ggcgtctggctgctaaaatagccggagcgtgtgccatcgccggccatgcatctgtggcgtggtaccgaccatcagggagagg cccgagattcggaggtacctcccgccctgggcgagcccttcacgtgacggcacaagtcccttgcatcggcccgcgagcacgga atacagagccccgtgctccccacgggccctcacatcatccactccattgttcttgccacaccgatcagc (SEQ ID NO:144)

[0756] Nucleotide sequence of Cysteine Endopeptidase allele 1 promoter contained in plasmid pSZ3512 (PmCEP1 promoter sequence):

ataacgaggcacaatgatcgatatttctatcgaacaactgtatttagccctgtacgtaccccgctcttgggccagcccgtccgtg cttgccttcggaaaattgcatggcgcctcatgcaaactcgcgctctcacagcagatctcgcccagctcccgggagagcaatcgc gggtggggcccggggcgaatccaggacgcgccccgcggggccgctccactcgccagggccaatgggcggcttatagtcctg gcatgggctctgcatgcacagtatcgcagtttgggcgaggtgttgcccccgcgatttcgaatacgcgacgcccggtactcgtgc gagaacagggttcttg (SEQ ID NO:145)

[0757] Nucleotide sequence of Fatty acid desaturase 2 allele 1 promoter contained in plasmid pSZ3375 and 3382 (PmFAD2-1 promoter sequence):

atcgcgatggtgcgcactcgtgcgcaatgaatatggggtcacgcggtggacgaacgcggaggggcctggccgaatctagg cttgcattcctcagatcactttctgccggcggtccggggtttgcgcgtcgcgcaacgctccgtctccctagccgctgcgcaccgcg cgtgcgacgcgaaggtcattttccagaacaacgaccatggcttgtcttagcgatcgctcgaatgactgctagtgagtcgtacgc tcgacccagtcgctcgcaggagaacgcggcaactgccgagcttcggcttgccagtcgtgactcgtatgtgatcaggaatcatt ggcattggtagcattataattcggcttccgcgctgtttatgggcatggcaatgtctcatgcagtcgaccttagtcaaccaattctg ggtggccagctccgggcgaccgggctccgtgtcgccgggcaccacctcctgccatgagtaacagggccgccctctcctcccgac gttggcccactgaataccgtgtcttggggccctacatgatgggctgcctagtcgggcgggacgcgcaactgcccgcgcaatct gggacgtggtctgaatcctccaggcgggtttccccgagaaagaaagggtgccgatttcaaagcagagccatgtgccgggccc tgtggcctgtgttggcgcctatgtagtcacccccccctcacccaattgtcgccagtttgcgcaatccataaactcaaaactgcagct tctgagctgcgctgttcaagaacacctctggggtttgctcacccgcgaggtcgacgcccagca (SEQ ID NO:146)

[0758] Nucleotide sequence of Fatty acid desaturase 2 allele 2 promoter contained in plasmid pSZ3376 and 3383 (PmFAD2-2 promoter sequence):

atcacgatggtgcgcattcgtgcaaagtgaatatggggtcacgcggtggacgaacgcggaggggcatgaccgaatctag gctcgcattcctcagatcacttcatgccggcggtccggggtttgcgcgtcgcgcaaggctacgtctccctagccgctgcgcacca cgcgtgcgacgcggaggccatcttccggagcaacgaccatggattgtcttagcgatcgcacgaatgagtgctagtgagtcgt acgctcgacccagtcgctcgcaggagaaggcggcagctgccgagcttcggcttaccagtcgtgactcgtatgtgatcaggaat cattggcattggtagcattataattcggcttccgcgctgcgtatgggcatggcaatgtctcatgcagtcgatcttagtcaaccaa ttttgggtggccaggtccgggcgaccgggctccgtgtcgccgggcaccacctcctgccaggagtagcagggccgccctctcgtc ccgacgttggcccactgaataccgtggcttcgagccctacatgatgggctgcctagtcgggcgggacgcgcaactgcccgcgc gatctgggggctggtctgaatccttcaggcgggtgttacccgagaaagaaagggtgccgatttcaaagcagacccatgtgcc gggccctgtggcctgtgttggcgcctatgtagtcacccccccctcacccaattgtcgccagtttgcgcactccataaactcaaaac agcagcttctgagctgcgctgttcaagaacacctctggggtttgctcacccgcgaggtcgacgcccagca (SEQ ID NO:147)

[0759] To determine their impact on growth and fatty acid profiles, the above-described constructs were independently transformed into a Δfad2 Δfata1 strain S5204. Primary transformants were clonally purified and grown under standard lipid production conditions at pH5.0 or at pH7.0. The resulting profiles from a set of representative clones arising from transformations are shown in Tables 84-114.

[0760] Table 84. Fatty acid profile in some representative complemented (D2087) and parent S5204 lines transformed with pSZ3375 DNA containing PmFAD2-1p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.1	0
pH5; S5204	0.39	5.67	1.36	91.13	0	0
pH7; S5204; T665; D2087-22	0.38	4.43	1.78	83.93	**7.58**	0.81
pH7; S5204; T665; D2087-16	0.41	4.92	1.94	83.21	**7.55**	0.84
pH7; S5204; T665; D2087-17	0.40	4.82	1.78	83.51	**7.52**	0.79
pH7; S5204; T665; D2087-26	1.30	8.06	2.54	79.03	**7.30**	0.82
pH7; S5204; T665; D2087-29	1.13	7.88	2.45	79.48	**7.26**	0.79

[0761] Table 85. Fatty acid profile in some representative complemented (D) and parent S5204 lines transformed with pSZ3382 DNA containing PmFAD2-1p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.1	0
pH5; S5204	0.39	5.67	1.36	91.13	0	0
pH7; S5204; T672; D2094-5	0.49	5.76	2.95	83.39	**5.08**	0.84
pH7; S5204; T672; D2094-25	0.35	5.01	2.41	85.10	**5.09**	0.64
pH7; S5204; T672; D2094-13	0.33	5.07	2.30	84.89	**5.30**	0.69
pH7; S5204; T672; D2094-11	0.38	4.33	1.78	85.63	**5.31**	0.85
pH7; S5204; T672; D2094-8	0.35	5.29	2.32	84.59	**5.34**	0.66

[0762] Table 86. Fatty acid profile in some representative complemented (D2088) and parent S5204 lines transformed with pSZ3376 DNA containing PmFAD2-2p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.1	0
pH5; S5204	0.39	5.67	1.36	91.13	0	0
pH7; S5204; T665; D2088-16	1.11	8.18	2.92	78.13	**6.96**	0.87
pH7; S5204; T665; D2088-20	1.06	7.78	2.95	78.65	**6.95**	0.84
pH7; S5204; T665; D2088-29	0.91	7.13	2.87	79.63	**6.93**	0.78
pH7; S5204; T665; D2088-6	1.18	8.29	2.98	77.90	**6.91**	0.88
pH7; S5204; T665; D2088-18	1.10	7.98	3.09	78.42	**6.78**	0.81

[0763] Table 87. Fatty acid profile in some representative complemented (D) and parent S5204 lines transformed with pSZ3383 DNA containing PmFAD2-2p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68

pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.1	0
pH5; S5204	0.39	5.67	1.36	91.13	0	0
pH7; S5204; T673; D2095-47	0.30	5.43	2.45	85.10	**4.62**	0.68
pH7; S5204; T673; D2095-14	0.38	5.16	2.48	84.46	**5.41**	0.68
pH7; S5204; T673; D2095-16	0.43	4.60	2.54	84.82	**5.47**	0.58
pH7; S5204; T673; D2095-6	0.34	5.41	2.57	84.21	**5.49**	0.66
pH7; S5204; T673; D2095-39	0.42	5.30	2.49	83.97	**5.57**	0.68

[0764] Table 88. Fatty acid profile in representative complemented (D2089) and parent S5204 lines transformed with pSZ3377 DNA containing PmCPS1p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH7; S5204; T672; D2089-40	0.35	4.73	2.29	88.94	**1.79**	0.39
pH7; S5204; T672; D2089-2	0.51	4.85	2.96	87.55	**2.05**	0.41
pH7; S5204; T672; D2089-14	0.56	5.00	3.04	87.24	**2.07**	0.36
pH7; S5204; T672; D2089-7	0.38	5.04	2.39	88.02	**2.39**	0.44
pH7; S5204; T672; D2089-18	0.38	5.00	2.37	87.93	**2.42**	0.43

[0765] Table 89. Fatty acid profile in some representative complemented (D2096) and parent S5204 lines transformed with pSZ3384 DNA containing PmCPS1p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH7; S5204; T673; D2096-6	0.33	4.18	1.10	92.91	**0.00**	0.00
pH7; S5204; T673;	0.36	4.14	1.33	92.42	**0.34**	0.12

D2096-12						
pH7; S5204; T673; D2096-14	0.32	4.35	1.64	92.12	**0.35**	0.14
pH7; S5204; T673; D2096-8	0.50	6.44	0.95	89.81	**0.46**	0.32
pH7; S5204; T673; D2096-1	0.29	3.93	1.79	91.19	**1.34**	0.37

[0766] Table 90. Fatty acid profile in some representative complemented (D2090) and parent S5204 lines transformed with pSZ3378 DNA containing PmCPS2p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH7; S5204; T672; D2090-5	0.33	4.73	1.84	91.24	**0.00**	0.00
pH7; S5204; T672; D2090-29	0.42	4.99	2.01	91.06	**0.00**	0.00
pH7; S5204; T672; D2090-22	0.43	4.31	1.87	90.44	**0.78**	0.16
pH7; S5204; T672; D2090-1	0.32	3.77	2.43	89.72	**1.68**	0.35
pH7; S5204; T672; D2090-32	0.49	5.01	1.97	88.48	**1.84**	0.38

[0767] Table 91. Fatty acid profile in some representative complemented (D2097) and parent S5204 lines transformed with pSZ3385 DNA containing PmCPS2p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH5; S5204; T680; D2097-1	0.50	5.73	1.97	87.12	**2.61**	0.76
pH5; S5204; T680; D2097-2	0.75	8.20	2.46	85.73	**0.89**	0.53

[0768] Table 92. Fatty acid profile in some representative complemented (D2091) and parent S5204 lines transformed with pSZ3379 DNA containing PmDPS1p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH7; S5204; T672; D2091-4	1.42	4.39	2.32	89.87	**0.00**	0.00
pH7; S5204; T672; D2091-14	0.27	4.79	2.24	90.94	**0.00**	0.00
pH7; S5204; T672; D2091-15	0.30	5.26	2.20	90.73	**0.00**	0.00
pH7; S5204; T672; D2091-19	0.31	4.51	1.77	91.65	**0.00**	0.00
pH7; S5204; T672; D2091-46	0.31	5.36	2.24	90.67	**0.00**	0.00

[0769] Table 93. Fatty acid profile in some representative complemented (D2098) and parent S5204 lines transformed with pSZ3386 DNA containing PmDPS1p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH7; S5204; T680; D2098-39	0.34	4.89	1.56	92.08	**0.00**	0.00
pH7; S5204; T680; D2098-7	0.30	4.31	1.61	9234	**0.30**	0.00
pH5; S5204; T680; D2098-3	0.33	3.89	1.58	92.65	**0.36**	0.00
pH7; S5204; T680; D2098-25	0.32	4.18	1.64	92.34	**0.36**	0.11
pH7; S5204; T680; D2098-13	0.32	4.36	1.50	92.10	**0.37**	0.12

[0770] Table 94. Fatty acid profile in some representative complemented (D2092) and parent S5204 lines transformed with pSZ3380 DNA containing PmDPS2p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH7; S5204; T672; D2092-35	0.29	5.13	1.59	92.16	0.00	0.00
pH7; S5204; T672; D2092-29	0.37	4.66	1.75	91.71	0.19	0.05
pH7; S5204; T672; D2092-15	0.24	3.47	1.84	93.19	**0.43**	0.11
pH7; S5204; T672; D2092-21	0.25	3.50	1.82	93.16	**0.44**	0.09
pH7; S5204; T672; D2092-16	0.28	3.18	1.50	93.59	**0.52**	0.12

[0771] Table 95. Fatty acid profile in some representative complemented (D2099) and parent S5204 lines transformed with pSZ3387 DNA containing PmDPS2p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	accccgc	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH7; S5204; T680; D2099-20	0.31	4.02	1.46	93.07	0.00	0.00
pH7; S5204; T680; D2099-24	0.28	4.67	1.50	92.38	0.00	0.00
pH5; S5204; T680; D2099-27	0.40	4.07	1.22	93.26	0.00	0.00
pH7; S5204; T680; D2099-30	0.32	4.59	1.57	92.40	0.00	0.00
pH7; S5204; T680; D2099-35	0.30	4.56	1.54	92.49	0.00	0.00

[0772] Table 96. Fatty acid profile in some representative complemented (D2259) and parent S5204 lines transformed with pSZ3480 DNA containing PmIPP1p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH5; S5204; T711 D2259-43	0.36	5.27	2.19	89.32	**1.51**	0.51
pH5; S5204; T711 D2259-22	0.35	4.88	2.17	86.34	**4.41**	0.70
pH5; S5204; T711 D2259-28	0.35	4.82	2.18	86.32	**4.45**	0.69
pH5; S5204; T711 D2259-21	0.33	4.90	2.08	86.33	**4.49**	0.74
pH5; S5204; T711 D2259-36	0.50	5.97	2.14	84.67	**4.49**	0.74

[0773] Table 97. Fatty acid profile in some representative complemented (D2260) and parent S5204 lines transformed with pSZ3481 DNA containing PmIPP1p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH5; S5204; T711;D2260-32	0.36	4.96	2.10	89.46	**1.55**	0.49
pH5; S5204; T711;D2260-10	0.33	4.83	1.99	89.40	**1.63**	0.58
pH5; S5204; T711;D2260-2	0.34	4.83	2.16	89.39	**1.64**	0.49
pH5; S5204; T711;D2260-30	0.37	4.81	2.11	89.51	**1.69**	0.26
pH5; S5204; T711;D2260-41	0.33	4.91	2.17	89.73	**1.72**	0.16

[0774] Table 98. Fatty acid profile in some representative complemented (D2434) and parent S5204 lines transformed with pSZ3509 DNA containing PmAHC1p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH5; S5204; T768; D2434-32	0.33	4.45	1.55	81.55	**8.51**	1.38
pH5; S5204; T768; D2434-27	0.62	7.27	1.58	78.65	**9.44**	1.49
pH5; S5204; T768; D2434-4	0.38	5.81	1.79	79.63	**10.01**	1.18
pH5; S5204; T768; D2434-23	0.5	5.93	1.5	78.7	**10.25**	1.56
pH5; S5204; T768; D2434-43	0.51	6.08	1.6	78.79	**10.25**	1.36

[0775] Table 99. Fatty acid profile in some representative complemented (D2266) and parent S5204 lines transformed with pSZ3516 DNA containing PmAHC1p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH5; S5204; T718; D2266-46	0.32	5.41	1.94	91.26	**0.11**	0.00
pH5; S5204; T718; D2266-36	0.36	5.33	1.90	91.17	**0.17**	0.00
pH5; S5204; T718; D2266-35	0.37	4.96	2.13	90.82	**0.41**	0.00
pH5; S5204; T718; D2266-41	0.38	5.33	2.10	90.31	**0.44**	0.31
pH5; S5204; T718; D2266-5	0.36	5.15	2.23	90.55	**0.48**	0.31

[0776] Table 100. Fatty acid profile in some representative complemented (D2435) and parent S5204 lines transformed with pSZ3510 DNA containing PmAHC2p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0	0.00
pH5; S5204; T768; D2435-37	0.35	6.09	1.90	78.52	**11.01**	1.18
pH5; S5204; T768; D2435-3	0.43	5.90	1.97	78.74	**10.97**	1.20
pH5; S5204; T768; D2435-20	0.40	6.01	1.89	79.00	**10.97**	1.14
pH5; S5204; T768; D2435-13	0.39	6.11	1.89	78.26	**10.84**	1.24
pH5; S5204; T768; D2435-34	0.46	6.02	1.97	79.48	**10.46**	1.19

[0777] Table 101. Fatty acid profile in some representative complemented (D2263) and parent S5204 lines transformed with pSZ3513 DNA containing PmPPI1p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH5; S5204; T718; D2263-13	0.75	9.44	1.98	87.09	0.00	0.00
pH5; S5204; T718; D2263-14	0.58	7.72	1.64	89.26	0.00	0.00
pH5; S5204; T718; D2263-19	0.62	7.92	1.56	89.25	0.00	0.00
pH5; S5204; T718; D2263-26	0.42	7.39	1.70	89.28	0.00	0.00
pH5; S5204; T718; D2263-29	0.58	7.32	1.30	90.07	0.00	0.00

[0778] Table 102. Fatty acid profile in some representative complemented (D2440) and parent S5204 lines transformed with pSZ3689 DNA containing PmPPI1p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH5; S5204; T770; D2440-23	0.31	6.24	1.41	90.42	**0.17**	0.05
pH5; S5204; T770; D2440-32	0.23	4.69	1.41	91.72	**0.17**	0.00
pH5; S5204; T770; D2440-38	0.30	6.31	1.49	90.21	**0.17**	0.00
pH5; S5204; T770; D2440-7	0.30	6.33	1.38	90.29	**0.18**	0.05
pH5; S5204; T770; D2440-36	0.29	6.38	1.36	90.39	**0.18**	0.05
pH5; S5204; T770; D2440-8	0.34	5.63	1.15	91.15	**0.19**	0.05

[0779] Table 103. Fatty acid profile in some representative complemented (D2264) and parent S5204 lines transformed with pSZ3514 DNA containing PmPPI2p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH7; S6207; T718; D2264-1	0.49	6.15	1.61	90.82	0.00	0.00
pH7; S6207; T718; D2264-6	0.38	5.36	1.5	91.58	0.00	0.00
pH7; S6207; T718; D2264-29	0.45	6.09	1.46	91.10	0.00	0.00
pH7; S6207; T718; D2264-4	0.40	5.42	2.28	89.86	**0.90**	0.00
pH7; S6207; T718; D2264-7	0.40	5.37	2.02	90.18	**1.04**	0.00

[0780] Table 104. Fatty acid profile in some representative complemented (D2268) and parent S5204 lines transformed with pSZ3518 DNA containing PmPPI2p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH5; S5204; T720; D2268-1	0.39	6.43	1.78	90.49	0.00	0.00
pH5; S5204; T720; D2268-2	0.38	6.49	1.74	90.38	0.00	0.00
pH5; S5204; T720; D2268-3	0.38	6.56	1.74	90.27	0.00	0.00
pH5; S5204; T720; D2268-4	0.45	5.73	1.52	91.75	0.00	0.00
pH5; S5204; T720; D2268-5	0.38	6.58	1.81	90.79	0.00	0.00

[0781] Table 105. Fatty acid profile in some representative complemented (D2265) and parent S5204 lines transformed with pSZ3515 DNA containing PmGMPS1p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH5; S5204; T718; D2265-16	0.46	7.02	1.71	90.06	**0.00**	0.00
pH5; S5204; T718; D2265-43	0.00	7.90	1.90	89.27	**0.00**	0.00
pH5; S5204; T718; D2265-14	0.46	5.53	1.68	91.28	**0.35**	0.00
pH5; S5204; T718; D2265-4	0.39	6.17	1.75	90.44	**0.42**	0.00
pH5; S5204; T718; D2265-9	0.49	5.87	1.77	90.51	**0.45**	0.00

[0782] Table 106. Fatty acid profile in some representative complemented (D2269) and parent S5204 lines transformed with pSZ3519 DNA containing PmGMPS1p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH5; S5204; T720; D2269-1	0.38	6.73	1.68	90.24	0.00	0.00
pH5; S5204; T720; D2269-3	0.36	6.76	1.71	90.17	0.00	0.00
pH5; S5204; T720; D2269-4	0.42	6.57	1.71	90.32	0.00	0.00
pH5; S5204; T720; D2269-5	0.59	8.81	1.93	87.97	0.00	0.00
pH5; S5204; T720; D2269-6	0.50	7.29	1.73	89.29	0.00	0.00

[0783] Table 107. Fatty acid profile in some representative complemented (D2270) and parent S5204 lines transformed with pSZ3520 DNA containing PmGMPS2p driving PmFAD2-1

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH5; S5204; T720; D2270-1	0.37	6.80	1.74	90.18	0.00	0.00
pH5; S5204; T720; D2270-2	0.46	6.76	1.83	89.90	0.00	0.00
pH5; S5204; T720; D2270-3	0.41	6.69	1.70	90.22	0.00	0.00
pH5; S5204; T720; D2270-4	0.43	7.44	1.72	89.31	0.00	0.00
pH5; S5204; T720; D2270-5	0.44	6.98	1.78	89.79	0.00	0.00

[0784] Table 108. Fatty acid profile in some representative complemented (D2436) and parent S5204 lines transformed with pSZ3684 DNA containing PmCS1p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH5; S5204; T768; D2436-48	7.59	1.57	88.88	0.18	**0.00**	0.00
pH5; S5204; T768; D2436-1	6.37	1.50	85.00	3.97	**1.04**	0.00
pH5; S5204; T768; D2436-16	9.40	1.86	81.13	4.11	**1.21**	0.00
pH5; S5204; T768; D2436-8	6.07	1.77	84.78	4.26	**0.94**	0.00
pH5; S5204; T768; D2436-32	5.97	1.62	85.28	4.50	**0.98**	0.00

[0785] Table 109. Fatty acid profile in some representative complemented (D2438) and parent S5204 lines transformed with pSZ3686 DNA containing PmCS1p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH5; S5204; T770; D2438-7	0.50	5.96	1.69	89.97	**1.30**	0.00
pH5; S5204; T770; D2438-11	0.41	6.05	1.86	87.88	**2.46**	0.00
pH5; S5204; T770; D2438-9	0.41	5.75	1.93	88.35	**2.50**	0.00
pH5; S5204; T770; D2438-15	0.45	6.18	1.85	87.86	**2.59**	0.00
pH5; S5204; T770; D2438-37	0.40	5.92	1.97	87.80	**2.59**	0.00

[0786] Table 110. Fatty acid profile in some representative complemented (D2437) and parent S5204 lines transformed with pSZ3685 DNA containing PmCSCp driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH5; S5204; T768; D2437-15	0.00	4.83	1.98	90.43	**1.17**	0.53
pH5; S5204; T768; D2437-35	0.45	6.03	1.81	88.69	**1.88**	0.31
pH5; S5204; T768; D2437-17	0.39	4.96	2.00	88.58	**3.24**	0.00
pH5; S5204; T768; D2437-26	0.90	9.55	2.07	82.29	**3.37**	1.24
pH5; S5204; T768; D2437-8	0.53	10.76	1.55	79.62	**4.46**	1.12

[0787] Table 111. Fatty acid profile in some representative complemented (D2439) and parent S5204 lines transformed with pSZ3688 DNA containing PmGGHp driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH5; S5204; T770; D2439-11	0.31	6.79	1.47	89.97	**0.00**	0.00
pH5; S5204; T768; D2439-22	0.27	4.19	0.94	92.91	**0.08**	0.00
pH5; S5204; T770; D2439-12	0.39	6.02	1.26	90.9	**0.16**	0.00
pH5; S5204; T770; D2439-34	0.64	6.50	1.10	89.53	**0.20**	0.00
pH5; S5204; T770; D2439-32	0.33	5.25	1.45	89.98	**1.08**	0.51

[0788] Table 112. Fatty acid profile in some representative complemented (D2261) and parent S5204 lines transformed with pSZ3511 DNA containing PmAHI2p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH5; S5204; T711; D2261-35	0.45	5.06	2.02	89.35	**1.73**	0.63
pH5; S5204; T711; D2261-8	0.46	5.12	2.19	88.92	**2.16**	0.19
pH5; S5204; T711; D2261-43	0.37	5.12	5.15	88.62	**2.30**	0.45
pH5; S5204; T711; D2261-2	0.42	5.27	5.14	88.23	**2.39**	0.30
pH5; S5204; T711; D2261-24	0.41	5.14	5.23	88.44	**2.39**	0.45

[0789] Table 113. Fatty acid profile in some representative complemented (D2267) and parent S5204 lines transformed with pSZ3517 DNA containing PmAHI1p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH5; S5204; T720; D2267-3	0.34	4.87	2.11	90.00	**1.20**	0.39
pH5;S5204;T720;D 2267-20	0.37	5.00	2.14	89.50	**1.46**	0.49
pH5;S5204;T720;D 2267-36	0.34	4.90	2.08	89.75	**1.67**	0.36
pH5;S5204;T720;D 2267-15	0.37	4.94	2.14	89.77	**1.69**	0.00
pH5;S5204;T720;D 2267-2	0.35	4.85	2.12	89.71	**1.72**	0.32

[0790] Table 114. Fatty acid profile in some representative complemented (D2262) and parent S5204 lines transformed with pSZ3512 DNA containing PmCEP1p driving PmFAD2-1.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
pH7; S3150	1.71	29.58	3.13	56.53	6.43	0.68
pH5; S3150	1.56	27.70	2.98	59.49	5.95	0.53
pH7; S5204	0.30	5.59	1.63	90.88	0.10	0.00
pH5; S5204	0.39	5.67	1.36	91.13	0.00	0.00
pH5; S5204; T711; D2262-3	0.48	5.50	2.08	90.58	**0.35**	0.00
pH5; S5204; T720; D2262-33	0.39	5.20	2.17	89.90	**1.08**	0.37
pH55204; T720; D2262-24	0.34	5.08	1.93	89.69	**1.34**	0.37
pH5; S5204; T720; D2262-32	0.40	4.89	2.19	89.88	**1.45**	0.27
pH5; S5204; T720; D2262-34	0.39	4.95	2.75	89.30	**1.47**	0.27

[0791] Combined baseline expression of endogenous *PmFAD2-1* and *PmFAD2-2* in wild type *Prototheca* strains (like S3150, S1920 or S1331) manifests as 5-7% C18:2. S5204 overexpresses *PmKASII* which results in the elongation of C16:0 to C18:0. This increased pool of C18:0 is eventually desaturated by *PmSAD2* resulting in elevated C18:1 levels. Additionally disruption of the both copies of *PmFAD2* (viz. *PmFAD2-1* and *PmFAD2-2*) in S5204 prevents further desaturation of C18:1 into C18:2 and results in a unique high oleic oil (C18:1) with 0% linoleic acid (C18:2). However as mentioned above any strain with 0% C18:2 grows very poorly and requires exogenous addition of linoleic acid to sustain growth/productivity. Complementation of a strain like S5204 with inducible *PmAMT03p* driven *PmFAD2-1* can rescue the growth phenotype while preserving the terminal high C18:1 with 0% C18:2 levels. However data suggests that *PmAMT03* shuts off in the early stages of fermentation thus severely compromising the ability of any complemented strain to achieve its full growth and productivity potential. The goal of this work was to identify promoter elements that would allow the complemented strains to grow efficiently in early stages of fermentation (T0-T30 hrs; irrespective of excess batched N in the fermenters) and then effectively shut off once the cells enter active lipid production (when N in the media gets depleted) so that the complemented strains would still finish with very high C18:1 and 0% C18:2 levels. As a comparator we also complemented S5204 with *PmFAD2-1* being driven by either *PmFAd2-1p* or *PmFAD2-2p* promoter elements.

[0792] Complementation of S5204 with PmFAD2-1 driven by either *PmFAD2-1p* or *PmFAD2-2p* promoter elements results in complete restoration of the C18:2 levels using vectors either designed to amplify *PmFAD2-1* copy number (e.g. pSZ3375 or pSZ3376) or the ones where *PmFAD2-1* copy number is restricted to one (pSZ3382 or pSZ3383). Copy number of the *PmFAD2-1* in these strains seems to have very marginal effect on the terminal C18:2 levels.

[0793] On the other hand expression of *PmFAD2-1* driven by any of new promoter elements results in marked decrease in terminal C18:2 levels. The representative profiles from various strains expressing new promoters driving FAD2-1 are shown in Tables 84-114. This reduction in C18:2 levels is even more pronounced in strains where the copy number of *PmFAD2-1* is limited to one . Promoter elements like *PmDPS1* (D2091 & D2098), *PmDPS2* (D2092 & D2099), *PmPPI1* (D2263 & D2440), *PmPPI2* (D2264 & D2268), *PmGMPS1* (D2265 & D2269), *PmGMPS2* (D2270) resulted in strains with 0% or less than 0.5% terminal C18:2 levels in both single or multiple copy *PmFAD2-1* versions. The rest of the promoters resulted in terminal C18:2 levels that ranged between 1-5%. One unexpected result was the data from *PmAHC1p* and *PmAHC2p* driving *PmFAD2-1* in D2434 and D2435. Both these promoters resulted in very high levels of C18:2 (9-20%) in multiple copy FAD2-1 versions. The levels of terminal C18:2 in single copy version in D2266 was more in line with the transcriptomic data suggesting that *PmAHC* promoter activity and the corresponding *PmAHC* transcription is severely downregulated when cells are actively producing lipid in depleted nitrogen environment. A quick look at the transcriptome revealed that the initial transcription of *PmAHC* is very high (4000 – 5500 TPM) which then suddenly drops down to ~ 250 TPM. Thus it is conceivable that in strains with multiple copies on *PmFAD2-1* (D2434 and D2435), the massive amount of *PmFAD2-1* protein produced earlier in the fermentation lingers and results in high C18:2 levels. In single copy *PmFAD2-1* strains this is not the case and thus we do not see elevated C18:2 levels in D2266.

[0794] In complemented strains with 0% terminal C18:2 levels, the key question was whether they were complemented in the first place. In order to ascertain that, representative strains along with parent S5204 and previously *AMT03p* driven *PmFAD2-1* complemented S2532 (viz S4695) strains were grown in seed medium in 96 well blocks. The cultures were seeded at 0.1 OD units per ml and the OD750 was checked at different time points. Compared to S5204, which grew very poorly, only S4695 and newly complemented strains grew to any meaningful OD's at 20 and 44 hrs (Table 115) demonstrating that the promoters

identified above are active early on and switch off once cells enter the active lipid production phase.

[0795] Table 115. Growth characteristics of Δ*fad2* Δ*fata1* strain S5204, S4695 and representative complemented S5204 lines in seed medium sorted by OD750 at 44 hrs. Note that in 1 ml 96 well blocks after initial rapid division and growth, cells stop growing efficiently because of lack of nutrients, aeration etc.

Sample ID	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α	OD750 @20 hrs	OD750 @44hrs	OD750 @68hrs
S5204							0.162	7.914	10.93
S5204							0.224	6.854	9.256
S4695							1.456	29.032	32.766
pH7; S5204; T672; D2091-46	0.31	5.36	2.24	90.67	0.00	0.00	1.38	33.644	33.226
pH5; S5204; T720; D2268-1	0.39	6.43	1.78	90.49	0.00	0.00	0.75	32.782	31.624
S5204; T720; D2270-47	0.39	6.69	1.81	90.05	0.00	0.00	1.204	32.752	31.602
pH5; S5204; T720; D2270-39	0.39	6.87	1.81	89.94	0.00	0.00	1.012	32.552	33.138
pH7; S5204; T680; D2099-35	0.30	4.56	1.54	92.49	0.00	0.00	0.48	32.088	31.92
pH5; S5204; T720; D2270-44	0.51	6.85	1.74	90.06	0.00	0.00	1.468	31.802	30.61
pH5; S5204; T720; D2270-41	0.00	7.85	1.65	89.18	0.00	0.00	1.576	31.35	30.69
pH5; S5204; T720; D2270-17	0.46	6.78	1.71	90.24	0.00	0.00	1.79	30.732	24.768
pH7; S5204; T680;	0.32	4.59	1.57	92.40	0.00	0.00	0.59	30.166	34.64

D2099-30									
pH5; S5204; T720; D2268-40	0.42	6.66	1.86	90.02	0.00	0.00	0.764	29.62	29
pH5; S5204; T720; D2270-23	0.39	6.52	1.72	90.35	0.00	0.00	1.334	29.604	27.518
pH5; S5204; T720; D2270-42	0.61	6.59	1.53	90.28	0.00	0.00	2.042	28.986	32.184
pH7; S5204; T672; D2090-5	0.33	4.73	1.84	91.24	0.00	0.00	1.326	28.976	35.508
pH7; S5204; T672; D2091-15	0.30	5.26	2.20	90.73	0.00	0.00	0.826	28.824	32.848
pH7; S5204; T680; D2099-20	0.31	4.02	1.46	93.07	0.00	0.00	1.31	28.732	26.61
pH5; S5204; T720; D2269-19	0.42	6.51	1.61	90.43	0.00	0.00	1.278	28.65	31.362
pH5; S5204; T720; D2269-29	0.43	7.36	1.72	89.35	0.00	0.00	1.342	28.376	28.66
pH5; S5204; T720; D2270-19	0.39	6.81	1.75	90.05	0.00	0.00	2.142	28.376	25.934
pH5; S5204; T720; D2270-43	0.80	7.64	1.66	88.93	0.00	0.00	1.896	28.174	32.376
pH5; S5204; T720; D2270-46	0.45	6.75	1.72	90.02	0.00	0.00	1.644	28.122	30.464
pH5; S5204; T720; D2268-3	0.38	6.56	1.74	90.27	0.00	0.00	0.926	28.114	31.552
pH5;	0.00	5.68	1.84	91.53	0.00	0.00	1.414	28.106	30.644

S5204; T720; D2268-12									
pH5; S5204; T720; D2269-37	0.54	7.12	1.75	89.80	0.00	0.00	1.268	28.078	30.014
pH5; S5204; T720; D2270-31	0.46	6.94	1.74	89.71	0.00	0.00	1.224	28.064	29.344
pH5; S5204; T720; D2270-48	0.00	7.21	1.87	90.16	0.00	0.00	1.352	28	28.21
pH5; S5204; T720; D2269-8	0.33	6.67	1.64	90.34	0.00	0.00	0.96	27.912	27.564
pH5; S5204; T720; D2268-32	0.44	6.59	1.85	90.11	0.00	0.00	0.78	27.834	31.952
pH5; S5204; T720; D2269-47	0.42	6.83	1.82	89.85	0.00	0.00	1.17	27.76	29.648
pH7; S5204; T672; D2091-19	0.31	4.51	1.77	91.65	0.00	0.00	1.568	27.682	25.828
pH5; S5204; T720; D2270-38	0.39	6.65	1.83	90.11	0.00	0.00	1.74	27.606	31.104
pH5; S5204; T720; D2268-2	0.38	6.49	1.74	90.38	0.00	0.00	0.95	27.564	32.254
pH5; S5204; T720; D2269-35	0.38	7.04	1.68	89.82	0.00	0.00	1.19	27.482	29.186
pH5; S5204; T720; D2269-20	0.36	7.01	1.73	89.86	0.00	0.00	0.966	27.47	28.284
pH5; S5204; T720;	0.39	6.76	1.89	89.98	0.00	0.00	0.936	27.39	33.464

D2269-13									
pH7; S5204; T680; D2099-24	0.28	4.67	1.50	92.38	0.00	0.00	0.8	27.28	27.35
pH5; S5204; T720; D2268-11	0.38	6.56	1.85	90.56	0.00	0.00	1.136	27.254	32.508
pH5; S5204; T720; D2270-3	0.41	6.69	1.70	90.22	0.00	0.00	0.872	27.214	30.23
pH5; S5204; T720; D2269-33	0.39	6.36	1.67	90.59	0.00	0.00	0.956	27.194	30.568
pH5; S5204; T720; D2268-10	0.45	6.93	1.70	90.16	0.00	0.00	0.612	27.126	31.616
pH5; S5204; T720; D2269-43	0.36	6.55	1.84	90.25	0.00	0.00	0.998	27.086	29.618
pH5; S5204; T720; D2270-1	0.37	6.80	1.74	90.18	0.00	0.00	2.428	27.004	31.044
pH5; S5204; T720; D2268-4	0.45	5.73	1.52	91.75	0.00	0.00	0.736	26.948	28.796
pH5; S5204; T720; D2270-9	0.38	6.88	1.74	90.22	0.00	0.00	2.68	26.944	29.92
pH5; S5204; T720; D2269-26	0.41	6.85	1.68	90.03	0.00	0.00	0.896	26.794	31.31
pH5; S5204; T720; D2270-24	0.39	6.51	1.78	90.33	0.00	0.00	1.51	26.682	27.486
pH5; S5204; T720; D2269-18	0.41	7.04	1.71	89.83	0.00	0.00	1.024	26.58	29.794
pH5;	0.38	6.81	1.72	90.06	0.00	0.00	1.214	26.48	29.478

S5204; T720; D2269-32									
pH5; S5204; T720; D2268-31	0.33	6.68	1.76	90.20	0.00	0.00	0.808	26.432	31.294
pH5; S5204; T720; D2269-7	0.29	5.33	1.69	91.59	0.00	0.00	1.1	26.41	28.754
pH5; S5204; T720; D2268-6	0.39	6.62	1.70	90.28	0.00	0.00	0.626	26.372	30.822
pH7; S5204; T680; D2099-27	0.40	4.07	1.22	93.26	0.00	0.00	0.936	26.116	29.75
pH5; S5204; T720; D2269-39	0.48	6.88	1.82	89.67	0.00	0.00	2.218	26.106	30.8
pH5; S5204; T720; D2269-12	0.35	6.39	1.80	90.47	0.00	0.00	1.18	26.032	28.19
pH5; S5204; T720; D2269-42	0.39	6.99	1.67	89.91	0.00	0.00	2.132	25.924	27.854
pH5; S5204; T720; D2268-8	0.56	6.77	1.49	90.20	0.00	0.00	0.96	25.702	29.788
pH5; S5204; T720; D2270-37	0.44	7.33	1.71	89.69	0.00	0.00	0.916	25.612	34.034
pH5; S5204; T720; D2270-40	0.00	9.30	1.62	88.12	0.00	0.00	2.072	25.552	29.474
pH5; S5204; T720; D2270-14	0.43	7.40	1.71	89.73	0.00	0.00	1.916	25.526	27.908
pH5; S5204; T720;	0.40	6.69	1.69	89.99	0.00	0.00	0.826	25.396	29

D2269-21									
pH5; S5204; T718; D2265-16	0.46	7.02	1.71	90.06	0.00	0.00	0.9	25.332	32.018
pH5; S5204; T720; D2270-15	0.40	6.90	1.68	90.32	0.00	0.00	1.594	25.32	26.794
pH5; S5204; T720; D2269-40	0.00	7.00	1.66	90.15	0.00	0.00	1.804	25.286	29.468
pH5; S5204; T720; D2268-5	0.38	6.58	1.81	90.79	0.00	0.00	0.678	25.156	33.066
pH5; S5204; T720; D2270-18	0.45	6.20	1.45	91.09	0.00	0.00	2.646	25.126	27.536
pH5; S5204; T720; D2269-25	0.44	7.02	1.69	89.91	0.00	0.00	0.868	25.018	32.104
pH5; S5204; T720; D2269-30	0.45	6.77	1.78	90.00	0.00	0.00	0.718	24.978	29.868
pH5; S5204; T720; D2270-25	0.31	6.82	1.68	90.09	0.00	0.00	2.32	24.814	36.024
pH5; S5204; T720; D2270-21	0.52	7.23	1.70	89.99	0.00	0.00	1.92	24.58	25.398
pH5; S5204; T720; D2269-38	0.00	7.45	1.50	90.19	0.00	0.00	1.494	24.578	30.178
pH5; S5204; T720; D2268-9	0.48	5.94	1.51	90.83	0.00	0.00	0.73	24.344	30.83
pH5; S5204; T720; D2268-37	0.44	6.35	1.84	90.31	0.00	0.00	0.548	24.306	32.848
pH5;	0.41	7.12	1.66	89.73	0.00	0.00	0.808	24.288	31.27

S5204; T720; D2269-28									
pH5; S5204; T720; D2270-5	0.44	6.98	1.78	89.79	0.00	0.00	2.328	24.14	30.186
pH5; S5204; T720; D2269-23	0.44	6.99	1.71	89.43	0.00	0.00	0.876	24.076	29.494
pH5; S5204; T720; D2269-9	0.38	6.84	1.71	90.32	0.00	0.00	0.806	24	26.844
pH5; S5204; T720; D2269-24	0.55	7.31	1.71	89.68	0.00	0.00	1.09	23.97	29.642
pH5; S5204; T720; D2270-35	0.36	6.58	1.72	90.38	0.00	0.00	1.554	23.71	28.868
pH5; S5204; T720; D2269-15	0.00	5.69	1.36	91.86	0.00	0.00	1.246	23.584	28.196
pH5; S5204; T720; D2270-28	0.39	7.15	1.82	89.92	0.00	0.00	1.648	23.486	30.858
pH7; S5204; T680; D2098-39	0.34	4.89	1.56	92.08	0.00	0.00	1.08	23.46	31.888
pH5; S5204; T720; D2269-27	0.33	6.87	1.68	89.98	0.00	0.00	1.3	23.262	33.112
pH5; S5204; T718; D2265-43	0.00	7.90	1.90	89.27	0.00	0.00	0.832	23.23	30.052
pH5; S5204; T720; D2270-30	0.41	7.00	1.68	89.83	0.00	0.00	2.144	23.1	30.97
pH5; S5204; T720;	0.00	7.05	1.94	90.20	0.00	0.00	0.716	23.088	29.922

D2268-25									
pH5; S5204; T720; D2270-29	0.34	6.81	1.74	90.11	0.00	0.00	2.542	22.98	31.402
pH5; S5204; T720; D2269-45	0.00	7.64	1.56	89.90	0.00	0.00	0.806	22.892	29.022
pH5; S5204; T720; D2270-27	0.72	9.32	1.99	87.35	0.00	0.00	2.352	22.81	29.996
pH5; S5204; T720; D2269-11	0.65	6.41	1.69	90.22	0.00	0.00	1.056	22.768	26.056
pH5; S5204; T720; D2270-36	0.00	5.45	1.59	91.60	0.00	0.00	1.886	22.738	24.69
pH5; S5204; T720; D2269-22	0.39	7.12	1.72	89.63	0.00	0.00	1.08	22.634	27.532
pH5; S5204; T718; D2263-30	0.54	7.58	1.57	89.47	0.00	0.00	0.71	22.564	29.996
pH7; S5204; T672; D2091-47	0.32	5.22	2.23	90.45	0.00	0.00	0.938	22.486	32.046
pH5; S5204; T720; D2269-1	0.38	6.73	1.68	90.24	0.00	0.00	1.154	22.48	29.994
pH7; S5204; T673; D2096-6	0.33	4.18	1.10	92.91	0.00	0.00	0.91	22.446	28.714
pH5; S5204; T720; D2270-33	0.40	6.95	1.76	89.89	0.00	0.00	2.28	22.408	29.656
pH5; S5204; T718; D2263-14	0.58	7.72	1.64	89.26	0.00	0.00	0.306	22.35	32.294
pH5;	0.36	6.75	1.77	90.10	0.00	0.00	2.398	22.3	28.958

S5204; T720; D2270-34									
pH7; S5204; T672; D2090-29	0.42	4.99	2.01	91.06	0.00	0.00	1.16	22.112	30.376
pH5; S5204; T720; D2269-14	0.00	7.86	1.80	89.57	0.00	0.00	0.574	21.802	31.558
pH5; S5204; T718; D2263-29	0.58	7.32	1.30	90.07	0.00	0.00	0.418	21.746	30.426
pH5; S5204; T718; D2263-19	0.62	7.92	1.56	89.25	0.00	0.00	0.574	21.692	29.514
pH5; S5204; T720; D2269-10	0.39	6.82	1.70	90.05	0.00	0.00	1.104	21.622	25.264
pH5; S5204; T720; D2269-4	0.42	6.57	1.71	90.32	0.00	0.00	1.082	21.466	29.698
pH5; S5204; T720; D2270-4	0.43	7.44	1.72	89.31	0.00	0.00	1.758	21.446	32.656
pH5; S5204; T720; D2269-34	0.00	6.69	1.78	90.64	0.00	0.00	0.946	21.438	28.538
pH5; S5204; T720; D2270-16	0.39	7.08	1.71	89.70	0.00	0.00	1.592	21.422	27.72
pH5; S5204; T718; D2263-26	0.42	7.39	1.70	89.28	0.00	0.00	0.514	21.328	29.746
pH5; S5204; T720; D2269-3	0.36	6.76	1.71	90.17	0.00	0.00	0.668	21.242	29.74
pH5; S5204; T720;	0.35	6.77	1.67	90.15	0.00	0.00	1.194	21.026	25.084

D2270-22									
pH5; S5204; T720; D2270-26	0.41	6.81	1.82	89.66	0.00	0.00	1.606	20.948	32.142
pH5; S5204; T720; D2270-10	0.46	6.98	1.80	90.03	0.00	0.00	0.792	20.728	28.264
pH5; S5204; T720; D2269-16	0.51	6.17	1.50	90.64	0.00	0.00	0.922	20.502	30.132
pH5; S5204; T720; D2270-8	0.50	6.95	1.42	90.34	0.00	0.00	2.252	20.486	28.34
pH5; S5204; T720; D2270-2	0.46	6.76	1.83	89.90	0.00	0.00	0.97	20.366	31.758
pH5; S5204; T720; D2269-36	0.00	7.43	1.66	89.88	0.00	0.00	0.754	20.006	29.648
pH5; S5204; T720; D2269-31	0.72	9.29	1.86	86.92	0.00	0.00	2.062	19.002	27.61
pH5; S5204; T720; D2269-44	0.00	9.45	1.58	88.16	0.00	0.00	1.378	18.576	22.52
pH7; S5204; T672; D2091-14	0.27	4.79	2.24	90.94	0.00	0.00	0.93	18.1	30.434
pH5; S5204; T720; D2270-32	0.40	7.14	1.74	89.63	0.00	0.00	1.668	17.966	27.06
pH5; S5204; T720; D2270-11	0.82	9.24	1.93	87.35	0.00	0.00	1.178	15.998	28.196
pH5; S5204; T720; D2269-48	0.72	9.05	2.14	88.08	0.00	0.00	1.172	14.694	25.384
pH5;	0.66	9.08	2.12	87.12	0.00	0.00	0.84	14.488	25.886

S5204; T720; D2269-17									
pH5; S5204; T720; D2270-20	0.62	8.35	1.97	88.43	0.00	0.00	1.37	14.168	23.794
pH5; S5204; T718; D2263-13	0.75	9.44	1.98	87.09	0.00	0.00	0.64	13.854	29.466
pH5; S5204; T720; D2269-46	0.43	6.87	1.71	89.81	0.00	0.00	0.646	10.452	31.464
pH5; S5204; T720; D2269-5	0.59	8.81	1.93	87.97	0.00	0.00	0.654	9.37	25.786
pH7; S5204; T672; D2091-4	1.42	4.39	2.32	89.87	0.00	0.00	0.686	8.182	16.454
pH5; S5204; T720; D2269-6	0.50	7.29	1.73	89.29	0.00	0.00	0.79	7.978	21.346
pH5; S5204; T720; D2270-45	0.00	9.16	1.65	88.19	0.00	0.00	0.464	3.448	16.796
Blank							0	0	0

[0796] It is comtemplated that these promoters, or variants thereof , discovered here can be used to regulate a fatty acid synthesis gene (e.g., any of the FATA, FATB, SAD, FAD2, KASI/IV, KASII, LPAAT or KCS genes disclosed herein) or other gene or gene-suppression element expressed in a cell including a microalgal cell. Variants can have for example 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99% or greater identity to the sequences disclosed here.

EXAMPLE 64: FRACTIONATION OF A HIGH SOS OIL TO INCREASE SOS CONCENTRATION AND REDUCE TRISATURATES.

[0797] Microalgal oil was fractionated using dry fractionation and solvent fractionation techniques. The starting material was an oil that was high in SOS triglycerides. The oil was

produced from *Prototheca moriformis* strain S7566, in which a the endogenous KASII gene was inserted into (and thereby knocking out) a SADII locus; additionally, the C18-preferring FATA1genefrom Garcinia mangostana was inserted and a FADII hairpin RNA was produced; as described above. After cultivation and extracted, the oil was refined, bleached and deodorized. The fatty acid profile of the oil is given in Table 115. The SOS TAG area% was about 62%. During the RBD processing, the total trisaturates (i.e. triglycerides with three fully saturated acyl chains such as SSS, PSS, PPS, PPP, etc.) in the oil decreased from 5.1% to 1.2%.

[0798] Table 116. Fatty acid profile of clarified oil from strain 7566.

Strain		**S7566**
Fatty Acid Area %	**C14:0**	0.49
	C16:0	3.12
	C18:0	54.77
	C18:1	35.88
	C18:2	2.16
	C18:3 α	0.23
	C20:0	1.64
	C22:0	0.19
	C24:0	0.11
	sum C18	93.05
	saturates	60.69
	unsaturates	38.55

[0799] The oil was fractionated using solvent (acetone or hexane) and dry fractionation. Acetone fractionation (1:1 oil-solvent, w/w; crystallization at 5 °C) gave excellent recovery of an SOS-enriched stearin fraction, with relatively little SOS in the olein fraction. SOS was at 77%, with total trisaturates <1% for the stearin fraction.

[0800] Hexane fractionation(1:1 oil-solvent, w/w; crystallization at 5 °C) gave a higher level (85%) of SOS, but also gave higher trisaturates (1.6%). Thus, using a single-step solvent fractionation, oils with over 75% SOS and less than 2% trisaturates were obtainable.

[0801] Dry fractionation was also successful in enriching SOS and decreasing trisaturates. The general approach was to remove trisaturates by crystallization at a higher temperature, then removing OOS at a lower temperature. The reverse order was also tried and yielded a superior result. It was also found that rinsing the SOS-enriched ("stearin") fraction with acetone helped in removing the olein fraction.

[0802] In one test, the oil was crystallized at 24°C and the stearin fraction was rinsed with acetone. Analysis showed that OOS levels decreased. The stearin fraction was heated and

allowed to cool and crystallize overnight at 29°C. The resulting liquid oil was separated from the crystallized trisaturates to afford a product with 84% SOS and < 0.5% total trisaturates. Lipase-based sn-2 profile analysis of revealed that over 96% of that position was occupied by unsaturated fatty acids (93.3% oleate, 3.2% linolate, and 0.2% linolenate), while only 2.2% stearate was located there.

[0803] The DSC heating curve thermogram and DSC-derived solid fat content curve of the two step dry fractionated oil was compared to those of kokum butter. The two oils have essentially identical maximum heat-flow temperatures and the DSC-derived SFC curves are super-imposable. The oil could be expected to behave functionally similarly to kokum butter.

EXAMPLE 65: PRODUCTION OF MICROBIAL OIL WITH OVER 60% SOS CONTENT.

[0804] Here, we demonstrate in the microalga Prototheca moriformis, that by disrupting an allele of the SAD2 gene, overexpressing KASII, knocking out endogenous FATA-1, overexpressing a more stearate-specific FATA (GarmFATA1 from Garcinia mangostana) relative to the endogenous FATA and activating FAD2 RNAi, we generate strains capable of accumulating over 60% SOS, useful as a structuring fat.

[0805] To reduce SAD activity, Strain S3150 was transformed with DNA constructs designed to recombine in the SAD2-1 and SAD2-2 alleles and express the selectable marker, Arabidopsis thialiana THIC (*AtTHIC*, codon-optimized for expression in *P. moriformis*). THIC encodes 4-amino-5-hydroxymethyl-2-methylpyrimidine synthase, thereby allowing growth in the absence of added thiamine. Transformants were selected in the absence of exogenous thiamine.

[0806] To make the *SAD2-1* ablation construct pSZ2601, the *Arabidopsis thaliana THIC* gene (*AtTHIC*, codon-optimized for expression in *P. moriformis*), was utilized as a selectable marker for transformation. The sequence of the transforming DNA is shown in SEQ ID NO:148. Relevant restriction sites are indicated in lowercase, bold, and are from 5'-3' BspQI, PmeI, KpnI, XbaI, MfeI, SacI, BspQI and PmeI. Underlined sequences at the 5' and 3' flanks of the construct represent genomic DNA from *P. moriformis* that enable targeted integration of the transforming DNA via homologous recombination at the *SAD2-1* locus. Proceeding in the 5' to 3' direction, the *Chlorella protothecoides ACT* promoter (*CpACT*) driving the expression of the *AtTHIC* gene (encoding 4-amino-5-hydroxymethyl-2-methylpyrimidine synthase activity, thereby permitting the strain to grow in the absence of exogenous thiamine) is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for *AtTHIC* are indicated by uppercase italics, while the coding region is indicated with

lowercase italics. The 3' UTR of the *Chlorella vulgaris nitrate reductase* (*CvNR*) gene is indicated by small capitals.

[0807] Nucleotide sequence of the transforming DNA from pSZ2601:

gaagagcgcccaat**gtttaaac**GCCGGTCACCACCCGCATGCTCGTACTACAGCGCACGCACCGCTTCGTG
ATCCACCGGGTGAACGTAGTCCTCGACGGAAACATCTGGTTCGGGCCTCCTGCTTGCACTCCCGCCC
ATGCCGACAACCTTTCTGCTGTTACCACGACCCACAATGCAACGCGACACGACCGTGTGGGACTGAT
CGGTTCACTGCACCTGCATGCAATTGTCACAAGCGCTTACTCCAATTGTATTCGTTTGTTTTCTGGGA
GCAGTTGCTCGACCGCCCGCGTCCCGCAGGCAGCGATGACGTGTGCGTGGCCTGGGTGTTTCGTCG
AAAGGCCAGCAACCCTAAATCGCAGGCGATCCGGAGATTGGGATCTGATCCGAGTTTGGACCAGAT
CCGCCCCGATGCGGCACGGGAACTGCATCGACTCGGCGCGGAACCCAGCTTTCGTAAATGCCAGAT
TGGTGTCCGATACCTGGATTTGCCATCAGCGAAACAAGACTTCAGCAGCGAGCGTATTTGGCGGGC
GTGCTACCAGGGTTGCATACATTGCCCATTTCTGTCTGGACCGCTTTACTGGCGCAGAGGGTGAGTT
GATGGGGTTGGCAGGCATCGAAACGCGCGTGCATGGTGTGCGTGTCTGTTTTCGGCTGCACGAATT
CAATAGTCGGATGGGCGACGGTAGAATTGGGTGTGGCGCTCGCGTGCATGCCTCGCCCCGTCGGGT
GTCATGACCGGGACTGGAATCCCCCCTCGCGACCATCTTGCTAACGCTCCCGACTCTCCCGACCGCG
CGCAGGATAGACTCTTGTTCAACCAATCGACA**ggtacc**agtttaggtccagcgtccgtgggggggggacgggctggga
gcttgggccgggaagggcaagacgatgcagtccctctggggagtcacagccgactgtgtgtgttgcactgtgcggcccgcagcact
cacacgcaaaatgcctggccgacaggcaggccctgtccagtgcaacatccacggtccctctcatcaggctcaccttgctcattgaca
taacggaatgcgtaccgctctttcagatctgtccatccagagaggggagcaggctccccaccgacgctgtcaaacttgcttcctgcc
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tgtggaacacagtgagtgcagtatgcagagcatgtatgctaggggtcagcgcaggaaggggccctttcccagtctcccatgccact
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gcgtgcaccgcaaaggccagccgatcgtgccgattcctgggtggaggatatgagtcagccaacttggggctcagagtgcacactgg
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cctgtttcgatcactgtttgggtcctttccttttcgtctcggatgcgcgtctcgaaacaggctgcgtcgggctttcggatccctttgctc
cctccgtcaccatcctgcgcgcgggcaagttgcttgaccctgggctgataccagggttggagggtattaccgcgtcaggccattccc
agcccggattcaattcaaagtctgggccaccaccctccgccgctctgtctgatcactccacattcgtgcatacactacgttcaagtcct
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gcgccacgctgacgttcgacccccccacgaccaactccgagcgcgccaagcagcgcaagcacaccatcgacccctcctccccc
gacttccagcccatcccctccttcgaggagtgcttccccaagtccacgaaggagcacaaggaggtggtgcacgaggagtccg
gccacgtcctgaaggtgcccttccgccgcgtgcacctgtccggcggcgagcccgccttcgacaactacgacacgtccggccccc
agaacgtcaacgcccacatcggcctggcgaagctgcgcaaggagtggatcgaccgccgcgagaagctgggcacgccccgct
acacgcagatgtactacgcgaagcagggcatcatcacggaggagatgctgtactgcgcgacgcgcgagaagctggaccccg
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ggccgcaagttcctggtgaaggtgaacgcgaacatcggcaactccgccgtggcctcctccatcgaggaggaggtctacaagg
tgcagtgggccaccatgtggggcgccgacaccatcatggacctgtccacgggccgccacatccacgagacgcgcgagtggat
cctgcgcaactccgcggtccccgtgggcaccgtccccatctaccaggcgctggagaaggtggacggcatcgcggagaacctg
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tgcgctacatccccctgaccgccaagcgcctgacgggcatcgtgtcccgcggcggctccatccacgcgaagtggtgcctggcct
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gacggcctgcgccccggctccatctacgacgccaacgacacggcccagttcgccgagctgctgacccagggcgagctgacgc
gccgcgcgtgggagaaggacgtgcaggtgatgaacgagggccccggccacgtgcccatgcacaagatccccgagaacatg
cagaagcagctggagtggtgcaacgaggcgcccttctacaccctgggccccctgacgaccgacatcgcgcccggctacgacc
acatcacctccgccatcggcgcggccaacatcggcgccctgggcaccgccctgctgtgctacgtgacgcccaaggagcacctg
ggcctgcccaaccgcgacgacgtgaaggcgggcgtcatcgcctacaagatcgccgcccacgcggccgacctggccaagcag
cacccccacgcccaggcgtgggacgacgcgctgtccaaggcgcgcttcgagttccgctggatggaccagttcgcgctgtccctg
gaccccatgacggcgatgtccttccacgacgagacgctgcccgcggacggcgcgaaggtcgcccacttctgctccatgtgcgg
ccccaagttctgctccatgaagatcacggaggacatccgcaagtacgccgaggagaacggctacggctccgccgaggaggc
catccgccagggcatggacgccatgtccgaggagttcaacatcgccaagaagacgatctccggcgagcagcacggcgaggt
*cggcggcgagatctacctgcccgagtcctacgtcaaggccgcgcagaagTGA***caattg**GCAGCAGCAGCTCGGATAGTATC
GACACACTCTGGACGCTGGTCGTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCCGCTTT
TATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGCGAGTTGCTAGCTGCTTGTGCTATTTGCGAATACCA
CCCCCAGCATCCCCTTCCCTCGTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCTATCCCTCAGCGCT
GCTCCTGCTCCTGCTCACTGCCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCTCCTGGTACTGCAACCTGTAAACC
AGCACTGCAATGCTGATGCACGGGAAGTAGTGGGATGGGAACACAAATGGA**GGATCGTA**gagctcTAGGGAGCGA
CGAGTGTGCGTGCGGGGCTGGCGGGAGTGGGACGCCCTCCTCGCTCCTCTCTGTTCTGAACGGAAC
AATCGGCCACCCCGCGCTACGCGCCACGCATCGAGCAACGAAGAAAACCCCCCGATGATAGGTTGC
GGTGGCTGCCGGGATATAGATCCGGCCGCACATCAAAGGGCCCCTCCGCCAGAGAAGAAGCTCCTT
TCCCAGCAGACTCCTTCTGCTGCCAAAACACTTCTCTGTCCACAGCAACACCAAAGGATGAACAGATC
AACTTGCGTCTCCGCGTAGCTTCCTCGGCTAGCGTGCTTGCAACAGGTCCCTGCACTATTATCTTCCT

GCTTTCCTCTGAATTATGCGGCAGGCGAGCGCTCGCTCTGGCGAGCGCTCCTTCGCGCCGCCCTCGC
TGATCGAGTGTACAGTCAATGAATGGTCCTGGGCGAAGAACGAGGGAATTTGTGGGTAAAACAAG
CATCGTCTCTCAGGCCCCGGCGCAGTGGCCGTTAAAGTCCAAGACCGTGACCAGGCAGCGCAGCGC
GTCCGTGTGCGGGCCCTGCCTGGCGGCTCGGCGTGCCAGGCTCGAGAGCAGCTCCCTCAGGTCGCC
TTGGACGGCCTCTGCGAGGCCGGTGAGGGCCTGCAGGAGCGCCTCGAGCGTGGCAGTGGCGGTCG
TATCCGGGTCGCCGGTCACCGCCTGCGACTCGCCATCC**gaagagcgtttaaac** (SEQ ID NO:148)

[0808] The sequence of the transforming DNA from the *SAD2-1* disruption construct, pSZ2607, is shown below in SEQ ID NO:149. Relevant restriction sites are indicated in lowercase, bold, and are from 5'-3' PmeI, KpnI, XbaI, MfeI, SacI, BspQI and PmeI. Underlined sequences at the 5' and 3' flanks of the construct represent genomic DNA from *P. moriformis* that enable targeted integration of the transforming DNA via homologous recombination at the *SAD2-1* locus. Proceeding in the 5' to 3' direction, the *Chlorella protothecoides ACT* promoter (*CpACT*) driving the expression of the *AtTHIC* gene (encoding 4-amino-5-hydroxymethyl-2-methylpyrimidine synthase activity, thereby permitting the strain to grow in the absence of exogenous thiamine) is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for *AtTHIC* are indicated by uppercase italics, while the coding region is indicated with lowercase italics. The 3' UTR of the *Chlorella vulgaris nitrate reductase* (*CvNR*) gene is indicated by small capitals.

[0809] Nucleotide sequence of the transforming DNA from pSZ2607:

gtttaaacGCCGGTCACCACCCGCATGCTCGTACTACAGCGCACGCACCGCTTCGTGATCCACCGGGTG
AACGTAGTCCTCGACGGAAACATCTGGTTCGGGCCTCCTGCTTGCACTCCCGCCCATGCCGACAACC
TTTCTGCTGTTACCACGACCCACAATGCAACGCGACACGACCGTGTGGGACTGATCGGTTCACTGCA
CCTGCATGCAATTGTCACAAGCGCTTACTCCAATTGTATTCGTTTGTTTTCTGGGAGCAGTTGCTCGA
CCGCCCGCGTCCCGCAGGCAGCGATGACGTGTGCGTGGCCTGGGTGTTTCGTCGAAAGGCCAGCAA
CCCTAAATCGCAGGCGATCCGGAGATTGGGATCTGATCCGAGTTTGGACCAGATCCGCCCCGATGC
GGCACGGGAACTGCATCGACTCGGCGCGGAACCCAGCTTTCGTAAATGCCAGATTGGTGTCCGATA
CCTGGATTTGCCATCAGCGAAACAAGACTTCAGCAGCGAGCGTATTTGGCGGGCGTGCTACCAGGG
TTGCATACATTGCCCATTTCTGTCTGGACCGCTTTACTGGCGCAGAGGGTGAGTTGATGGGGTTGGC
AGGCATCGAAACGCGCGTGCATGGTGTGCGTGTCTGTTTTCGGCTGCACGAATTCAATAGTCGGAT
GGGCGACGGTAGAATTGGGTGTGGCGCTCGCGTGCATGCCTCGCCCCGTCGGGTGTCATGACCGG
GACTGGAATCCCCCCTCGCGACCATCTTGCTAACGCTCCCGACTCTCCCGACCGCGCGCAGGATAGA
CTCTTGTTCAACCAATCGACA**ggtacc**agtttaggtccagcgtccgtgggggggacgggctgggagcttgggccgggaa

gggcaagacgatgcagtccctctggggagtcacagccgactgtgtgtgttgcactgtgcggcccgcagcactcacacgcaaaatgc

ctggccgacaggcaggccctgtccagtgcaacatccacggtccctctcatcaggctcaccttgctcattgacataacggaatgcgta

ccgctctttcagatctgtccatccagagaggggagcaggctccccaccgacgctgtcaaacttgcttcctgcccaaccgaaaacatt

attgtttgaggggggggggggggggcagattgcatggcgggatatctcgtgaggaacatcactgggacactgtggaacacagtg

agtgcagtatgcagagcatgtatgctaggggtcagcgcaggaaggggccctttcccagtctcccatgccactgcaccgtatccacg

actcaccaggaccagcttcttgatcggcttccgctcccgtggacaccagtgtgtagcctctggactccaggtatgcgtgcaccgcaa

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aacaacatctacaccgtgtcctccatgctgacacaccacagcttcgctccacctgaatgtgggcgcatgggcccgaatcacagcca

atgtcgctgctgccataatgtgatccagaccctctccgcccagatgccgagcggatcgtgggcgctgaatagattcctgtttcgatca

ctgtttgggtcctttccttttcgtctcggatgcgcgtctcgaaacaggctgcgtcgggctttcggatccctttgctccctccgtcaccat

cctgcgcgcgggcaagttgcttgaccctgggctgataccagggttggagggtattaccgcgtcaggccattcccagcccggattcaa

ttcaaagtctgggccaccaccctccgccgctctgtctgatcactccacattcgtgcatacactacgttcaagtcctgatccaggcgtgt

ctcgggacaaggtgtgcttgagtttgaatctcaaggacccactccagcacagctgctggttgaccccgccctcgcaa**tctaga***ATG*

gccgcgtccgtccactgcaccctgatgtccgtggtctgcaacaacaagaaccactccgcccgccccaagctgcccaactcctccc

tgctgcccggcttcgacgtggtggtccaggccgcggccacccgcttcaagaaggagacgacgaccacccgcgccacgctgac

gttcgacccccccacgaccaactccgagcgcgccaagcagcgcaagcacaccatcgacccctcctccccgacttccagcccat

cccctccttcgaggagtgcttccccaagtccacgaaggagcacaaggaggtggtgcacgaggagtccggccacgtcctgaag

gtgcccttccgccgcgtgcacctgtccggcggcgagcccgccttcgacaactacgacacgtccggcccccagaacgtcaacgcc

cacatcggcctggcgaagctgcgcaaggagtggatcgaccgccgcgagaagctgggcacgccccgctacacgcagatgtac

tacgcgaagcagggcatcatcacggaggagatgctgtactgcgcgacgcgcgagaagctggaccccgagttcgtccgctccg

aggtcgcgcggggccgcgccatcatcccctccaacaagaagcacctggagctggagcccatgatcgtgggccgcaagttcct

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ccatcggcgcggccaacatcggcgccctgggcaccgccctgctgtgctacgtgacgcccaaggagcacctgggcctgcccaac

cgcgacgacgtgaaggcgggcgtcatcgcctacaagatcgccgcccacgcggccgacctggccaagcagcacccccacgccc
aggcgtgggacgacgcgctgtccaaggcgcgcttcgagttccgctggatggaccagttcgcgctgtccctggaccccatgacg
gcgatgtccttccacgacgagacgctgcccgcggacggcgcgaaggtcgcccacttctgctccatgtgcggccccaagttctgc
tccatgaagatcacggaggacatccgcaagtacgccgaggagaacggctacggctccgccgaggaggccatccgccaggg
catggacgccatgtccgaggagttcaacatcgccaagaagacgatctccggcgagcagcacggcgaggtcggcggcgagat
*ctacctgcccgagtcctacgtcaaggccgcgcagaagTGA***caattg**GCAGCAGCAGCTCGGATAGTATCGACACACTCTGG
ACGCTGGTCGTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCCGCTTTTATCAAACAGCCT
CAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGCGAGTTGCTAGCTGCTTGTGCTATTTGCGAATACCACCCCCAGCATCCC
CTTCCCTCGTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCTATCCCTCAGCGCTGCTCCTGCTCCTG
CTCACTGCCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCTCCTGGTACTGCAACCTGTAAACCAGCACTGCAATGC
TGATGCACGGGAAGTAGTGGGATGGGAACACAAATGGA**GGATCGTA**gagctcCAGCCACGGCAACACCGCGCG
CCTTGCGGCCGAGCACGGCGACAAGAACCTGAGCAAGATCTGCGGGCTGATCGCCAGCGACGAGG
GCCGGCACGAGATCGCCTACACGCGCATCGTGGACGAGTTCTTCCGCCTCGACCCCGAGGGCGCCG
TCGCCGCCTACGCCAACATGATGCGCAAGCAGATCACCATGCCCGCGCACCTCATGGACGACATGG
GCCACGGCGAGGCCAACCCGGGCCGCAACCTCTTCGCCGACTTCTCCGCGGTCGCCGAGAAGATCG
ACGTCTACGACGCCGAGGACTACTGCCGCATCCTGGAGCACCTCAACGCGCGCTGGAAGGTGGACG
AGCGCCAGGTCAGCGGCCAGGCCGCCGCGGACCAGGAGTACGTCCTGGGCCTGCCCCAGCGCTTCC
GGAAACTCGCCGAGAAGACCGCCGCCAAGCGCAAGCGCGTCGCGCGCAGGCCCGTCGCCTTCTCCT
GGATCTCCGGGCGCGAGATCATGGTCTAGGGAGCGACGAGTGTGCGTGCGGGGCTGGCGGGAGT
GGGACGCCCTCCTCGCTCCTCTCTGTTCTGAACGGAACAATCGGCCACCCCGCGCTACGCGCCACGC
ATCGAGCAACGAAGAAAACCCCCCGATGATAGGTTGCGGTGGCTGCCGGGATATAGATCCGGCCGC
ACATCAAAGGGCCCCTCCGCCAGAGAAGAAGCTCCTTTCCCAGCAGACTCCT**gaagagcgtttaaac**
(SEQ ID NO:149)

[0810] The sequence of the transforming DNA from the *SAD2-2* disruption construct,pSZ2622, is shown below in SEQ ID NO:150. Relevant restriction sites are indicated in lowercase, bold, and are from 5'-3' BspQI, PmeI, KpnI, XbaI, MfeI, SacI, BspQI and PmeI. Underlined sequences at the 5' and 3' flanks of the construct represent genomic DNA from *P. moriformis* that enable targeted integration of the transforming DNA via homologous recombination at the *SAD2-1* locus. Proceeding in the 5' to 3' direction, the *Chlorella protothecoides ACT* promoter (*CpACT*) driving the expression of the *AtTHIC* gene (encoding 4-amino-5-hydroxymethyl-2-methylpyrimidine synthase activity, thereby permitting the strain to grow in the absence of exogenous thiamine) is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for *AtTHIC* are indicated by

uppercase italics, while the coding region is indicated with lowercase italics. The 3' UTR of the *Chlorella vulgaris nitrate reductase* (*CvNR*) gene is indicated by small capitals.

[0811] Nucleotide sequence of the transforming DNA from pSZ2622:

gaagagcgcccaat**gtttaaac**GCCGGTCACCATCCGCATGCTCATATTACAGCGCACGCACCGCTTCGTGA
TCCACCGGGTGAACGTAGTCCTCGACGGAAACATCTGGCTCGGGCCTCGTGCTGGCACTCCCTCCCA
TGCCGACAACCTTTCTGCTGTCACCACGACCCACGATGCAACGCGACACGACCCGGTGGGACTGATC
GGTTCACTGCACCTGCATGCAATTGTCACAAGCGCATACTCCAATCGTATCCGTTTGATTTCTGTGAA
AACTCGCTCGACCGCCCGCGTCCCGCAGGCAGCGATGACGTGTGCGTGACCTGGGTGTTTCGTCGA
AAGGCCAGCAACCCCAAATCGCAGGCGATCCGGAGATTGGGATCTGATCCGAGCTTGGACCAGATC
CCCCACGATGCGGCACGGGAACTGCATCGACTCGGCGCGGAACCCAGCTTTCGTAAATGCCAGATT
GGTGTCCGATACCTTGATTTGCCATCAGCGAAACAAGACTTCAGCAGCGAGCGTATTTGGCGGGCG
TGCTACCAGGGTTGCATACATTGCCCATTTCTGTCTGGACCGCTTTACCGGCGCAGAGGGTGAGTTG
ATGGGGTTGGCAGGCATCGAAACGCGCGTGCATGGTGTGTGTGTCTGTTTTCGGCTGCACAATTTCA
ATAGTCGGATGGGCGACGGTAGAATTGGGTGTTGCGCTCGCGTGCATGCCTCGCCCCGTCGGGTGT
CATGACCGGGACTGGAATCCCCCCTCGCGACCCTCCTGCTAACGCTCCCGACTCTCCCGCCCGCGCG
CAGGATAGACTCTAGTTCAACCAATCGACA**ggtacc**agtttaggtccagcgtccgtgggggggggacgggctgggagc
ttgggccgggaagggcaagacgatgcagtccctctggggagtcacagccgactgtgtgtgttgcactgtgcggcccgcagcactca
cacgcaaaatgcctggccgacaggcaggccctgtccagtgcaacatccacggtccctctcatcaggctcaccttgctcattgacata
acggaatgcgtaccgctctttcagatctgtccatccagagaggggagcaggctccccaccgacgctgtcaaacttgcttcctgccca
accgaaaacattattgtttgaggggggggggggggggcagattgcatggcgggatatctcgtgaggaacatcactgggacactg
tggaacacagtgagtgcagtatgcagagcatgtatgctaggggtcagcgcaggaaggggccctttcccagtctcccatgccactgc
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aatcacagccaatgtcgctgctgccataatgtgatccagaccctctccgcccagatgccgagcggatcgtgggcgctgaatagattc
ctgtttcgatcactgtttgggtccctttccttttcgtctcggatgcgcgtctcgaaacaggctgcgtcgggctttcggatccctttgctcc
ctccgtcaccatcctgcgcgcgggcaagttgcttgaccctgggctgataccagggttggagggtattaccgcgtcaggccattccca
gcccggattcaattcaaagtctgggccaccaccctccgccgctctgtctgatcactccacattcgtgcatacactacgttcaagtcctg
atccaggcgtgtctcgggacaaggtgtgcttgagtttgaatctcaaggacccactccagcacagctgctggttgaccccgccctcgc
aa**tctaga***ATGgccgcgtccgtccactgcaccctgatgtccgtggtctgcaacaacaagaaccactccgcccgccccaagctg*
cccaactcctccctgctgcccggcttcgacgtggtggtccaggccgcggccacccgcttcaagaaggagacgacgaccacccg

cgccacgctgacgttcgaccccccccacgaccaactccgagcgcgccaagcagcgcaagcacaccatcgacccctcctcccccg

acttccagcccatcccctccttcgaggagtgcttccccaagtccacgaaggagcacaaggaggtggtgcacgaggagtccggc

cacgtcctgaaggtgcccttccgccgcgtgcacctgtccggcggcgagcccgccttcgacaactacgacacgtccggcccccag

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acgcagatgtactacgcgaagcagggcatcatcacggaggagatgctgtactgcgcgacgcgcgagaagctggaccccga

gttcgtccgctccgaggtcgcgcggggccgcgccatcatcccctccaacaagaagcacctggagctggagcccatgatcgtgg

gccgcaagttcctggtgaaggtgaacgcgaacatcggcaactccgccgtggcctcctccatcgaggaggaggtctacaaggt

gcagtgggccaccatgtggggcgccgacaccatcatggacctgtccacgggccgccacatccacgagacgcgcgagtggatc

ctgcgcaactccgcggtccccgtgggcaccgtccccatctaccaggcgctggagaaggtggacggcatcgcggagaacctga

actgggaggtgttccgcgagacgctgatcgagcaggccgagcagggcgtggactacttcacgatccacgcgggcgtgctgct

gcgctacatccccctgaccgccaagcgcctgacgggcatcgtgtcccgcggcggctccatccacgcgaagtggtgcctggccta

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acggcctgcgccccggctccatctacgacgccaacgacacggcccagttcgccgagctgctgacccagggcgagctgacgcg

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agaagcagctggagtggtgcaacgaggcgcccttctacaccctgggccccctgacgaccgacatcgcgcccggctacgacca

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acccccacgcccaggcgtgggacgacgcgctgtccaaggcgcgcttcgagttccgctggatggaccagttcgcgctgtccctg

gaccccatgacggcgatgtccttccacgacgagacgctgcccgcggacggcgcgaaggtcgcccacttctgctccatgtgcgg

ccccaagttctgctccatgaagatcacggaggacatccgcaagtacgccgaggagaacggctacggctccgccgaggaggc

catccgccagggcatggacgccatgtccgaggagttcaacatcgccaagaagacgatctccggcgagcagcacggcgaggt

*cggcggcgagatctacctgcccgagtcctacgtcaaggccgcgcagaagTGA***caattg**GCAGCAGCAGCTCGGATAGTATC

GACACACTCTGGACGCTGGTCGTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCCGCTTT

TATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGCGAGTTGCTAGCTGCTTGTGCTATTTGCGAATACCA

CCCCCAGCATCCCCTTCCCTCGTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCTATCCCTCAGCGCT

GCTCCTGCTCCTGCTCACTGCCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCTCCTGGTACTGCAACCTGTAAACC

AGCACTGCAATGCTGATGCACGGGAAGTAGTGGGATGGGAACACAAATGGA**GGATCGTA**gagctcCAGCCACGGC

AACACCGCGCGCCTGGCGGCCGAGCACGGCGACAAGGGCCTGAGCAAGATCTGCGGGCTGATCGC

CAGCGACGAGGGCCGGCACGAGATCGCCTACACGCGCATCGTGGACGAGTTCTTCCGCCTCGACCC

CGAGGGCGCCGTCGCCGCCTACGCCAACATGATGCGCAAGCAGATCACCATGCCCGCGCACCTCAT

GGACGACATGGGCCACGGCGAGGCCAACCCGGGCCGCAACCTCTTCGCCGACTTCTCCGCCGTCGC

CGAGAAGATCGACGTCTACGACGCCGAGGACTACTGCCGCATCCTGGAGCACCTCAACGCGCGCTG

GAAGGTGGACGAGCGCCAGGTCAGCGGCCAGGCCGCCGCGGACCAGGAGTACGTTCTGGGCCTGC
CCCAGCGCTTCCGGAAACTCGCCGAGAAGACCGCCGCCAAGCGCAAGCGCGTCGCGCGCAGGCCC
GTCGCCTTCTCCTGGATCTCCGGACGCGAGATTATGGTCTAGGGAGGTACGAGCGCGCGCGAGGGA
TTGGTGGGAGTGGGACGCGCTCGTCGCTCCTTTCTATTCTGAAGGGAAGATTGGCCACCCCGCTCCA
CGCGCCACGCATCGAGCAACGAAGAAAACCCCCCGATGATAGGTTGCAGTGGCTGCCGAGATATAG
ATCCGGCTGCACGTCAAAGGGCCCCTCGGCCAGAGAAGAAGCTCTTTTCCCAGCGACCGCAGACTCC
Tgaagagcgtttaaac (SEQ ID NO:150)

[0812] Constructs D1557, D1565 and D1566, derived from pSZ2601, pSZ2607 and pSZ2622, respectively, were transformed into S3150 as described previously. Primary transformants were clonally purified and grown under low-nitrogen lipid production conditions at pH 5. The resulting fatty acid profiles from representative clones are summarized in Table 117. *SAD2-1* disruption strains derived from D1557 and D1565 transformants accumulated up to 13.4% C18:0 at the expense of C18:1, indicating that SAD activity was significantly reduced in these strains. C18:0 levels only increased to 8.5% in *SAD2-2* disruption strains, suggesting that the expression or activity of *SAD2-2* was lower than that of *SAD2-1*. We also observed that C20:0 levels increased up to 1.1% in strains with elevated C18:0, demonstrating that C18:0 was an effective primer for fatty acid elongation reactions in the endoplasmic reticulum (ER).

[0813] Table 117. Fatty acid profiles from representative clones.

	Strain	S3150	D1557-2	D1557-3	D1565-10	D1565-3	D1565-8	D1566-5	D1566-6	D1566-1
Fatty Acid Area %	C14:0	1.30	1.14	1.20	1.08	1.12	1.11	1.18	1.12	1.21
	C16:0	28.71	29.32	29.74	28.84	29.34	29.11	29.21	29.13	28.46
	C16:1	0.76	0.21	0.23	0.21	0.21	0.21	0.32	0.31	0.31
	C17:0	0.12	0.14	0.15	0.15	0.14	0.14	0.14	0.16	0.14
	C18:0	2.93	13.42	11.92	14.29	14.14	14.04	8.47	8.47	7.68
	C18:1	58.08	46.29	47.65	45.75	45.31	45.69	51.29	51.33	53.38
	C18:2	6.81	7.15	6.96	7.09	7.18	7.19	7.25	7.34	6.92
	C18:3 α	0.59	0.69	0.63	0.72	0.72	0.73	0.71	0.73	0.62
	C20:0	0.24	0.93	0.84	**1.10**	**1.09**	**1.04**	0.75	0.77	0.63
	C22:0	0.05	0.16	0.15	0.19	0.19	0.18	0.14	0.14	0.11
	C24:0	0.06	0.16	0.16	0.20	0.20	0.20	0.17	0.17	0.14
	sum C18	68.40	67.55	67.16	67.85	67.35	67.65	67.72	67.87	68.60
	saturates	33.4	45.35	44.24	45.94	46.32	45.93	40.18	40.04	38.48

		9								
	unsaturates	66.52	54.62	55.76	54.04	53.68	54.07	59.83	59.97	61.50

[0814] In order to increase C18:0 accumulation at the expense of C16:0 we generated DNA constructs which simultaneously ablated *SAD2-1* and over-expressed a codon-optimized version of the endogenous *β-ketoacyl-ACP synthase II* (*PmKASII*) gene. The sequence of the transforming DNA from the *SAD2-1* ablation, PmKASII over-expression construct, pSZ2624, is shown below in SEQ ID NO:151. Relevant restriction sites are indicated in lowercase, bold, and are from 5'-3' PmeI, SpeI, AscI, ClaI, SacI, AvrII, EcoRV, AflII, KpnI, XbaI, MfeI, BamHI, BspQI and PmeI. Underlined sequences at the 5' and 3' flanks of the construct represent genomic DNA from *P. moriformis* that enable targeted integration of the transforming DNA via homologous recombination at the *SAD2-1* locus. The *SAD2-1* 5' integration flank contained the endogeneous *SAD2-1* promoter, enabling the *in situ* activation of the *PmKASII* gene. Proceeding in the 5' to 3' direction, the region encoding the PmKASII plastid targeting sequence is indicated by lowercase, underlined italics. The sequence that encodes the mature PmKASII polypeptide is indicated with lowercase italics, while a 3xFLAG epitope encoding sequence is in bold italics. The initiator ATG and terminator TGA for *PmKASII-FLAG* are indicated by uppercase italics. Two spacer regions are represented by lowercase text. The *CpACT* promoter driving the expression of the *AtTHIC* gene is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for *AtTHIC* are indicated by uppercase italics, while the coding region is indicated with lowercase italics. The 3' UTR of the *Chlorella vulgaris nitrate reductase* (*CvNR*) gene is indicated by small capitals.

[0815] Nucleotide sequence of the transforming DNA from pSZ2624:

gtttaaacGCCGGTCACCACCCGCATGCTCGTACTACAGCGCACGCACCGCTTCGTGATCCACCGGGTG
AACGTAGTCCTCGACGGAAACATCTGGTTCGGGCCTCCTGCTTGCACTCCCGCCCATGCCGACAACC
TTTCTGCTGTTACCACGACCCACAATGCAACGCGACACGACCGTGTGGGACTGATCGGTTCACTGCA
CCTGCATGCAATTGTCACAAGCGCTTACTCCAATTGTATTCGTTTGTTTTCTGGGAGCAGTTGCTCGA
CCGCCCGCGTCCCGCAGGCAGCGATGACGTGTGCGTGGCCTGGGTGTTTCGTCGAAAGGCCAGCAA
CCCTAAATCGCAGGCGATCCGGAGATTGGGATCTGATCCGAGTTTGGACCAGATCCGCCCCGATGC
GGCACGGGAACTGCATCGACTCGGCGCGGAACCCAGCTTTCGTAAATGCCAGATTGGTGTCCGATA
CCTGGATTTGCCATCAGCGAAACAAGACTTCAGCAGCGAGCGTATTTGGCGGGCGTGCTACCAGGG
TTGCATACATTGCCCATTTCTGTCTGGACCGCTTTACTGGCGCAGAGGGTGAGTTGATGGGGTTGGC
AGGCATCGAAACGCGCGTGCATGGTGTGCGTGTCTGTTTTCGGCTGCACGAATTCAATAGTCGGAT

GGGCGACGGTAGAATTGGGTGTGGCGCTCGCGTGCATGCCTCGCCCCGTCGGGTGTCATGACCGG
GACTGGAATCCCCCCTCGCGACCATCTTGCTAACGCTCCCGACTCTCCCGACCGCGCGCAGGATAGA
CTCTTGTTCAACCAATCGACA**actagt***ATG*cagaccgcccaccagcgcccccccaccgagggccactgcttcggcgcc
cgcctgcccaccgcctcccgccgcgccgtgcgccgcgcctggtcccgcatcgcccgcg**ggcgcgcc***gccgccgccgccgacgcc*
aaccccgcccgccccgagcgccgcgtggtgatcaccggccagggcgtggtgacctccctgggccagaccatcgagcagttcta
ctcctccctgctggagggcgtgtccggcatctcccagatccagaagttcgacaccaccggctacaccaccaccatcgccggcga
gatcaagtccctgcagctggacccctacgtgcccaagcgctgggccaagcgcgtggacgacgtgatcaagtacgtgtacatcg
ccggcaagcaggccctggagtccgccggcctgcccatcgaggccgccggcctggccggcgccggcctggaccccgccctgtgc
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tgctggccggcggcgccgacgccgccatcatcccctccggcatcggcggcttcatcgcctgcaaggccctgtccaagcgcaacg
acgagcccgagcgcgcctcccgcccctgggacgccgaccgcgacggcttcgtgatgggcgagggcgccggcgtgctggtgct
ggaggagctggagcacgccaagcgccgcggcgccaccatcctggccgagctggtgggcggcgccgccacctccgacgccca
ccacatgaccgagcccgacccccagggccgcggcgtgcgcctgtgcctggagcgcgccctggagcgcgcccgcctggcccccg
agcgcgtgggctacgtgaacgcccacggcacctccacccccgccggcgacgtggccgagtaccgcgccatccgcgccgtgatc
ccccaggactccctgcgcatcaactccaccaagtccatgatcggccacctgctgggcggcgccggcgccgtggaggccgtggc
cgccatccaggccctgcgcaccggctggctgcaccccaacctgaacctggagaaccccgcccccggcgtggaccccgtggtgc
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*gtgatcttccgcaagtacgacgag**atggactacaaggaccacgacggcgactacaaggaccacgacatcgactacaagg***
acgacgacgacaag*TGA***atcgat**AGATCTCTTAAGGCAGCAGCAGCTCGGATAGTATCGACACACTCTGGACGCTGGTC
GTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCCGCTTTTATCAAACAGCCTCAGTGTGTT
TGATCTTGTGTGTACGCGCTTTTGCGAGTTGCTAGCTGCTTGTGCTATTTGCGAATACCACCCCCAGCATCCCCTTCCCTCG
TTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCTATCCCTCAGCGCTGCTCCTGCTCCTGCTCACTGCC
CCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCTCCTGGTACTGCAACCTGTAAACCAGCACTGCAATGCTGATGCAC
GGGAAGTAGTGGGATGGGAACACAAATGGAAAGCTTAATTAA**gagctc**cgcgtctcgaacagagcgcgcagaggaacgctg
aaggtctcgcctctgtcgcacctcagcgcggcatacaccacaataaccacctgacgaatgcgcttggttcttcgtccattagcgaagc
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gatatccat**cttaag**gatctaagtaagattcgaagcgctcgaccgtgccggacggactgcagccccatgtcgtagtgaccgccaat
gtaagtgggctggcgtttccctgtacgtgagtcaacgtcactgcacgcgcaccaccctctcgaccggcaggaccaggcatcgcgag
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ccaagctgcccaactcctccctgctgcccggcttcgacgtggtggtccaggccgcggccacccgcttcaagaaggagacgacg

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agcacctgggcctgcccaaccgcgacgacgtgaaggcgggcgtcatcgcctacaagatcgccgcccacgcggccgacctggc

caagcagcacccccacgcccaggcgtgggacgacgcgctgtccaaggcgcgcttcgagttccgctggatggaccagttcgcg
ctgtccctggaccccatgacggcgatgtccttccacgacgagacgctgcccgcggacggcgcgaaggtcgcccacttctgctcc
atgtgcggccccaagttctgctccatgaagatcacggaggacatccgcaagtacgccgaggagaacggctacggctccgccg
aggaggccatccgccagggcatggacgccatgtccgaggagttcaacatcgccaagaagacgatctccggcgagcagcacg
*gcgaggtcggcggcgagatctacctgcccgagtcctacgtcaaggccgcgcagaagTGA***caattg**ACGGAGCGTCGTGCG
GGAGGGAGTGTGCCGAGCGGGGAGTCCCGGTCTGTGCGAGGCCCGGCAGCTGACGCTGGCGAGCCGTACGCCCCGAG
GGTCCCCCTCCCCTGCACCCTCTTCCCCTTCCCTCTGACGGCCGCGCCTGTTCTTGCATGTTCAGCGAC**ggatcc**TAGGGA
GCGACGAGTGTGCGTGCGGGGCTGGCGGGAGTGGGACGCCCTCCTCGCTCCTCTCTGTTCTGAACG
GAACAATCGGCCACCCCGCGCTACGCGCCACGCATCGAGCAACGAAGAAAACCCCCCGATGATAGG
TTGCGGTGGCTGCCGGGATATAGATCCGGCCGCACATCAAAGGGCCCCTCCGCCAGAGAAGAAGCT
CCTTTCCCAGCAGACTCCTTCTGCTGCCAAAACACTTCTCTGTCCACAGCAACACCAAAGGATGAACA
GATCAACTTGCGTCTCCGCGTAGCTTCCTCGGCTAGCGTGCTTGCAACAGGTCCCTGCACTATTATCT
TCCTGCTTTCCTCTGAATTATGCGGCAGGCGAGCGCTCGCTCTGGCGAGCGCTCCTTCGCGCCGCCCT
CGCTGATCGAGTGTACAGTCAATGAATGGTCCTGGGCGAAGAACGAGGGAATTTGTGGGTAAAACA
AGCATCGTCTCTCAGGCCCCGGCGCAGTGGCCGTTAAAGTCCAAGACCGTGACCAGGCAGCGCAGC
GCGTCCGTGTGCGGGCCCTGCCTGGCGGCTCGGCGTGCCAGGCTCGAGAGCAGCTCCCTCAGGTCG
CCTTGGACGGCCTCTGCGAGGCCGGTGAGGGCCTGCAGGAGCGCCTCGAGCGTGGCAGTGGCGGT
CGTATCCGGGTCGCCGGTCACCGCCTGCGACTCGCCATCC**gaagagc**gtttaaac (SEQ ID NO:151)

[0816] Using the methods of this example, by overexpressing KASII, and Garcinia mangostana FATA, and by reducing expression of endogenous SAD, FAD2, and FATA, we produced a strain of P. moriformis that produced and oil with greater than 55% SOS with Sat-O-Sat (where O is oleate and Sat is any saturated fatty acid) of about 70-75% and trisaturatated TAGs of less than 6.5%.

EXAMPLE 66: COMBINING KASII, FATA AND LPAAT TRANSGENES TO PRODUCE AN OIL HIGH IN SOS.

[0817] In Prototheca moriformis, we overexpressed the P. moriformis KASII, knocked out an endogenous SAD2 allele, knocked out the endogenous FATA allele, and overexpressed both a LPAAT from Brassica napus and a FATA gene from Garcinia mangostana ("GarmFAT1"). The resulting strain produced an oil with over 55% SOS, over 70% Sat-O-Sat, and less than 8% trisaturated TAGs.

[0818] A base strain was transformed with a linearized plasmid with flanking regions designed for homologous recombination at the SAD2 site. As in examples above, the

construct ablated SAD2 and overexpressed P. moriformis KASII. A ThiC selection marker was used. This strain was further transformed with a construct designed to overexpress GarmFATA1 with a P. moriformis SASD1 plastid targeting peptide via homologous recombination at the 6S chromosomal site using invertase as a selection marker. The resulting strain, produced oil with about 62% stearate, 6% palmitate, 5% linoleate, 45% SOS and 20% trisaturates.

[0819] The sequence of the transforming DNA from the *GarmFATA1* expression construct (pSZ3204) is shown below in SEQ ID NO:152. Relevant restriction sites are indicated in lowercase, bold, and are from 5'-3' BspQI, KpnI, XbaI, MfeI, BamHI, AvrII, EcoRV, SpeI, AscI, ClaI, AflII, SacI and BspQI. Underlined sequences at the 5' and 3' flanks of the construct represent genomic DNA from *P. moriformis* that enable targeted integration of the transforming DNA via homologous recombination at the *6S* locus. Proceeding in the 5' to 3' direction, the *CrTUB2* promoter driving the expression of *Saccharomyces cerevisiae SUC2* (*ScSUC2*) gene, enabling strains to utilize exogenous sucrose, is indicated by lowercase, boxed text. The initiator ATG and terminator TGA of *ScSUC2* are indicated by uppercase italics, while the coding region is represented by lowercase italics. The 3' UTR of the *CvNR* gene is indicated by small capitals. A spacer region is represented by lowercase text. The *P. moriformis SAD2-2* (*PmSAD2-2*) promoter driving the expression of the chimeric *CpSAD1tp_GarmFATA1_FLAG* gene is indicated by lowercase, boxed text. The initiator ATG and terminator TGA are indicated by uppercase italics; the sequence encoding CpSAD1tp is represented by lowercase, underlined italics; the sequence encoding the GarmFATA1 mature polypeptide is indicated by lowercase italics; and the 3X FLAG epitope tag is represented by uppercase, bold italics. A second *CvNR* 3' UTR is indicated by small capitals.

[0820] Nucleotide sequence of the transforming DNA from pSZ3204:

gctcttcGCCGCCGCCACTCCTGCTCGAGCGCGCCCGCGCGTGCGCCGCCAGCGCCTTGGCCTTTTCGC
CGCGCTCGTGCGCGTCGCTGATGTCCATCACCAGGTCCATGAGGTCTGCCTTGCGCCGGCTGAGCCA
CTGCTTCGTCCGGGCGGCCAAGAGGAGCATGAGGGAGGACTCCTGGTCCAGGGTCCTGACGTGGT
CGCGGCTCTGGGAGCGGGCCAGCATCATCTGGCTCTGCCGCACCGAGGCCGCCTCCAACTGGTCCT
CCAGCAGCCGCAGTCGCCGCCGACCCTGGCAGAGGAAGACAGGTGAGGGGGGTATGAATTGTACA
GAACAACCACGAGCCTTGTCTAGGCAGAATCCCTACCAGTCATGGCTTTACCTGGATGACGGCCTGC
GAACAGCTGTCCAGCGACCCTCGCTGCCGCCGCTTCTCCCGCACGCTTCTTTCCAGCACCGTGATGGC
GCGAGCCAGCGCCGCACGCTGGCGCTGCGCTTCGCCGATCTGAGGACAGTCGGGGAACTCTGATCA

GTCTAAACCCCCTTGCGCGTTAGTGTTGCCATCCTTTGCAGACCGGTGAGAGCCGACTTGTTGTGCG
CCACCCCCCACACCACCTCCTCCCAGACCAATTCTGTCACCTTTTTGGCGAAGGCATCGGCCTCGGCC
TGCAGAGAGGACAGCAGTGCCCAGCCGCTGGGGGTTGGCGGATGCACGCTCA**ggtacc**ctttcttgcgct
atgacacttccagcaaaaggtagggcgggctgcgagacggcttcccggcgctgcatgcaacaccgatgatgcttcgacccccga
agctccttcggggctgcatgggcgctccgatgccgctccagggcgagcgctgtttaaatagccaggccccgattgcaaagacatta
tagcgagctaccaaagccatattcaaacacctagatcactaccacttctacacaggccactcgagcttgtgatcgcactccgctaag
ggggcgcctcttcctcttcgtttcagtcacaacccgcaaac**tctaga**atatca*ATGctgctgcaggccttcctgttcctgctggccg*
gcttcgccgccaagatcagcgcctccatgacgaacgagacgtccgaccgccccctggtgcacttcacccccaacaagggctgg
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cccaccaaccctggcgctcctccatgtccctcgtgcgcaagttctccctcaacaccgagtaccaggccaacccggagacggag
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CTCGGATAGTATCGACACACTCTGGACGCTGGTCGTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATA
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ATCCCTCAGCGCTGCTCCTGCTCCTGCTCACTGCCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCTCCTGGTACTG
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acagagcgcgcagaggaacgctgaaggtctcgcctctgtcgcacctcagcgcggcatacaccacaataaccacctgacgaatgcg

cttggttcttcgtccattagcgaagcgtccggttcacacacgtgccacgttggcgaggtggcaggtgacaatgatcggtggagctgat

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<u>*cgcccagcgaggcccctccccgtgcgcg*</u>***ggcgcgcc****atccccccccgcatcatcgtggtgtcctcctcctcctccaaggtgaaccc*

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caacgtgtccgccaacgaccacggctgccgcaacttcctgcacctgctgcgcctgtccggcaacggcctggagatcaaccgcg

*gccgcaccgagtggcgcaagaagcccacccgc**ATGGACTACAAGGACCACGACGGCGACTACAAGGACCAC***

***GACATCGACTACAAGGACGACGACGACAAG**TGA*atcgatagatct**cttaag**GCAGCAGCAGCTCGGATAGTAT

CGACACACTCTGGACGCTGGTCGTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCCGCTT

TTATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGCGAGTTGCTAGCTGCTTGTGCTATTTGCGAATACC

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CAGCACTGCAATGCTGATGCACGGGAAGTAGTGGGATGGGAACACAAATGGAaagcttaattaa**gagctc**TTGTTTTCC

AGAAGGAGTTGCTCCTTGAGCCTTTCATTCTCAGCCTCGATAACCTCCAAAGCCGCTCTAATTGTGGA

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CGAGCACCTCCATGCTCTGAGTGGCCACCCCCCGGCCCTGGTGCTTGCGGAGGGCAGGTCAACCGG

CATGGGGCTACCGAAATCCCCGACCGGATCCCACCACCCCCGCGATGGGAAGAATCTCTCCCCGGG

ATGTGGGCCCACCACCAGCACAACCTGCTGGCCCAGGCGAGCGTCAAACCATACCACACAAATATCC

TTGGCATCGGCCCTGAATTCCTTCTGCCGCTCTGCTACCCGGTGCTTCTGTCCGAAGCAGGGGTTGCT

AGGGATCGCTCCGAGTCCGCAAACCCTTGTCGCGTGGCGGGGCTTGTTCGAGCTT**gaagagc** (SEQ

ID NO:152)

[0821] The resulting strain was further transformed with a construct designed to recombine at (and thereby disrupt) the endogenous FATA and also express the LPAAT from B. napus under control of the UAPA1 promoter and using alpha galactosidase as a selectable marker with selection on melbiose. The resulting strain showed increased production of SOS (about 57-60%) and Sat-O-Sat (about 70-76%) and lower amounts of trisaturates (4.8 to 7.6%).

[0822] Strains were generated in the high-C18:0 S6573 background in which we maximized SOS production and minimized the formation of trisaturated TAGs by targeting both the *Brassica napus LPAT2(Bn1.13)* gene and the *PmFAD2hpA* RNAi construct to the *FATA-1* locus. The sequence of the transforming DNA from the *PmFAD2hpA* expression construct pSZ4164 is shown below in SEQ ID NO:153. Relevant restriction sites are indicated in lowercase, bold, and are from 5'-3' BspQI, KpnI, SpeI, SnaBI, BamHI, NdeI, NsiI, AflII, EcoRI, SpeI, BsiWI, XhoI, SacI and BspQI. Underlined sequences at the 5' and 3' flanks of the construct represent genomic DNA from *P. moriformis* that enable targeted

integration of the transforming DNA via homologous recombination at the *FATA-1* locus. Proceeding in the 5' to 3' direction, the *PmHXT1* promoter driving the expression of *Saccharomyces carlbergensis MEL1* (*ScarMEL1*) gene, enabling strains to utilize exogenous melibiose, is indicated by lowercase, boxed text. The initiator ATG and terminator TGA of *ScarMEL1* are indicated by uppercase italics, while the coding region is represented by lowercase italics. The 3' UTR of the *P. moriformis PGK* gene is indicated by small capitals. A spacer region is represented by lowercase text. The *P. moriformis UAPA1* promoter driving the expression of the *BnLPAT2(Bn1.13)* gene is indicated by lowercase, boxed text. The initiator ATG and terminator TGA are indicated by uppercase italics; the sequence encoding *BnLPAT2(Bn1.13)* is represented by lowercase, underlined italics. The 3' UTR of the *CvNR* gene is indicated by small capitals. A second spacer region is represented by lowercase text. The *C. reinhardtii CrTUB2* promoter driving the expression of the *PmFAD2hpA* hairpin sequence is indicated by lowercase, boxed text. The *FAD2* exon 1 sequence in the forward orientation is indicated with lowercase italics; the *FAD2* intron 1 sequence is represented with lowercase, bold italics; a short linker region is indicated with lowercase text, and the *FAD2* exon 1 sequence in the reverse orientation is indicated with lowercase, underlined italics. A second *CvNR* 3' UTR is indicated by small capitals.

[0823] Nucleotide sequence of the transforming DNA from pSZ4164:

gctcttcCCAACTCAGATAATACCAATACCCCTCCTTCTCCTCCTCATCCATTCAGTACCCCCCCCCTTCTC
TTCCCAAAGCAGCAAGCGCGTGGCTTACAGAAGAACAATCGGCTTCCGCCAAAGTCGCCGAGCACT
GCCCGACGGCGGCGCGCCCAGCAGCCCGCTTGGCCACACAGGCAACGAATACATTCAATAGGGGG
CCTCGCAGAATGGAAGGAGCGGTAAAGGGTACAGGAGCACTGCGCACAAGGGGCCTGTGCAGGA
GTGACTGACTGGGCGGGCAGACGGCGCACCGCGGGCGCAGGCAAGCAGGGAAGATTGAAGCGGC
AGGGAGGAGGATGCTGATTGAGGGGGGCATCGCAGTCTCTCTTGGACCCGGGATAAGGAAGCAAA
TATTCGGCCGGTTGGGTTGTGTGTGTGCACGTTTTCTTCTTCAGAGTCGTGGGTGTGCTTCCAGGGA
GGATATAAGCAGCAGGATCGAATCCCGCGACCAGCGTTTCCCCATCCAGCCAACCACCCTGTC**ggtac**
cgcggtgagaatcgaaaatgcatcgtttctaggttcggagacggtcaattccctgctccggcgaatctgtcggtcaagctggccagt
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*tgcgcagcgtcccgatcgtgaacgaaggcttctccacaggctgcctgttcgtcttgatagccat***ctcgag**GCAGCAGCAGCTCG

GATAGTATCGACACACTCTGGACGCTGGTCGTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATATCCC

TGCCGCTTTTATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGCGAGTTGCTAGCTGCTTGTGCTATTTG

CGAATACCACCCCCAGCATCCCCTTCCCTCGTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCTATCC

CTCAGCGCTGCTCCTGCTCCTGCTCACTGCCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCTCCTGGTACTGCAAC

CTGTAAACCAGCACTGCAATGCTGATGCACGGGAAGTAGTGGGATGGGAACACAAATGGAAAGCTGTA**gagctc**ttgtttt

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AGAGGTGAAGGAACGCATCCCTATGCCCTTGCAATGGACAGTGTTTCTGGCCACCGCCACCAAGACT

TCGTGTCCTCTGATCATCATGCGATTGATTACGTTGAATGCGACGGCCGGTCAGCCCCGGACCTCCA

CGCACCGGTGCTCCTCCAGGAAGATGCGCTTGTCCTCCGCCATCTTGCAGGGCTCAAGCTGCTCCCA

AAACTCTTGGGCGGGTTCCGGACGGACGGCTACCGCGGGTGCGGCCCTGACCGCCACTGTTCGGAA

GCAGCGGCGCTGCATGGGCAGCGGCCGCTGCGGTGCGCCACGGACCGCATGATCCACCGGAAAAG

CGCACGCGCTGGAGCGCGCAGAGGACCACAGAGAAGCGGAAGAGACGCCAGTACTGGCAAGCAG

GCTGGTCGGTGCCATGGCGCGCTACTACCCTCGCTATGACTCGGGTCCTCGGCCGGCTGGCGGTGCT

GACAATTCGTTTAGTGGAGCAGCGACTCCATTCAGCTACCAGTCGAACTCAGTGGCACAGTGACTcc

gctcttc (SEQ ID NO:153)

[0824] The described embodiments of the invention are intended to be merely exemplary and numerous variations and modifications will be apparent to those skilled in the art. All such variations and modifications are intended to be within the scope of the present invention. For example, the various triglyceride oils can be tailored in for a mixture of midchain and long chain fatty acids in order to adjust parameters such as polarity, solvency, and foam-height of the oils or chemicals made from the oils. In addition, where a knockout

of a gene is called for, an equivalent result may be reached using knockdown techniques including mutation and expression of inhibitory substances such as RNAi or antisense.

WHAT IS CLAIMED IS:

1. A method comprising:

(a) cultivating a recombinant cell, the cell

(i) expressing an exogenous KASI or KASIV gene, optionally encoding a protein having at least 60, 65, 70, 75, 80, 85, 90, or 95% amino acid sequence identity to an enzyme encoded by any of SEQ ID NOs: 46-49, and at least one FATB acyl-ACP thioesterase gene optionally encoding a protein having at least 60, 65, 70, 75, 80, 85, 90, or 95% nucleic acid sequence identity to SEQ ID NOs: 11, 87, 89, 159, 162 or 163;

(ii) expressing a gene encoding a FATA, FATB, KASI, KASII, LPAAT, SAD, or FAD2 under the control of a nitrogen-sensitive promoter having at least 60, 65, 70, 75, 80, 85, 90, or 95% sequence identity to any of SEQ ID NOs: 129 to 147; or

(iii) having a knockout or knockdown of a SAD gene, a FAD2 gene, and a FATA gene, an overexpressing an exogenous C18-preferring FATA gene, an oleoyl-preferring LPAAT gene, and a KASII gene; and

(b) extracting oil from the cell.

2. The method of claim 1, wherein the cell is of type (i).

3. The method of claim 2, wherein the cell comprises at least a second acyl-ACP thioesterase, optionally encoding a protein having at least 60, 65, 70, 75, 80, 85, 90, or 95% nucleic acid sequence identity to any of SEQ ID NOS: : 11, 87, 89, 159, 162 or 163.

4. The method of claim 2 or 3, wherein the oil comprises at least 30% C10:0 and at least 30% C12:0.

5. The method of any of claims 2 to 4, wherein the oil has a viscosity of less than 30 cS and optionally of 25 cS ± 20% at 40°C as measured by ASTM D445.
6. The method of any of claims 2 to 5, wherein C10:0 and C12:0 fatty acids are balanced to within 20%, 10% or 5%.
7. The method of claim 1, wherein the cell is of type (iii).
8. The method of claim 7, wherein the cell oil comprises at least 60% Stearate-oleate-stearate (SOS).
9. The method of claim 7, wherein:
    (a) the C18-preferring FATA gene encodes a protein with at least 60, 65, 70, 75, 80, 85, 90, or 95% amino acid identity to SEQ ID NO: 156
    (b) the LPAAT gene encodes a protein with at least 60, 65, 70, 75, 80, 85, 90, or 95% amino acid identity to SEQ ID NO: 157; and/or
    (c) the KASII gene encodes a protein with at least 60, 65, 70, 75, 80, 85, 90, or 95% amino acid identity to SEQ ID NO 160 or 161.
10. The method of any of the above claims wherein the cell is a microalga, optionally of Trebouxiophyceae, and optionally of the genus Prototheca.
11. An oil, soap, oleochemical, foodstuff, or other oil-derived product produced according to any of the above claims.
12. A method comprising cultivating an oleaginous recombinant cell, optionally of an oleaginous recombinant eukaryotic microalga, wherein the cell comprises an exogenous gene encoding a palmitate ACP-desaturase enzyme active to produce an oil having a fatty acid profile characterized by a ratio of palmitoleic acid to palmitic acid of at least 0.1 and/or palmitoleic acid levels of 0.5 % or more, as determined by FAME GC/FID analysis.
13. The method of claim 12, wherein the exogenous gene encodes a palmitoyl-ACP desaturase (PAD) having desaturating activity toward ACP-palmitate.
14. The method of claim 13, wherein the exogenous gene encodes a stearoyl-ACP desaturase variant having increased activity toward ACP-palmitate.

15. The method of claim 14 wherein the stearoyl-ACP desaturase variant is a L118W mutant.
16. The method of any of claims 12-15, wherein the gene is in operable linkage with a promoter, plastid-targeting transit peptide, and 5'UTR active to express the gene product in a eukaryotic oleaginous microalga.
17. The method of claim 16, wherein the microalga is of Trebouxiophyceae, and optionally of the genus Chlorella or Prototheca.
18. The method of claim 16, wherein the microalga has 23S rRNA with at least 65, 70, 75, 80, 85, 90 or 95% nucleotide sequence identity to SEQ ID NO: 76.
19. The method of any of the above claims wherein the fatty acid profile is further characterized by less than 3.5% saturated fatty acids.
20. The method of any of the claims 12-19, wherein the cell is cultivated to at least 40% oil by dry cell weight.
21. The method of any of claims 12-20, wherein the microalga further comprises a knockout or knockdown of an endogenous acyl-ACP thioesterase and/or an exogenous KASII gene.
22. The method of claim 21, wherein the oil comprises reduced amounts of saturated fatty acids as a result of the knockout or knockdown of an endogenous acyl-ACP thioesterase and/or an exogenous KASII gene.
23. The method of claim 21, wherein the exogenous KASII gene is inserted into the coding region of the endogenous acyl-ACP thioesterase.
24. The method of claim 23, wherein the inserted KASII gene is inverted in orientation relative to the endogenous acyl-ACP thioesterase.
25. The method of any of the claims 12-24, wherein the oil is produced by heterotrophically cultivating the microalga on sucrose and the microalga comprises an exogenous invertase gene that allows it to metabolize the sucrose.
26. The method of any of claims 12-25, wherein the oil has a fatty acid profile with at least 90% oleic acid, less than 3% saturated fat, and more oleic acid than linoleic acid.
27. The method of any of claims 12-26, further comprising recovering the oil.
28. A method comprising using the oil of claim 27 for frying or as an ingredient in a prepared food.
29. An oil produced by the method of the above claims, comprising a microalgal sterol profile.

30. The oil of claim 27, wherein the microalgal sterol profile is characterized by an excess of ergosterol over β-sitosterol and/or the presence of 22, 23-dihydrobrassicasterol, poriferasterol or clionasterol.
31. A method comprising cultivating an oleaginous recombinant cell, optionally of an oleaginous recombinant eukaryotic microalga, wherein the cell produces an oil having greater than 20 % linoleic acid and less than 10% linolenic acid.
32. The method of claim 31, wherein the cell comprises an exogenous KASII gene and/or a FATA knockout or knockdown.
33. The method of claim 32, wherein the cell further comprises an overexpressed FAD2 gene, optionally under control of a promoter regulatable via environmental condition.
34. The method of any of claims 31-33, wherein the cell is a microalga of Trebouxiophyceae, and optionally of the genus Chlorella or Prototheca or has 23S rRNA with at least 65, 70, 75, 80, 85, 90 or 95% nucleotide sequence identity to SEQ ID NO: 76.
35. The method of any of claims 31-34, wherein the cell is cultivated to at least 40% oil by dry cell weight.
36. The method of any of claims 31-35, wherein the cell is cultivated on sucrose and the cell comprises an exogenous invertase gene that allows it to metabolize the sucrose.
37. The method of any of any of claims 31-36, further comprising recovering the oil.
38. An oil produced by the method of claim 37, comprising a microalgal sterol profile.
39. The oil of claim 38, wherein the microalgal sterol profile is characterized by an excess of ergosterol over β-sitosterol and/or the presence of 22, 23-dihydrobrassicasterol, poriferasterol or clionasterol.
40. A method comprising cultivating an oleaginous cell, optionally a microalga, so that the cell produces an oil with less than 10% palmitic acid, greater than 85% oleic acid, 1% or less polyunsaturated fatty acids, and less than 7% saturated fatty acids.
41. The method of claim 40, wherein the cell is a microalga with FAD and FATA knockouts and expresses an exogenous KASII gene.
42. The method of claim 40 or 41, further comprising extracting the oil from the cell.
43. The oil produced by the method of claim 42.
44. The oil of claim 43, comprise a microalgal sterol profile.
45. A foodstuff or chemical produced from the oil of claim 43 or 44.
46. A method comprising cultivating an oleaginous cell, optionally a microalga, so that the cell produces an oil with a fatty acid profile in which:

(a) the sum of lauric and myristic acids is at least 50%;

(b) total saturated fatty acids are at least 50% and levels of capric and lauric fatty acids are balanced to within 20%.

(c) capric acid is at least 45% and lauric acid is at least 45%.

47. The method of claim 46, wherein the sum of lauric and myristic acids is at least 60%.

48. The method of claim 46, wherein the sum of lauric and myristic acids is at least 70%.

49. The method of claim 46, wherein the sum of lauric and myristic acids is at least 75%.

50. The method of any of claims 46-49 wherein the cell comprises an exogenous plant FATB gene.

51. The method of any of claims 46-50, wherein the cell comprises an exogenous KASI or KASIV gene.

52. The method of any of claims 46-51, further comprising extracting the oil from the cell.

53. An oil produced by the method of claim 52.

54. The oil of claim 53, comprising a microalgal sterol profile.

55. A foodstuff or chemical produced from the oil of claim 53 or 54.

56. A method comprising cultivating an oleaginous cell, optionally a microalga, so that the cell produces an oil with a fatty acid profile characterized by 10% or less linolenic acid and 20% or more linoleic acid.

57. The method of claim 56, wherein the cell comprises an overexpressed KASII gene and a FAD gene replacement and optionally,

(a) an exogenous gene encoding an oleate-specific acyl-ACP thioesterase; or

(b) a knockout of one or more FATA alleles, together with an exogenous gene encoding an oleate-specific acyl-ACP thioesterase.

58. The method of claim 57, wherein the overexpression of the FAD gene is by environmental control of a regulatable promoter.

59. The method of any of claims 56 to 58, further comprising extracting the oil from the cell.

60. An oil produced by the method of claim 59.

61. The oil of claim 60, comprising a microalgal sterol profile.

62. A foodstuff or chemical produced from the oil of claim 61 or 62.

63. A method for producing a triglyceride oil, the method comprising:

(a) cultivating an oleaginous cell under nitrogen-replete conditions, thereby increasing the number of cells, then;

(b) cultivating the cells under nitrogen-poor conditions thereby causing the cells to accumulate triglycerides to at least 20% by dry cell weight; comprising a FADc allele, optionally a sole allele, under control of a promoter that is active under the nitrogen replete conditions and inactive under the nitrogen-starved conditions, the promoter retaining at least half of its activity at pH 5.0 as compared to pH 7.0; and

(c) obtaining the oil, wherein the oil comprises reduced linoleic acid due to the downregulation of the FADc gene under the nitrogen-starved conditions.

64. The method of claim 63, where in the cell is cultivated at a pH of less than 6.5 using sucrose in the presence of invertase.

65. The method of claim 63, wherein the invertase is produced by the cell.

66. The method of claim 64 or 65, wherein the invertase is produced from an exogenous gene expressed by the cell.

67. The method of any of claims 64-66, wherein the oil obtained has a fatty acid profile with less than 3%, 2%, 1%, or .5% linoleic acid.

68. The method of any of claims 64-67, wherein the cell further comprises a FADc knockout so as to amplify the change in linoleic acid.

69. The method of any of claims 64-68, wherein the transcript level of FADc decreases by a factor of 10 or more between the nitrogen-replete and nitrogen-starved conditions.

70. A method for producing a triglyceride cell oil comprising cultivating a recombinant cell comprising an exogenous FATB gene and an exogenous KASI gene, wherein the expression of the KASI gene causes the oil to have a shorter chain distribution relative to a control cell with the FATB gene but without the KASI gene.

71. A recombinant cell comprising a FATB acyl-ACP thioesterase gene having at least 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 88% nucleotide identity to SEQ ID NOs:90 or 91 or equivalent sequence due to the degeneracy of the genetic code, or encoding an enzyme having at least 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 88% amino acid identity to SEQ ID NOs:90 or 91.

72. The cell of claim 71, wherein the cell produces triglycerides that are shifted in fatty acid profile due to expression of the FATB gene.

73. A process for producing a oil, the process comprising obtaining a cell oil from a genetically engineered microbe, optionally a microalga, and fractionating the cell oil to produce a stearin fraction characterized by a TAG profile having at least 70% SOS

with no more than 4% trisaturates and an sn-2 profile characterized by at least 90% oleate at the sn-2 position.

74. The process of claim 73, wherein the microbe is a microalga comprising one or more of an overexpressed KASII gene, a SAD knockout or knockdown, or an exogenous C18-preferring FATA gene, an exogenous LPAAT, and a FAD2 knockout or knockdown.
75. The process of claim 73 or 74, wherein the stearin fraction has a maximum heat-flow temperature or DSC-derived SFC curve that is an essentially identical to the equivalent curve of Kokum butter.
76. The process of any of claims 73-75, wherein the fractionation is a two step fractionation performed at a first temperature that removes OOS, optionally about 24°C, and a second temperature that removes trisaturates, optionally about 29°C.
77. A method for producing a triglyceride oil characterized by a TAG profile, the method comprising, consisting of, or consisiting essentially of (a) providing an oleaginous plastidic host cell overexpressing a KASII gene, an exogenous FATA gene and an exogenous LPAAT gene, (b) cultivating the cell so as to produce the oil, and (c) isolating the oil, wherein the TAG profile has greater than 50% SOS an less than 10% trisaturates.
78. The method of claim 77, wherein the cell further comprises a knockdown or knockout of an endogenous SAD2 gene.
79. The method of claim 77 or 78, wherein the cell further comprises a knockdown or knockout of an endogenous FATA gene.
80. The method of any of claims 77 to 79, wherein the exogenous FATA gene encodes a functional FATA acyl-ACP thioesterase protein with at least 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity to SEQ ID NO: 92.
81. The method of any of claims 77 to 80, wherein the exogenous LPAAT gene encodes a functional Lysophosphatidic acid acyltransferase protein with at least 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity to SEQ ID NO: 93.
82. The method of any of claims 77 to 81, wherein the host cell is a microalga, optionally of Trebouxiophyceae, and optionally of the genus Chlorella or Prototheca, and optionally having 23S rRNA with at least 65, 70, 75, 80, 85, 90 or 95% nucleotide sequence identity to SEQ ID NO: 76.
83. A recombinant microlagal host cell optionally of Trebouxiophyceae, and optionally of the genus Chlorella or Prototheca, and optionally having 23S rRNA with at least 65,

70, 75, 80, 85, 90 or 95% nucleotide sequence identity to SEQ ID NO: 76, the host cell expressing an exogenous FATA gene encodes a functional FATA acyl-ACP thioesterase protein with at least 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity to SEQ ID NO: 92.

84. A recombinant microlagal host cell optionally of Trebouxiophyceae, and optionally of the genus Chlorella or Prototheca, and optionally having 23S rRNA with at least 65, 70, 75, 80, 85, 90 or 95% nucleotide sequence identity to SEQ ID NO: 76, the host cell expressing an exogenous LPAAT gene encodes a functional Lysophosphatidic acid acyltransferase protein with at least 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% sequence identity to SEQ ID NO: 93.