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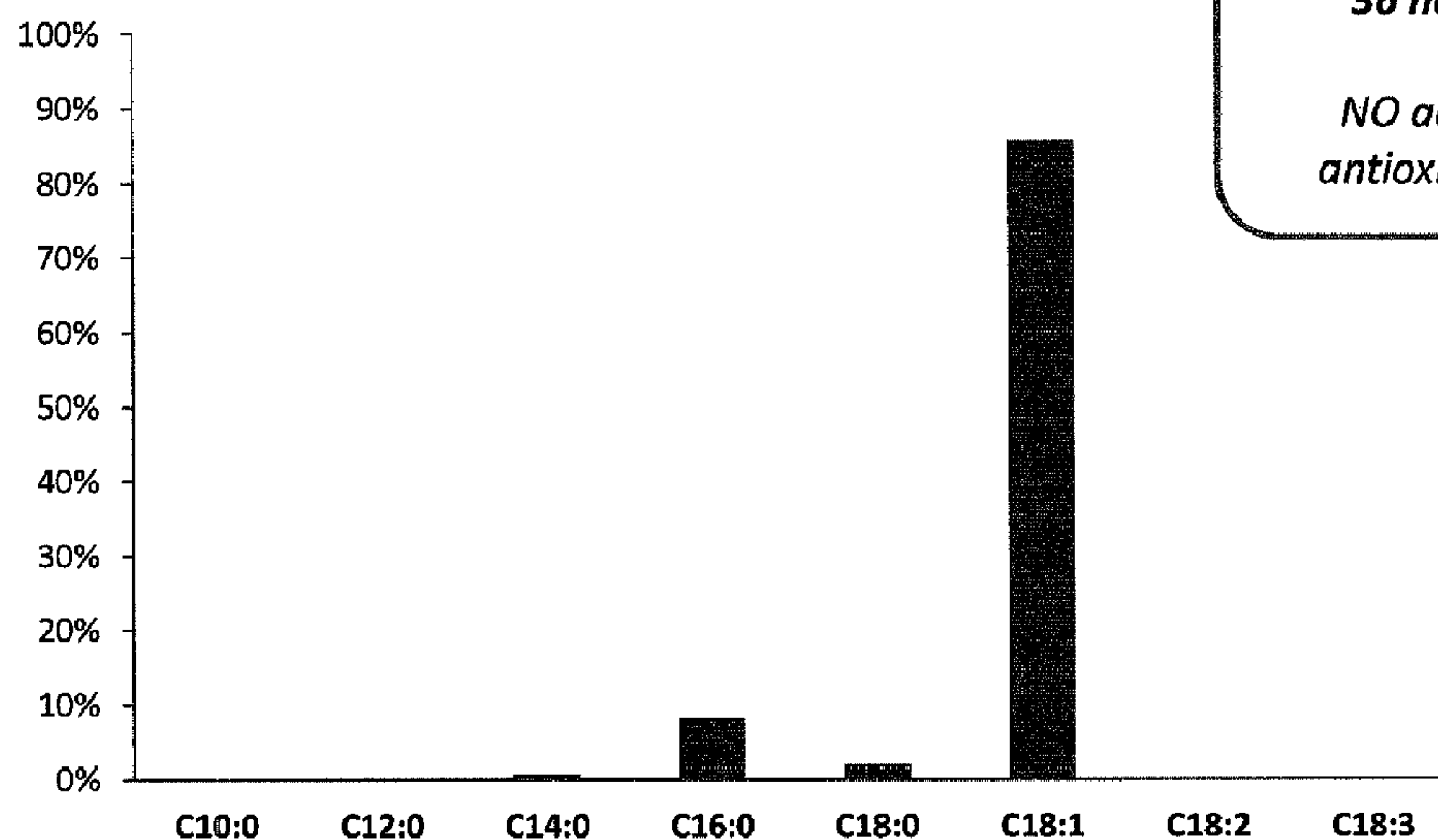
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(54) Titre : HUILES FORMULEES SUR MESURE

(54) Title: TAILORED OILS

High-Stability Oleic Algal Oil

Composition



OSI at 110 ° C
36 hours

NO added
antioxidants

Fatty Acid Chain Length & Degree of Unsaturation

RBD-6

Figure 11

(57) Abrégé/Abstract:

Recombinant DNA techniques are used to produce oleaginous recombinant cells that produce triglyceride oils having desired fatty acid profiles and regiospecific or stereospecific profiles. Genes manipulated include those encoding stearoyl-ACP desturase, delta

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(57) **Abrégé(suite)/Abstract(continued):**

12 fatty acid desaturase, acyl-ACP thioesterase, ketoacyl-ACP synthase, and lysophosphatidic acid acyltransferase. The oil produced can have enhanced oxidative or thermal stability, or can be useful as a frying oil, shortening, roll-in shortening, tempering fat, cocoa butter replacement, as a lubricant, or as a feedstock for various chemical processes. The fatty acid profile can be enriched in midchain profiles or the oil can be enriched in triglycerides of the saturated-unsaturated-saturated type.

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[Continued on next page]

- (54) Title: TAILORED OILS

High-Stability Oleic Algal Oil

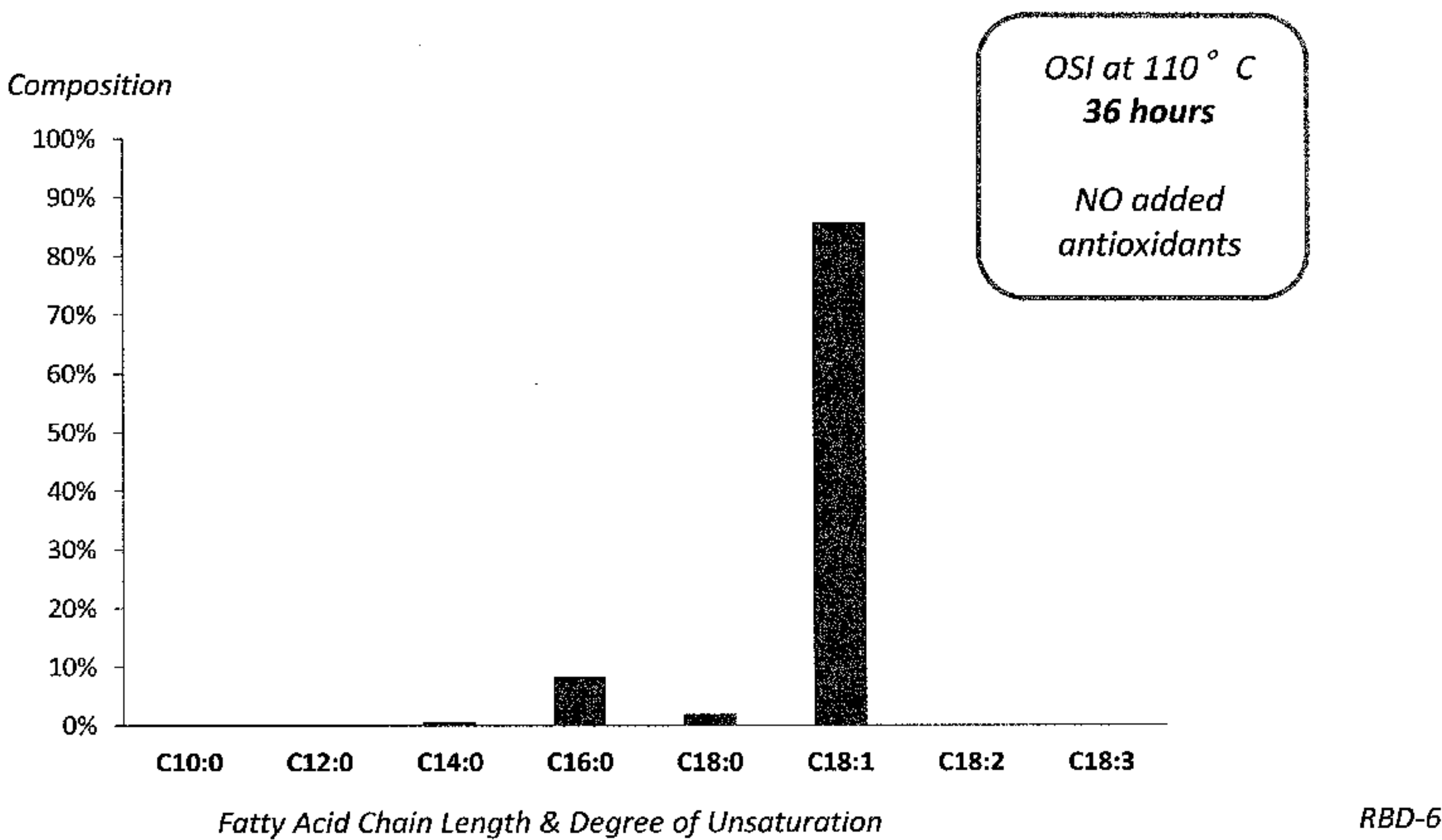


Figure 11

- (57) Abstract: Recombinant DNA techniques are used to produce oleaginous recombinant cells that produce triglyceride oils having desired fatty acid profiles and regiospecific or stereospecific profiles. Genes manipulated include those encoding stearyl-ACP desaturase, delta 12 fatty acid desaturase, acyl-ACP thioesterase, ketoacyl-ACP synthase, and lysophosphatidic acid acyltransferase. The oil produced can have enhanced oxidative or thermal stability, or can be useful as a frying oil, shortening, roll-in shortening, tempering fat, cocoa butter replacement, as a lubricant, or as a feedstock for various chemical processes. The fatty acid profile can be enriched in midchain profiles or the oil can be enriched in triglycerides of the saturated-unsaturated-saturated type.

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TAILORED OILS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 USC 119(e) of US Provisional Patent Application No. 61/635,285, filed April 18, 2012, US Provisional Patent Application No. 61/639,838, filed April 27, 2012, US Provisional Patent Application No. 61/655,469, filed June 4, 2012, US Provisional Patent Application No. 61/672,196, filed July 16, 2012, US Provisional Patent Application No. 61/679,026, filed August 2, 2012, US Provisional Patent Application No. 61/715,998, filed October 19, 2012, US Provisional Patent Application No. 61/769,678, filed February 26, 2013, US Provisional Patent Application No. 61/778,963, filed March 13, 2013, and US Provisional Patent Application No. 61/809,213, filed April 5, 2013, all of which are incorporated by reference in relevant part, with the proviso that the definitions of terms herein shall be the complete and controlling definitions.

REFERENCE TO A SEQUENCE LISTING

[0002] This application includes a sequence listing as shown at the end of the detailed description.

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 oleyl-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, *Protetheca 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 one aspect, the present invention provides an oleaginous microalgal cell, optionally comprising 23S rRNA having at least 65% nucleotide sequence identity to SEQ ID NO: 76, optionally obligately heterotrophic, and optionally comprising an exogenous sucrose invertase gene so that the cell can grow on sucrose as a sole carbon source, wherein the cell comprises an exogenous gene encoding an active LPAAT enzyme, and the cell produces an oil comprising triglycerides, wherein the oil is, by virtue of the LPAAT activity: (a) enriched in triglycerides with midchain fatty acids; or (b) enriched in triglycerides of the saturated-unsaturated-saturated type.

[0010] In some cases, the triglycerides of the oil comprise 40, 50, 60, 70, or 80% or more of C8:0, C10:0, C12:0, C14:0, or C16:0 fatty acids. In some cases, the cell further comprises

an exogenous gene encoding an active FATB acyl-ACP thioesterase. In some cases, the triglycerides of the oil are enriched in midchain fatty acids by greater than 70% as a result of the expression of the exogenous LPAAT and acyl-ACP thioesterase. In some cases, the cell further comprises recombinant nucleic acids operable to encode an exogenous KAS I or KAS IV enzyme to reduce the activity of an endogenous KAS I enzyme. In some cases, the cell further comprises nucleic acids operable to reduce the expression of a delta 12 fatty acid desaturase, optionally via a regulatable promoter, so as to produce an oil with linoleic and linolenic acids totaling 5 area percent or less by FAME GC/FID. In some cases, the oil is enriched in triglycerides of the saturated-unsaturated-saturated type. In some cases, the oil is enriched in SOS, POS, and/or POP. In some cases, the oil comprises triglycerides comprising at least 50% SOS, and optionally less than 10% SSS.

[0011] In some cases, the cell further comprises a knockout or knockdown of a stearyl-ACP desaturase gene, FatA gene, or both. In some cases, the cell further comprises recombinant nucleic acids operable to increase beta-ketoacyl CoA synthase activity. In some cases, the nucleic acids operable to increase beta-ketocyl synthase activity comprise an exogenous gene encoding a beta-ketoacyl CoA synthase.

[0012] In some cases, the ratio of stearate to oleate in oil is $3:1 \pm 20\%$. In some cases, POP, SOS, and POS in the oil comprise at least 30% in sum. In some cases, the oil comprises at least 30% POS. In some cases, the oil comprises POP at $16\% \pm 20\%$, POS at $38\% \pm 20\%$, and SOS at $23\% \pm 20\%$. In some cases, the fatty acid profile of the oil comprises 1 to 4% arachidic acid.

[0013] In some cases, the cell further comprises nucleic acids operable to reduce the expression of a delta 12 fatty acid desaturase, optionally via a regulatable promoter, so as to produce an oil with linoleic and linolenic acids totaling 5 area percent or less. In some cases, the oil has 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 acids.

[0014] In some cases, the LPAAT has the amino acid sequence of SEQ ID NO: 78 or SEQ ID NO: 79 or a sequence having at least 95% identity to SEQ ID NO:78 or SEQ ID NO: 79.

[0015] In another aspect, the present invention provides a method for producing an oil comprising providing or cultivating a cell as discussed above, and extracting the oil, wherein the cell is optionally cultivated heterotrophically.

[0016] In another aspect, the present invention provides an oil comprising triglycerides produced by the method discussed above. In some cases, the oil comprises one or more of: at least 10% ergosterol; ergosterol and b-sitosterol, wherein the ratio of ergosterol to b-sitosterol

is greater than 25:1; ergosterol and brassicasterol; ergosterol, brassicasterol, and poriferasterol, and the oil is optionally free from one or more of β -sitosterol, campesterol, and stigmasterol.

[0017] In some cases, the oil forms β polymorph crystals. In some cases, the crystals have a 2L lamellae structure. In some cases, the crystals have a 3L lamellae structure.

[0018] In some cases, the oil forms β' polymorph crystals. In some cases, the crystals have a 2L lamellae structure. In some cases, the crystals have a 3L lamellae structure.

[0019] In some cases, wherein the triglycerides of the oil have a fatty acid profile characterized in that the sum of the percentage of stearate and palmitate is equal to the percentage of oleate multiplied by 2.0 +/- 40%. In some cases, the oil has greater than 65% SOS triglycerides, less than 45% unsaturated fatty acid, less than 5% unsaturated fatty acids, less than 1% lauric acid, and less than 2% trans fatty acid. In some cases, the sum of the percent stearate and palmitate in the fatty acid profile of the oil is twice the percentage of oleate, $\pm$ 20%. In some cases, the sn-2 profile of the oil has at least 40% oleate. In some cases, the oil is at least 40, 50, 60, 70, 80, or 90% SOS.

[0020] In some cases, the oil is a roll-in shortening having a melting temperature of between 30°C and 40°C. In some cases, the oil comprises β' polymorphic crystals. In some cases, the oil has a solid fat content of less than 15% at 35°C. In some cases, the oil comprises 15 to 20% C8 to C14 fatty acids, 45-50% fatty acids of C16 and higher, and/or 30-25% unsaturated fatty acids.

[0021] In another aspect, the present invention provides a food, fuel or chemical product produced using the oil discussed above.

[0022] In another aspect, the present invention provides a natural oil or RBD oil produced from a natural oil, in which the oil comprises 3.5% or less saturated fatty acids, and optionally comprises greater than 50% oleic acid and/or greater than 1% palmitoleic acid. In some cases, the oil has between 0.1 and 3.5% saturated fatty acids. In some cases, the oil comprises at least 90% oleic acid. In some cases, the oil comprises at least 3% polyunsaturated fatty acids.

[0023] In another aspect, the present invention provides an oleaginous cell, optionally comprising 23S rRNA having at least 65% nucleotide sequence identity to SEQ ID NO: 76 and optionally obligately heterotrophic, wherein the cell produces an oil comprising 3.5% or less saturated fatty acids.

[0024] In some cases, the cell is a microalgal cell, optionally of the genus *Prototheca*. In some cases, the cell further comprises a FATA knockout or knockdown. In some cases, the cell comprises an exogenous gene encoding an enzyme active to desaturate palmitoyl-CoA to

plamitoyl-CoA. In some cases, the exogenous gene is a PAD gene. In some cases, the exogenous gene is a SAD gene having desaturase activity toward palmitoyl-ACP. In some cases, the cell further comprises an overexpressed KAS II enzyme.

[0025] In some cases, the cell further comprises nucleic acids operable to reduce the expression of a delta 12 fatty acid desaturase, optionally via a regulatable promoter, so as to produce an oil with linoleic and linolenic acids totaling 5 area percent or less.

[0026] In another aspect, the present invention provides an oil produced by the cells discussed above, optionally refined, bleached and deodorized, wherein the oil comprises one or more of: at least 10% ergosterol; ergosterol and b-sitosterol, wherein the ratio of ergosterol to b-sitosterol is greater than 25:1; ergosterol and brassicasterol; and ergosterol, brassicasterol, and poriferasterol, and wherein the oil is optionally free from one or more of β -sitosterol, campesterol, and stigmasterol.

[0027] In another aspect, the present invention provides a method for producing an oil having 3.5% or less saturated fatty acids, wherein the method comprises providing or cultivating a cell as discussed above or herein, and extracting the oil from the cell.

[0028] In another aspect, the present invention provides a method for producing a food, wherein the method comprises incorporating an oil produced by the methods discussed above or herein into the food, wherein the finished food product has 3.5% or less saturated fat.

[0029] In another aspect, the present invention provides a recombinant oleaginous cell, optionally comprising 23S rRNA having at least 65% nucleotide sequence identity to SEQ ID NO: 76 and optionally obligately heterotrophic, wherein the cell comprises an exogenous gene encoding an active ketoacyl-CoA synthase enzyme.

[0030] In some cases, the cell produces an oil comprising greater than 20% erucic acid. In some cases, the cell produces an oil comprising greater than 60% erucic acid. In some cases, the cell comprises at least 40% oil. In some cases, the cell is of the genus *Prototheca*, and optionally of the species *Prototheca moriformis*. In some cases, the oil produced by the cell comprises one or more of: at least 10% ergosterol; ergosterol and b-sitosterol, wherein the ratio of ergosterol to b-sitosterol is greater than 25:1; ergosterol and brassicasterol; and ergosterol, brassicasterol, and poriferasterol, wherein the oil is optionally free from one or more of β -sitosterol, campesterol, and stigmasterol.

[0031] In another aspect, the present invention provides a chemical produced from the oil discussed above.

[0032] In another aspect, the present invention provides a method for producing an oil, wherein the method comprises providing or cultivating a cell as discussed above, and extracting an oil from the cell.

[0033] In another aspect, the present invention provides a recombinant oleaginous cell comprising recombinant nucleic acids operable to suppress the activity of a delta 12 fatty acid desaturase gene product so that the cell produces an oil with a triacylglycerol profile having less than 5% linoleic acid. In some cases, the cell produces an oil with a triacylglycerol profile having less than 3% linoleic acid. In some cases, the cell produces an oil with a triacylglycerol profile having less than 2% linoleic acid.

[0034] In some cases, the cell is a linoleic acid auxotroph or activity of the delta 12 fatty acid desaturase can be suppressed via environmental conditions so as to produce the oil. In some cases, the delta 12 fatty acid desaturase is regulatable via environmental conditions due to a regulatable promoter in operable linkage to the delta 12 fatty acid desaturase gene. In some cases, the regulatable promoter is regulatable by change in media pH or nitrogen levels.

[0035] In some cases, the cell further comprises recombinant nucleic acids operable to express an exogenous KAS II, LPAAT, or FATB enzyme. In some cases, the cell further comprises recombinant nucleic acids operable to knockout or knockdown the expression of a stearyl ACP desaturase enzyme. In some cases, the cell further comprises recombinant nucleic acids operable to knockout or knockdown the expression of an endogenous FatA-encoded acyl-ACP thioesterase.

[0036] In some cases, the oil is stable at 110°C so that the inflection point in conductance is not yet reached by 20 hours under conditions of the AOCS Cd 12b-92 Rancimat test. In some cases, the oil is stable at 110°C so that the inflection point in conductance is not yet reached by 5 days under conditions of the AOCS Cd 12b-92 Rancimat test, when 1050ppm of tocopherol and 500 pm of ascorbyl palmitate are added to the oil.

[0037] In another aspect, the present invention provides a method comprising: (a) providing a recombinant oleaginous cell, optionally comprising 23S rRNA having at least 65% nucleotide sequence identity to SEQ ID NO: 76, optionally obligately heterotrophic, wherein the cell comprises recombinant nucleic acids operable to modify the amount of a fatty acid made by the cell in response to a change in an environmental condition; (b) cultivating the cell under a first environmental condition that is permissive to synthesis of the fatty acid so as to allow for cell division and increase the number of cells; (c) cultivating the cell under a second environmental condition that, due to the recombinant nucleic acids,

reduces the synthesis of the fatty acid and thus the amount of that fatty acid in an oil produced by the cell; and (d) extracting the oil from the cell.

[0038] In some cases, the cell comprises exogenous nucleic acids operable to reduce the activity of a delta 12 fatty acid desaturase so as to reduce the amount of linoleic acid in the oil. In some cases, the linoleic acid is depleted in the oil by at least than 50, 60, 70, 80, or 90%.

[0039] In some cases, the cell is cultivated heterotrophically. In some cases, the cell is a microalgal cell. In some cases, the cell produces at least 40, 50, 60, 70, 80, or 90% oil by dry cell weight.

[0040] In some cases, the first environmental condition is a first pH and the second environmental condition is a second pH of cultivation media.

[0041] In some cases, the oil, when extracted from the cell, is stable at 110°C so that the inflection point in conductance is not yet reached by 20 hours under conditions of the AOCS Cd 12b-92 Rancimat test. In some cases, the oil, when extracted from the cell, is stable at 110°C so that the inflection point in conductance is not yet reached by 5 days under conditions of the AOCS Cd 12b-92 Rancimat test, when 1050ppm of tocopherol and 500 pm of ascorbyl palmitate are added to the oil.

[0042] In some cases, the cell comprises an exogenous gene encoding a KAS II enzyme and optionally a knockout or knockdown of a FatA gene. In some cases, the recombinant nucleic acids operable to modify the amount of a fatty acid made by the cell comprise an inhibitory RNA targeting a FAD gene, the production of the inhibitory RNA being under control of a regulatable promoter.

[0043] In some cases, the oil is characterized by a fatty acid profile with greater than 60% oleic acid and less than 3% polyunsaturates. In some cases, the oil is characterized by a fatty acid profile with greater than 70% oleic acid and less than 2% polyunsaturates. In some cases, the oil is characterized by a fatty acid profile with greater than 80% oleic acid and less than 1% polyunsaturates.

[0044] In another aspect, the present invention provides an oil produced by the method discussed above. In some cases, the oil comprises 0.01 to 2% linoleic acid and (i) 80 to 95% oleic acid or (ii) more than 40% of C8, C10, C12, C14 or C16 fatty acids. In some cases, the oil further comprises one or more of: at least 10% ergosterol; ergosterol and β -sitosterol, wherein the ratio of ergosterol to β -sitosterol is greater than 25:1; ergosterol and brassicasterol; and ergosterol, brassicasterol, and poriferasterol.

[0045] In another aspect, the present invention provides a product produced by the oil discussed above. In some cases, the product is a food, fuel or chemical. In some cases, the product is a frying oil, lubricating oil, cleaning solvent, surfactant, foam or lubricant. In some cases, the product is an oleic acid dimer.

[0046] In another aspect, the present invention provides a construct, vector, chromosome or host cell comprising nucleic acids encoding a protein having at least 90% identity to SEQ ID NOs: 77 to 79. In some cases, the nucleic acids encode a protein having at least 95% identity to SEQ ID NOs: 77 to 79. In some cases, the nucleic acids encode a protein having at least 98% identity to SEQ ID NOs: 77 to 79. In some cases, the nucleic acids have at least 80, 90, 95, 96, 97, 98, or 99% sequence identity to SEQ ID NOs:80-85 or equivalent sequences by virtue of the degeneracy of the genetic code.

[0047] 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

[0048] 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;

[0049] Figure 15 shows the stability of different oils as a function of antioxidant concentration, as discussed in Example 5;

[0050] Figure 16 shows various properties of natural oils with very low levels of polyunsaturated fatty acids in accordance with an embodiment of the invention; and

[0051] 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% milkfat; (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% milkfat; and (f) Malaysian cocoa butter. The cocoa butter and cocoa butter milkfat values are literature values (Bailey's Industrial Oils and Fat Products, 6th ed.).

[0052] 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.

[0053] Figure 19 shows various properties of high-oleic and high-stability high-oleic algal oils.

[0054] Figure 20 shows TAG composition of S4495, S5665 and S5675 oils from flask and fermentor 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.

[0055] Figure 21 shows TAG composition of S5774, S5775 and S5776 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.

[0056] 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 morformis* strain as discussed in Example 52.

[0057] Figure 23 shows the pairwise alignment of heterologous FAE proteins expressed in STRAIN Z.

DETAILED DESCRIPTION OF THE INVENTION

I. DEFINITIONS

[0058] An “allele” is any of one or more alternative forms of a gene which relate to one trait or characteristic.

[0059] A “natural oil” or “natural 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 natural oil or natural 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. In connection with a natural oil or natural 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 “natural oil” and “natural fat” encompass such oils obtained from an organism, where the oil has undergone minimal processing, including refining, bleaching and/or degumming, that does not substantially change its triglyceride profile. A natural oil can also be a “noninteresterified natural oil”, which means that the natural 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.

[0060] “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.

[0061] “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.

[0062] “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.

[0063] “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.

[0064] “Microalgae” are 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*.

[0065] 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.

[0066] 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.

[0067] 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. 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.

[0068] 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.

[0069] In connection with a natural 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). 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.

[0070] “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.

[0071] The terms “triglyceride”, “triacylglyceride” and “TAG” are used interchangeably as is known in the art.

II. GENERAL

[0072] 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. Specific examples of cells include heterotrophic or obligate heterotrophic microalgae of the phylum Chlorophyta, the class Trebouxiophytae, the order Chlorellales, or the family Chlorellaceae. Examples of oleaginous microalgae 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, $\pm 5\%$. 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.

[0073] The oleaginous cells express one or more exogenous genes encoding fatty acid biosynthesis enzymes. As a result, some embodiments feature natural oils that were not obtainable from a non-plant or non-seed oil, or not obtainable at all.

[0074] 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. WO2008/151149, WO2010/06032, WO2011/150410, and WO2011/1504 disclose heterotrophic cultivation and oil isolation techniques. 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. 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.

[0075] 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.

[0076] 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, and (v) genetic constructs or cells encoding a newly discovered gene encoding an LPAAT enzyme from *Cuphea* PSR23 (see Example 43). 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.

[0077] 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). 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 natural oil produced can be low in chlorophyll or other colorants. For example, the natural oil can have less than 100, 50, 10, 5, 1, 0.05 ppm of chlorophyll without substantial purification.

[0078] The stable carbon isotope value $\delta^{13}\text{C}$ is an expression of the ratio of $^{13}\text{C}/^{12}\text{C}$ 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 $\delta^{13}\text{C}$ (‰) of the oils can be related to the $\delta^{13}\text{C}$ 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 $\delta^{13}\text{C}$ (‰) of the oil is from -10 to -17 ‰ or from -13 to -16 ‰.

[0079] 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.

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

[0080] 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. 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.

[0081] 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. 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.

[0082] 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 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 thioesterase. Example 36 details the use of RNA hairpin constructs to reduce the expression of FATA1 in *Prototheca*.

[0083] 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 natural 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. In specific embodiments, these transformants produce natural 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.

[0084] In an embodiment the oleaginous cells are cultured. 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).

[0085] 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.

[0086] 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).

[0087] 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 natural oils produced by oleaginous cells.

[0088] 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 stearyl-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. LOW POLYUNSATURATED NATURAL OILS

[0089] In an embodiment of the present invention, the natural oil produced by the cell has very low levels of polyunsaturated fatty acids. As a result, the natural oil can have improved stability, including oxidative stability. The natural 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 $\Delta 12$ desaturase(s) responsible for converting oleic acid (18:1) into linoleic acid (18:2) and/or one or more fatty acyl $\Delta 15$ 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.

[0090] In a specific embodiment, fatty acid desaturase (e.g., $\Delta 12$ 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 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.

[0091] In a specific embodiment, a cell is cultivated using a modulation of linoleic acid levels within the cell. In particular, the natural 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 $\Delta 12$ 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 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, microorganism, 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.

[0092] 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, but predominantly or only during the cell division 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). For example, the majority, and preferably all, of the fatty acid $\Delta 12$ 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.

[0093] 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.

[0094] 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.

[0095] Using one or more of these desaturase regulation methods, it is possible to obtain a natural 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.

[0096] 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 natural 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 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 natural 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.

[0097] 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 natural 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 natural oil produced by RBD treatment of a natural 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 natural oil produced by RBD treatment of a natural 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.

[0098] 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 $\Delta 12$ 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.

[0099] 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).

[0100] 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.

[0101] In a specific embodiment, a high oleic natural 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 natural oil is produced by a cell having recombinant nucleic acids operable to decrease fatty acid $\Delta 12$ desaturase activity and optionally fatty acid $\Delta 15$ 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 that 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. 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.

[0102] 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 natural oils of these embodiments can have tocopherols and tocotrienols levels of 1 g/L or less. In a specific embodiment, a natural 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 natural 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.

[0103] Any of the high-stability (low-polyunsaturate) natural 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 natural 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.

[0104] In another related embodiment, the fatty acid profile of a natural 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.

[0105] 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%.

[0106] 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).

[0107] Blends of the low polyunsaturated natural 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.

[0108] 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.

[0109] 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.

V. CELLS WITH EXOGENOUS ACYLTRANSFERASES

[0110] 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 natural 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.

[0111] 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.

[0112] 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 oleyl-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.

[0113] 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-octadecanoyl-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.

[0114] According to certain embodiments of the present invention, oleaginous cells are transformed with recombinant nucleic acids so as to produce natural 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 natural 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 natural 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.

[0115] 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.

[0116] 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. 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.

[0117] Table 1. Glycerol-3-phosphate acyltransferases and GenBank accession numbers.

glycerol-3-phosphate acyltransferase	<i>Arabidopsis thaliana</i>	BAA00575
glycerol-3-phosphate acyltransferase	<i>Chlamydomonas reinhardtii</i>	EDP02129
glycerol-3-phosphate acyltransferase	<i>Chlamydomonas reinhardtii</i>	Q886Q7
acyl-(acyl-carrier-protein): glycerol-3-phosphate acyltransferase	<i>Cucurbita moschata</i>	BAB39688
glycerol-3-phosphate acyltransferase	<i>Elaeis guineensis</i>	AAF64066
glycerol-3-phosphate acyltransferase	<i>Garcinia mangostana</i>	ABS86942
glycerol-3-phosphate acyltransferase	<i>Gossypium hirsutum</i>	ADK23938
glycerol-3-phosphate acyltransferase	<i>Jatropha curcas</i>	ADV77219

plastid glycerol-3-phosphate acyltransferase	<i>Jatropha curcas</i>	ACR61638
plastidial glycerol-phosphate acyltransferase	<i>Ricinus communis</i>	EEF43526
glycerol-3-phosphate acyltransferase	<i>Vicia faba</i>	AAD05164
glycerol-3-phosphate acyltransferase	<i>Zea mays</i>	ACG45812

[0118] Lysophosphatidic acid acyltransferases suitable for use with the microbes and methods of the invention include, without limitation, those listed in Table 2.

[0119] Table 2. Lysophosphatidic acid acyltransferases and GenBank accession numbers.

1-acyl-sn-glycerol-3-phosphate acyltransferase	<i>Arabidopsis thaliana</i>	AEE85783
1-acyl-sn-glycerol-3-phosphate acyltransferase	<i>Brassica juncea</i>	ABQ42862
1-acyl-sn-glycerol-3-phosphate acyltransferase	<i>Brassica juncea</i>	ABM92334
1-acyl-sn-glycerol-3-phosphate acyltransferase	<i>Brassica napus</i>	CAB09138
lysophosphatidic acid acyltransferase	<i>Chlamydomonas reinhardtii</i>	EDP02300
lysophosphatidic acid acyltransferase	<i>Limnanthes alba</i>	AAC49185
1-acyl-sn-glycerol-3-phosphate acyltransferase (putative)	<i>Limnanthes douglasii</i>	CAA88620
acyl-CoA:sn-1-acylglycerol-3-phosphate acyltransferase	<i>Limnanthes douglasii</i>	ABD62751
1-acylglycerol-3-phosphate O-acyltransferase	<i>Limnanthes douglasii</i>	CAA58239
1-acyl-sn-glycerol-3-phosphate acyltransferase	<i>Ricinus communis</i>	EEF39377

[0120] Diacylglycerol acyltransferases suitable for use with the microbes and methods of the invention include, without limitation, those listed in Table 3.

[0121] Table 3. Diacylglycerol acyltransferases and GenBank accession numbers.

diacylglycerol acyltransferase	<i>Arabidopsis thaliana</i>	CAB45373
diacylglycerol acyltransferase	<i>Brassica juncea</i>	AAV40784
putative diacylglycerol acyltransferase	<i>Elaeis guineensis</i>	AEQ94187
putative diacylglycerol acyltransferase	<i>Elaeis guineensis</i>	AEQ94186
acyl CoA:diacylglycerol acyltransferase	<i>Glycine max</i>	AAT73629

diacylglycerol acyltransferase	<i>Helianthus annuus</i>	ABX61081
acyl-CoA:diacylglycerol acyltransferase 1	<i>Olea europaea</i>	AAS01606
diacylglycerol acyltransferase	<i>Ricinus communis</i>	AAR11479

[0122] Phospholipid diacylglycerol acyltransferases suitable for use with the microbes and methods of the invention include, without limitation, those listed in Table 4.

[0123] Table 4. Phospholipid diacylglycerol acyltransferases and GenBank accession numbers.

phospholipid:diacylglycerol acyltransferase	<i>Arabidopsis thaliana</i>	AED91921
putativephospholipid:diacylglycerol acyltransferase	<i>Elaeis guineensis</i>	AEQ94116
phospholipid:diacylglycerol acyltransferase 1-like	<i>Glycine max</i>	XP_003541296
phospholipid:diacylglycerol acyltransferase	<i>Jatropha curcas</i>	AEZ56255
phospholipid:diacylglycerol acyltransferase	<i>Ricinus communis</i>	ADK92410
phospholipid:diacylglycerol acyltransferase	<i>Ricinus communis</i>	AEW99982

[0124] In 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 more 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 (seperately or in sum).

[0125] 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 particular, specific embodiment, the LPAAT gene has a coding sequence have at least 80, 85, 90, 95, or 98% 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. Plants expressing the novel LPAAT are expressly included in the embodiments and can be produced using known genetic engineering techniques.

VI. CELLS WITH EXOGENOUS ELONGASES OR ELONGASE COMPLEX ENZYMES

[0126] 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 natural 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*.

[0127] 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.

[0128] Table 5. Beta-Ketoacyl-CoA synthases and elongases listed with GenBank accession numbers.

Trypanosoma brucei elongase 3 (GenBank Accession No. AAX70673), *Marchantia 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)

[0129] 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 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.

[0130] 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. In a specific embodiment, the cell is a microalgal cell, optionally cultivated heterotrophically. As in the other embodiments, the 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 Chlorellaceae. 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*.

VII. REGIOSPECIFIC AND STEREOSPECIFIC OILS/FATS

[0131] In an embodiment, a recombinant cell produces a natural 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 form 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 natural 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 semisolid yet free of artificially produced trans-fatty acids. Such fats can also be useful in skin care and other consumer or industrial products.

[0132] As in the other embodiments, the 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 Chlorellaceae. 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.

[0133] In an embodiment, the natural 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.

[0134] In a specific related embodiment, a natural 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 natural 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 natural 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 natural 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.

[0135] In yet another specific embodiment, the sum of the percent stearate and palmitate in the fatty acid profile of the natural fat is twice the percentage of oleate, $\pm 10, 20, 30$ or 40% [e.g., $(\%P + \%S)/\%O = 2.0 \pm 20\%$]. Optionally, the sn-2 profile of this fat is at least 40%, and

preferably at least 50, 60, 70, or 80% oleate. 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.

[0136] 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 than 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.

[0137] In various embodiments the fat forms polymorphs of the β form, β' form, or both, when heated above melting temperature and then 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.

[0138] Optionally, any of these fats can have less than 2 mole % diacylglycerol, or less than 2 mole% mono and diacylglycerols, in sum.

[0139] 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.

[0140] 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 can increase the levels mid-chain saturates. Alternately a gene encoding a SAD enzyme can be overexpressed to increase unsaturates.

[0141] 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.

[0142] 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 produce (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.

[0143] 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).

[0144] 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 be may 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 *Prototheca moriformis* (UTEX 1435) from about 3.7% to about 30.4%. Example 41 provides additional examples of C18:0-preferring acyl-ACP thioesterases function to elevate C18:0 levels in *Prototheca*. Introduction of the 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 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.

[0145] 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. 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.

[0146] 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 $3:1 \pm 30\%$ (i.e., in the range of 2.7:1 to 3.3:1), $3:1 \pm 20\%$ or $3:1 \pm 10\%$.

[0147] 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.

[0148] 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% $\pm$ 20%, POS can be 38% $\pm$ 20%, and SOS can be 23% $\pm$ 20%. Or, POP can be 16% $\pm$ 15%, POS can be 38% $\pm$ 15%, an SOS can be 23% $\pm$ 15%. Or, POP can be 16% $\pm$ 10%, POS can be 38% $\pm$ 10%, an SOS can be 23% $\pm$ 10%.

[0149] 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.

[0150] 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.

[0151] In an embodiment, the natural 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%.

[0152] 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 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. 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.

[0153] 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.

[0154] In an embodiment of the invention there is a natural 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 natural 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 1×10^5 Pa or 1×10^6 Pa. (see Example 46)

[0155] 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 natural fat having greater than 65% SOS can be blended with palm mid-fraction to make a cocoa butter equivalent.

[0156] 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.

[0157] 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.

[0158] In an embodiment, a fat comprises a natural (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 $\leq 15\%$ at 35°C ,
 - (l) a solid fat content of $>15\%$ at 10°C and a solid fat content $\leq 25\%$ 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.

[0159] 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.

[0160] In related embodiments there is a spread, margarine or other food product made with the natural oil or natural oil blend. For example, the natural fat can be used to make an edible W/O 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 natural 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.

[0161] 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 natural oil comprises at least 1, 2, or 5% SRS, where S is stearate and R is ricinoleic acid.

[0162] In an alternate embodiment, a natural oil that is a cocoa butter mimetic as described above 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. In specific embodiments, the melting temperature of the fractionated natural 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% $\pm 20\%$, POS can be 38% $\pm 20\%$, an SOS can be 23% $\pm 20\%$. Or, POP can be 16% $\pm 15\%$, POS can be 38% $\pm 15\%$, an SOS can be 23% $\pm 15\%$. Or, POP can be 16% $\pm 10\%$, POS can be

38%±10%, an SOS can be 23%±10%. In addition, the tristearin levels can be less than 5% of the triacylglycerides.

VIII. HIGH MID-CHAIN OILS

[0163] 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. 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 natural 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.

[0164] Any of these natural 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 natural 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 (natural or interesterified) can optionally be a high stability oil comprising, for example, less than 2% polyunsaturated fatty acids.

[0165] 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.

[0166] 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 chlorophyte 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.

[0167] 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%. 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%.

[0168] 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 cooa-butter/milkfat 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 desturases, 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.

[0169] The high mid-chain oils or fatty acids derived from hydrolysis of these oils may be particularly useful in food, fule 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.

[0170] In some embodiments, the natural oil is interesterified and the kinematic viscosity of the interesterified natural 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 natural 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.

[0171] 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.

IX. HIGH OLEIC/PALMITIC OIL

[0172] 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 an 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

[0173] In an embodiment, a natural oil is produced from a recombinant cell. The oil produced has a fatty acid profile that has less than 4%, 3%, 2%, 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 natural 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 natural 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.

[0174] As described in Example 51, levels of saturated fats may also be reduced by introduction of an exogenous 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, see Example 55) and/or a mutation in an endogenous SAD gene to give PAD activity. Such a 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.

[0175] 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 $\Delta 12$ 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.

[0176] 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% saturated fat.

[0177] 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.

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.

XI. COCOA BUTTER/MILK-FAT BLEND MIMETICS

[0178] In certain embodiments, the cell produces a natural oil that has a temperature-dependent solid fat content (“SFC-curve”) that approximates a blend of cocoa butter and milkfat. Such oils may be used where the cocoa butter/milkfat 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 natural oil approximates. Accordingly, an embodiment of the invention is using a natural oil from a recombinant microalgal cell to replace a cocoa butter/milkfat blend in a recipe. In a related embodiment,

[0179] 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% milkfat, (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% milkfat, and (f) Malaysian cocoa butter. The cocoa butter and cocoa butter milkfat values are literature values (Bailey's Industrial Oils and Fat Products, 6th ed.)

[0180] In an embodiment of the present invention, a natural oil that is similar in thermal properties to a 75% cocoa butter/25% milkfat 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 $38\% \pm 30\%$ at 20°C, $32\% \pm 30\%$ at 25°C, $17\% \pm 30\%$ at 30°C, and less than $5\% \pm 30\%$ at 35°C. For the sake of clarity, $\pm 10\%$ 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 $38\% \pm 20\%$ at 20°C, $32\% \pm 20\%$ at 25°C, $17\% \pm 20\%$ at 30°C, and less than $5\% \pm 20\%$ at 35°C or the oil has a solid fat content (e.g., as determined by NMR) of $38\% \pm 10\%$ at 20°C, $32\% \pm 10\%$ at 25°C, $17\% \pm 10\%$ at 30°C, and less than $5\% \pm 10\%$ at 35°C.

XII. MINOR OIL COMPONENTS

[0181] 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 C24 β stereochemistry. Thus, it is believed that the

molecules detected as campesterol, stigmasterol, and β -sitosterol, are actually 22,23-dihydrobrassicasterol, proferasterol and clionasterol, respectively. Thus, the oils produced by the microalgae described above can be distinguished from plant oils by the presence of sterols with C24 β stereochemistry and the absence of C24 α 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.

[0182] 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.

[0183] Sterol profile analysis is a particularly well-known method for determining the biological source of organic matter. Campesterol, β -sitosterol, and stigmasterol are common plant sterols, with β -sitosterol being a principle plant sterol. For example, β -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).

[0184] 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):

	Sterol	Crude	Clarified	Refined & bleached	Refined, bleached, & deodorized
1	Ergosterol	384	398	293	302

		(56%)	(55%)	(50%)	(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 (Campersterol 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

[0185] 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.

[0186] 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.

[0187] 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.

[0188] 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.

[0189] 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.

[0190] 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.

[0191] 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.

[0192] 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.

[0193] In some embodiments the ratio of ergosterol to brassicasterol is at least 5:1, 10:1, 15:1, or 20:1.

[0194] 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.

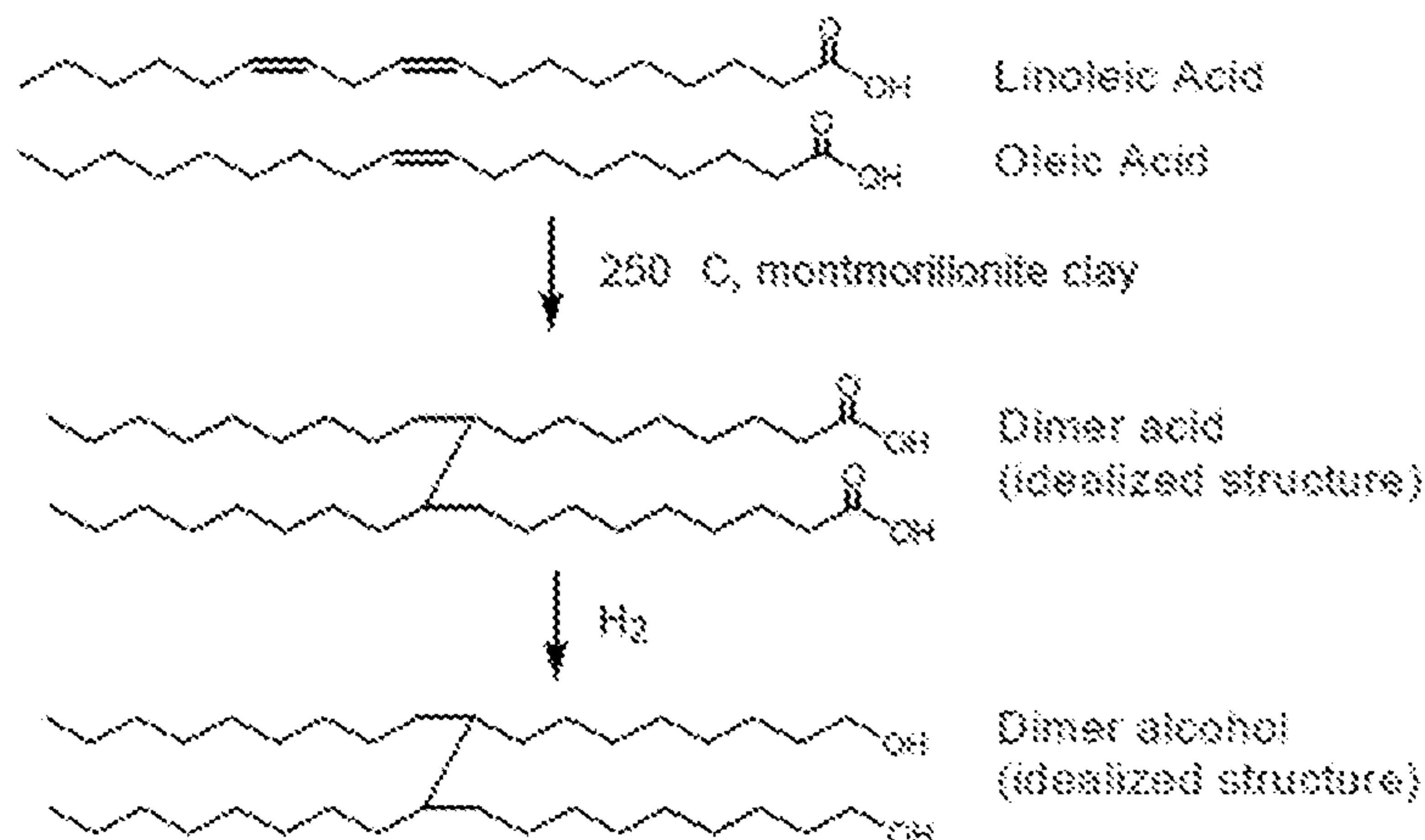
XIII. FUELS AND CHEMICALS

[0195] 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.

[0196] The oils can be converted to alkanes (e.g., renewable diesel) or esters (e.g., methyl or ethyl esters for biodiesel 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.

[0197] 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.

[0198] 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.



[0199] 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.

[0200] 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.

[0201] 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 CO₂ 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.

[0202] 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.

[0203] 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.

[0204] 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.

[0205] 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.

[0206] 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.

[0207] 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.

[0208] 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).

[0209] 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.

[0210] 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.

[0211] 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.

[0212] 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.

[0213] 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.

[0214] 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.

[0215] 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.

[0216] 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.

[0217] 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.

[0218] 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.

[0219] 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.

[0220] 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).

[0221] 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.

[0222] 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.

[0223] 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.

[0224] Suitable methods for isomerization include using alkali isomerization and other oil isomerization known in the art.

[0225] 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.

[0226] 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).

[0227] 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.

[0228] 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.

[0229] 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.

[0230] 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.

[0231] 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.

[0232] 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.

[0233] 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 naphta-type Aeroplane fuel (including Jet B) typically has a carbon number distribution between about 5 and 15 carbons.

[0234] 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).

[0235] 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.

[0236] 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 C₂-C₅ olefins.

[0237] 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 AmberlystTM-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.

[0238] 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 C₂-C₅ olefins are described in U.S. Pat. Nos. 6,538,169, 7,288,685, which are incorporated in their entirety by reference.

[0239] 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.

[0240] 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 about 2 seconds or less.

[0241] 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.

[0242] 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.

[0243] 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.

[0244] 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.

[0245] 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 sulphur and nitrogen compounds are removed. Sulphur 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.

[0246] 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/HDS 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.

[0247] 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 C₂-C₅ olefins are described in U.S. Pat. No. 7,232,935, which is incorporated in its entirety by reference.

[0248] 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 sulphur 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.

[0249] 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/Al₂O₃ and CoMo/Al₂O₃ catalysts are used.

[0250] 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.

[0251] A gaseous stream from the HDO step containing hydrogen is cooled and then carbon monoxide, carbon dioxide, nitrogen, phosphorus and sulphur 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.

[0252] 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 vapour 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.

[0253] 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.

[0254] 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 Al₂O₃ or SiO₂. Typical isomerization catalysts are, for example, Pt/SAPO-11/Al₂O₃, Pt/ZSM-22/Al₂O₃, Pt/ZSM-23/Al₂O₃ and Pt/SAPO-11/SiO₂. 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.

[0255] 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/M³ and 840K/M³. 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.

[0256] 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, 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, 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.

[0257] 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 repellent compositions); 6,924,333 (Paint additives); 6,596,768 (Lipid-enriched ruminant feedstock); and 6,380,410 (Surfactants for detergents and cleaners).

[0258] 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).

[0259] 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.

[0260] 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.

[0261] 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).

[0262] 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.

[0263] 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.

[0264] 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) nitrosyl nitrate, 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.

[0265] 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.

[0266] 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.

[0267] 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.

[0268] 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.

[0269] 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.

[0270] 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.

[0271] 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, the 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.

[0272] 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.

[0273] 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.

[0274] 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.

[0275] 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 is used in conjunction with a separate support suitable for suspending the catalyst. In an embodiment, the condensation catalyst support is silica.

[0276] 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.

[0277] 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-dimethylhexane, 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.

[0278] 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.

[0279] 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.

[0280] 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.

[0281] 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.

[0282] 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%.

[0283] 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.

[0284] 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).

[0285] 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.

[0286] 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.

[0287] 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.

[0288] 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).

[0289] 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).

[0290] 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 alkylidene 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.

[0291] 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.

[0292] 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.

[0293] 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).

[0294] 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).

[0295] 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.

[0296] 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

[0297] Lipid samples were prepared from dried biomass. 20-40 mg of dried biomass was resuspended in 2 mL of 5% H₂SO₄ in MeOH, and 200 μ L 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% K₂CO₃ (aq) to

neutralize the acid. The mixture was agitated vigorously, and a portion of the upper layer was transferred to a vial containing Na₂SO₄ (anhydrous) for gas chromatography analysis using standard FAME GC/FID (fatty acid methyl ester gas chromatography flame ionization detection) methods.

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

[0298] 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 dichloromethane-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 °C 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

[0299] 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.

[0300] 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 (*CwTE2*) 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 *CwTE2* 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.

[0301] 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.

[0302] 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.

[0303] 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

[0304] As shown in Table 6, the fatty acid profile of Strain B expressing *CwTE2* 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.

[0305] The untransformed *P. moriformis* Strain A is 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 CnLPAAT 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.

[0306] 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 analysed 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.”

[0307] 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

[0308] As shown in Table 7, the fatty acid composition of triglycerides (TAGs) isolated from Strain B expressing *CwTE2* 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.

[0309] 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.)

[0310] 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.

[0311] 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.

[0312] 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.

[0313] Table 8, below, gives details of the genetic engineering of the strains identified in Figures 1-13.

[0314] 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 <i>Carthamus tinctorius</i> oleoyl thioesterase

EXAMPLE 5: CHARACTERISTICS OF PROCESSED OIL PRODUCED FROM ENGINEERED MICROORGANISMS

[0315] 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.

[0316] 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* stearyl-ACP desaturase transit peptide (SEQ ID NO: 9). The protein coding regions of CtOTE 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.

[0317] 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.

[0318] 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.

[0319] 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	Tricylglycerides	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 Tricylglycerides	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	%

[0320] 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.

[0321] 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

[0322] Table 11. Solid Fat Content of RBD469 Oil

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

AOCS Cd 12b-93	Solid Fat Content 35 °C	0.25%
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[0323] 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

[0324] RBD469 oil was subjected to transesterification to produce fatty acid methyl esters (FAMES). The resulting FAME profile of RBD469 is shown in Table 12.

[0325] 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

[0326] 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 astericks (*), are the average of multiple OSI runs. Those samples not analyzed are indicated (NA).

[0327] Table 14. Oil Stability Index at 110°C of RBD oil samples with and without antioxidants.

		OSI (hours) for each RBD Sample					
		RBD437	RBD469	RBD502	RBD501	RBD503	RBD529
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

[0328] 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

[0329] 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.

[0330] 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. 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* 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. protothecoides* stearyl-ACP desaturase transit peptide (SEQ ID NO: 9). The codon-optimized sequence of PmKASII comprising a *C. protothecoides* S106 stearyl-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.

[0331] 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.

[0332] 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.

Strain	Transformation Construct(s)	pH	Area % Fatty Acid				
			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

[0333] 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.

[0334] 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

[0335] 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 natural 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.

[0336] 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 *PmFAD2* 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.

[0337] 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 *PmAmt03* 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.

[0338] Table 16. C18:2 fatty acid profiles of *Prototheca moriformis* (UTEX 1435) Strains A, D, and F.

Strain	Transformation Construct (s)	Area % C18:2	
		5.0	7.0
A	None	07	26
D	pSZ1500	01	01
F-1	pSZ1500 + pSZ2413	37	29
F-2	pSZ1500 + pSZ2413	45	87
F-3	pSZ1500 + pSZ2413	50	79
F-4	pSZ1500 + pSZ2413	57	06
F-5	pSZ1500 + pSZ2413	57	58
F-6	pSZ1500 + pSZ2413	60	88
F-7	pSZ1500 + pSZ2413	62	52
F-8	pSZ1500 + pSZ2413	63	79
F-9	pSZ1500 + pSZ2413	77	53

[0339] As shown in Table 16 the impact of regulated expression of the *PmFAD2* 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.

[0340] 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

[0341] 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

[0342] This example describes the use of recombinant polynucleotides that encode a C18:0-preferring *Brassica napus* thioesterase (*BnOTE*) enzyme to engineer a microorganism in which the triacylglyceride distribution of the transformed microorganism has been enriched in SOS, POS, and POP triacylglycerides.

[0343] 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 (*BnOTE*) 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 *BnOTE* 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 *BnOTE* 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.

[0344] 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 *BnOTE* from the *PmAmt03* 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.

[0345] Table 17. Effect of *BnOTE* 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

[0346] Table 18. Effect of *BnOTE* 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

[0347] As shown in Table 17, the fatty acid composition of TAGs isolated from Strain G expressing *BnOTE* 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.

[0348] 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.

[0349] 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

[0350] 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.

[0351] 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 saccharophila*, *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 microadriaticum*, *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.

[0352] 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.

[0353] Tables 19A-D. Codon preference listing.

Amino Acid	Codon	<i>Chlorella sorokiniana</i>	<i>Chlorella vulgaris</i>	<i>Chlorella ellipsoidea</i>	<i>Chlorella kessleri</i>	<i>Dunaliella tertiolecta</i>	<i>Volvox carteri</i>	<i>Haemato coccus pluvialis</i>
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	<i>Closterium peracerosum-strigosum-littorale complex</i>	<i>Dunaliella viridis</i>	<i>Dunaliella salina</i>	<i>Gonium pectorale</i>	<i>Phaeodactylum tricornutum</i>	<i>Chaetoceros compressum</i>
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	<i>Cylindro-theca fusiformis</i>	<i>Amphidinium carterae</i>	<i>Symbiodinium microadriaticum</i>	<i>Nannochloropsis</i> sp	<i>Cyclotella cryptica</i>	<i>Navicula pelliculosa</i>	<i>Thalassiosira pseudonana</i>	<i>C. reinhardtii</i>
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

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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

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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	<i>Yarrowia lipolytica</i>	<i>Mortierella alpina</i>	<i>Rhodococcus opacus</i>
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

[0354] Table 20. Lipid biosynthesis pathway proteins.

<i>3-Ketoacyl ACP synthase</i> <i>Cuphea hookeriana</i> 3-ketoacyl-ACP synthase (GenBank Acc. No. AAC68861.1), <i>Cuphea wrightii</i> beta-ketoacyl-ACP synthase II (GenBank Acc. No. AAB37271.1), <i>Cuphea lanceolata</i> beta-ketoacyl-ACP synthase IV (GenBank Acc. No. CAC59946.1), <i>Cuphea wrightii</i> beta-ketoacyl-ACP synthase II (GenBank Acc. No. AAB37270.1), <i>Ricinus communis</i> ketoacyl-ACP synthase (GenBank Acc. No. XP_002516228), <i>Gossypium hirsutum</i> ketoacyl-ACP synthase (GenBank Acc. No. ADK23940.1), <i>Glycine max</i> plastid 3-keto-acyl-ACP synthase II-A (GenBank Acc No. AAW88763.1), <i>Elaeis guineensis</i> beta-ketoacyl-ACP synthase II (GenBank Acc. No. AAF26738.2), <i>Helianthus annuus</i> plastid 3-keto-acyl-ACP synthase I (GenkBank Acc. No. ABM53471.1), <i>Glycine max</i> 3-keto-acyl-ACP synthase I (GenkBank Acc. No. NP_001238610.1), <i>Helianthus annuus</i> plastid 3-keto-acyl-ACP synthase II (GenBank Acc ABI18155.1), <i>Brassica napus</i> beta-ketoacyl-ACP synthetase 2

(GenBank Acc. No. AAF61739.1), *Perilla frutescens* beta-ketoacyl-ACP synthase II (GenBank Acc. No. AAC04692.1), *Helianthus annuus* 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 curcas* beta-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), *Garcinia 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), *Vicia 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 annuus* 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*

[0355] 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 $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$ 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.

[0356] In an embodiment of the present invention, vector pSV72-NRg, comprising nucleotide sequence encoding the *Chlorella vulgaris* nitrate reductase (CvNR) 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 CvNR promoter upstream of the protein-coding sequence and operably linked to the CvNR 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 CvNR 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 KH₂PO₄, 0.5g/L K₂HPO₄, 0.25 g/L MgSO₄·7H₂O, 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*

[0357] 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 Renouvelables*, 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 Renouvelables*, Volume 14 (2011), pp. 21-26.

[0358] 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 NaNO_3 . 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 , $1.25 \text{ g l}^{-1} \text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $20 \text{ mg l}^{-1} \text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ with micronutrients and low-nitrogen medium (comprising 203 mg/l $(\text{NH}_4)_2\text{HPO}_4$, 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*

[0359] 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 pBinU Ω NP-1 into *Chlorella ellipsoidea*. The nucleotide sequence of pBinU Ω NP-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 pBinU Ω NP-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 pBinU Ω NP-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 K₂HPO₄, 0.2 g/L MgSO₄·7H₂O, 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 pBinU Ω NP-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 pBinU Ω NP-1 was suitable for use as a selectable marker in *Chlorella ellipsoidea*.

[0360] In an embodiment of the present invention, vector pBinU Ω NP-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*

[0361] 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 Plantarum*, 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 (*nptII*) 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 pBI121 plasmid, transformants of *Chlorella kessleri* were obtained that were propagated in selective culture medium comprising 15 mg/L kanamycin. The expression of the *nptII* 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*.

[0362] 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*

[0363] 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*.

[0364] 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 *rbcS1* promoter upstream of the protein-coding sequence and operably linked to the *rbcS1* 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*

[0365] 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).

[0366] 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*

[0367] 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 KNO₃, 0.03 g/L Na₂HPO₄, 0.246 g/L MgSO₄·7H₂O, 0.11 g/L CaCl₂·2H₂O, 2.62 mg/L Fe_(III)citrate x H₂O, 0.011 mg/L CoCl₂·6H₂O, 0.012 mg/L CuSO₄·5H₂O, 0.075 mg/L Cr₂O₃, 0.98 mg/L MnCl₂·4H₂O, 0.12 mg/L Na₂MoO₄ x 2H₂O, 0.005 mg/L SeO₂ 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).

[0368] 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

[0369] 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 KNO₃, 0.015 g/L Ca(NO₃)₂·4H₂O, 0.05 g/L glycerophosphate-Na₂, 0.04 g/L MgSO₄·7H₂O, 0.5 g/L Tris (hydroxymethyl) aminomethane, trace minerals, biotin, vitamins B₁ and B₁₂) 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).

[0370] 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*

[0371] 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 (NO₃⁻) 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 KNO₃. 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.

[0372] In an embodiment of the present invention, vector pDVNR, comprising the nucleotide sequence encoding the *Dunaliella viridis* nitrate reductase (*DvNR*) 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*

[0373] 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 pU Ω HBsAg-CAT plasmid into *Dunaliella salina*. pU Ω HBsAg-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. pU Ω HBsAg-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 pU Ω HBsAg-CAT, *Dunaliella salina* was unable to propagate on medium comprising 60 mg/L chloramphenicol. Upon transformation with the pU Ω HBsAg-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 reporteds that the CAT resistance cassette encoded on pUΩHBsAg-CAT was suitable for use as a selectable marker in *Dunaliella salina*.

[0374] 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*

[0375] 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 $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 12.4 mg/L KH_2PO_4 , 50 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 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 $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 1 mg/L $(\text{NH}_4)_6\text{MO}_7\text{O}_2 \cdot 4.4\text{H}_2\text{O}$, 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 $\text{Na}_4\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) 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*.

[0376] 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 Jawaorski'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*

[0377] 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 (*fcgA*) upstream of the *ble* protein-coding region and operably linked to the 3' UTR/terminator of the *Phaeodactylum tricornutum* *fcgA* 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*.

[0378] 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.*

[0379] 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 pTpfc_p/nat into *Chaetoceros sp.* pTpfc_p/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 (*fc_p*) promoter upstream of the *nat* protein-coding region and operably linked to the *Thalassiosira pseudonana* *fc_p* 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 pTpfc_p/nat, *Chaetoceros sp.* was unable to propagate on medium comprising 500 ug/ml nourseothricin. Upon transformation with pTpfc_p/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 Guillard, 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 pTpfcfcp/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 pTpfcfcp/nat was suitable for use as a selectable marker in *Chaetoceros* sp.

[0380] In an embodiment of the present invention, vector pTpfcfcp/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*

[0381] 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*.

[0382] 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.

[0383] 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.*

[0384] 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 microadriaticum*

[0385] Expression of recombinant genes in accordance with the present invention in *Symbiodinium microadriaticum* 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 microadriaticum* with plasmid DNA. Using the transformation technique of silicon fibre-mediated microinjection, ten Lohuis introduced the plasmid pMT NPT/GUS into *Symbiodinium microadriaticum*. 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 microadriaticum* was unable to be propagated on medium comprising 3 mg/ml G418. Upon transformation with pMT NPT/GUS, transformants of *Symbiodinium microadriaticum* were obtained that were propagated in selective culture medium comprising 3 mg/ml G418. The expression of the *nptII* gene product in *Symbiodinium microadriaticum* 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 microadriaticum*. Detectable activity of the GUS reporter gene indicated that CaMV 35S promoter and 3'UTR are suitable for enabling gene expression in *Symbiodinium microadriaticum*. Evaluation of the genomic DNA of the stable transformants was performed by Southern analysis. ten Lohuis and Miller reported liquid propagation of *Symbiodinium microadriaticum* 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 microadriaticum* transformants on agar medium comprising seawater supplemented with F/2 enrichment solution and 3 mg/ml G418. Propagation of *Symbiodinium microadriaticum* 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 microadriaticum* 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 microadriaticum*. 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 microadriaticum*.

[0386] 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 microadriaticum* to reflect the codon bias inherent in nuclear genes of *Symbiodinium microadriaticum* 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 microadriaticum* 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 microadriaticum* 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 microadriaticum* transformed with the transformation vector in, but not limited to, seawater agar medium comprising G418. Growth media suitable for *Symbiodinium microadriaticum* 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 microadriaticum* lipids can be assessed through standard lipid extraction and analytical methods described herein.

EXAMPLE 26: Engineering *Nannochloropsis* sp.

[0387] 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.

[0388] 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*

[0389] 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*.

[0390] 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*

[0391] 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*.

[0392] 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*

[0393] 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 pTpfcf/nat in to *Thalassiosira pseudonana*. pTpfcf/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 pTpfcfcp/nat, *Thalassiosira pseudonana* was unable to propagate on medium comprising 10 ug/ml nourseothricin. Upon transformation with pTpfcfcp/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 pTpfcfcp/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 pTpfcfcp/nat was suitable for use as a selectable marker in *Thalassiosira pseudonana*.

[0394] In an embodiment of the present invention, vector pTpfcfcp/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*

[0395] 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*.

[0396] 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* *RbcS2* promoter upstream of the protein-coding sequence and operably linked to the *Chlamydomonas reinhardtii* *RbcS2* 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*

[0397] 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*. Springer-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*.

[0398] 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 alpina*

[0399] Expression of recombinant genes in accordance with the present invention in *Mortierella alpina* 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'-phosphoribosyl)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% NH₄SO₄, 0.02% MgSO₄·7H₂O, 0.0001% FeCl₃·6H₂O, 0.1%, trace elements, 10 mM K₂HPO₄-NaH₂PO₄), 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*.

[0400] 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

[0401] 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 pNC9501, 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 Alvarez *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.

[0402] 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

[0403] Strain B of Example 3, *Prototheca moriformis* (UTEX 1435) engineered to express a *Cuphea wrightii* thioesterase (CwTE2), 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.

[0404] 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. 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 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

[0405] 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).

[0406] 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.

[0407] 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

[0408] 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.

[0409] 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 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

[0410] 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).

[0411] 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

[0412] 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.

[0413] 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.

Construct	Area % Fatty Acid						
	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

[0414] 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.

[0415] 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

[0416] 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.

[0417] 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 (CwTE2), 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.

[0418] 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.

[0419] Table 24. Plasmid constructs used to transform *Prototheca moriformis* (UTEX 1435) Strain B.

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

pSZD1133	<i>Cuphea hookeriana</i>	Native	SEQ ID NO: 49
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[0420] Table 25. Fatty acid profiles of *Prototheca moriformis* (UTEX 1435) Strain Bengineered for increased C10, lauric acid, and total saturated fatty acids.

Plasmid construct(s)	No.	Fatty Acid (Area%)								
		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

pSZD1133										
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[0421] 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 of 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.

[0422] 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

[0423] 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.

[0424] 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 (CwTE2), 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. protothecoides* 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.

[0425] 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.

[0426] 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.

Strain	Transformation plasmid(s)	Fatty Acid (Area%)							
		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, pSZ2302	0.16	1.43	7.49	38.88	1.30	37.58	8.73	9.08
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

[0427] 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.

Strain	Transformation plasmid(s)	Fatty Acid (Area%)							
		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-5	pSZ1283, pSZ2302	14.75	47.28	10.32	6.04	0.59	15.50	4.65	72.35
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

[0428] 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).

[0429] 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.

[0430] 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

[0431] 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.

[0432] 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.

[0433] 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

[0434] 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.

[0435] Table 29. Fatty acid profiles of *Prototheca moriformis* (UTEX 1435) cells engineered to express hairpin RNA constructs targeting KASI gene/gene products.

Strain	Plasmid	Number	Fatty Acid (Area%)							
			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

[0436] 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.

[0437] 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 ELONGASE OVEREXPRESSION

[0438] This example describes the use of recombinant polynucleotides that encode 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.

[0439] 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.

[0440] Table 30. Plasmid constructs used to transform *Prototheca moriformis* (UTEX 1435) Strain J.

Plasmid construct	Elongase source	GenBank Accession No.	SEQ ID NO:
pSZ2328	<i>Marchantia polymorpha</i>	AAP74370	58, 59
pSZ2324	<i>Trypanosoma brucei</i>	AAX70673	60, 61
pSZ2323	<i>Saccharomyces cerevisiae</i>	P39540	62, 63

[0441] 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.

[0442] 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	C18:3 α	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

[0443] 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.

[0444] 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

[0445] 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.

[0446] 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.

[0447] 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:
pSZD581	FATA, CAA52070	<i>Brassica napus</i>	native	64, 65
pSZD643	FATA, CAA52070	<i>Brassica napus</i>	UTEX 250 SAD	66, 67
pSZD645	FATA, AAA33019	<i>C. tinctorius</i>	UTEX 250 SAD	68, 69
pSZD644	FATA, ABS30422	<i>Ricinis communis</i>	native	70, 71
pSZD1323	FATA, AAB51523	<i>G. mangostana</i>	native	72, 73
pSZD1320	FATA	<i>Theobroma cacao</i>	native	74, 75

[0448] 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.

[0449] Table 33. Fatty acid profiles of *Prototheca moriformis* (UTEX 1435) Strain J cells engineered to overexpress exogenous acyl-ACP thioesterase enzymes.

Strain	Plasmid construct	No.	Fatty Acid Area %					
			C14:0	C16:0	C18:0	C18:1	C18:2	C18:3 α
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

[0450] 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.

[0451] 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

[0452] 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.

[0453] 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

[0454] 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 (*CuPSR23*) were identified by analysis of a combination of *CuPSR23* 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.

[0455] 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 *CuPSR23* *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 is indicated by lowercase, boxed text. The initiator ATG and terminator TGA for the *CuPSR23 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.

[0456] pSZ2299 Transforming Construct

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[0457] pSZ2300 Transforming Construct

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(SEQ ID NO:91)

[0458] To determine the impact of the *CuPSR23 LPAAT2* and *LPAAT3* genes on mid-chain fatty acid accumulation, the above constructs containing the codon optimized *CuPSR23 LPAAT2* or *LPAAT3* genes driven by the UTEX 1453 *AMT3* promoter were transformed into strain B.

[0459] 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 *CuPSR23 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 *CuPSR23 LPAAT2* and D1521 represents clones of Strain B with *CuPSR23 LPAAT3*.

[0460] 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

[0461] The transgenic *CuPSR23 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 *CuPSR23 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 *CuPSR23 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.

[0462] 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*

[0463] 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.

[0464] 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 *CuPSR23 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.

[0465] pSZ2575 Transforming Construct

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cgctcgtcgtccgggagtcaggaccggcggaataagaggcaacacactccgcttcttagctcttcg (SEQ ID
NO:92)

[0466] To determine the impact of the *CuPSR23 LPAATx* gene on fatty acid accumulation, the above construct containing the codon optimized *CuPSR23 LPAATx* gene driven by the UTEX 1453 *AMT3* promoter was transformed into strain B.

[0467] 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 *CuPSR23 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 *CuPSR23 LPAATx*.

[0468] 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

[0469] The transgenic *CuPSR23 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 *CuPSR23 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 *CuPSR23 LPAATx* appears to favor incorporation of longer chain fatty acids into TAGs.

[0470] 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, *CuPSR23 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

[0471] 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.

[0472] **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*

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 cgccgtggccgtgcgccccgagcccccccaggagttctgggagcagctggagccctgcaagatggccgaggacaagcgcac
 ttcttgaggagcaccgcacccgcggcaacgaggtgggcccctcccagcgctgaccatcacccgcgtggccaacatcctgca
 ggaggccgcccggcaaccacgccgtggccatgtggggccgctcctccgagggttcgccaccgacccccgagctgcaggaggcc
 ggccgtatcttcgtgatgaccgcattgcagatccagatgtaccgctacccccgctggggcgacctgatgcaggtggagacctggt
 ccagaccgcccggcaagctggggcgccccagcgcgagtgggtgctgcgcgacaagctgaccggcgaggccctgggcgcccgcac
 ctctcctgggtgatgatcaacatccgcacccgcccctgcccattgcccgagctgggtgcgcgtgaagtccgccttcttcgcc
 gcgagccccccgctggccctgcccccgccgtgaccgcgccaagctgccaacatcgccacccccgccccctgcgcggc
 caccgccaggtggccccgcgcaccgacatggacatgaacggccacgtgaacaacgtggcctacctggcctggtgctggaggc
 cgtgcccgagcacgtgttctccgactaccacctgtaccagatggagatcgacttcaaggccgagtgcacgcggcgacgtgatc
 tcctcccaggccgagcagatcccccccaggaggccctgaccacaaacggcgccggccgcaacccctcctgcttcgtgcactcc
 atcctgcgcgcccagaccgagctgggtgcgcgccccgaccacctgggtccgcccccatcgacgccccgcccgaagcccccaa
 ggccctccacatggactacaaggaccacgacggcgactacaaggaccacgacatcgactacaaggacgacgacgacaag**T**
GAatcgatagatctcttaaggcagcagcagctcggatagtatcgacacactctggacgctggtcgtgtgatggactgttccgcccac
 acttgctgccttgacctgtgaatatccctgccgcttttatcaaacagcctcagtggtgttgatcttgtgtgtacgcgcttttgcgagttgctag
 ctgcttgtgtatattgcgaataaccacccccagcatcccccttcctcgtttcataatcgcttgcacccaaccgcaacttatctacgtgtcctg
 ctatccctcagcgctgctcctgctcctgctcactgccccctgcacagccttggttgggctccgctgtattctcctgggtactgcaacctgt
 aaaccagcactgcaatgctgatgcacgggaagtagtgggatgggaacacaaatggaaagcttaattaa**gagctcttgtttccagaa**
ggagttgctccttgagcctttcattctcagcctcgataacctccaaagccgctctaattgtggagggggttcgaatttaaaagctt
ggaatgttggttcgtgcgtctggaacaagcccagacttggtgctcactgggaaaaggaccatcagctccaaaaaacttgccgc
tcaaacgcgtacctctgcttgcgcgaatctgccctgttgaaatgccaccacattcatattgtgacgcttgagcagctctgtaat
tgcctcagaatgtggaatcatctgccccctgtgcgagcccatgccaggcatgtcgcgggcgaggacacccgccactcgtacag

cagaccattatgctacctcacaatagttcataacagtgaccatatttctcgaagctccccaacgagcacctccatgctctgagtg
gccaccccccgccctgggtgcttgcggagggcaggtcaaccggcatggggctaccgaaatccccgaccggatcccaccaccc
ccgcgatgggaagaatctctccccgggatgtgggcccaccaccagcacaacctgctggcccaggcgagcgtcaaaccatacc
acacaaatatecttggcatcgccctgaattccttctgccgctctgctaccgggtgcttctgtccgaagcaggggtgctaggga
tcgctccgagtcgcgaacccctgtcgcgtggcggggcttgttcgagcttgaagagc (SEQ ID NO:93)

[0476] 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.

[0477] 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.

[0478] 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 stearyl-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 STRAIN J.

[0479] 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
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[0480] 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

[0481] 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: NATURAL OILS SUITABLE FOR USE AS ROLL-IN SHORTENINGS

[0482] 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 7×10^6 Pa- 1.8×10^7 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.

[0483] 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

[0484] 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 analysed 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.

[0485] 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 REPRESSSION OF A SECOND SAD ALLELE

[0486] The oleaginous, non-photosynthetic alga, *Protetheca 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.

[0487] 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.

[0488] 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 S1920. A version of the *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.

[0489] Nucleotide sequence of the the transforming DNA from pSZ2282:

gctcttcgggtcgccgctgcctcgcgctcccctgggtggtgcgcgcggtcgccagcgaggccccgctgggcggttcgccctcggtgca
gcgccccctccccgtggtctactccaagctggacaagcagcaccgcctgacgcccagcgctggagctggtgcagagcatggggc
agtttgcgaggagagggtgctgcccgtgctgcaccccgtggacaagctgtggcagccgcaggacttttgcggacccccgagtcgc
ccgacttcgaggatcaggtggcgagctgcgcgcgcgcgccaaggacctgcccagcagctactttgtggtgctggtgggggacatg
atcacggaggaggcgctgccgacctacatggccatgctcaacacgctggacggcgctgcgcgacgacacggggcgcgccgaccacc
cgtgggcgcgctggacgcggcagtggtggccgaggagaaccggcacggcgacctgctgaacaagtactgctggctgacggggc
gcgtcaacatgcgggccgtggaggtgacctcaacaacctgatcaagagcgccatgaacccgcagacggacaacaaccttattt
ggggttcgtctacacctcctccaggagcgcgccaccaagtaggtacccttcttgcgctatgacacttcagcaaaaggtaggcg
ggctgcgagacggcttcccggcgctgcatgcaacaccgatgatgcttcgacccccgaagctccttcggggctgcatgggcgctccg
atgccgctccaggcgagcgctgtttaatagccaggccccgattgcaaagacattatagcgagctaccaaagccatattcaaac
acntagatcactaccacttctacacaggccactcgagcttgtgatcgactccgctaagggggcgccctcttctcttcgtttcagtcac
aacccgcaaacggcgcgccATGctgctgcaggccttcctgttcctgctggccggcttcgccgccaagatcagcgccctccatgac

gaacgagacgtccgaccgccccctggtgcacttcaccccccaacaagggctggatgaacgaccccaacggcctgtggtacgac
 gagaaggacgccaagtggcacctgtacttccagtacaacccgaacgacaccgtctgggggacgcccttgttctggggccacg
 ccacgtccgacgacctgaccaactgggaggaccagcccatcgccatcgccccgaagcgcaacgactccggcgcttctccggc
 tccatggtggtggactacaacaacacctccggcttcttaacgacaccatcgacccgcgccagcgctgctggtggccatctggacc
 tacaacacccccggagtccgaggagcagtacatctctacagcctggacggcggttacaccttcaccgagtaccagaagaacc
 ccgtgctggccgccaactccacccagttccgcgacccgaaggcttctggtacgagccctcccagaagtggatcatgaccgcgg
 ccaagtcccaggactacaagatcgagatctactcctccgacgacctgaagtctggaagctggagtccgcgttcgccaacgag
 ggcttctcggctaccagtacgagtgccccggcctgatcgagggtccccaccgagcaggacccagcaagtctactgggtgat
 gttcatctccatcaacccccggcgccccggcgggcctccttaaccagtacttcgtcggcagcttcaacggcaccacttcgag
 gccttcgacaaccagtcccgcgtggtggacttcggcaaggactactacgccctgcagaccttcttaacacccgacccgacctac
 gggagcgccctgggcatcgctggtggcctccaactgggagtactccgccttcgtgcccaccaacccctggcgctcctccatgtccc
 tcgtgcgcaagttctccctcaacacccgagtaccaggccaacccggagacggagctgatcaacctgaaggccgagccgatcct
 gaacatcagcaacgccggccccctggagccgggttcgccaccaacaccacggttgacgaaggccaacagctacaacgtcgacctg
 tccaacagcaccggcaccctggagttcgagctggtgtacgccgtcaacacccaccagacgatctccaagtccgtgttcgcgga
 cctctccctctggttcaagggcctggaggaccccgaggagtacctccgcatgggcttcgaggtgtccgcgtcctccttcttctgg
 accgcgggaacagcaaggtgaagttcgtgaaggagaacccctacttcaccaaccgcatgagcgtgaacaaccagcccttca
 agagcgagaacgacctgtcctactacaaggtgtacggcttgctggaccagaacatcctggagctgtacttcaacgacggcga
 cgctcgtgtccaccaacacctacttcatgaccaccgggaacgccctgggctccgtgaacatgacgacgggggtggacaacctgt
 tctacatcgacaagttccaggtgcgcgaggtcaag**TGAcaattg**GCAGCAGCAGCTCGGATAGTATCGACACACTCTGGAC
 GCTGGTCGTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCCGCTTTTATCAAACAGCCTCA
 GTGTGTTTGATCTTGTGTGTACGCGCTTTTGCAGATTGCTAGCTGCTTGTGCTATTTGCGAATACCACCCCCAGCATCCCCT
 TCCCTCGTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCTATCCCTCAGCGCTGCTCCTGCTCCTGCT
 CACTGCCCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCTCCTGGTACTGCAACCTGTAAACCAGCACTGCAATGCT
 GATGCACGGGAAGTAGTGGGATGGGAACACAAATGGAG**ggatccc**gcgtctcgaacagagcgcgagaggaacgctgaaggt
 ctcgcctctgtcgacctcagcgcggcatacaccacaataaccacctgacgaatgcgcttggttcttcgtccattagcgaagcgtccg
 gttcacacacgtgccacgttggcgaggtggcaggtgacaatgatcggtggagctgatggtcgaaacgttcacag**cctagggatatc**
gaattcctttcttgcgctatgacacttcagcaaaaaggtagggcgggctgcgagacggcttccggcgctgcatgcaacaccgatga
 tgcttcgacccccgaagctccttcggggctgcatggcgctccgatgccgtccagggcgagcgctgtttaatagccaggccccc
 gattgcaaagacattatagcgagctaccaaagccatattcaaacacctagatcactaccacttctacacaggccactcgagcttgtg
 atcgacactccgctaagggggcgctccttctccttcgtttcagtcacaacccgcaaac**actagtGCGCTGGACGCGGCAGTG**
GGTGGCCGAGGAGAACCGGCACGGCGACCTGCTGAACAAGTACTGTTGGCTGACGGGGCGCGTC
AACATGCGGGCCGTGGAGGTGACCATCAACAACCTGATCAAGAGCGGCATGAACCCGCAGACGG

ACAACAACCCTTACTTGGGCTTCGTCTACACCTCCTTCCAGGAGCGCGCGACCAAGTACAGCCACGG
CAACACCGCGCGCCTTGCGGCCGAGCAGTGTGTTTGAGGGTTTTGGTTGCCCGTATCGAGGTCCTGG
TGGCGCGCATGGGGGAGAAGGCGCCTGTCCCGCTGACCCCCCGGCTACCCTCCCGGCACCTTCCAG
GGCGCGTACGggatccTGCTCGGCCGCAAGGCGCGCGGTGTTGCCGTGGCTGTACTTGGTCGCGCGC
TCCTGGAAGGAGGTGTAGACGAAGCCCAAGTAAGGGTTGTTGTCCGTCTGCGGGTTCATGCCGCT
CTTGATCAGGTTGTTGATGGTCACCTCCACGGCCCGCATGTTGACGCGCCCCGTACGCCAACAGTAC
TTGTTACAGAGGTCGCCGTGCCGTTCTCCTCGGCCACCCACTGCCGCGTCCAGCGCaagcttGCAGCAG
 CAGCTCGGATAGTATCGACACACTCTGGACGCTGGTCGTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGA
 ATATCCCTGCCGCTTTTATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGCGAGTTGCTAGCTGCTTGTG
 CTATTTGCGAATACCACCCCCAGCATCCCCTTCCCTCGTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCT
 GCTATCCCTCAGCGCTGCTCCTGCTCCTGCTCACTGCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCTCCTGGTA
 CTGCAACCTGTAAACCAGCACTGCAATGCTGATGCACGGGAAGTAGTGGGATGGGAACACAAATGGAAAGCTG**gagctc**
cagccacggcaacaccgcgccttgccggccgagcacggcgacaagaacctgagcaagatctgcgggctgatcgccagcgacga
gggcccggcacgagatcgctacacgcgcacatcgctggacgagttcttcgcctcgaccccaggggcgccgtcgccgcctacgccaaca
tgatgcgcaagcagatcacatgcccgcgcacctcatggacgacatgggcccagggcaggccaacccgggcccgaacctcttcgc
cgacttctccgcggtcgccgagaagatcgacgtctacgacgccgaggactactgccgcatcctggagcacctcaacgcgcgctgga
aggtggacgagcgccaggtcagcggccaggccgcccgcggaccaggagtagctcctgggctgccccagcgcttccggaaactcgc
cgagaagaccgcccgaagcgcaagcgcgctcgcgcgagggccgctcgcttctcctggatctccgggcccgcgagatcatggtctagg
gagcgacgagtgctgctgccccgctggcgggagtgggacgccctcctcgctcctctctgttctgaacggaacaatcgggcaccg
cgctacgcgccacgcatcgagcaacgaagaaaacccccgatgataggttgcggtggctgccgggatatagatccggccgcacat
caaagggcccctccgccagagaagaagctcctttccagcagactcctgaagagc (SEQ ID NO: 94)

[0490] Identification and analysis of SAD2 knockout/knockdown strains: Construct D1283, derived from pSZ2282, was transformed into S1920 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 with pSZ2282 into S1920 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.

[0491] Table 39. Fatty acid profiles of D1283 [pSZ2282] primary transformants, compared to the wild-type parental strain, S1920.

Strain		S1920	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
	C18:3 α	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

[0492] In Table 39, Stearate (C18:0) levels greater than the wild-type level are highlighted with bold text.

[0493] 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. S4495 had the highest level of C18:0 (~44%) and the best lipid titer (~26%) relative to the S1920 parent, and so was selected for further fermentation development.

[0494] 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, S1920.

Primary		T342:D1283-4				T342:D1283-7		
Strain		S1920	S4490	S4491	S4492	S4493	S4494	S4495
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	29.58	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	38.99	41.41	44.42
	C18:1	56.96	13.52	15.58	13.49	13.57	12.98	15.64
	C18:2	5.50	8.01	7.85	7.65	10.37	9.47	5.72
	C18:3 α	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

[0495] In Table 40, Stearate (C18:0) levels greater than the wild-type level are highlighted with bold text.

[0496] We optimized the performance of S4495 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 S4495 fermentations are summarized in Table 41, below. Fermentation of S4495 under optimal conditions yielded nearly 44% C18:0, which was similar to the stearate level that accumulated in shake flask assays. S4495 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.

[0497] Table 41. Fatty acid profiles and lipid titers of S4495, compared to a control transgenic strain S2074.

Strain		S2074	S4495	S4495	S4495
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
	C18:3 α	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

[0498] In Table 41, Stearate (C18:0) levels greater than the control are highlighted with bold text. S2074 contains *S. cerevisiae* SUC2, encoding sucrose invertase, integrated at the 6S locus, and has a fatty acid profile that is indistinguishable from the S1920 wild-type parent.

[0499] **Constructs used for *KASII* overexpression in S4495:** DNA construct pSZ2734 was made to overexpress a codon-optimized *P. moriformis* *KASII* gene in S4495. 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, BglIII, AflIII, 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, underlined, uppercase italics, while a 3xFLAG epitope encoding sequence is in bold italics. A second *C. vulgaris* NR 3' UTR is indicated by small capitals.

[0500] Nucleotide sequence of the the transforming DNA from pSZ2734:

gctcttcgccgcccactcctgctcgagcgcgcccgcgctgcgccgcccagcgccttggccttttcgccgcgctcgtgcgcgtcgct
gatgtccatcaccaggtccatgaggtctgccttgcgccggctgagccactgcttcgtccgggcgcccaagaggagcatgagggagg
actcctggtccagggctctgacgtggtcgcggtctgggagcgggccagcatcatctggctctgcgcaccgaggccgcctccaact
ggtcctccagcagccgcagtcgcccgcaccctggcagaggaagacaggtgaggggtgtatgaattgtacagaacaaccacgagc
cttgtctaggcagaatccctaccagtcattggctttacctggatgacggcctgcgaacagctgtccagcgaccctcgctgccgccgctt
ctccgcacgcttctttccagcaccgtgatggcgcgagccagcgccgcacgctggcgctgcgcttcgccgatctgaggacagtcggg
gaactctgatcagtcctaaaccccttgcgcgttagtggtgccatcctttgcagaccggtgagagccgacttggtgtgcgccacccccca
caccacctctccagaccaattctgtcaccttttggcgaaggcatcgccctcgccctgcagagaggacagcagtgcccagccgct
gggggttggcggtgacgcgctcaggtacccttcttgcgctatgacacttcagcaaaaggtagggcgggctgcgagacggcttccc
ggcgctgcatgcaacaccgatgatgcttcgacccccgaagctccttcggggctgcatggcgctccgatgccgctccagggcgag
cgctgtttaaatagccaggccccgattgcaaagacattatagcgagctaccaaagccatattcaaacacctagatcactaccactt
ctacacaggccactcgagcttgatgcactccgctaagggggcgccctcttctcttcgtttcagtcacaacccgcaaadtctagaa
 tatcaATGatcgagcaggacggcctccacgcccggctccccgcccgcctgggtggagcgccctgttcggctacgactgggcccag
 cagaccatcggtgctccgacgcccgcgtgttccgctgtccgcccaggggccgccccgtgctgttcgtgaagaccgacctgtccg
 gcgcctgaacgagctgcaggacgaggccgcccgcctgtcctggctggccaccaccggcgctgccctgcgcgcccgtgctggac
 gtggtgaccgaggccggccgcgactggctgctgctgggaggtgcccggccaggacctgctgtcctccacctggccccgc
 cgagaagggtgtccatcatggccgacgccatgcgcgcctgcacacctggacccccgccacctgccccttcgaccaccaggcca
 agcaccgcatcgagcgcgcccgcacccgcatggaggccggcctggtggaccaggacgacctggacgaggagcaccagggc
 ctggccccgcccagctgttcgcccgcctgaaggcccgcagtcggcgagggacctggtggtgaccacggcgacgcctg
 cctgcccacatcatggtggagaaacggccgcttctccggcttcacgactgcggccgcctgggctggccgaccgctaccagg
 acatcgccctggccacccgcgacatcgccgaggagctgggcccgcagtgggccgaccgcttctggtgctgtacggcatcgcc
 gccccgactcccagcgcatcgcccttctaccgctgctggacgagttcttcTGAcaattgGCAGCAGCAGCTCGGATAG
 TATCGACACACTCTGGACGCTGGTCGTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGT
 GAATATCCCTGCCGCTTTTATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGCGAG
 TTGCTAGCTGCTTGTGCTATTTGCGAATACCACCCCCAGCATCCCCTTCCCTCGTTTCATATCGCTTGC
 ATCCCAACCGCAACTTATCTACGCTGTCTGCTATCCCTCAGCGCTGCTCCTGCTCCTGCTCACTGCCC

CTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCTCCTGGTACTGCAACCTGTAAACCAGCACTGCA
 ATGCTGATGCACGGGAAGTAGTGGGATGGGAACACAAATGG**Aggatccc**gcgtctcgaacagagcgcgcaga
 ggaacgctgaaggctcgcctctgtcgcacctcagcgcggcatacaccacaataaccacctgacgaatgcgcttggttcttcgtccat
 tagcgaagcgtccggttcacacacgtgccacgttggcgaggtggcaggtgacaatgatcggaggagctgatggcgaaacgttcac
 ag**cctagggatato**CTGAAGAATGGGAGGCAGGTGTTGTTGATTATGAGTGTGTAAAAGAAAGGGGTA
 GAGAGCCGTCCTCAGATCCGACTACTATGCAGGTAGCCGCTCGCCCATGCCCGCCTGGCTGAATATT
 GATGCATGCCCATCAAGGCAGGCAGGCATTTCTGTGCACGCACCAAGCCCACAATCTTCCACAACAC
 ACAGCATGTACCAACGCACGCGTAAAAGTTGGGGTGCTGCCAGTGCGTCATGCCAGGCATGATGTG
 CTCCTGCACATCCGCCATGATCTCCTCCATCGTCTCGGGTGTTTCCGGCGCCTGGTCCGGGAGCCGTT
 CCGCCAGATACCCAGACGCCACCTCCGACCTCACGGGGTACTTTTCGAGCGTCTGCCGGTAGTCGAC
 GATCGCGTCCACCATGGAGTAGCCGAGGCGCCGGAAGTGGCGTGACGGAGGGAGGAGAGGGGAGG
 AGAGAGAGGGGGGGGGGGGGGGGGGGGATGATTACACGCCAGTCTCACAACGCATGCAAGACCCGT
 TTGATTATGAGTACAATCATGCACTACTAGATGGATGAGCGCCAGGCATAAGGCACACCGACGTTG
 ATGGCATGAGCAACTCCCGCATCATATTTCTATTGTCCTCACGCCAAGCCGGTCACCATCCGCATGC
 TCATATTACAGCGCACGCACCGCTTCGTGATCCACCGGGTGAACGTAGTCCTCGACGGAAACATCTG
 GCTCGGGCCTCGTGCTGGCACTCCCTCCCATGCCGACAACCTTTCTGCTGTCACCACGACCCACGATG
 CAACGCGACACGACCCGGTGGGACTGATCGGTTCACTGCACCTGCATGCAATTGTCACAAGCGCAT
 ACTCCAATCGTATCCGTTTGATTTCTGTGAAAACCTCGCTCGACCGCCCGCGTCCCGCAGGCAGCGAT
 GACGTGTGCGTGACCTGGGTGTTTCGTGAAAGGCCAGCAACCCCAAATCGCAGGCGATCCGGAGA
 TTGGGATCTGATCCGAGCTTGGACCAGATCCCCCACGATGCGGCACGGGAACTGCATCGACTCGGC
 GCGGAACCCAGCTTTCGTAAATGCCAGATTGGTGTCCGATACCTTGATTGTCATCAGCGAAACAAG
 ACTTCAGCAGCGAGCGTATTTGGCGGGCGTGCTACCAGGGTTGCATACATTGCCCATTTCTGTCTGG
 ACCGCTTTACCGGCGCAGAGGGTGAGTTGATGGGGTTGGCAGGCATCGAAACGCGCGTGTCATGGT
 GTGTGTGTCTGTTTTCGGCTGCACAATTTCAATAGTCGGATGGGCGACGGTAGAATTGGGTGTTGC
 GCTCGCGTGTCATGCCTCGCCCCGTGGGTGTCATGACCGGGACTGGAATCCCCCCTCGCGACCCTCC
 TGCTAACGCTCCCGACTCTCCCGCCCGCGCGCAGGATAGACTCTAGTTCAACCAATCGACA**actagtAT**
 GCAGACCGCCACCAGCGCCCCCCCCACCGAGGGCCACTGCTTCGGCGCCCGCCTGCCACCGCCTCCC
 GCCGCGCCGTGCGCCGCGCCTGGTCCCGCATCGCCCGCG**ggcgcgccGCCGCCGCCGCCGACGCCAAC**
CCCGCCCGCCCCGAGCGCCGCGTGGTGATCACCGGCCAGGGCGTGGTGACCTCCCTGGGGCCAGACC
ATCGAGCAGTTCTACTCCTCCCTGCTGGAGGGCGTGTCCGGCATCTCCAGATCCAGAAGTTTCGACA

CCACCGGCTACACCACCACCATCGCCGGCGAGATCAAGTCCCTGCAGCTGGACCCCTACGTGCCCAA
 GCGCTGGGCCAAGCGCGTGGACGACGTGATCAAGTACGTGTACATCGCCGGCAAGCAGGCCCTGG
 AGTCCGCCGGCCTGCCCATCGAGGCCGCCGGCCTGGCCGGCGCCGGCCTGGACCCCGCCCTGTGCG
 GCGTGCTGATCGGCACCGCCATGGCCGGCATGACCTCCTTCGCCGGCGGTGGAGGCCCTGACCC
 GCGGCGGCGTGCGCAAGATGAACCCCTTCTGCATCCCCTTCTCCATCTCCAACATGGGCGGGGCCAT
 GCTGGCCATGGACATCGGCTTCATGGGCCCCAACTACTCCATCTCCACCGCCTGCGCCACCGGCAAC
 TACTGCATCCTGGGCGCCGCCGACCACATCCGCCGCGGCGACGCCAACGTGATGCTGGCCGGCGGC
 GCCGACGCCGCCATCATCCCCTCCGGCATCGGCGGCTTCATCGCCTGCAAGGCCCTGTCCAAGCGCA
 ACGACGAGCCCGAGCGCGCCTCCCGCCCCCTGGGACGCCGACCGCGACGGCTTCGTGATGGGCGAG
 GCGCGCCGGCGTGCTGGTGCTGGAGGAGCTGGAGCACGCCAAGCGCCGCGGCGCCACCATCCTGGC
 CGAGCTGGTGGGCGGCGCCGACCTCCGACGCCACCATGACCGAGCCCGACCCCGAGGGCCG
 CGGCGTGCGCCTGTGCCTGGAGCGCGCCCTGGAGCGCGCCCGCCTGGCCCCCGAGCGCGTGGGCTA
 CGTGAACGCCACGGCACCTCCACCCCGCCGGCGACGTGGCCGAGTACCGCGCCATCCGCGCCGT
 GATCCCCAGGACTCCCTGCGCATCAACTCCACCAAGTCCATGATCGGCCACCTGCTGGGCGGGCGCC
 GGCGCCGTGGAGGCCGTGGCCGCCATCCAGGCCCTGCGCACCGGCTGGCTGCACCCCAACCTGAAC
 CTGGAGAACCCCGCCCCCGGCGTGGACCCCGTGGTGCTGGTGGGCCCCCGCAAGGAGCGCGCCGA
 GGACCTGGACGTGGTGCTGTCCAACCTCCTTCGGCTTCGGCGGCCACAACCTGCGTGATCTTCCGC
 AAGTACGACGAGATGGACTACAAGGACCACGACGGCGACTACAAGGACCACGACATCGACTACA
AGGACGACGACGACAAGTGAAatcgatagatctcttaagGCAGCAGCAGCTCGGATAGTATCGACACACTC
 TGGACGCTGGTTCGTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCC
 GCTTTTATCAACAGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGCGAGTTGCTAGCTGCTT
 GTGCTATTTGCGAATACCACCCCGAGCATCCCCTTCCCTCGTTTCATATCGCTTGCATCCCAACCGCAA
 CTTATCTACGCTGTCCTGCTATCCCTCAGCGCTGCTCCTGCTCCTGCTCACTGCCCTCGCACAGCCTT
 GGTTTGGGCTCCGCCTGTATTCTCCTGGTACTGCAACCTGTAAACCAGCACTGCAATGCTGATGCAC
 GGAAGTAGTGGGATGGGAACACAAATGGAAagcttaattaagagctcttgttttccagaaggagttgctccttgag
cctttcattctcagcctcgataacctccaaagccgctctaattgtggagggggttcgaatttaaaagcttggaatgttggttcgtgcgt
ctggaacaagcccagacttggtgctcactgggaaaaggaccatcagctccaaaaaacttgccgctcaaaccgctacctctgcttc
gcgcaatctgccctgttgaaatcgccaccacattcatattgtgacgcttgagcagctctgtaattgcctcagaatgtggaatcatctgcc
ccctgtgcgagcccatgccaggcatgtcgggcgaggacacccgccactcgtacagcagaccattatgctacctcacaatagttca
taacagtgaccatatttctcgaagctccccaacgagcacctccatgctctgagtggccacccccggccctggtgcttgccggagggc
aggtcaaccggcatggggctaccgaaatccccgaccggatcccaccacccccgcgatgggaagaatctctccccgggatgtgggcc
caccaccagcacaacctgctggcccaggcgagcgtcaaaccataccacacaaatatccttggcatcgccctgaattccttctgccg

ctctgctacccggtgcttctgtccgaagcaggggttgctagggatcgctccgagtcgcaaaccttgtcgcggtggcggggcttggtc
gagcttgaagagc (SEQ ID NO:95)

[0501] Overexpression of *KASII* in Strain X: Construct D1643 derived from pSZ2734 was transformed into S4495 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 S4495 with D1643 are summarized in Table 42, below. Overexpression of *KASII* in the *SAD2* knockout/knock-down S4495 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 S4495.

[0502] Table 42. Fatty acid profiles of D1653 [pSZ2734] primary transformants, compared to the S4495 base strain and the wild-type parental strain, S1920.

Strain	S1920	S4495	D1653-83	D1653-10A	D1653-28	D1653-38	D1653-7A	D1653-75	D1653-90	D1653-26	D1653-72	D1653-68	D1653-82	D1653-66
Fatty Acid Area %	C12:0	0.04	0.06	0.27	0.13	0.20	0.18	0.24	0.13	0.12	0.27	0.16	0.18	0.25
	C14:0	1.44	1.06	1.55	1.55	1.79	1.67	1.70	1.53	1.58	1.74	1.57	1.64	1.58
	C16:0	29.23	29.83	8.16	11.45	10.68	10.11	9.37	11.14	11.08	9.40	9.78	9.95	8.32
	C16:1	0.88	0.10	0.04	0.10	0.00	0.00	0.00	0.04	0.04	0.00	0.04	0.00	0.05
	C18:0	2.97	40.17	54.25	53.87	53.61	53.46	53.32	53.15	52.43	52.20	51.23	50.52	50.02
	C18:1	58.07	20.15	23.92	23.12	22.10	23.48	24.02	22.73	23.45	23.94	23.21	26.07	28.00
	C18:2	0.25	0.25	0.75	0.75	0.42	0.23	0.56	0.19	0.98	0.88	0.28	0.31	0.31
	C18:3 α	0.50	0.56	0.79	0.88	0.70	0.79	0.79	0.26	0.82	0.88	0.78	0.78	0.76
	C20:0	0.21	1.38	3.11	3.81	3.01	2.91	3.02	2.88	2.77	3.21	2.74	2.80	2.80
	C20:1	0.02	0.07	0.19	0.21	0.38	0.27	0.28	0.12	0.11	0.41	0.14	0.30	0.28
	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
	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
sum C18	67.76	66.24	85.31	82.92	83.01	83.98	84.60	83.09	83.35	84.11	84.47	84.30	85.89	85.45
saturates	33.07	73.52	68.94	70.63	70.11	69.12	68.37	69.72	69.33	67.93	67.30	66.54	64.01	64.03
unsaturates	66.71	26.23	31.29	29.26	29.74	30.79	31.65	29.83	30.38	32.09	32.46	33.46	35.70	35.75

[0503] In Table 42, Stearate (C18:0) levels greater than the wild-type level are highlighted with bold text. Palmitate (C16:0) levels lower than S4495 or S1920 are highlighted with bold. For three strains the ratio of saturated to unsaturated fatty acids is $\leq 2:1$; these are highlighted with bold, italicized text.

[0504] 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 S4495 parent. The saturates:unsaturates ratios were substantially reduced compared to S4495. Strains S5665 and S5675 were selected for evaluation in 3-L high-density fermentations.

[0505] Table 43. Shake flask assays of strains derived from D1653, expressing *KASII*, driven by the *PmSAD2-2* promoter, targeted to the 6S locus.

Primary				D1653-68		D1653-98		D1653-10A		D1653-72		D1653-89	
Strain		S1920	S4495	S5664	S5665	S5666	S5670	S5671	S5672	S5673	S5674	S5675	S5677
Fatty Acid Area%	C10:0	0.02	0.04	0.08	0.09	0.12	0.06	0.06	0.08	0.09	0.12	0.12	0.12
	C12:0	0.04	0.09	0.28	0.29	0.35	0.20	0.20	0.23	0.26	0.32	0.32	0.33
	C14:0	1.42	1.12	1.81	1.66	1.73	1.75	1.72	1.50	1.61	1.38	1.43	1.38
	C16:0	25.59	28.56	8.39	8.61	9.44	9.99	10.11	8.26	8.85	6.81	7.21	6.53
	C16:1	1.93	0.10	0.06	0.03	0.08	0.06	0.06	0.04	0.04	0.03	0.03	0.03
	C18:0	1.60	40.13	47.60	52.47	55.12	50.25	49.73	54.56	54.01	52.96	53.68	52.12
	C18:1	62.08	29.74	27.78	23.93	21.31	25.37	25.70	22.86	22.87	24.37	23.99	25.17
	C18:2	6.16	5.83	7.98	7.52	7.72	7.55	7.64	7.20	7.24	8.11	7.83	8.04
	C18:3 α	0.40	0.89	1.21	1.22	1.49	1.17	1.07	1.20	1.29	1.28	1.24	1.31
	C20:0	0.18	1.82	2.62	2.93	2.76	2.63	2.66	2.97	2.72	3.43	3.10	3.59
	C20:1	0.04	0.13	0.37	0.36	0.39	0.34	0.34	0.35	0.34	0.48	0.41	0.47
	C20:1	0.07	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.09
	C20:1	0.15	0.06	0.11	0.09	0.11	0.10	0.10	0.09	0.10	0.12	0.10	0.12
	C22:0	0.02	0.20	0.28	0.30	0.24	0.29	0.28	0.30	0.27	0.32	0.29	0.35
	C24:0	0.09	0.09	0.16	0.29	0.00	0.03	0.15	0.16	0.02	0.05	0.04	0.07
sum C18		71.23	67.68	84.57	85.13	85.63	84.34	84.13	85.81	85.40	86.71	86.73	86.63
saturates		29.86	71.97	62.22	66.63	68.74	65.20	64.90	68.06	67.90	65.37	66.17	64.57
unsaturates		69.91	27.76	37.50	33.15	31.07	34.57	34.89	31.73	31.67	34.37	33.59	35.13
% parent lipid titer		100.0	88.2	43.4	48.8	27.5	44.1	47.4	59.0	47.6	43.2	48.4	44.4

[0506] In Table 43, S4495 is the parent strain; S1920 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 S1920 and S4495 are highlighted bold. Bold italics indicate that the saturates:unsaturates ratio is $\leq 2:1$.

[0507] The fatty acid profiles and performance metrics of strains S5665 and S5675 are detailed in Table 44, below. The fatty acid profile of the parent strain S4495, grown under the same fermentation conditions, is presented for comparison. The strains that over-express *KASII* accumulate about 11% more C18:0 than the S4495 parent. C16:0 is reduced to 7-9%, and levels of unsaturated fatty acids increase by 4-5%. The lipid titers of S5665 and S5675 were comparable to S4495, indicating that *KASII* over-expression did not have deleterious effects on lipid production.

[0508] Table 44. End point fatty acid profiles of biomass from S4495, S5665 and S5775 fermentations.

Strain	S4495	S5665	S5675
Fermentation	120580F1	130097F3	130098F4
pH	5	5	5
temp (°C)	32	32	32
[N] (mM)	300	300	300
N/P	1.4	1.4	1.4
DO%	30	30	30
sugar	S70	S70	S70
Fe (μM)	557.5	557.5	557.5
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	43.87	56.88	57.24
C18:1	17.63	21.57	21.66
C18:2	9.58	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

[0509] The fermentations were cultured for 6 days using a fed-batch process. The S4495 fatty acid profile from fermentation 120580F1 was presented in Table 41, and is shown again in Table 44 for comparison with S5665 and S5675. All fermentations were carried out at 32°C, pH 5, with a nitrogen/phosphorus (N/P) ratio of 1.4, 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.

[0510] 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 S5665 and S5675, 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 S4495 oils. These results demonstrate that *KASII* over-expression in a high-stearate background significantly improves SOS accumulation, and reduces the accumulation of trisaturated TAGs.

[0511] Constructs used for *FATA-1* disruption, *KASII* over-expression and *FAD2*

RNAi in S1920: 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 S1920. 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, AflIII, 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, underlined, uppercase italics, while a 3xFLAG epitope encoding sequence is in bold 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.

[0512] Nucleotide sequence of the the transforming DNA from pSZ2419:

gctcttcggagtcactgtgccactgagttcgactggtagctgaatggagtcgctgctccactaaacgaattgtcagcaccgccagcc
ggccgaggacccgagtcatagcgagggtagtagcgcgccatggcaccgaccagcctgcttgccagtactggcgtctctccgcttct
ctgtggtcctctgcgcgctccagcgcggtgcgctttccggtggatcatgcggtccgtggcgaccgcagcggccgctgccatgcagc
gccgctgcttccgaacagtggcggtcagggccgcacccgcggtagccgtccgtccggaacccgcccaagagttttgggagcagctt
gagccctgcaagatggcggaggacaagcgcatcttctggaggagcaccgggtgcgtggagggtccggggctgaccggccgctcgcat
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atgcgttccttcacctctcatttctcatttctgaatccctccctgctcactcttctcctccttcccgttcacgcagcattcggggtaacc
cttcttgcgctatgacacttccagcaaaaggtagggcggtgcgagacgggttcccggcgctgcatgcaacaccgatgatgcttc
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ctccgctaagggggcgcttctccttctggttcagtcacaacccgcaaac**ggcgcgcc****ATG**ctgctgcaggccttctgttctgtct
ggccggcttcgccgccaagatcagcgctccatgacgaacgagacgtccgaccgccccctggtgcacttcacccccaaacagg
gctggatgaacgacccccaacggcctgtggtacgacgagaaggacgccaagtggcacctgtacttccagtacaacccgaacg
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gccccgaagcgcaacgactccggcgcttctccggctccatggtggtggactacaacaacacctccggcttcttcaacgacacc
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gtacgagccctcccagaagtggatcatgaccgcggccaagtcccaggactacaagatcgagatctactcctccgacgacctga
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accgagcaggacccccagcaagtcctactgggtgatgttcatctccatcaacccggcgccccggcgggcgtccttcaaccag
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cggagctgatcaacctgaaggccgagccgatcctgaacatcagcaacgccggccccctggagccggttcgccaccaacaccac
gttgacgaaggccaacagctacaacgtcgacctgtccaacagcaccggcaccctggaggttcgagctggtgtacgccgtcaac
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accaaccgcatgagcgtgaacaaccagccctcaagagcgagaacgacctgtcctactacaaggtgtacggcttgctggacc
agaacatcctggagctgtacttcaacgacggcgacgtcgtgtccaccaacacctacttcatgaccacggggaacgccctgggc
tccgtgaacatgacgacgggggtggacaacctgttctacatcgacaagttccaggtgcgcgaggtcaag**TGA****caattg****GCA**
GCAGCAGCTCGGATAGTATCGACACACTCTGGACGCTGGTCGTGTGATGGACTGTTGCCGCCACACT

TGCTGCCTTGACCTGTGAATATCCCTGCCGCTTTTATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGT
 ACGCGCTTTTGCGAGTTGCTAGCTGCTTGTGCTATTTGCGAATACCACCCCCAGCATCCCCTTCCCTC
 GTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCTATCCCTCAGCGCTGCTCCTG
 CTCCTGCTCACTGCCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCTCCTGGTACTGCAACCT
 GTAAACCAGCACTGCAATGCTGATGCACGGGAAGTAGTGGGATGGGAACACAAATGG**Aggatcccgc**
 gtctcgaacagagcgcgagaggaacgctgaagggtctgcctctgtgcacctcagcgcgccatacaccacaataaccacctgacg
 aatgcgcttggttcttcgtccattagcgaagcggtccggtcacacacgtgccacgttggcgaggtggcaggtgacaatgatcggtgg
 agctgatggtcgaaacggtcacag**cctagggatatcgaattc**ggccgacaggacgcgctcaaaggtgctggtcgtgtatgccctg
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 caaagcaccggtgtatcaggtccgtgtcatccactctaaagaactcgactacgacctactgatggccctagattcttcatcaaaaac
 gcctgagacacttgcccaggattgaaactccctgaaggggaccaccagggggccctgagttgttccttcccccggtggcgagctgccag
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 ccttcgtgtacgggcccttcctcaaccctaggtatgcgcgcatgcggtgccgcgcaactgcgcgagggccgaggggttgggacg
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 gcctgacgccagcgtccacttttgtgcacacattccattcgtgcccaagacatttcatttggtgcgaagcgtcccagttacgtcad
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 TTTCTCGGCGTTCAATGCCCCGCTGCGGCGACCTGCGTCGCTCGGCGGGCTCCGGGCCCCGGCGCCCA
 GCGAGGCCCCCTCCCCGTGCGCG**ggcgcgccGCCGCCGCCGCCGACGCCAACCCCGCCCCGCCCCGAGCG**
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CTCCCTGCTGGAGGGCGTGTCCGGCATCTCCAGATCCAGAAGTTCGACACCACCGGCTACACCACC
ACCATCGCCGGCGAGATCAAGTCCCTGCAGCTGGACCCCTACGTGCCCAAGCGCTGGGCCAAGCGC
GTGGACGACGTGATCAAGTACGTGTACATCGCCGGCAAGCAGGCCCTGGAGTCCGCCGGCCTGCC
CATCGAGGCCGCCGGCCTGGCCGGCGCCGGCCTGGACCCCGCCCTGTGCGGCGTGCTGATCGGCAC
CGCCATGGCCGGCATGACCTCCTTCGCCGCCGGCGTGAGGCCCTGACCCGCGGCGGCGTGCGCAA
GATGAACCCCTTCTGCATCCCCTTCTCCATCTCCAACATGGGCGGGCGCCATGCTGGCCATGGACATC
GGCTTCATGGGCCCCAACTACTCCATCTCCACCGCCTGCGCCACCGGCAACTACTGCATCCTGGGCG
CCGCCGACCACATCCGCCGCGGCGACGCCAACGTGATGCTGGCCGGCGGCGCCGACGCCGCCATCA

TCCCCTCCGGCATCGGCGGCTTCATCGCCTGCAAGGCCCTGTCCAAGCGCAACGACGAGCCCGAGC
GCGCCTCCCGCCCCCTGGGACGCCGACCGCGACGGCTTCGTGATGGGCGAGGGCGCCGGCGTGCTG
GTGCTGGAGGAGCTGGAGCACGCCAAGCGCCGCGGCCACCATCCTGGCCGAGCTGGTGGGCG
GCGCCGCCACCTCCGACGCCCACCACATGACCGAGCCCGACCCCCAGGGCCGCGGCGTGCGCCTGT
GCCTGGAGCGCGCCCTGGAGCGCGCCCGCCTGGCCCCCGAGCGCGTGGGCTACGTGAACGCCCAC
GGCACCTCCACCCCCGCCGGCGACGTGGCCGAGTACCGCGCCATCCGCGCCGTGATCCCCAGGACT
CCCTGCGCATCAACTCCACCAAGTCCATGATCGGCCACCTGCTGGGCGGCGCCGGCGCCGTGGAGG
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CCCCGGCGTGGAACCCGTGGTGCTGGTGGGCCCCCGCAAGGAGCGCGCCGAGGACCTGGACGTG
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TGGACTACAAGGACCACGACGGCGACTACAAGGACCACGACATCGACTACAAGGACGACGAC
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 GTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCCGCTTTTATCAAACAG
 CCTCAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGCGAGTTGCTAGCTGCTTGTGCTATTTGCGAAT
 ACCACCCCCAGCATCCCCTTCCCTCGTTTCATATCGCTTGATCCCAACCGCAACTTATCTACGCTGTC
 CTGCTATCCCTCAGCGCTGCTCCTGCTCCTGCTCACTGCCCTCGCACAGCCTTGGTTTGGGCTCCGC
 CTGTATTCTCCTGGTACTGCAACCTGTAAACCAGCACTGCAATGCTGATGCACGGGAAGTAGTGGGA
 TGGGAACACAAATGGAA**agctta**atta**agagctc**ctttcttgcgctatgacacttcagcaaaaggtagggcgggctgcga
 gacggcttcccggcgctgcatgcaacaccgatgatgcttcgacccccgaagctccttcggggctgcatgggcgctccgatgccgct
 ccagggcgagcgctgtttaaatagccaggcccccgattgcaaagacattatagcgagctaccaaagccatattcaaacacctagat
 cactaccacttctacacaggccactcgagcttgtgatcgactccgctaagggggcgccctcttcctcttcgtttcagtcacaacccgc
aaacactag**TGGCTATCAAGACGAACAGGCAGCCTGTGGAGAAGCCTCCGTTCACGATCGGGACG**
CTGCGCAAGGCCATCCCCGCGCACTGTTTCGAGCGCTCGGCGCTTCGTAGCAGCATGTACCTGGCCT
TTGACATCGCGGTCATGTCCCTGCTCTACGTCGCGTCGACGTACATCGACCCTGCACCGGTGCCTAC
GTGGGTCAAGTACGGCATCATGTGGCCGCTCTACTGGTTCTTCCAGGTGTGTTTGAGGGTTTTGGTT
GCCCGTATTGAGGTCCTGGTGGCGCGCATGGAGGAGAAGGCGCCTGTCCCGCTGACCCCCCGGCT
ACCCTCCCGGCACCTTCCAGGGCGCGTACGGGAAGAACCAGTAGAGCGGCCACATGATGCCGTACT
TGACCCACGTAGGCACCGGTGCAGGGTCGATGTACGTCGACGCGACGTAGAGCAGGGACATGACC
GCGATGTCAAAGGCCAGGTACATGCTGCTACGAAGCGCCGAGCGCTCGAAACAGTGCGCGGGGA
TGGCCTTGCGCAGCGTCCCGATCGTGAACGGAGGCTTCTCCACAGGCTGCCTGTTGCTCTTGATAGC
CATctcgagGCAGCAGCAGCTCGGATAGTATCGACACACTCTGGACGCTGGTCGTGTGATGGACTGTT

[0513] Identification and analysis of *FATA-1* knockout, *KASII* over-expression and *FAD2* RNAi strains: Construct D1358, derived from pSZ2419, was transformed into S1920 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 S1920 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.

[0514] Table 45. Fatty acid profiles of D1358 [pSZ2419] primary transformants, compared to the wild-type parental strain, S1920.

Strain		S1920	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	20.63	16.27	18.4	19.1	18.18	15.6	16.42	11.24
	C16:1	0.84	0.74	0.79	0.97	0.80	0.77	1.17	0.79	0.56	0.61	0.97
	C18:0	3.19	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	68.76	71.46	69.55	69.02	68.93	70.44	69.54	75.27
	C18:2	7.39	1.97	5.87	5.61	6.22	6.31	6.42	6.8	7.68	7.78	8.51
	C18:3 α	0.95	0.23	0.59	0.51	0.26	0.39	0.46	0.38	0.24	0.27	0.24
	C20:0	0.24	0.12	0.20	0.13	0.32	0.20	0.03	0.20	0.33	0.31	0.12
	C20:1	0.11	0.40	0.29	0.37	0.23	0.33	0.33	0.39	0.35	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		79.11	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	14.87
unsaturates		67.96	78.12	76.68	76.24	78.79	77.35	77.4	77.25	79.28	79.57	84.99

[0515] 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 S1920 parent are highlighted with bold text.

[0516] 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, S5003 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.

[0517] 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, S1920.

Primary		1589.01358-13													
Strain		S1828	S5003	S5004	S5005	S5006	S5007	S5008	S5009	S5010	S5011	S5012	S5013	S5101	S5102
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	1.05	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	0.38	0.35	0.27	0.27
	C18:0	2.78	2.92	3.00	3.16	1.71	2.88	2.85	2.91	3.21	3.08	3.10	3.20	1.77	1.71
	C18:1	98.45	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	5.23	1.89	1.88	1.80	2.01	1.83	1.89	1.88	1.77	1.73	1.75	1.76	1.94	1.98
	C18:3a	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	0.16	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		65.83	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	100.0	75.5	77.5	56.7	54.5

[0518] 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.

[0519] **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 S5003. 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.

[0520] Nucleotide sequence of the the transforming DNA from pSZ2283:

gctcttcgggtcgccgctgcctcgctcccctggtggtgcgcgcggtgccagcgaggccccgctgggcgttccgccctcgtgca
gcgccccctccccgtggtctactccaagctggacaagcagcaccgcctgacgcccagcgctggagctggtgcagagcatggggc
agtttgccgaggagagggtgctgcccgtgctgcaccccgtggacaagctgtggcagccgcaggacttttgccgaccccgagtcgc
ccgacttcgaggatcagggtggcggagctgcgcgcgcgcgccaaggacctgcccagcagtagtactttgtggtgctggtgggggacatg
atcacggaggaggcgctgccgacctacatggccatgctcaacacgctggacggcgctgcgcgacgacacgggcgcggccgaccacc
cgtgggcgcgctggacgcggcagtggtggccgaggagaaccggcacggcgacctgctgaacaagtactgctggctgacggggc
gcgtcaacatgcgggccgtggaggtgaccatcaacaacctgatcaagagcggcagtaacccgcagacggacaacaacccttattt
ggggttcgtctacacctcctccaggagcgcgccaccaagtaggtagccctttcttgcgctatgacacttcagcaaaaggtaggcg
ggctgcgagacggcttcccggcgctgcatgcaacaccgatgatgcttcgacccccgaagctccttcggggctgcatgggcgctccg
atgccgctccaggcgagcgctgtttaaatagccaggcccccgattgcaaagacattatagcgagctaccaaagccatattcaaac
acctagatcactaccacttctacacaggccactcgagcttgtgatcgactccgctaagggggcgcttcttcttctgtttcagtcac
aaccgcgaacttctagaatatcaATGatcgagcaggacggcctccacgccggctccccgccgctgggtggagcgctgttc
ggctacgactgggcccagcagaccatcggtgctccgacgccgcccgtgttcgcctgtccgcccaggggccgccccgtgctgttc
gtgaagaccgacctgtccggcgccctgaacgagctgcaggacgaggccgcccgcctgtcctggctggccaccaccggcgctgc
cctgcgccgcccgtgctggacgtggtgaccgaggccggccgagctggctgctgctgggagaggtgcccggccaggacctgct
gtcctcccacctggcccccgccgagaagggtgtccatcatggccgacgccatgcgccgctgcacacctggaccccgccacctg
ccccttcgaccaccaggccaagcaccgcatcgagcgcgcccgcacccgcatggaggccggcctggtggaccaggacgacctg
gacgaggagcaccagggcctggcccccgccgagctgttcgcccgcctgaaggcccgcatgcccgacggcgaggacctggtg
gtgaccacggcgacgcctgcctgcccacatcatggtggagaaacggccgcttctccggcttcatcgactgcggccgcctgggc
gtggccgaccgctaccaggacatcgccctggccacccgcgacatcgccgaggagctgggcggcgagtgggccgaccgcttcc
tggtgctgtacggcatcgccgccccgactcccagcgcatcgcttctaccgcctgctggacgagttcttcTGAcattgGCAG
CAGCAGCTCGGATAGTATCGACACACTCTGGACGCTGGTCGTGTGATGGACTGTTGCCGCCACACTT
GCTGCCTTGACCTGTGAATATCCCTGCCGCTTTTATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGT
ACGCGCTTTTGCGAGTTGCTAGCTGCTTGTGCTATTTGCGAATACCACCCCAGCATCCCCTTCCCTC
GTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCTATCCCTCAGCGCTGCTCCTG
CTCCTGCTCACTGCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCTCCTGGTACTGCAACCT
GTAAACCAGCACTGCAATGCTGATGCACGGGAAGTAGTGGGATGGGAACACAAATGGAggatccgcg
gtctcgaacagagcgcgagaggaacgctgaagggtctgcctctgtcgacctcagcgcggcatacaccacaataaccacctgacg

aatgcgcttggttcttcgtccattagcgaagcgtccggttcacacacgtgccacgttggcgaggtggcaggtgacaatgatcggtgg
 agctgatggtcgaaacgttcacag**cctagggatatcgaattc**ctttcttcgctatgacacttcagcaaaaggtagggcgggctgc
gagacggcttcccggcgctgcatgcaacaccgatgatgcttcgacccccgaagctccttcggggctgcatgggcgctccgatgccg
ctccagggcgagcgctgtttaaatagccaggccccgattgcaaagacattatagcgagctaccaagccatattcaaacacctag
atcactaccacttctacacaggccactcgagcttgtgatcgactccgctaagggggcgctcttctcttcgtttcagtcacaacccg
caaac**actagtGCGCTGGACGCGGCAGTGGGTGGCCGAGGAGAACCGGCACGGCGACCTGCTGAAC**
AAGTACTGTTGGCTGACGGGGCGCGTCAACATGCGGGCCGTGGAGGTGACCATCAACAACCTGAT
CAAGAGCGGCATGAACCCGCAGACGGACAACAACCTTACTTGGGCTTCGTCTACACCTCCTTCCAG
GAGCGCGCGACCAAGTACAGCCACGGCAACACCGCGCGCCTTGCGGCCGAGCAGTGTGTTTGAGG
GTTTTGGTTGCCCGTATCGAGGTCTGGTGGCGCGCATGGGGGAGAAGGCGCCTGTCCCGCTGACC
CCCCGGCTACCCTCCCGGCACCTTCCAGGGCGCGTACG**ggatccTGCTCGGCCGCAAGGCGCGCGGT**
GTTGCCGTGGCTGTACTTGGTCGCGCGCTCCTGGAAGGAGGTGTAGACGAAGCCCAAGTAAGGGT
TGTTGTCCGTCTGCGGGTTCATGCCGCTCTTGATCAGGTTGTTGATGGTCACCTCCACGGCCCGCAT
GTTGACGCGCCCCGTACGCCAACAGTACTTGTTCAGCAGGTGCGCGTGCCGTTCTCCTCGGCCACC
CACTGCCGCGTCCAGCGC**Caagctt**GCAGCAGCAGCTCGGATAGTATCGACACACTCTGGACGCTGGTC
 GTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCCGCTTTTATCAAAC
 AGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGCGAGTTGCTAGCTGCTTGTGCTATTTGCGA
 ATACCACCCCCAGCATCCCCTTCCCTCGTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTG
 TCCTGCTATCCCTCAGCGCTGCTCCTGCTCCTGCTCACTGCCCTCGCACAGCCTTGGTTTGGGCTCCG
 CCTGTATTCTCCTGGTACTGCAACCTGTAAACCAGCACTGCAATGCTGATGCACGGGAAGTAGTGGG
 ATGGGAACACAAATGGA**aagctggagctc**cagccacggcaacaccgcgccttgcgccgagcacggcgacaagaacc
tgagcaagatctcggggctgatcgccagcgacgagggccggcacgagatcgctacacgcgcatcgtggacgagttcttcgcctc
gaccccgagggcgccgtcgccgcctacgccaacatgatgcgcaagcagatcaccatgcccgcgcacctcatggacgacatgggcc
acggcgaggccaacccgggcccgaacctcttcgcccacttctccgcggtcgccgagaagatcgacgtctacgacgccgaggactac
tgccgcatcctggagcacctcaacgcgcgctggaaggtggacgagcgccaggtcagcggccaggccgcccggaccaggagtac
gtcctgggcctgccccagcgcttccggaaactcgccgagaagaccgcccgaagcgcaagcgcgctcgcgcgaggcccgctgcctt
ctcctggatctccgggcgcgagatcatggtctagggagcgacgagtgctgcgtcggggctggcgggagtgaggacgccctcctcgct
cctctctgttctgaacggaacaatcggccaccccgcgctacgcgccacgcatcgagcaacgaagaaaacccccgatgataggttg
cggtggctgccgggatatagatccggccgcacatcaaagggcccctccgccagagaagaagctcctttcccagcagactcctgaag
agc (SEQ ID NO:97)

[0521] 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.

[0522] Nucleotide sequence of the the transforming DNA from pSZ2697:

atgcatgccggtcaccacccgcatgctcgctactacagcgacgcaccgcttcgtgatccaccgggtgaacgtagtcctcgacggaa
acatctgggttcgggcctcctgcttgactcccggccatgccgacaacctttctgctgttaccacgacccacaatgcaacgcgacacga
ccgtgtgggactgatcggttcactgcacctgcatgcaattgtcacaagcgcttactccaattgtattcgtttgtttctgggagcagttg
ctcgaccgccccgcgtcccgaggcagcgatgacgtgtgcgtggcctgggtgtttcgtcgaaaggccagcaaccctaaatcgaggc
gatccggagattgggatctgatccgagtttgaccagatccgccccgatgcggcacgggaactgcatcgactcggcgcggaacca
gctttcgtaaataccagattggtgtccgatacctggatttgccatcagcgaaacaagacttcagcagcgagcgatatttgccgggcgt
gctaccagggttgcatatcattgcccatttctgtctggaccgcttactggcgagagggtgagttgatggggtggcaggcatcgaaa
cgcgctgcatggtgtgcgtgtctgtttcggctgcacgaattcaatagtcggatgggagcgagtagaattgggtgtggcgctcgct
gcatgcctcgccccgtcgggtgtcatgaccgggactggaatccccctcgcgaccatcttgtaacgctcccgactctcccgactagt
GCGCTGGACGCGGCAGTGGGTGGCCGAGGAGAACCGGCACGGCGACCTGCTGAACAAGTACTGT
TGGCTGACGGGGCGCGTCAACATGCGGGCCGTGGAGGTGACCATCAACAACCTGATCAAGAGCG
GATGAACCCGACAGACGGACAACAACCTTACTTGGGCTTCGTCTACACCTCCTTCCAGGAGCGCGC
GACCAAGTACAGCCACGGCAACACCGCGCGCCTTGCGGCCGAGCAGTGTGTTTGAGGGTTTTGGTT
GCCCGTATCGAGGTCTGGTGGCGCGCATGGGGGAGAAGGCGCCTGTCCCGCTGACCCCCCGGCT
ACCCTCCCGGCACCTTCCAGGGCGCGTACGggatc**TGCTCGGCCGCAAGGCGCGCGGTGTTGCCGTG**
GCTGTACTTGGTCGCGCGCTCCTGGAAGGAGGTGTAGACGAAGCCCAAGTAAGGGTTGTTGTCCG

TCTGCGGGTTCATGCCGCTCTTGATCAGGTTGTTGATGGTCACCTCCACGGCCCGCATGTTGACGCG
CCCCGTCAGCCAACAGTACTTGTTGAGCAGGTCGCCGTGCCGGTTCTCCTCGGCCACCCACTGCCGC
GTCCAGCGC**Caagctt**GCAGCAGCAGCTCGGATAGTATCGACACACTCTGGACGCTGGTCGTGTGATG
 GACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCCGCTTTTATCAAACAGCCTCAG
 TGTGTTTGATCTTGTGTGTACGCGCTTTTGCAGAGTTGCTAGCTGCTTGTGCTATTTGCGAATACCACC
 CCCAGCATCCCCTTCCCTCGTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCTA
 TCCCTCAGCGCTGCTCCTGCTCCTGCTCACTGCCCTCGCACAGCCTTGTTTTGGGCTCCGCCTGTATT
 CTCCTGGTACTGCAACCTGTAAACCAGCACTGCAATGCTGATGCACGGGAAGTAGTGGGATGGGAA
 CACAAATGGAAAGCTG**gagct**caaa**gatat**caacttaattaacca**aggtacc****cgctgcaacgaaggcgagccacagcc**
gctcccacccgccgctgaaccgacacgtgcttgggcgcctgccgcctgcctgccgcatgcttgctggtgaggctgggcagtgtg
ccatgctgattgaggcttggttcacgggtggaagcttatgtgtgtgctgggcttgcctgccgggcaatgcgcatggtggcaagagg
gcggcagcacttgctggagctgccgcggtgcctccaggtggttaatcgcggcagccagagggatttcagatgatcgcgctacag
gttgagcagcagtgctcagcaaaggtagcagtttgccagaatgatcggttcagctgttaataatgccagcaagagaaggggtcaag
tgcaaacacgggcatgccacagcacgggcacccggggagtggaatggcaccaccaagtgtgtgagccagcatcgccgcctggct
gtttcagctacaacggcaggagtcaccaacgtaacatgagctgatcaacactgcaatcatcgggcgggcgatgcaagcatgc
ctggcgaagacacatggtgtgcggatgctgccggctgctgcctgctgcgcacgccgttgagttggcagcaggctcagccatgcactg
gatggcagctgggctgccactgcaatgtggtggataggatgcaagtggagcgaatacacaacccctctggctgcttgctgggttgc
ggcatcgacccatcagcaggagcgcatgcgaagggaactggcccatgcacgccatgcaaaccggagcgacccagtggtccaca
ctgtcaccaggcccgaagctttgcagaacatgctcatggacgcatgtagcgctgacgtcccttgacggcgctcctctcgggtgtg
ggaaacgcaatgcagcacaggcagcagaggcgggcggcagcagagcgggcggcagcagcgggcgggggccacccttctgcgggggt
cgcgccccagccagcggtgatgcgctgatcccaaacgagttcacattcatttgcatgcctggagaagcgaggctggggcctttgggc
tggtgcagcccgcaatggaatgcgggaccgccaggctagcagcaaaggcgctcccctactccgcatcgatgttccatagtgcatt
ggactgcatttgggtggggcgccggctgtttctttcgtgttgcaaaacgcgccagctcagcaacctgtcccgtgggtccccgtgcc
gatgaaatcgtgtgcacgccgatcagctgattgccggctcgcgaagtaggcgcctccttctgctgcctctctccgtcccgcctc
tagaatatca**ATGatcgagcaggacggcctccacgccggctccccgccgctgggtggagcgctgttcggctacgactggg**
cccagcagaccatcggctgctccgacgccgctgttccgcctgtccgccagggccgccccgtgctgttcgtgaagaccgacct
gtccggcgccctgaacgagctgcaggacgaggccgccgctgtcctggctggccaccaccggcgctgccctgcgccgctgtc
tggacgtggtgaccgaggccggcgcgactggctgctgctgggcgaggtgccggccaggacctgctgtcctccacctggcc
cccgccgagaaggtgtccatcatggccgacgccatgcgccgctgcacaccctggacccccgccacctgccccttcgaccaccag
gccaagcaccgcatcgagcgcgcccgacccgcatggaggccggcctggtggaccaggacacctggacgaggagacca

gggcctggccccgcccagctgttcgcccgcctgaaggcccgcatgccgcgagggacactggtggtgacccacggcgac
 gcctgcctgcccacatcatggtggagaacggccgcttctccggcttcatcgactgcgccgcctgggctggccgaccgctac
 caggacatcgccctggccacccgagacatcgccgaggagctgggcggcgagtgggccgaccgcttctggtgctgtacggca
 tcgccgccccgactcccagcgcatcgcttctaccgctgctggacgagttcttcTGA**caattg**GCAGCAGCAGCTCGGA
 TAGTATCGACACACTCTGGACGCTGGTCGTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACC
 TGTGAATATCCCTGCCGCTTTTATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGC
 GAGTTGCTAGCTGCTTGTGCTATTTGCGAATACCAACCCAGCATCCCCTTCCCTCGTTTCATATCGCT
 TGCATCCCAACCGCAACTTATCTACGCTGTCCTGCTATCCCTCAGCGCTGCTCCTGCTCCTGCTCACTG
 CCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCTCCTGGTACTGCAACCTGTAAACCAGCACT
 GCAATGCTGATGCACGGGAAGTAGTGGGATGGGAACACAAATGG**Aggat**cccgctctcgaacagagcgcg
 cagaggaacgctgaaggtctgcctctgtgcacctcagcgcgccatacaccacaataaccacctgacgaatgcgcttggttcttcg
 tccattagcgaagcgctccggttcacacacgtgccacgttggcgaggtggcaggtgacaatgatcggtggagctgatggtcgaacg
 ttcacag**cctagggatatcgaattc**cgggtcgccgcgctgcctcgcgtcccctggtggtgcgcgcggtcgccagcgaggccccgctg
ggcgttcgccctcggtgcagcgccccctccccgtggtctactccaagctggacaagcagcaccgcctgacgcccagcgccctgga
gctggtgcagagcatggggcagtttgccgaggagaggggtgctgcccgtgctgcaccccgtggacaagctgtggcagccgcaggac
ttttgcccgaccccgagtcgcccgacttcgaggatcaggtggcgagctgcgcgcgcgcgccaaggacctgcccgacgagtacttt
gtggtgctggtgggggacatgatcacggaggaggcgctgccgacctacatggccatgctcaacacgctggacggcggtgcgcgacg
acacgggcgccgaccacccgtgggcgcgctggacgcggcagtggtggccgaggagaaccggcacggcgacctgctgaaca
agtactgctggctgacggggcgcgctcaacatgcgggcccgtggaggtgacctcaacaacctgatcaagagcggcacgaacccgca
gacggacaacaacccttatttggggttcgtctacacctccttcaggagcgcgccaccaagtat**tctaga** (SEQ ID NO:98)

[0523] 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 S5003 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 S5003 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.

[0524] Table 47. Fatty acid profiles of D1639 [pSZ2697] and D1682 [pSZ2283] primary transformants, compared to the wild-type strain, S1920, and the S5003 parental base strain.

Strain		S1920	S5003	D1682-4	D1682-17	D1682-7	D1682-6	D1639-7	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	1.54	1.39	1.51	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	0.05	0.07	0.07	0.10	0.00	0.00	0.00	0.00
	C18:0	3.38	3.60	35.46	29.92	24.66	22.50	55.86	53.38	43.46	37.30
	C18:1	57.80	34.14	48.39	52.45	61.04	63.60	23.70	25.79	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
	C18:3 α	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	1.73	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	0.30	0.19	0.28
	C24:0	0.09	0.02	0.33	0.26	0.34	0.25	0.84	0.45	0.32	0.31
sum C18		69.55	80.14	86.72	85.42	89.39	89.35	85.34	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

[0525] 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.

[0526] 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.

[0527] 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		S1920	S5003	S5774	S5775	S5776	S5777
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
	C18:3 α	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

[0528] In Table 48, S5003 is the parent strain; S1920 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 S5003 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.

[0529] Lab scale oils were prepared from biomass collected from the S5774, S5775 and S5776 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 S5665 and S5675 oils, described above. S5774-S5776 oils contained 12-13% trisaturated TAGs (S-S-S), similar to the amounts that accumulated in the S5665 and S5775 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.

[0530] 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.

[0531] 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 Strain Q. The fatty acid profile of the oil produced by Strain Q is listed below in Table 49.

[0532] 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

<u>C20:0</u>	<u>0.02</u>
C20:1	0.38
C22:0	0.04
C24:0	0.03

[0533] 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 selection 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 acy-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.

[0534] 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

[0535] The HO and HSAO oils were metylated 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.

[0536] 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.

[0537] 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.

[0538] The high oleic oil and the high-stability high-oleic algal oils can have the properties shown in Figure 19 or these values $\pm 20\%$ for the measured parameters.

[0539] 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.

[0540] 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).

[0541] 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 Physiol (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 vulgaris* *SAD* gene).

[0542] 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.

[0543] 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%.

[0544] Constructs for over expression of *Macfadyena unguis* C16:0 desaturase (MuPAD) follow:

[0545] **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. *BspQ*I 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 stearyl-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.

[0546] Nucleotide sequence of transforming DNA contained in pSZ3142:

gctcttcgccgccgccaactcctgctcgagcgcgcccgcgctgcgccgagcgccttggcctttcgccgcgctcgtgcgcgtcgtgatgtccatcaccaggtccatgaggtctgccttgcgccggctgagccactgcttcgtccggcgcccaagaggagcatgagggaggactcctggtccagggctcctgacgtggtcgcggctctgggagcgggcccagcatcatctggctctgccgcaccgaggccgcctccaa
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agccttgtctaggcagaatccctaccagtcattggctttacctggatgacggcctgcgaacagctgtccagcgaccctcgtgccgcc

gcttctccgcacgcttcttccagcaccgtgatggcgcgagccagcgccgcacgctggcgcgtgcgcttcgccgatctgaggacagt
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 ccccaacaccacctcctcccagaccaattctgtcaccttttggcgaaggcatcggcctcggcctgcagagaggacagcagtgccc
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 gacgccaagtggcacctgtacttcagttacaacccgaacgacaccgtctgggggacgcccttggtctggggccacgccacgtc
 cgacgacctgaccaactgggaggaccagcccatcgccatcgccccgaagcgcaacgactccggcgcttctccggctccatgg
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cccccggtggcgagctgccagccaggctgtacctgtgatcgaggctggcgggaaaataggcttcgtgtgctcaggtcatggg
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ccacggcgacccccctgaacaagtacctgtacctgtccggccgcgtggacatgaagcagatcgagaagaccatccagtacct
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 tcctcagcgctgctcctgctcctgctcactgcccctcgcacagccttggttgggctccgcctgtattctcctggtactgcaacctgta
 aaccagcactgcaatgctgatgcacgggaagtagtgggatgggaacacaaatggaaagcttaattaag**gagctcttgtttccagaa**
ggagttgctccttgagcctttcattctcagcctcgataacctccaaagccgctctaattgtggaggggttcgaatttaaaagcttgg
aatgttggtcgtgcgtctggaacaagcccagacttggtgctcactgggaaaaggaccatcagctccaaaaaacttgccgctcaaa
ccgctacctctgctttcgcgcaatctgccctgttgaaatcgccaccacattcatattgtgacgcttgagcagctgtaattgcctca
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atgctacctcacaatagttcataacagtgaccatatttctgaagctccccaacgagcacctccatgctctgagtggccaccccccg
gccctggtgcttgaggaggcaggtcaaccggcatggggctaccgaaatccccgaccggatcccaccacccccgcgatgggaag
aatctctccccgggatgtgggcccaccaccagcacaaacctgctggcccaggcgagcgtcaaaccataccacacaaatatccttgg
catcgccctgaattccttctgccgctctgctacccggtgcttctgtccgaagcaggggttgctagggatcgctccgagtcgcaaa
cccttgcgcgtggcggggcttggtcgagcttgaagagc (SEQ ID NO:99)

[0547] 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. *BspQ* I 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.

[0548] Nucleotide sequence of transforming DNA contained in pSZ3145:

gctcttcgcccgcgccaactcctgctcgagcgcgcccgcgctgcccgcgccaagcgcccttgcccttttcgcccgcgctcgtgcccgcgct
 gatgtccatcaccaggtccatgaggtctgccttgcgcccggctgagccactgcttcgtccggcgcccaagaggagcatgaggag
 gactcctggtccagggctcgtgacgtggtcgcggtctgggagcgggccaagcatcatctggctctgcccaccgaggccgcctcaa
 ctggtcctccagcagccgcagtcgcccgcgaccctggcagaggaagacaggtgaggggggtatgaattgtacagaacaaccacg
 agccttgtctaggcagaatccctaccagtcattggctttacctggatgacggcctggaacagctgtccagcgaacctcgctgcccgc
 gcttctcccgacgcttcttccagcaccgtgatggcgcgagccagcggcgacgctggcgctgcgcttcgccgatctgaggacagt
 cggggaactctgatcagctctaaaccccccttgcgcgtagtggtgccatcctttgcagaccggtgagagccgacttggtgcccac
 cccccacaccacctcctcccagaccaattctgtcaccttttggcgaaggcatcggcctcggcctgcagagaggacagcagtgccc
 agccgctgggggttgcggtatgcagctcaggtacccttcttgcgctatgacacttcagcaaaaggtagggcgggctgcgagac
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cttctgccgctctgctacccggtgcttctgtccgaagcaggggttgctagggatcgctccgagtcgcaaaccttgtcgcgtggcg
gggcttgttcgagcttgaagagc (SEQ ID NO:100)

[0549] 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. *BspQ*I 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 stearyl-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.

[0550] Nucleotide sequence of transforming DNA contained in pSZ3137:

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agccttgtctaggcagaatccctaccagtcattggctttacctggatgacggcctgcgaacagctgtccagcgaccctcgctgccgcc
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 aatctctccccgggatgtgggcccaccaccagcacaacctgctggcccaggcgagcgtcaaaccataccacacaaatatccttgg
 catcgccctgaattccttctgccgctctgctacccggtgcttctgtccgaagcaggggttgctagggatcgctccgagtcgcaaa
 cccttgcgcgtggcggggcttggtcgagcttgaagagc (SEQ ID NO:101)

EXAMPLE 52: MYRISTATE RICH OIL PRODUCED BY OVEREXPRESSING A *CUPHEA PALUSTRIS* THIOESTERASE

[0551] Here, we demonstrate that over expression of a *Cuphea palustris* thioesterase (*Cpal* FATB1, accession AAC49180) UTEX1435 results in a large increase in C14:0.

[0552] Constructs used for the overexpression of the *Cpal* FATB1 gene were codon optimized for expression in *P. moriformis* as described herein. The construct can be written as 6SA::CrTUB2-ScSUC2-CvNR:PmAMT3-CpSAD1tpExt_CpalFATB2FLAG_ExtA-CvNR::6SB.

[0553] In this high-myristate strain, the myristate content was 65.70 percent, as shown in Table 51, below. This is a very large increase from the myristate content of the wild-type oil produced by the base strain which has a myristate content of approximately 1%.

[0554] Table 51. The fatty acid profile of the high myristate strain.

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

[0555] Figure 22 shows that at 25°C, the solid fat content is over 80%. At 25°C, the base strain oil has no or negligible solid fat. As a comparison, the solid fat content of a refined, bleached, deodorized oil (RBD-3), as shown in Figure 4, is reproduced. RBD-3 is oil from a *P. moriformis* strain engineered to produce high amounts of C16:0. The construct for the high C16:0 producing strain can be written as 6SA-bTub-Inv-nr::Amt03-Native Ch16TE-nr-6SB. This construct encodes for a C16:0 preferring *Cuphea hookeriana* thioesterase.

EXAMPLE 53: MYRISTATE RICH OIL PRODUCED BY OVEREXPRESSING A *CUPHEA PALUSTRIS* THIOESTERASE

[0556] 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).

[0557] 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- *Cpal*FATB2ExtA-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-*Cpal*FATB2FLAG_ExtA-CvNR::6SB. The nucleic acid sequence and amino acid sequence are given as SEQ ID NO: 88 and SEQ ID NO: 89.

[0558] *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%.

[0559] The fatty acid profile of the high myristate strain is shown in the Table 52 below.

[0560] Table 52. 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 54: PRODUCTION OF EICOSENOIC AND ERUCIC FATTY ACIDS

[0561] 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^{Δ11}) and erucic (22 : 1^{Δ13}) 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^{Δ11}), 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.

[0562] Construct used for the expression of the *Cramble abyssinica* fatty acid elongase (CaFAE) in *P. moriformis* (UTEX 1435 strain 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.

[0563] 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' *BspQI*, *KpnI*, *XbaI*, *MfeI*, *BamHI*, *EcoRI*, *SpeI*, *AflIII*, *SacI*, *BspQI*, respectively. *BspQI* 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.

[0564] Nucleotide sequence of transforming DNA contained in plasmid pSZ3070:

gctcttcgcccgcgcactcctgctcgagcgccccgcgctgcgcgcgcagcgcttggccttttcgcccgcctcgtgcgcgtcgtgatgt
ccatcaccagggtccatgaggtctgccttgcgcggctgagccactgcttcgtccggcgcccaagaggagcatgagggaggactcctggt
ccagggtcctgacgtggtgcggctctgggagcgccagcatcatctggctctgccgcaccgaggccgctccaactggtcctccagca
gccgcagtcgcgcgcaccctggcagaggaagacaggtgaggggggtatgaattgtacagaacaaccagagccttgtctaggcagaa
tcctaccagtcattggtttacctggatgacggcctgcgaacagctgtccagcgacctcgtgcgcgccttctccgcacgcttcttcca
gcaccgtgatggcgagccagcgccgcacgtggcgctgcgttcgccgatctgaggacagtcggggaaactctgatcagcttaaacc
cttgcgcgttagtgttgcctccttgcagaccggtgagagccgacttgttgcgcacccccacaccacctctccagaccaattctgt
caccttttggcgaaggcctcggcctgcagagaggacagcagtgcccagccgctgggggttggcggtatgcacgctcaggtacc
ttcttgcgctatgacactccagcaaaaggtagggcggtgcgagacggctccccggcgctgcatgcaacaccgatgatgcttcgacccccg
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ctaccaaaagccatattcaaacacctagatcactaccattctacacaggccactcgagcttgatgcactccgctaagggggcgcttctctt
cgttccagtcacaaccgcgaaactctagaatatca***ATG****ctgctgcaggccttctgttctgctggccggcttcgccgccaagatcagcgctcc*
atgacgaacgagacgtccgaccgccccctggtgcacttcacccccaaacagggtgatgaacgaccccaacggcctgtgttacgacgag
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caacacctcgggttcttcaacgacaccatcgacccgcgcagcgctgcgtggccatctggacctacaacaccccgagtcaggagcag
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ggacccagcaagtcctactgggtgatgtcatctcatcaaccccgcgccccggcgcggtccttcaaccagtacttcgtcggcagcttc

aacggcaccacttcgagggccttcgacaaccagtcgccgctgggtggacttcggcaaggactactacgccctgcagaccttctcaacaccgac
 ccgacctacgggagcgccctgggcatcgctgggacctcaactgggagtactccgcttcgtgccaccaacccctggcgctcctccatgtcc
 ctgctgcgaagtctccctcaacaccgagtaccaggccaacccggagacggagctgatcaacctgaaggccgagccgatcctgaacatca
 gcaacgccggccctggagccggttcgccaccaacaccacgttgacgaaggccaacagctacaacgtcgacctgtccaacagcaccggca
 ccctggagttcgagctgggtgtacgccgtcaacaccacccagacgatctccaagtccgtgttcgaggacctctccctctggttcaagggcctgga
 ggaccccgaggagtacctccgcatgggcttcgaggtgtccgctcctcttctctggaccgcgggaacagcaaggtgaagttcgtgaagga
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 gaacagagcgcgagaggaacgctgaaggtctgcctctgtcgcacctcagcgcggcatacaccacaataaccacctgacgaatgcgcttggtt
 ctctgctcattagcgaagcgtccggttcacacacgtgccacgttggcgaggtggcaggtgacaatgatcggtggagctgatggtcgaaacgttcac
 agcctagggatata**gaattc**ggccgacaggacgcgcgtcaaaaggtgctgtcgtgtatgccctggccggcaggtcgttgctgctgctggttagt
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ccggaatccaaggaggcaagagcgcccggtcagttgaagggtttacgcgcaaggtacagccgctcctgcaaggctgcgtgggtggaatt
ggacgtgcaggtcctgctgaagtctccaccgcctcaccagcggacaaagcaccgggtgatcaggtccgtgtcatccactctaagaactcg
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 acctgcaccacctgtactactcctacctgcagcacaacgtgatcaccatcgccccctgttcgccttcaccgtgttcggctccatcct
 gtacatcgtgacccgccccaaagcccggtgtacctgggtggagtactcctgctacctgcccccaaccagtgccgctcctccatctccaa
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 cgccgcgccccgcgaggagaccgagcaggtgatcgtgggcgcctgaagaacctgttcgagaacaccaaggtgaaccccaa
 ggacatcggcatcctggtggtgaactcctcatgttcaacccacccccctcctgtccgcatggtggtgaacaccttcaagctgcg
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gtgcacaagaacacactacgcctgggtgggtgccaccgagaacatcacctacaacatctacgccggcgacaaccgctccatgatg
gtgtccaactgcctgttccgcgtggggcggcgccgccatcctgctgtccaacaagccccgcgaccgccgctccaagtacgagc
tggtgcacaccgtgcgcacccacaccggcgccgacgacaagtccttccgctgcgtgcagcagggcgacgacgagaacggcaa
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tcctgcccctgtccgagaagctgctgttcttcgtgaccttcatggccaagaagctgttcaaggacaaggtgaagcactactacgtgc
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ttggttcgtgcgtctggaacaagcccagacttgttgcctactgggaaaaggaccatcgactccaaaaaacttgccgctcaaaccgctacc
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tgccccctgtgcgagcccatgccaggcatgtcgcgggcgaggacacccgccactcgtacagcagaccattatgctacctcacaatagtcca
taacagtgaccatatttctcgaagctccccaacgagcacctccatgctctgagtggccacccccggccctggtgcttgcggaggggcaggt
caaccggcatggggctaccgaaatccccgaccggatcccaccacccccgcgatgggaagaatctctccccgggatgtgggcccaccacc
agcacaacctgctggcccaggegagcgtcaaaccataccacacaaatatccttggcatcgccctgaattccttctgcegetctgctaccgg
gtgcttctgtccgaagcaggggttgctagggatcgctccgagtcgcgaaaccttgtcgcgtggcggggcttgttcgagcttgaagagc
(SEQ ID NO:102)

[0565] 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

[0566] 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 *SpeI* and *AflIII* are shown 5'-3' respectively.

[0567] Nucleotide sequence of *LaFAE* contained in pSZ3071:

actagtATGacctccatcaacgtgaagctgctgtaccactacgtgatcaccaacttctcaacctgtgcttcttccccctgaccgccat
cctggccggcaaggcctcccgcctgaccaccaacgacctgcaccacttctactcctacctgcagcacaacctgatcacctgacc
ctgctgttcgccttcaccgtgttcggctccgtgctgtacttcgtgacccgcccccaagcccggtgtacctgggtggactactcctgctacctg
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gccgtgggctccggcttcaagtgcactccgcccgtgtgggtggccctgcgcaacgtgaaggcctccgccaactccccctgggagc
actgcatccacaagtaccccgctgcagatgtactccggctcctccaagtccgagaccgcgcccagaacggccgctccTGActta
ag (SEQ ID NO:103)

[0568] Nucleotide sequence of *CgFAE* contained in pSZ3072:

actagtATGacctccatcaacgtgaagctgctgtaccactacgtgctgaccaacttcttcaacctgtgcttcttccccctgaccgctt
ccccgccggcaaggcctcccagctgaccaccaacgacctgcaccacctgtactcctacctgcaccacaacctgatcacctgac
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tgcccccccgccacctgtcctgcggcatctcccgcgtgatggagatcttctacgagatccgcaagtcgacccccctcccgcgaggtg
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ggcctgggtgcacgacatgccccctgcgcatgaacttcgcccgcgcccgcgaggagaccgagcaggtgatcaacggcgccctgga
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 cgccatcggtcctcggttcaagtgaactccgctgtgggtggccctgtgcaacgtgaagccctccgtgaactccccctgggag
 cactgcatcgaccgctaccccggtggagatcaactacggctcctccaagtccgagacccgcgccagaaacggccgctccTGActt
aag (SEQ ID NO:104)

[0569] Nucleotide sequence of *TmFAE* contained in pSZ3067:

actagtATGtccggcaccaaggccacctccgtgtccgtgccccgtcccgaactcaagcagtcctgaacctgaagtacgtgaagc
 tgggtaccactactccatcacccacgccatgtacctgttctgacccccctgctgctgatcatgtccgccagatctccaccttctcc
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 gcgaccgctgtggcagatcgcttcggctccggcttcaagtgaactccgctgtggaaggccctgcgcaacgtgaaccccg
 ccgaggagaagaaccttggtggacgagatccacctgttccccgtggaggtggccctgaacTGActtaag (SEQ ID
 NO:105)

[0570] Nucleotide sequence of *BnFAE1* contained in pSZ3068:

actagtATGacctccatcaacgtgaagctgctgtaccactacgtgatcaccaacctgttcaacctgtgtcttctccccctgaccgcc
 atcgtggccggcaaggcctacctgaccatcgacgacctgcaccacctgtactactcctacctgcagcacaacctgatcaccatcg
 cccccctgctggccttcaccgtgttcggctccgtgctgtacatcgccaccgcccccaagcccggtgtacctggaggagtactcctgcta
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 cctgtccgccatgggtggtgaacaccttcaagctgcgctccaacgtgcgctccttcaacctggggcgccatgggctgctccgcccggcg
 tgatcgccatcgacctggccaaggacctgctgcacgtgcacaagaacacctacgcccctgggtggtgtccaccgagaacatcacct
 acaacatctacgccggcgacaaccgctccatgatgggtgtccaactgcctgttccgctggggcgccgcccacatcctgtgtccaac
 aagccccgcgaccgcccgcctccaagtaagagctgggtgcacaccgtgcgcacccacacccggcgccgacgacaagtccttcc
 gctgcgtgcagcagggcgacgacgagaacggccagaccggcggtgtccctgtccaaggacatcacccgacgtggccggcgccgac
 cgtgaagaagaacatcgccaccctggggccccctgatcctgcccctgtccgagaagctgctgttcttctgtgaccttcatgggcaaga
 agctgttcaaggacgagatcaagcactactacgtgcccgaacttcaagctggccatcgaccacttctgcateccacgccggcgga
 ggccgtgatcgacgtgctggagaagaacctgggcctggcccccatcgacgtggaggcctcccgctccaccctgcaccgcttcgg
 caacacctcctcctcctccatctggtacgagctggcctacatcgagcccaagggccgcatgaagaagggcaacaaggtgtggca
 gategccctgggctccggcttcaagtgcactcggcctgtgggtggccctgaacaacgtgaaggcctccaccaactccccctgg
 gagcactgcategaccgctaccccgtaagatcgactccgactccggcaagtccgagaccgcgtgcccacggccgctccTG
 Acttaag (SEQ ID NO:106)

[0571] Nucleotide sequence of *BnFAE2* contained in pSZ3069:

actagtATGgagcgcaccaactccatcgagatggaccaggagcgctgaccgccgagatggccttcaaggactcctcctccgc
 cgtgatccgcatccgccggcctgcccgaacttctgacctccgtgaagctgaagtacgtgaagctgggcctgcacaactccttca
 acttaccaccttctgttctgctgacatcctgcccctgaccggcacccgtgctgggtgcagctgaccggcctgacctcgagaccttcc
 tccgagctgtggtacaaccacgcgcgccagctggacggcggtgacccgcctggcctgctggtgtccctgtgcttctgctgacatc
 tacgtgaccaaccgctccaagccggtgtacctgggtgacttctcctgctacaagcccgaggacgagcgcaagatgtccgtggact
 ccttctgaagatgaccgagcagaacggcgcccttaccgacgacaccgtgcagttccagcagcgcatctccaaccgcgcgggc
 ctggggcgacgagacctacctgccccgcggcatcacctccacccccccaagctgaacatgtccgaggcccgccgaggccga
 ggccgtgatgttcggcgccctggactccctgttcgagaagaccggcatcaagcccgccgaggtgggcatcctgatcgtgtcctgct
 ccctgttcaacccccccccctcctgtccgcatgatcgtgaaccactacaagatgcgcgaggacatcaagtcctacaacctggg
 cggcatgggctgctccgcccggcctgatctccatcgacctggccaacaacctgctgaaggccaaccccaactcctacgccgtgggtg
 gtgtccaccgagaacatcacctgaactggtacttcggcaacgaccgctccatgctgctgtgcaactgcatcttccgcatggggcg
 cgccgccatcctgctgtccaaccgcgcggcaggaccgctccaagtccaagtacgagctgggtgaacgtgggtgcgcacccacaagg
 gctccgacgacaagaactacaactgcgtgtaccagaaggaggacgagcgcgccaccatcggcggtgtccctggccccgcgagct

*gatgtccgtggccggcgacgccctgaagaccaacatcaccaccctgggcccctggtgctgccctgtccggccagctgatgttct
ccgtgtccctggtgaagcgcaagctgctgaagctgaaggtgaagccctacatccccgacttcaagctggccttcgagcacttctgc
atccacgccggcgccgcgctgctggacgaggtgcagaagaacctggacctggaggactggcacatggagccctcccgca
tgacctgcaccgcttcggcaacacctctctctctccctgtggtacgagatggcctacaccgaggccaagggccgctgaaggc
cggcgaccgctgtggcagatgccttcggctccggcttcaagtgcactccgctgtggaaggccctgcgctggtgtccacc
gaggagctgaccggcaacgcctgggcccgtccatcgagaactaccccgtaagatcgtgcagTGActtaag* (SEQ ID
NO:107)

[0572] 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.

[0573] Primary transformants were clonally purified and grown under standard 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 53-58, respectively, below.

[0574] All the transgenic STRAIN Z strains expressing heterologous FAE genes show an increased accumulation of C20:1 and C22:1 fatty acid (see Tables 53-58). The increase in eicosenoic (20 : 1^{Δ11}) and erucic (22 : 1^{Δ13}) 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.

[0575] Table 53. 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	57.99	6.62	0.56	0.19	0.00	0.06	0.05

pH 7

Cntrl STRAIN Z

pH 5 57.70 7.08 0.54 0.11 0.00 0.05 0.05

[0576] Table 54. 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	C22:2 n6	C22: :5
STRAIN Z; T588;	54.6						
D1829-36	6	7.04	0.52	1.82	0.84	0.12	0.09
STRAIN Z; T588;	56.2						
D1829-24	7	6.72	0.51	1.70	0.72	0.09	0.00
STRAIN Z; T588;	56.6						
D1829-11	5	8.36	0.54	2.04	0.67	0.00	0.00
STRAIN Z; T588;	55.5						
D1829-35	7	7.71	0.53	0.10	0.66	0.00	0.00
STRAIN Z; T588;	56.0						
D1829-42	3	7.06	0.54	1.54	0.51	0.06	0.08
Cntrl STRAIN Z	57.7						
pH 7	0	7.08	0.54	0.11	0.00	0.06	0.05
Cntrl STRAIN Z	57.9						
pH 5	9	6.62	0.56	0.19	0.00	0.05	0.05

[0577] Table 55. 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	C22:2 n6	C22: 5
STRAIN Z; T588;	57.7						
D1830-47	4	7.79	0.52	1.61	0.25	0.11	0.05
STRAIN Z; T588;	58.0						
D1830-16	6	7.39	0.55	1.64	0.22	0.07	0.06
STRAIN Z; T588;	57.7						
D1830-12	7	6.86	0.51	1.34	0.19	0.09	0.00
STRAIN Z; T588;	58.4						
D1830-37	5	7.54	0.49	1.65	0.19	0.06	0.00
STRAIN Z; T588;	57.1						
D1830-44	0	7.28	0.56	1.43	0.19	0.07	0.00
Cntrl STRAIN Z	57.7						
pH 7	0	7.08	0.54	0.11	0.00	0.06	0.05
Cntrl STRAIN Z	57.9						
pH 5	9	6.62	0.56	0.19	0.00	0.05	0.05

[0578] Table 56. Unsaturated fatty acid profile in S3067 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	C22:2n 6	C22: 5
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STRAIN Z; T588;	59.9					
D1825-47	7	7.44	0.56	0.57	0.00	0.00
STRAIN Z; T588;	58.7					
D1825-35	7	7.16	0.51	0.50	0.09	0.11
STRAIN Z; T588;	60.4					
D1825-27	0	7.82	0.47	0.44	0.07	0.07
STRAIN Z; T588;	58.0					
D1825-14	7	7.32	0.54	0.41	0.05	0.05
STRAIN Z; T588;	58.6					
D1825-40	6	7.74	0.46	0.39	0.08	0.00
Cntrl STRAIN Z	57.9					
pH 7	9	6.62	0.56	0.19	0.05	0.05
Cntrl STRAIN Z	57.7					
pH 5	0	7.08	0.54	0.11	0.06	0.05

[0579] Table 57. 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	C18:3a	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

[0580] Table 58. 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	C18:3a	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;	60.66	8.21	0.59	0.29	0.04	0.00

D1827-43**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 55: Elevating total unsaturated fatty acids level by expressing heterologous desaturase genes

[0581] 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 expect the total saturates will be further diminished by expressing desaturase genes in Strain N.

[0582] In the examples below, we demonstrated the ability to reduce stearic and palmitic levels in wild type strain UTEX1435 by over expression of heterologous stearyl-ACP desaturase genes, including desaturases from *Olea europaea*, *Ricinus communis*, and *Chlorella protothecoides*.

[0583] Construct used for the expression of the *Olea europaea* stearyl-ACP

desaturase: To introduce the *O. europaea* stearyl-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.

[0584] 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. *BspQ*I 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 stearyl-ACP desaturase coding region is indicated by bold italics. The *Chlorella protothecoides* stearyl-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.

[0585] Nucleotide sequence of transforming DNA contained in pSZ 1377:

gctcttcgcccgcgccaactcctgctcgagcgcgcccgcgctgcccgcagcgcttggccttttcgcccgcctcgtgcgctcgtgatgtcca
tcaccaggtccatgaggtctgccttgcgccggtgagccactgcttcgtccggcgcccaagaggagcatgaggaggactcctgggtccaggg
tcctgacgtgggtcgccgctctgggagcgccgagcatcatctggctctgccgcaccgaggccgctccaactggctcctccagcagccgcagtcg
ccgccgaccctggcagaggaagacaggtgaggggggtatgaattgtacagaacaaccacgagccttgtctaggcagaatccctaccagtcac
ggctttacctggatgacggcctgcgaacagctgtccagcgaccctcgctgccgccgttctccgcacgcttctttccagcaccgtgatggcgcg
agccagcgccgcacgctggcgctgcgcttcgccgatctgaggacagtcggggaaactctgatcagtctaaaccccttgcgcgttagtggtgcca
tcctttgcagaccggtgagagccgacttgtgtgcccacccccacacacctcctccagaccaattctgtcacctttttggcgaaggcatcgg
cctcggcctgcagagaggacagcagtgcccagccgctgggggttggcggatgcacgctcaggtacc
ctttcttgcgctatgacacttcagcaa
aaggtagggcggtgcgagacggcttcccgcgctgcatgcaacaccgatgatgcttcgacccccgaagctccttcggggctgcatggcgcd
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gacggcggctacaccttaccgagtagcagaagaaccccgctgctggccgccaactccacccagttccgcgacccgaaggtcttctggtacg
agccctcccagaagtggatcatgaccgcggccaagtcccaggactacaagatcgagatctactcctccgacgacctgaagtcttgaagc
tgaggtccgcgttcgccaacgagggcttctcggctaccagtacgagtccccggcctgatcgaggtccccaccgagcaggacccagca
agtcctactgggtgatgttcatctccatcaaccccgcgccccggcgcggtccttcaaccagtagttcgtcggcagcttcaacggcaccc
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gggagcgccctgggcatcgctgggctccaactgggagtactccgccttcgtgccaccaacccctggcgctcctccatgtccctcgtgcgc
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cggccccctggagccggttcgccaccaacacacggttgacgaaggccaacagctacaacgtcgacctgtccaacagcaccggcacccctgga
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acccctacttcaaccaaccgcatgagcgtgaacaaccagcccttcaagagcgagaaacgacctgtcctactacaaggtgtacggcttgctgga

ccagaacatcctggagctgtacttcaacgacggcgacgtcgtgtccaccaacacacttcatgaccaccgggaacgccctgggctccgtg
 aacatgacgacgggggtggacaacctgttctacatcgacaagttccaggtgcgcgaggtcaag**TGA**caattggcagcagcagctcggat
 agtatcgacacactctggacgctggcgtgtgatggactgttgccgccacacttctgccttgacctgtgaatatccctgccgcttttatcaaacag
 cctcagtgtgttgatcttgtgtgtacgcgcttttgcgagttgctagctgcttgtgtctatttgcgaataccacccccagcatccccttccctcgtttcat
 atcgcttgcacccaaccgcaacttatctacgctgtcctgctatccctcagcgctgctcctgctcctgctcactgcccctgcacagccttggttgg
 gctccgcctgtattctcctggtactgcaacctgtaaaccagcactgcaatgctgatgcacgggaagtagtgggatgggaacacaaatggaggat
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gctgcatgcaacaccgatgatgcttcgacccccgaagctccttcggggctgcatgggcgctccgatgccgctccaggggcgagcgctgttta
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ctcgagcttgtgatcgactccgctaagggggcgcttctcctcttctgtttcagtcacaaacccgcaaa**actagtATG**gccaccgcatccact
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gatggagctgcgcaagcgtgcaaggagatccccgacgactacttcatcgtgctggtgggcgacatgatcaccgaggaggccctgccc
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atgatgcgcaagaaggtgtccatgccgcccacctgatgtacgacggccaggacgacaacctgttcgagaacttctcctcgtggccca
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gaaggcctccgccaccccccttctcctggatcttcggccgcgagatcaacctgatggactacaaggaccacgacggcgactacaaggacc
acgacatcgactacaaggacgacgacgacaagTGAatcgatagatctcttaaggcagcagcagctcggatagtatcgacacactctggac
 gctggtcgtgtgatggactgttgccgccacacttctgccttgacctgtgaatatccctgccgcttttatcaaacagcctcagtgtgttgatcttgtg
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 acttatctacgctgtcctgctatccctcagcgctgctcctgctcctgctcactgcccctgcacagccttggttgggctccgcctgtattctcctggt
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 cagaaggagttgctccttgagcctttcattctcagcctcgataacctccaaagccgctctaattgtggagggggttcgaatttaaaagcttgaa
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 ctgctttcgcgcaatctgcctgttgaaatcgccaccacattcatattgtgacgcttgagcagctgtgaattgcctcagaatgtggaatcatctgc
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gtgaccatatttctcgaagctccccaacgagcacctccatgctctgagtgccacccccggccctgggtgcttcggaggggcaggtcaaccggc
 atggggctaccgaaatccccgaccggatcccaccacccccgcgatgggaagaatctctccccgggatgtgggcccaccaccagcacaaacctgc
 tggcccaggcgagcgtcaaaccataccacacaaatatccttggcatcggccctgaattccttctgccgctctgctacccgggtgcttctgtccgaa
 gcaggggttgctagggatcgctccgagtcgcaaacccttgtcgcgtggcggggcttggtcgagcttgaagagc (SEQ ID NO:108)

[0586] 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.

[0587] 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. *BspQ*I 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 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.

[0588] Nucleotide sequence of transforming DNA contained in pSZ1454 :

gctcttcgccgccgcccactcctgctcgagcgcgcccgcgctgcgccgagcgcttggccttttcgccgctcgtgcgcgtcgtgatgtcca
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[0589] Construct used for the expression of the *Chlorella protothecoides* stearyl-ACP

desaturase: To introduce the *Chlorella protothecoides* stearyl-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.

[0590] 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. *BspQ*I 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 stearyl-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.

[0591] Nucleotide sequence of transforming DNA contained in pSZ3144 :

gctcttcgccgccgcccactcctgctcgagcgcgcccgcgctgcgccgagcgcttggccttttcgccgctcgtgcgcgtcgctgatgtcca
tcaccaggtccatgaggtctgccttgcgccggctgagccactgcttcgtccgggcggaagaggagcatgagggaggactcctgggtccaggg
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ctacccggtgcttctgtccgaagcaggggttgtagggatcgctccgagtcgcaaaccttgtcgcggtggcggggcttgttcgagcttgaaga
gc (SEQ ID NO:110)

[0592] Results: 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 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 59, 60 and 61, respectively, below. The 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 stearyl-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. 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. The impact of these desaturase genes in the high oleic strain S5587 will be determined.

[0593] Table 59. 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

[0594] Table 60. 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

[0595] Table 61. 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

[0596] 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.

SEQUENCE LISTING

SEQ ID NO: 1
6S 5' genomic donor sequence

GCTCTTCGCCGCCGCGCCACTCCTGCTCGAGCGCGCCCGCGCGTGCGCCGCCAGCGCCTTGGCCTTTTCG
CCGCGCTCGTGCGCGTCGCTGATGTCCATCACCAGGTCCATGAGGTCTGCCTTGCGCCGGCTGAGCCA
CTGCTTCGTCCGGGCGGCCAAGAGGAGCATGAGGGAGGACTCCTGGTCCAGGGTCCTGACGTGGTCGC
GGCTCTGGGAGCGGGCCAGCATCATCTGGCTCTGCCGCACCGAGGCCGCCTCCAAGTGGTCTCCAGC
AGCCGCAGTCGCCGCCGACCCTGGCAGAGGAAGACAGGTGAGGGGGGTATGAATTGTACAGAACAACC
ACGAGCCTTGTCTAGGCAGAATCCCTACCAGTCATGGCTTTACCTGGATGACGGCCTGCGAACAGCTG
TCCAGCGACCCTCGCTGCCGCCGCTTCTCCCGCACGCTTCTTTCCAGCACCGTGATGGCGCGAGCCAG
CGCCGCACGCTGGCGCTGCGCTTCGCCGATCTGAGGACAGTCGGGGAAGTCTGATCAGTCTAAACCCC
CTTGCGCGTTAGTGTTGCCATCCTTTGCAGACCGGTGAGAGCCGACTTGTTGTGCGCCACCCCCACA
CCACCTCCTCCCAGACCAATTCTGTCACCTTTTTGGCGAAGGCATCGGCCTCGGCCTGCAGAGAGGAC
AGCAGTGCCCAGCCGCTGGGGGTTGGCGGATGCACGCTCAGGTACC

SEQ ID NO: 2
6S 3' genomic donor sequence

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 252

NOTE : Pour les tomes additionels, veuillez contacter le Bureau canadien des brevets

JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 252

NOTE: For additional volumes, please contact the Canadian Patent Office

NOM DU FICHER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

WHAT IS CLAIMED IS:

1. An oleaginous microalgal cell, optionally comprising 23S rRNA having at least 65% nucleotide sequence identity to SEQ ID NO: 76, optionally obligately heterotrophic, and optionally comprising an exogenous sucrose invertase gene so that the cell can grow on sucrose as a sole carbon source, the cell comprising an exogenous gene encoding an active LPAAT enzyme, the cell producing an oil comprising triglycerides, wherein the oil is, by virtue of the LPAAT activity:
 - (a) enriched in triglycerides with midchain fatty acids; or
 - (b) enriched in triglycerides of the saturated-unsaturated-saturated type.
2. The cell of claim 1, wherein the triglycerides of the oil comprise 40, 50, 60, 70, or 80% or more of C8:0, C10:0, C12:0, C14:0, or C16:0 fatty acids.
3. The cell of claim 1 or 2, wherein the cell further comprises an exogenous gene encoding an active FATB acyl-ACP thioesterase.
4. The cell of claim 3, wherein the triglycerides of the oil are enriched in midchain fatty acids by greater than 70% as a result of the expression of the exogenous LPAAT and acyl-ACP thioesterase.
5. The cell of any one of claims 1 to 4, wherein the cell further comprises recombinant nucleic acids operable to encode an exogenous KAS I or KAS IV enzyme or to reduce the activity of an endogenous KAS I enzyme.
6. The cell of any one of claims 1 to 5, wherein the cell further comprises nucleic acids operable to reduce the expression of a delta 12 fatty acid desaturase, optionally via a regulatable promoter, so as to produce an oil with linoleic and linolenic acids totaling 5 area percent or less by FAME GC/FID.
7. The cell of claim 1, wherein the oil is enriched in triglycerides of the saturated-unsaturated-saturated type.
8. The cell of claim 7, wherein the oil is enriched in SOS, POS, and/or POP.
9. The cell of claim 8, wherein the oil comprises triglycerides comprising at least 50% SOS, and optionally less than 10% SSS.

10. The cell of claim 8 or 9, further comprising a knockout or knockdown of a stearyl-ACP desaturase gene, FatA gene, or both.

11. The cell of any of claims 7 to 9, further comprising recombinant nucleic acids operable to increase beta-ketoacyl CoA synthase activity.

12. The cell of claim 11, wherein the nucleic acids operable to increase beta-ketocyl synthase activity comprise an exogenous gene encoding a beta-ketoacyl CoA synthase.

13. The cell of any one of claims 7 to 12, wherein the ratio of stearate to oleate in oil is $3:1 \pm 20\%$.

14. The cell of any one of claims 7 to 13, wherein POP, SOS, and POS in the oil comprise at least 30% in sum.

15. The cell of any one of claims 7 to 14, wherein the oil comprises at least 30% POS.

16. The cell of any one of claims 7 to 15, wherein the oil comprises POP at $16\% \pm 20\%$, POS at $38\% \pm 20\%$, and SOS at $23\% \pm 20\%$.

17. The cell of any one of claims 7 to 16, wherein the fatty acid profile of the oil comprises 1 to 4% arachidic acid.

18. The cell of any one of claims 7 to 17, wherein the cell further comprises nucleic acids operable to reduce the expression of a delta 12 fatty acid desaturase, optionally via a regulatable promoter, so as to produce an oil with linoleic and linolenic acids totaling 5 area percent or less.

19. The cell of any one of claims 7 to 17, wherein the oil has 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 acids.

20. The cell of any one of claims 1 to 19, wherein the LPAAT has the amino acid sequence of SEQ ID NO: 78 or SEQ ID NO: 79 or a sequence having at least 95% identity to SEQ ID NO:78 or SEQ ID NO: 79.

21. A method for producing an oil comprising providing or cultivating a cell according to any one of claims 1 to 20, and extracting the oil, the cell optionally being cultivated heterotrophically.
22. An oil comprising triglycerides produced according to claim 21.
23. The oil of claim 22, comprising one or more of:
at least 10% ergosterol;
ergosterol and β -sitosterol, wherein the ratio of ergosterol to β -sitosterol is greater than 25:1;
ergosterol and brassicasterol;
ergosterol, brassicasterol, and poriferasterol;
and wherein the oil is optionally free from one or more of β -sitosterol, campesterol, and stigmasterol.
24. The oil of claim 22 wherein the oil forms β polymorph crystals.
25. The oil of claim 24, wherein the crystals have a 2L lamellae structure.
26. The oil of claim 24, wherein the crystals have a 3L lamellae structure.
27. The oil of claim 22, wherein the oil forms β' polymorph crystals.
28. The oil of claim 24, wherein the crystals have a 2L lamellae structure.
29. The oil of claim 24, wherein the crystals have a 3L lamellae structure.
30. The oil of any one of claims 22 to 29, wherein the triglycerides have a fatty acid profile characterized in that the sum of the percentage of stearate and palmitate is equal to the percentage of oleate multiplied by 2.0 +/- 40%.
31. The oil of any one of claims 22 to 30, wherein the oil has greater than 65% SOS triglycerides, less than 45% unsaturated fatty acid, less than 5% unsaturated fatty acids, less than 1% lauric acid, and less than 2% trans fatty acid.
32. The oil of any one of claims 22 to 31, wherein the sum of the percent stearate and palmitate in the fatty acid profile is twice the percentage of oleate, $\pm$ 20% .
33. The oil of claim 32, wherein the sn-2 profile has at least 40% oleate.

34. The oil of any one of claims 22 to 33, wherein the oil is at least 40, 50, 60, 70, 80, or 90% SOS.

35. The oil of any one of claims 22 to 33, wherein the oil is a roll-in shortening having a melting temperature of between 30°C and 40°C.

36. The oil of claim 35, comprising β' polymorphic crystals.

37. The oil of claim 35 or 36, having a solid fat content of less than 15% at 35°C.

38. The oil of any one of claims 35 to 37, comprising 15 to 20% C8 to C14 fatty acids, 45-50% fatty acids of C16 and higher, and/or 30-25% unsaturated fatty acids.

39. A food, fuel or chemical product produced using the oil of any one of claims 22 to 38.

40. A natural oil or RBD oil produced from a natural oil, the oil comprising 3.5% or less saturated fatty acids, and optionally comprising greater than 50% oleic acid and/or greater than 1% palmitoleic acid.

41. The oil of claim 40, having between 0.1 and 3.5% saturated fatty acids.

42. The oil of claim 40 or 41, comprising at least 90% oleic acid.

43. The oil of claim 42, comprising at least 3% polyunsaturated fatty acids.

44. An oleaginous cell, optionally comprising 23S rRNA having at least 65% nucleotide sequence identity to SEQ ID NO: 76 and optionally obligately heterotrophic, that produces an oil comprising 3.5% or less saturated fatty acids.

45. The cell of claim 44, wherein the cell is of the genus *Prototheca*.

46. The cell of claim 44, further comprising a FATA knockout or knockdown.

47. The cell of any one of claims 44 to 46, comprising an exogenous gene encoding an enzyme active to desaturate palmitoyl-CoA to plamitoyl-CoA.

48. The cell of claim 47, wherein the exogenous gene is a PAD gene.

49. The cell of claim 47, wherein the exogenous gene is a SAD gene having desaturase activity toward palmitoyl-ACP.

50. The cell of any one of claims 44 to 49, further comprising an overexpressed KASII enzyme.

51. The cell of any one of claims 44 to 50, further comprising nucleic acids operable to reduce the expression of a delta 12 fatty acid desaturase, optionally via a regulatable promoter, so as to produce an oil with linoleic and linolenic acids totaling 5 area percent or less.

52. An oil produced by the cell of any one of claims 44 to 51, optionally refined, bleached and deodorized, the oil comprising one or more of:
at least 10% ergosterol;
ergosterol and b-sitosterol, wherein the ratio of ergosterol to b-sitosterol is greater than 25:1;
ergosterol and brassicasterol; and
ergosterol, brassicasterol, and poriferasterol;
wherein the oil is optionally free from one or more of -sitosterol, campesterol, and stigmasterol.

53. A method for producing an oil having 3.5% or less saturated fatty acids, the method comprising providing or cultivating a cell of any one of claims 5 to 12, and extracting the oil from the cell.

54. A method for producing a food comprising incorporating an oil produced by the method of claim 53 into the food, wherein the finished food product has 3.5% or less saturated fat.

55. A recombinant oleaginous cell, optionally comprising 23S rRNA having at least 65% nucleotide sequence identity to SEQ ID NO: 76 and optionally obligately heterotrophic, the cell comprising an exogenous gene encoding an active ketoacyl-CoA synthase enzyme.

56. The cell of claim 55, wherein the cell produces an oil comprising greater than 20% erucic acid.

57. A cell according to claim 56, wherein the cell produces an oil comprising greater than 60% erucic acid.

58. The cell of any one of claims 55 to 57, wherein the cell comprises at least 40% oil.

59. The cell of any one of claims 55 to 58, wherein the cell is of the genus *Prototheca*, and optionally of the species *Prototheca moriformis*.

60. An oil produced by the cell of any one of claims 55 to 59, comprising one or more of:
at least 10% ergosterol;
ergosterol and β -sitosterol, wherein the ratio of ergosterol to β -sitosterol is greater than 25:1;
ergosterol and brassicasterol; and
ergosterol, brassicasterol, and poriferasterol;
wherein the oil is optionally free from one or more of β -sitosterol, campesterol, and stigmasterol.

61. A chemical produced from the oil of claim 60.

62. A method for producing an oil comprising providing or cultivating a cell according to any one of claims 55 to 59 and extracting an oil from the cell.

63. A recombinant oleaginous cell comprising recombinant nucleic acids operable to suppress the activity of a delta 12 fatty acid desaturase gene product so that the cell produces an oil with a triacylglycerol profile having less than 5% linoleic acid.

64. The cell of claim 63, wherein the cell produces an oil with a triacylglycerol profile having less than 3% linoleic acid.

65. The cell of claim 64, wherein the cell produces an oil with a triacylglycerol profile having less than 2% linoleic acid.

66. The cell of claim 63, wherein the cell is a linoleic acid auxotroph or activity of the delta 12 fatty acid desaturase can be suppressed via environmental conditions so as to produce the oil.

67. The cell of claim 66, wherein the delta 12 fatty acid desaturase is regulatable via environmental conditions due to a regulatable promoter in operable linkage to the delta 12 fatty acid desaturase gene.

68. The cell of claim 67, wherein the regulatable promoter is regulatable by change in media pH or nitrogen levels.

69. The cell of any one of claims 64 to 68, further comprising recombinant nucleic acids operable to express an exogenous KASII, LPAAT, or FATB enzyme.

70. The cell of any one of claims 64 to 69, further comprising recombinant nucleic acids operable to knockout or knockdown the expression of a stearyl ACP desaturase enzyme.

71. The cell of any one of claims 64 to 70, further comprising recombinant nucleic acids operable to knockout or knockdown the expression of an endogenous FatA-encoded acyl-ACP thioesterase.

72. The cell of any one of claims 64 to 71, wherein the oil is stable at 110°C so that the inflection point in conductance is not yet reached by 20 hours under conditions of the AOCS Cd 12b-92 Rancimat test.

73. The cell of any one of claims 64 to 71, wherein the oil is stable at 110°C so that the inflection point in conductance is not yet reached by 5 days under conditions of the AOCS Cd 12b-92 Rancimat test, when 1050ppm of tocopherol and 500 pm of ascorbyl palmitate are added to the oil.

74. A method comprising:

(a) providing a recombinant oleaginous cell, optionally comprising 23S rRNA having at least 65% nucleotide sequence identity to SEQ ID NO:76, optionally obligately heterotrophic, the cell comprising recombinant nucleic acids operable to modify the amount of a fatty acid made by the cell in response to a change in an environmental condition;

(b) cultivating the cell under a first environmental condition that is permissive to synthesis of the fatty acid so as to allow for cell division and increase the number of cells;

(c) cultivating the cell under a second environmental condition that, due to the recombinant nucleic acids, reduces the synthesis of the fatty acid and thus the amount of that fatty acid in an oil produced by the cell; and

(d) extracting the oil from the cell.

75. The method of claim 74, wherein the cell comprises exogenous nucleic acids operable to reduce the activity of a delta 12 fatty acid desaturase so as to reduce the amount of linoleic acid in the oil.

76. The method of claim 74 or 75, wherein the linoleic acid is depleted in the oil by at least than 50, 60, 70, 80, or 90%.

77. The method of any one of claims 74 to 76 wherein the cell is cultivated heterotrophically.

78. The method of any one of claims 74 to 77, wherein the cell is a microalgal cell.

79. The method of any one of claims 74 to 78, wherein the cell produces at least 40, 50, 60, 70, 80, or 90% oil by dry cell weight.

80. The method of any one of claims 74 to 79, wherein the first environmental condition is a first pH and the second environmental condition is a second pH of cultivation media.

81. The method of any one of claims 74 to 80, wherein, when extracted from the cell, the oil is stable at 110°C so that the inflection point in conductance is not yet reached by 20 hours under conditions of the AOCS Cd 12b-92 Rancimat test.

82. The method of any one of claims 74 to 81, wherein when extracted from the cell, the oil is stable at 110°C so that the inflection point in conductance is not yet reached by 5 days under conditions of the AOCS Cd 12b-92 Rancimat test, when 1050ppm of tocopherol and 500 pm of ascorbyl palmitate are added to the oil.

83. The method of any one of claims 74 to 82, wherein the cell comprises an exogenous gene encoding a KAS II enzyme and optionally a knockout or knockdown of a FatA gene.

84. The method of any one of claims 74 to 83, wherein the recombinant nucleic acids operable to modify the amount of a fatty acid made by the cell comprise an inhibitory RNA targeting a FAD gene, the production of the inhibitory RNA being under control of a regulatable promoter.

85. The method of claim 83 or 84, wherein the oil is characterized by a fatty acid profile with greater than 60% oleic acid and less than 3% polyunsaturates.

86. The method of claim 85, wherein the oil is characterized by a fatty acid profile with greater than 70% oleic acid and less than 2% polyunsaturates.

87. The method of claim 86, wherein the oil is characterized by a fatty acid profile with greater than 80% oleic acid and less than 1% polyunsaturates.

88. An oil produced by the method of any one of claims 74 to 87.

89. The oil of claim 88 comprising 0.01 to 2% linoleic acid and (i) 80 to 95% oleic acid or (ii) more than 40% of C8, C10, C12, C14 or C16 fatty acids.

90. The oil of claim 88 or 89, further comprising one or more of:
at least 10% ergosterol;
ergosterol and b-sitosterol, wherein the ratio of ergosterol to b-sitosterol is greater than 25:1;
ergosterol and brassicasterol; and
ergosterol, brassicasterol, and poriferasterol.

91. A product produced from the oil of any one of claims 88 to 90.

92. The product of claim 91, wherein the product is a food, fuel or chemical.

93. The product of claim 91, wherein the product is a frying oil, lubricating oil, cleaning solvent, surfactant, foam or lubricant.

94. The product of claim 91, wherein the product is an oleic acid dimer.

95. A construct, vector, chromosome or host cell comprising nucleic acids encoding a protein having at least 90% identity to SEQ ID NOs: 77 to 79.

96. The construct, vector, chromosome or host cell of claim 95, encoding a protein having at least 95% identity to SEQ ID NOs: 77 to 79.

97. The construct, vector, chromosome or host cell of claim 95, encoding a protein having at least 98% identity to SEQ ID NOs: 77 to 79.

98. The construct, vector, chromosome, or host cell of any of claims 95 to 97, comprising nucleic acids having at least 80, 90, 95, 96, 97, 98, or 99% sequence identity to SEQ ID NOs:80-85 or equivalent sequences by virtue of the degeneracy of the genetic code.

SAMPLE OIL PROFILES: LOW TO HIGH OLEIC CONTENT

	capric-rich	lauric-rich	myristic-palmitic	high palmitic	balanced	stearic-palmitic-oleic	mixed palmitic-oleic	mixed palmitic-oleic-2	high oleic	high-stability oleic
<i>Oil</i>	<i>RB Z</i>	<i>RBD-1</i>	<i>RBD-2</i>	<i>RBD-3</i>	<i>RBD Y</i>	<i>RBD X</i>	<i>RBD W</i>	<i>RBD-4</i>	<i>RDB-5</i>	<i>RBD-6</i>
C8:0	4.5	0.2	0.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C10:0	29.3	16.8	13.2	0.0	2.4	0.0	0.0	0.0	0.0	0.0
C12:0	4.4	47.2	2.8	0.2	0.9	0.0	0.1	0.1	0.0	0.0
C14:0	23.6	11.3	24.8	6.0	15.3	0.7	2.0	1.7	0.5	0.6
C16:0	21.5	5.1	31.6	49.1	35.9	24.0	28.7	25.0	6.9	8.2
C16:1	0.1	0.1	0.1	0.4	0.3	0.2	0.4	1.0	0.7	0.8
C18:0	2.3	0.9	4.8	5.1	3.5	21.4	9.5	3.6	1.4	1.9
C18:1	8.5	12.8	13.9	28.7	29.6	43.5	48.9	59.4	79.5	85.9
C18:2	4.6	4.2	4.8	8.3	10.3	7.8	8.0	7.4	8.9	0.05
C18:3	0.3	0.3	0.2	0.3	0.2	0.1	0.2	0.2	0.2	0.0

Figure 1

SOLID FAT CONTENT RANGE: SOLAZYME TAILORED OILS

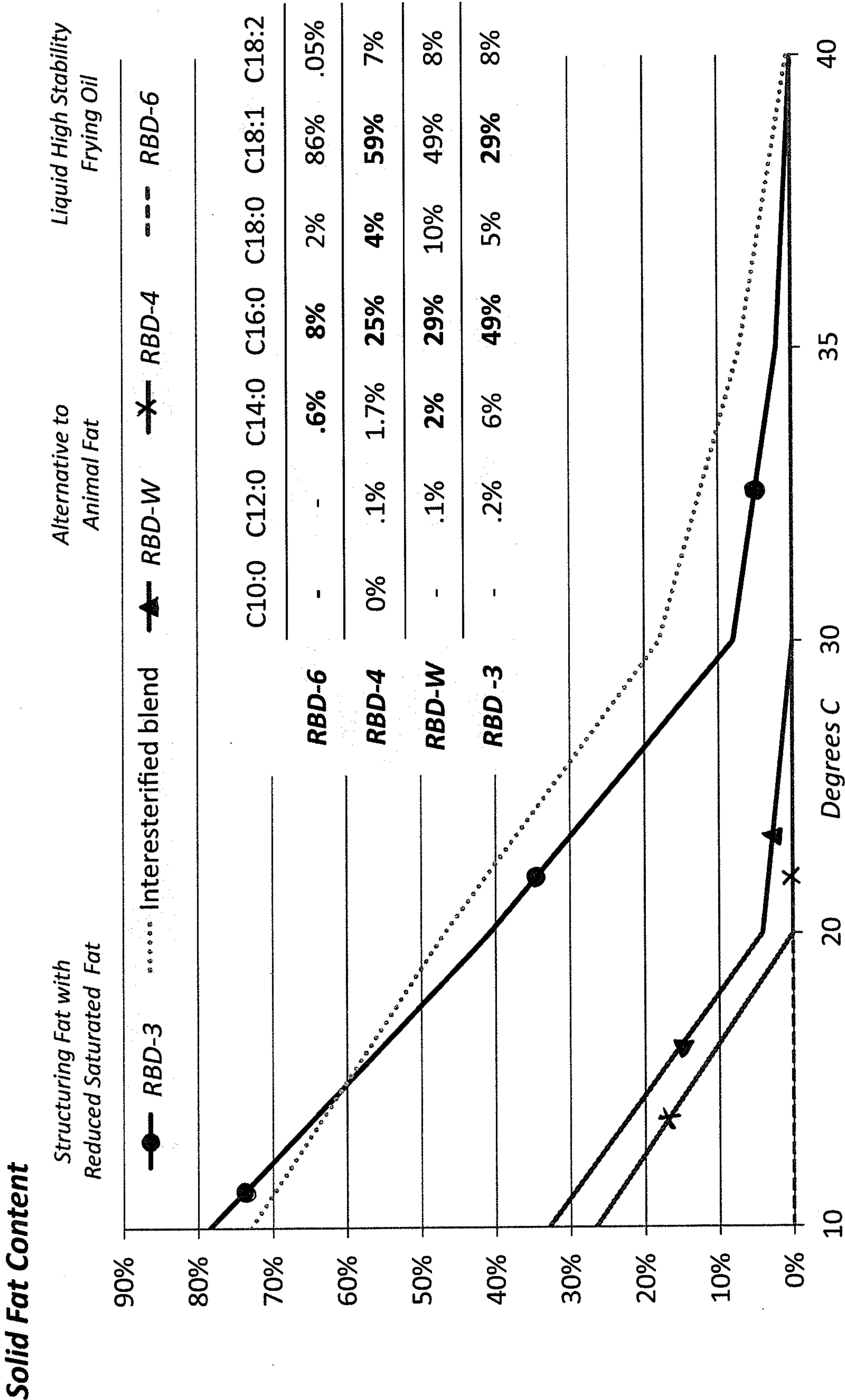


Figure 2

ALTERNATIVE COMPOSITIONS: SIMILAR SFC PROFILES

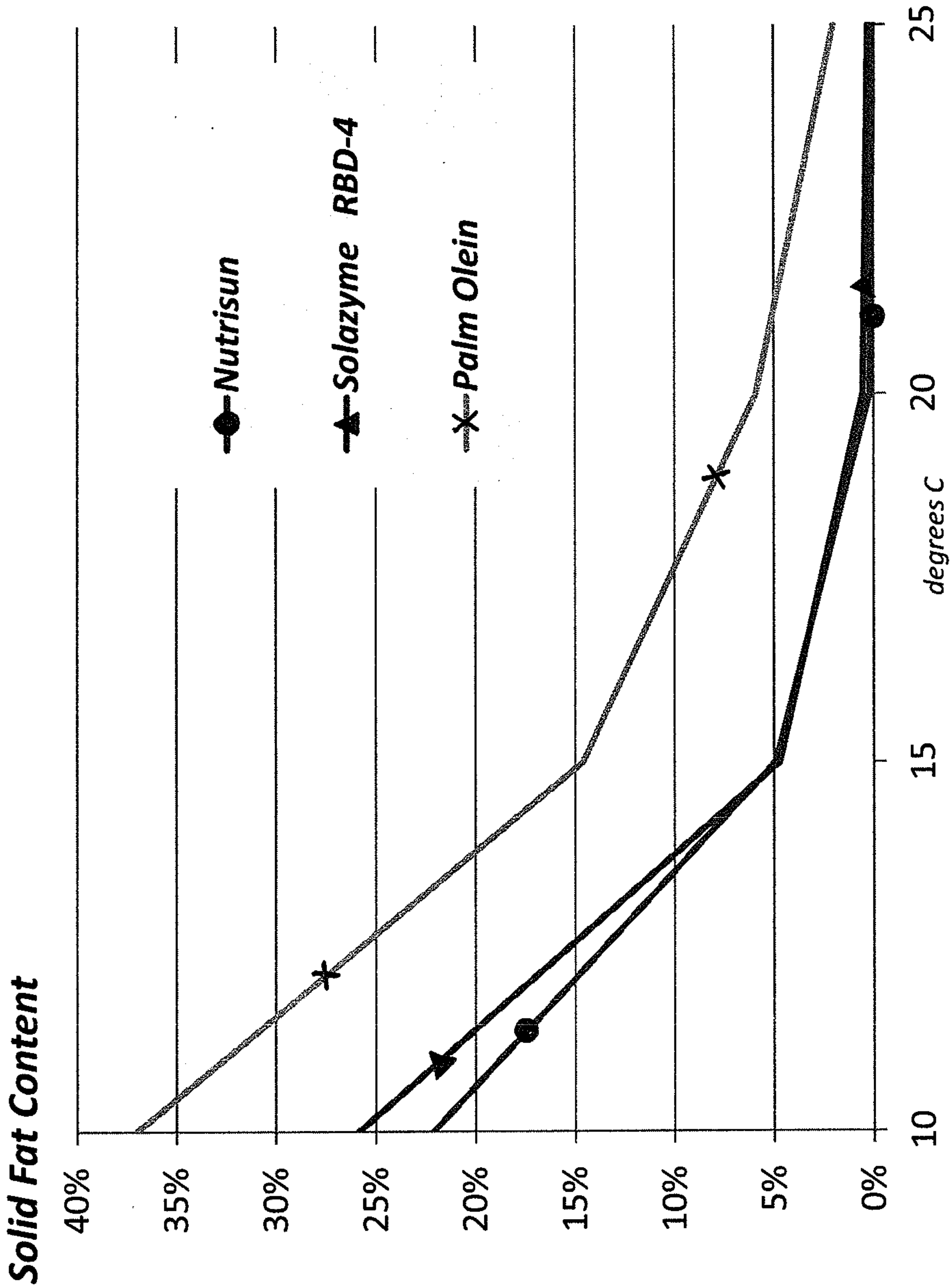


Figure 3

STRUCTURING FAT COMPOSITIONS

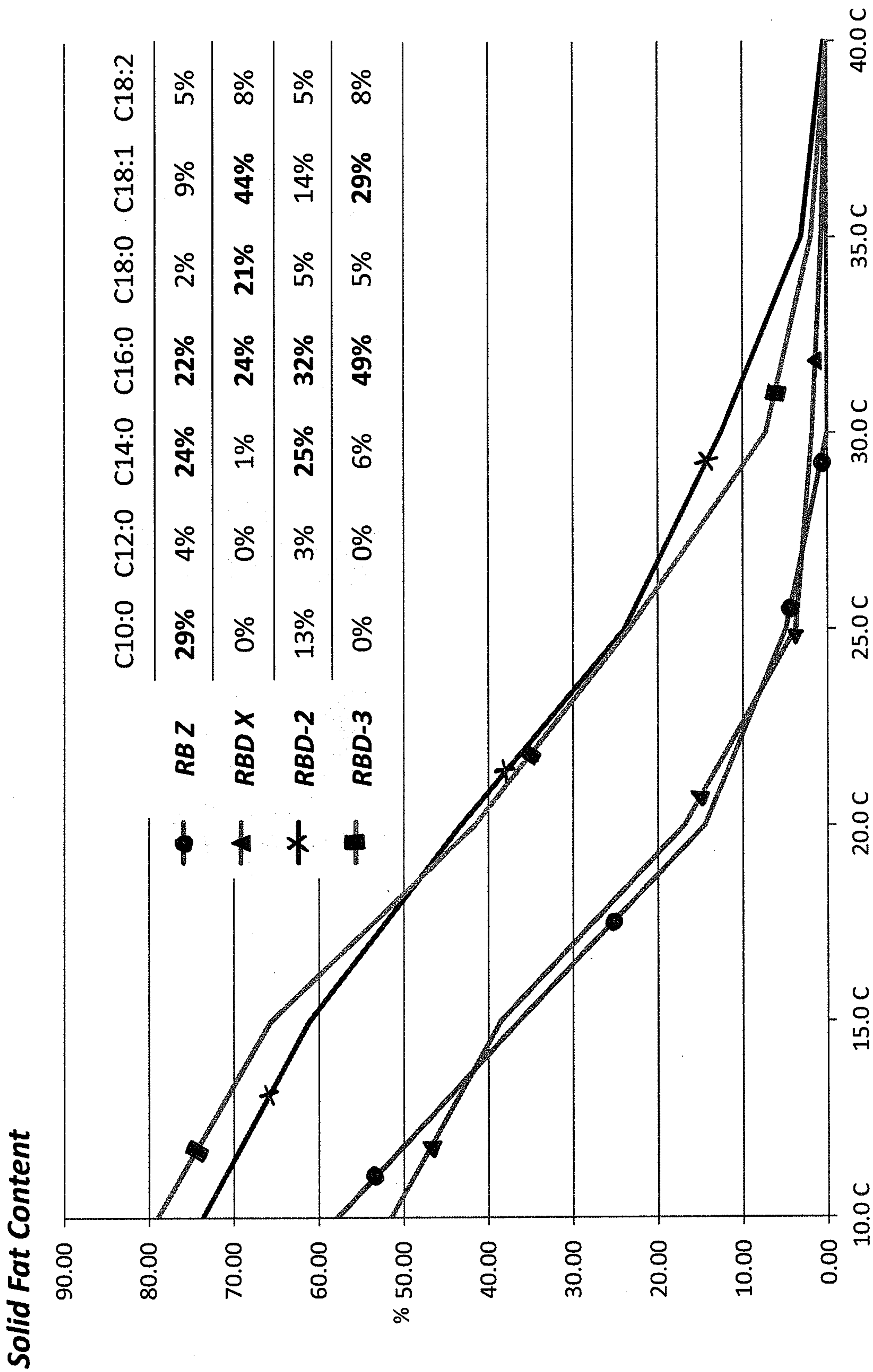


Figure 4

SOLAZYME'S TAILORED OILS: UNIQUE PHYSICAL PROPERTIES

- *Similar fatty acid profile to palm but...*
 - Higher solids & sharper melting
 - Completely melted by body temp
- *~20% lower saturated fat vs. shortening blend*

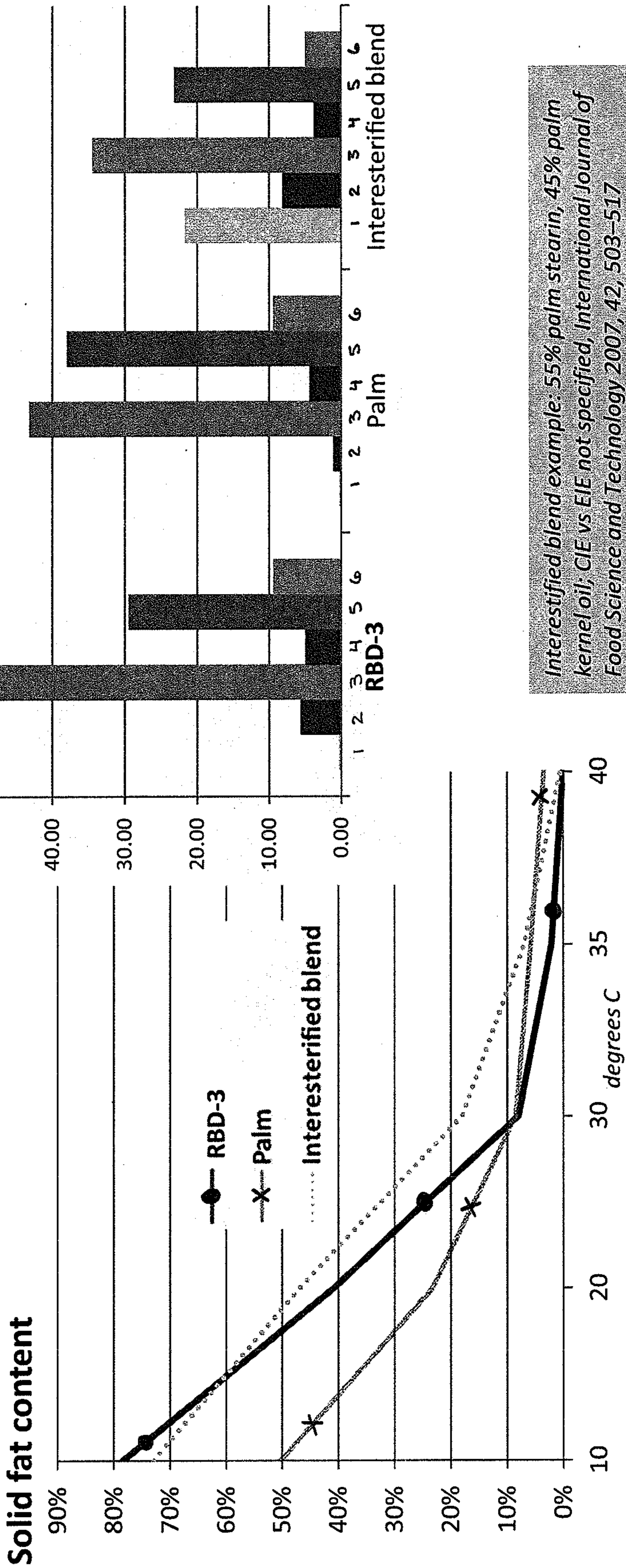


Figure 5

Lauric-Rich Algal Oil

WO 2013/158938

CA 02870364 2014-10-10

PCT/US2013/037261

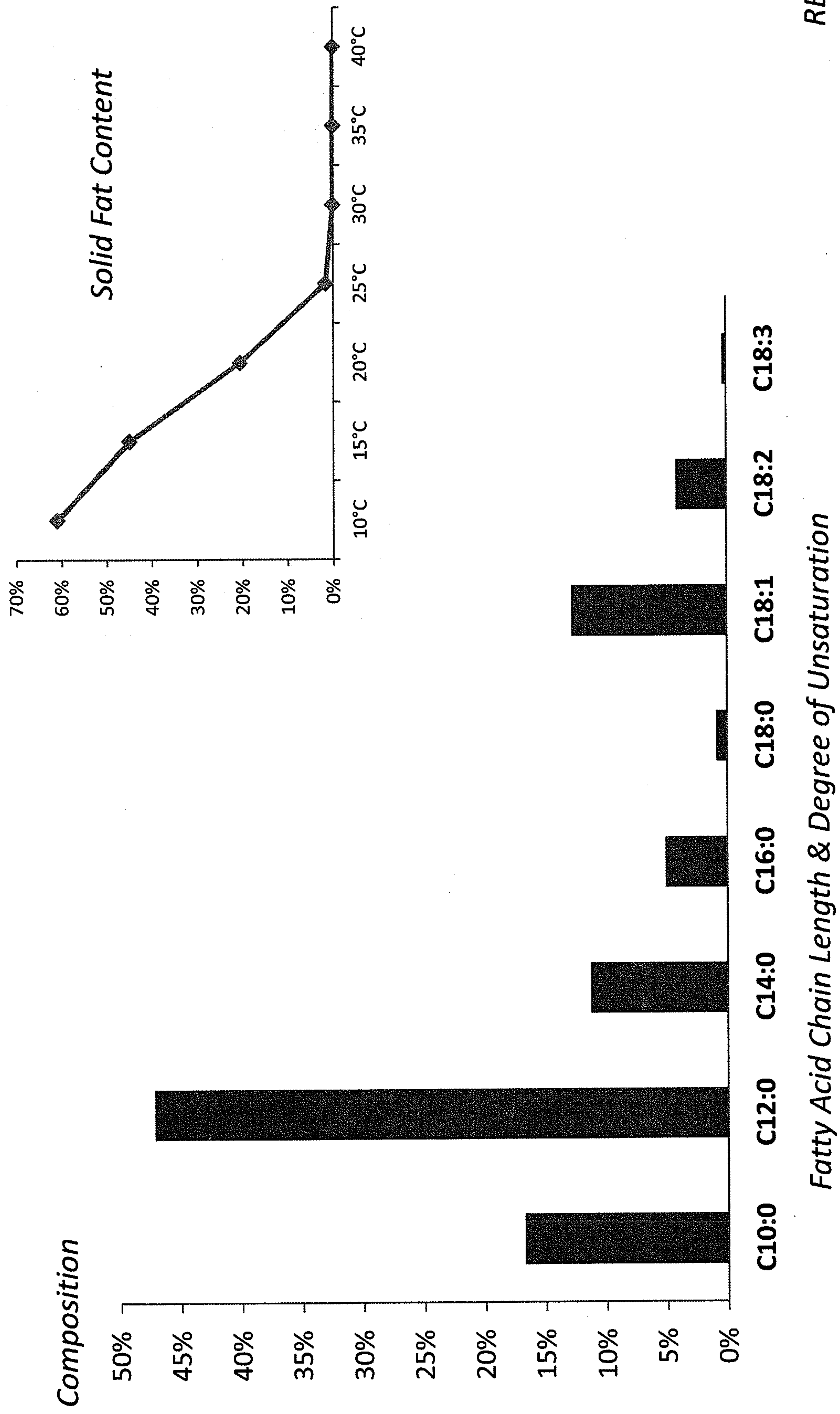


Figure 6

Myristic-Palmitic Algal Oil

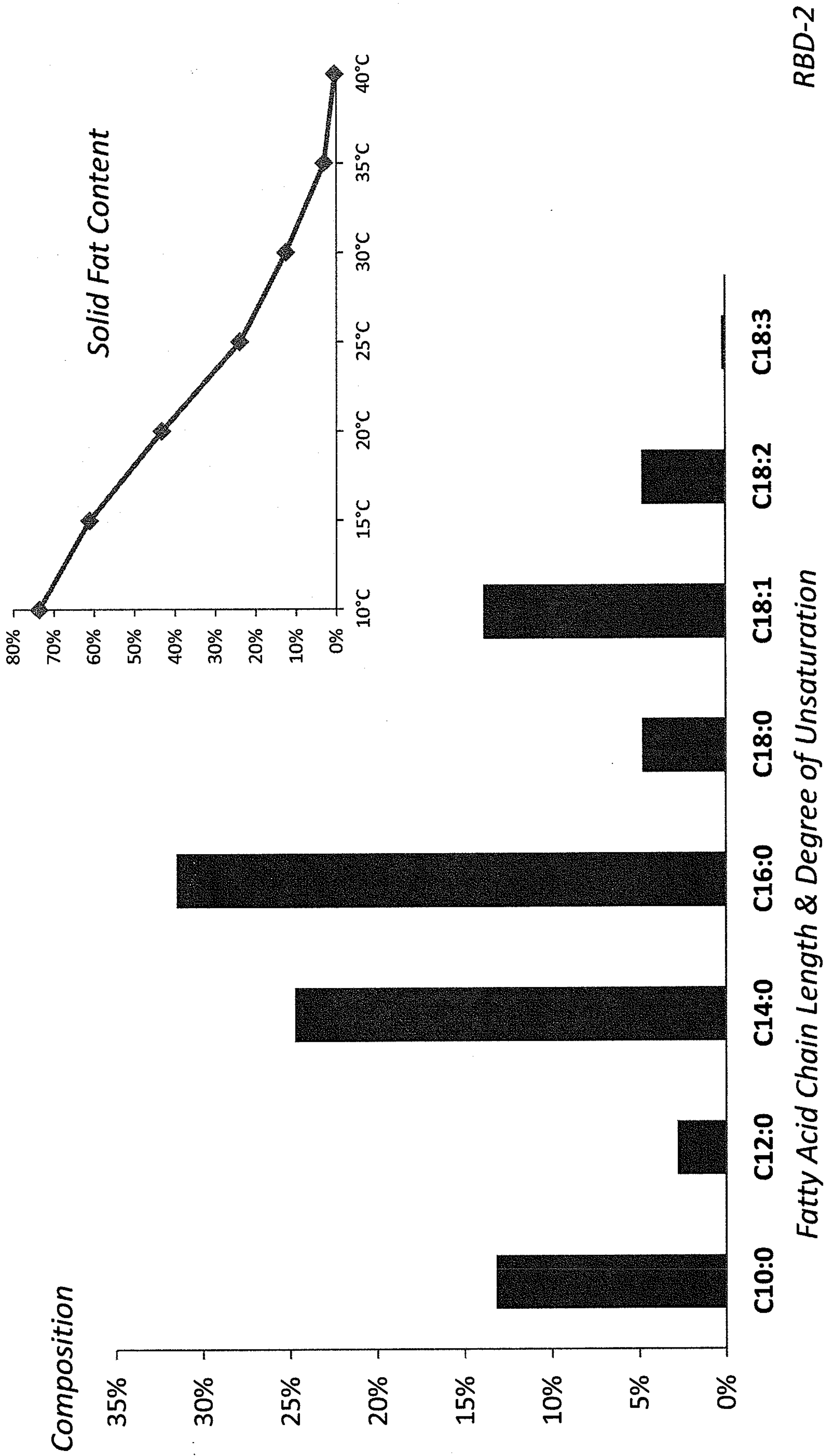


Figure 7

High-Palmitic Algal Oil

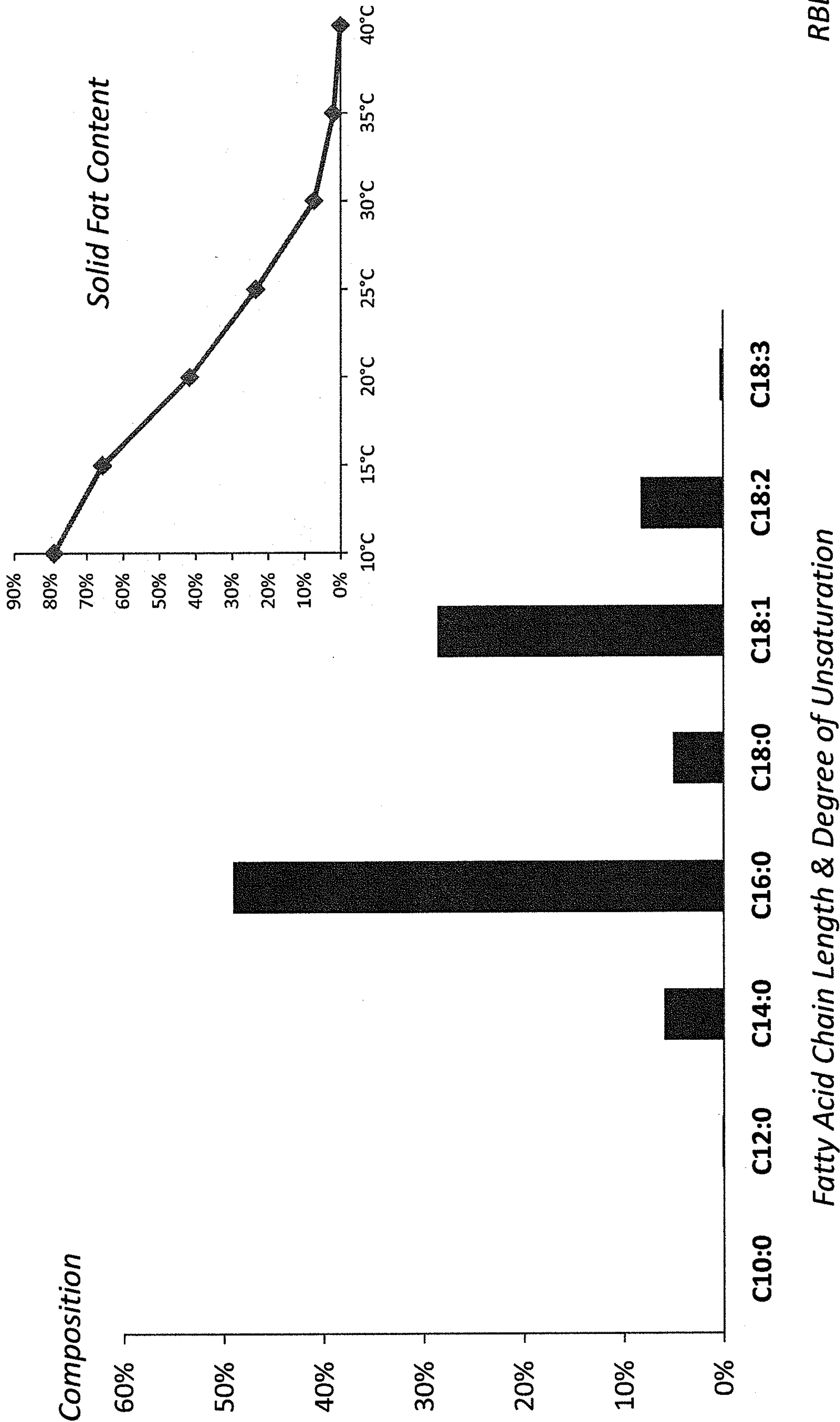


Figure 8

High-Oleic Mid-Palmitic Algal Oil

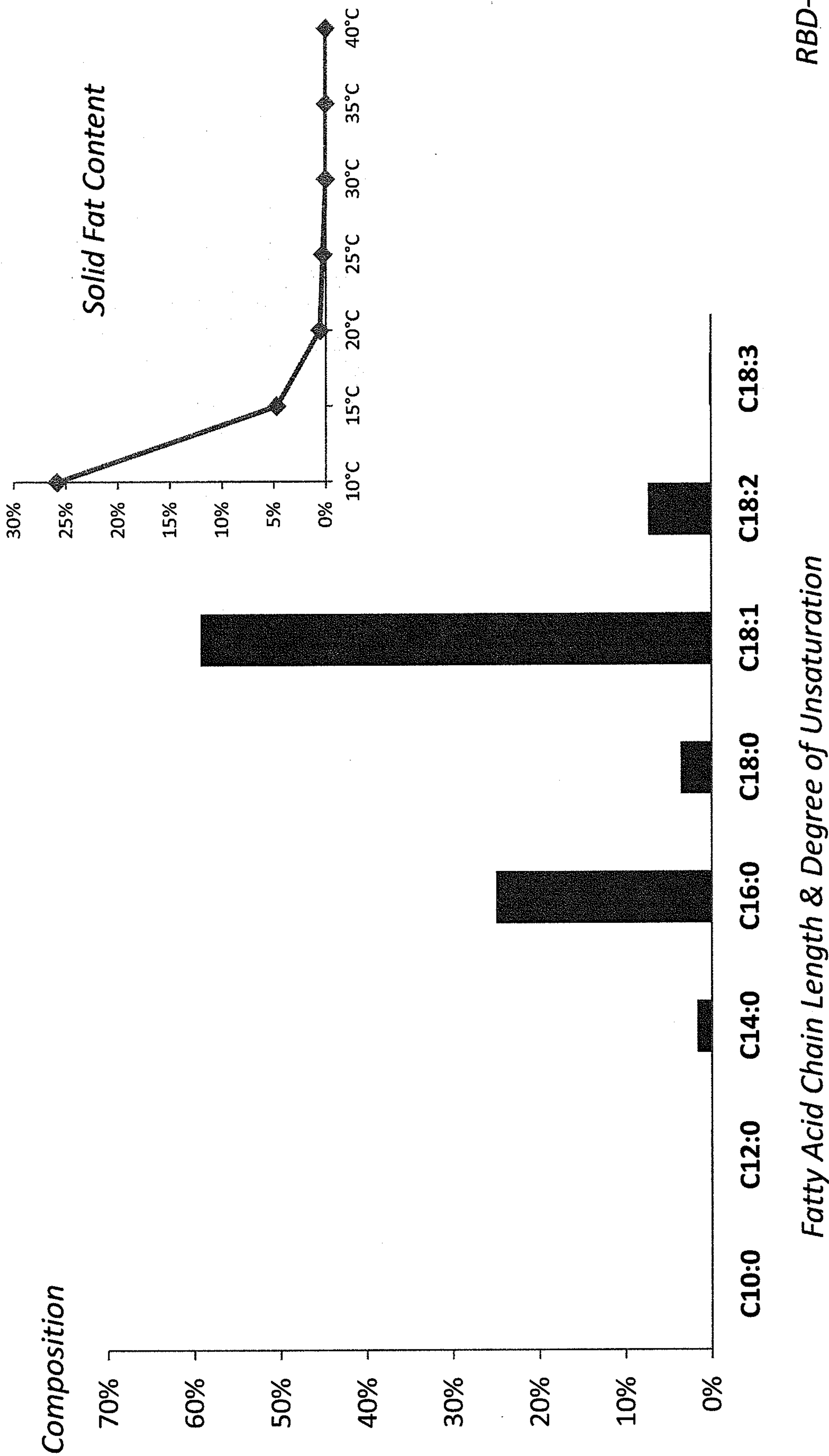


Figure 9

High-Oleic Algal Oil

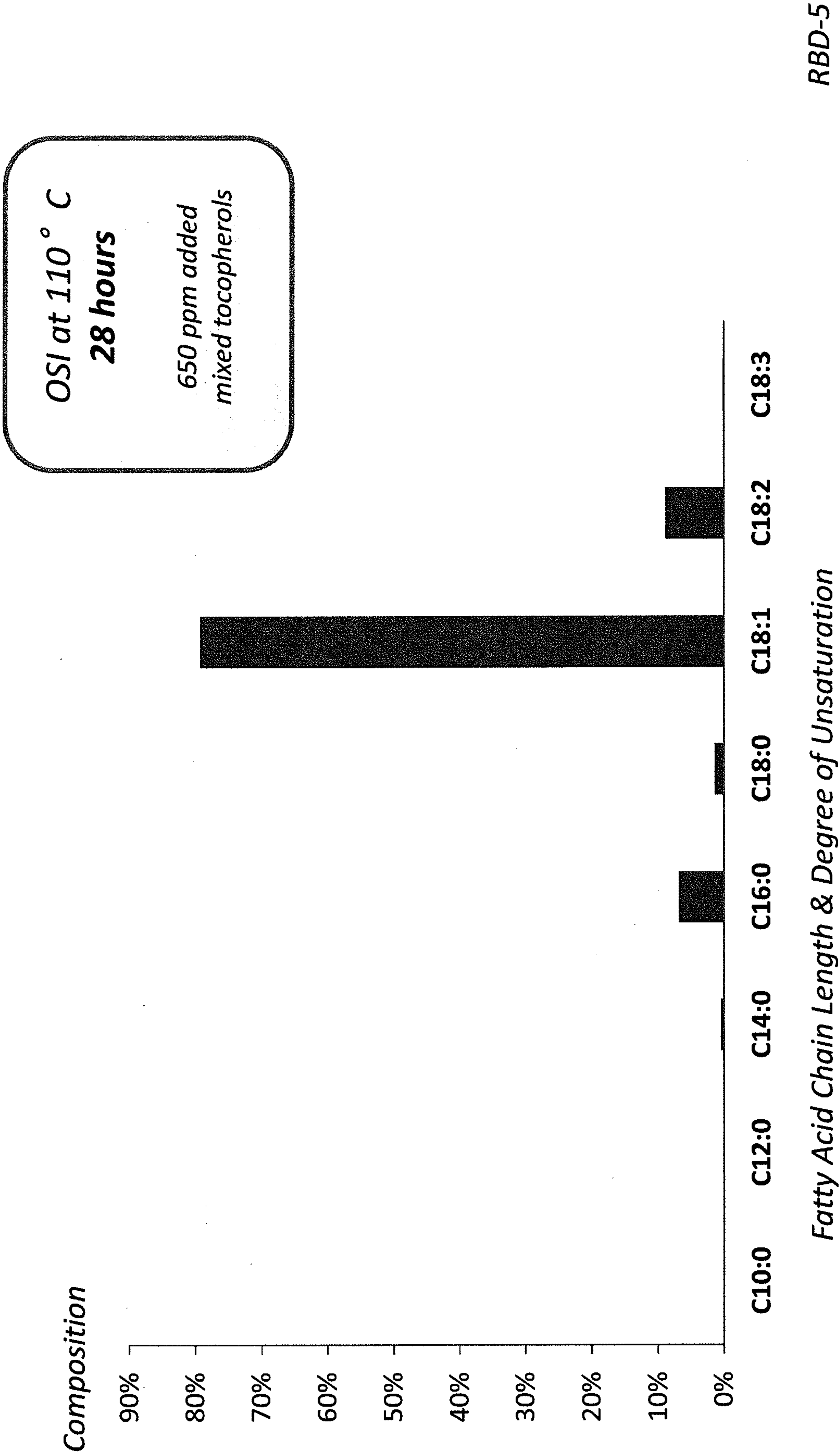


Figure 10

High-Stability Oleic Algal Oil

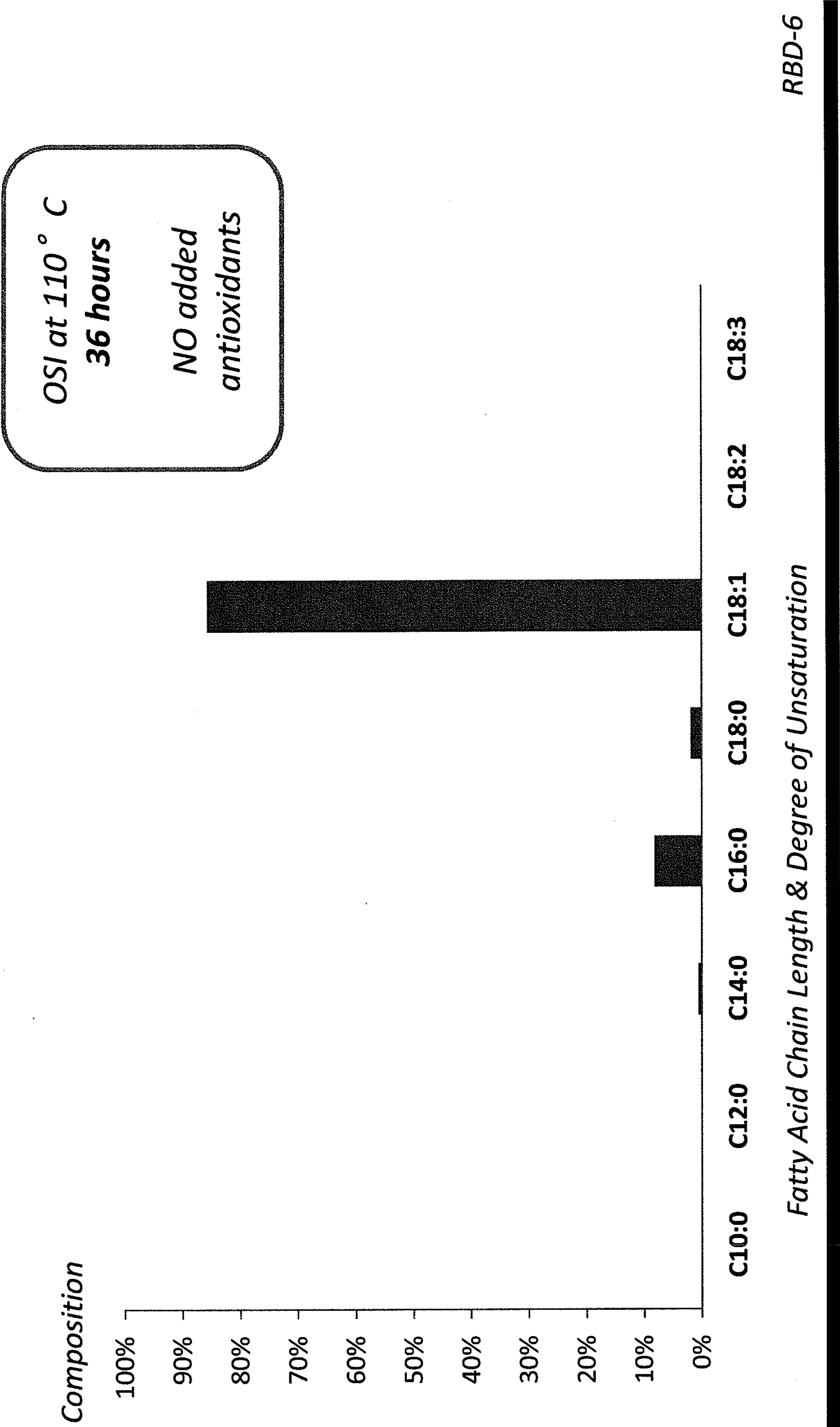


Figure 11

TEMPERING FAT / MULTIPLE TAG STRUCTURES

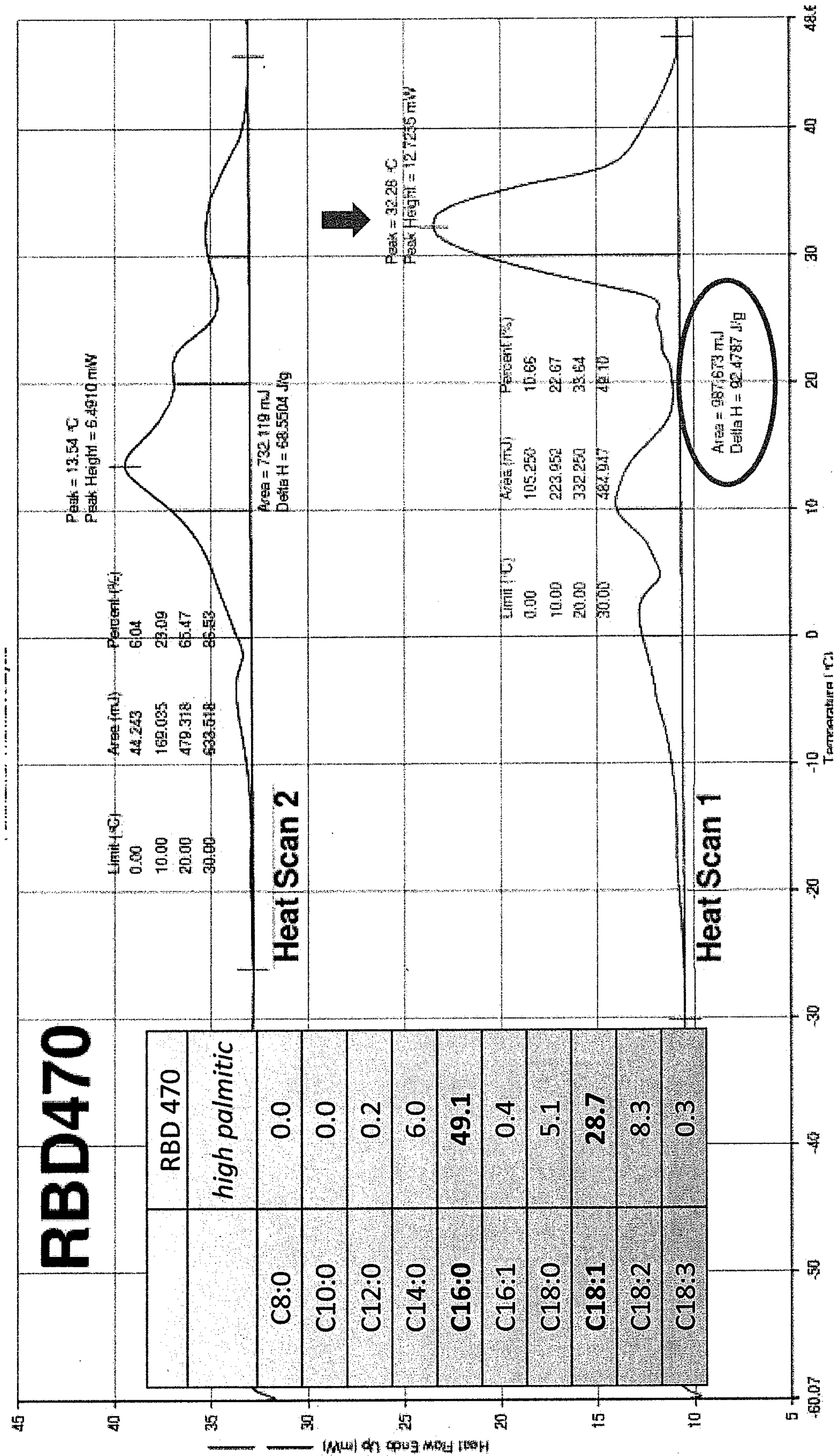


Figure 12

Lot	Name	Features	Benefits
RBD-6	High Stability Oleic Rich	Very low levels of polyunsaturates < .1% combined with very high levels of mono unsaturates (oleic) >85%	Unprecedented low levels of polyunsaturates creates perhaps the most stable natural oleic-rich oil available anywhere in the world for use in industrial and food applications. Outstanding stability and low (oxidative) reactivity is ideal for use in snack foods or spray-coating applications in foods. Combination of low pour point and oxidative stability is attractive for industrial uses such as bio-based functional fluids and lubricants. Remarkable stability minimizes need for antioxidants to be added to the oil.
	High Oleic Sunflower Oil Mimetic	Low levels of polyunsaturates <10% combined with high levels of mono unsaturates (oleic) ~80%	Sugar-derived alternative to high oleic sunflower with better GHG profile (Brazilian production). Ideal oil for cooking and frying; balances stability with health benefits of polyunsaturates. The higher level of polyunsaturates (than high stability algal oil) lowers the pour point for food and industrial applications e.g. functional fluids.
RBD-1	Lauric Rich Alternative to Palm Kernel Oil, Coconut oil	High levels of mid-chain length saturated fatty acids (C ₁₀ -C ₁₄)	Sugar-derived alternative to palm kernel oil and coconut oil with enhanced sustainability and fatty acid profiles for the oleochemical and food industries. Enhanced mid-chain fatty acid (C10 – C14) concentration will create sharper melting fat for confectionary coating application, and efficient foaming properties in soaps. Lauric-rich oil also interesting as food oil catching wave of coconut oil as lauric is neutral-to-heart-healthy; solid sat for baking and/or confectionery.

Figure 13

Lot	Name	Features	Benefits
RBD-3	High Palmitic / Low Oleic	50% palmitic and 30% oleic; similar profile to a specialty palm mid fraction used to create structure in food products	Very efficient structuring fat for food products maximizes structuring while minimizing saturated fat content. Sharp melting profile minimizes negative impact on sensory properties of food products. Replacement for specialty palm fractions in food products; enhanced sustainability profile compared to palm
	Myristic / Palmitic Rich	Highest level of myristic than any existing oil	Uniquely high concentration of myristic acid combined with capric and palmitic creates sharp melting profiles and higher solid fat content at low temperature than palm oil; enhanced sustainability profile compared to palm. Very unique solid fat content profile
RBD-2			
RBD-4	High Oleic Mid Palmitic	60% oleic and 25% palmitic; higher oleic & lower palmitic than palm oil	High oleic content combined with mid palmitic levels produces very stable oil with solid fat content that makes it an excellent frying oil alternative: food product texture may be positively impacted by reduced liquid oil pick up. 30% saturated fats in range for shortening applications. Potentially good alternative to trans-fat-containing oils.

Figure 14

SOLAZYME'S HIGH OLEIC OIL

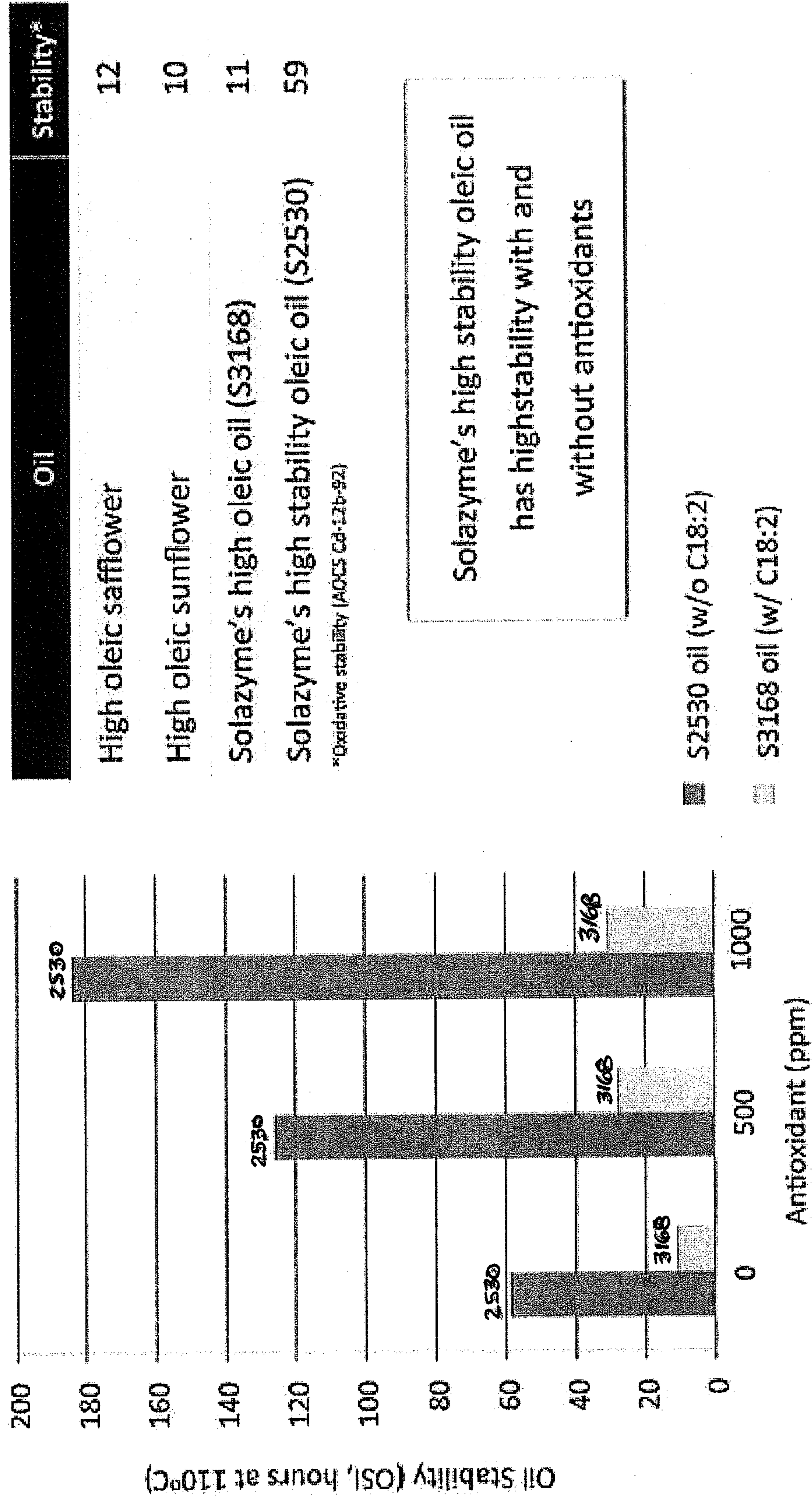


Figure 15

<i>test</i>	<i>RBD oil tested</i>	<i>result</i>	<i>test protocol</i>
OSI	RBD 502	current max: 242 hours at 110 °C	AOCS Method Cd 12b-92 (modified)
pour point	RBD 437	-19.5 °C	D97
cloud point	RBD 437	7.5 °C	AOCS Cc 6-25
flash point	RBD 437	245 °C	AOCS Cc 9b-55
viscosity	average of RBD 437 and RBD 469	41.6 cSt	Viscosity (D445) at 40 °C
viscosity index	"	195.5	Viscosity Index (D445)
color	RBD 437	2.0 Red, 18.0 Yellow	Lovibond Color AOCS Cc 13j-97 (5 1/4 inch cell)
color (converted)	RBD 437	87.3, -8.7, 32.3	Hunter Transmittance: L, a, b
bio-degradeability		Ultimate Biodegradation*	OECD 301 B

Figure 16

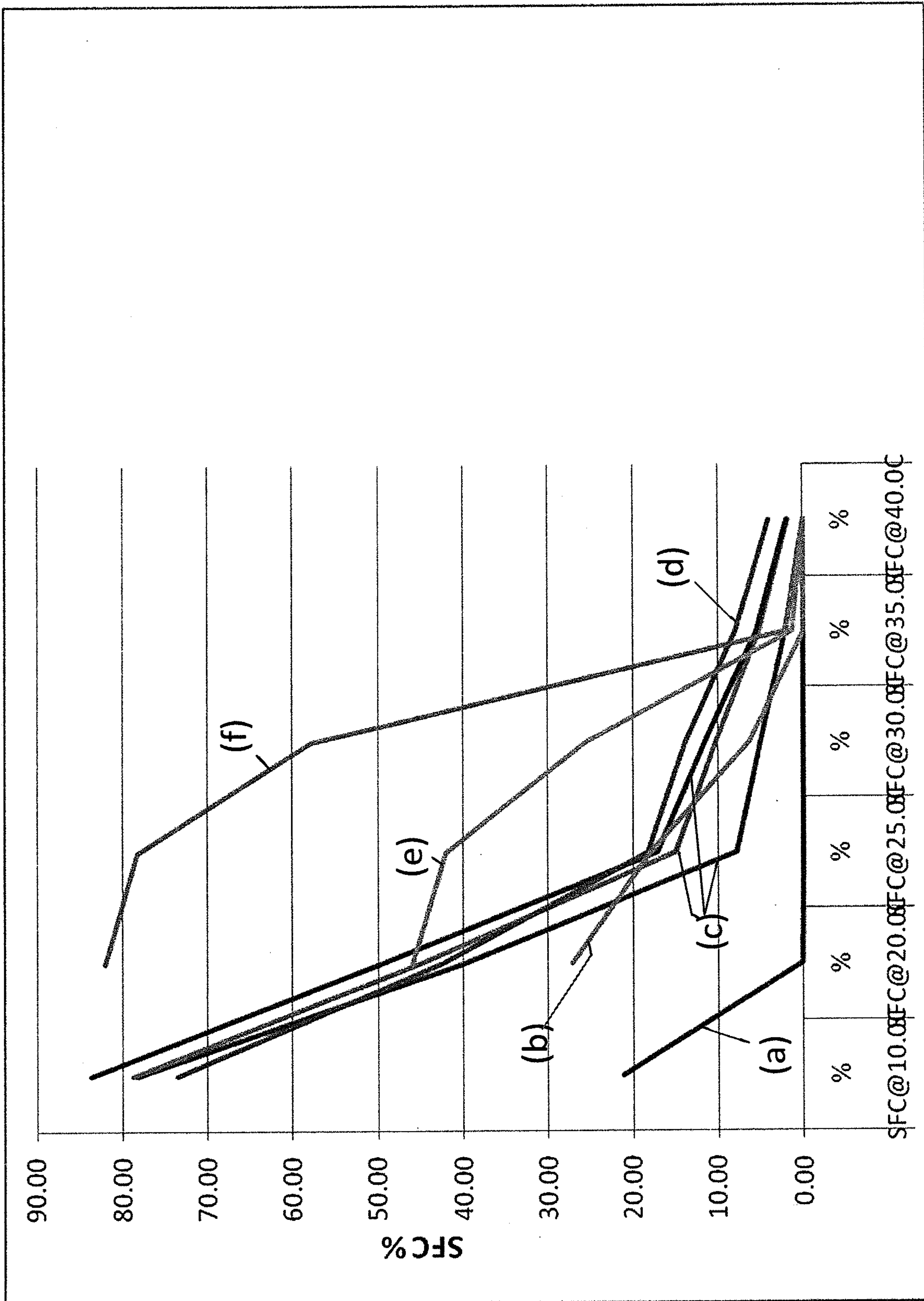


Figure 17

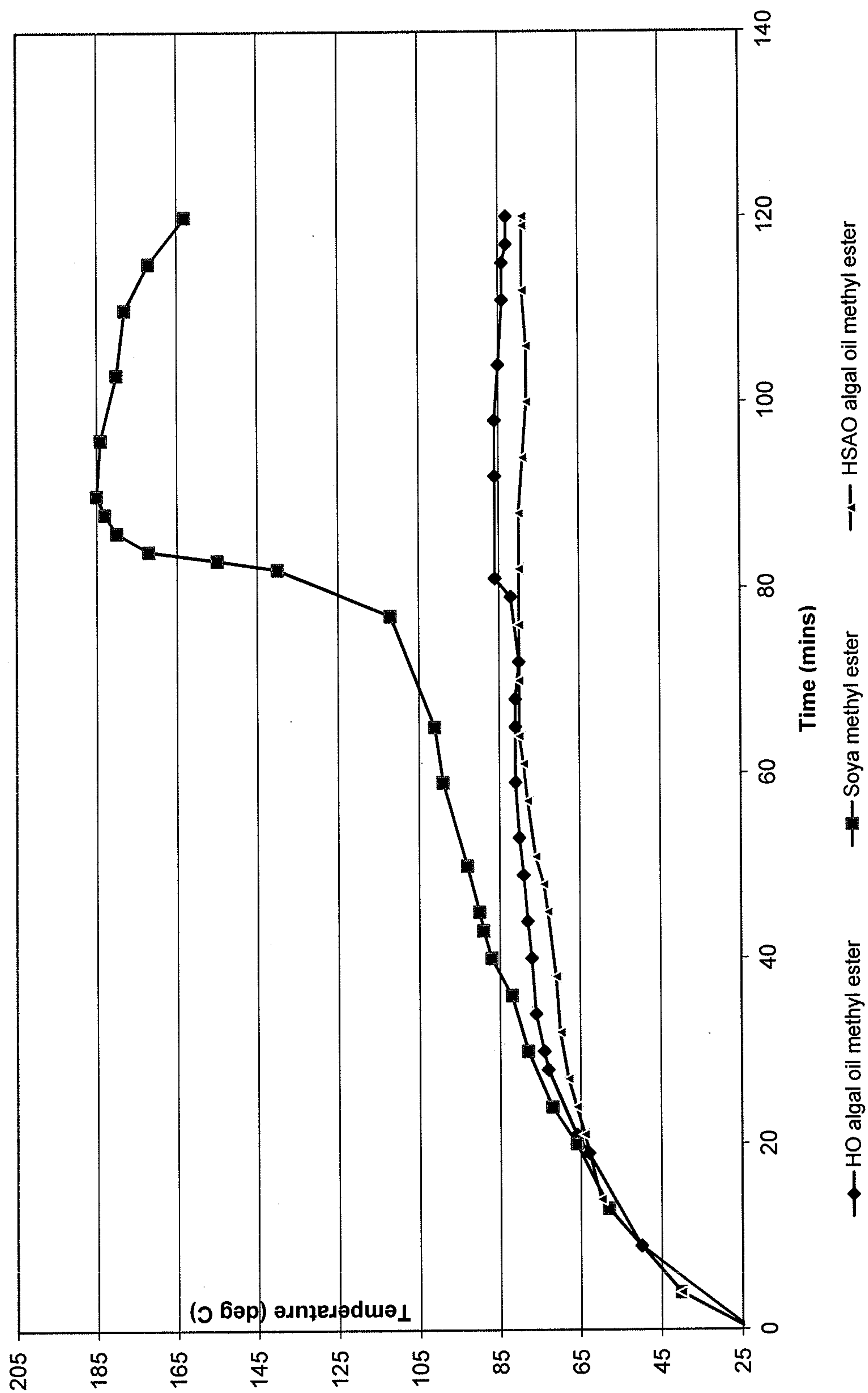


Figure 18

TEST	TEST PROTOCOL	HIGH STABILITY ALGAL OIL	HIGH OLEIC ALGAL OIL	COMMENTS ON HIGH STABILITY ALGAL OIL
OSI at 110 °C	AOCS Method Cd 12b-92	41.0 – 56.6 hours (neat) 242 hours (natural antioxidants)	14.4 hours (neat) --	HSAO outperforms natural plant-based oils with same additives
RPVOT	ASTM D2272	33 min (neat) 500 min (prelim. formulation)	30 minutes (neat) --	Neat oil outperforms neat PAO; HSAO outperforms natural plant-based oils with same additives
4-Ball wear	@ 40kg; unformulated	0.64 mm	0.60 mm	
Copper Strip Corrosion	ASTM D130, 24 hrs	1A	1A	
Pour point	ASTM D6749/D97; unformulated	-19.5 °C (neat) -29 °C (w/ pp depressant)	-21 °C (neat) --	Low pour point with high oxidative stability
Cloud point	ASTM D7683/D2500	-14 °C	-18 °C	
Flash point	D92 Cleveland Open Cup	315 °C	330 °C	Outperforms mineral oils
Viscosity	Viscosity (D445) at 40 °C	41.6 cSt	38.6 cSt	
Viscosity index	Viscosity Index (D445)	196	202	
VOCs	ASTM E1868-10	0.37% (near-zero)	nm	Outperforms mineral oils
Biodegradability	OECD 301 B	Ultimate Biodegradation: 96% degradation by day 28	Ultimate Biodegradation: 94% degradation by day 28	Outperforms mineral oils

Figure 19

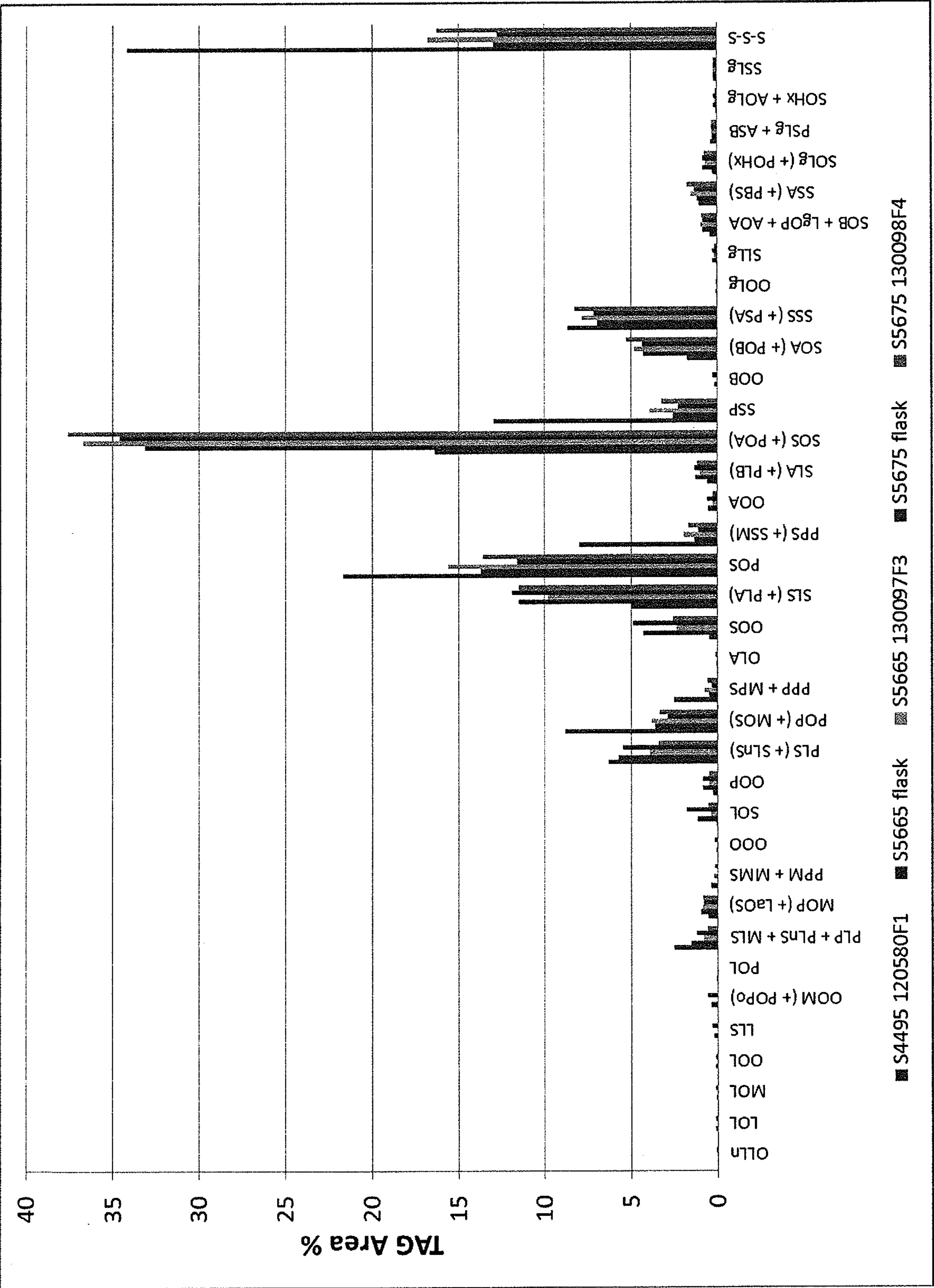


Figure 20

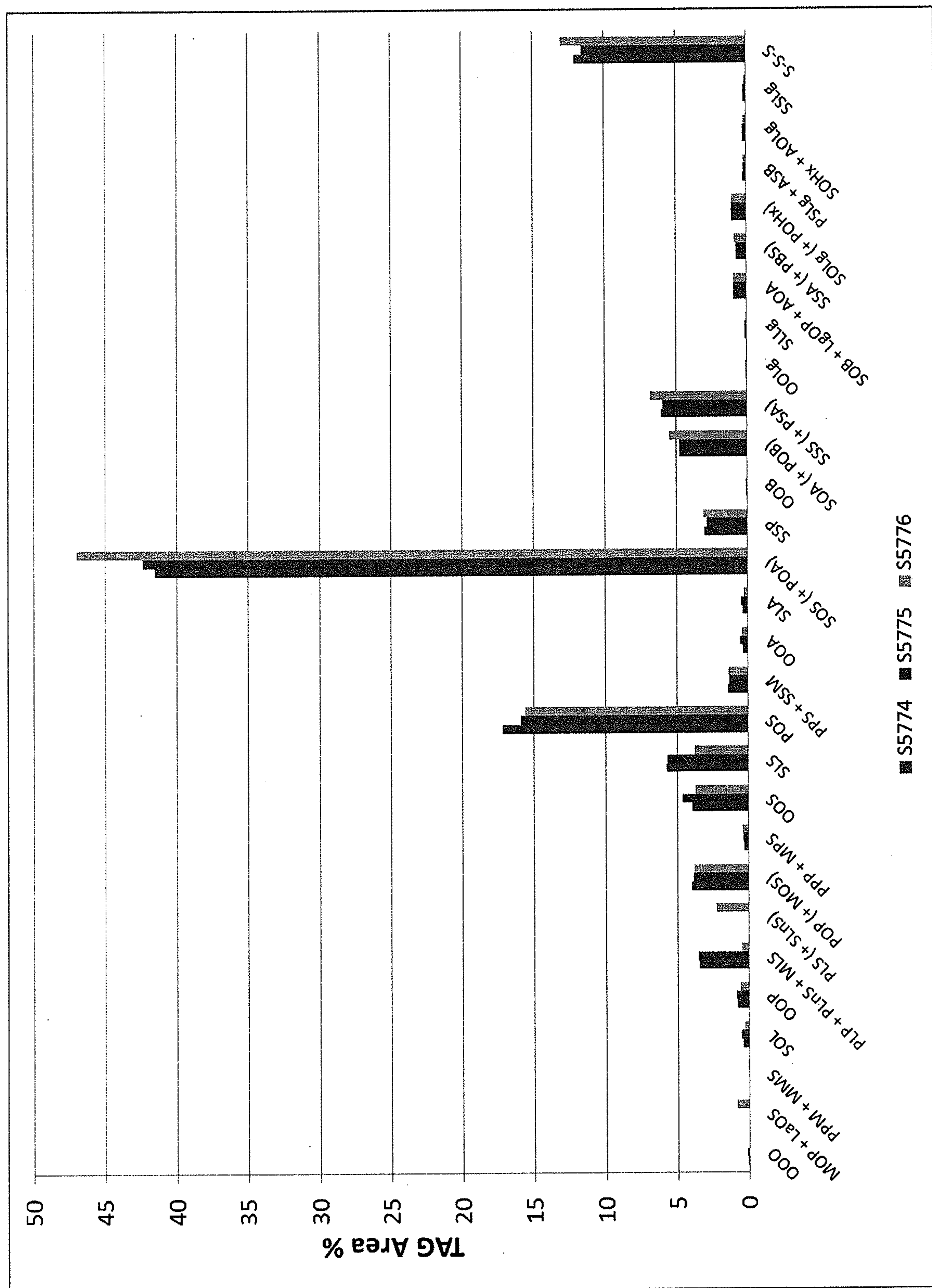


Figure 21

CHAIN LENGTH: WIDE RANGE OF ACCESSIBLE MELTING PROFILES

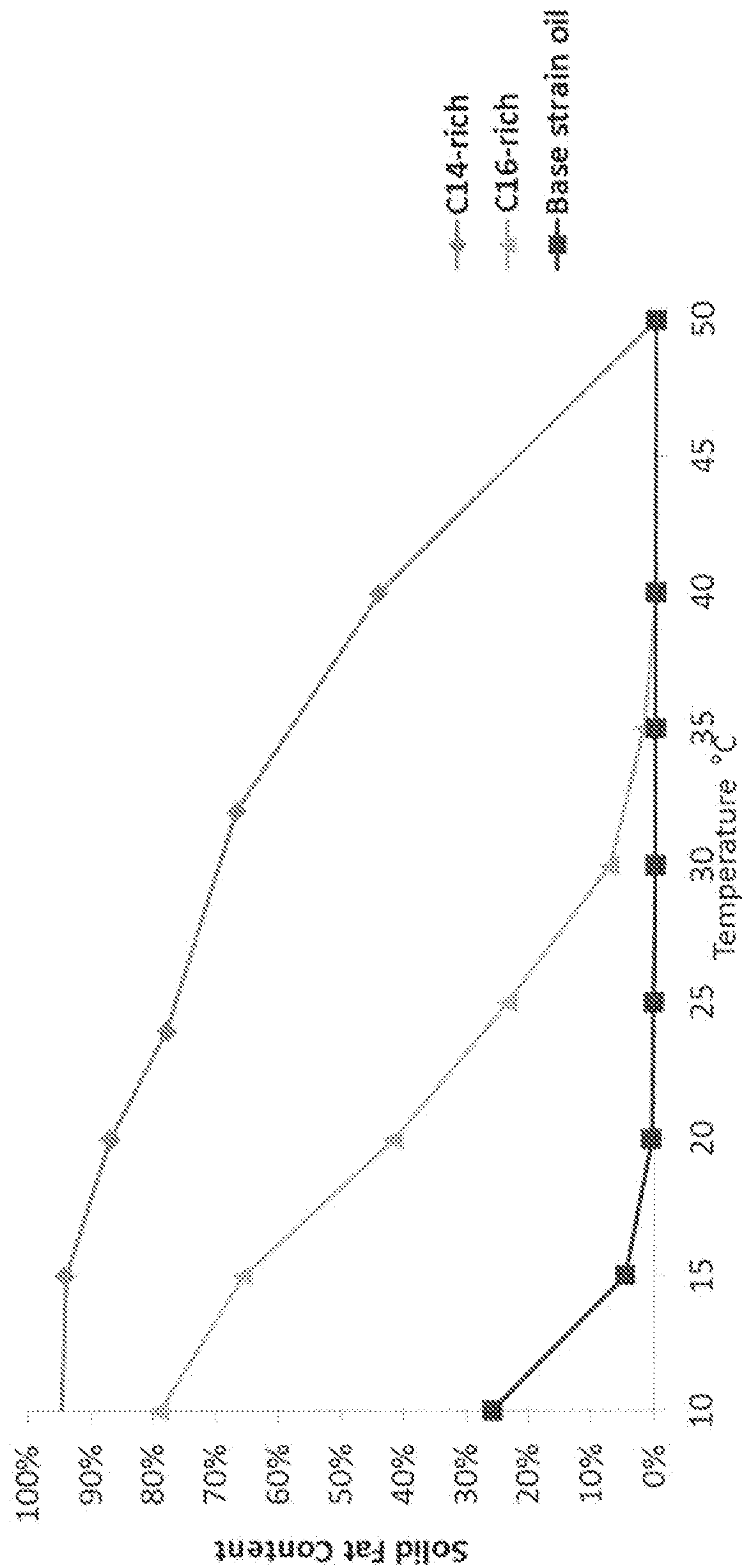


Figure 22

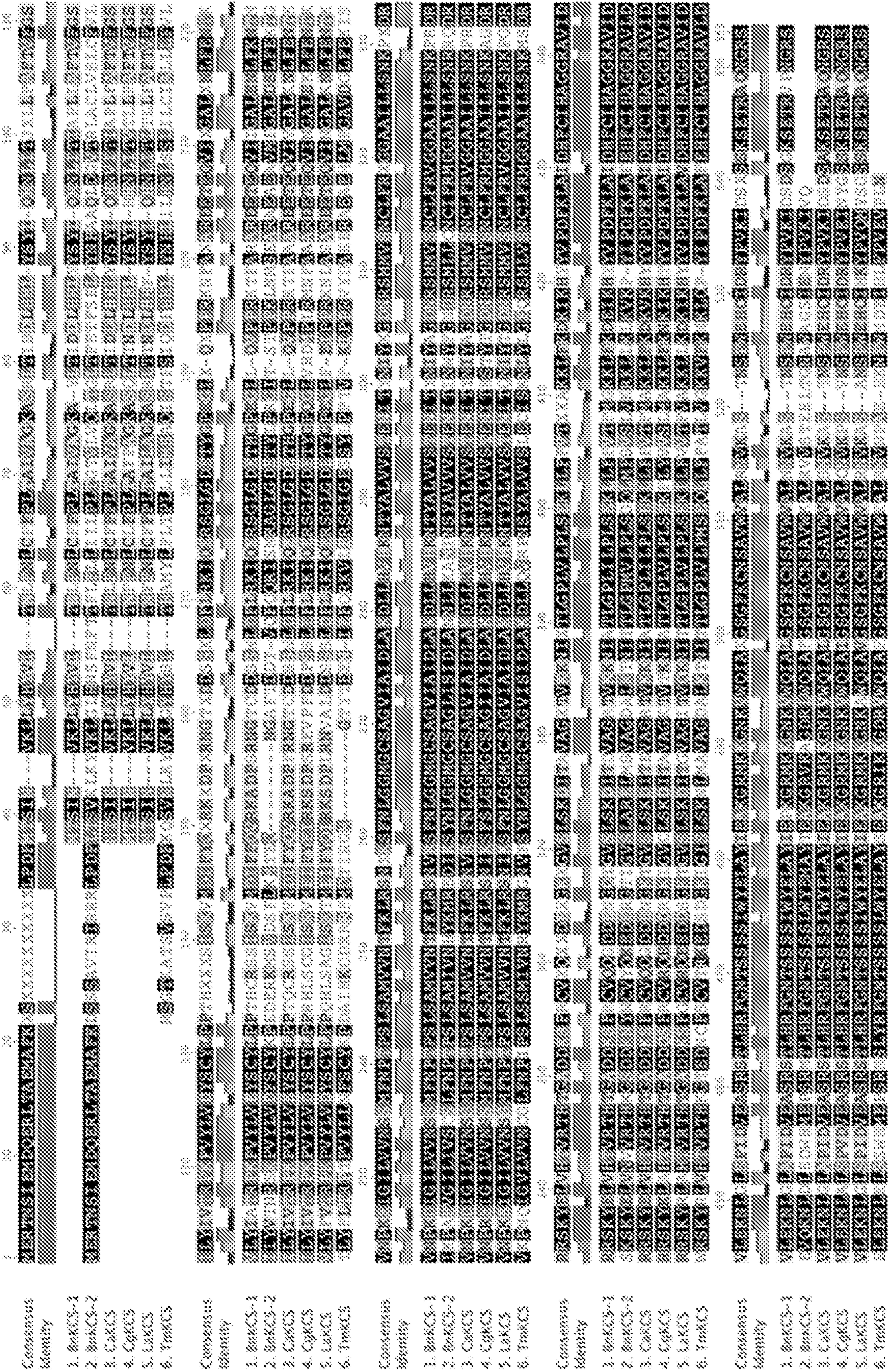
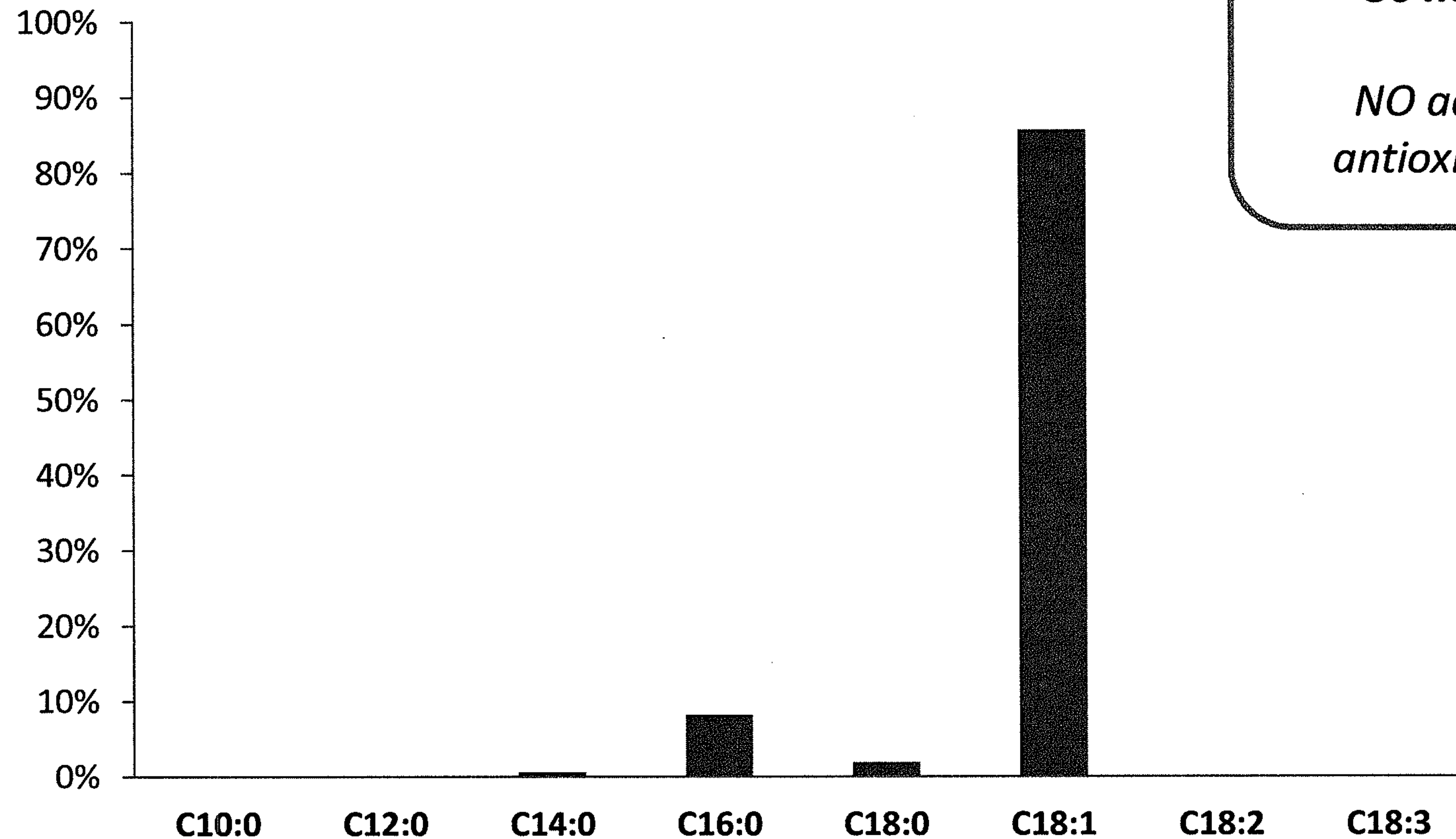


Figure 23

High-Stability Oleic Algal Oil

Composition



*OSI at 110° C
36 hours*

*NO added
antioxidants*

Fatty Acid Chain Length & Degree of Unsaturation

RBD-6

Figure 11