

MANUFACTURING OF TAILORED OILS IN RECOMBINANT HETEROTROPHIC MICROORGANISMS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. 119(e) of US Provisional Patent Application No. 61/118,590, filed November 28, 2008, US Provisional Patent Application No. 61/118,994, filed December 1, 2008, US Provisional Patent Application No. 61/174,357, filed April 30, 2009, and US Provisional Patent Application No. 61/219,525, filed June 23, 2009. Each of these applications is incorporated herein by reference in its entirety for all purposes.

REFERENCE TO A SEQUENCE LISTING

[0002] This application includes a sequence listing as shown in pages 1-72, appended hereto.

FIELD OF THE INVENTION

[0003] The present invention relates to the production of oils, fuels, and oleochemicals made from microorganisms. In particular, the disclosure relates to oil-bearing microalgae, methods of cultivating them for the production of useful compounds, including lipids, fatty acid esters, fatty acids, aldehydes, alcohols, and alkanes, and methods and reagents for genetically altering them to improve production efficiency and alter the type and composition of the oils produced by them.

BACKGROUND OF THE INVENTION

[0004] Fossil fuel is a general term for buried combustible geologic deposits of organic materials, formed from decayed plants and animals that have been converted to crude oil, coal, natural gas, or heavy oils by exposure to heat and pressure in the earth's crust over hundreds of millions of years. Fossil fuels are a finite, non-renewable resource.

[0005] Increased demand for energy by the global economy has also placed increasing pressure on the cost of hydrocarbons. Aside from energy, many industries, including plastics and chemical manufacturers, rely heavily on the availability of hydrocarbons as a feedstock for their manufacturing processes. Cost-effective alternatives to current sources of supply could help mitigate the upward pressure on energy and these raw material costs.

[0006] PCT Pub. No. 2008/151149 describes methods and materials for cultivating microalgae for the production of oil and particularly exemplifies the production of diesel fuel from oil produced by the microalgae *Chlorella protothecoides*. There remains a need for improved methods for producing oil in microalgae, particularly for methods that produce oils

with shorter chain length and a higher degree of saturation and without pigments, with greater yield and efficiency. The present invention meets this need.

SUMMARY OF THE INVENTION

[0007] In a first aspect, the present invention refers to a codon-optimized recombinant nucleic acid comprising a fatty acyl-ACP thioesterase coding sequence codon-optimized for expression in *Prototheca*, wherein the coding sequence contains the most or second most preferred codon of Table 1 for at least 60% of the codons of the coding sequence, such that the codon-optimized sequence is more efficiently translated in *Prototheca* than a native sequence encoding the fatty acyl-ACP thioesterase, the fatty acyl-ACP thioesterase having hydrolysis activity towards one or more fatty acyl-ACP substrates, the recombinant nucleic acid further comprising a plastid targeting sequence from microalgae that is in-frame with the codon-optimized fatty acyl-ACP thioesterase coding sequence. In another aspect, the present invention refers to a codon-optimized recombinant nucleic acid comprising a fatty acyl-ACP thioesterase coding sequence codon-optimized for expression in *Prototheca*, wherein the coding sequence contains the most or second most preferred codon of Table 1 for at least 60% of the codons of the coding sequence, such that the codon-optimized sequence is more efficiently translated in *Prototheca* than a native sequence encoding the fatty acyl-ACP thioesterase, the fatty acyl-ACP thioesterase having hydrolysis activity towards one or more fatty acyl-ACP substrates, the recombinant nucleic acid further comprising a plastid targeting sequence in-frame with the codon-optimized fatty acyl-ACP thioesterase coding sequence, the recombinant nucleic acid further comprising first and second flanking nucleic acid sequences endogenous to the nuclear genome of a cell of the genus *Prototheca*, wherein the first and second flanking sequences are each at least 200 nucleotides long and the first and second flanking sequences flank the codon-optimized recombinant nucleic acid for integration of the recombinant nucleic acid into the nuclear genome by homologous recombination with a corresponding genomic sequence. In still another aspect, the present invention refers to a codon-optimized recombinant nucleic acid comprising a fatty acyl-ACP thioesterase coding sequence codon-optimized for expression in *Prototheca*, wherein the coding sequence contains the most or second most preferred codon of Table 1 for at least 60% of the codons of the coding sequence, such that the codon-optimized sequence is more efficiently translated in *Prototheca* than a native sequence encoding the fatty acyl-ACP thioesterase, the fatty acyl-ACP thioesterase having hydrolysis activity towards one or more fatty acyl-ACP substrates, the recombinant nucleic acid further comprising a plastid targeting sequence from microalgae that is in-frame with the codon-optimized fatty acyl-ACP

thioesterase coding sequence, and wherein the amino acid sequence of the fatty acyl-ACP thioesterase comprises an amino acid sequence of a fatty acyl-ACP thioesterase from an organism selected from the group consisting of *Cuphea hookeriana*, *Umbellularia californica*, *Cinnamomum camphora*, *Cuphea palustris* and *Ulmus americana*. In another aspect, the present invention refers to a codon-optimized recombinant nucleic acid comprising a fatty acyl-ACP thioesterase coding sequence and a sucrose invertase coding sequence, each coding sequence codon-optimized for expression in *Prototheca*, wherein each coding sequence contains the most or second most preferred codon of Table 1 for at least 60% of the codons of the coding sequence, such that each codon-optimized sequence is more efficiently translated in *Prototheca* than a native sequence encoding the fatty acyl-ACP thioesterase or the native sequence encoding the sucrose invertase, the fatty acyl-ACP thioesterase having hydrolysis activity towards one or more fatty acyl-ACP substrates, the recombinant nucleic acid further comprising a plastid targeting sequence in-frame with the codon-optimized fatty acyl-ACP thioesterase coding sequence. In another aspect, the present invention refers to a host cell of the genus *Prototheca* comprising a recombinant nucleic acid comprising a fatty acyl-ACP thioesterase coding sequence codon-optimized for expression in *Prototheca*, wherein the coding sequence contains the most or second most preferred codon of Table 1 for at least 60% of the codons of the coding sequence, such that the codon-optimized sequence is more efficiently translated in *Prototheca* than a native sequence encoding the fatty acyl-ACP thioesterase, and wherein a plastid targeting sequence is in-frame with the codon-optimized fatty acyl-ACP thioesterase coding sequence and the fatty acyl-ACP thioesterase has hydrolysis activity towards one or more fatty acyl-ACP substrates. In another aspect, the present invention refers to a host cell of the genus *Prototheca* comprising a codon optimized recombinant nucleic acid comprising a fatty acyl-ACP thioesterase coding sequence codon-optimized for expression in *Prototheca*, wherein the coding sequence contains the most or second most preferred codon of Table 1 for at least 60% of the codons of the coding sequence, such that the codon-optimized sequence is more efficiently translated in *Prototheca* than a native sequence encoding the fatty acyl-ACP thioesterase, the fatty acyl-ACP thioesterase having hydrolysis activity towards a fatty acyl-ACP substrate of chain length C8, C10, C12 or C14, the recombinant nucleic acid further comprising a plastid targeting sequence that is in-frame with the codon-optimized fatty acyl-ACP thioesterase coding sequence. In still another aspect, the present invention refers to a host cell of the genus *Prototheca* comprising the recombinant nucleic acid of the present invention. In one other aspect, the present invention refers to a codon-optimized recombinant

nucleic acid comprising a fatty acyl-ACP thioesterase coding sequence codon-optimized for expression in *Prototheca*, wherein the coding sequence contains the most or second most preferred codon of Table 1 for at least 60% of the codons of the coding sequence, such that the codon-optimized sequence is more efficiently translated in *Prototheca* than a native sequence encoding the fatty acyl-ACP thioesterase, the fatty acyl-ACP thioesterase having hydrolysis activity towards one or more fatty acyl-ACP substrates, further comprising a promoter comprising the nucleotide sequence of any of SEQ ID NOS: 91-102. The disclosure provides cells of the genus *Prototheca* comprising an exogenous gene, and in some examples the cell is a strain of the species *Prototheca moriformis*, *Prototheca krugani*, *Prototheca stagnora* or *Prototheca zopfii* and in other example the cell has a 23S rRNA sequence with at least 70, 75, 80, 85 or 95% nucleotide identity to one or more of SEQ ID NOS: 11-19. In some cells the exogenous gene is coding sequence and is in operable linkage with a promoter, and in some examples the promoter is from a gene endogenous to a species of the genus *Prototheca*. In further examples the coding sequence encodes a protein selected from the group consisting of a sucrose invertase, a fatty acyl-ACP thioesterase, a fatty acyl-CoA/aldehyde reductase, a fatty acyl-CoA reductase, a fatty aldehyde reductase, a fatty aldehyde decarbonylase, an acyl carrier protein and a protein that imparts resistance to an antibiotic. Some examples of a fatty acyl-ACP thioesterase that has hydrolysis activity towards one or more fatty acyl-ACP substrates of chain length C8, C10, C12 or C14, including acyl-ACP thioesterases with at least 50, 60, 70, 80, or 90% amino acid identity with one or more sequences selected from the group consisting of SEQ ID NOS: 59, 61, 63 and 138-140. In further examples the coding sequence comprises a plastid targeting sequence from microalgae, and in some examples the microalgae is a species of the genus *Prototheca* or *Chlorella* as well as other genera from the family *Chlorellaceae*. In some examples the plastid targeting sequence has at least 20, 25, 35, 45, or 55% amino acid sequence identity to one or more of SEQ ID NOS: 127-133 and is capable of targeting a protein encoded by an exogenous gene not located in the plastid genome to the plastid. In other examples the promoter is upregulated in response to reduction or elimination of nitrogen in the culture media of the cell, such as at least a 3-fold upregulation as determined by transcript abundance in a cell of the genus *Prototheca* when the extracellular environment changes from containing at least 10mM or 5mM nitrogen to containing no nitrogen. In further examples the promoter comprises a segment of 50 or more nucleotides of one of SEQ ID NOS: 91-102. In other examples the

cell has a 23S rRNA sequence with at least 70, 75, 80, 85 or 95% nucleotide identity to one or more of SEQ ID NOs: 11-19. In other examples the exogenous gene is integrated into a chromosome of the cell.

[0008] In additional examples of cells as described herein, the cell is of the genus *Prototheca* and comprises an exogenous fatty acyl-ACP thioesterase gene and a lipid profile

of at least 4% C8-C14 of total lipids of the cell, an amount of C8 that is at least 0.3% of total lipids of the cell, an amount of C10 that is at least 2% of total lipids of the cell, an amount of C12 that is at least 2% of total lipids of the cell, an amount of C14 that is at least 4% of total lipids of the cell, and an amount of C8-C14 that is 10-30%, 20-30%, or at least 10, 20, or 30% of total lipids of the cell. In some examples the cell further comprises an exogenous sucrose invertase gene. In some examples the cell is a strain of the species *Prototheca moriformis*, *Prototheca krugani*, *Prototheca stagnora* or *Prototheca zopfii*, and in other example the cell has a 23S rRNA sequence with at least 70, 75, 80, 85 or 95% nucleotide identity to one or more of SEQ ID NOs: 11-19. In other examples the exogenous fatty acyl-ACP thioesterase gene is integrated into a chromosome of the cell. Other examples described herein comprise methods of making triglyceride compositions of a lipid profile of at least 4% C8-C14 w/w or area percent of the triglyceride composition, an amount of C8 that is at least 0.3% w/w or area percent, an amount of C10 that is at least 2% w/w or area percent, an amount of C12 that is at least 2% w/w or area percent, an amount of C14 that is at least 4% w/w or area percent, and an amount of C8-C14 that is 10-30%, 20-30%, or at least 10, 20, or 30% w/w or area percent. The disclosure also comprises methods of making triglyceride compositions comprising cultivating the foregoing cells, wherein the cells also comprise an exogenous gene encoding a sucrose invertase and sucrose is provided as a carbon source. In some examples the sucrose invertase has at least 50, 60, 70, 80, or 90% amino acid identity to one or more of SEQ ID NOs: 3, 20-29 and 90.

[0009] Examples described herein include triglyceride oil compositions as well as cells containing triglyceride oil compositions comprising a lipid profile of at least 4% C8-C14 and one or more of the following attributes: 0.1-0.4 micrograms/ml total carotenoids, less than 0.4 micrograms/ml total carotenoids, less than 0.001 micrograms/ml lycopene; less than 0.02 micrograms/ml beta carotene, less than 0.02 milligrams of chlorophyll per kilogram of oil; 0.40-0.60 milligrams of gamma tocopherol per 100 grams of oil; 0.2-0.5 milligrams of total tocotrienols per gram of oil, less than 0.4 milligrams of total tocotrienols per gram of oil, 4-8 mg per 100 grams of oil of campesterol, and 40-60 mg per 100 grams of oil of stigmasterol. In some examples described herein the triglyceride oil compositions have a lipid profile of at least 4% C8-C14 w/w or area percent of the triglyceride composition, an amount of C8 that is at least 0.3% w/w or area percent, an amount of C10 that is at least 2% w/w or area percent, an amount of C12 that is at least 2% w/w or area percent, an amount of C14 that is at least 4% w/w or area percent, and an amount of C8-C14 that is 10-30%, 20-30%, or at least 10, 20, or 30% w/w or area percent. In other examples the triglyceride oil composition is

blended with at least one other composition selected from the group consisting of soy, rapeseed, canola, palm, palm kernel, coconut, corn, waste vegetable, Chinese tallow, olive, sunflower, cotton seed, chicken fat, beef tallow, porcine tallow, microalgae, macroalgae, *Cuphea*, flax, peanut, choice white grease, lard, *Camelina sativa*, mustard seed, cashew nut, oats, lupine, kenaf, calendula, hemp, coffee, linseed (flax), hazelnut, euphorbia, pumpkin seed, coriander, camellia, sesame, safflower, rice, tung tree, cocoa, copra, pium poppy, castor beans, pecan, jojoba, jatropha, macadamia, Brazil nuts, avocado, petroleum, or a distillate fraction of any of the preceding oils.

[0010] Methods of the disclosure also include processing the aforementioned oils by performing one or more chemical reactions from the list consisting of transesterification, hydrogenation, hydrocracking, deoxygenation, isomerization, interesterification, hydroxylation, hydrolysis to yield free fatty acids, and saponification. The disclosure also describes hydrocarbon fuels made from hydrogenation and isomerization of the aforementioned oils and fatty acid alkyl esters made from transesterification of the aforementioned oils. In some examples the hydrocarbon fuel is made from triglyceride isolated from cells of the genus *Prototheca* wherein the ASTM D86 T10-T90 distillation range is at least 25°C. In other examples the fatty acid alkyl ester fuel is made from triglyceride isolated from cells of the genus *Prototheca*, wherein the composition has an ASTM D6751 A1 cold soak time of less than 120 seconds.

[0011] The disclosure also describes a composition comprising (a) polysaccharide comprising one or more monosaccharides from the list consisting of 20-30 mole percent galactose; 55-65 mole percent glucose; and 5-15 mole percent mannose; (b) protein; and (c) DNA comprising a 23S rRNA sequence with at least 70, 75, 80, 85 or 95% nucleotide identity to one or more of SEQ ID NOs: 11-19; and (d) an exogenous gene. In some examples the exogenous gene is selected from a sucrose invertase and a fatty acyl-ACP thioesterase, and in further examples the composition further comprises lipid with a lipid profile of at least 4% C8-C14. In other examples the composition is formulated for consumption as an animal feed.

[0012] The disclosure describes recombinant nucleic acids encoding promoters that are upregulated in response to reduction or elimination of nitrogen in the culture media of a cell of the genus *Prototheca*, such as at least a 3-fold upregulation as determined by transcript abundance when the extracellular environment changes from containing at least 10mM or 5mM nitrogen to containing no nitrogen. In some examples the recombinant nucleic acid comprises a segment of 50 or more nucleotides of one of SEQ ID NOs: 91-102. The

disclosure also describes nucleic acid vectors comprising an expression cassette comprising (a) a promoter that is active in a cell of the genus *Prototheca*; and (b) a coding sequence in operable linkage with the promoter wherein the coding sequence contains the most or second most preferred codons of Table 1 for at least 20, 30, 40, 50, 60, or 80% of the codons of the coding sequence. In some vectors the coding sequence comprises a plastid targeting sequence in-frame with a fatty acyl-ACP thioesterase, including thioesterase that have hydrolysis activity towards one or more fatty acyl-ACP substrates of chain length C8, C10, C12 or C14. Some vectors include plastid targeting sequences that encode peptides that are capable of targeting a protein to the plastid of a cell of the genus *Prototheca*, including those from microalgae and those wherein the plastid targeting sequence has at least 20, 25, 35, 45, or 55% amino acid sequence identity to one or more of SEQ ID NOs. 127-133 and is capable of targeting a protein to the plastid of a cell of the genus *Prototheca*. Additional vectors described herein comprise nucleic acid sequences endogenous to the nuclear genome of a cell of the genus *Prototheca*, wherein the sequence is at least 200 nucleotides long, and some vectors comprise first and second nucleic acid sequences endogenous to the nuclear genome of a cell of the genus *Prototheca*, wherein the first and second sequences (a) are each at least 200 nucleotides long; (b) flank the expression cassette; and (c) are located on the same *Prototheca* chromosome no more than 5, 10, 15, 20, and 50kB apart.

[0013] The disclosure also describes a recombinant nucleic acid with at least 80, 90, 95 or 98% nucleotide identity to one or both of SEQ ID NOs: 134-135 and a recombinant nucleic acid encoding a protein with at least 80, 90, 95 or 98% amino acid identity to one or both of SEQ ID NOs: 136-137.

[0014] The disclosure also describes methods of producing triglyceride compositions, comprising (a) culturing a population of cells of the genus *Prototheca* in the presence of a fixed carbon source, wherein: (i) the cells contain an exogenous gene; (ii) the cells accumulate at least 10, 20, 30, 40, 60, or 70% of their dry cell weight as lipid; and (iii) the fixed carbon source is selected from the group consisting of sorghum and depolymerized cellulosic material; and (b) isolating lipid components from the cultured microorganisms. In some examples the the fixed carbon source is depolymerized cellulosic material selected from the group consisting of corn stover, *Miscanthus*, forage sorghum, sugar beet pulp and sugar cane bagasse, optionally that has been subjected to washing with water prior to the culturing step. In some methods the fixed carbon source is depolymerized cellulosic material and the glucose level of the depolymerized cellulosic material is concentrated to a level of at least 300 g/liter, at least 400 g/liter, at least 500 g/liter, or at least 600 g/liter of prior to the

culturing step and is fed to the culture over time as the cells grow and accumulate lipid. In some methods the exogenous gene encodes a fatty acyl-ACP thioesterase that has hydrolysis activity towards one or more fatty acyl-ACP substrates of chain length C8, C10, C12 or C14, and in some methods the triglyceride has a lipid profile of at least 4% C8-C14 and one or more of the following attributes: 0.1-0.4 micrograms/ml total carotenoids; less than 0.02 milligrams of chlorophyll per kilogram of oil; 0.40-0.60 milligrams of gamma tocopherol per 100 grams of oil; 0.2-0.5 milligrams of total tocotrienols per gram of oil, 4-8 mg per 100 grams of oil of campesterol, and 40-60 mg per 100 grams of oil of stigmasterol.

[0015] Further methods described herein include producing a triglyceride composition, comprising: (a) culturing a population of microorganisms in the presence of depolymerized cellulosic material, wherein: (i) the depolymerized cellulosic material is subjected to washing with water prior to the culturing step; (ii) the cells accumulate at least 10, 20, 30, 40, 60, or 70% of their dry cell weight as lipid; and (iii) the depolymerized cellulosic material is concentrated to at least 300, 400, 500, or 600 g/liter of glucose prior to the cultivation step; (iv) the microorganisms are cultured in a fed-batch reaction in which depolymerized cellulosic material of at least 300, 400, 500, or 600 g/liter of glucose is fed to the microorganisms; and (b) isolating lipid components from the cultured microorganisms. In some examples the the fixed carbon source is depolymerized cellulosic material selected from the group consisting of corn stover, *Miscanthus*, forage sorghum, sugar beet pulp and sugar cane bagasse. In further examples the microorganisms are a species of the genus *Prototheca* and contain an exogenous gene, including a fatty acyl-ACP thioesterase that has hydrolysis activity towards one or more fatty acyl-ACP substrates of chain length C8, C10, C12 or C14. A further method described herein comprises manufacturing triglyceride oil comprising cultivating a cell that has a 23S rRNA sequence with at least 90 or 96% nucleotide identity to SEQ ID NO: 30 in the presence of sucrose as a carbon source.

[0016] The disclosure also describes methods of manufacturing a chemical comprising performing one or more chemical reactions from the list consisting of transesterification, hydrogenation, hydrocracking, deoxygenation, isomerization, interesterification, hydroxylation, hydrolysis, and saponification on a triglyceride oil, wherein the oil has a lipid profile of at least 4% C8-C14 and one or more of the following attributes: 0.1-0.4 micrograms/ml total carotenoids; less than 0.02 milligrams of chlorophyll per kilogram of oil; 0.10-0.60 milligrams of gamma tocopherol per 100 grams of oil; 0.1-0.5 milligrams of total tocotrienols per gram of oil, 1-8 mg per 100 grams of oil of campesterol, and 10-60 mg per 100 grams of oil of stigmasterol. Some methods are performed by manufacturing the oil by

cultivating a cell of the genus *Prototheca* that comprises an exogenous fatty acyl-ACP thioesterase gene that encodes a fatty acyl-ACP thioesterase having hydrolysis activity towards one or more fatty acyl-ACP substrates of chain length C8, C10, C12 or C14. In some methods the hydrolysis reaction is selected from the group consisting of saponification, acid hydrolysis, alkaline hydrolysis, enzymatic hydrolysis, catalytic hydrolysis, and hot-compressed water hydrolysis, including a catalytic hydrolysis reaction wherein the oil is split into glycerol and fatty acids. In further methods the fatty acids undergo an amination reaction to produce fatty nitrogen compounds or an ozonolysis reaction to produce mono- and dibasic-acids. In some examples the oil undergoes a triglyceride splitting method selected from the group consisting of enzymatic splitting and pressure splitting. In some methods a condensation reaction follows the hydrolysis reaction. Other methods include performing a hydroprocessing reaction on the oil, optionally wherein the product of the hydroprocessing reaction undergoes a deoxygenation reaction or a condensation reaction prior to or simultaneous with the hydroprocessing reaction. Some methods additionally include a gas removal reaction. Additional methods include processing the aforementioned oils by performing a deoxygenation reaction selected from the group consisting of: a hydrogenolysis reaction, hydrogenation, a consecutive hydrogenation-hydrogenolysis reaction, a consecutive hydrogenolysis-hydrogenation reaction, and a combined hydrogenation-hydrogenolysis reaction. In some methods a condensation reaction follows the deoxygenation reaction. Other methods include performing an esterification reaction on the aforementioned oils, optionally an interestification reaction or a transesterification reaction. Other methods include performing a hydroxylation reaction on the aforementioned oils, optionally wherein a condensation reaction follows the hydroxylation reaction.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] Figures 1 and 2 illustrate the growth curves of *Prototheca* species and *Chlorella luteoviridis* strain SAG 2214 grown on sorghum as the carbon source.

[0018] Figure 3 shows time course growth of SAG 2214 on glucose and sucrose.

[0019] Figure 4 shows maps of the cassettes using in *Prototheca* transformations, as described in Example 3.

[0020] Figure 5 shows the results of Southern blot analysis on three transformants of UTEX strain 1435, as described in Example 3.

[0021] Figure 6 shows a schematic of the codon optimized and non-codon optimized *suc2* (yeast sucrose invertase (yInv)) transgene construct. The relevant restriction cloning sites are indicated and arrows indicate the direction of transcription.

[0022] Figure 7a shows the results of *Prototheca moriformis* grown on cellulosic-derived sugars (corn stover, beet pulp, sorghum cane, *Miscanthus* and glucose control). Growth is expressed in optical density measurements (A750 readings).

[0023] Figure 7b shows the results of growth experiments using *Prototheca moriformis* using different levels of corn stover-derived cellulosic sugar as compared to glucose/xylose control.

[0024] Figure 7c shows the impact that xylose has on the lipid production in *Prototheca* cultures.

[0025] Figure 7d shows the impact of salt concentration (Na_2SO_4) and antifoam on the growth (in dry cell weight (DCW)) of *Prototheca*.

[0026] Figure 8 shows the impact of hydrothermal treatment of various cellulosic materials (sugar cane bagasse, sorghum cane, *Miscanthus* and beet pulp) and the resulting sugar stream on the growth of *Prototheca*.

[0027] Figure 9 shows decreasing levels of hydroxymethyl furfurals (HMF) and furfurals in cellulosic biomass (sugar cane bagasse, sorghum cane, *Miscanthus* and beet pulp) after repeated cycles of hydrothermal treatment.

[0028] Figure 10 shows a schematic of a saccharification process of cellulosic materials to generate sugar streams suitable for use in heterotrophic oil production in a fermentor.

[0029] Figure 11 shows decreasing levels of HMF and furfurals in exploded sugar cane bagasse after repeated cycles of hydrothermal treatment.

[0030] Figure 12 shows a schematic of thioesterase constructs used in *Prototheca* transformations. The heterologous beta-tubulin (driving Neo^R) and glutamate dehydrogenase promoters are derived from *Chlamydomonas reinhardtii* and *Chlorella sorokiniana*, respectively. The nitrate reductase 3'UTR was derived from *Chlorella vulgaris*. The relevant restriction cloning sites are indicated and arrows indicate the direction of transcription.

[0031] Figure 13 shows a chromatogram of renewable diesel produced from *Prototheca* triglyceride oil.

DETAILED DESCRIPTION OF THE INVENTION

[0032] The present invention arises from the discovery that *Prototheca* and certain related microorganisms have unexpectedly advantageous properties for the production of oils, fuels, and other hydrocarbon or lipid compositions economically and in large quantities, as well as from the discovery of methods and reagents for genetically altering these microorganisms to improve these properties. The oils produced by these microorganisms can be used in the transportation fuel, petrochemical, and/or food and cosmetic industries, among other

applications. Transesterification of lipids yields 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 invention 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.

[0033] This detailed description of the invention is divided into sections for the convenience of the reader. Section I provides definitions of terms used herein. Section 2 provides a description of culture conditions useful in the methods of the invention. Section 3 provides a description of genetic engineering methods and materials. Section 4 provides a description of genetic engineering of *Prototheca* to enable sucrose utilization. Section 5 provides a description of genetic engineering of *Prototheca* to modify lipid biosynthesis. Section 6 describes methods for making fuels and chemicals. Section 7 discloses examples and embodiments of the invention. The detailed description of the invention is followed by examples that illustrate the various aspects and embodiments of the invention.

I. DEFINITIONS

[0034] Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton *et al.*, *Dictionary of Microbiology and Molecular Biology* (2nd ed. 1994); *The Cambridge Dictionary of Science and Technology* (Walker ed., 1988); *The Glossary of Genetics*, 5th Ed., R. Rieger *et al.* (eds.), Springer Verlag (1991); and Hale & Marham, *The Harper Collins Dictionary of Biology* (1991). As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0035] "Active in microalgae" refers to a nucleic acid that is functional in microalgae. For example, a promoter that has been used to drive an antibiotic resistance gene to impart antibiotic resistance to a transgenic microalgae is active in microalgae.

[0036] "Acyl carrier protein" or "ACP" is a protein that binds a growing acyl chain during fatty acid synthesis as a thiol ester at the distal thiol of the 4'-phosphopantetheine moiety and comprises a component of the fatty acid synthase complex.

[0037] "Acyl-CoA molecule" or "acyl-CoA" is a molecule comprising an acyl moiety covalently attached to coenzyme A through a thiol ester linkage at the distal thiol of the 4'-phosphopantetheine moiety of coenzyme A.

[0038] “Area Percent” refers to the area of peaks observed using FAME GC/FID detection methods in which every fatty acid in the sample is converted into a fatty acid methyl ester (FAME) prior to detection. For example, a separate peak is observed for a fatty acid of 14 carbon atoms with no unsaturation (C14:0) compared to any other fatty acid such as C14:1. The peak area for each class of FAME is directly proportional to its percent composition in the mixture and is calculated based on the sum of all peaks present in the sample (i.e. [area under specific peak/ total area of all measured peaks] X 100). When referring to lipid profiles of oils and cells of the invention, “at least 4% C8-C14” means that at least 4% of the total fatty acids in the cell or in the extracted glycerolipid composition have a chain length that includes 8, 10, 12 or 14 carbon atoms.

[0039] “Axenic” is a culture of an organism free from contamination by other living organisms.

[0040] “Biodiesel” is a biologically produced fatty acid alkyl ester suitable for use as a fuel in a diesel engine.

[0041] “Biomass” is material produced by growth and/or propagation of cells. Biomass may contain cells and/or intracellular contents as well as extracellular material, includes, but is not limited to, compounds secreted by a cell.

[0042] “Bioreactor” is an enclosure or partial enclosure in which cells are cultured, optionally in suspension.

[0043] “Catalyst” is an agent, such as a molecule or macromolecular complex, capable of facilitating or promoting a chemical reaction of a reactant to a product without becoming a part of the product. A catalyst increases the rate of a reaction, after which, the catalyst may act on another reactant to form the product. A catalyst generally lowers the overall activation energy required for the reaction such that it proceeds more quickly or at a lower temperature. Thus, a reaction equilibrium may be more quickly attained. Examples of catalysts include enzymes, which are biological catalysts; heat, which is a non-biological catalyst; and metals used in fossil oil refining processes.

[0044] “Cellulosic material” is the product of digestion of cellulose, including glucose and xylose, and optionally additional compounds such as disaccharides, oligosaccharides, lignin, furfurals and other compounds. Nonlimiting examples of sources of cellulosic material include sugar cane bagasses, sugar beet pulp, corn stover, wood chips, sawdust and switchgrass.

[0045] “Co-culture”, and variants thereof such as “co-cultivate” and “co-ferment”, refer to the presence of two or more types of cells in the same bioreactor. The two or more types of

cells may both be microorganisms, such as microalgae, or may be a microalgal cell cultured with a different cell type. The culture conditions may be those that foster growth and/or propagation of the two or more cell types or those that facilitate growth and/or proliferation of one, or a subset, of the two or more cells while maintaining cellular growth for the remainder.

[0046] “Cofactor” is any molecule, other than the substrate, required for an enzyme to carry out its enzymatic activity.

[0047] “Complementary DNA” or “cDNA” is a DNA copy of mRNA, usually obtained by reverse transcription of messenger RNA (mRNA) or amplification (e.g., via polymerase chain reaction (“PCR”)).

[0048] “Cultivated”, and variants thereof such as “cultured” and “fermented”, refer to the intentional fostering of growth (increases in cell size, cellular contents, and/or cellular activity) and/or propagation (increases in cell numbers via mitosis) of one or more cells by use of selected and/or controlled conditions. The combination of both growth and propagation may be termed proliferation. Examples of selected and/or controlled conditions include the use of a defined medium (with known characteristics such as pH, ionic strength, and carbon source), specified temperature, oxygen tension, carbon dioxide levels, and growth in a bioreactor. Cultivate does not refer to the growth or propagation of microorganisms in nature or otherwise without human intervention; for example, natural growth of an organism that ultimately becomes fossilized to produce geological crude oil is not cultivation.

[0049] “Cytolysis” is the lysis of cells in a hypotonic environment. Cytolysis is caused by excessive osmosis, or movement of water, towards the inside of a cell (hyperhydration). The cell cannot withstand the osmotic pressure of the water inside, and so it explodes.

[0050] “Delipidated meal” and “delipidated microbial biomass” is microbial biomass after oil (including lipids) has been extracted or isolated from it, either through the use of mechanical (i.e., exerted by an expeller press) or solvent extraction or both. Delipidated meal has a reduced amount of oil/lipids as compared to before the extraction or isolation of oil/lipids from the microbial biomass but does contain some residual oil/lipid.

[0051] “Expression vector” or “expression construct” or “plasmid” or “recombinant DNA construct” refer to a nucleic acid that has been generated via human intervention, including by recombinant means or direct chemical synthesis, with a series of specified nucleic acid elements that permit transcription and/or translation of a particular nucleic acid in a host cell. The expression vector can be part of a plasmid, virus, or nucleic acid fragment. Typically, the expression vector includes a nucleic acid to be transcribed operably linked to a promoter.

[0052] "Exogenous gene" is a nucleic acid that codes for the expression of an RNA and/or protein that has been introduced ("transformed") into a cell. A transformed cell 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 or as an episomal molecule.

[0053] "Exogenously provided" refers to a molecule provided to the culture media of a cell culture.

[0054] "Expeller pressing" is a mechanical method for extracting oil from raw materials such as soybeans and rapeseed. An expeller press is a screw type machine, which presses material through a caged barrel-like cavity. Raw materials enter one side of the press and spent cake exits the other side while oil seeps out between the bars in the cage and is collected. The machine uses friction and continuous pressure from the screw drives to move and compress the raw material. The oil seeps through small openings that do not allow solids to pass through. As the raw material is pressed, friction typically causes it to heat up.

[0055] "Fatty acyl-ACP thioesterase" is an enzyme that catalyzes the cleavage of a fatty acid from an acyl carrier protein (ACP) during lipid synthesis.

[0056] "Fatty acyl-CoA/aldehyde reductase" is an enzyme that catalyzes the reduction of an acyl-CoA molecule to a primary alcohol.

[0057] "Fatty acyl-CoA reductase" is an enzyme that catalyzes the reduction of an acyl-CoA molecule to an aldehyde.

[0058] "Fatty aldehyde decarbonylase" is an enzyme that catalyzes the conversion of a fatty aldehyde to an alkane.

[0059] "Fatty aldehyde reductase" is an enzyme that catalyzes the reduction of an aldehyde to a primary alcohol.

[0060] "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.

[0061] "Homogenate" is biomass that has been physically disrupted.

[0062] "Hydrocarbon" is (a) a molecule containing only hydrogen and carbon atoms wherein the carbon atoms are covalently linked to form a linear, branched, cyclic, or partially

cyclic backbone to which the hydrogen atoms are attached. The molecular structure of hydrocarbon compounds varies from the simplest, in the form of methane (CH₄), which is a constituent of natural gas, to the very heavy and very complex, such as some molecules such as asphaltenes found in crude oil, petroleum, and bitumens. Hydrocarbons may be in gaseous, liquid, or solid form, or any combination of these forms, and may have one or more double or triple bonds between adjacent carbon atoms in the backbone. Accordingly, the term includes linear, branched, cyclic, or partially cyclic alkanes, alkenes, lipids, and paraffin. Examples include propane, butane, pentane, hexane, octane, and squalene.

[0063] “Hydrogen:carbon ratio” is the ratio of hydrogen atoms to carbon atoms in a molecule on an atom-to-atom basis. The ratio may be used to refer to the number of carbon and hydrogen atoms in a hydrocarbon molecule. For example, the hydrocarbon with the highest ratio is methane CH₄ (4:1).

[0064] “Hydrophobic fraction” is the portion, or fraction, of a material that is more soluble in a hydrophobic phase in comparison to an aqueous phase. A hydrophobic fraction is substantially insoluble in water and usually non-polar.

[0065] “Increase lipid yield” refers to an increase in the productivity of a microbial culture by, for example, increasing dry weight of cells per liter of culture, increasing the percentage of cells that constitute lipid, or increasing the overall amount of lipid per liter of culture volume per unit time.

[0066] “Inducible promoter” is a promoter that mediates transcription of an operably linked gene in response to a particular stimulus.

[0067] “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.

[0068] “In situ” means “in place” or “in its original position”.

[0069] “Limiting concentration of a nutrient” is a concentration of a compound in a culture that limits the propagation of a cultured organism. A “non-limiting concentration of a nutrient” is a concentration that supports maximal propagation during a given culture period. Thus, the number of cells produced during a given culture period is lower in the presence of a limiting concentration of a nutrient than when the nutrient is non-limiting. A nutrient is said to be “in excess” in a culture, when the nutrient is present at a concentration greater than that which supports maximal propagation.

[0070] “Lipase” is a water-soluble enzyme that catalyzes the hydrolysis of ester bonds in water-insoluble, lipid substrates. Lipases catalyze the hydrolysis of lipids into glycerols and fatty acids.

[0071] “Lipid modification enzyme” refers to an enzyme that alters the covalent structure of a lipid. Examples of lipid modification enzymes include a lipase, a fatty acyl-ACP thioesterase, a fatty acyl-CoA/aldehyde reductase, a fatty acyl-CoA reductase, a fatty aldehyde reductase, and a fatty aldehyde decarbonylase.

[0072] “Lipid pathway enzyme” is any enzyme that plays a role in lipid metabolism, i.e., either lipid synthesis, modification, or degradation, and any proteins that chemically modify lipids, as well as carrier proteins.

[0073] “Lipids” are a class of molecules that are soluble in nonpolar solvents (such as ether and chloroform) and are relatively or completely insoluble in water. Lipid molecules have these properties, because they consist largely of long hydrocarbon tails which are hydrophobic in nature. Examples of lipids include fatty acids (saturated and unsaturated); glycerides or glycerolipids (such as monoglycerides, diglycerides, triglycerides or neutral fats, and phosphoglycerides or glycerophospholipids); nonglycerides (sphingolipids, sterol lipids including cholesterol and steroid hormones, prenol lipids including terpenoids, fatty alcohols, waxes, and polyketides); and complex lipid derivatives (sugar-linked lipids, or glycolipids, and protein-linked lipids). “Fats” are a subgroup of lipids called “triacylglycerides.”

[0074] “Lysate” is a solution containing the contents of lysed cells.

[0075] “Lysis” is the breakage of the plasma membrane and optionally the cell wall of a biological organism sufficient to release at least some intracellular content, often by mechanical, viral or osmotic mechanisms that compromise its integrity.

[0076] “Lysing” is disrupting the cellular membrane and optionally the cell wall of a biological organism or cell sufficient to release at least some intracellular content.

[0077] “Microalgae” is a eukaryotic microbial organism that contains a chloroplast or 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*.

[0078] “Microorganism” and “microbe” are microscopic unicellular organisms.

[0079] “Naturally co-expressed” with reference to two proteins or genes means that the proteins or their genes are co-expressed naturally in a tissue or organism from which they are derived, e.g., because the genes encoding the two proteins are under the control of a common regulatory sequence or because they are expressed in response to the same stimulus.

[0080] “Osmotic shock” is the rupture of cells in a solution following a sudden reduction in osmotic pressure. Osmotic shock is sometimes induced to release cellular components of such cells into a solution.

[0081] “Polysaccharide-degrading enzyme” is any enzyme capable of catalyzing the hydrolysis, or saccharification, of any polysaccharide. For example, cellulases catalyze the hydrolysis of cellulose.

[0082] “Polysaccharides” or “glycans” are carbohydrates made up of monosaccharides joined together by glycosidic linkages. Cellulose is a polysaccharide that makes up certain plant cell walls. Cellulose can be depolymerized by enzymes to yield monosaccharides such as xylose and glucose, as well as larger disaccharides and oligosaccharides.

[0083] “Promoter” is a nucleic acid control sequence that directs transcription of a nucleic acid. As used herein, a promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements, which can be located as much as several thousand base pairs from the start site of transcription.

[0084] “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 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. A “recombinant nucleic acid” is a nucleic acid originally formed in vitro, in general, by the manipulation of nucleic acid, e.g., using polymerases and endonucleases, 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.

[0085] "Renewable diesel" is a mixture of alkanes (such as C10:0, C12:0, C14:0, C16:0 and C18:0) produced through hydrogenation and deoxygenation of lipids.

[0086] "Saccharification" is a process of converting biomass, usually cellulosic or lignocellulosic biomass, into monomeric sugars, such as glucose and xylose. "Saccharified" or "depolymerized" cellulosic material or biomass refers to cellulosic material or biomass that has been converted into monomeric sugars through saccharification.

[0087] "Sonication" is a process of disrupting biological materials, such as a cell, by use of sound wave energy.

[0088] "Species of furfural" is 2-furancarboxaldehyde or a derivative that retains the same basic structural characteristics.

[0089] "Stover" is the dried stalks and leaves of a crop remaining after a grain has been harvested.

[0090] "Sucrose utilization gene" is a gene that, when expressed, aids the ability of a cell to utilize sucrose as an energy source. Proteins encoded by a sucrose utilization gene are referred to herein as "sucrose utilization enzymes" and include sucrose transporters, sucrose invertases, and hexokinases such as glucokinases and fructokinases.

II. CULTIVATION

[0091] The present invention generally relates to cultivation of *Prototheca* strains, particularly recombinant *Prototheca* strains, for the production of lipid. For the convenience of the reader, this section is subdivided into subsections. Subsection 1 describes *Prototheca* species and strains and how to identify new *Prototheca* species and strains and related microalgae by genomic DNA comparison. Subsection 2 describes bioreactors useful for cultivation. Subsection 3 describes media for cultivation. Subsection 4 describes oil production in accordance with illustrative cultivation methods of the invention.

1. Prototheca species and strains

[0092] *Prototheca* is a remarkable microorganism for use in the production of lipid, because it can produce high levels of lipid, particularly lipid suitable for fuel production. The

lipid produced by *Prototheca* has hydrocarbon chains of shorter chain length and a higher degree of saturation than that produced by other microalgae. Moreover, *Prototheca* lipid is generally free of pigment (low to undetectable levels of chlorophyll and certain carotenoids) and in any event contains much less pigment than lipid from other microalgae. Moreover, recombinant *Prototheca* cells provided by the invention can be used to produce lipid in greater yield and efficiency, and with reduced cost, relative to the production of lipid from other microorganisms. Illustrative *Prototheca* strains for use in the methods of the invention include In addition, this microalgae grows heterotrophically and can be genetically engineered as *Prototheca wickerhamii*, *Prototheca stagnora* (including UTEX 327), *Prototheca portoricensis*, *Prototheca moriformis* (including UTEX strains 1441, 1435), and *Prototheca zopfii*. Species of the genus *Prototheca* are obligate heterotrophs.

[0093] Species of *Prototheca* for use in the invention can be identified by amplification of certain target regions of the genome. For example, identification of a specific *Prototheca* species or strain can be achieved through amplification and sequencing of nuclear and/or chloroplast DNA using primers and methodology using any region of the genome, for example using the methods described in Wu *et al.*, *Bot. Bull. Acad. Sin.* (2001) 42:115-121 Identification of *Chlorella spp.* isolates using ribosomal DNA sequences. Well established methods of phylogenetic analysis, such as amplification and sequencing of ribosomal internal transcribed spacer (ITS1 and ITS2 rDNA), 23S rRNA, 18S rRNA, and other conserved genomic regions can be used by those skilled in the art to identify species of not only *Prototheca*, but other hydrocarbon and lipid producing organisms with similar lipid profiles and production capability. For examples of methods of identification and classification of algae also see for example *Genetics*, 2005 Aug;170(4):1601-10 and *RNA*, 2005 Apr;11(4):361-4.

[0094] Thus, genomic DNA comparison can be used to identify suitable species of microalgae to be used in the present invention. Regions of conserved genomic DNA, such as but not limited to DNA encoding for 23S rRNA, can be amplified from microalgal species and compared to consensus sequences in order to screen for microalgal species that are taxonomically related to the preferred microalgae used in the present invention. Examples of such DNA sequence comparison for species within the *Prototheca* genus are shown below. Genomic DNA comparison can also be useful to identify microalgal species that have been misidentified in a strain collection. Often a strain collection will identify species of microalgae based on phenotypic and morphological characteristics. The use of these characteristics may lead to miscategorization of the species or the genus of a microalgae. The

use of genomic DNA comparison can be a better method of categorizing microalgae species based on their phylogenetic relationship.

[0095] Microalgae for use in the present invention typically have genomic DNA sequences encoding for 23S rRNA that have at least 99%, least 95%, at least 90%, or at least 85% nucleotide identity to at least one of the sequences listed in SEQ ID NOs: 11-19.

[0096] For sequence comparison to determine percent nucleotide or amino acid identity, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are input into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. The sequence comparison algorithm then calculates the percent sequence identity for the test sequence(s) relative to the reference sequence, based on the designated program parameters.

[0097] Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection (see generally Ausubel *et al.*, *supra*).

[0098] Another example algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (at the web address www.ncbi.nlm.nih.gov). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score

falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. For identifying whether a nucleic acid or polypeptide is within the scope of the invention, the default parameters of the BLAST programs are suitable. The BLASTN program (for nucleotide sequences) uses as defaults a word length (W) of 11, an expectation (E) of 10, $M=5$, $N=-4$, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a word length (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix. The TBLASTN program (using protein sequence for nucleotide sequence) uses as defaults a word length (W) of 3, an expectation (E) of 10, and a BLOSUM 62 scoring matrix. (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)).

[0099] In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability ($P(N)$), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

[0100] Other considerations affecting the selection of microorganisms for use in the invention include, in addition to production of suitable lipids or hydrocarbons for production of oils, fuels, and oleochemicals: (1) high lipid content as a percentage of cell weight; (2) ease of growth; (3) ease of genetic engineering; and (4) ease of biomass processing. In particular embodiments, the wild-type or genetically engineered microorganism yields cells that are at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, or at least 70% or more lipid. Preferred organisms grow heterotrophically (on sugars in the absence of light).

2. Bioreactor

[0101] Microorganisms are cultured both for purposes of conducting genetic manipulations and for production of hydrocarbons (e.g., lipids, fatty acids, aldehydes, alcohols, and alkanes). The former type of culture is conducted on a small scale and initially, at least, under conditions in which the starting microorganism can grow. Culture for purposes of hydrocarbon production is usually conducted on a large scale (e.g., 10,000 L, 40,000 L,

100,000 L or larger bioreactors) in a bioreactor. *Prototheca* are typically cultured in the methods of the invention in liquid media within a bioreactor. Typically, the bioreactor does not allow light to enter.

[0102] The bioreactor or fermentor is used to culture microalgal cells through the various phases of their physiological cycle. Bioreactors offer many advantages for use in heterotrophic growth and propagation methods. To produce biomass for use in food, microalgae are preferably fermented in large quantities in liquid, such as in suspension cultures as an example. Bioreactors such as steel fermentors can accommodate very large culture volumes (40,000 liter and greater capacity bioreactors are used in various embodiments of the invention). Bioreactors also typically allow for the control of culture conditions such as temperature, pH, oxygen tension, and carbon dioxide levels. For example, bioreactors are typically configurable, for example, using ports attached to tubing, to allow gaseous components, like oxygen or nitrogen, to be bubbled through a liquid culture. Other culture parameters, such as the pH of the culture media, the identity and concentration of trace elements, and other media constituents can also be more readily manipulated using a bioreactor.

[0103] Bioreactors can be configured to flow culture media through the bioreactor throughout the time period during which the microalgae reproduce and increase in number. In some embodiments, for example, media can be infused into the bioreactor after inoculation but before the cells reach a desired density. In other instances, a bioreactor is filled with culture media at the beginning of a culture, and no more culture media is infused after the culture is inoculated. In other words, the microalgal biomass is cultured in an aqueous medium for a period of time during which the microalgae reproduce and increase in number; however, quantities of aqueous culture medium are not flowed through the bioreactor throughout the time period. Thus in some embodiments, aqueous culture medium is not flowed through the bioreactor after inoculation.

[0104] Bioreactors equipped with devices such as spinning blades and impellers, rocking mechanisms, stir bars, means for pressurized gas infusion can be used to subject microalgal cultures to mixing. Mixing may be continuous or intermittent. For example, in some embodiments, a turbulent flow regime of gas entry and media entry is not maintained for reproduction of microalgae until a desired increase in number of said microalgae has been achieved.

[0105] Bioreactor ports can be used to introduce, or extract, gases, solids, semisolids, and liquids, into the bioreactor chamber containing the microalgae. While many bioreactors have

more than one port (for example, one for media entry, and another for sampling), it is not necessary that only one substance enter or leave a port. For example, a port can be used to flow culture media into the bioreactor and later used for sampling, gas entry, gas exit, or other purposes. Preferably, a sampling port can be used repeatedly without altering compromising the axenic nature of the culture. A sampling port can be configured with a valve or other device that allows the flow of sample to be stopped and started or to provide a means of continuous sampling. Bioreactors typically have at least one port that allows inoculation of a culture, and such a port can also be used for other purposes such as media or gas entry.

[0106] Bioreactors ports allow the gas content of the culture of microalgae to be manipulated. To illustrate, part of the volume of a bioreactor can be gas rather than liquid, and the gas inlets of the bioreactor to allow pumping of gases into the bioreactor. Gases that can be beneficially pumped into a bioreactor include air, air/CO₂ mixtures, noble gases, such as argon, and other gases. Bioreactors are typically equipped to enable the user to control the rate of entry of a gas into the bioreactor. As noted above, increasing gas flow into a bioreactor can be used to increase mixing of the culture.

[0107] Increased gas flow affects the turbidity of the culture as well. Turbulence can be achieved by placing a gas entry port below the level of the aqueous culture media so that gas entering the bioreactor bubbles to the surface of the culture. One or more gas exit ports allow gas to escape, thereby preventing pressure buildup in the bioreactor. Preferably a gas exit port leads to a “one-way” valve that prevents contaminating microorganisms from entering the bioreactor.

3. Media

[0108] Microalgal culture media typically contains components such as a fixed nitrogen source, a fixed carbon source, trace elements, optionally a buffer for pH maintenance, and phosphate (typically provided as a phosphate salt). Other components can include salts such as sodium chloride, particularly for seawater microalgae. Nitrogen sources include organic and inorganic nitrogen sources, including, for example, without limitation, molecular nitrogen, nitrate, nitrate salts, ammonia (pure or in salt form, such as, (NH₄)₂SO₄ and NH₄OH), protein, soybean meal, cornsteep liquor, and yeast extract. Examples of trace elements include zinc, boron, cobalt, copper, manganese, and molybdenum in, for example, the respective forms of ZnCl₂, H₃BO₃, CoCl₂·6H₂O, CuCl₂·2H₂O, MnCl₂·4H₂O and (NH₄)₆Mo₇O₂₄·4H₂O.

[0109] Microorganisms useful in accordance with the methods of the present invention are found in various locations and environments throughout the world. As a consequence of their isolation from other species and their resulting evolutionary divergence, the particular growth medium for optimal growth and generation of lipid and/or hydrocarbon constituents can be difficult to predict. In some cases, certain strains of microorganisms may be unable to grow on a particular growth medium because of the presence of some inhibitory component or the absence of some essential nutritional requirement required by the particular strain of microorganism.

[0110] Solid and liquid growth media are generally available from a wide variety of sources, and instructions for the preparation of particular media that is suitable for a wide variety of strains of microorganisms can be found, for example, online at <http://www.utex.org/>, a site maintained by the University of Texas at Austin, 1 University Station A6700, Austin, Texas, 78712-0183, for its culture collection of algae (UTEX). For example, various fresh water and salt water media include those described in PCT Pub. No. 2008/151149, incorporated herein by reference.

[0111] In a particular example, Proteose Medium is suitable for axenic cultures, and a 1L volume of the medium (pH ~6.8) can be prepared by addition of 1g of proteose peptone to 1 liter of Bristol Medium. Bristol medium comprises 2.94 mM NaNO₃, 0.17 mM CaCl₂·2H₂O, 0.3 mM MgSO₄·7H₂O, 0.43 mM, 1.29 mM KH₂PO₄, and 1.43 mM NaCl in an aqueous solution. For 1.5% agar medium, 15 g of agar can be added to 1 L of the solution. The solution is covered and autoclaved, and then stored at a refrigerated temperature prior to use. Another example is the *Prototheca* isolation medium (PIM), which comprises 10g/L potassium hydrogen phthalate (KHP), 0.9g/L sodium hydroxide, 0.1g/L magnesium sulfate, 0.2g/L potassium hydrogen phosphate, 0.3g/L ammonium chloride, 10g/L glucose 0.001g/L thiamine hydrochloride, 20g/L agar, 0.25g/L 5-fluorocytosine, at a pH in the range of 5.0 to 5.2 (see Pore, 1973, App. Microbiology, 26: 648-649). Other suitable media for use with the methods of the invention can be readily identified by consulting the URL identified above, or by consulting other organizations that maintain cultures of microorganisms, such as SAG, CCAP, or CCALA. SAG refers to the Culture Collection of Algae at the University of Göttingen (Göttingen, Germany), CCAP refers to the culture collection of algae and protozoa managed by the Scottish Association for Marine Science (Scotland, United Kingdom), and CCALA refers to the culture collection of algal laboratory at the Institute of Botany (Třeboň, Czech Republic). Additionally, US Patent No. 5,900,370 describes media formulations and conditions suitable for heterotrophic fermentation of *Prototheca* species.

[0112] For oil production, selection of a fixed carbon source is important, as the cost of the fixed carbon source must be sufficiently low to make oil production economical. Thus, while suitable carbon sources include, for example, acetate, floridoside, fructose, galactose, glucuronic acid, glucose, glycerol, lactose, mannose, N-acetylglucosamine, rhamnose, sucrose, and/or xylose, selection of feedstocks containing those compounds is an important aspect of the methods of the invention. Suitable feedstocks useful in accordance with the methods of the invention include, for example, black liquor, corn starch, depolymerized cellulosic material, milk whey, molasses, potato, sorghum, sucrose, sugar beet, sugar cane, rice, and wheat. Carbon sources can also be provided as a mixture, such as a mixture of sucrose and depolymerized sugar beet pulp. The one or more carbon source(s) can be supplied at a concentration of at least about 50 μ M, at least about 100 μ M, at least about 500 μ M, at least about 5 mM, at least about 50 mM, and at least about 500 mM, of one or more exogenously provided fixed carbon source(s). Carbon sources of particular interest for purposes of the present invention include cellulose (in a depolymerized form), glycerol, sucrose, and sorghum, each of which is discussed in more detail below.

[0113] In accordance with the present invention, microorganisms can be cultured using depolymerized cellulosic biomass as a feedstock. Cellulosic biomass (e.g., stover, such as corn stover) is inexpensive and readily available; however, attempts to use this material as a feedstock for yeast have failed. In particular, such feedstocks have been found to be inhibitory to yeast growth, and yeast cannot use the 5-carbon sugars produced from cellulosic materials (e.g., xylose from hemi-cellulose). By contrast, microalgae can grow on processed cellulosic material. Cellulosic materials generally include about 40-60% cellulose; about 20-40% hemicellulose; and 10-30% lignin.

[0114] Suitable cellulosic materials include residues from herbaceous and woody energy crops, as well as agricultural crops, i.e., the plant parts, primarily stalks and leaves, not removed from the fields with the primary food or fiber product. Examples include agricultural wastes such as sugarcane bagasse, rice hulls, corn fiber (including stalks, leaves, husks, and cobs), wheat straw, rice straw, sugar beet pulp, citrus pulp, citrus peels; forestry wastes such as hardwood and softwood thinnings, and hardwood and softwood residues from timber operations; wood wastes such as saw mill wastes (wood chips, sawdust) and pulp mill waste; urban wastes such as paper fractions of municipal solid waste, urban wood waste and urban green waste such as municipal grass clippings; and wood construction waste. Additional cellulose include dedicated cellulosic crops such as switchgrass, hybrid poplar

wood, and miscanthus, fiber cane, and fiber sorghum. Five-carbon sugars that are produced from such materials include xylose.

[0115] Cellulosic materials are treated to increase the efficiency with which the microbe can utilize the sugar(s) contained within the materials. The invention provides novel methods for the treatment of cellulosic materials after acid explosion so that the materials are suitable for use in a heterotrophic culture of microbes (e.g., microalgae and oleaginous yeast). As discussed above, lignocellulosic biomass is comprised of various fractions, including cellulose, a crystalline polymer of beta 1,4 linked glucose (a six-carbon sugar), hemicellulose, a more loosely associated polymer predominantly comprised of xylose (a five-carbon sugar) and to a lesser extent mannose, galactose, arabinose, lignin, a complex aromatic polymer comprised of sinapyl alcohol and its derivatives, and pectins, which are linear chains of an alpha 1,4 linked polygalacturonic acid. Because of the polymeric structure of cellulose and hemicellulose, the sugars (e.g., monomeric glucose and xylose) in them are not in a form that can be efficiently used (metabolized) by many microbes. For such microbes, further processing of the cellulosic biomass to generate the monomeric sugars that make up the polymers can be very helpful to ensuring that the cellulosic materials are efficiently utilized as a feedstock (carbon source).

[0116] Cellulose or cellulosic biomass is subjected to a process, termed “explosion”, in which the biomass is treated with dilute sulfuric (or other) acid at elevated temperature and pressure. This process conditions the biomass such that it can be efficiently subjected to enzymatic hydrolysis of the cellulosic and hemicellulosic fractions into glucose and xylose monomers. The resulting monomeric sugars are termed cellulosic sugars. Cellulosic sugars can subsequently be utilized by microorganisms to produce a variety of metabolites (e.g., lipid). The acid explosion step results in a partial hydrolysis of the hemicellulose fraction to constituent monosaccharides. These sugars can be completely liberated from the biomass with further treatment. In some embodiments, the further treatment is a hydrothermal treatment that includes washing the exploded material with hot water, which removes contaminants such as salts. This step is not necessary for cellulosic ethanol fermentations due to the more dilute sugar concentrations used in such processes. In other embodiments, the further treatment is additional acid treatment. In still other embodiments, the further treatment is enzymatic hydrolysis of the exploded material. These treatments can also be used in any combination. The type of treatment can affect the type of sugars liberated (e.g., five carbon sugars versus six carbon sugars) and the stage at which they are liberated in the process. As a consequence, different streams of sugars, whether they are predominantly five-carbon or six-

carbon, can be created. These enriched five-carbon or six-carbon streams can thus be directed to specific microorganisms with different carbon utilization capabilities.

[0117] The methods of the present invention typically involve fermentation to higher cell densities than what is achieved in ethanol fermentation. , Because of the higher densities of the cultures for heterotrophic cellulosic oil production, the fixed carbon source (e.g., the cellulosic derived sugar stream(s)) is preferably in a concentrated form. The glucose level of the depolymerized cellulosic material is preferably at least 300 g/liter, at least 400 g/liter, at least 500 g/liter or at least 600 g/liter prior to the cultivation step, which is optionally a fed batch cultivation in which the material is fed to the cells over time as the cells grow and accumulate lipid. Cellulosic sugar streams are not used at or near this concentration range in the production of cellulosic ethanol. Thus, in order to generate and sustain the very high cell densities during the production of lignocellulosic oil, the carbon feedstock(s) must be delivered into the heterotrophic cultures in a highly concentrated form. However, any component in the feedstream that is not a substrate for, and is not metabolized by, the oleaginous microorganism will accumulate in the bioreactor, which can lead to problems if the component is toxic or inhibitory to production of the desired end product. While ligin and lignin-derived by-products, carbohydrate-derived byproducts such as furfurals and hydroxymethyl furfurals and salts derived from the generation of the cellulosic materials (both in the explosion process and the subsequent neutralization process), and even non-metabolized pentose/hexose sugars can present problems in ethanolic fermentations, these effects are amplified significantly in a process in which their concentration in the initial feedstock is high. To achieve sugar concentrations in the 300g/L range (or higher) for six-carbon sugars that may be used in large scale production of lignocellulosic oil described in the present invention, the concentration of these toxic materials can be 20 times higher than the concentrations typically present in ethanolic fermentations of cellulosic biomass.

[0118] The explosion process treatment of the cellulosic material utilizes significant amounts of sulfuric acid, heat and pressure, thereby liberating by-products of carbohydrates, namely furfurals and hydroxymethyl furfurals. Furfurals and hydroxymethyl furfurals are produced during hydrolysis of hemicellulose through dehydration of xylose into furfural and water. In some embodiments of the present invention, these by-products (e.g., furfurals and hydroxymethyl furfurals) are removed from the saccharified lignocellulosic material prior to introduction into the bioreactor. In certain embodiments of the present invention, the process for removal of the by-products of carbohydrates is hydrothermal treatment of the exploded cellulosic materials. In addition, the present invention provides methods in which strains

capable of tolerating compounds such as furfurals or hydroxymethyl furfurals are used for lignocellulosic oil production. In another embodiment, the present invention also provides methods and microorganisms that are not only capable of tolerating furfurals in the fermentation media, but are actually able to metabolize these by-products during the production of lignocellulosic oil.

[0119] The explosion process also generates significant levels of salts. For example, typical conditions for explosion can result in conductivities in excess of 5 mS/cm when the exploded cellulosic biomass is resuspended at a ratio of 10:1 water:solids (dry weight). In certain embodiments of the present invention, the diluted exploded biomass is subjected to enzymatic saccharification, and the resulting supernatant is concentrated up to 25 fold for use in the bioreactor. The salt level (as measured by conductivity) in the concentrated sugar stream(s) can be unacceptably high (up to 1.5 M Na⁺ equivalents). Additional salts are generated upon neutralization of the exploded materials for the subsequent enzymatic saccharification process as well. The present invention provides methods for removing these salts so that the resulting concentrated cellulosic sugar stream(s) can be used in heterotrophic processes for producing lignocellulosic oil. In some embodiments, the method of removing these salts is deionization with resins, such as, but not limited to, DOWEX Marathon MR3. In certain embodiments, the deionization with resin step occurs before sugar concentration or pH adjustment and hydrothermal treatment of biomass prior to saccharification, or any combination of the preceding; in other embodiments, the step is conducted after one or more of these processes. In other embodiments, the explosion process itself is changed so as to avoid the generation of salts at unacceptably high levels. For example, a suitable alternative to sulfuric acid (or other acid) explosion of the cellulosic biomass is mechanical pulping to render the cellulosic biomass receptive to enzymatic hydrolysis (saccharification). In still other embodiments, native strains of microorganisms resistant to high levels of salts or genetically engineered strains with resistance to high levels of salts are used.

[0120] A preferred embodiment for the process of preparing of exploded cellulosic biomass for use in heterotrophic lignocellulosic oil production using oleaginous microbes is diagramed in Figure 10. Step I. comprises adjusting the pH of the resuspended exploded cellulosic biomass to the range of 5.0-5.3 followed by washing the cellulosic biomass three times. This washing step can be accomplished by a variety of means including the use of desalting and ion exchange resins, reverse osmosis, hydrothermal treatment (as described above), or just repeated re-suspension and centrifugation in deionized water. This wash step results in a cellulosic stream whose conductivity is between 100-300 μ S/cm and the removal

of significant amounts of furfurals and hydroxymethyl furfurals. Decants from this wash step can be saved to concentrate five-carbon sugars liberated from the hemicellulose fraction. Step II comprises enzymatic saccharification of the washed cellulosic biomass. In a preferred embodiment, Accellerase (Genencor) is used. Step III comprises the recovery of sugars via centrifugation or decanting and rinsing of the saccharified biomass. The resulting biomass (solids) is an energy dense, lignin rich component that can be used as fuel or sent to waste. The recovered sugar stream in the centrifugation/decanting and rinse process is collected. Step IV comprises microfiltration to remove contaminating solids with recovery of the permeate. Step V comprises a concentration step which can be accomplished using a vacuum evaporator. This step can optionally include the addition of antifoam agents such as P'2000 (Sigma/Fluka), which is sometimes necessary due to the protein content of the resulting sugar feedstock.

[0121] In another embodiment of the methods of the invention, the carbon source is glycerol, including acidulated and non-acidulated glycerol byproduct from biodiesel transesterification. In one embodiment, the carbon source includes glycerol and at least one other carbon source. In some cases, all of the glycerol and the at least one other fixed carbon source are provided to the microorganism at the beginning of the fermentation. In some cases, the glycerol and the at least one other fixed carbon source are provided to the microorganism simultaneously at a predetermined ratio. In some cases, the glycerol and the at least one other fixed carbon source are fed to the microbes at a predetermined rate over the course of fermentation.

[0122] Some microalgae undergo cell division faster in the presence of glycerol than in the presence of glucose (see PCT Pub. No. 2008/151149). In these instances, two-stage growth processes in which cells are first fed glycerol to rapidly increase cell density, and are then fed glucose to accumulate lipids can improve the efficiency with which lipids are produced. The use of the glycerol byproduct of the transesterification process provides significant economic advantages when put back into the production process. Other feeding methods are provided as well, such as mixtures of glycerol and glucose. Feeding such mixtures also captures the same economic benefits. In addition, the invention provides methods of feeding alternative sugars to microalgae such as sucrose in various combinations with glycerol.

[0123] In another embodiment of the methods of the invention, the carbon source is sucrose, including a complex feedstock containing sucrose, such as thick cane juice from sugar cane processing. In one embodiment, the culture medium further includes at least one sucrose utilization enzyme. In some cases, the culture medium includes a sucrose invertase.

In one embodiment, the sucrose invertase enzyme is a secretable sucrose invertase enzyme encoded by an exogenous sucrose invertase gene expressed by the population of microorganisms. Thus, in some cases, as described in more detail in Section IV, below, the microalgae has been genetically engineered to express a sucrose utilization enzyme, such as a sucrose transporter, a sucrose invertase, a hexokinase, a glucokinase, or a fructokinase.

[0124] Complex feedstocks containing sucrose include waste molasses from sugar cane processing; the use of this low-value waste product of sugar cane processing can provide significant cost savings in the production of hydrocarbons and other oils. Another complex feedstock containing sucrose that is useful in the methods of the invention is sorghum, including sorghum syrup and pure sorghum. Sorghum syrup is produced from the juice of sweet sorghum cane. Its sugar profile consists of mainly glucose (dextrose), fructose and sucrose.

4. Oil production

[0125] For the production of oil in accordance with the methods of the invention, it is preferable to culture cells in the dark, as is the case, for example, when using extremely large (40,000 liter and higher) fermentors that do not allow light to strike the culture. *Prototheca* species are grown and propagated for the production of oil in a medium containing a fixed carbon source and in the absence of light; such growth is known as heterotrophic growth.

[0126] As an example, an inoculum of lipid-producing microalgal cells are introduced into the medium; there is a lag period (lag phase) before the cells begin to propagate. Following the lag period, the propagation rate increases steadily and enters the log, or exponential, phase. The exponential phase is in turn followed by a slowing of propagation due to decreases in nutrients such as nitrogen, increases in toxic substances, and quorum sensing mechanisms. After this slowing, propagation stops, and the cells enter a stationary phase or steady growth state, depending on the particular environment provided to the cells. For obtaining lipid rich biomass, the culture is typically harvested well after the end of the exponential phase, which may be terminated early by allowing nitrogen or another key nutrient (other than carbon) to become depleted, forcing the cells to convert the carbon sources, present in excess, to lipid. Culture condition parameters can be manipulated to optimize total oil production, the combination of lipid species produced, and/or production of a specific oil.

[0127] As discussed above, a bioreactor or fermentor is used to allow cells to undergo the various phases of their growth cycle. As an example, an inoculum of lipid-producing cells can be introduced into a medium followed by a lag period (lag phase) before the cells begin

growth. Following the lag period, the growth rate increases steadily and enters the log, or exponential, phase. The exponential phase is in turn followed by a slowing of growth due to decreases in nutrients and/or increases in toxic substances. After this slowing, growth stops, and the cells enter a stationary phase or steady state, depending on the particular environment provided to the cells. Lipid production by cells disclosed herein can occur during the log phase or thereafter, including the stationary phase wherein nutrients are supplied, or still available, to allow the continuation of lipid production in the absence of cell division.

[0128] Preferably, microorganisms grown using conditions described herein and known in the art comprise at least about 20% by weight of lipid, preferably at least about 40% by weight, more preferably at least about 50% by weight, and most preferably at least about 60% by weight. Process conditions can be adjusted to increase the yield of lipids suitable for a particular use and/or to reduce production cost. For example, in certain embodiments, a microalgae is cultured in the presence of a limiting concentration of one or more nutrients, such as, for example, nitrogen, phosphorous, or sulfur, while providing an excess of fixed carbon energy such as glucose. Nitrogen limitation tends to increase microbial lipid yield over microbial lipid yield in a culture in which nitrogen is provided in excess. In particular embodiments, the increase in lipid yield is at least about: 10%, 50%, 100%, 200%, or 500%. The microbe can be cultured in the presence of a limiting amount of a nutrient for a portion of the total culture period or for the entire period. In particular embodiments, the nutrient concentration is cycled between a limiting concentration and a non-limiting concentration at least twice during the total culture period. Lipid content of cells can be increased by continuing the culture for increased periods of time while providing an excess of carbon, but limiting or no nitrogen.

[0129] In another embodiment, lipid yield is increased by culturing a lipid-producing microbe (e.g., microalgae) in the presence of one or more cofactor(s) for a lipid pathway enzyme (e.g., a fatty acid synthetic enzyme). Generally, the concentration of the cofactor(s) is sufficient to increase microbial lipid (e.g., fatty acid) yield over microbial lipid yield in the absence of the cofactor(s). In a particular embodiment, the cofactor(s) are provided to the culture by including in the culture a microbe (e.g., microalgae) containing an exogenous gene encoding the cofactor(s). Alternatively, cofactor(s) may be provided to a culture by including a microbe (e.g., microalgae) containing an exogenous gene that encodes a protein that participates in the synthesis of the cofactor. In certain embodiments, suitable cofactors include any vitamin required by a lipid pathway enzyme, such as, for example: biotin, pantothenate. Genes encoding cofactors suitable for use in the invention or that participate in

the synthesis of such cofactors are well known and can be introduced into microbes (e.g., microalgae), using constructs and techniques such as those described above.

[0130] The specific examples of bioreactors, culture conditions, and heterotrophic growth and propagation methods described herein can be combined in any suitable manner to improve efficiencies of microbial growth and lipid and/or protein production.

[0131] Microalgal biomass with a high percentage of oil/lipid accumulation by dry weight has been generated using different methods of culture, which are known in the art (see PCT Pub. No. 2008/151149). Microalgal biomass generated by the culture methods described herein and useful in accordance with the present invention comprises at least 10% microalgal oil by dry weight. In some embodiments, the microalgal biomass comprises at least 25%, at least 50%, at least 55%, or at least 60% microalgal oil by dry weight. In some embodiments, the microalgal biomass contains from 10-90% microalgal oil, from 25-75% microalgal oil, from 40-75% microalgal oil, or from 50-70% microalgal oil by dry weight.

[0132] The microalgal oil of the biomass described herein, or extracted from the biomass for use in the methods and compositions of the present invention can comprise glycerolipids with one or more distinct fatty acid ester side chains. Glycerolipids are comprised of a glycerol molecule esterified to one, two or three fatty acid molecules, which can be of varying lengths and have varying degrees of saturation. The length and saturation characteristics of the fatty acid molecules (and the microalgal oils) can be manipulated to modify the properties or proportions of the fatty acid molecules in the microalgal oils of the present invention via culture conditions or via lipid pathway engineering, as described in more detail in Section IV, below. Thus, specific blends of algal oil can be prepared either within a single species of algae by mixing together the biomass or algal oil from two or more species of microalgae, or by blending algal oil of the invention with oils from other sources such as soy, rapeseed, canola, palm, palm kernel, coconut, corn, waste vegetable, Chinese tallow, olive, sunflower, cottonseed, chicken fat, beef tallow, porcine tallow, microalgae, macroalgae, microbes, Cuphea, flax, peanut, choice white grease, lard, Camelina sativa, mustard seed, cashew nut, oats, lupine, kenaf, calendula, hemp, coffee, linseed (flax), hazelnut, euphorbia, pumpkin seed, coriander, camellia, sesame, safflower, rice, tung tree, cocoa, copra, pium poppy, castor beans, pecan, jojoba, macadamia, Brazil nuts, avocado, petroleum, or a distillate fraction of any of the preceding oils.

[0133] The oil composition, *i.e.*, the properties and proportions of the fatty acid constituents of the glycerolipids, can also be manipulated by combining biomass or oil from at least two distinct species of microalgae. In some embodiments, at least two of the distinct

species of microalgae have different glycerolipid profiles. The distinct species of microalgae can be cultured together or separately as described herein, preferably under heterotrophic conditions, to generate the respective oils. Different species of microalgae can contain different percentages of distinct fatty acid constituents in the cell's glycerolipids.

[0134] Generally, *Prototheca* strains have very little or no fatty acids with the chain length C8-C14. For example, *Prototheca moriformis* (UTEX 1435), *Prototheca krugani* (UTEX 329), *Prototheca stagnora* (UTEX 1442) and *Prototheca zopfii* (UTEX 1438) contains no (or undetectable amounts) C8 fatty acids, between 0-0.01% C10 fatty acids, between 0.03-2.1% C12 fatty acids and between 1.0-1.7% C14 fatty acids.

[0135] In some cases, the *Prototheca* strains containing a transgene encoding a fatty acyl-ACP thioesterase that has activity towards fatty acyl-ACP substrate of chain lengths C8-10 has at least 0.3%, at least 0.8%, at least 1.5% or more fatty acids of chain length C8 and at least 0.3%, at least 1.0%, at least 3.0%, at least 5% or more fatty acids of chain length C10. In other instances, the *Prototheca* strains containing a transgene encoding a fatty acyl-ACP thioesterase that has activity towards fatty acyl-ACP substrate of chain length C12 has at least 3.0%, at least 5%, at least 7%, at least 10%, at least 13% or more fatty acids of the chain length C12 and at least 1.5%, at least 2%, or at least 3% or more fatty acids of the chain length C14. In other cases, the *Prototheca* strains containing a transgene encoding a fatty acyl-ACP thioesterase that has activity towards fatty acyl-ACP substrate of chain length C14 has at least 4.0%, at least 7%, at least 10%, at least 15%, at least 20%, at least 25% or more fatty acids of the chain length C14, and at least 0.4%, at least 1%, at least 1.5%, or more fatty acids of the chain length C12.

[0136] In non-limiting examples, the *Prototheca* strains containing a transgene encoding a fatty acyl-ACP thioesterase that has activity towards fatty acyl-ACP substrate of chain length C8 and C10 has between 0.3-1.58 % fatty acids of chain length C8 and between 0.35-6.76 % fatty acids of the chain length C10. In other non-limiting examples, *Prototheca* strains containing a transgene encoding a fatty acyl-ACP thioesterase that has activity towards fatty acyl-ACP substrate of chain length C12 has between 3.9-14.11% fatty acids of the chain length C12 and between 1.95-3.05% fatty acids of the chain length C14. In other non-limiting examples, *Prototheca* strains containing a transgene encoding a fatty acyl-ACP thioesterase that has activity towards fatty acyl-ACP substrate of chain length C14 has between 4.40-17.35 % fatty acids of the chain length C14 and between 0.4-1.83 Area % fatty acids of the chain length C12. In some cases, the *Prototheca* strains containing a transgene encoding a fatty acyl-ACP thioesterase that has activity towards fatty acyl-ACP substrate of chain

lengths between C8 and C14 have between 3.5- 20% medium chain (C8-C14) fatty acids. In some instances, keeping the transgenic *Prototheca* strains under constant and high selective pressure to retain exogenous genes is advantageous due to the increase in the desired fatty acid of a specific chain length. In a non-limiting example, Example 5 demonstrates a two fold increase in C14 chain length fatty acids (more than 30% C8-C14 chain length fatty acids) when the culture of *Prototheca moriformis* containing a C14 preferring thioesterase exogenous gene is retained. High levels of exogenous gene retention can also be achieved by inserting exogenous genes into the nuclear chromosomes of the cells using homologous recombination vectors and methods disclosed herein. Recombinant cells containing exogenous genes integrated into nuclear chromosomes are an object of the invention.

[0137] Microalgal oil can also include other constituents produced by the microalgae, or incorporated into the microalgal oil from the culture medium. These other constituents can be present in varying amount depending on the culture conditions used to culture the microalgae, the species of microalgae, the extraction method used to recover microalgal oil from the biomass and other factors that may affect microalgal oil composition. Non-limiting examples of such constituents include carotenoids, present from 0.1-0.4 micrograms/ml, chlorophyll present from 0-0.02 milligrams/kilogram of oil, gamma tocopherol present from 0.4-0.6 milligrams/100 grams of oil, and total tocotrienols present from 0.2-0.5 milligrams/gram of oil.

[0138] The other constituents can include, without limitation, phospholipids, tocopherols, tocotrienols, carotenoids (*e.g.*, alpha-carotene, beta-carotene, lycopene, etc.), xanthophylls (*e.g.*, lutein, zeaxanthin, alpha-cryptoxanthin and beta-cryptoxanthin), and various organic or inorganic compounds.

[0139] In some cases, the oil extracted from *Prototheca* species comprises no more than 0.02mg/kg chlorophyll. In some cases, the oil extracted from *Prototheca* species comprises no more than 0.4 mcg/ml total carotenoids. In some cases the *Prototheca* oil comprises between 0.40-0.60 milligrams of gamma tocopherol per 100 grams of oil. In other cases, the *Prototheca* oil comprises between 0.2-0.5 milligrams of total tocotrienols per gram of oil.

III. GENETIC ENGINEERING METHODS AND MATERIALS

[0140] The present invention provides methods and materials for genetically modifying *Prototheca* cells and recombinant host cells useful in the methods of the present invention, including but not limited to recombinant *Prototheca moriformis*, *Prototheca zopfii*, *Prototheca krugani*, and *Prototheca stagnora* host cells. The description of these methods and materials is divided into subsections for the convenience of the reader. In subsection 1,

transformation methods are described. In subsection 2, genetic engineering methods using homologous recombination are described. In subsection 3, expression vectors and components are described.

1. Engineering Methods - Transformation

[0141] Cells can be transformed by any suitable technique including, e.g., biolistics, electroporation (see Maruyama et al. (2004), *Biotechnology Techniques* 8:821-826), glass bead transformation and silicon carbide whisker transformation. Another method that can be used involves forming protoplasts and using CaCl_2 and polyethylene glycol (PEG) to introduce recombinant DNA into microalgal cells (see Kim et al. (2002), *Mar. Biotechnol.* 4:63-73, which reports the use of this method for the transformation of *Chorella ellipsoidea*). Co-transformation of microalgae can be used to introduce two distinct vector molecules into a cell simultaneously (see for example Protist 2004 Dec;155(4):381-93).

[0142] Biolistic methods (see, for example, Sanford, *Trends In Biotech.* (1988) 6:299 302, U.S. Patent No. 4,945,050; electroporation (Fromm et al., *Proc. Nat'l. Acad. Sci. (USA)* (1985) 82:5824 5828); use of a laser beam, microinjection or any other method capable of introducing DNA into a microalgae can also be used for transformation of a *Prototheca* cell.

2. Engineering Methods - Homologous Recombination

[0143] Homologous recombination is the ability of complementary DNA sequences to align and exchange regions of homology. Transgenic DNA ("donor") containing sequences homologous to the genomic sequences being targeted ("template") is introduced into the organism and then undergoes recombination into the genome at the site of the corresponding genomic homologous sequences. The mechanistic steps of this process, in most cases, include: (1) pairing of homologous DNA segments; (2) introduction of double-stranded breaks into the donor DNA molecule; (3) invasion of the template DNA molecule by the free donor DNA ends followed by DNA synthesis; and (4) resolution of double-strand break repair events that result in final recombination products.

[0144] The ability to carry out homologous recombination in a host organism has many practical implications for what can be carried out at the molecular genetic level and is useful in the generation of an oleaginous microbe that can produced tailored oils. By its very nature homologous recombination is a precise gene targeting event, hence, most transgenic lines generated with the same targeting sequence will be essentially identical in terms of phenotype, necessitating the screening of far fewer transformation events. Homologous recombination also targets gene insertion events into the host chromosome, resulting in excellent genetic stability, even in the absence of genetic selection. Because different

chromosomal loci will likely impact gene expression, even from heterologous promoters/UTRs, homologous recombination can be a method of querying loci in an unfamiliar genome environment and to assess the impact of these environments on gene expression.

[0145] Particularly useful genetic engineering applications using homologous recombination is to co-opt specific host regulatory elements such as promoters/UTRs to drive heterologous gene expression in a highly specific fashion. For example, precise ablation of the endogenous stearoyl ACP desaturase gene with a heterologous C12:0 specific FATB (thioesterase) gene cassette and suitable selective marker, might be expected to dramatically decrease endogenous levels of C18:1 fatty acids concomitant with increased levels of the C12:0 fatty acids. Example 13 describes the homologous recombination targeting construct that is suitable for the ablation of an endogenous *Prototheca moriformis* stearoyl ACP desaturase gene.

[0146] Because homologous recombination is a precise gene targeting event, it can be used to precisely modify any nucleotide(s) within a gene or region of interest, so long as sufficient flanking regions have been identified. Therefore, homologous recombination can be used as a means to modify regulatory sequences impacting gene expression of RNA and/or proteins. It can also be used to modify protein coding regions in an effort to modify enzyme activities such as substrate specificity, affinities and K_m , and thus affecting the desired change in metabolism of the host cell. Homologous recombination provides a powerful means to manipulate the host genome resulting in gene targeting, gene conversion, gene deletion, gene duplication, gene inversion and exchanging gene expression regulatory elements such as promoters, enhancers and 3'UTRs.

[0147] Homologous recombination can be achieved by using targeting constructs containing pieces of endogenous sequences to "target" the gene or region of interest within the endogenous host cell genome. Such targeting sequences can either be located 5' of the gene or region of interest, 3' of the gene/region of interest or even flank the gene/region of interest. Such targeting constructs can be transformed into the host cell either as a supercoiled plasmid DNA with additional vector backbone, a PCR product with no vector backbone, or as a linearized molecule. In some cases, it may be advantageous to first expose the homologous sequences within the transgenic DNA (donor DNA) with a restriction enzyme. This step can increase the recombination efficiency and decrease the occurrence of undesired events. Other methods of increasing recombination efficiency include using PCR to generate transforming transgenic DNA containing linear ends homologous to the genomic sequences being targeted.

3. Vectors and Vector Components

[0148] Vectors for transformation of microorganisms in accordance with the present invention can be prepared by known techniques familiar to those skilled in the art in view of the disclosure herein. A vector typically contains one or more genes, in which each gene codes for the expression of a desired product (the gene product) and is operably linked to one or more control sequences that regulate gene expression or target the gene product to a particular location in the recombinant cell. To aid the reader, this subsection is divided into subsections. Subsection A describes control sequences typically contained on vectors as well as novel control sequences provided by the present invention. Subsection B describes genes typically contained in vectors as well as novel codon optimization methods and genes prepared using them provided by the invention.

A. Control Sequences

[0149] Control sequences are nucleic acids that regulate the expression of a coding sequence or direct a gene product to a particular location in or outside a cell. Control sequences that regulate expression include, for example, promoters that regulate transcription of a coding sequence and terminators that terminate transcription of a coding sequence. Another control sequence is a 3' untranslated sequence located at the end of a coding sequence that encodes a polyadenylation signal. Control sequences that direct gene products to particular locations include those that encode signal peptides, which direct the protein to which they are attached to a particular location in or outside the cell.

[0150] Thus, an exemplary vector design for expression of an exogenous gene in a microalgae contains a coding sequence for a desired gene product (for example, a selectable marker, a lipid pathway modification enzyme, or a sucrose utilization enzyme) in operable linkage with a promoter active in microalgae. Alternatively, if the vector does not contain a promoter in operable linkage with the coding sequence of interest, the coding sequence can be transformed into the cells such that it becomes operably linked to an endogenous promoter at the point of vector integration. The promoterless method of transformation has been proven to work in microalgae (see for example Plant Journal 14:4, (1998), pp.441-447).

[0151] Many promoters are active in microalgae, including promoters that are endogenous to the algae being transformed, as well as promoters that are not endogenous to the algae being transformed (i.e., promoters from other algae, promoters from higher plants, and promoters from plant viruses or algae viruses). Illustrative exogenous and/or endogenous promoters that are active in microalgae (as well as antibiotic resistance genes functional in microalgae) are described in PCT Pub. No. 2008/151149 and references cited therein).

[0152] The promoter used to express an exogenous gene can be the promoter naturally linked to that gene or can be a heterologous gene. Some promoters are active in more than one species of microalgae. Other promoters are species-specific. Illustrative promoters include promoters such as β -tubulin from *Chlamydomonas reinhardtii*, used in the Examples below, and viral promoters, such as cauliflower mosaic virus (CMV) and chlorella virus, which have been shown to be active in multiple species of microalgae (see for example Plant Cell Rep. 2005 Mar;23(10-11):727-35; J Microbiol. 2005 Aug;43(4):361-5; Mar Biotechnol (NY). 2002 Jan;4(1):63-73). Another promoter that is suitable for use for expression of exogenous genes in *Prototheca* is the *Chlorella sorokiniana* glutamate dehydrogenase promoter/5'UTR (SEQ ID NO: 69). Optionally, at least 10, 20, 30, 40, 50, or 60 nucleotides or more of these sequences containing a promoter are used. Illustrative promoters useful for expression of exogenous genes in *Prototheca* are listed in the sequence listing of this application, such as the promoter of the *Chlorella* HUP1 gene (SEQ ID NO:1) and the *Chlorella ellipsoidea* nitrate reductase promoter (SEQ ID NO:2). Chlorella virus promoters can also be used to express genes in *Prototheca*, such as SEQ ID NOs: 1-7 of U.S. Patent 6,395,965. Additional promoters active in *Prototheca* can be found, for example, in Biochem Biophys Res Commun. 1994 Oct 14;204(1):187-94; Plant Mol Biol. 1994 Oct;26(1):85-93; Virology. 2004 Aug 15;326(1):150-9; and Virology. 2004 Jan 5;318(1):214-23.

[0153] A promoter can generally be characterized as either constitutive or inducible. Constitutive promoters are generally active or function to drive expression at all times (or at certain times in the cell life cycle) at the same level. Inducible promoters, conversely, are active (or rendered inactive) or are significantly up- or down-regulated only in response to a stimulus. Both types of promoters find application in the methods of the invention. Inducible promoters useful in the invention include those that mediate transcription of an operably linked gene in response to a stimulus, such as an exogenously provided small molecule (e.g., glucose, as in SEQ ID NO:1), temperature (heat or cold), lack of nitrogen in culture media, etc. Suitable promoters can activate transcription of an essentially silent gene or upregulate, preferably substantially, transcription of an operably linked gene that is transcribed at a low level.

[0154] Inclusion of termination region control sequence is optional, and if employed, then the choice is primarily one of convenience, as the termination region is relatively interchangeable. The termination region may be native to the transcriptional initiation region (the promoter), may be native to the DNA sequence of interest, or may be obtainable from another source. See, for example, Chen and Orozco, Nucleic Acids Res. (1988) 16:8411.

[0155] The present invention also provides control sequences and recombinant genes and vectors containing them that provide for the compartmentalized expression of a gene of interest. Organelles for targeting are chloroplasts, plastids, mitochondria, and endoplasmic reticulum. In addition, the present invention provides control sequences and recombinant genes and vectors containing them that provide for the secretion of a protein outside the cell.

[0156] Proteins expressed in the nuclear genome of *Prototheca* can be targeted to the plastid using plastid targeting signals. Plastid targeting sequences endogenous to *Chlorella* are known, such as genes in the *Chlorella* nuclear genome that encode proteins that are targeted to the plastid; see for example GenBank Accession numbers AY646197 and AF499684, and in one embodiment, such control sequences are used in the vectors of the present invention to target expression of a protein to a *Prototheca* plastid.

[0157] The Examples below describe the use of algal plastid targeting sequences to target heterologous proteins to the correct compartment in the host cell. cDNA libraries were made using *Prototheca moriformis* and *Chlorella protothecoides* cells and are described in Examples 12 and Example 11 below. Sequences were BLASTed and analyzed for homology to known proteins that traffic to the plastid/chloroplast. The cDNAs encoding these proteins were cloned and plastid targeting sequences were isolated from these cDNAs. The amino acid sequences of the algal plastid targeting sequences identified from the cDNA libraries and the amino acid sequences of plant fatty acyl-ACP thioesterases that are used in the heterologous expression Examples below are listed in SEQ ID NOs: 127-133.

[0158] In another embodiment of the present invention, the expression of a polypeptide in *Prototheca* is targeted to the endoplasmic reticulum. The inclusion of an appropriate retention or sorting signal in an expression vector ensure that proteins are retained in the endoplasmic reticulum (ER) and do not go downstream into Golgi. For example, the IMPACTVECTOR1.3 vector, from Wageningen UR- Plant Research International, includes the well known KDEL retention or sorting signal. With this vector, ER retention has a practical advantage in that it has been reported to improve expression levels 5-fold or more. The main reason for this appears to be that the ER contains lower concentrations and/or different proteases responsible for post-translational degradation of expressed proteins than are present in the cytoplasm. ER retention signals functional in green microalgae are known. For example, see Proc Natl Acad Sci U S A. 2005 Apr 26;102(17):6225-30.

[0159] In another embodiment of the present invention, a polypeptide is targeted for secretion outside the cell into the culture media. See Hawkins et al., Current Microbiology

Vol. 38 (1999), pp. 335–341 for examples of secretion signals active in *Chlorella* that can be used, in accordance with the methods of the invention, in *Prototheca*.

B. Genes and Codon Optimization

[0160] Typically, a gene includes a promoter, coding sequence, and termination control sequences. When assembled by recombinant DNA technology, a gene may be termed an expression cassette and may be flanked by restriction sites for convenient insertion into a vector that is used to introduce the recombinant gene into a host cell. The expression cassette can be flanked by DNA sequences from the genome or other nucleic acid target to facilitate stable integration of the expression cassette into the genome by homologous recombination. Alternatively, the vector and its expression cassette may remain unintegrated, in which case, the vector typically includes an origin of replication, which is capable of providing for replication of the heterologous vector DNA.

[0161] A common gene present on a vector is a gene that codes for a protein, the expression of which allows the recombinant cell containing the protein to be differentiated from cells that do not express the protein. Such a gene, and its corresponding gene product, is called a selectable marker. Any of a wide variety of selectable markers can be employed in a transgene construct useful for transforming *Prototheca*. Examples of suitable selectable markers include the G418 resistance gene, the nitrate reductase gene (see Dawson et al. (1997), *Current Microbiology* 35:356-362), the hygromycin phosphotransferase gene (HPT; see Kim et al. (2002), *Mar. Biotechnol.* 4:63-73), the neomycin phosphotransferase gene, and the ble gene, which confers resistance to phleomycin (Huang et al. (2007), *Appl. Microbiol. Biotechnol.* 72:197-205). Methods of determining sensitivity of microalgae to antibiotics are well known. For example, *Mol Gen Genet.* 1996 Oct 16;252(5):572-9.

[0162] For purposes of the present invention, the expression vector used to prepare a recombinant host cell of the invention will include at least two, and often three, genes, if one of the genes is a selectable marker. For example, a genetically engineered *Prototheca* of the invention can be made by transformation with vectors of the invention that comprise, in addition to a selectable marker, one or more exogenous genes, such as, for example, sucrose invertase gene or acyl ACP-thioesterase gene. One or both genes can be expressed using an inducible promoter, which allows the relative timing of expression of these genes to be controlled to enhance the lipid yield and conversion to fatty acid esters. Expression of the two or more exogenous genes may be under control of the same inducible promoter or under control of different inducible (or constitutive) promoters. In the latter situation, expression of a first exogenous gene can be induced for a first period of time (during which expression of a

second exogenous gene may or may not be induced) and expression of a second exogenous gene can be induced for a second period of time (during which expression of a first exogenous gene may or may not be induced).

[0163] In other embodiments, the two or more exogenous genes (in addition to any selectable marker) are: a fatty acyl-ACP thioesterase and a fatty acyl-CoA/aldehyde reductase, the combined action of which yields an alcohol product. Further provided are other combinations of exogenous genes, including without limitation, a fatty acyl-ACP thioesterase and a fatty acyl-CoA reductase to generate aldehydes. In one embodiment, the vector provides for the combination of a fatty acyl-ACP thioesterase, a fatty acyl-CoA reductase, and a fatty aldehyde decarbonylase to generate alkanes. In each of these embodiments, one or more of the exogenous genes can be expressed using an inducible promoter.

[0164] Other illustrative vectors of the invention that express two or more exogenous genes include those encoding both a sucrose transporter and a sucrose invertase enzyme and those encoding both a selectable marker and a secreted sucrose invertase. The recombinant *Prototheca* transformed with either type of vector produce lipids at lower manufacturing cost due to the engineered ability to use sugar cane (and sugar cane-derived sugars) as a carbon source. Insertion of the two exogenous genes described above can be combined with the disruption of polysaccharide biosynthesis through directed and/or random mutagenesis, which steers ever greater carbon flux into lipid production. Individually and in combination, trophic conversion, engineering to alter lipid production and treatment with exogenous enzymes alter the lipid composition produced by a microorganism. The alteration can be a change in the amount of lipids produced, the amount of one or more hydrocarbon species produced relative to other lipids, and/or the types of lipid species produced in the microorganism. For example, microalgae can be engineered to produce a higher amount and/or percentage of TAGs.

[0165] For optimal expression of a recombinant protein, it is beneficial to employ coding sequences that produce mRNA with codons preferentially used by the host cell to be transformed. Thus, proper expression of transgenes can require that the codon usage of the transgene matches the specific codon bias of the organism in which the transgene is being expressed. The precise mechanisms underlying this effect are many, but include the proper balancing of available aminoacylated tRNA pools with proteins being synthesized in the cell, coupled with more efficient translation of the transgenic messenger RNA (mRNA) when this need is met. When codon usage in the transgene is not optimized, available tRNA pools are

not sufficient to allow for efficient translation of the heterologous mRNA resulting in ribosomal stalling and termination and possible instability of the transgenic mRNA.

[0166] The present invention provides codon-optimized nucleic acids useful for the successful expression of recombinant proteins in *Prototheca*. Codon usage in *Prototheca* species was analyzed by studying cDNA sequences isolated from *Prototheca moriformis*. This analysis represents the interrogation over 24, 000 codons and resulted in Table 1 below.

[0167] Table 1. Preferred codon usage in *Prototheca* strains.

Ala	GCG	345 (0.36)	Asn	AAT	8 (0.04)
	GCA	66 (0.07)		AAC	201 (0.96)
	GCT	101 (0.11)	Pro	CCG	161 (0.29)
	GCC	442 (0.46)		CCA	49 (0.09)
Cys	TGT	12 (0.10)		CCT	71 (0.13)
	TGC	105 (0.90)		CCC	267 (0.49)
Asp	GAT	43 (0.12)	Gln	CAG	226 (0.82)
	GAC	316 (0.88)		CAA	48 (0.18)
Glu	GAG	377 (0.96)	Arg	AGG	33 (0.06)
	GAA	14 (0.04)		AGA	14 (0.02)
Phe	TTT	89 (0.29)		CGG	102 (0.18)
	TTC	216 (0.71)		CGA	49 (0.08)
				CGT	51 (0.09)
Gly	GGG	92 (0.12)		CGC	331 (0.57)
	GGA	56 (0.07)	Ser	AGT	16 (0.03)
	GGT	76 (0.10)		AGC	123 (0.22)
	GGC	559 (0.71)		TCG	152 (0.28)
His	CAT	42 (0.21)		TCA	31 (0.06)
	CAC	154 (0.79)		TCT	55 (0.10)
Ile	ATA	4 (0.01)	Thr	TCC	173 (0.31)
	ATT	30 (0.08)		ACG	184 (0.38)
	ATC	338 (0.91)		ACA	24 (0.05)
Lys	AAG	284 (0.98)		ACT	21 (0.05)
	AAA	7 (0.02)	Val	ACC	249 (0.52)
Leu	TTG	26 (0.04)		GTG	308 (0.50)
	TTA	3 (0.00)		GTA	9 (0.01)
	CTG	447 (0.61)		GTT	35 (0.06)
	CTA	20 (0.03)		GTC	262 (0.43)
	CTT	45 (0.06)	Trp	TGG	107 (1.00)
	CTC	190 (0.26)		TAT	10 (0.05)
			Tyr	TAC	180 (0.95)

Met	ATG	191 (1.00)	Stop	TGA/TAG/TAA
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[0168] In other embodiments, the gene in the recombinant vector has been codon-optimized with reference to a microalgal strain other than a *Prototheca* strain. For example, methods of recoding genes for expression in microalgae are described in U.S. Patent 7,135,290. Additional information for codon optimization is available, e.g., at the codon usage database of GenBank.

[0169] While the methods and materials of the invention allow for the introduction of any exogenous gene into *Prototheca*, genes relating to sucrose utilization and lipid pathway modification are of particular interest, as discussed in the following sections.

IV. SUCROSE UTILIZATION

[0170] In embodiment, the recombinant *Prototheca* cell of the invention further contains one or more exogenous sucrose utilization genes. In various embodiments, the one or more genes encode one or more proteins selected from the group consisting of a fructokinase, a glucokinase, a hexokinase, a sucrose invertase, a sucrose transporter. For example, expression of a sucrose transporter and a sucrose invertase allows *Prototheca* to transport sucrose into the cell from the culture media and hydrolyze sucrose to yield glucose and fructose. Optionally, a fructokinase can be expressed as well in instances where endogenous hexokinase activity is insufficient for maximum phosphorylation of fructose. Examples of suitable sucrose transporters are Genbank accession numbers CAD91334, CAB92307, and CAA53390. Examples of suitable fructokinases are Genbank accession numbers P26984, P26420 and CAA43322.

[0171] In one embodiment, the present invention provides a *Prototheca* host cell that secretes a sucrose invertase. Secretion of a sucrose invertase obviates the need for expression of a transporter that can transport sucrose into the cell. This is because a secreted invertase catalyzes the conversion of a molecule of sucrose into a molecule of glucose and a molecule of fructose, both of which can be transported and utilized by microbes provided by the invention. For example, expression of a sucrose invertase (such as SEQ ID NO:3) with a secretion signal (such as that of SEQ ID NO: 4 (from yeast), SEQ ID NO: 5 (from higher plants), SEQ ID NO: 6 (eukaryotic consensus secretion signal), and SEQ ID NO: 7 (combination of signal sequence from higher plants and eukaryotic consensus) generates invertase activity outside the cell. Expression of such a protein, as enabled by the genetic

engineering methodology disclosed herein, allows cells already capable of utilizing extracellular glucose as an energy source to utilize sucrose as an extracellular energy source.

[0172] *Prototheca* species expressing an invertase in media containing sucrose are a preferred microalgal species for the production of oil. Example 3 illustrates how the methods and reagents of the invention can be used to express a recombinant yeast invertase and secrete it from a recombinant *Prototheca* cell. The expression and extracellular targeting of this fully active protein allows the resulting host cells to grow on sucrose, whereas their non-transformed counterparts cannot. Thus, the present invention provides *Prototheca* recombinant cells with a codon-optimized invertase gene, including but not limited to the yeast invertase gene, integrated into their genome such that the invertase gene is expressed as assessed by invertase activity and sucrose hydrolysis. The present invention also provides invertase genes useful as selectable markers in *Prototheca* recombinant cells, as such cells are able to grow on sucrose, while their non-transformed counterparts cannot; and methods for selecting recombinant host cells using an invertase as a powerful, selectable marker for algal molecular genetics.

[0173] The successful expression of a sucrose invertase in *Prototheca* also illustrates another aspect of the present invention in that it demonstrates that heterologous (recombinant) proteins can be expressed in the algal cell and successfully transit outside of the cell and into the culture medium in a fully active and functional form. Thus, the present invention provides methods and reagents for expressing a wide and diverse array of heterologous proteins in microalgae and secreting them outside of the host cell. Such proteins include, for example, industrial enzymes such as, for example, lipases, proteases, cellulases, pectinases, amylases, esterases, oxidoreductases, transferases, lactases, isomerases, and invertases, as well as therapeutic proteins such as, for example, growth factors, cytokines, full length antibodies comprising two light and two heavy chains, Fabs, scFvs (single chain variable fragment), camellid-type antibodies, antibody fragments, antibody fragment-fusions, antibody-receptor fusions, insulin, interferons, and insulin-like growth factors.

[0174] The successful expression of a sucrose invertase in *Prototheca* also illustrates another aspect of the present invention in that it provides methods and reagents for the use of fungal transit peptides in algae to direct secretion of proteins in *Prototheca*; and methods and reagents for determining if a peptide can function, and the ability of it to function, as a transit peptide in *Prototheca* cells. The methods and reagents of the invention can be used as a tool and platform to identify other transit peptides that can successfully traffic proteins outside of a cell, and that the yeast invertase has great utility in these methods. As demonstrated in this

example, removal of the endogenous yeast invertase transit peptide and its replacement by other transit peptides, either endogenous to the host algae or from other sources (eukaryotic, prokaryotic and viral), can identify whether any peptide of interest can function as a transit peptide in guiding protein egress from the cell.

[0175] Examples of suitable sucrose invertases include those identified by Genbank accession numbers CAB95010, NP_012104 and CAA06839. Non-limiting examples of suitable invertases are listed below in Table 2. Amino acid sequences for each listed invertase are included in the Sequence Listing below. In some cases, the exogenous sucrose utilization gene suitable for use in the methods and vectors of the invention encodes a sucrose invertase that has at least 40, 50, 60, 75, or 90% or higher amino acid identity with a sucrose invertase selected from Table 2.

[0176] Table 2. Sucrose invertases.

Description	Organism	GenBank Accession No.	SEQ ID NO:
Invertase	<i>Chicorium intybus</i>	Y11124	SEQ ID NO:20
Invertase	<i>Schizosaccharomyces pombe</i>	AB011433	SEQ ID NO:21
beta-fructofuranosidase (invertase)	<i>Pichia anomala</i>	X80640	SEQ ID NO:22
Invertase	<i>Debaryomyces occidentalis</i>	X17604	SEQ ID NO:23
Invertase	<i>Oryza sativa</i>	AF019113	SEQ ID NO:24
Invertase	<i>Allium cepa</i>	AJ006067	SEQ ID NO:25
Invertase	<i>Beta vulgaris subsp. Vulgaris</i>	AJ278531	SEQ ID NO:26
beta-fructofuranosidase (invertase)	<i>Bifidobacterium breve UCC2003</i>	AAT28190	SEQ ID NO:27
Invertase	<i>Saccharomyces cerevisiae</i>	NP_012104	SEQ ID NO:8 (nucleotide)
			SEQ ID NO:28 (amino acid)
Invertase A	<i>Zymomonas mobilis</i>	AAO38865	SEQ ID NO:29

[0177] The secretion of an invertase to the culture medium by *Prototheca* enable the cells to grow as well on waste molasses from sugar cane processing as they do on pure reagent-grade glucose; the use of this low-value waste product of sugar cane processing can provide significant cost savings in the production of lipids and other oils. Thus, the present invention provides a microbial culture containing a population of *Prototheca* microorganisms, and a culture medium comprising (i) sucrose and (ii) a sucrose invertase enzyme. In various embodiments the sucrose in the culture comes from sorghum, sugar beet, sugar cane, molasses, or depolymerized cellulosic material (which may optionally contain lignin). In another aspect, the methods and reagents of the invention significantly increase the number and type of feedstocks that can be utilized by recombinant *Prototheca*. While the microbes exemplified here are altered such that they can utilize sucrose, the methods and reagents of the invention can be applied so that feedstocks such as cellulose are utilizable by an engineered host microbe of the invention with the ability to secrete cellulases, pectinases, isomerases, or the like, such that the breakdown products of the enzymatic reactions are no longer just simply tolerated but rather utilized as a carbon source by the host.

V. LIPID PATHWAY ENGINEERING

[0178] In addition to altering the ability of *Prototheca* to utilize feedstocks such as sucrose-containing feedstocks, the present invention also provides recombinant *Prototheca* that have been modified to alter the properties and/or proportions of lipids produced. The pathway can further, or alternatively, be modified to alter the properties and/or proportions of various lipid molecules produced through enzymatic processing of lipids and intermediates in the fatty acid pathway. In various embodiments, the recombinant *Prototheca* cells of the invention have, relative to their untransformed counterparts, optimized lipid yield per unit volume and/or per unit time, carbon chain length (e.g., for renewable diesel production or for industrial chemicals applications requiring lipid feedstock), reduced number of double or triple bonds, optionally to zero, and increasing the hydrogen:carbon ratio of a particular species of lipid or of a population of distinct lipid.

[0179] In particular embodiments, one or more key enzymes that control branch points in metabolism to fatty acid synthesis have been up-regulated or down-regulated to improve lipid production. Up-regulation can be achieved, for example, by transforming cells with expression constructs in which a gene encoding the enzyme of interest is expressed, e.g., using a strong promoter and/or enhancer elements that increase transcription. Such constructs can include a selectable marker such that the transformants can be subjected to selection, which can result in amplification of the construct and an increase in the expression level of

the encoded enzyme. Examples of enzymes suitable for up-regulation according to the methods of the invention include pyruvate dehydrogenase, which plays a role in converting pyruvate to acetyl-CoA (examples, some from microalgae, include Genbank accession numbers NP_415392; AAA53047; Q1XDM1; and CAF05587). Up-regulation of pyruvate dehydrogenase can increase production of acetyl-CoA, and thereby increase fatty acid synthesis. Acetyl-CoA carboxylase catalyzes the initial step in fatty acid synthesis. Accordingly, this enzyme can be up-regulated to increase production of fatty acids (examples, some from microalgae, include Genbank accession numbers BAA94752; AAA75528; AAA81471; YP_537052; YP_536879; NP_045833; and BAA57908). Fatty acid production can also be increased by up-regulation of acyl carrier protein (ACP), which carries the growing acyl chains during fatty acid synthesis (examples, some from microalgae, include Genbank accession numbers A0T0F8; P51280; NP_849041; YP_874433). Glycerol-3-phosphate acyltransferase catalyzes the rate-limiting step of fatty acid synthesis. Up-regulation of this enzyme can increase fatty acid production (examples, some from microalgae, include Genbank accession numbers AAA74319; AAA33122; AAA37647; P44857; and ABO94442).

[0180] Up- and/or down-regulation of genes can be applied to global regulators controlling the expression of the genes of the fatty acid biosynthetic pathways. Accordingly, one or more global regulators of fatty acid synthesis can be up- or down-regulated, as appropriate, to inhibit or enhance, respectively, the expression of a plurality of fatty acid synthetic genes and, ultimately, to increase lipid production. Examples include sterol regulatory element binding proteins (SREBPs), such as SREBP-1a and SREBP-1c (for examples see Genbank accession numbers NP_035610 and Q9WTN3).

[0181] The present invention also provides recombinant *Prototheca* cells that have been modified to contain one or more exogenous genes encoding lipid modification enzymes such as, for example, fatty acyl-ACP thioesterases (see Table 3), fatty acyl-CoA/aldehyde reductases (see Table 4), fatty acyl-CoA reductases (see Table 5), fatty aldehyde decarbonylase (see Table 6), fatty aldehyde reductases, and squalene synthases (see GenBank Accession number AF205791). In some embodiments, genes encoding a fatty acyl-ACP thioesterase and a naturally co-expressed acyl carrier protein are transformed into a *Prototheca* cell, optionally with one or more genes encoding other lipid modification enzymes. In other embodiments, the ACP and the fatty acyl-ACP thioesterase may have an affinity for one another that imparts an advantage when the two are used together in the microbes and methods of the present invention, irrespective of whether they are or are not

naturally co-expressed in a particular tissue or organism. Thus, the present invention contemplates both naturally co-expressed pairs of these enzymes as well as those that share an affinity for interacting with one another to facilitate cleavage of a length-specific carbon chain from the ACP.

[0182] In still other embodiments, an exogenous gene encoding a desaturase is transformed into the *Prototheca* cell in conjunction with one or more genes encoding other lipid modification enzymes to provide modifications with respect to lipid saturation. Stearoyl-ACP desaturase (see, e.g., GenBank Accession numbers AAF15308; ABM45911; and AAY86086), for example, catalyzes the conversion of stearoyl-ACP to oleoyl-ACP. Up-regulation of this gene can increase the proportion of monounsaturated fatty acids produced by a cell; whereas down-regulation can reduce the proportion of monounsaturates. Similarly, the expression of one or more glycerolipid desaturases can be controlled to alter the ratio of unsaturated to saturated fatty acids such as ω -6 fatty acid desaturase, ω -3 fatty acid desaturase, or ω -6-oleate desaturase. In some embodiments, the desaturase can be selected with reference to a desired carbon chain length, such that the desaturase is capable of making location specific modifications within a specified carbon-length substrate, or substrates having a carbon-length within a specified range.

[0183] Thus, in particular embodiments, microbes of the present invention are genetically engineered to express one or more exogenous genes selected from an acyl-ACP thioesterase, an acyl-CoA/aldehyde reductase, a fatty acyl-CoA reductase, a fatty aldehyde reductase, a fatty aldehyde decarbonylase, or a naturally co-expressed acyl carrier protein. Suitable expression methods are described above with respect to the expression of a lipase gene, including, among other methods, inducible expression and compartmentalized expression. A fatty acyl-ACP thioesterase cleaves a fatty acid from an acyl carrier protein (ACP) during lipid synthesis. Through further enzymatic processing, the cleaved fatty acid is then combined with a coenzyme to yield an acyl-CoA molecule. This acyl-CoA is the substrate for the enzymatic activity of a fatty acyl-CoA reductase to yield an aldehyde, as well as for a fatty acyl-CoA/aldehyde reductase to yield an alcohol. The aldehyde produced by the action of the fatty acyl-CoA reductase identified above is the substrate for further enzymatic activity by either a fatty aldehyde reductase to yield an alcohol, or a fatty aldehyde decarbonylase to yield an alkane or alkene.

[0184] In some embodiments, fatty acids, glycerolipids, or the corresponding primary alcohols, aldehydes, alkanes or alkenes, generated by the methods described herein, contain 8, 10, 12, or 14 carbon atoms. Preferred fatty acids for the production of diesel, biodiesel,

renewable diesel, or jet fuel, or the corresponding primary alcohols, aldehydes, alkanes and alkenes, for industrial applications contain 8 to 14 carbon atoms. In certain embodiments, the above fatty acids, as well as the other corresponding hydrocarbon molecules, are saturated (with no carbon-carbon double or triple bonds); mono unsaturated (single double bond); poly unsaturated (two or more double bonds); are linear (not cyclic) or branched. For fuel production, greater saturation is preferred.

[0185] The enzymes described directly above have a preferential specificity for hydrolysis of a substrate containing a specific number of carbon atoms. For example, a fatty acyl-ACP thioesterase may have a preference for cleaving a fatty acid having 12 carbon atoms from the ACP. In some embodiments, the ACP and the length-specific thioesterase may have an affinity for one another that makes them particularly useful as a combination (e.g., the exogenous ACP and thioesterase genes may be naturally co-expressed in a particular tissue or organism from which they are derived). Therefore, in various embodiments, the recombinant *Prototheca* cell of the invention can contain an exogenous gene that encodes a protein with specificity for catalyzing an enzymatic activity (e.g., cleavage of a fatty acid from an ACP, reduction of an acyl-CoA to an aldehyde or an alcohol, or conversion of an aldehyde to an alkane) with regard to the number of carbon atoms contained in the substrate. The enzymatic specificity can, in various embodiments, be for a substrate having from 8 to 34 carbon atoms, preferably from 8 to 18 carbon atoms, and more preferably from 8 to 14 carbon atoms. A preferred specificity is for a substrate having fewer, i.e., 12, rather than more, i.e., 18, carbon atoms.

[0186] In non-limiting but illustrative examples, the present invention provides vectors and *Prototheca* host cells that express an exogenous thioesterase and accordingly produce lipid enriched, relative to the lipid profile of untransformed *Prototheca* cells, in the chain length for which the thioesterase is specific. The thioesterases illustrated are (i) *Cinnamomum camphorum* FatB1 (GenBank Accension No. Q39473, amino acid sequence is in SEQ ID NO: 59, amino acid sequence without plastid targeting sequence (PTS) is in SEQ ID NO: 139, and codon optimized cDNA sequence based on Table 1 is in SEQ ID NO: 60), which has a preference for fatty acyl-ACP substrate with a carbon chain length of 14; (ii) *Cuphea hookeriana* FatB2 (GenBank Accension No. AAC49269, amino acid sequence is in SEQ ID NO: 61, amino acid sequence without PTS is in SEQ ID NO: 138, and codon optimized cDNA sequence based on Table 1 is in SEQ ID NO: 62), which has a preference for a fatty acyl-ACP substrate with a carbon chain length of 8-10; and (iii) *Umbellularia* Fat B1 (GenBank Accession No. Q41635, amino acid sequence is included in SEQ ID NO: 63, amino

acid sequence without PTS is in SEQ ID NO: 139, and codon optimized cDNA sequence based on Table 1 is included in SEQ ID NO: 64), which has a preference for a fatty acyl-ACP substrate with a carbon chain length of 12.

[0187] Other fatty acyl-ACP thioesterases suitable for use with the microbes and methods of the invention include, without limitation, those listed in Table 3.

[0188] Table 3. Fatty acyl-ACP thioesterases and GenBank accession numbers.

<i>Umbellularia californica</i> fatty acyl-ACP thioesterase (GenBank #AAC49001)
<i>Cinnamomum camphora</i> fatty acyl-ACP thioesterase (GenBank #Q39473)
<i>Umbellularia californica</i> fatty acyl-ACP thioesterase (GenBank #Q41635)
<i>Myristica fragrans</i> fatty acyl-ACP thioesterase (GenBank #AAB71729)
<i>Myristica fragrans</i> fatty acyl-ACP thioesterase (GenBank #AAB71730)
<i>Elaeis guineensis</i> fatty acyl-ACP thioesterase (GenBank #ABD83939)
<i>Elaeis guineensis</i> fatty acyl-ACP thioesterase (GenBank #AAD42220)
<i>Populus tomentosa</i> fatty acyl-ACP thioesterase (GenBank #ABC47311)
<i>Arabidopsis thaliana</i> fatty acyl-ACP thioesterase (GenBank #NP_172327)
<i>Arabidopsis thaliana</i> fatty acyl-ACP thioesterase (GenBank #CAA85387)
<i>Arabidopsis thaliana</i> fatty acyl-ACP thioesterase (GenBank #CAA85388)
<i>Gossypium hirsutum</i> fatty acyl-ACP thioesterase (GenBank #Q9SQI3)
<i>Cuphea lanceolata</i> fatty acyl-ACP thioesterase (GenBank #CAA54060)
<i>Cuphea hookeriana</i> fatty acyl-ACP thioesterase (GenBank #AAC72882)
<i>Cuphea calophylla</i> subsp. <i>mesostemon</i> fatty acyl-ACP thioesterase (GenBank #ABB71581)
<i>Cuphea lanceolata</i> fatty acyl-ACP thioesterase (GenBank #CAC19933)
<i>Elaeis guineensis</i> fatty acyl-ACP thioesterase (GenBank #AAL15645)
<i>Cuphea hookeriana</i> fatty acyl-ACP thioesterase (GenBank #Q39513)
<i>Gossypium hirsutum</i> fatty acyl-ACP thioesterase (GenBank #AAD01982)
<i>Vitis vinifera</i> fatty acyl-ACP thioesterase (GenBank #CAN81819)
<i>Garcinia mangostana</i> fatty acyl-ACP thioesterase (GenBank #AAB51525)
<i>Brassica juncea</i> fatty acyl-ACP thioesterase (GenBank #ABI18986)
<i>Madhuca longifolia</i> fatty acyl-ACP thioesterase (GenBank #AAX51637)
<i>Brassica napus</i> fatty acyl-ACP thioesterase (GenBank #ABH11710)
<i>Oryza sativa</i> (indica cultivar-group) fatty acyl-ACP thioesterase (GenBank #EAY86877)
<i>Oryza sativa</i> (japonica cultivar-group) fatty acyl-ACP thioesterase (GenBank #NP_001068400)
<i>Oryza sativa</i> (indica cultivar-group) fatty acyl-ACP thioesterase (GenBank #EAY99617)
<i>Cuphea hookeriana</i> fatty acyl-ACP thioesterase (GenBank #AAC49269)
<i>Ulmus Americana</i> fatty acyl-ACP thioesterase (GenBank #AAB71731)
<i>Cuphea lanceolata</i> fatty acyl-ACP thioesterase (GenBank #CAB60830)
<i>Cuphea palustris</i> fatty acyl-ACP thioesterase (GenBank #AAC49180)
<i>Iris germanica</i> fatty acyl-ACP thioesterase (GenBank #AAG43858)
<i>Cuphea palustris</i> fatty acyl-ACP thioesterase (GenBank #AAC49179)
<i>Myristica fragrans</i> fatty acyl-ACP thioesterase (GenBank# AAB71729)
<i>Cuphea hookeriana</i> fatty acyl-ACP thioesterase (GenBank #U39834)
<i>Umbellularia californica</i> fatty acyl-ACP thioesterase (GenBank # M94159)
<i>Cinnamomum camphora</i> fatty acyl-ACP thioesterase (GenBank #U31813)

[0189] The Examples below describe the successful targeting and expression of heterologous fatty acyl-ACP thioesterases from *Cuphea hookeriana*, *Umbellularia californica*, *Cinnamomun camphora* in *Prototheca* species. Additionally, alterations in fatty acid profiles were confirmed in the host cells expression these heterologous fatty acyl-ACP thioesterases. These results were quite unexpected given the lack of sequence identity between algal and higher plant thioesterases in general, and between *Prototheca moriformis* fatty acyl-ACP thioesterase and the above listed heterologous fatty acyl-ACP thioesterases. Two *Prototheca moriformis* acyl-ACP thioesterases were isolated and sequenced. The sequences of the two cDNAs showed a high degree of identity between each other, differing in only 12 positions at the nucleotide level and five positions at the amino acid level, four of these in the plastid transit peptide. Further analysis of genomic sequence from *Prototheca moriformis* confirmed that these two cDNAs were indeed encoded on separate contigs, and although highly homologous, are encoded by two distinct genes. The cDNA and amino acid sequence of the two *Prototheca moriformis* fatty acyl-ACP thioesterase, *P.moriformis* fatty acyl-ACP thioesterase-1 and *P.moriformis* fatty acyl-ACP thioesterase-2, are listed as SEQ ID NOS: 134-137.

[0190] When the amino acid sequences of these two cDNAs were BLASTed against the NCBI database, the two most homologous sequences were fatty acyl-ACP thioesterases from *Chlamydomonas reinhardtii* and *Arabidopsis thaliana*. Surprisingly, the level of amino acid identity between the *Prototheca moriformis* fatty acyl-ACP thioesterases and higher plant thioesterases was fairly low, at only 49 and 37% identity. In addition, there also is a subtle difference in the sequences surrounding the amino terminal portion of the catalytic triad (NXHX₃₆C) among these fatty acyl-ACP thioesterases. Thirty nine of forty higher plant fatty acyl-ACP thioesterases surveyed showed the sequence LDMNQH surrounding the N and H residues at the amino terminus of the triad, while all of the algal sequences identified had the sequence MDMNGH. Given the low amino acid sequence identity and the differences surrounding the catalytic triad of the thioesterases, the successful results of expression of exogenous fatty acyl-ACP thioesterases obtained and described in the Examples were unexpected, particularly given the fact that activity of the exogenous fatty acyl-ACP thioesterases was dependent on a functional protein-protein interaction with the endogenous *Prototheca* acyl carrier protein.

[0191] Fatty acyl-CoA/aldehyde reductases suitable for use with the microbes and methods of the invention include, without limitation, those listed in Table 4.

[0192] Table4. Fatty acyl-CoA/aldehyde reductases listed by GenBank accession numbers.

AAC45217, YP_047869, BAB85476, YP_001086217, YP_580344, YP_001280274, YP_264583, YP_436109, YP_959769, ZP_01736962, ZP_01900335, ZP_01892096, ZP_01103974, ZP_01915077, YP_924106, YP_130411, ZP_01222731, YP_550815, YP_983712, YP_001019688, YP_524762, YP_856798, ZP_01115500, YP_001141848, NP_336047, NP_216059, YP_882409, YP_706156, YP_001136150, YP_952365, ZP_01221833, YP_130076, NP_567936, AAR88762, ABK28586, NP_197634, CAD30694, NP_001063962, BAD46254, NP_001030809, EAZ10132, EAZ43639, EAZ07989, NP_001062488, CAB88537, NP_001052541, CAH66597, CAE02214, CAH66590, CAB88538, EAZ39844, AAZ06658, CAA68190, CAA52019, and BAC84377

[0193] Fatty acyl-CoA reductases suitable for use with the microbes and methods of the invention include, without limitation, those listed in Table 5.

[0194] Table 5. Fatty acyl-CoA reductases listed by GenBank accession numbers.

NP_187805, ABO14927, NP_001049083, CAN83375, NP_191229, EAZ42242, EAZ06453, CAD30696, BAD31814, NP_190040, AAD38039, CAD30692, CAN81280, NP_197642, NP_190041, AAL15288, and NP_190042

[0195] Fatty aldehyde decarboxylases suitable for use with the microbes and methods of the invention include, without limitation, those listed in Table 6.

[0196] Table 6. Fatty aldehyde decarboxylases listed by GenBank accession numbers.

NP_850932, ABN07985, CAN60676, AAC23640, CAA65199, AAC24373, CAE03390, ABD28319, NP_181306, EAZ31322, CAN63491, EAY94825, EAY86731, CAL55686, XP_001420263, EAZ23849, NP_200588, NP_001063227, CAN83072, AAR90847, and AAR97643

[0197] Combinations of naturally co-expressed fatty acyl-ACP thioesterases and acyl carrier proteins are suitable for use with the microbes and methods of the invention.

[0198] Additional examples of hydrocarbon or lipid modification enzymes include amino acid sequences contained in, referenced in, or encoded by nucleic acid sequences contained or referenced in, any of the following US patents: 6,610,527; 6,451,576; 6,429,014; 6,342,380; 6,265,639; 6,194,185; 6,114,160; 6,083,731; 6,043,072 ; 5,994,114; 5,891,697; 5,871,988; 6,265,639, and further described in GenBank Accession numbers: AAO18435;

ZP_00513891; Q38710; AAK60613; AAK60610; AAK60611; NP_113747; CAB75874; AAK60612; AAF20201; BAA11024; AF205791; and CAA03710.

[0199] Other suitable enzymes for use with the microbes and the methods of the invention include those that have at least 70% amino acid identity with one of the proteins listed in Tables 3-6, and that exhibit the corresponding desired enzymatic activity (e.g., cleavage of a fatty acid from an acyl carrier protein, reduction of an acyl-CoA to an aldehyde or an alcohol, or conversion of an aldehyde to an alkane). 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 sequences, all of which are hereby incorporated by reference as if fully set forth.

[0200] By selecting the desired combination of exogenous genes to be expressed, one can tailor the product generated by the microbe, which may then be extracted from the aqueous biomass. For example, the microbe can contain: (i) an exogenous gene encoding a fatty acyl-ACP thioesterase; and, optionally, (ii) a naturally co-expressed acyl carrier protein or an acyl carrier protein otherwise having affinity for the fatty acyl-ACP thioesterase (or conversely); and, optionally, (iii) an exogenous gene encoding a fatty acyl-CoA/aldehyde reductase or a fatty acyl-CoA reductase; and, optionally, (iv) an exogenous gene encoding a fatty aldehyde reductase or a fatty aldehyde decarbonylase. The microbe, under culture conditions described herein, synthesizes a fatty acid linked to an ACP and the fatty acyl-ACP thioesterase catalyzes the cleavage of the fatty acid from the ACP to yield, through further enzymatic processing, a fatty acyl-CoA molecule. When present, the fatty acyl-CoA/aldehyde reductase catalyzes the reduction of the acyl-CoA to an alcohol. Similarly, the fatty acyl-CoA reductase, when present, catalyzes the reduction of the acyl-CoA to an aldehyde. In those embodiments in which an exogenous gene encoding a fatty acyl-CoA reductase is present and expressed to yield an aldehyde product, a fatty aldehyde reductase, encoded by the third exogenous gene, catalyzes the reduction of the aldehyde to an alcohol. Similarly, a fatty aldehyde decarbonylase catalyzes the conversion of the aldehyde to an alkane or an alkene, when present.

[0201] Genes encoding such enzymes can be obtained from cells already known to exhibit significant lipid production such as *Chlorella protothecoides*. Genes already known to have a role in lipid production, e.g., a gene encoding an enzyme that saturates double bonds, can be transformed individually into recipient cells. However, to practice the invention it is not necessary to make *a priori* assumptions as to which genes are required. Methods for

identifying genes that can alter (improve) lipid production in microalgae are described in PCT Pub. No. 2008/151149.

[0202] Thus, the present invention provides a *Prototheca* cell that has been genetically engineered to express a lipid pathway enzyme at an altered level compared to a wild-type cell of the same species. In some cases, the cell produces more lipid compared to the wild-type cell when both cells are grown under the same conditions. In some cases, the cell has been genetically engineered and/or selected to express a lipid pathway enzyme at a higher level than the wild-type cell. In some cases, the lipid pathway enzyme is selected from the group consisting of pyruvate dehydrogenase, acetyl-CoA carboxylase, acyl carrier protein, and glycerol-3 phosphate acyltransferase. In some cases, the cell has been genetically engineered and/or selected to express a lipid pathway enzyme at a lower level than the wild-type cell. In at least one embodiment in which the cell expresses the lipid pathway enzyme at a lower level, the lipid pathway enzyme comprises citrate synthase.

[0203] In some embodiments, the cell has been genetically engineered and/or selected to express a global regulator of fatty acid synthesis at an altered level compared to the wild-type cell, whereby the expression levels of a plurality of fatty acid synthetic genes are altered compared to the wild-type cell. In some cases, the lipid pathway enzyme comprises an enzyme that modifies a fatty acid. In some cases, the lipid pathway enzyme is selected from a stearoyl-ACP desaturase and a glycerolipid desaturase.

[0204] In other embodiments, the present invention is directed to an oil-producing microbe containing one or more exogenous genes, wherein the exogenous genes encode protein(s) selected from the group consisting of a fatty acyl-ACP thioesterase, a fatty acyl-CoA reductase, a fatty aldehyde reductase, a fatty acyl-CoA/aldehyde reductase, a fatty aldehyde decarbonylase, and an acyl carrier protein. In one embodiment, the exogenous gene is in operable linkage with a promoter, which is inducible or repressible in response to a stimulus. In some cases, the stimulus is selected from the group consisting of an exogenously provided small molecule, heat, cold, and limited or no nitrogen in the culture media. In some cases, the exogenous gene is expressed in a cellular compartment. In some embodiments, the cellular compartment is selected from the group consisting of a chloroplast, a plastid and a mitochondrion. In some embodiments the microbe is *Prototheca moriformis*, *Prototheca krugani*, *Prototheca stagnora* or *Prototheca zopfii*.

[0205] In one embodiment, the exogenous gene encodes a fatty acid acyl-ACP thioesterase. In some cases, the thioesterase encoded by the exogenous gene catalyzes the cleavage of an 8 to 18-carbon fatty acid from an acyl carrier protein (ACP). In some cases, the thioesterase

encoded by the exogenous gene catalyzes the cleavage of a 10 to 14-carbon fatty acid from an ACP. In one embodiment, the thioesterase encoded by the exogenous gene catalyzes the cleavage of a 12-carbon fatty acid from an ACP.

[0206] In one embodiment, the exogenous gene encodes a fatty acyl-CoA/aldehyde reductase. In some cases, the reductase encoded by the exogenous gene catalyzes the reduction of an 8 to 18-carbon fatty acyl-CoA to a corresponding primary alcohol. In some cases, the reductase encoded by the exogenous gene catalyzes the reduction of a 10 to 14-carbon fatty acyl-CoA to a corresponding primary alcohol. In one embodiment, the reductase encoded by the exogenous gene catalyzes the reduction of a 12-carbon fatty acyl-CoA to dodecanol.

[0207] The present invention also provides a recombinant *Prototheca* cell containing two exogenous genes, wherein a first exogenous gene encodes a fatty acyl-ACP thioesterase and a second exogenous gene encodes a protein selected from the group consisting of a fatty acyl-CoA reductase, a fatty acyl-CoA/aldehyde reductase, and an acyl carrier protein. In some cases, the two exogenous genes are each in operable linkage with a promoter, which is inducible in response to a stimulus. In some cases, each promoter is inducible in response to an identical stimulus, such as limited or no nitrogen in the culture media. Limitation or complete lack of nitrogen in the culture media stimulates oil production in some microorganisms such as *Prototheca* species, and can be used as a trigger to induce oil production to high levels. When used in combination with the genetic engineering methods disclosed herein, the lipid as a percentage of dry cell weight can be pushed to high levels such as at least 30%, at least 40%, at least 50%, at least 60%, at least 70% and at least 75%; methods disclosed herein provide for cells with these levels of lipid, wherein the lipid is at least 4% C8-C14, at least 0.3% C8, at least 2% C10, at least 2% C12, and at least 2% C14. In some embodiments the cells are over 25% lipid by dry cell weight and contain lipid that is at least 10% C8-C14, at least 20% C8-C14, at least 30% C8-C14, 10-30% C8-C14 and 20-30% C8-C14.

[0208] The novel oils disclosed herein are distinct from other naturally occurring oils that are high in mic-chain fatty acids, such as palm oil, palm kernel oil, and coconut oil. For example, levels of contaminants such as carotenoids are far higher in palm oil and palm kernel oil than in the oils of the invention. Palm and palm kernel oils in particular contain alpha and beta carotenes and lycopene in much higher amounts than is in the oils of the invention. In addition, over 20 different carotenoids are found in palm and palm kernel oil, whereas the Examples demonstrate that the oils of the invention contain very few carotenoids

species and very low levels. In addition, the levels of vitamin E compounds such as tocotrienols are far higher in palm, palm kernel, and coconut oil than in the oils of the invention.

[0209] In one embodiment, the thioesterase encoded by the first exogenous gene catalyzes the cleavage of an 8 to 18-carbon fatty acid from an ACP. In some embodiments, the second exogenous gene encodes a fatty acyl-CoA/aldehyde reductase which catalyzes the reduction of an 8 to 18-carbon fatty acyl-CoA to a corresponding primary alcohol. In some cases, the thioesterase encoded by the first exogenous gene catalyzes the cleavage of a 10 to 14-carbon fatty acid from an ACP, and the reductase encoded by the second exogenous gene catalyzes the reduction of a 10 to 14-carbon fatty acyl-CoA to the corresponding primary alcohol, wherein the thioesterase and the reductase act on the same carbon chain length. In one embodiment, the thioesterase encoded by the first exogenous gene catalyzes the cleavage of a 12-carbon fatty acid from an ACP, and the reductase encoded by the second exogenous gene catalyzes the reduction of a 12-carbon fatty acyl-CoA to dodecanol. In some embodiments, the second exogenous gene encodes a fatty acyl-CoA reductase which catalyzes the reduction of an 8 to 18-carbon fatty acyl-CoA to a corresponding aldehyde. In some embodiments, the second exogenous gene encodes an acyl carrier protein that is naturally co-expressed with the fatty acyl-ACP thioesterase.

[0210] In some embodiments, the second exogenous gene encodes a fatty acyl-CoA reductase, and the microbe further contains a third exogenous gene encoding a fatty aldehyde decarbonylase. In some cases, the thioesterase encoded by the first exogenous gene catalyzes the cleavage of an 8 to 18-carbon fatty acid from an ACP, the reductase encoded by the second exogenous gene catalyzes the reduction of an 8 to 18-carbon fatty acyl-CoA to a corresponding fatty aldehyde, and the decarbonylase encoded by the third exogenous gene catalyzes the conversion of an 8 to 18-carbon fatty aldehyde to a corresponding alkane, wherein the thioesterase, the reductase, and the decarbonylase act on the same carbon chain length.

[0211] In some embodiments, the second exogenous gene encodes an acyl carrier protein, and the microbe further contains a third exogenous gene encoding a protein selected from the group consisting of a fatty acyl-CoA reductase and a fatty acyl-CoA/aldehyde reductase. In some cases, the third exogenous gene encodes a fatty acyl-CoA reductase, and the microbe further contains a fourth exogenous gene encoding a fatty aldehyde decarbonylase.

[0212] The present invention also provides methods for producing an alcohol comprising culturing a population of recombinant *Prototheca* cells in a culture medium, wherein the cells

contain (i) a first exogenous gene encoding a fatty acyl-ACP thioesterase, and (ii) a second exogenous gene encoding a fatty acyl-CoA/aldehyde reductase, and the cells synthesize a fatty acid linked to an acyl carrier protein (ACP), the fatty acyl-ACP thioesterase catalyzes the cleavage of the fatty acid from the ACP to yield, through further processing, a fatty acyl-CoA, and the fatty acyl-CoA/aldehyde reductase catalyzes the reduction of the acyl-CoA to an alcohol.

[0213] The present invention also provides methods of producing a lipid molecule in a *Prototheca* cell. In one embodiment, the method comprises culturing a population of *Prototheca* cells in a culture medium, wherein the cells contain (i) a first exogenous gene encoding a fatty acyl-ACP thioesterase, and (ii) a second exogenous gene encoding a fatty acyl-CoA reductase, and wherein the microbes synthesize a fatty acid linked to an acyl carrier protein (ACP), the fatty acyl-ACP thioesterase catalyzes the cleavage of the fatty acid from the ACP to yield, through further processing, a fatty acyl-CoA, and the fatty acyl-CoA reductase catalyzes the reduction of the acyl-CoA to an aldehyde.

[0214] The present invention also provides methods of producing a fatty acid molecule having a specified carbon chain length in a *Prototheca* cell. In one embodiment, the method comprises culturing a population of lipid-producing *Prototheca* cells in a culture medium, wherein the microbes contain an exogenous gene encoding a fatty acyl-ACP thioesterase having an activity specific or preferential to a certain carbon chain length, such as 8, 10, 12 or 14 carbon atoms, and wherein the microbes synthesize a fatty acid linked to an acyl carrier protein (ACP) and the thioesterase catalyzes the cleavage of the fatty acid from the ACP when the fatty acid has been synthesized to the specific carbon chain length.

[0215] In the various embodiments described above, the *Prototheca* cell can contain at least one exogenous gene encoding a lipid pathway enzyme. In some cases, the lipid pathway enzyme is selected from the group consisting of a stearyl-ACP desaturase, a glycerolipid desaturase, a pyruvate dehydrogenase, an acetyl-CoA carboxylase, an acyl carrier protein, and a glycerol-3 phosphate acyltransferase. In other cases, the *Prototheca* cell contains a lipid modification enzyme selected from the group consisting of a fatty acyl-ACP thioesterase, a fatty acyl-CoA/aldehyde reductase, a fatty acyl-CoA reductase, a fatty aldehyde reductase, a fatty aldehyde decarbonylase, and/or an acyl carrier protein.

VI. FUELS AND CHEMICALS PRODUCTION

[0216] 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 as described above. Intracellular lipids produced in microorganisms are, in some embodiments, extracted after lysing the cells of the microorganism. Once extracted, the lipids are further refined to produce oils, fuels, or oleochemicals.

[0217] After completion of culturing, the microorganisms can be separated from the fermentation broth. Optionally, the separation is effected by centrifugation to generate a concentrated paste. Centrifugation does not remove significant amounts of intracellular water from the microorganisms and is not a drying step. The biomass can then optionally be washed with a washing solution (e.g., DI water) to get rid of the fermentation broth and debris. Optionally, the washed microbial biomass may also be dried (oven dried, lyophilized, etc.) prior to cell disruption. Alternatively, cells can be lysed without separation from some or all of the fermentation broth when the fermentation is complete. For example, the cells can be at a ratio of less than 1:1 v:v cells to extracellular liquid when the cells are lysed.

[0218] Microorganisms containing a lipid can be lysed to produce a lysate. As detailed herein, the step of lysing a microorganism (also referred to as cell lysis) can be achieved by any convenient means, including heat-induced lysis, adding a base, adding an acid, using enzymes such as proteases and polysaccharide degradation enzymes such as amylases, using ultrasound, mechanical lysis, using osmotic shock, infection with a lytic virus, and/or expression of one or more lytic genes. Lysis is performed to release intracellular molecules which have been produced by the microorganism. Each of these methods for lysing a microorganism can be used as a single method or in combination simultaneously or sequentially. The extent of cell disruption can be observed by microscopic analysis. Using one or more of the methods described herein, typically more than 70% cell breakage is observed. Preferably, cell breakage is more than 80%, more preferably more than 90% and most preferred about 100%.

[0219] In particular embodiments, the microorganism is lysed after growth, for example to increase the exposure of cellular lipid and/or hydrocarbon for extraction or further processing. The timing of lipase expression (e.g., via an inducible promoter) or cell lysis can be adjusted to optimize the yield of lipids and/or hydrocarbons. Below are described a number of lysis techniques. These techniques can be used individually or in combination.

[0220] In one embodiment of the present invention, the step of lysing a microorganism comprises heating of a cellular suspension containing the microorganism. In this embodiment, the fermentation broth containing the microorganisms (or a suspension of

microorganisms isolated from the fermentation broth) is heated until the microorganisms, i.e., the cell walls and membranes of microorganisms degrade or breakdown. Typically, temperatures applied are at least 50°C. Higher temperatures, such as, at least 30°C at least 60°C, at least 70°C, at least 80°C, at least 90°C, at least 100°C, at least 110°C, at least 120°C, at least 130°C or higher are used for more efficient cell lysis. Lysing cells by heat treatment can be performed by boiling the microorganism. Alternatively, heat treatment (without boiling) can be performed in an autoclave. The heat treated lysate may be cooled for further treatment. Cell disruption can also be performed by steam treatment, i.e., through addition of pressurized steam. Steam treatment of microalgae for cell disruption is described, for example, in U.S. Patent No. 6,750,048. In some embodiments, steam treatment may be achieved by sparging steam into the fermentor and maintaining the broth at a desired temperature for less than about 90 minutes, preferably less than about 60 minutes, and more preferably less than about 30 minutes.

[0221] In another embodiment of the present invention, the step of lysing a microorganism comprises adding a base to a cellular suspension containing the microorganism. The base should be strong enough to hydrolyze at least a portion of the proteinaceous compounds of the microorganisms used. Bases which are useful for solubilizing proteins are known in the art of chemistry. Exemplary bases which are useful in the methods of the present invention include, but are not limited to, hydroxides, carbonates and bicarbonates of lithium, sodium, potassium, calcium, and mixtures thereof. A preferred base is KOH. Base treatment of microalgae for cell disruption is described, for example, in U.S. Patent No. 6,750,048.

[0222] In another embodiment of the present invention, the step of lysing a microorganism comprises adding an acid to a cellular suspension containing the microorganism. Acid lysis can be effected using an acid at a concentration of 10-500 mM or preferably 40-160 mM. Acid lysis is preferably performed at above room temperature (e.g., at 40-160°, and preferably a temperature of 50-130°. For moderate temperatures (e.g., room temperature to 100°C and particularly room temperature to 65°, acid treatment can usefully be combined with sonication or other cell disruption methods.

[0223] In another embodiment of the present invention, the step of lysing a microorganism comprises lysing the microorganism by using an enzyme. Preferred enzymes for lysing a microorganism are proteases and polysaccharide-degrading enzymes such as hemicellulase (e.g., hemicellulase from *Aspergillus niger*; Sigma Aldrich, St. Louis, MO; #H2125), pectinase (e.g., pectinase from *Rhizopus sp.*; Sigma Aldrich, St. Louis, MO; #P2401), Mannaway 4.0 L (Novozymes), cellulase (e.g., cellulase from *Trichoderma viride*; Sigma

Aldrich, St. Louis, MO; #C9422), and driselase (e.g., driselase from *Basidiomycetes sp.*; Sigma Aldrich, St. Louis, MO; #D9515).

[0224] In other embodiments of the present invention, lysis is accomplished using an enzyme such as, for example, a cellulase such as a polysaccharide-degrading enzyme, optionally from *Chlorella* or a *Chlorella* virus, or a protease, such as *Streptomyces griseus* protease, chymotrypsin, proteinase K, proteases listed in Degradation of Polylactide by Commercial Proteases, Oda Yet al., Journal of Polymers and the Environment, Volume 8, Number 1, January 2000 , pp. 29-32(4), Alcalase 2.4 FG (Novozymes), and Flavourzyme 100 L (Novozymes). Any combination of a protease and a polysaccharide-degrading enzyme can also be used, including any combination of the preceding proteases and polysaccharide-degrading enzymes.

[0225] In another embodiment, lysis can be performed using an expeller press. In this process, biomass is forced through a screw-type device at high pressure, lysing the cells and causing the intracellular lipid to be released and separated from the protein and fiber (and other components) in the cell.

[0226] In another embodiment of the present invention, the step of lysing a microorganism is performed by using ultrasound, i.e., sonication. Thus, cells can also be lysed with high frequency sound. The sound can be produced electronically and transported through a metallic tip to an appropriately concentrated cellular suspension. This sonication (or ultrasonication) disrupts cellular integrity based on the creation of cavities in cell suspension.

[0227] In another embodiment of the present invention, the step of lysing a microorganism is performed by mechanical lysis. Cells can be lysed mechanically and optionally homogenized to facilitate hydrocarbon (e.g., lipid) collection. For example, a pressure disrupter can be used to pump a cell containing slurry through a restricted orifice valve. High pressure (up to 1500 bar) is applied, followed by an instant expansion through an exiting nozzle. Cell disruption is accomplished by three different mechanisms: impingement on the valve, high liquid shear in the orifice, and sudden pressure drop upon discharge, causing an explosion of the cell. The method releases intracellular molecules. Alternatively, a ball mill can be used. In a ball mill, cells are agitated in suspension with small abrasive particles, such as beads. Cells break because of shear forces, grinding between beads, and collisions with beads. The beads disrupt the cells to release cellular contents. Cells can also be disrupted by shear forces, such as with the use of blending (such as with a high speed or Waring blender as examples), the french press, or even centrifugation in case of weak cell walls, to disrupt cells.

[0228] In another embodiment of the present invention, the step of lysing a microorganism is performed by applying an osmotic shock.

[0229] In another embodiment of the present invention, the step of lysing a microorganism comprises infection of the microorganism with a lytic virus. A wide variety of viruses are known to lyse microorganisms suitable for use in the present invention, and the selection and use of a particular lytic virus for a particular microorganism is within the level of skill in the art. For example, *paramecium bursaria chlorella* virus (PBCV-1) is the prototype of a group (family Phycodnaviridae, genus Chlorovirus) of large, icosahedral, plaque-forming, double-stranded DNA viruses that replicate in, and lyse, certain unicellular, eukaryotic chlorella-like green algae. Accordingly, any susceptible microalgae can be lysed by infecting the culture with a suitable chlorella virus. Methods of infecting species of *Chlorella* with a chlorella virus are known. See for example *Adv. Virus Res.* 2006;66:293-336; *Virology*, 1999 Apr 25;257(1):15-23; *Virology*, 2004 Jan 5;318(1):214-23; *Nucleic Acids Symp. Ser.* 2000;(44):161-2; *J. Virol.* 2006 Mar;80(5):2437-44; and *Annu. Rev. Microbiol.* 1999;53:447-94.

[0230] In another embodiment of the present invention, the step of lysing a microorganism comprises autolysis. In this embodiment, a microorganism according to the invention is genetically engineered to produce a lytic protein that will lyse the microorganism. This lytic gene can be expressed using an inducible promoter so that the cells can first be grown to a desirable density in a fermentor, followed by induction of the promoter to express the lytic gene to lyse the cells. In one embodiment, the lytic gene encodes a polysaccharide-degrading enzyme. In certain other embodiments, the lytic gene is a gene from a lytic virus. Thus, for example, a lytic gene from a Chlorella virus can be expressed in an algal cell; see *Virology* 260, 308-315 (1999); *FEMS Microbiology Letters* 180 (1999) 45-53; *Virology* 263, 376-387 (1999); and *Virology* 230, 361-368 (1997). Expression of lytic genes is preferably done using an inducible promoter, such as a promoter active in microalgae that is induced by a stimulus such as the presence of a small molecule, light, heat, and other stimuli.

[0231] Various methods are available for separating lipids from cellular lysates produced by the above methods. 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.

[0232] Thus, lipids, lipid derivatives and hydrocarbons generated by the microorganisms of the present invention 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.

[0233] Lipids and lipid derivatives such as fatty aldehydes, fatty alcohols, and hydrocarbons such as alkanes produced by cells as described herein can be modified by the use of one or more enzymes, including a lipase, as described above. When the hydrocarbons are in the extracellular environment of the cells, the one or more enzymes can be added to that environment under conditions in which the enzyme modifies the hydrocarbon or completes its synthesis from a hydrocarbon precursor. Alternatively, the hydrocarbons can be partially, or completely, isolated from the cellular material before addition of one or more catalysts such as enzymes. Such catalysts are exogenously added, and their activity occurs outside the cell or *in vitro*.

[0234] Thus, lipids and hydrocarbons produced by cells *in vivo*, or enzymatically modified *in vitro*, as described herein can be optionally further processed by conventional means. The processing can include “cracking” to reduce the size, and thus increase the hydrogen:carbon ratio, of hydrocarbon molecules. Catalytic and thermal cracking methods are routinely used in hydrocarbon and triglyceride oil processing. Catalytic methods involve the use of a catalyst, such as a solid acid catalyst. The catalyst can be silica-alumina or a zeolite, which result in the heterolytic, or asymmetric, breakage of a carbon-carbon bond to result in a carbocation and a hydride anion. These reactive intermediates then undergo either rearrangement or hydride transfer with another hydrocarbon. The reactions can thus regenerate the intermediates to result in a self-propagating chain mechanism. Hydrocarbons

can also be processed to reduce, optionally to zero, the number of carbon-carbon double, or triple, bonds therein. Hydrocarbons can also be processed to remove or eliminate a ring or cyclic structure therein. Hydrocarbons can also be processed to increase the hydrogen:carbon ratio. This can include the addition of hydrogen ("hydrogenation") and/or the "cracking" of hydrocarbons into smaller hydrocarbons.

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

[0236] Catalytic and thermal methods are standard in plants for hydrocarbon processing and oil refining. Thus hydrocarbons produced by cells as described herein can be collected and processed or refined via conventional means. See Hillen et al. (Biotechnology and Bioengineering, Vol. XXIV:193-205 (1982)) for a report on hydrocracking of microalgae-produced hydrocarbons. In alternative embodiments, the fraction is treated with another catalyst, such as an organic compound, heat, and/or an inorganic compound. For processing of lipids into biodiesel, a transesterification process is used as described in Section IV herein.

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

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

lipids generated by the methods described herein as a biological material to produce biodiesel, renewable diesel, and jet fuel.

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

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

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

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

[0243] Biodiesel can also be used as a heating fuel in domestic and commercial boilers. Existing oil boilers may contain rubber parts and may require conversion to run on biodiesel. The conversion process is usually relatively simple, involving the exchange of rubber parts for synthetic parts due to biodiesel being a strong solvent. Due to its strong solvent power,

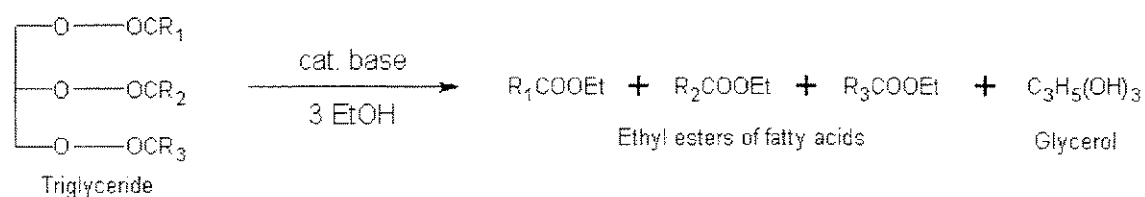
burning biodiesel will increase the efficiency of boilers. Biodiesel can be used as an additive in formulations of diesel to increase the lubricity of pure Ultra-Low Sulfur Diesel (ULSD) fuel, which is advantageous because it has virtually no sulfur content. Biodiesel is a better solvent than petrodiesel and can be used to break down deposits of residues in the fuel lines of vehicles that have previously been run on petrodiesel.

[0244] 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.

[0245] The lipid profile of the biodiesel is usually highly similar to the lipid profile of the feedstock oil. Other oils provided by the methods and compositions of the invention can be subjected to transesterification to yield biodiesel with lipid profiles including (a) at least 4% C8-C14; (b) at least 0.3% C8; (c) at least 2% C10; (d) at least 2% C12; and (3) at least 30% C8-C14.

[0246] 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.

[0247] Animal and plant oils are typically made of triglycerides which are esters of free fatty acids with the trihydric alcohol, glycerol. In transesterification, the glycerol in a triacylglyceride (TAG) is replaced with a short-chain alcohol such as methanol or ethanol. A typical reaction scheme is as follows:



In this reaction, the alcohol is deprotonated with a base to make it a stronger nucleophile. Commonly, ethanol or methanol is used in vast excess (up to 50-fold). Normally, this reaction will proceed either exceedingly slowly or not at all. Heat, as well as an acid or base can be used to help the reaction proceed more quickly. The acid or base are not consumed by the transesterification reaction, thus they are not reactants but catalysts. Almost all biodiesel has been produced using the base-catalyzed technique as it requires only low temperatures and pressures and produces over 98% conversion yield (provided the starting oil is low in moisture and free fatty acids).

[0248] 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 7. 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.

[0249] Table 7. Lipases suitable for use in transesterification.

Aspergillus niger lipase ABG73614, *Candida antarctica* lipase B (Novozym-435) CAA83122, *Candida cylindracea* lipase AAR24090, *Candida lipolytica* lipase (Lipase L; Amano Pharmaceutical Co., Ltd.), *Candida rugosa* lipase (e.g., Lipase-OF; Meito Sangyo Co., Ltd.), *Mucor miehei* lipase (Lipozyme IM 20), *Pseudomonas fluorescens* lipase AAA25882, *Rhizopus japonicus* lipase (Lilipase A-10FG) Q7M4U7_1, *Rhizomucor miehei* lipase B34959, *Rhizopus oryzae* lipase (Lipase F) AAF32408, *Serratia marcescens* lipase (SM Enzyme) ABI13521, *Thermomyces lanuginosa* lipase CAB58509, Lipase P (Nagase ChemteX Corporation), and Lipase QLM (Meito Sangyo Co., Ltd., Nagoya, Japan)

[0250] One challenge to using a lipase for the production of fatty acid esters suitable for biodiesel is that the price of lipase is much higher than the price of sodium hydroxide (NaOH) used by the strong base process. This challenge has been addressed by using an immobilized lipase, which can be recycled. However, the activity of the immobilized lipase must be maintained after being recycled for a minimum number of cycles to allow a lipase-

based process to compete with the strong base process in terms of the production cost. Immobilized lipases are subject to poisoning by the lower alcohols typically used in transesterification. U.S. Patent No. 6,398,707 (issued June 4, 2002 to Wu et al.) describes methods for enhancing the activity of immobilized lipases and regenerating immobilized lipases having reduced activity. Some suitable methods include immersing an immobilized lipase in an alcohol having a carbon atom number not less than 3 for a period of time, preferably from 0.5-48 hours, and more preferably from 0.5-1.5 hours. Some suitable methods also include washing a deactivated immobilized lipase with an alcohol having a carbon atom number not less than 3 and then immersing the deactivated immobilized lipase in a vegetable oil for 0.5-48 hours.

[0251] In particular embodiments, a recombinant lipase is expressed in the same microorganisms that produce the lipid on which the lipase acts. Suitable recombinant lipases include those listed above in Table 7 and/or having GenBank Accession numbers listed above in Table 7, or a polypeptide that has at least 70% amino acid identity with one of the lipases listed above in Table 7 and that exhibits lipase 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 sequences, all of which are hereby incorporated by reference as if fully set forth. DNA encoding the lipase and selectable marker is preferably codon-optimized cDNA. Methods of recoding genes for expression in microalgae are described in US Patent 7,135,290.

[0252] The common international standard for biodiesel is EN 14214. ASTM D6751 is the most common biodiesel standard referenced in the United States and Canada. Germany uses DIN EN 14214 and the UK requires compliance with BS EN 14214. Basic industrial tests to determine whether the products conform to these standards typically include gas chromatography, HPLC, and others. Biodiesel meeting the quality standards is very non-toxic, with a toxicity rating (LD_{50}) of greater than 50 mL/kg.

[0253] Although biodiesel that meets the ASTM standards has to be non-toxic, there can be contaminants which tend to crystallize and/or precipitate and fall out of solution as sediment. Sediment formation is particularly a problem when biodiesel is used at lower temperatures. The sediment or precipitates may cause problems such as decreasing fuel flow, clogging fuel lines, clogging filters, etc. Processes are well-known in the art that specifically deal with the removal of these contaminants and sediments in biodiesel in order to produce a higher quality product. Examples for such processes include, but are not limited to, pretreatment of the oil to

remove contaminants such as phospholipids and free fatty acids (*e.g.*, degumming, caustic refining and silica adsorbant filtration) and cold filtration. Cold filtration is a process that was developed specifically to remove any particulates and sediments that are present in the biodiesel after production. This process cools the biodiesel and filters out any sediments or precipitates that might form when the fuel is used at a lower temperature. Such a process is well known in the art and is described in US Patent Application Publication No. 2007-0175091. Suitable methods may include cooling the biodiesel to a temperature of less than about 38°C so that the impurities and contaminants precipitate out as particulates in the biodiesel liquid. Diatomaceous earth or other filtering material may then added to the cooled biodiesel to form a slurry, which may then filtered through a pressure leaf or other type of filter to remove the particulates. The filtered biodiesel may then be run through a polish filter to remove any remaining sediments and diatomaceous earth, so as to produce the final biodiesel product.

[0254] Example 14 described the production of biodiesel using triglyceride oil from *Prototheca moriformis*. The Cold Soak Filterability by the ASTM D6751 A1 method of the biodiesel produced in Example 14 was 120 seconds for a volume of 300ml. This test involves filtration of 300 ml of B100, chilled to 40°F for 16 hours, allowed to warm to room temp, and filtered under vacuum using 0.7 micron glass fiber filter with stainless steel support. Oils of the invention can be transesterified to generate biodiesel with a cold soak time of less than 120 seconds, less than 100 seconds, and less than 90 seconds.

[0255] 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.

[0256] 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.

[0257] 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.

[0258] 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.

[0259] 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.

[0260] 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).

[0261] 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.

[0262] 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.

[0263] 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.

[0264] Suitable methods for isomerization includes using alkali isomerization and other oil isomerization known in the art.

[0265] 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.

[0266] 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).

[0267] The distillation properties of a diesel fuel is described in terms of T10-T90 (temperature at 10% and 90%, respectively, volume distilled). Renewable diesel was produced from *Prototheca moriformis* triglyceride oil and is described in Example 14. The T10-T90 of the material produced in Example 14 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.

[0268] The T10 of the material produced in Example 14 was 242.1°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 values, such as T10 between 180 and 295, between 190 and 270, between 210 and 250, between 225 and 245, and at least 290.

[0269] The T90 of the material produced in Example 14 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 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.

[0270] The FBP of the material produced in Example 14 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.

[0271] 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 4% C8-C14; (b) at least 0.3% C8; (c) at least 2% C10; (d) at least 2% C12; and (3) at least 30% C8-C14.

[0272] 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.

[0273] The present invention also provides methods to produce jet fuel. Jet fuel is clear to straw colored. The most common fuel is an unleaded/paraffin oil-based fuel classified as Aeroplane A-1, which is produced to an internationally standardized set of specifications. Jet fuel is a mixture of a large number of different hydrocarbons, possibly as many as a thousand or more. The range of their sizes (molecular weights or carbon numbers) is restricted by the requirements for the product, for example, freezing point or smoke point. Kerosene-type Aeroplane fuel (including Jet A and Jet A-1) has a carbon number distribution between about 8 and 16 carbon numbers. Wide-cut or naphtha-type Aeroplane fuel (including Jet B) typically has a carbon number distribution between about 5 and 15 carbons.

[0274] Both Aeroplanes (Jet A and Jet B) may contain a number of additives. Useful additives include, but are not limited to, antioxidants, antistatic agents, corrosion inhibitors,

and fuel system icing inhibitor (FSII) agents. Antioxidants prevent gumming and usually, are based on alkylated phenols, for example, AO-30, AO-31, or AO-37. Antistatic agents dissipate static electricity and prevent sparking. Stadis 450 with dinonylnaphthylsulfonic acid (DINNSA) as the active ingredient, is an example. Corrosion inhibitors, e.g., DCI-4A is used for civilian and military fuels and DCI-6A is used for military fuels. FSII agents, include, e.g., Di-EGME.

[0275] 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).

[0276] 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.

[0277] 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.

[0278] 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.

[0279] 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.

[0280] 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 an Y-type zeolite, an active alumina material, a binder material, comprising either silica or alumina and an inert filler such as kaolin.

[0281] 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.

[0282] 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.

[0283] 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.

[0284] 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.

[0285] 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.

[0286] 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.

[0287] 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.

[0288] 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.

[0289] 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.

[0290] 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.

[0291] 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.

[0292] 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.

[0293] 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.

[0294] 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.

[0295] 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.

[0296] Thus, in one 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, that 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.

[0297] 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. 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 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, and higher alkanes.

[0298] 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).

[0299] With regard to hydrolysis, in one embodiment of the invention, a triglyceride oil is optionally first hydrolyzed in a liquid medium such as water or sodium hydroxide so as to obtain glycerol and soaps. There are various suitable triglyceride hydrolysis methods, including, but not limited to, saponification, acid hydrolysis, alkaline hydrolysis, enzymatic hydrolysis (referred herein as splitting), and hydrolysis using hot-compressed water. One

skilled in the art will recognize that a triglyceride oil need not be hydrolyzed in order to produce an oleochemical; rather, the oil may be converted directly to the desired oleochemical by other known process. For example, the triglyceride oil may be directly converted to a methyl ester fatty acid through esterification.

[0300] In some embodiments, catalytic hydrolysis of the oil produced by methods disclosed herein occurs by splitting the oil into glycerol and fatty acids. As discussed above, the fatty acids may then be further processed through several other modifications to obtain derivative oleochemicals. For example, in one embodiment the fatty acids may undergo an amination reaction to produce fatty nitrogen compounds. In another embodiment, the fatty acids may undergo ozonolysis to produce mono- and dibasic-acids.

[0301] In other embodiments hydrolysis may occur via the, splitting of oils produced herein to create oleochemicals. In some preferred embodiments of the invention, a triglyceride oil may be split before other processes is performed. One skilled in the art will recognize that there are many suitable triglyceride splitting methods, including, but not limited to, enzymatic splitting and pressure splitting.

[0302] Generally, enzymatic oil splitting methods use enzymes, lipases, as biocatalysts acting on a water/oil mixture. Enzymatic splitting then splits the oil or fat, respectively, into glycerol and free fatty acids. The glycerol may then migrate into the water phase whereas the organic phase enriches with free fatty acids.

[0303] The enzymatic splitting reactions generally take place at the phase boundary between organic and aqueous phase, where the enzyme is present only at the phase boundary. Triglycerides that meet the phase boundary then contribute to or participate in the splitting reaction. As the reaction proceeds, the occupation density or concentration of fatty acids still chemically bonded as glycerides, in comparison to free fatty acids, decreases at the phase boundary so that the reaction is slowed down. In certain embodiments, enzymatic splitting may occur at room temperature. One of ordinary skill in the art would know the suitable conditions for splitting oil into the desired fatty acids.

[0304] By way of example, the reaction speed can be accelerated by increasing the interface boundary surface. Once the reaction is complete, free fatty acids are then separated from the organic phase freed from enzyme, and the residue which still contains fatty acids chemically bonded as glycerides is fed back or recycled and mixed with fresh oil or fat to be subjected to splitting. In this manner, recycled glycerides are then subjected to a further enzymatic splitting process. In some embodiments, the free fatty acids are extracted from an oil or fat partially split in such a manner. In that way, if the chemically bound fatty acids

(triglycerides) are returned or fed back into the splitting process, the enzyme consumption can be drastically reduced.

[0305] The splitting degree is determined as the ratio of the measured acid value divided by the theoretically possible acid value which can be computed for a given oil or fat. Preferably, the acid value is measured by means of titration according to standard common methods. Alternatively, the density of the aqueous glycerol phase can be taken as a measure for the splitting degree.

[0306] In one embodiment, the splitting process as described herein is also suitable for splitting the mono-, di- and triglyceride that are contained in the so-called soap-stock from the alkali refining processes of the produced oils. In this manner, the soap-stock can be quantitatively converted without prior saponification of the neutral oils into the fatty acids. For this purpose, the fatty acids being chemically bonded in the soaps are released, preferably before splitting, through an addition of acid. In certain embodiments, a buffer solution is used in addition to water and enzyme for the splitting process.

[0307] In one embodiment, oils produced in accordance with the methods of the invention can also be subjected to saponification as a method of hydrolysis. Animal and plant oils are typically made of triacylglycerols (TAGs), which are esters of fatty acids with the trihydric alcohol, glycerol. In an alkaline hydrolysis reaction, the glycerol in a TAG is removed, leaving three carboxylic acid anions that can associate with alkali metal cations such as sodium or potassium to produce fatty acid salts. In this scheme, the carboxylic acid constituents are cleaved from the glycerol moiety and replaced with hydroxyl groups. The quantity of base (e.g., KOH) that is used in the reaction is determined by the desired degree of saponification. If the objective is, for example, to produce a soap product that comprises some of the oils originally present in the TAG composition, an amount of base insufficient to convert all of the TAGs to fatty acid salts is introduced into the reaction mixture. Normally, this reaction is performed in an aqueous solution and proceeds slowly, but may be expedited by the addition of heat. Precipitation of the fatty acid salts can be facilitated by addition of salts, such as water-soluble alkali metal halides (e.g., NaCl or KCl), to the reaction mixture. Preferably, the base is an alkali metal hydroxide, such as NaOH or KOH. Alternatively, other bases, such as alkanolamines, including for example triethanolamine and aminomethylpropanol, can be used in the reaction scheme. In some cases, these alternatives may be preferred to produce a clear soap product.

[0308] 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).

[0309] 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.

[0310] 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.

[0311] 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).

[0312] 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.

[0313] 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.

[0314] 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.

[0315] 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.

[0316] 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.

[0317] 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.

[0318] 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.

[0319] 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.

[0320] 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.

[0321] 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.

[0322] 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.

[0323] In some embodiments, the reaction products discussed above may be converted into higher hydrocarbons through a condensation reaction in a condensation reactor (shown schematically as condensation reactor 110 in Figure 1). 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.

[0324] 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.

[0325] 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.

[0326] 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.

[0327] 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.

[0328] 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.

[0329] 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.

[0330] 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.

[0331] 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.

[0332] 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%.

[0333] 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.

[0334] 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).

[0335] 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.

[0336] 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.

[0337] 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.

[0338] 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).

[0339] 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., *JAACS* 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).

[0340] 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).

[0341] 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.

[0342] 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.

VII. EXAMPLES

[0343] EXAMPLE 1: Methods for Culturing *Prototheca*

[0344] *Prototheca* strains were cultivated to achieve a high percentage of oil by dry cell weight. Cryopreserved cells were thawed at room temperature and 500 ul of cells were added to 4.5 ml of medium (4.2 g/L K_2HPO_4 , 3.1 g/L NaH_2PO_4 , 0.24 g/L $MgSO_4 \cdot 7H_2O$, 0.25 g/L Citric Acid monohydrate, 0.025 g/L $CaCl_2 \cdot 2H_2O$, 2g/L yeast extract) plus 2% glucose and grown for 7 days at 28°C with agitation (200 rpm) in a 6-well plate. Dry cell weights were determined by centrifuging 1 ml of culture at 14,000 rpm for 5 min in a pre-weighed Eppendorf tube. The culture supernatant was discarded and the resulting cell pellet washed with 1 ml of deionized water. The culture was again centrifuged, the supernatant discarded, and the cell pellets placed at -80°C until frozen. Samples were then lyophilized for 24 hrs and dry cell weights calculated. For determination of total lipid in cultures, 3 ml of culture was removed and subjected to analysis using an Ankom system (Ankom Inc., Macedon, NY) according to the manufacturer's protocol. Samples were subjected to solvent extraction with an Amkom XT10 extractor according to the manufacturer's protocol. Total lipid was determined as the difference in mass between acid hydrolyzed dried samples and solvent extracted, dried samples. Percent oil dry cell weight measurements are shown in Table 8.

[0345] Table 8. Percent oil by dry cell weight

Species	Strain	% Oil
<i>Prototheca stagnora</i>	UTEX 327	13.14
<i>Prototheca moriformis</i>	UTEX 1441	18.02
<i>Prototheca moriformis</i>	UTEX 1435	27.17

[0346] Microalgae samples from the strains listed in Table 22 above were genotyped. Genomic DNA was isolated from algal biomass as follows. Cells (approximately 200 mg) were centrifuged from liquid cultures 5 minutes at 14,000 x g. Cells were then resuspended in sterile distilled water, centrifuged 5 minutes at 14,000 x g and the supernatant discarded. A single glass bead ~2mm in diameter was added to the biomass and tubes were placed at -80°C for at least 15 minutes. Samples were removed and 150 µl of grinding buffer (1% Sarkosyl, 0.25 M Sucrose, 50 mM NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, RNase A 0.5

ug/ul) was added. Pellets were resuspended by vortexing briefly, followed by the addition of 40 µl of 5M NaCl. Samples were vortexed briefly, followed by the addition of 66 µl of 5% CTAB (Cetyl trimethylammonium bromide) and a final brief vortex. Samples were next incubated at 65°C for 10 minutes after which they were centrifuged at 14,000 x g for 10 minutes. The supernatant was transferred to a fresh tube and extracted once with 300 µl of Phenol:Chloroform:Isoamyl alcohol 12:12:1, followed by centrifugation for 5 minutes at 14,000 x g. The resulting aqueous phase was transferred to a fresh tube containing 0.7 vol of isopropanol (~190 µl), mixed by inversion and incubated at room temperature for 30 minutes or overnight at 4°C. DNA was recovered via centrifugation at 14,000 x g for 10 minutes. The resulting pellet was then washed twice with 70% ethanol, followed by a final wash with 100% ethanol. Pellets were air dried for 20-30 minutes at room temperature followed by resuspension in 50 µl of 10mM TrisCl, 1mM EDTA (pH 8.0).

[0347] Five µl of total algal DNA, prepared as described above, was diluted 1:50 in 10 mM Tris, pH 8.0. PCR reactions, final volume 20 µl, were set up as follows. Ten µl of 2 x iProof HF master mix (BIO-RAD) was added to 0.4 µl primer SZ02613 (5'-TGTTGAAGAATGAGCCGCGAC-3' (SEQ ID NO:9) at 10mM stock concentration). This primer sequence runs from position 567-588 in Gen Bank accession no. L43357 and is highly conserved in higher plants and algal plastid genomes. This was followed by the addition of 0.4 µl primer SZ02615 (5'-CAGTGAGCTATTACGCACTC-3' (SEQ ID NO:10) at 10 mM stock concentration). This primer sequence is complementary to position 1112-1093 in Gen Bank accession no. L43357 and is highly conserved in higher plants and algal plastid genomes. Next, 5 µl of diluted total DNA and 3.2 µl dH₂O were added. PCR reactions were run as follows: 98°C, 45''; 98°C, 8''; 53°C, 12''; 72°C, 20'' for 35 cycles followed by 72°C for 1 min and holding at 25°C. For purification of PCR products, 20 µl of 10 mM Tris, pH 8.0, was added to each reaction, followed by extraction with 40 µl of Phenol:Chloroform:isoamyl alcohol 12:12:1, vortexing and centrifuging at 14,000 x g for 5 minutes. PCR reactions were applied to S-400 columns (GE Healthcare) and centrifuged for 2 minutes at 3,000 x g. Purified PCR products were subsequently TOPO cloned into PCR8/GW/TOPO and positive clones selected for on LB/Spec plates. Purified plasmid DNA was sequenced in both directions using M13 forward and reverse primers. In total, twelve *Prototheca* strains were selected to have their 23S rRNA DNA sequenced and the sequences are listed in the Sequence Listing. A summary of the strains and Sequence Listing Numbers is included below. The sequences were analyzed for overall divergence from the UTEX 1435 (SEQ ID NO: 15) sequence. Two pairs emerged (UTEX 329/UTEX 1533 and UTEX

329/UTEX 1440) as the most divergent. In both cases, pairwise alignment resulted in 75.0% pairwise sequence identity. The percent sequence identity to UTEX 1435 is also included below.

<u>Species</u>	<u>Strain</u>	<u>% nt identity</u>	<u>SEQ ID NO.</u>
<i>Prototheca kruegani</i>	UTEX 329	75.2	SEQ ID NO: 11
<i>Prototheca wickerhamii</i>	UTEX 1440	99	SEQ ID NO: 12
<i>Prototheca stagnora</i>	UTEX 1442	75.7	SEQ ID NO: 13
<i>Prototheca moriformis</i>	UTEX 288	75.4	SEQ ID NO: 14
<i>Prototheca moriformis</i>	UTEX 1439; 1441; 1435; 1437	100	SEQ ID NO: 15
<i>Prototheca wickerhamii</i>	UTEX 1533	99.8	SEQ ID NO: 16
<i>Prototheca moriformis</i>	UTEX 1434	75.9	SEQ ID NO: 17
<i>Prototheca zopfii</i>	UTEX 1438	75.7	SEQ ID NO: 18
<i>Prototheca moriformis</i>	UTEX 1436	88.9	SEQ ID NO: 19

[0348] Lipid samples from a subset of the above-listed strains were analyzed for lipid profile using HPLC. Results are shown below in Table 9.

[0349] Table 9. Diversity of lipid chains in microalgal species

<u>Strain</u>	<u>C14:0</u>	<u>C16:0</u>	<u>C16:1</u>	<u>C18:0</u>	<u>C18:1</u>	<u>C18:2</u>	<u>C18:3</u>	<u>C20:0</u>	<u>C20:1</u>
UTEX 327	0	12.01	0	0	50.33	17.14	0	0	0
UTEX 1441	1.41	29.44	0.70	3.05	57.72	12.37	0.97	0.33	0
UTEX 1435	1.09	25.77	0	2.75	54.01	11.90	2.44	0	0

[0350] Algal plastid transit peptides were identified through the analysis of UTEX 1435 (*Prototheca moriformis*) or UTEX 250 (*Chlorella protothecoides*) cDNA libraries as described in Examples 12 and Example 11 below. cDNAs encoding potentially plastid targeted proteins based upon BLAST hit homology to other known plastid targeted proteins were subjected to further analysis by the software programs PSORT (<http://psort.ims.u-tokyo.ac.jp/form.html>), ChloroP (<http://www.cbs.dtu.dk/services/ChloroP/>) or TargetP (<http://www.cbs.dtu.dk/services/TargetP/>). Candidate plastid transit peptides identified through at least one of these three programs were then PCR amplified from the appropriate genomic DNA. Below is a summary of the amino acid sequences algal plastid targeting sequences (PTS) that were identified from this screen. Also included are the amino acid sequences of plant fatty acyl-ACP thioesterases that are used in the heterologous expression Examples below.

<u>cDNA</u>	<u>SEQ ID NO.</u>
<i>P.moriformis</i> isopentenyl diphosphate synthase PTS	SEQ ID NO: 127
<i>P.moriformis</i> delta 12 fatty acid desaturase PTS	SEQ ID NO: 128

<i>P.moriformis</i> stearoyl ACP desaturase PTS	SEQ ID NO: 129
<i>C.protothecoides</i> stearoyl ACP desaturase PTS	SEQ ID NO: 130
<i>Cuphea hookeriana</i> fatty acyl-ACP thioesterase (C8-10)	SEQ ID NO: 131
<i>Umbellularia californica</i> fatty acyl-ACP thioesterase (C12)	SEQ ID NO: 132
<i>Cinnamomum camphora</i> fatty acyl-ACP thioesterase (C14)	SEQ ID NO: 133

[0351] EXAMPLE 2: Culturing *Prototheca* on Various Feedstocks

A. Sorghum

[0352] The following strains were shown to be capable of utilizing sorghum as a sole carbon source: *Prototheca moriformis* strains UTEX 1435, UTEX 1437, UTEX 288, UTEX 1439, UTEX 1441 and UTEX 1434, and *Prototheca stagnora* strain UTEX 1442. The “UTEX” designation indicates the strain number from the algal culture collection of the University of Texas, 1 University State A6700, Austin, Texas 78712-0183.

[0353] Pure sorghum was purchased from Maasdam Sorghum Mills (Lynnville, Iowa) with a sugar profile of fructose 21.0% w/w, dextrose 28.0% w/w, sucrose 16.0% w/w and maltose <0.5%w/w. The cultures were grown in liquid medium containing 2%, 5%, or 7% (v/v) pure sorghum (diluted from the pure stock) as the sole carbon source and the cultures were grown heterotrophically in the dark, agitating at ~ 350 rpm. Samples from the cultures were pulled at 24, 40, 48, 67 and 89 hours and growth was measured using A750 readings on a spectrophotometer. Growth was observed for each of the strains tested as shown in Figures 1-2.

B. Cellulose

[0354] Wet, exploded corn stover, *Miscanthus*, forage sorghum, beet pulp and sugar cane bagasse were prepared by The National Renewable Energy Laboratory (Golden, CO) by cooking in a 1.4% sulfuric acid solution and dewatering the resultant slurry. Percent solids were determined gravimetrically by drying and were as follows: corn stover, 25% solids; *Miscanthus*, 28.7% solids; forage sorghum, 26.7% solids; and sugar cane bagasse, 26% solids.

[0355] 100 gram wet samples of exploded cellulosic materials (corn stover or switch grass) were resuspended in deionized water to a final volume of 420 mL and the pH was adjusted to 4.8 using 10N NaOH. For beet pulp, 9.8 grams dry solids were brought to 350 mL with deionized water and pH was adjusted to 4.8 with 10 N NaOH. For all of the above feedstocks, Accellerase 1000 (Genencor, New York) was used at a ratio of 0.25 ml enzyme per gram of dry biomass for saccharification of the cellulosic materials. Samples were incubated with

agitation (110 rpm) at 50°C for 72 hours. The pH of each of the samples was adjusted to 7.0 with NaOH (with negligible volume change), filter sterilized through a 0.22 µm filter and used in the processes detailed below. For larger scale processes, the same procedure for saccharification was followed except an additional step of tangential flow filtration (TFF) or microfiltration step was performed to aid in filter sterilization of feedstocks. A sample from each of the feedstocks prepared was reserved for determination of glucose and xylose concentration using an HPLC/ELSD-based system or a hexokinase-based kit (Sigma). Additionally, for beet pulp, the material was initially brought to volume as with the other feedstocks, the pH was then adjusted to 4.0 and a pectinase treatment was carried out at 50°C for 24 hours. The pH was then adjusted to 4.8 if no washing steps were conducted or 5.3 if washing steps were conducted. Enzymatic saccharification was then performed with the same procedure used for the other feedstocks as described above.

[0356] Microalgae *Prototheca moriformis* strain UTEX 1435 was assessed for its ability to grow on a series of cellulosic feedstocks prepared as described above (corn stover, beet pulp, sorghum cane, *Miscanthus* and glucose control). The microalgae culture was grown in conditions described in Example 1 above with the exception of the carbon source. The carbon source was either 4% glucose (for control conditions) or 4% glucose as measured by available glucose in the cellulosic materials. Growth was assessed by A750 readings and the culturing time was 168 hours, with A750 readings at 48, 72, 96, 120, 144 and 168 hours after initiation of the culture. As can be seen in Figure 7a, the *Prototheca moriformis* culture grew best in corn stover. The other cellulosic feedstocks used, *Miscanthus*, sorghum cane and beet pulp, all exhibited inhibition of growth.

[0357] Based on the above results with corn stover derived cellulosic sugars, lipid accumulation was also assessed in *Prototheca moriformis* using different levels of corn stover derived cellulosic sugars and reagent glucose as a control. Cultures were grown in 18g/L glucose that was completely from corn stover derived cellulosic sugars (100% corn stover condition in Figure 7b), 9g/L glucose from corn stover derived cellulosic sugars supplemented with 9g/L reagent glucose (50% corn stover supplemented with glucose to 18g/L condition in Figure 7b), 9g/L glucose from corn stover derived cellulosic sugars (50% corn stover, not supplemented; glucose at 9g/L condition in Figure 7b) and a control culture of 42g/L reagent glucose and 13 g/L reagent xylose for osmolarity control. All cultures were fed with cellulosic sugars to maintain the glucose concentration at 20 g/L, except for the control culture, which was fed with reagent glucose to maintain the glucose concentration at 20 g/L. Growth was measured based on the dry cell weight of the culture and lipid

productivity was determined as a percent dry cell weight. Total lipids were determined gravimetrically using an Ankom acid hydrolysis/solvent extraction system as described in Example 1 above.

[0358] As can be seen in Figure 7b, based on biomass accumulation (as measured by DCW), all concentrations of the corn stover derived cellulosics out-performed (higher DCW) the control media that was fed glucose alone. Lipid production as a percentage of DCW was also calculated for all of the conditions. In addition to the higher biomass accumulation seen for growth on corn stover, lipid accumulation was also higher in the corn stover derived cellulosics conditions as compared to the glucose control condition. These data demonstrate that, in addition to providing cellulosic derived sugars, corn stover provides additional nutrients/components that contribute to an increased biomass accumulation (growth) and increased product yield.

[0359] Because the cellulosic feedstocks contain components in addition to glucose, some of these additional components can accumulate to undesirable levels during culture as more cellulosic derived sugars are fed into the culture as the main carbon source (usually, but not limited to, glucose) is consumed. For example, the xylose present in the cellulosic derived sugar feedstock may build up during the high density cultivation of microalgae to levels inhibitory to growth and end product production. To test the effects of xylose build up during *Prototheca* cultivation, cultures were grown with 4% glucose in the media and supplemented with 0, 10 g/L, 25 g/L, 50 g/L and 100 g/L xylose. After 6 days of culture, growth and lipid accumulation were assessed using the methods described above. As seen in Figure 7c, surprisingly, the highest concentrations of xylose tested were not inhibitory to *Prototheca moriformis*' ability to grow and accumulate lipid, and the culture actually grew better and accumulated more lipids at the highest xylose concentrations. To explore this phenomenon, a similar experiment was carried out with sucrose, a carbon source which wild type *Prototheca moriformis* is unable to metabolize. No positive impact was observed with sucrose, suggesting that the increased growth and lipid accumulation seen with xylose is attributable to a mechanism other than the osmotic stress from high concentrations of unmetabolized components in the media and is xylose-specific.

[0360] In addition to non-metabolized sugars, salts may accumulate to inhibitory levels as a result of concentrating lignocellulosic derived sugars. Due to the acid hydrolysis step with H_2SO_4 during the typical preparation of cellulosic materials followed by neutralization of the acid with NaOH, Na_2SO_4 is formed during the generation of lignocellulosic sugars. To assess the impact of salt concentration on growth and lipid production, *Prototheca moriformis*

cultures were grown at Na₂SO₄ concentrations ranging from 0-700 mM in media supplemented with 4% glucose. As shown in Figure 7d, a significant inhibition of growth was observed, as measured by DCW accumulation, where Na₂SO₄ concentrations exceeded 25 mM, specifically at the 80 mM, 240 mM and 700 mM concentrations. In addition, the impact of antifoam P2000 was assessed in the same test. The antifoam compound had a significant, positive impact on biomass productivity. Lipid productivity was also assessed for each condition, and Na₂SO₄ concentrations above 80 mM, specifically 240 mM and 700 mM, were inhibitory while the addition of antifoam P2000 significantly increased lipid productivity. Thus, in one embodiment, the culturing steps of the methods of the present invention include culturing in media containing an antifoaming agent.

[0361] Based on the results discussed above and summarized in Figure 7a, inhibitors were likely present in the cellulosic feedstocks exhibiting poor growth. The present invention provides means of removing such compounds by washing the materials with hot water (hydrothermal treatment). Figure 8 summarizes the growth results, as measured by A750, using sugar derived from cellulosic feedstock with a single hot water wash. The culture conditions were identical to those used in the processes summarized in Figure 7a. Compared to the results shown in Figure 7a, after just one hot water wash, *Prototheca moriformis* cultures grew better in all cellulosic feedstocks tested, specifically sugar cane bagasse, sorghum cane, *Miscanthus* and beet pulp, as compared to glucose control. Lipid productivity was also assessed in each of the conditions. Except for the beet pulp condition, which was comparable to the glucose control, cultures grown in sugars derived from cellulosic materials subjected to one hot water wash exhibited better lipid productivity than the glucose control.

[0362] One potential impact of hydrothermal treatment (hot water washing) of cellulosic biomass is the removal of furfurals and hydroxymethyl furfurals released by acid explosion of the material. The presence of furfurals and hydroxymethyl furfurals may have contributed to limited growth observed in some of the processes summarized in Figure 7a. To assess how hydrothermal treatment affected the levels of furfurals (FA) and hydroxymethyl furfurals (HMF), supernatants resulting from one to three washes of cellulosic biomass derived from sugarcane bagasse (B), sorghum cane (S), *Miscanthus* (M) or beet pulp (BP) were assayed for FA and HMF by HPLC. As shown in Figure 8, FA and HMF levels decrease significantly with each washing step. This result is consistent with the observation that FA and HMF can be inhibitory to microalgal growth (as seen in Figure 7a) and that hydrothermal treatment removes these compounds and results in improved microalgal growth, even better than the growth in the control glucose conditions (as seen in Figure 8).

[0363] The impact on the lipid profile of *Prototheca moriformis* cultures grown on the various hydrothermally treated lignocellulosic derived sugars was assessed. *Prototheca moriformis* cultures were grown on the following 4x-washed cellulosic feedstocks: *Miscanthus*, sugar cane bagasse and sorghum cane, with glucose levels maintained at 20 g/L through feeding of the cellulosic sugars. At the conclusion of the culturing, microalgae biomass from each condition was analyzed for lipid profile using the methods described in Example 1. The results of the lipid profile analysis (expressed in Area %) are summarized in Table 10 below. Each condition was tested in duplicates, and the results from each of the duplicate test conditions are included. Growth on cellulosic feedstocks resulted in a significant re-distribution in the lipid profile as compared to the glucose control. For example, there was a significant increase in C18:0 Area % in all of the cellulosic feedstock conditions as compared to the glucose control condition.

[0364] Table 10. Lipid profile of *Prototheca moriformis* grown on glucose and cellulose derived sugars.

	glucose 1 (ctrl)	glucose 2 (ctrl)	bagasse 1	bagasse 2	sorgh 1	sorgh 2	Miscan 1	Miscan 2
C10:0	n.d.	n.d.	0.03	0.02	n.d.	n.d.	n.d.	n.d.
C12:0	0.04	0.05	0.04	0.04	0.05	0.04	0.04	0.04
C14:0	1.64	1.64	1.07	1.10	1.17	1.14	1.08	1.12
C14:1	0.03	0.04	0.04	0.04	0.06	0.06	0.03	0.03
C15:0	0.04	0.05	0.07	0.05	0.08	0.08	0.06	0.06
C16:0	26.80	26.81	22.32	22.81	22.09	22.19	23.45	23.62
C16:1	0.75	0.82	1.68	1.70	1.92	2.12	1.38	1.23
C17:0	0.14	0.16	0.28	0.17	0.29	0.27	0.21	0.19
C17:1	0.07	0.06	0.10	0.10	0.13	0.12	0.10	0.09
C18:0	3.56	3.64	15.88	10.40	15.30	12.37	10.15	8.69
C18:1	54.22	54.01	49.87	53.87	49.35	50.80	54.05	55.26
C18:2	11.23	11.11	6.54	7.91	7.47	8.80	7.71	7.88
C18:3 alpha	0.84	0.85	0.39	0.56	0.47	0.53	0.56	0.60
C20:0	0.31	0.30	0.85	0.63	0.76	0.69	0.63	0.56
C20:1	0.15	0.15	0.33	0.28	0.32	0.32	0.27	0.25
C20:3	0.06	0.06	0.13	0.12	0.14	0.12	0.11	0.11
C24:0	0.12	0.12	0.22	0.19	0.22	0.20	0.18	0.15

n.d. denotes none detected

[0365] Cellulosic sugar stream was generated from exploded corn stover, saccharified using Accellerase enzyme and concentrated using vacuum evaporation. This sugar stream was tested in *Prototheca moriformis* growth assays at a 4% glucose concentration. The results of the growth assays showed very poor growth and the cellulosic sugar stream was tested for conductivity (salt content). The conductivity was very high, far greater than 700 mM sodium equivalents, a level that was shown to be inhibitory to growth as described above and shown in Figure 7d. Methods of the invention include methods in which salt is reduced or removed

from lignocellulosic derived sugars prior to utilizing these feedstocks in the production of lignocellulosic derived microalgal oil. Surprisingly, however, one cannot use resins to desalt concentrated sugar streams, one must first dilute the concentrated sugar stream. To demonstrate this embodiment of the invention, cellulosic sugars derived from corn stover material were diluted eight-fold prior to removing contaminating salts with the resin. The initial conductivity of the concentrated starting material was 87 mS/cm while that of the eight-fold diluted stream was 10990 μ S/cm at a pH of 5.61. Previous studies had indicated that failure to dilute the concentrated sugar stream prior to de-ionization resulted in an inability to remove salts quantitatively as well as a significant loss of glucose from the sugar stream. Three different bed volumes of IEX resin (DOWEX Marathon MR3) were used (1:2, 1:4 and 1:10). Table 11 summarizes results demonstrating the ability of a mixed bed ion exchange (IEX) resin to reduce salts (as measured by conductivity) significantly in a previously concentrated corn stover derived cellulosic sugar stream in diluted feedstocks.

[0366] Table 11. Ability of IEX resin to reduce salts.

Bed volume resin: cellulosics	pH post-deionization	Conductivity post-deionization (μ S/cm)	Calculated conductivity post deionization and 8x re-concentration (μ S/cm)	Na ⁺ equivalents (based on std curve) in mM
1:2	3.1	74	592	7.42
1:4	3.1	97	776	9.7
1:10	5.25	6320	50560	634

[0367] A process employing a 1:4 bed volume:cellulosic feedstock and re-concentration of the material eight-fold would result in a sodium concentration is well within the range for normal biomass and lipid accumulation. Alternatively, deionization or salt removal can be performed prior to saccharification or after saccharification, but before concentration of the sugar stream. If salt removal is performed before the concentration of the sugar stream, a dilution step of the sugar stream before salt removal would likely not be necessary.

[0368] This example demonstrates the efficacy of washing of exploded cellulosic material for the use in cellulosic oil production. As described above, concentration of cellulosically derived sugars without the removal of salts (inherent to the production of exploded cellulosic material and subsequent treatment) results in less than optimal fermentations. The materials treated in the process described below were of the appropriate pH for subsequent saccharification. In addition, the conductivity of this material was significantly reduced (over 100 fold) from the starting feedstock. Therefore, the subsequent concentrated sugars to be used in fermentations were not inhibitory due to the presence of excessive salts. An

additional advantage is seen by the removal of furfurals from the cellulosic material. Any xylose or glucose removed in the hemicellulosic fraction can either be discarded or preferably re-concentrated to be used in fermentations.

[0369] Wet, exploded sugar cane bagasse (NREL, Colorado) with an initial starting mass of 65 kg wet weight and conductivity of 15,000 $\mu\text{S}/\text{cm}$, pH 2.4 was brought to 128 kg with deionized water and the pH adjusted to 4.6 with 10 N NaOH, making the resulting conductivity 6,800 $\mu\text{S}/\text{cm}$). The percent solids were assessed by removal of an aliquot of the suspended materials to a tared (weight = t) aluminum pan, recording the wet weight (weight = w) followed by drying for three hours at 110°C. After drying samples were removed to a desiccator and allowed to come to room temperature (25 °C) at which point, they were weighed again (weight = d). Percent solids were calculated as: % solids = [(d-t/w-t)]x 100. Conductivities were measured on a Thermo Electron Orion 3 Star Conductivity meter.

[0370] The sugar cane bagasse was washed in a semi-continuous fashion by continuously mixing the cellulosic slurry (initial percent solids of 8.2%) at a temperature of 50°C in a stainless steel reactor (150L capacity). Cellulosics were discharged from the reactor vessel via a rotary load pump at a flow rate of 1.9-3.8 kg/min to a Sharples Model 660 decanter centrifuge. Liquid permeate was retained batch wise (ca. 35-175 kg aliquots, see Table 12 below) and homogenous aliquots removed for assessment of total sugars (glucose and xylose) and percent solids as described in Table 12. Conductivity and pH of the cellulosic material were controlled via the addition of de-ionized water and 10 N NaOH, respectively. Samples 1-10 in Table 12 represent decanted centrifuge permeate, and as such, solids and sugars present in these fractions are removed from the final, washed cellulosic materials. A mass balance calculation of total solids compared to solids removed minus solids lost plus final solids for saccharification, resulted in a 99% recovery in the above process. Figure 8 summarizes the furfural and hydroxymethyl furfurals concentration (mg/L) in each of the 11 centrifuge permeates collected and described in Table 12. These data demonstrate a clear removal of furfurals and hydroxymethyl furfurals from the sugar cane bagasse.

[0371] Table 12. Mass balance for semi-continuous hydrothermal treatment of sugar cane bagasse.

Sample	kg (wet)	kg (dry)	pH	Conductivity $\mu\text{S}/\text{cm}$	total xylose removed (g)	total glucose removed (g)
1 (initial material)	128	10.50	4.60	6,880	0	0
2	81.8	2.03		3,280	1030.68	286.3
3	76.5	0.49		2,500	298.35	76.50
4	106	0.41			254.40	63.60
5	173.9	0.30	3.74	1,260	226.07	69.56

6	101.8	0.08	4.40	791	71.26	20.36
7	110.6	0.04	4.86	327	44.24	0
8	77.2	0			0	0
9	108.6	0.02	4.7	221	0	0
10	101.5	0			0	0
11	34.8	0	4.7	146	0	0
Solids removed (samples 1-10) lost in process		3.37				
Total xylose removed					1925.00	
Total glucose removed						516.32
Final solids for saccharification	7.03					

[0372] In another demonstration of the ability of *Prototheca* to utilize cellulosic-derived feedstock, *Prototheca moriformis* (UTEX 1435) was cultivated in three-liter bioreactors using cellulosic derived sugar as a fixed carbon feedstock. The inoculum was prepared from cryopreserved cells, which were thawed at room temperature and 1 mL of cells were added to 300 mL of inoculum medium based on the basal microalgae medium described in Example 1 with 1 g/L (NH₄)₂SO₄, 4 g/L yeast extract and a trace element solution, plus 4% glucose and grown for 1 day at 28°C with agitation (200 rpm). This culture was used to inoculate a three-liter bioreactor containing 1L medium plus 0.26mL of Antifoam 204 (Sigma, USA). The fermentor was controlled at 28°C and pH was maintained at 6.8 by addition of KOH. Dissolved oxygen was maintained at 30% saturation by cascading agitation and airflow. Cellulosic sugar feedstock from corn stover was fed to the culture to maintain 0-10g/L glucose. Desalination of cellulosic sugar feedstocks to less than 300 mM salt was essential to assure similar dry cell weight and lipid accumulation performance as compared to purified sugar feedstock controls. Desalination of the cellulosic sugar feedstock was performed using the methods described above. Fermentor samples were removed to monitor fermentation performance. Cell mass accumulation was monitored by optical density and dry cell weight. Glucose, xylose, ammonia, potassium, sodium and furfural concentrations were also determined and monitored throughout the fermentation time course. Lipid concentration was determined by gravimetric methods discussed above.

[0373] **EXAMPLE 3: Methods for Transforming *Prototheca***

A. General Method for Biolistic transformation of *Prototheca*

[0374] S550d gold carriers from Seashell Technology were prepared according to the protocol from manufacturer. Linearized plasmid (20 µg) was mixed with 50 µl of binding buffer and 60 µl (30 mg) of S550d gold carriers and incubated in ice for 1 min. Precipitation

buffer (100 μ l) was added, and the mixture was incubated in ice for another 1 min. After vortexing, DNA-coated particles were pelleted by spinning at 10,000 rpm in an Eppendorf 5415C microfuge for 10 seconds. The gold pellet was washed once with 500 μ l of cold 100% ethanol, pelleted by brief spinning in the microfuge, and resuspended with 50 μ l of ice-cold ethanol. After a brief (1-2 sec) sonication, 10 μ l of DNA-coated particles were immediately transferred to the carrier membrane.

[0375] *Prototheca* strains were grown in proteose medium (2g/L yeast extract, 2.94mM NaNO₃, 0.17mM CaCl₂•2H₂O, 0.3mM MgSO₄•7H₂O, 0.4mM K₂HPO₄, 1.28mM KH₂PO₄, 0.43mM NaCl) on a gyratory shaker until it reaches a cell density of 2x10⁶ cells/ml. The cells were harvested, washed once with sterile distilled water, and resuspended in 50 μ l of medium. 1 x 10⁷ cells were spread in the center third of a non-selective proteose media plate. The cells were bombarded with the PDS-1000/He Biolistic Particle Delivery system (Bio-Rad). Rupture disks (1100 and 1350 psi) were used, and the plates are placed 9 and 12 cm below the screen/macrocarrrier assembly. The cells were allowed to recover at 25°C for 12–24 h. Upon recovery, the cells were scraped from the plates with a rubber spatula, mixed with 100 μ l of medium and spread on plates containing the appropriate antibiotic selection. After 7-10 days of incubation at 25°C, colonies representing transformed cells were visible on the plates from 1100 and 1350 psi rupture discs and from 9 and 12 cm distances. Colonies were picked and spotted on selective agar plates for a second round of selection.

B. Transformation of *Prototheca* with G418 Resistance Gene

[0376] *Prototheca moriformis* and other *Prototheca* strains sensitive to G418 can be transformed using the methods described below. G418 is an aminoglycoside antibiotic that inhibits the function of 80S ribosomes and thereby inhibits protein synthesis. The corresponding resistance gene functions through phosphorylation, resulting in inactivation of G418. *Prototheca* strains UTEX 1435, UTEX 1439 and UTEX 1437 were selected for transformation. All three *Prototheca* strains were genotyped using the methods described above. All three *Prototheca* strains had identical 23s rRNA genomic sequences (SEQ ID NO:15).

[0377] All transformation cassettes were cloned as EcoRI-SacI fragments into pUC19. Standard molecular biology techniques were used in the construction of all vectors according to Sambrook and Russell, 2001. The *C. reinhardtii* beta-tubulin promoter/5'UTR was obtained from plasmid pHyg3 (Berthold *et al.*, (2002) Protist: 153(4), pp 401-412) by PCR as an EcoRI-AscI fragment. The *Chlorella vulgaris* nitrate reductase 3'UTR was obtained

from genomic DNA isolated from UTEX strain 1803 via PCR using the following primer pairs:

Forward:

5' TGACCTAGGTGATTAATTAAGTCTGAGGCAGCAGCAGCTCGGATAGTATCG 3'
(SEQ ID NO:35)

Reverse:

5' CTACGAGCTCAAGCTTTCCATTTGTGTTC CCATCCCCTACTTCC 3'
(SEQ ID NO:36)

[0378] The *Chlorella sorokiniana* glutamate dehydrogenase promoter/UTR was obtained via PCR of genomic DNA isolated from UTEX strain 1230 via PCR using the following primer pairs:

Forward: 5' GATCAGAATTCCGCCTGCAACGCAAGG GCAGC 3' (SEQ ID NO:37)

Reverse: 5' GCATACTAGTGGCGGGACGGAGAGA GGGCG 3' (SEQ ID NO:38)

[0379] Codon optimization was based on the codons in Table 1 for *Prototheca moriformis*. The sequence of the non-codon optimized neomycin phosphotransferase (nptII) cassette was synthesized as an AscI-XhoI fragment and was based on upon the sequence of Genbank Accession No. YP_788126. The codon optimized nptII cassette was also based on this Genbank Accession number.

[0380] The three *Prototheca* strains were transformed using biolistic methods described above. Briefly, the *Prototheca* strains were grown heterophically in liquid medium containing 2% glucose until they reached the desired cell density (1×10^7 cells/mL to 5×10^7 cells/mL). The cells were harvested, washed once with sterile distilled water and resuspended at 1×10^8 cells/mL. 0.5mL of cells were then spread out on a non-selective solid media plate and allowed to dry in a sterile hood. The cells were bombarded with the PDS-1000/He Biolistic Particle Delivery System (BioRad). The cells were allowed to recover at 25° C for 24 hours. Upon recovery, the cells were removed by washing plates with 1 mL of sterile media and transferring to fresh plates containing 100 µg/mL G418. Cells were allowed to dry in a sterile hood and colonies were allowed to form on the plate at room temperature for up to three weeks. Colonies of UTEX 1435, UTEX 1439 and UTEX 1437 were picked and spotted on selective agar plates for a second round of selection.

[0381] A subset of colonies that survived a second round of selection described above, were cultured in small volume and genomic DNA and RNA were extracted using standard molecular biology methods. Southern blots were done on genomic DNA extracted from untransformed (WT), the transformants and plasmid DNA. DNA from each sample was run

on 0.8% agarose gels after the following treatments: undigested (U), digested with AvrII (A), digested with NcoI (N), digested with SacI (S). DNA from these gels was blotted on Nylon+ membranes (Amersham). These membranes were probed with a fragment corresponding to the entire coding region of the nptII gene (NeoR probe). Figure 4 shows maps of the cassettes used in the transformations. Figure 5 shows the results of Southern blot analysis on three transformants (all generated in UTEX strain 1435) (1, 2, and 3) transformed with either the beta-tubulin::neo::nit (SEQ ID NO: 39) (transformants 1 and 2) or glutamate dehydrogenase::neo::nit (SEQ ID NO: 40) (transformant 3). The glutamate dehydrogenase::neo::nit transforming plasmid was run as a control and cut with both NcoI and SacI. AvrII does not cut in this plasmid. Genomic DNA isolated from untransformed UTEX strain 1435 shows no hybridization to the NeoR probe.

[0382] Additional transformants containing the codon-optimized glutamate dehydrogenase::neo::nit (SEQ ID NO: 41) and codon-optimized β -tubulin::neo::nit (SEQ ID NO:42) constructs were picked and analyzed by Southern blot analysis. As expected, only digests with SacI show linearization of the transforming DNA. These transformation events are consistent with integration events that occur in the form of oligomers of the transforming plasmid. Only upon digestion with restriction enzymes that cut within the transforming plasmid DNA do these molecules collapse down the size of the transforming plasmid.

[0383] Southern blot analysis was also performed on transformants generated upon transformation of *Prototheca* strains UTEX 1437 and UTEX 1439 with the glutamate dehydrogenase::neo::nit cassette. The blot was probed with the NeoR probe and the results are similar to the UTEX 1435 transformants. The results are indicative of integration events characterized by oligomerization and integration of the transforming plasmid. This type of integration event is known to occur quite commonly in *Dictyostelium discoideum* (see, for example, Kuspa, A. and Loomis, W. (1992) *PNAS*, 89:8803-8807 and Morio *et al.*, (1995) *J. Plant Res.* 108:111-114).

[0384] To further confirm expression of the transforming plasmid, Northern blot analysis and RT-PCR analysis were performed on selected transformants. RNA extraction was performed using Trizol Reagent according to manufacturer's instructions. Northern blot analysis were run according to methods published in Sambrook and Russel, 2001. Total RNA (15 μ g) isolated from five UTEX 1435 transformants and untransformed UTEX 1435 (control lanes) was separated on 1% agarose-formaldehyde gel and blotted on nylon membrane. The blot was hybridized to the neo-non-optimized probe specific for transgene sequences in transformants 1 and 3. The two other transformants RNAs express the codon-optimized

version of the neo-transgene and, as expected, based on the sequence homology between the optimized and non-optimized neo genes, showed significantly lower hybridization signal.

[0385] RNA (1 µg) was extracted from untransformed *Prototheca* strain UTEX 1435 and two representative UTEX 1435 transformants and reverse transcribed using an oligo dT primer or a gene specific primer. Subsequently these cDNAs (in duplicate) were subjected to qPCR analysis on ABI Veriti Thermocycler using SYBR-Green qPCR chemistry using the following primers (nptII):

Forward: 5' GCCGCGACTGGCTGCTGCTGG 3' (SEQ ID NO:43)

Reverse: 5' AGGTCCTCGCCGTCGGGCATG 3' (SEQ ID NO:44)

[0386] Possible genomic DNA contamination was ruled out by a no reverse transcriptase negative control sample. The results indicated that the NeoR genes used to transform these strains is actively transcribed in the transformants.

C. Transformation of *Prototheca* with Secreted Heterologous Sucrose

Invertase

[0387] All of the following experiments were performed using liquid medium/agar plates based on the basal medium described in Ueno et al., (2002) *J Bioscience and Bioengineering* 94(2):160-65, with the addition of trace minerals described in US Patent No. 5,900,370, and 1x DAS Vitamin Cocktail (1000x solution): tricaine: 9g, thiamine HCL: 0.67g, biotin: 0.01g, cyannocobalamin (vitamin B12): 0.008g, calcium pantothenate: 0.02g and p-aminobenzoic acid: 0.04g).

[0388] Two plasmid constructs were assembled using standard recombinant DNA techniques. The yeast sucrose invertase genes (one codon optimized and one non-codon optimized), *suc2*, were under the control of the *Chlorella reinhardtii* beta-tubulin promoter/5'UTR and had the *Chlorella vulgaris* nitrate reductase 3'UTR. The sequences (including the 5'UTR and 3'UTR sequences) for the non-codon optimized (Crβ-tub::NCO-*suc2*::CvNitRed) construct, SEQ ID NO: 57, and codon optimized (Crβ-tub::CO-*suc2*::CvNitRed) construct, SEQ ID NO: 58, are listed in the Sequence Listing. Codon optimization was based on Table 1 for *Prototheca* sp. Figure 6 shows a schematic of the two constructs with the relevant restriction cloning sites and arrows indicating the direction of transcription. Selection was provided by Neo R (codon optimized using Table 1).

[0389] Preparation of the DNA/gold microcarrier: DNA/gold microcarriers were prepared immediately before use and stored on ice until applied to macrocarriers. The plasmid DNA (in TE buffer) was added to 50 µl of binding buffer. Saturation of the gold beads was achieved at 15 µg plasmid DNA for 3 mg gold carrier. The binding buffer and DNA were

mixed well via vortexing. The DNA and binding buffer should be pre-mix prior to gold addition to ensure uniformed plasmid binding to gold carrier particles. 60 µl of S550d (Seashell Technologies, San Diego, CA) gold carrier was added to the DNA/binding buffer mixture. For a gold stock at 50 mg/ml, addition of 60 µl results in an optimal ratio of 15 µg DNA/3 mg gold carrier. The gold carrier/DNA mixture was allowed to incubate on ice for 1 minute and then 100 µl of precipitation buffer was added. The mixture was allowed to incubate again on ice for 1 minute and then briefly vortexed and centrifuged at 10,000 rpm at room temperature for 10 seconds to pellet the gold carrier. The supernatant was carefully removed with a pipette and the pellet was washed with 500 µl of ice cold 100% ethanol. The gold particles were re-pelleted by centrifuging again at 10,000 rpm for 10 seconds. The ethanol was removed and 50 µl of ice cold ethanol was added to the gold mixture. Immediately prior to applying the gold to macrocarriers, the gold/ethanol was resuspended with a brief 1-2 second pulse at level 2 on a MISONIX sonicator using the micro tip. Immediately after resuspension, 10 µl of the dispersed gold particles was transferred to the macrocarrier and allowed to dry in a sterile hood.

[0390] The two *Prototheca moriformis* strains (UTEX 1435 and 1441) were grown heterotrophically in liquid medium containing 2% glucose from cryopreserved vials. Each strain was grown to a density of 10^7 cells/ml. This seed culture was then diluted with fresh media to a density of 10^5 cells/ml and allowed to grow for 12-15 hours to achieve a final cell density of approximately 10^6 cells/ml. The microalgae were aliquoted into 50 ml conical tubes and centrifuged for 10 minutes at 3500 rpm. The cells were washed with fresh medium and centrifuged again for 10 minutes at 3500 rpm. The cells were then resuspended at a density of 1.25×10^8 cells/ml in fresh medium.

[0391] In a sterile hood, 0.4ml of the above-prepared cells were removed and placed directly in the center of an agar plate (without selection agent). The plate was gently swirled with a level circular motion to evenly distribute the cells to a diameter of no more than 3cm. The cells were allowed to dry onto the plates in the sterile hood for approximately 30-40 minutes and then were bombarded at a rupture disk pressure of 1350 psi and a plate to macrocarrier distance of 6 cm. The plates were then covered and wrapped with parafilm and allowed to incubate under low light for 24 hours.

[0392] After the 24 hour recovery, 1ml of sterile medium (with no glucose) was added to the lawn of cells. The cells were resuspended using a sterile loop, applied in a circular motion to the lawn of cells and the resuspended cells were collected using a sterile pipette. The cells were then plated onto a fresh agar plate with 2% glucose and 100 µg/ml G418. The

appearance of colonies occurred 7-12 days after plating. Individual colonies were picked and grown in selective medium with 2% glucose and 100 µg/ml G418. The wildtype (untransformed) and transgenic cells were then analyzed for successful introduction, integration and expression of the transgene.

[0393] Genomic DNA from transformed *Prototheca moriformis* UTEX 1435 and 1441 and their wildtype (untransformed) counterparts were isolated using standard methods. Briefly, the cells were centrifuged for 5 minutes at 14,000 rpm in a standard table top Eppendorf centrifuge (model 5418) and flash frozen prior to DNA extraction. Cell pellets were lysed by adding 200 µL of Lysis buffer (100 mM Tris HCl, pH 8.0, 1% Lauryl Sarcosine, 50 mM NaCl, 20 mM EDTA, 0.25 M sucrose, 0.5 mg/ml RNase A) for every 100-200 mg of cells (wet weight) and vortexing for 30-60 seconds. Cetyl trimethylammonium bromide (CTAB) and NaCl were brought to 1% and 1 M, respectively, and cell extracts were incubated at 60-65°C for 10 minutes. Subsequently, extracts were clarified via centrifugation at 14,000 rpm for 10 minutes and the resulting supernatant was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Samples were then centrifuged for 5 minutes at 14,000 rpm and the aqueous phase removed. DNA was precipitated with 0.7 volumes of isopropanol. DNA was pelleted via centrifugation at 14,000 rpm for 10 minutes and washed twice with 80% ethanol, and once with ethanol. After drying, DNA was resuspended in 10 mM Tris HCl, pH 8.0 and DNA concentrations were determined by using PicoGreen fluorescence quantification assay (Molecular Probes).

[0394] RNA from transformed *Prototheca moriformis* UTEX 1435 and 1441 and their wildtype (untransformed) counterparts were isolated using standard methods. Briefly, the cells were centrifuged for 5 minutes at 14,000 rpm in a standard table top Eppendorf centrifuge (model 5418) and flash frozen before RNA extraction. Cell pellets were lysed by addition of 1 mL of Trizol reagent (Sigma) for every 100 mg of cells (wet weight) and by vortexing for 1-2 minutes. Samples were incubated at room temperature for 5 minutes and subsequently adjusted with 200 µL of chloroform per 1 mL of Trizol reagent. After extensive shaking, cells were incubated at room temperature for 15 minutes and then subjected to centrifugation at 14000 rpm for 15 minutes in a refrigerated table top microcentrifuge. RNA partitioning to the upper aqueous phase was removed and precipitated by addition of isopropanol (500 µL per 1ml of Trizol reagent). RNA was collected by centrifugation for 10 minutes and the resulting pellet washed twice with 1 mL of 80% ethanol, dried, and resuspended in RNase free water. RNA concentration was estimated by RiboGreen fluorescence quantification assay (Molecular Probes).

[0395] Expression of neomycin phosphotransferase gene conferring G418 antibiotic resistance and yeast invertase was assayed in non-transformed *Prototheca moriformis* UTEX 1435 and 1441 and transformants T98 (UTEX 1435 transformant) and T97 (UTEX 1441 transformant) using reverse transcription quantitative PCR analysis (RT-qPCR). 20 ng total RNA (isolated as described above) was subjected to one step RT-qPCR analysis using iScript SYBR Green RT-PCR kit (BioRad Laboratories) and primer pairs targeting the neomycin resistance gene (forward primer 5'CCGCCGTGCTGGACGTGGTG 3' and reverse primer 5'GGTGGCGGGGTCCAGGGTGT 3'; SEQ ID NOs: 65 and 66, respectively) and *suc2* invertase transcripts (forward primer 5' CGGCCGGCGGCTCCTTCAAC 3' and reverse primer 5' GGCGTCCCGTAGGTCTGGGT 3'; SEQ ID NO: 67 and 68, respectively). Endogenous beta-tubulin transcripts served as an internal positive control for PCR amplification and as a normalization reference to estimate relative transcript levels.

[0396] Both codon optimized and non-codon optimized constructs were transformed into UTEX 1435 and 1441 *Prototheca moriformis* cells as described above. Initially, transformants were obtained with both constructs and the presence of the transgene was verified by Southern blot analysis followed by RTPCR to confirm the presence of the DNA and mRNA from the transgene. For the Southern blot analysis, genomic DNA isolated as described above was electrophoresed on 0.7% agarose gels in 1x TAE buffer. Gels were processed as described in Sambrook et al. (Molecular Cloning; A Laboratory Manual, 2nd Edition. Cold Spring Harbor Laboratory Press, 1989). Probes were prepared by random priming and hybridizations carried out as described in Sambrook et al. Transformants from both the codon optimized and the non-codon optimized constructs showed the presence of the invertase cassette, while the non-transformed control was negative. Invertase mRNA was also detected in transformants with both the codon optimized and non-codon optimized constructs.

[0397] To confirm that the transformants were expressing an active invertase protein, the transformants were plated on sucrose plates. The transformants containing the non-codon optimized cassette failed to grow on the sucrose containing plates, indicating that, while the gene and the mRNA encoding the SUC2 protein were present, the protein was either (1) not being translated, or (2) being translated, but not accumulating to levels sufficient to allow for growth on sucrose as the sole carbon source. The transformants with the codon optimized cassette grew on the sucrose containing plates. To assess the levels of invertase being expressed by these transformants, two clones (T98 and T97) were subjected to an invertase assay of whole cells scraped from solid medium and direct sampling and quantitation of

sugars in the culture supernatants after 48 hours of growth in liquid medium containing 2% sucrose as the sole carbon source.

[0398] For the invertase assay, the cells (T98 and T97) were grown on plates containing 2% sucrose, scraped off and assayed for invertase activity. 10 μ l of the scraped cells was mixed with 40 μ l of 50mM NaOAc pH 5.1. 12.5 μ l of 0.5M sucrose was added to the cell mixture and incubated at 37°C for 10-30 minutes. To stop the reaction, 75 μ l of 0.2M K_2HPO_4 was added. To assay for glucose liberated, 500 μ l of reconstituted reagent (glucose oxidase/peroxidase + o-Dianisidine) from Sigma (GAGO-20 assay kit) was added to each tube and incubated at 37°C for 30 minutes. A glucose standard curve was also created at this time (range: 25 μ g to 0.3 μ g glucose). After incubation, 500 μ l of 6N HCl was added to stop the reaction and to develop the color. The samples were read at 540nm. The amount of glucose liberated was calculated from the glucose standard curve using the formula $y=mx+c$, where y is the 540nm reading, and x is μ g of glucose. Weight of glucose was converted to moles of glucose, and given the equimolar relationship between moles of sucrose hydrolyzed to moles of glucose generated, the data was expressed as nmoles of sucrose hydrolyzed per unit time. The assay showed that both T98 and T97 clones were able to hydrolyze sucrose, indicating that a functional sucrose invertase was being produced and secreted by the cells.

[0399] For the sugar analysis on liquid culture media after 48 hours of algal growth, T97 and T98 cells were grown in 2% sucrose containing medium for 48 hours and the culture media were processed for sugar analysis. Culture broths from each transformant (and negative non-transformed cell control) were centrifuged at 14,000 rpm for 5 minutes. The resulting supernatant was removed and subjected to HPLC/ELSD (evaporative light scattering detection). The amount of sugar in each sample was determined using external standards and liner regression analysis. The sucrose levels in the culture media of the transformants were very low (less than 1.2g/L, and in most cases 0g/L). In the negative controls, the sucrose levels remained high, at approximately 19g/L after 48 hours of growth.

[0400] These results were consistent with the invertase activity results, and taken together, indicated that the codon optimized transformants, T97 and T98, secreted an active sucrose invertase that allowed the microalgae to utilize sucrose as the sole carbon source in contrast to (1) the non-codon optimized transformants and (2) the non-transformed wildtype microalgae, both of which could not utilize sucrose as the sole carbon source in the culture medium.

[0401] *Prototheca moriformis* strains, T98 and T97, expressing a functional, secreted sucrose invertase (SUC2) transgene were assayed for growth and lipid production using sucrose as the sole carbon source.

[0402] Wild type (untransformed), T98 and T97 strains were grown in growth media (as described above) containing either 4% glucose or 4% sucrose as the sole carbon source under heterotrophic conditions for approximately 6 days. Growth, as determined by A750 optical density readings were taken of all four samples every 24 hours and the dry cell weight of the cultures and lipid profiles were determined after the 6 days of growth. The optical density readings of the transgenic strains grown in both the glucose and sucrose conditions were comparable to the wildtype strains grown in the glucose conditions. These results indicate that the transgenic strains were able to grow on either glucose or sucrose as the sole carbon source at a rate equal to wildtype strains in glucose conditions. The non-transformed, wildtype strains did not grow in the sucrose-only condition.

[0403] The biomass for the wildtype strain grown on glucose and T98 strain grown on sucrose was analyzed for lipid profile. Lipid samples were prepared from dried biomass (lyophilized) using an Acid Hydrolysis System (Ankom Technology, NY) according to manufacturer's instructions. Lipid profile determinations were carried as described in Example 4. The lipid profile for the non-transformed *Prototheca moriformis* UTEX 1435 strain, grown on glucose as the sole carbon source and two clonal T98 strains (UTEX 1435 transformed with a sucrose invertase transgene), grown on sucrose as the sole carbon source, are disclosed in Table 13 (wildtype UTEX 1435 and T98 clone 8 and clone 11 below. C:19:0 lipid was used as an internal calibration control.

[0404] Table 13. Lipid profile of wildtype UTEX 1435 and UTEX 1435 clones with *suc2* transgene.

Name	wildtype (Area % - ISTD)	T98 clone 11 (Area % - ISTD)	T98 clone 8 (Area % - ISTD)
C 12:0	0.05	0.05	0.05
C 14:0	1.66	1.51	1.48
C 14:1	0.04	nd	nd
C 15:0	0.05	0.05	0.04
C 16:0	27.27	26.39	26.50
C 16:1	0.86	0.80	0.84
C 17:0	0.15	0.18	0.14
C 17:1	0.05	0.07	0.05
C 18:0	3.35	4.37	4.50
C 18:1	53.05	54.48	54.50
C 18:2	11.79	10.33	10.24
C 19:0 (ISTD)	--	--	--
C 18:3 alpha	0.90	0.84	0.81
C 20:0	0.32	0.40	0.38

C 20:1	0.10	0.13	0.12
C 20:1	0.04	0.05	0.04
C 22:0	0.12	0.16	0.12
C 20:3	0.07	0.08	0.07
C 24:0	0.12	0.11	0.10
nd - denotes none detected			

[0405] Oil extracted from wildtype *Prototheca moriformis* UTEX 1435 (via solvent extraction or using an expeller press (see methods in Example 44 above) was analyzed for carotenoids, chlorophyll, tocopherols, other sterols and tocotrienols. The results are summarized below in Table 14.

[0406] Table 14. Carotenoid, chlorophyll, tocopherol/sterols and tocotrienol analysis in oil extracted from *Prototheca moriformis* (UTEX 1435).

	Pressed oil (mcg/ml)	Solvent extracted oil (mcg/ml)
cis-Lutein	0.041	0.042
trans-Lutein	0.140	0.112
trans-Zeaxanthin	0.045	0.039
cis-Zeaxanthin	0.007	0.013
t-alpha-Cryptoxanthin	0.007	0.010
t-beta-Cryptoxanthin	0.009	0.010
t-alpha-Carotene	0.003	0.001
c-alpha-Carotene	none detected	none detected
t-beta-Carotene	0.010	0.009
9-cis-beta-Carotene	0.004	0.002
Lycopene	none detected	none detected
Total Carotenoids	0.267	0.238
Chlorophyll	<0.01 mg/kg	<0.01 mg/kg
Tocopherols and Sterols		
	Pressed oil (mg/100g)	Solvent extracted oil (mg/100g)
gamma Tocopherol	0.49	0.49
Campesterol	6.09	6.05
Stigmasterol	47.6	47.8
Beta-sitosterol	11.6	11.5
Other sterols	445	446
Tocotrienols		
	Pressed oil (mg/g)	Solvent extracted oil (mg/g)
alpha Tocotrienol	0.26	0.26
beta Tocotrienol	<0.01	<0.01
gamma Tocotrienol	0.10	0.10
delta Tocotrienol	<0.01	<0.01
Total Tocotrienols	0.36	0.36

[0407] The ability of using sucrose as the sole carbon source as the selection factor for clones containing the *suc2* transgene construct instead of G418 (or another antibiotic) was assessed using the positive *suc2* gene transformants. A subset of the positive transformants was grown on plates containing sucrose as the sole carbon source and without antibiotic

selection for 24 doublings. The clones were then challenged with plates containing glucose as the sole carbon source and G418. There was a subset of clones that did not grow on the glucose + G418 condition, indicating a loss of expression of the transgene. An additional experiment was performed using a plate containing sucrose as the sole carbon source and no G418 and streaking out a *suc2* transgene expressing clone on one half of the plate and wild-type *Prototheca moriformis* on the other half of the plate. Growth was seen with both the wild-type and transgene-containing *Prototheca moriformis* cells. Wild-type *Prototheca moriformis* has not demonstrated the ability to grow on sucrose, therefore, this result shows that unlike antibiotic resistance, the use of sucrose/invertase selection is not cell-autonomous. It is very likely that the transformants were secreting enough sucrose invertase into the plate/media to support wildtype growth as the sucrose was hydrolyzed into fructose and glucose.

[0408] EXAMPLE 4: Recombinant *Prototheca* with Exogenous TE Gene

[0409] As described above, *Prototheca* strains can be transformed with exogenous genes. *Prototheca moriformis* (UTEX 1435) was transformed, using methods described above, with either *Umbellularia californica* C12 thioesterase gene or *Cinnamomum camphora* C14 thioesterase gene (both codon optimized according to Table 1). Each of the transformation constructs contained a *Chlorella sorokiniana* glutamate dehydrogenase promoter/5'UTR region (SEQ ID NO: 69) to drive expression of the thioesterase transgene. The thioesterase transgenes coding regions of *Umbellularia californica* C12 thioesterase (SEQ ID NO: 70) or *Cinnamomum camphora* C14 thioesterase (SEQ ID NO: 71), each with the native putative plastid targeting sequence. Immediately following the thioesterase coding sequence is the coding sequence for a c-terminal 3x-FLAG tag (SEQ ID NO: 72), followed by the *Chlorella vulgaris* nitrate reductase 3'UTR (SEQ ID NO: 73). A diagram of the thioesterase constructs that were used in the *Prototheca moriformis* transformations is shown in Figure 9.

[0410] Preparation of the DNA, gold microcarrier and *Prototheca moriformis* (UTEX 1435) cells were performed using the methods described above in Example 3. The microalgae were bombarded using the gold microcarrier—DNA mixture and plated on selection plates containing 2% glucose and 100µg/ml G418. The colonies were allowed to develop for 7 to 12 days and colonies were picked from each transformation plate and screened for DNA construct incorporation using Southern blots assays and expression of the thioesterase constructs were screened using RT-PCR.

[0411] Positive clones were picked from both the C12 and C14 thioesterase transformation plates and screened for construct incorporation using Southern blot assays. Southern blot

assays were carried out using standard methods (and described above in Example 3) using an optimized c probes, based on the sequence in SEQ ID NO: 70 and SEQ ID NO: 71.

Transforming plasmid DNA was run as a positive control. Out of the clones that were positive for construct incorporation, a subset was selected for reverse transcription quantitative PCR (RT-qPCR) analysis for C12 thioesterase and C14 thioesterase expression.

[0412] RNA isolation was performed using methods described in Example 3 above and RT-qPCR of the positive clones were performed using 20 ng of total RNA from each clone using the below-described primer pair and iScript SYBR Green RT-PCR kit (Bio-Rad Laboratories) according to manufacturer's protocol. Wildtype (non-transformed) *Prototheca moriformis* total RNA was included as a negative control. mRNA expression was expressed as relative fold expression (RFE) as compared to negative control. . The primers that were used in the C12 thioesterase transformation RT-qPCR screening were:

[0413] *U. californica* C12 thioesterase PCR primers:

Forward: 5' CTGGGCGACGGCTTCGGCAC 3' (SEQ ID NO: 74)

Reverse: 5' AAGTCGCGGCGCATGCCGTT 3' (SEQ ID NO: 75)

[0414] The primers that were used in the C14 thioesterase transformation RT-qPCR screening were:

[0415] *Cinnamomum camphora* C14 thioesterase PCR primers:

Forward: 5' TACCCCGCCTGGGGCGACAC 3' (SEQ ID NO: 76)

Reverse: 5' CTTGCTCAGGCGGCGGGTGC 3' (SEQ ID NO: 77)

[0416] RT-qPCR results for C12 thioesterase expression in the positive clones showed an increased RFE of about 40 fold to over 2000 fold increased expression as compared to negative control. Similar results were seen with C14 thioesterase expression in the positive clones with an increase RFE of about 60-fold to over 1200 fold increased expression as compared to negative control.

[0417] A subset of the positive clones from each transformation (as screened by Southern blotting and RT-qPCR assays) were selected and grown under nitrogen-replete conditions and analyzed for total lipid production and profile. Lipid samples were prepared from dried biomass from each clone. 20-40 mg of dried biomass from each transgenic clone was resuspended in 2 mL of 3% H₂SO₄ in MeOH, and 200ul 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 65-70°C for two 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. Lipid profile (expressed as Area %) of the positive clones as compared to wildtype negative control are summarized in Tables 15 and 16 below. As shown in Table 15, the fold increase of C12 production in the C12 transformants ranged from about a 5-fold increase (clone C12-5) to over 11-fold increase (clone C12-1). Fold increase of C14 production in the C14 transformants ranged from about a 1.5 fold increase to about a 2.5 fold increase.

[0418] Table 15. Summary of total lipid profile of the *Prototheca moriformis* C12 thioesterase transformants.

	Wildtype	C12-1	C12-2	C12-3	C12-4	C12-5	C12-6	C12-7	C12-8
C6:0	0.03	nd	nd	nd	nd	nd	nd	nd	nd
C8:0	0.11	0.09	nd	0.11	nd	nd	nd	nd	nd
C10:0	nd	nd	nd	0.01	0.01	nd	nd	0.01	nd
C12:0	0.09	1.04	0.27	0.72	0.71	0.50	0.67	0.61	0.92
C14:0	2.77	2.68	2.84	2.68	2.65	2.79	2.73	2.56	2.69
C14:1	0.01	nd	nd	0.02	nd	nd	nd	0.01	nd
C15:0	0.30	0.09	0.10	0.54	0.19	0.09	0.13	0.97	0.09
C15:1	0.05	nd	nd	0.02	nd	nd	nd	nd	nd
C16:0	24.13	23.12	24.06	22.91	22.85	23.61	23.14	21.90	23.18
C16:1	0.57	0.62	0.10	0.52	0.69	0.63	0.69	0.49	0.63
C17:0	0.47	0.24	0.27	1.02	0.36	0.17	0.26	2.21	0.19
C17:1	0.08	nd	0.09	0.27	0.10	0.05	0.09	0.80	0.05
C18:0	nd	nd	2.14	1.75	2.23	2.16	2.38	1.62	2.47
C18:1	22.10	23.15	24.61	21.90	23.52	19.30	22.95	20.22	22.85
C18:1	nd	0.33	0.24	nd	nd	0.09	0.09	nd	0.11
C18:2	37.16	34.71	35.29	35.44	35.24	36.29	35.54	36.01	35.31
C18:3 alpha	11.68	11.29	9.26	11.62	10.76	13.61	10.64	11.97	10.81
C20:0	0.15	0.16	0.19	0.16	0.16	0.14	0.18	0.14	0.18
C20:1	0.22	0.17	0.19	0.20	0.21	0.19	0.21	0.20	0.21
C20:2	0.05	nd	0.04	0.05	0.05	0.05	0.04	0.05	0.04
C22:0	nd	nd	nd	0.01	nd	nd	nd	0.02	nd
C22:1	nd	nd	nd	nd	nd	0.01	nd	0.01	nd
C20:3	0.05	nd	0.07	0.06	0.06	0.10	0.07	0.05	0.06
C20:4	nd	nd	nd	nd	nd	0.02	nd	nd	nd
C24:0	nd	nd	0.24	0.01	0.20	0.19	0.19	0.14	0.20

[0419] Table 16. Summary of total lipid profile of the *Prototheca moriformis* C14 thioesterase transformants.

	Wildtype	C14-1	C14-2	C14-3	C14-4	C14-5	C14-6	C14-7
C6:0	0.03	nd	nd	nd	nd	nd	nd	nd
C8:0	0.11	nd	nd	nd	nd	nd	nd	nd
C10:0	nd	0.01	nd	0.01	nd	0.01	nd	nd
C12:0	0.09	0.20	0.16	0.25	0.21	0.19	0.40	0.17
C14:0	2.77	4.31	4.76	4.94	4.66	4.30	6.75	4.02
C14:1	0.01	nd	0.01	nd	nd	0.01	nd	nd
C15:0	0.30	0.43	0.45	0.12	0.09	0.67	0.10	0.33
C15:1	0.05	nd	nd	nd	nd	nd	nd	nd

C16:0	24.13	22.85	23.20	23.83	23.84	23.48	24.04	23.34
C16:1	0.57	0.65	0.61	0.60	0.60	0.47	0.56	0.67
C17:0	0.47	0.77	0.76	0.21	0.19	1.11	0.18	0.54
C17:1	0.08	0.23	0.15	0.06	0.05	0.24	0.05	0.12
C18:0	nd	1.96	1.46	2.48	2.34	1.84	2.50	2.06
C18:1	22.10	22.25	19.92	22.36	20.57	19.50	20.63	22.03
C18:1	nd	nd	nd	nd	nd	nd	0.10	nd
C18:2	37.16	34.97	36.11	34.35	35.70	35.49	34.03	35.60
C18:3	11.68	10.71	12.00	10.15	11.03	12.08	9.98	10.47
alpha								
C20:0	0.15	0.16	0.19	0.17	0.17	0.14	0.18	0.16
C20:1	0.22	0.20	0.12	.019	0.19	0.19	0.17	0.20
C20:2	0.05	0.04	0.02	0.03	0.04	0.05	0.03	0.04
C22:0	nd	nd	nd	nd	0.02	0.01	nd	nd
C22:1	nd	0.01	nd	nd	nd	nd	nd	0.01
C20:3	0.05	0.08	0.03	0.06	0.09	0.05	0.05	0.07
C20:4	nd	0.01	nd	nd	nd	nd	0.02	nd
C24:0	nd	0.17	0.14	0.19	0.20	0.16	0.22	0.17

[0420] The above-described experiments indicate the successful transformation of *Prototheca moriformis* (UTEX 1435) with transgene constructs of two different thioesterases (C12 and C14), which involved not only the successful expression of the transgene, but also the correct targeting of the expressed protein to the plastid and a functional effect (the expected change in lipid profile) as a result of the transformation. The same transformation experiment was performed using an expression construct containing a codon-optimized (according to Table 1) *Cuphea hookeriana* C8-10 thioesterase coding region with the native plastid targeting sequence (SEQ ID NO: 78) yielded no change in lipid profile. While the introduction of the *Cuphea hookeriana* C8-10 transgene into *Prototheca moriformis* (UTEX 1435) was successful and confirmed by Southern blot analysis, no change in C8 or C10 fatty acid production was detected in the transformants compared to the wildtype strain.

[0421] EXAMPLE 5: Generation of *Prototheca moriformis* strain with exogenous plant TE with algal plastid targeting sequence

[0422] In order to investigate whether the use of algal chloroplast/plastid targeting sequences would improve medium chain (C8-C14) thioesterase expression and subsequent medium chain lipid production in *Prototheca moriformis* (UTEX 1435), several putative algal plastid targeting sequences were cloned from *Chlorella protothecoides* and *Prototheca moriformis*. Thioesterase constructs based on *Cuphea hookeriana* C8-10 thioesterase, *Umbellularia californica* C12 thioesterase, and *Cinnamomum camphora* C14 thioesterase were made using made with a *Chlorella sorokiniana* glutamate dehydrogenase promoter/5'UTR and a *Chlorella vulgaris* nitrate reductase 3'UTR. The thioesterase coding sequences were modified by removing the native plastid targeting sequences and replacing

them with plastid targeting sequences from the *Chlorella protothecoides* and the *Prototheca moriformis* genomes. The thioesterase expression constructs and their corresponding sequence identification numbers are listed below. Each transformation plasmid also contained a Neo resistance construct that was identical to the ones described in Example 3 above. Additionally, another algal-derived promoter, the *Chlamydomonas reinhardtii* β -tubulin promoter, was also tested in conjunction with the thioesterase constructs. “Native” plastid targeting sequence refers to the higher plant thioesterase plastid targeting sequence. A summary of the constructs used in these experiments is provided below:

Construct Name	Promoter/ 5'UTR	Plastid targeting seq	Gene	3'UTR	SEQ ID NO.
Construct 1	<i>C. sorokiniana</i> glutamate dehydrogenase	<i>C. protothecoides</i> stearyl ACP desaturase	<i>Cuphea hookeriana</i> C8-10 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 79
Construct 2	<i>C. sorokiniana</i> glutamate dehydrogenase	<i>P. moriformis</i> delta 12 fatty acid desaturase	<i>Cuphea hookeriana</i> C8-10 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 80
Construct 3	<i>C. sorokiniana</i> glutamate dehydrogenase	<i>P. moriformis</i> isopentenyl diphosphate synthase	<i>Cuphea hookeriana</i> C8-10 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 81
Construct 4	<i>C. sorokiniana</i> glutamate dehydrogenase	<i>P. moriformis</i> isopentenyl diphosphate synthase	<i>Umbellularia californica</i> C12 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 82
Construct 5	<i>C. sorokiniana</i> glutamate dehydrogenase	<i>P. moriformis</i> stearyl ACP desaturase	<i>Umbellularia californica</i> C12 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 83
Construct 6	<i>C. sorokiniana</i> glutamate dehydrogenase	<i>C. protothecoides</i> stearyl ACP desaturase	<i>Umbellularia californica</i> C12 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 84
Construct 7	<i>C. sorokiniana</i> glutamate dehydrogenase	<i>P. moriformis</i> delta 12 fatty acid desaturase	<i>Umbellularia californica</i> C12 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 85
Construct 8	<i>C. sorokiniana</i> glutamate dehydrogenase	<i>C. protothecoides</i> stearyl ACP desaturase	<i>Cinnamomum camphora</i> C14 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 86
Construct 9	<i>Chlamydomonas reinhardtii</i> β -tubulin	Native	<i>Cuphea hookeriana</i> C8-10 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 113
Construct 10	<i>Chlamydomonas reinhardtii</i> β -tubulin	<i>P. moriformis</i> isopentenyl diphosphate synthase	<i>Cuphea hookeriana</i> C8-10 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 114
Construct 11	<i>Chlamydomonas</i>	<i>P. moriformis</i>	<i>Cuphea</i>	<i>C. vulgaris</i>	SEQ ID NO: 115

	<i>reinhardtii</i> β -tubulin	delta 12 fatty acid desaturase	<i>hookeriana</i> C8-10 TE	nitrate reductase	
Construct 12	<i>Chlamydomonas reinhardtii</i> β -tubulin	<i>C. protothecoides</i> stearoyl ACP desaturase	<i>Cuphea hookeriana</i> C8-10 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 116
Construct 13	<i>Chlamydomonas reinhardtii</i> β -tubulin	<i>P. moriformis</i> stearoyl ACP desaturase	<i>Cuphea hookeriana</i> C8-10 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 117
Construct 14	<i>Chlamydomonas reinhardtii</i> β -tubulin	Native	<i>Umbellularia californica</i> C12 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 118
Construct 15	<i>Chlamydomonas reinhardtii</i> β -tubulin	<i>P. moriformis</i> isopentenyl diphosphate	<i>Umbellularia californica</i> C12 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 119
Construct 16	<i>Chlamydomonas reinhardtii</i> β -tubulin	<i>P. moriformis</i> delta 12 fatty acid desaturase	<i>Umbellularia californica</i> C12 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 120
Construct 17	<i>Chlamydomonas reinhardtii</i> β -tubulin	<i>C. protothecoides</i> stearoyl ACP desaturase	<i>Umbellularia californica</i> C12 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 121
Construct 18	<i>Chlamydomonas reinhardtii</i> β -tubulin	<i>P. moriformis</i> stearoyl ACP desaturase	<i>Umbellularia californica</i> C12 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 122
Construct 19	<i>Chlamydomonas reinhardtii</i> β -tubulin	Native	<i>Cinnamomum camphora</i> C14 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 123
Construct 20	<i>Chlamydomonas reinhardtii</i> β -tubulin	<i>P. moriformis</i> isopentenyl diphosphate synthase	<i>Cinnamomum camphora</i> C14 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 124
Construct 21	<i>Chlamydomonas reinhardtii</i> β -tubulin	<i>P. moriformis</i> delta 12 fatty acid desaturase	<i>Cinnamomum camphora</i> C14 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO:
Construct 22	<i>Chlamydomonas reinhardtii</i> β -tubulin	<i>C. protothecoides</i> stearoyl ACP desaturase	<i>Cinnamomum camphora</i> C14 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 87
Construct 23	<i>Chlamydomonas reinhardtii</i> β -tubulin	<i>P. moriformis</i> stearoyl ACP desaturase	<i>Cinnamomum camphora</i> C14 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 88

[0423] Each construct was transformed into *Prototheca moriformis* (UTEX 1435) and selection was performed using G418 using the methods described in Example 4 above. Several positive clones from each transformation were picked and screened for the presence thioesterase transgene using Southern blotting analysis. Expression of the thioesterase

transgene was confirmed using RT-PCR. A subset of the positive clones (as confirmed by Southern blotting analysis and RT-PCR) from each transformation was selected and grown for lipid profile analysis. Lipid samples were prepared from dried biomass samples of each clone and lipid profile analysis was performed using acid hydrolysis methods described in Example 4. Changes in area percent of the fatty acid corresponding to the thioesterase transgene were compared to wildtype levels, and clones transformed with a thioesterase with the native plastid targeting sequence.

[0424] As mentioned in Example 4, the clones transformed with *Cuphea hookeriana* C8-10 thioesterase constructs with the native plastid targeting sequence had the same level of C8 and C10 fatty acids as wildtype. The clones transformed with *Cuphea hookeriana* C8-10 thioesterase constructs (Constructs 1-3) with algal plastid targeting sequences had over a 10-fold increase in C10 fatty acids for Construct 3 and over 40-fold increase in C10 fatty acids for Constructs 1 and 2 (as compared to wildtype). The clones transformed with *Umbellularia californica* C12 thioesterase constructs with the native plastid targeting sequence had a modest 6-8 fold increase in C12 fatty acid levels as compared to wildtype. The clones transformed with the *Umbellularia californica* C12 thioesterase constructs with the algal plastid targeting constructs (Constructs 4-7) had over an 80-fold increase in C12 fatty acid level for Construct 4, about a 20-fold increase in C12 fatty acid level for Construct 6, about a 10-fold increase in C12 fatty acid level for Construct 7 and about a 3-fold increase in C12 fatty acid level for Construct 5 (all compared to wildtype). The clones transformed with *Cinnamomum camphora* C14 thioesterase with either the native plastid targeting sequence or the construct 8 (with the *Chlorella protothecoides* stearyl ACP desaturase plastid targeting sequence) had about a 2-3 fold increase in C14 fatty acid levels as compared to wildtype. In general clones transformed with an algal plastid targeting sequence thioesterase constructs had a higher fold increase in the corresponding chain-length fatty acid levels than when using the native higher plant targeting sequence.

A. *Chlamydomonas reinhardtii* β -tubulin promoter

[0425] Additional heterologous thioesterase expression constructs were prepared using the *Chlamydomonas reinhardtii* β - tubulin promoter instead of the *C. sorokiniana* glutamate dehydrogenase promoter. The construct elements and sequence of the expression constructs are listed above. Each construct was transformed into *Prototheca moriformis* UTEX 1435 host cells using the methods described above. Lipid profiles were generated from a subset of positive clones for each construct in order to assess the success and productivity of each construct. The lipid profiles compare the fatty acid levels (expressed in area %) to wildtype

host cells. The “Mean” column represents the numerical average of the subset of positive clones. The “Sample” column represents the best positive clone that was screened (best being defined as the sample that produced the greatest change in area % of the corresponding chain-length fatty acid production). The “low-high” column represents the lowest area % and the highest area % of the fatty acid from the clones that were screened. The lipid profiles results of Constructs 9-23 are summarized below.

Construct 9. <i>Cuphea hookeriana</i> C8-10 TE				
Fatty Acid	wildtype	Mean	Sample	low/high
C 8:0	0	0.05	0.30	0-0.29
C 10:0	0.01	0.63	2.19	0-2.19
C 12:0	0.03	0.06	0.10	0-0.10
C 14:0	1.40	1.50	1.41	1.36-3.59
C 16:0	24.01	24.96	24.20	
C 16:1	0.67	0.80	0.85	
C 17:0	0	0.16	0.16	
C 17:1	0	0.91	0	
C 18:0	4.15	17.52	3.19	
C 18:1	55.83	44.81	57.54	
C 18:2	10.14	7.58	8.83	
C 18:3 α	0.93	0.68	0.76	
C 20:0	0.33	0.21	0.29	
C 24:0	0	0.05	0.11	

Construct 10. <i>Cuphea hookeriana</i> C8-10 TE				
Fatty Acid	wildtype	Mean	Sample	low/high
C 8:0	0	0.01	0.02	0-0.03
C 10:0	0	0.16	0.35	0-0.35
C 12:0	0.04	0.05	0.07	0-0.07
C 14:0	1.13	1.62	1.81	0-0.05
C 14:1	0	0.04	0.04	
C 15:0	0.06	0.05	0.05	
C 16:0	19.94	26.42	28.08	
C 16:1	0.84	0.96	0.96	
C 17:0	0.19	0.14	0.13	
C 17:1	0.10	0.06	0.05	
C 18:0	2.68	3.62	3.43	
C 18:1	63.96	54.90	53.91	
C 18:2	9.62	9.83	9.11	
C18:3 γ	0	0.01	0	
C 18:3 α	0.63	0.79	0.73	
C 20:0	0.26	0.35	0.33	
C 20:1	0.06	0.08	0.09	
C 20:1	0.08	0.06	0.07	
C 22:0	0	0.08	0.09	
C 24:0	0.13	0.13	0.11	

Construct 11. <i>Cuphea hookeriana</i> C8-10 TE				
Fatty Acid	wildtype	Mean	Sample	low/high
C 8:0	0	0.82	1.57	0-1.87
C 10:0	0	3.86	6.76	0-6.76

C 12:0	0.04	0.13	0.20	0.03-0.20
C 14:0	1.13	1.80	1.98	1.64-2.05
C 14:1	0	0.04	0.04	
C 15:0	0.06	0.06	0.06	
C 16:0	19.94	25.60	25.44	
C 16:1	0.84	1.01	1.02	
C 17:0	0.19	0.13	0.11	
C 17:1	0.10	0.06	0.05	
C 18:0	2.68	2.98	2.38	
C 18:1	63.96	51.59	48.85	
C 18:2	9.62	9.85	9.62	
C18:3 γ	0	0.01	0	
C 18:3 α	0.63	0.91	0.92	
C 20:0	0.26	0.29	0.26	
C 20:1	0.06	0.06	0	
C 20:1	0.08	0.06	0.03	
C 22:0	0	0.08	0.08	
C 24:0	0.13	0.06	0	

Construct 12. <i>Cuphea hookeriana</i> C8-10 TE				
Fatty Acid	wildtype	Mean	Sample	low/high
C 8:0	0	0.31	0.85	0-0.85
C 10:0	0	2.16	4.35	0.20-4.35
C 12:0	0.04	0.10	0.15	0-0.18
C 14:0	1.13	1.96	1.82	1.66-2.97
C 14:1	0	0.03	0.04	
C 15:0	0.06	0.07	0.07	
C 16:0	19.94	26.08	25.00	
C 16:1	0.84	1.04	0.88	
C 17:0	0.19	0.16	0.16	
C 17:1	0.10	0.05	0.07	
C 18:0	2.68	3.02	3.19	
C 18:1	63.96	51.08	52.15	
C 18:2	9.62	11.44	9.47	
C18:3 γ	0	0.01	0	
C 18:3 α	0.63	0.98	0.90	
C 20:0	0.26	0.30	0.28	
C 20:1	0.06	0.06	0.05	
C 20:1	0.08	0.04	0	
C 22:0	0	0.07	0	
C 24:0	0.13	0.05	0	

Construct 14. <i>Umbellularia californica</i> C12 TE				
Fatty Acid	wildtype	Mean	Sample	low/high
C 10:0	0.01	0.02	0.03	0.02-0.03
C 12:0	0.03	2.62	3.91	0.04-3.91
C 14:0	1.40	1.99	2.11	1.83-2.19
C 16:0	24.01	27.64	27.01	
C 16:1	0.67	0.92	0.92	

C 18:0	4.15	2.99	2.87	
C 18:1	55.83	53.22	52.89	
C 18:2	10.14	8.68	8.41	
C 18:3 α	0.93	0.78	0.74	
C 20:0	0.33	0.29	0.27	

Construct 15. <i>Umbellularia californica</i> C12 TE				
Fatty Acid	wildtype	Mean	Sample	low/high
C 10:0	0	0.05	0.08	0-0.08
C 12:0	0.04	8.12	12.80	4.35-12.80
C 13:0	0	0.02	0.03	0-0.03
C 14:0	1.13	2.67	3.02	2.18-3.37
C 14:1	0	0.04	0.03	0.03-0.10
C 15:0	0.06	0.07	0.06	
C 16:0	19.94	25.26	23.15	
C 16:1	0.84	0.99	0.86	
C 17:0	0.19	0.14	0.14	
C 17:1	0.10	0.05	0.05	
C 18:0	2.68	2.59	2.84	
C 18:1	63.96	46.91	44.93	
C 18:2	9.62	10.59	10.01	
C 18:3 α	0.63	0.92	0.83	
C 20:0	0.26	0.27	0.24	
C 20:1	0.06	0.06	0.06	
C 20:1	0.08	0.05	0.04	
C 22:0	0	0.07	0.09	
C 24:0	0.13	0.13	0.12	

Construct 16. <i>Umbellularia californica</i> C12 TE				
Fatty Acid	wildtype	Mean	Sample	low/high
C 10:0	0	0.03	0.04	0.02-0.04
C 12:0	0.04	2.43	5.32	0.98-5.32
C 13:0	0	0.01	0.02	0-0.02
C 14:0	1.13	1.77	1.93	1.62-1.93
C 14:1	0	0.03	0.02	0.02-0.04
C 15:0	0.06	0.06	0.05	
C 16:0	19.94	24.89	22.29	
C 16:1	0.84	0.91	0.82	
C 17:0	0.19	0.16	0.15	
C 17:1	0.10	0.06	0.06	
C 18:0	2.68	3.81	3.67	
C 18:1	63.96	53.19	52.82	
C 18:2	9.62	10.38	10.57	
C 18:3 α	0.63	0.80	0.77	
C 20:0	0.26	0.35	0.32	
C 20:1	0.06	0.06	0.07	
C 20:1	0.08	0.07	0.08	
C 22:0	0	0.08	0.07	
C 24:0	0.13	0.15	0.14	

Construct 17. <i>Umbellularia californica</i> C12 TE				
Fatty Acid	wildtype	Mean	Sample	low/high
C 10:0	0	0.04	0.07	0.03-0.08
C 12:0	0.04	7.02	14.11	4.32-14.11
C 13:0	0	0.03	0.04	0.01-0.04
C 14:0	1.13	2.25	3.01	1.95-3.01
C 14:1	0	0.03	0.03	0.02-0.03
C 15:0	0.06	0.06	0.06	
C 16:0	19.94	23.20	21.46	
C 16:1	0.84	0.82	0.77	
C 17:0	0.19	0.15	0.14	
C 17:1	0.10	0.06	0.06	
C 18:0	2.68	3.47	2.93	
C 18:1	63.96	50.30	45.17	
C 18:2	9.62	10.33	9.98	
C18:3 γ	0	0.01	0	
C 18:3 α	0.63	0.84	0.86	
C 20:0	0.26	0.32	0.27	
C 20:1	0.06	0.07	0.06	
C 20:1	0.08	0.06	0.06	
C 22:0	0	0.08	0.09	
C 24:0	0.13	0.14	0.13	

Construct 18. <i>Umbellularia californica</i> C12 TE				
Fatty Acid	wildtype	Mean	Sample	low/high
C 10:0	0	0.03	0.05	0.01-0.05
C 12:0	0.04	5.06	7.77	0.37-7.77
C 13:0	0	0.02	0	0-0.03
C 14:0	1.13	2.11	2.39	1.82-2.39
C 14:1	0	0.03	0.03	0.02-0.05
C 15:0	0.06	0.06	0.06	
C 16:0	19.94	24.60	23.95	
C 16:1	0.84	0.86	0.83	
C 17:0	0.19	0.15	0.14	
C 17:1	0.10	0.06	0.05	
C 18:0	2.68	3.31	2.96	
C 18:1	63.96	51.26	49.70	
C 18:2	9.62	10.18	10.02	
C18:3 γ	0	0.01	0.02	
C 18:3 α	0.63	0.86	0.86	
C 20:0	0.26	0.32	0.29	
C 20:1	0.06	0.05	0.05	
C 20:1	0.08	0.07	0.04	
C 22:0	0	0.08	0.08	
C 24:0	0.13	0.13	0.13	

Construct 19. <i>Cinnamomum camphora</i> C14 TE				
Fatty Acid	wildtype	Mean	Sample	low/high
C 10:0	0.02	0.01	0.01	0.01-0.02
C 12:0	0.05	0.27	0.40	0.08-0.41
C 14:0	1.52	4.47	5.81	2.10-5.81
C 16:0	25.16	28.14	28.55	
C 16:1	0.72	0.84	0.82	
C 18:0	3.70	3.17	2.87	
C 18:1	54.28	51.89	51.01	
C 18:2	12.24	9.36	8.62	
C 18:3 α	0.87	0.74	0.75	
C 20:0	0.33	0.33	0.31	

Construct 20. <i>Cinnamomum camphora</i> C14 TE				
Fatty Acid	wildtype	Mean	Sample	low/high
C 10:0	0.01	0.01	0.02	0.01-0.02
C 12:0	0.03	0.39	0.65	0.08-0.65
C 13:0	0	0.01	0.01	0.01-0.02
C 14:0	1.40	5.61	8.4	2.1-8.4
C 14:1	0	0.03	0.03	0.02-0.03
C 15:0	0	0.06	0.07	
C 16:0	24.01	25.93	25.57	
C 16:1	0.67	0.75	0.71	
C 17:0	0	0.13	0.12	
C 17:1	0	0.05	0.05	
C 18:0	4.15	3.30	3.23	
C 18:1	55.83	51.00	48.48	
C 18:2	10.14	10.38	10.35	
C 18:3 α	0.93	0.91	0.88	
C 20:0	0.33	0.35	0.32	
C 20:1	0	0.08	0.08	
C 20:1	0	0.07	0.07	
C 22:0	0	0.08	0.08	
C 24:0	0	0.14	0.13	

Construct 21. <i>Cinnamomum camphora</i> C14 TE				
Fatty Acid	wildtype	Mean	Sample	low/high
C 10:0	0.01	0.01	0.01	0-0.01
C 12:0	0.03	0.10	0.27	0.04-0.27
C 14:0	1.40	2.28	4.40	1.47-4.40
C 16:0	24.01	26.10	26.38	
C 16:1	0.67	0.79	0.73	
C 17:0	0	0.15	0.16	
C 17:1	0	0.06	0.06	
C 18:0	4.15	3.59	3.51	
C 18:1	55.83	53.53	50.86	
C 18:2	10.14	10.83	11.11	

C 18:3 α	0.93	0.97	0.87	
C 20:0	0.33	0.36	0.37	
C 20:1	0	0.09	0.08	
C 20:1	0	0.07	0.07	
C 22:0	0	0.09	0.09	

Construct 22. <i>Cinnamomum camphora</i> C14 TE				
Fatty Acid	wildtype	Mean	Sample	low/high
C 10:0	0.01	0.02	0.02	0.02-0.02
C 12:0	0.03	1.22	1.83	0.59-1.83
C 13:0	0	0.02	0.03	0.01-0.03
C 14:0	1.40	12.77	17.33	7.97-17.33
C 14:1	0	0.02	0.02	0.02-0.04
C 15:0	0	0.07	0.08	
C 16:0	24.01	24.79	24.22	
C 16:1	0.67	0.64	0.58	
C 17:0	0	0.11	0.10	
C 17:1	0	0.04	0.04	
C 18:0	4.15	2.85	2.75	
C 18:1	55.83	45.16	41.23	
C 18:2	10.14	9.96	9.65	
C 18:3 α	0.93	0.91	0.85	
C 20:0	0.33	0.30	0.30	
C 20:1	0	0.07	0.06	
C 20:1	0	0.06	0.05	
C 22:0	0	0.08	0.08	

Construct 23. <i>Cinnamomum camphora</i> C14 TE				
Fatty Acid	wildtype	Mean	Sample	low/high
C 10:0	0.01	0.01	0.02	0-0.02
C 12:0	0.05	0.57	1.08	0.16-1.08
C 13:0	0	0.02	0.02	0-0.02
C 14:0	1.45	7.18	11.24	2.96-11.24
C 14:1	0.02	0.03	0.03	0.02-0.03
C 15:0	0.06	0.07	0.07	
C 16:0	24.13	25.78	25.21	
C 16:1	0.77	0.72	0.66	
C 17:0	0.19	0.13	0.11	
C 17:1	0.08	0.05	0.04	
C 18:0	3.53	3.35	3.12	
C 18:1	56.15	49.65	46.35	
C 18:2	11.26	10.17	9.72	
C 18:3 α	0.84	0.95	0.83	
C 20:0	0.32	0.34	0.32	
C 20:1	0.09	0.08	0.09	
C 20:1	0.07	0.05	0.06	
C 22:0	0.07	0.08	0.08	
C 24:0	0.13	0.13	0.12	

[0426] Constructs 9-13 were expression vectors containing the *Cuphea hookeriana* C8-10 thioesterase construct. As can be seen in the data summaries above, the best results were seen with Construct 11, with the Sample C8 fatty acid being 1.57 Area % (as compared to 0 in wildtype) and C10 fatty acid being 6.76 Area % (as compared to 0 in wildtype). There was also a modest increase in C12 fatty acids (approximately 2-5 fold increase). While the native plastid targeting sequence produced no change when under the control of the *C.sorokinana* glutamate dehydrogenase promoter, the same expression construct driven by the *C.reinhardtii* β -tubulin promoter produced significant changes in C8-10 fatty acids in the host cell. This is further evidence of the idiosyncrasies of heterologous expression of thioesterases in *Prototheca* species. All of the clones containing the *C.reinhardtii* β -tubulin promoter C8-10 thioesterase construct had greater increases in C8-10 fatty acids than the clones containing the *C.sorokinana* glutamate dehydrogenase promoter C8-10 thioesterase construct. Lipid profile data for Construct 13 was not obtained and therefore, not included above.

[0427] Constructs 14-18 were expression vectors containing the *Umbellularia californica* C12 thioesterase construct. As can be seen in the data summaries above, the best results were seen with Constructs 15 (*P.moriformis* isopentenyl diphosphate synthase plastid targeting sequence) and 17 (*C.protothecoides* stearoyl ACP desaturase plastid targeting sequence). The greatest change in C12 fatty acid production was seen with Construct 17, with C12 fatty acids levels of up to 14.11 area %, as compared to 0.04 area % in wildtype. Modest changes (about 2-fold) were also seen with C14 fatty acid levels. When compared to the same constructs with the *C.sorokinana* glutamate dehydrogenase promoter, the same trends were true with the *C.reinhardtii* β -tubulin promoter—the *C.protothecoides* stearoyl ACP desaturase and *P.moriformis* isopentenyl diphosphate synthase plastid targeting sequences produced the greatest change in C12 fatty acid levels with both promoters.

[0428] Constructs 19-23 were expression vectors containing the *Cinnamomum camphora* C14 thioesterase construct. As can be seen in the data summaries above, the best results were seen with Constructs 22 and Construct 23. The greatest change in C14 fatty acid production was seen with Construct 22, with C14 fatty acid levels of up to 17.35 area % (when the values for C140 and C141 are combined), as compared to 1.40% in wildtype. Changes in C12 fatty acids were also seen (5-60 fold). When compared to the same constructs with the *C.sorokinana* glutamate dehydrogenase promoter, the same trends were true with the *C.reinhardtii* β -tubulin promoter—the *C.protothecoides* stearoyl ACP desaturase and *P.moriformis* stearoyl ACP desaturase plastid targeting sequences produced the greatest

change in C14 fatty acid levels with both promoters. Consistently with all thioesterase expression constructs, the *C.reinhardtii* β -tubulin promoter constructs produced greater changes in C8-14 fatty acid levels than the *C.sorokiniana* glutamate dehydrogenase

[0429] Two positive clones from the Construct 22 were selected and grown under high selective pressure (50mg/L G418). After 6 days in culture, the clones were harvested and their lipid profile was determined using the methods described above. The lipid profile data is summarized below and is expressed in area %.

Construct 22 clones + 50mg/L G418		
Fatty Acid	Construct 22 A	Construct 22 B
C 12:0	3.21	3.37
C 14:0	27.55	26.99
C 16:0	25.68	24.37
C 16:1	0.99	0.92
C 18:0	1.37	1.23
C 18:1	28.35	31.07
C 18:2	11.73	11.05
C 18:3 α	0.92	0.81
C 20:0	0.16	0.17

[0430] Both clones, when grown under constant, high selective pressure, produced an increased amount of C14 and C12 fatty acids, about double the levels seen with Construct 22 above. These clones yielded over 30 area % of C12-14 fatty acids, as compared to 1.5 area % of C12-14 fatty acids seen in wildtype cells.

[0431] **EXAMPLE 6: Heterologous Expression of *Cuphea palustris* and *Ulmus americana* Thioesterase in *Prototheca***

[0432] Given the success of the above-described heterologous expression thioesterases in *Prototheca* species, expression cassettes containing codon-optimized (according to Table 1) sequences encoding fatty acyl-ACP thioesterases from *Cuphea palustris* and *Ulmus americana* were constructed and described below.

Construct Name	Promoter/ 5'UTR	Plastid targeting seq	Gene	3'UTR	SEQ ID NO.
Construct 27	<i>C.reinhardtii</i> β -tubulin	<i>C.protothecoides</i> stearoyl ACP desaturase	<i>Cuphea palustris</i> thioesterase	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 107

[0433] The *Ulmus americana* (codon-optimized coding sequence) can be inserted into the expression cassette. The codon-optimized coding sequence without the native plastid targeting sequence for the *Ulmus americana* thioesterase is listed as SEQ ID NO: 108 and can be fused any desired plastid targeting sequence and expression element (i.e., promoter/5'UTR and 3'UTR).

[0434] These expression cassettes can be transformed in to *Prototheca* species using the methods described above. Positive clones can be screened with the inclusion of an antibiotic resistance gene (e.g, neoR) on the expression construct and screened on G418-containing plates/media. Positive clones can be confirmed using Southern blot assays with probes specific to the heterologous thioesterase coding region and expression of the construct can also be confirmed using RT-PCR and primers specific to the coding region of the heterologous thioesterase. Secondary confirmation of positive clones can be achieved by looking for changes in levels of fatty acids in the host cell's lipid profile. As seen in the above Examples, heterologous expression in *Prototheca* species of thioesterase can be idiosyncratic to the particular thioesterase. Promoter elements and plastid targeting sequences (and other expression regulatory elements) can be interchanged until the expression of the thioesterase (and the subsequent increase in the corresponding fatty acid) reaches a desired level.

[0435] EXAMPLE 7: Dual Transformants—Simultaneous Expression of Two Heterologous Proteins

[0436] Microalgae strain *Prototheca moriformis* (UTEX 1435) was transformed using the above disclosed methods with a expression construct containing the yeast sucrose invertase *suc2* gene encoding the secreted form of the *S. cerevisiae* invertase. Successful expression of this gene and targeting to the periplasm results in the host cell's ability to grow on (and utilize) sucrose as a sole carbon source in heterotrophic conditions (as demonstrated in Example 3 above). The second set of genes expressed are thioesterases which are responsible for the cleavage of the acyl moiety from the acyl carrier protein. Specifically, thioesterases from *Cuphea hookeriana* (a C8-10 preferring thioesterase), *Umbellularia californica* (a C12 preferring thioesterase), and *Cinnamomum camphora* (a C14 preferring thioesterase). These thioesterase expression cassettes were cloned as fusions with N-terminal microalgal plastid targeting sequences from either *Prototheca moriformis* or *Chlorella protothecoides*, which have been shown (in the above Examples) to be more optimal than the native higher plant plastid targeting sequences. The successful expression of the thioesterase genes and the targeting to the plastid resulted in measurable changes in the fatty acid profiles within the host cell. These changes in profiles are consistent with the enzymatic specificity or preference of each thioesterase. Below is a summary of dual expression constructs that were assembled and transformed into *Prototheca moriformis* (UTEX 1435). Each construct contained the yeast *suc2* gene under the control of the *C. reinhardtii* β -tubulin 5'UTR/promoter and contained the *C. vulgaris* nitrate reductase 3'UTR and a higher plant thioesterase with a

microalgal plastid targeting sequence replacing the native sequence under the control of *C. sorokiniana* glutamate dehydrogenase 5'UTR and contained the *C. vulgaris* nitrate reductase 3'UTR. Below is a summary of the thioesterase portion of the constructs that were assembled and transformed into *Prototheca moriformis* (UTEX 1435). The entire dual expression cassette with the *suc2* gene and the thioesterase gene and the is listed in the Sequence Identification Listing.

Construct Name	Promoter/ 5'UTR	Plastid targeting seq	Gene	3'UTR	SEQ ID NO.
Construct 24	<i>C. sorokiniana</i> glutamate dehydrogenase	<i>C. protothecoides</i> stearyl ACP desaturase	<i>Cuphea hookeriana</i> C8-10 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 109
Construct 25	<i>C. sorokiniana</i> glutamate dehydrogenase	<i>P. moriformis</i> isopentenyl diphosphate synthase	<i>Umbellularia californica</i> C12 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 110
Construct 26	<i>C. sorokiniana</i> glutamate dehydrogenase	<i>C. protothecoides</i> stearyl ACP desaturase	<i>Cinnamomum camphora</i> C14 TE	<i>C. vulgaris</i> nitrate reductase	SEQ ID NO: 111

Similar dual expression constructs with the thioesterase cassettes described in Example 5 (e.g., under the control of a different promoter such as *C. reinhardtii* β -tubulin promoter/5'UTR) can also be generated using standard molecular biology methods and methods described herein.

[0437] Positive clones containing each of expression constructs were screened using their ability to grow on sucrose-containing plates, where sucrose is the sole-carbon source, as the selection factor. A subset of these positive clones from each construct transformation was selected and the presence of the expression construct was confirmed using Southern blot assays. The function of the yeast sucrose invertase was also confirmed using a sucrose hydrolysis assay. Positive clones were selected and grown in media containing sucrose as the sole carbon source at a starting concentration of 40g/L. A negative control of wildtype *Prototheca moriformis* (UTEX 1435) grown in media containing glucose as the sole carbon source at the same 40 g/L starting concentration was also included. Utilization of sucrose was measured throughout the course of the experiment by measuring the level of sucrose in the media using a YSI 2700 Biochemistry Analyzer with a sucrose-specific membrane. After six days in culture, the cultures were harvested and processed for lipid profile using the same methods as described above. The lipid profile results are summarized below in Table 17 and are show in area %.

[0438] Table 17. Lipid profiles of dual transformants with *suc2* sucrose invertase and thioesterase.

Fatty Acid	Wt	C24 A	C24 B	C24 C	C25 A	C25 B	C25 C	C26 A	C26 B	C26 C
C 10:0	0.01	0.03	0.04	0.08	0.01	0.01	0.01	0.01	0.01	0.0
C 12:0	0.04	0.04	0.04	0.04	0.28	0.40	0.10	0.04	0.04	0.13
C 14:0	1.6	1.55	1.53	1.56	1.59	1.59	1.60	1.65	1.56	2.69
C 14:1	0.03	0.03	0.03	0.02	0.03	0.03	0.03	0.03	0.03	0.03
C 15:0	0.04	0.03	0.03	0.04	0.04	0.03	0.03	0.03	0.03	0.04
C 16:0	29.2	29.1	29.0	28.6	28.9	28.6	29.0	28.8	29.5	27.5
C 16:1	0.86	0.80	0.79	0.82	0.77	0.81	0.82	0.79	0.79	0.86
C 17:0	0.1	0.08	0.08	0.09	0.09	0.08	0.09	0.08	0.08	0.09
C 17:1	0.04	0.03	0.03	0.04	0.03	0.03	0.03	0.03	0.03	0.04
C 18:0	3.26	3.33	3.37	3.27	3.36	3.28	3.18	3.33	3.36	3.03
C 18:1	54.5	53.9	54.1	53.9	53.5	53.7	53.5	54.2	53.9	52.7
C 18:2	8.72	9.35	9.22	9.45	9.68	9.65	9.87	9.31	9.06	10.8
C 18:3 alpha	0.63	0.71	0.69	0.73	0.74	0.73	0.75	0.71	0.66	0.83
C 20:0	0.29	0.31	0.31	0.31	0.32	0.32	0.31	0.32	0.31	0.29

[0439] All of the positive clones selected for the sucrose utilization assay were able to hydrolyze the sucrose in the media and at the end of the 6 day culture period, there were no measurable levels of sucrose in the media. This data, in addition to the successful use of sucrose as a selection tool for positive clones, indicates that the exogenous yeast *suc2* sucrose invertase gene was targeted correctly and expressed in the transformants. As show in Table 17 above, the clones expressing Construct 24 (C8-10 thioesterase) had a measurable increase in C10 fatty acids (as high as an eight-fold increase). Likewise there were measurable increases in clones expressing Construct 25 (C12 thioesterase) and Construct 26 (C14 thioesterase) in the corresponding medium chain fatty acids. Taken together, the data shows the successful simultaneous expression in *Prototheca moriformis* two recombinant proteins (e.g., sucrose invertase and a fatty acid acyl-ACP thioesterase), both of which confer useful and quantifiable phenotypic changes on the host organism.

[0440] **EXAMPLE 8: Effects of glycerol on C10-C14 fatty acid production in C14 thioesterase transformants**

[0441] Clones from all the thioesterase transformations were selected and further evaluated. One clone expressing Construct 8 (*Cinnamomum camphora* C14 TE) was grown heterotrophically using different carbon sources: glucose only, fructose only and glycerol only. The glucose only condition resulted in higher cell growth and total lipid production when compared to the fructose only and glycerol only conditions. However, the proportion of C12-14 fatty acids produced in the glycerol only condition was two-fold higher than that attained in the glucose only condition.

[0442] EXAMPLE 9: Expression of *Arabidopsis thaliana* invertase in *Prototheca moriformis*

[0443] Microalgae strain *Prototheca moriformis* (UTEX 1435) was transformed using methods described above, with an expression construct containing a codon-optimized (according to Table 1) cell wall associated invertase from *Arabidopsis thaliana*. The *Arabidopsis* invertase sequence was modified to include the the N-terminal 39 amino acids from yeast invertase (SUC2 protein) to ensure efficient targeting to the ER and ultimately the periplasm. To aid detection, a Flag epitope was added to the C-terminus of the recombinant protein. The transgene was cloned into an expression vector with a *Chlorella sorokiniana* glutamate dehydrogenase promoter/5'UTR region and a *Chlorella vulgaris* nitrate reductase 3'UTR region. The DNA sequence of this transgene cassette is listed as SEQ ID NO: 89 and the translated amino acid sequence is listed as SEQ ID NO: 90. Positive clones were screened and selected using sucrose-containing media/plates. A subset of the positive clones were confirmed for the presence of the transgene and expression of invertase using Southern blot analysis and Western blot analysis for the Flag-tagged invertase. From these screens, 10 positive clones were chosen for lipid productivity and sucrose utilization assays. All 10 clones were grown on media containing sucrose as the sole carbon source and a positive control suc2 invertase transformant was also included. The negative control, wildtype *Prototheca moriformis*, was also grown but on glucose containing media. After six days, the cells were harvested and dried and the total percent lipid by dry cell weight was determined. The media was also analyzed for total sucrose consumption.

[0444] All ten positive clones were able to hydrolyze sucrose, however, most clones grew about half as well as either wildtype or the positive control suc2 yeast invertase transformant as determined by dry cell weight at the end of the experiment. Similarly, all ten positive clones produced about half as much total lipid when compared to wildtype or the positive control transformant. This data demonstrate the successful heterologous expression of diverse sucrose invertases in *Prototheca*.

[0445] EXAMPLE 10: Heterologous expression of yeast invertase (suc2) in *Prototheca krugani*, *Prototheca stagnora* and *Prototheca zopfii*

[0446] To test the general applicability of the transformation methods for use in species of the genus *Prototheca*, three other *Prototheca* species were selected: *Prototheca krugani* (UTEX 329), *Prototheca stagnora* (UTEX 1442) and *Prototheca zopfii* (UTEX 1438). These three strains were grown in the media and conditions described in Example 1 and their lipid

profiles were determined using the above described methods. A summary of the lipid profiles from the three *Prototheca* strains are summarized below in Area %.

Fatty Acid	<i>P. krugani</i> (UTEX 329)	<i>P. stagnora</i> (UTEX 1442)	<i>P. zopfii</i> (UTEX 1438)
C 10:0	0.0	0.0	0.0
C 10:1	0.0	0.0	0.0
C 12:0	1.5	0.8	2.1
C 14:0	1.2	0.9	1.7
C 16	15.1	17.1	19.7
C 18:0	3.3	4.1	5.4
C 18:1	66.0	61.5	53.8
C 18:2	12.9	15.6	17.3

[0447] These three strains were transformed with a yeast invertase (suc2) expression cassette (SEQ ID NO: 58) using the methods described in Example 3 above. This yeast invertase (suc2) expression cassette has been demonstrated to work in *Prototheca moriformis* (UTEX 1435) above in Example 3. The transformants were screened using sucrose containing plates/media. A subset of the positive clones for each *Prototheca* species was selected and the presence of the transgene was confirmed by Southern blot analysis. Ten of confirmed positive clones from each species were selected for sucrose hydrolysis analysis and lipid productivity. The clones were grown in media containing sucrose as the sole carbon source and compared to its wildtype counterpart grown on glucose. After 6 days, the cultures were harvested and dried and total percent lipid and dry cell weight was assessed. The media from each culture was also analyzed for sucrose hydrolysis using a YSI2700 Biochemistry Analyzer for sucrose content over the course of the experiment. Clones from all three species were able to hydrolyze sucrose, with *Prototheca stagnora* and *Prototheca zopfii* transformants being able to hydrolyze sucrose more efficiently than *Prototheca krugani*. Total lipid production and dry cell weight of the three species of transformants were comparable to their wildtype counterpart grown on glucose. This data demonstrates the successful transformation and expression exogenous genes in multiple species of the genus *Prototheca*.

[0448] EXAMPLE 11: Algal-derived Promoters and Genes for Use in Microalgae

A. 5'UTR and Promoter Sequences from *Chlorella protothecoides*

[0449] A cDNA library was generated from mixotrophically grown *Chlorella protothecoides* (UTEX 250) using standard techniques. Based upon the cDNA sequences, primers were designed in certain known housekeeping genes to “walk” upstream of the coding regions using Seegene’s DNA Walking kit (Rockville, MD). Sequences isolated include an actin (SEQ ID NO:31) and elongation factor-1a (EF1a) (SEQ ID NO:32)

promoter/UTR, both of which contain introns (as shown in the lower case) and exons (upper case italicized) and the predicted start site (in bold) and two beta-tubulin promoter/UTR elements: Isoform A (SEQ ID NO:33) and Isoform B (SEQ ID NO:34).

B. Lipid Biosynthesis Enzyme and Plastid Targeting Sequences from *C. protothecoides*

[0450] From the cDNA library described above, three cDNAs encoding proteins functional in lipid metabolism in *Chlorella protothecoides* (UTEX 250) were cloned using the same methods as described above. The nucleotide and amino acid sequences for an acyl ACP desaturase (SEQ ID NOS: 45 and 46) and two geranyl geranyl diphosphate synthases (SEQ ID NOS:47-50) are included in the Sequence Listing below. Additionally, three cDNAs with putative signal sequences targeting to the plastid were also cloned. The nucleotide and amino acid sequences for a glyceraldehyde-3-phosphate dehydrogenase (SEQ ID NOS:51 and 52), an oxygen evolving complex protein OEE33 (SEQ ID NOS:53 and 54) and a Clp protease (SEQ ID NOS:55 and 56) are included in the Sequence Listing below. The putative plastid targeting sequence has been underlined in both the nucleotide and amino acid sequence. The plastid targeting sequences can be used to target the products of transgenes to the plastid of microbes, such as lipid modification enzymes.

[0451] EXAMPLE 12: 5'UTR/promoters that are nitrogen responsive from *Prototheca moriformis*

[0452] A cDNA library was generated from *Prototheca moriformis* (UTEX 1435) using standard techniques. The *Prototheca moriformis* cells were grown for 48 hours under nitrogen replete conditions. Then a 5% inoculum (v/v) was then transferred to low nitrogen and the cells were harvested every 24 hours for seven days. After about 24 hours in culture, the nitrogen supply in the media was completely depleted. The collected samples were immediately frozen using dry ice and isopropanol. Total RNA was subsequently isolated from the frozen cell pellet samples and a portion from each sample was held in reserve for RT-PCR studies. The rest of the total RNA harvested from the samples was subjected to polyA selection. Equimolar amounts of polyA selected RNA from each condition was then pooled and used to generate a cDNA library in vector pcDNA 3.0 (Invitrogen). Roughly 1200 clones were randomly picked from the resulting pooled cDNA library and subjected to sequencing on both strands. Approximately 68 different cDNAs were selected from among these 1200 sequences and used to design cDNA-specific primers for use in real-time RT-PCR studies.

[0453] RNA isolated from the cell pellet samples that were held in reserve was used as substrate in the real time RT-PCR studies using the cDNA-specific primer sets generated above. This reserved RNA was converted into cDNA and used as substrate for RT-PCR for each of the 68 gene specific primer sets. Threshold cycle or C_T numbers were used to indicate relative transcript abundance for each of the 68 cDNAs within each RNA sample collected throughout the time course. cDNAs showing significant increase (greater than three fold) between nitrogen replete and nitrogen-depleted conditions were flagged as potential genes whose expression was up-regulated by nitrogen depletion. As discussed in the specification, nitrogen depletion/limitation is a known inducer of lipogenesis in oleaginous microorganisms.

[0454] In order to identify putative promoters/5'UTR sequences from the cDNAs whose expression was upregulated during nitrogen depletion/limitation, total DNA was isolated from *Prototheca moriformis* (UTEX 1435) grown under nitrogen replete conditions and were then subjected to sequencing using 454 sequencing technology (Roche). cDNAs flagged as being up-regulated by the RT-PCR results above were compared using BLAST against assembled contigs arising from the 454 genomic sequencing reads. The 5' ends of cDNAs were mapped to specific contigs, and where possible, greater than 500bp of 5' flanking DNA was used to putatively identify promoters/UTRs. The presence of promoters/5'UTR were subsequently confirmed and cloned using PCR amplification of genomic DNA. Individual cDNA 5' ends were used to design 3' primers and 5' end of the 454 contig assemblies were used to design 5' gene-specific primers.

[0455] As a first screen, one of the putative promoter, the 5'UTR/promoter isolated from Aat2 (Ammonium transporter, SEQ ID NO: 99), was cloned into the *Cinnamomum camphora* C14 thioesterase construct with the *Chlorella protothecoides* stearyl ACP desaturase transit peptide described in Example 5 above, replacing the *C.sorokinana* glutamate dehydrogenase promoter. This construct is listed as SEQ ID NO: 112. To test the putative promoter, the thioesterase construct is transformed into *Prototheca moriformis* cells to confirm actual promoter activity by screening for an increase in C14/C12 fatty acids under low/no nitrogen conditions, using the methods described above. Similar testing of the putative nitrogen-regulated promoters isolated from the cDNA/genomic screen can be done using the same methods.

[0456] Other putative nitrogen-regulated promoters/5'UTRs that were isolated from the cDNA/genomic screen were:

<u>Promoter/5'UTR</u>	<u>SEQ ID NO.</u>	<u>Fold increased</u>
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FatB/A promoter/5'UTR	SEQ ID NO: 91	n/a
NRAMP metal transporter promoter/5'UTR	SEQ ID NO: 92	9.65
Flap Flagellar-associated protein promoter/5'UTR	SEQ ID NO: 93	4.92
SulfRed Sulfite reductase promoter/5'UTR	SEQ ID NO: 94	10.91
SugT Sugar transporter promoter/5'UTR	SEQ ID NO: 95	17.35
Amt03-Ammonium transporter 03 promoter/5'UTR	SEQ ID NO: 96	10.1
Amt02-Ammonium transporter 02 promoter/5'UTR	SEQ ID NO: 97	10.76
Aat01-Amino acid transporter 01 promoter/5'UTR	SEQ ID NO: 98	6.21
Aat02-Amino acid transporter 02 promoter/5'UTR	SEQ ID NO: 99	6.5
Aat03-Amino acid transporter 03 promoter/5'UTR	SEQ ID NO: 100	7.87
Aat04-Amino acid transporter 04 promoter/5'UTR	SEQ ID NO: 101	10.95
Aat05-Amino acid transporter 05 promoter/5'UTR	SEQ ID NO: 102	6.71

Fold increase refers to the fold increase in cDNA abundance after 24 hours of culture in low nitrogen medium.

[0457] EXAMPLE 13: Homologous Recombination in *Prototheca* species

[0458] Homologous recombination of transgenes has several advantages over the transformation methods described in the above Examples. First, the introduction of transgenes without homologous recombination can be unpredictable because there is no control over the number of copies of the plasmid that gets introduced into the cell. Also, the introduction of transgenes without homologous recombination can be unstable because the plasmid may remain episomal and is lost over subsequent cell divisions. Another advantage of homologous recombination is the ability to “knock-out” gene targets, introduce epitope tags, switch promoters of endogenous genes and otherwise alter gene targets (e.g., the introduction of point mutations).

[0459] Two vectors were constructed using a specific region of the *Prototheca moriformis* (UTEX 1435) genome, designated KE858. KE858 is a 1.3 kb, genomic fragment that encompasses part of the coding region for a protein that shares homology with the transfer RNA (tRNA) family of proteins. Southern blots have shown that the KE858 sequence is present in a single copy in the *Prototheca moriformis* (UTEX 1435) genome. The first type of vector that was constructed, designated SZ725 (SEQ ID NO: 103), consisted of the entire 1.3 kb KE858 fragment cloned into a pUC19 vector backbone that also contains the optimized yeast invertase (*suc2*) gene used in Example 3 above. The KE858 fragment contains an unique SnaB1 site that does not occur anywhere else in the targeting construct. The second type of vector that was constructed, designated SZ726 (SEQ ID NO: 126),

consisted of the KE858 sequence that had been disrupted by the insertion of the yeast invertase gene (*suc2*) at the SnaB1 site within the KE858 genomic sequence. The entire DNA fragment containing the KE858 sequences flanking the yeast invertase gene can be excised from the vector backbone by digestion with EcoRI, which cuts at either end of the KE858 region.

[0460] Both vectors were used to direct homologous recombination of the yeast invertase gene (*suc2*) into the corresponding KE858 region of the *Prototheca moriformis* (UTEX 1435) genome. The linear DNA ends homologous to the genomic region that was being targeted for homologous recombination were exposed by digesting the vector construct SZ725 with SnaB1 and vector construct SZ726 with EcoRI. The digested vector constructs were then introduced into *Prototheca moriformis* cultures using methods described above in Example 3. Transformants from each vector construct were then selected using sucrose plates. Ten independent, clonally pure transformants from each vector transformation were analyzed for successful recombination of the yeast invertase gene into the desired genomic location (using Southern blots) and for transgene stability.

[0461] Southern blot analysis of the SZ725 transformants showed that 4 out of the 10 transformants picked for analysis contained the predicted recombinant bands, indicating that a single crossover event had occurred between the KE858 sequences on the vector and the KE858 sequences in the genome. In contrast, all ten of the SZ726 transformants contained the predicted recombinant bands, indicating that double crossover events had occurred between the EcoRI fragment of pSZ726 carrying KE858 sequence flanking the yeast invertase transgene and the corresponding KE858 region of the genome.

[0462] Sucrose invertase expression and transgene stability were assessed by growing the transformants for over 15 generations in the absence of selection. The four SZ725 transformants and the ten SZ726 transformants that were positive for the transgene by Southern blotting were selected and 48 single colonies from each of the transformants were grown serially: first without selection in glucose containing media and then with selection in media containing sucrose as the sole carbon source. All ten SZ726 transformants (100%) retained their ability to grow on sucrose after 15 generations, whereas about 97% of the SZ725 transformants retained their ability to grow on sucrose after 15 generations. Transgenes introduced by a double crossover event (SZ726 vector) have extremely high stability over generation doublings. In contrast, transgenes introduced by a single cross over event (SZ725 vector) can result in some instability over generation doublings because is

tandem copies of the transgenes were introduced, the repeated homologous regions flanking the transgenes may recombine and excise the transgenic DNA located between them.

[0463] These experiments demonstrate the successful use of homologous recombination to generate *Prototheca* transformants containing a heterologous sucrose invertase gene that is stably integrated into the nuclear chromosomes of the organism. The success of the homologous recombination enables other genomic alterations in *Prototheca*, including gene deletions, point mutations and epitope tagging a desired gene product. These experiments also demonstrate the first documented system for homologous recombination in the nuclear genome of an eukaryotic microalgae.

A. Use of Homologous Recombination to Knock-out an Endogenous *Prototheca moriformis* gene

[0464] In the *Prototheca moriformis* cDNA/genomic screen described in Example 11 above, an endogenous stearyl ACP desaturase (SAPD) cDNA was identified. Stearyl ACP desaturase enzymes are part of the lipid synthesis pathway and they function to introduce double bonds into the fatty acyl chains. In some cases, it may be advantageous to knock-out or reduce the expression of lipid pathway enzymes in order to alter a fatty acid profile. A homologous recombination construct was created to assess whether the expression of an endogenous stearyl ACP desaturase enzyme can be reduced (or knocked out) and if a corresponding reduction in unsaturated fatty acids can be observed in the lipid profile of the host cell. An approximately 1.5kb coding sequence of a stearyl ACP desaturase gene from *Prototheca moriformis* (UTEX 1435) was identified and cloned (SEQ ID NO: 104). The homologous recombination construct was constructed using 0.5kb of the SAPD coding sequence at the 5' end (5' targeting site), followed by the *Chlamydomonas reinhardtii* β -tubulin promoter driving a codon-optimized yeast sucrose invertase *suc2* gene with the *Chlorella vulgaris* 3' UTR. The rest (~1kb) of the *Prototheca moriformis* SAPD coding sequence was then inserted after the *C. vulgaris* 3' UTR to make up the 3' targeting site. The sequence for this homologous recombination cassette is listed in SEQ ID NO: 105. As shown above, the success-rate for integration of the homologous recombination cassette into the nuclear genome can be increased by linearizing the cassette before transforming the microalgae, leaving exposed ends. The homologous recombination cassette targeting an endogenous SAPD enzyme in *Prototheca moriformis* is linearized and then transformed into the host cell (*Prototheca moriformis*, UTEX 1435). A successful integration will eliminate the endogenous SAPD enzyme coding region from the host genome via a double reciprocal recombination event, while expression of the newly inserted *suc2* gene will be regulated by

the *C.reinhardtii* β -tubulin promoter. The resulting clones can be screened using plates/media containing sucrose as the sole carbon source. Clones containing a successful integration of the homologous recombination cassette will have the ability to grow on sucrose as the sole carbon source and changes in overall saturation of the fatty acids in the lipid profile will serve as a secondary confirmation factor. Additionally, Southern blotting assays using a probe specific for the yeast sucrose invertase *suc2* gene and RT-PCR can also confirm the presence and expression of the invertase gene in positive clones. As an alternative, the same construct without the β -tubulin promoter can be used to excise the endogenous SAPD enzyme coding region. In this case, the newly inserted yeast sucrose invertase *suc2* gene will be regulated by the endogenous SAPD promoter/5'UTR.

[0465] EXAMPLE 14: Fuel Production

A. Extraction of oil from microalgae using an expeller press and a press aid

[0466] Microalgal biomass containing 38% oil by DCW was dried using a drum dryer resulting in resulting moisture content of 5-5.5%. The biomass was fed into a French L250 press. 30.4 kg (67 lbs.) of biomass was fed through the press and no oil was recovered. The same dried microbial biomass combined with varying percentage of switchgrass as a press aid was fed through the press. The combination of dried microbial biomass and 20% w/w switchgrass yielded the best overall percentage oil recovery. The pressed cakes were then subjected to hexane extraction and the final yield for the 20% switchgrass condition was 61.6% of the total available oil (calculated by weight). Biomass with above 50% oil dry cell weight did not require the use of a pressing aid such as switchgrass in order to liberate oil.

B. Monosaccharide Composition of Delipidated *Prototheca moriformis* biomass

[0467] *Prototheca moriformis* (UTEX 1435) was grown in conditions and nutrient media (with 4% glucose) as described in Example 45 above. The microalgal biomass was then harvested and dried using a drum dryer. The dried algal biomass was lysed and the oil extracted using an expeller press as described in Example 44 above. The residual oil in the pressed biomass was then solvent extracted using petroleum ether. Residual petroleum ether was evaporated from the delipidated meal using a Rotovapor (Buchi Labortechnik AG, Switzerland). Glycosyl (monosaccharide) composition analysis was then performed on the delipidated meal using combined gas chromatography/mass spectrometry (GC/MS) of the per-O-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis. A sample of delipidated meal was subjected to methanolysis in 1M HCl in methanol at 80°C for approximately 20 hours, followed by re-N-

acetylation with pyridine and acetic anhydride in methanol (for detection of amino sugars). The samples were then per-O-trimethylsilylated by treatment with Tri-Sil (Pierce) at 80°C for 30 minutes (see methods in Merkle and Poppe (1994) *Methods Enzymol.* 230: 1-15 and York et al., (1985) *Methods Enzymol.* 118:3-40). GC/MS analysis of the TMS methyl glycosides was performed on an HP 6890 GC interfaced to a 5975b MSD, using a All Tech EC-1 fused silica capillary column (30m x 0.25 mm ID). The monosaccharides were identified by their retention times in comparison to standards, and the carbohydrate character of these are authenticated by their mass spectra. 20 micrograms per sample of inositol was added to the sample before derivatization as an internal standard. The monosaccharide profile of the delipidated *Prototheca moriformis* (UTEX 1435) biomass is summarized in Table 18 below. The total percent carbohydrate from the sample was calculated to be 28.7%.

[0468] Table 18. Monosaccharide (glycosyl) composition analysis of *Prototheca moriformis* (UTEX 1435) delipidated biomass.

	Mass (µg)	Mole % (of total carbohydrate)
Arabinose	0.6	1.2
Xylose	n.d.	n.d.
Galacturonic acid (GalUA)	n.d.	n.d.
Mannose	6.9	11.9
Galactose	14.5	25.2
Glucose	35.5	61.7
N Acetyl Galactosamine (GalNAc)	n.d.	n.d.
N Acetyl Glucosamine (GlcNAc)	n.d.	n.d.
Heptose	n.d.	n.d.
3 Deoxy-2-manno-2 Octulsonic acid (KDO)	n.d.	n.d.
Sum	57	100

n.d. = none detected

[0469] The carbohydrate content and monosaccharide composition of the delipidated meal makes it suitable for use as an animal feed or as part of an animal feed formulation. Thus, in one aspect, the present invention provides delipidated meal having the product content set forth in the table above.

C. Production of biodiesel from *Prototheca* oil

[0470] Degummed oil from *Prototheca moriformis* UTEX 1435, produced according to the methods described above, was subjected to transesterification to produce fatty acid methyl esters. Results are shown below:

The lipid profile of the oil was:

C10:0 0.02
C12:0 0.06
C14:0 1.81
C14:1 0.07

C16:0 24.53
 C16:1 1.22
 C18:0 2.34
 C18:1 59.21
 C18:2 8.91
 C18:3 0.28
 C20:0 0.23
 C20:1 0.10
 C20:1 0.08
 C21:0 0.02
 C22:0 0.06
 C24:0 0.10

[0471] Table 19. Biodiesel profile from *Prototheca moriformis* triglyceride oil.

Method	Test		Result	Units
ASTM D6751 A1	Cold Soak Filterability of Biodiesel Blend Fuels	Filtration Time	120	sec
		Volume Filtered	300	ml
ASTM D93	Pensky-Martens Closed Cup Flash Point	Procedure Used	A	
		Corrected Flash Point	165.0	°C
ASTM D2709	Water and Sediment in Middle Distillate Fuels (Centrifuge Method)	Sediment and Water	0.000	Vol %
EN 14538	Determination of Ca and Mg Content by ICP OES	Sum of (Ca and Mg)	<1	mg/kg
EN 14538	Determination of Ca and Mg Content by ICP OES	Sum of (Na and K)	<1	mg/kg
ASTM D445	Kinematic / Dynamic Viscosity	Kinematic Viscosity @ 104 °F/ 40 °C	4.873	mm ² /s
ASTM D874	Sulfated Ash from Lubricating Oils and Additives	Sulfated Ash	< 0.005	Wt %
ASTM D5453	Determination of Total Sulfur in Light Hydrocarbons, Spark Ignition Engine Fuel, Diesel Engine Fuel, and Engine Oil by Ultraviolet Fluorescence.	Sulfur, mg /kg	1.7	mg/kg
ASTM D130	Corrosion - Copper Strip	Biodiesel-Cu Corrosion 50°C (122°F)/3 hr	1a	
ASTM D2500	Cloud Point	Cloud Point	6	°C
ASTM D4530	Micro Carbon Residue	Average Micro Method Carbon Residue	< 0.10	Wt %
ASTM D664	Acid Number of Petroleum Products by Potentiometric Titration	Procedure Used	A	
		Acid Number	0.20	mg KOH/g
ASTM	Determination of Free and	Free Glycerin	< 0.005	Wt %

Method	Test		Result	Units
D6584	Total Glycerin in B-100 Biodiesel Methyl Esters By Gas Chromatography	Total Glycerin	0.123	Wt %
ASTM D4951	Additive Elements in Lubricating Oils by ICP-AES	Phosphorus	0.000200	Wt %
ASTM D1160	Distillation of Petroleum Products at Reduced Pressure	IBP	248	°C
		AET @ 5% Recovery	336	°C
		AET @ 10% Recovery	338	°C
		AET @ 20% Recovery	339	°C
		AET @ 30% Recovery	340	°C
		AET @ 40% Recovery	342	°C
		AET @ 50% Recovery	344	°C
		AET @ 60% Recovery	345	°C
		AET @ 70% Recovery	347	°C
		AET @ 80% Recovery	349	°C
		AET @ 90% Recovery	351	°C
		AET @ 95% Recovery	353	°C
		FBP	362	°C
		% Recovered	98.5	%
		% Loss	1.5	%
		% Residue	0.0	%
		Cold Trap Volume	0.0	ml
		IBP	248	°C
EN 14112	Determination of Oxidation Stability (Accelerated Oxidation Test)	Oxidation Stability	> 12	hr
		Operating Temp (usually 110 deg C)	110	°C
ASTM D4052	Density of Liquids by Digital Density Meter	API Gravity @ 60°F	29.5	°API
ASTM D 6890	Determination of Ignition Delay (ID) and Derived Cetane Number (DCN)	Derived Cetane Number (DCN)	> 61.0	

[0472] The lipid profile of the biodiesel was highly similar to the lipid profile of the feedstock oil. Other oils provided by the methods and compositions of the invention can be subjected to transesterification to yield biodiesel with lipid profiles including (a) at least 4%

C8-C14; (b) at least 0.3% C8; (c) at least 2% C10; (d) at least 2% C12; and (3) at least 30% C8-C14.

[0473] The Cold Soak Filterability by the ASTM D6751 A1 method of the biodiesel produced was 120 seconds for a volume of 300ml. This test involves filtration of 300 ml of B100, chilled to 40°F for 16 hours, allowed to warm to room temp, and filtered under vacuum using 0.7 micron glass fiber filter with stainless steel support. Oils of the invention can be transesterified to generate biodiesel with a cold soak time of less than 120 seconds, less than 100 seconds, and less than 90 seconds.

D. Production of Renewable Diesel

[0474] Degummed oil from *Prototheca moriformis* UTEX 1435, produced according to the methods described above and having the same lipid profile as the oil used to make biodiesel in Example X above, was subjected to transesterification to produce renewable diesel.

[0475] The oil was first hydrotreated to remove oxygen and the glycerol backbone, yielding n-paraffins. The n-paraffins were then subjected to cracking and isomerization. A chromatogram of the material is shown in Figure 13. The material was then subjected to cold filtration, which removed about 5% of the C18 material. Following the cold filtration the total volume material was cut to flash point and evaluated for flash point, ASTM D-86 distillation distribution, cloud point and viscosity. Flash point was 63°C; viscosity was 2.86 cSt (centistokes); cloud point was 4°C. ASTM D86 distillation values are shown in Table 20:

[0476] Table 20. Readings in °C:

Volume	Temperature
IBP	173
5	217.4
10	242.1
15	255.8
20	265.6
30	277.3
40	283.5
50	286.6
60	289.4
70	290.9
80	294.3
90	300
95	307.7
FBP	331.5

[0477] The T10-T90 of the material produced 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.

[0478] The T10 of the material produced was 242.1°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 values, such as T10 between 180 and 295, between 190 and 270, between 210 and 250, between 225 and 245, and at least 290.

[0479] The T90 of the material produced 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 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.

[0480] The FBP of the material produced 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.

[0481] 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 4% C8-C14; (b) at least 0.3% C8; (c) at least 2% C10; (d) at least 2% C12; and (3) at least 30% C8-C14.

[0482] **EXAMPLE 15: Utilization of Sucrose by *Chlorella luteoviridis***

A. SAG 2214 Growth on Glucose and Sucrose

[0483] SAG 2214 (designated as *Chlorella luteoviridis*) was tested for growth in the dark on media containing either glucose or sucrose. Heterotrophic liquid cultures were initiated using inoculum from a frozen vial in either media containing 4% glucose or 4% sucrose as the sole carbon source. Cultures were grown in the dark, shaking at 200 rpm. Samples from the cultures were taken at 0, 24, 48 and 72 hour timepoints and growth was measured by relative absorbance at 750nm (UV Mini1240, Shimadzu). SAG 2214 grew equally well on glucose as on sucrose, showing that this microalgae can utilize sucrose as effectively as glucose as a sole carbon source. The result of this experiment is represented graphically in Figure 3.

B. Lipid Productivity and Fatty Acid Profile for SAG 2214

[0484] Microalgal strain SAG 2214 was cultivated in liquid medium containing either glucose or sucrose as the sole carbon source in similar conditions as described in Example 32 above. After 7 days, cells were harvested for dry cell weight calculation. Cells were centrifuged and lyophilized for 24 hours. The dried cell pellets were weighed and the dry cell weight per liter was calculated. Cells for lipid analysis were also harvested and centrifuged at 4000 x g for 10 minutes at room temperature. The supernatant was discarded and the samples were processed for lipid analysis and fatty acid profile using standard gas chromatography (GC/FID) procedures. The results are summarized below in Tables 21 and 22.

[0485] Table 21. Lipid productivity and DCW for SAG 2214.

Sample	Lipid (g/L)	DCW (g/L)	% Lipid DCW
SAG 2214 glucose	2.43	5.73	42.44%
SAG 2214 sucrose	0.91	2.00	45.56%

[0486] Table 22. Fatty acid profile for SAG 2214.

Fatty Acid	Percent (w/w)
C:16:0	21
C:18:1	38
C:18:2	41

C. Genomic Comparison of SAG 2214 to other *Chlorella luteoviridis* strains

[0487] Microalgal strain SAG 2214 proved to be of general interest due to its ability to grow on sucrose as a carbon source (illustrated above). In addition to the growth characteristics of this strain, its taxonomic relationship to other microalgal species was also of interest. Designated by the SAG collection as a *Chlorella luteoviridis* strain, the 23s rRNA gene of SAG 2214 was sequenced and compared to the 23s rRNA genomic sequence of nine other strains also identified by the SAG and UTEX collections as *Chlorella luteoviridis*. These strains were UTEX 21, 22, 28, 257 and 258, and SAG strains 2133, 2196, 2198 and 2203. The DNA genotyping methods used were the same as the methods described above in Example 1. Sequence alignments and unrooted trees were generated using Geneious DNA analysis software. Out of the nine other strains that were genotypes, UTEX 21, 22, 28 and 257 had identical 23s rRNA DNA sequence (SEQ ID NO: 106). The other five *Chlorella luteoviridis* strains had 23s rRNA sequences that were highly homologous to UTEX 21, 22, 28, and 257.

[0488] The 23s rRNA gene sequence from SAG 2214 (SEQ ID NO: 30) is decidedly different from that of the other nine *C. luteoviridis* strains, having a large insertion that was not found in the other strains. Further analysis of this 23s rRNA gene sequence using BLAST indicated that it shared the greatest homology with members of the genus *Leptosira* and *Trebouxia* (members of phycobiont portion of lichens). These results indicate that SAG 2214 may not be *Chlorella luteoviridis* strain as categorized by the strain collection, but instead shares significant 23S rRNA nucleotide identity to algal symbionts found in lichen. The genomic analysis along with the growth characteristics indicate that SAG 2214 may be a source for genes and proteins involved in the metabolism of sucrose, as well as signaling and transit peptides responsible for the correct localization of such enzymes. SAG 2214 and other strains with a high degree of genomic similarity may also be strains useful for oil production using sucrose as a source of fixed carbon.

[0489] Although this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth.

[0490] All references cited herein, including patents, patent applications, and publications, are hereby incorporated by reference in their entireties, whether previously specifically incorporated or not. The publications mentioned herein are cited for the purpose of describing and disclosing reagents, methodologies and concepts that may be used in connection with the present invention. Nothing herein is to be construed as an admission that these references are prior art in relation to the inventions described herein. In particular, the following patent applications are hereby incorporated by reference in their entireties for all purposes: US Provisional Application No. 60/941,581, filed June 1, 2007, entitled "Production of Hydrocarbons in Microorganisms"; US Provisional Application No. 60/959,174, filed July 10, 2007, entitled "Production of Hydrocarbons in Microorganisms"; US Provisional Application No. 60/968,291, filed August 27, 2007, entitled "Production of Hydrocarbons in Microorganisms"; US Provisional Application No. 61/024,069, filed January 28, 2008, entitled "Production of Hydrocarbons in Microorganisms"; PCT Application No. PCT/US08/65563, filed June 2, 2008, entitled "Production of Oil in Microorganisms"; US Patent Application No. 12/131,783, filed June 2, 2008, entitled "Use of Cellulosic Material for Cultivation of Microorganisms"; US Patent Application No. 12/131,773, filed June 2,

2008, entitled “Renewable Diesel and Jet Fuel from Microbial Sources”; US Patent Application No. 12/131,793, filed June 2, 2008, entitled “Sucrose Feedstock Utilization for Oil-Based Fuel Manufacturing”; US Patent Application No. 12/131,766, filed June 2, 2008, entitled “Glycerol Feedstock Utilization for Oil-Based Fuel Manufacturing”; US Patent Application No. 12/131,804, filed June 2, 2008, entitled “Lipid Pathway Modification in Oil-Bearing Microorganisms”; US Patent Application No. 61/118,590, filed November 28, 2008, entitled “Production of Oil in Microorganisms”; US Provisional Patent Application No. 61/118,994, filed December 1, 2008, entitled “Production of Oil in Microorganisms”; US Provisional Patent Application No. 61/174,357, filed April 3, 2009, entitled “Production of Oil in Microorganisms”; US Provisional Patent Application No. 61/219,525, filed June 23, 2009, entitled “Production of Oil in Microorganisms”; and PCT Application No. _____ [[Attorney Docket No. 026172-005010PC]], filed November 30, 2009, entitled “Production of Tailored Oils in Heterotrophic Microorganisms”.

WHAT IS CLAIMED IS:

1. A codon-optimized recombinant nucleic acid comprising a fatty acyl-ACP thioesterase coding sequence codon-optimized for expression in *Prototheca*, wherein the coding sequence contains the most or second most preferred codon of Table 1 for at least 60% of the codons of the coding sequence, such that the codon-optimized sequence is more efficiently translated in *Prototheca* than a native sequence encoding the fatty acyl-ACP thioesterase, the fatty acyl-ACP thioesterase having hydrolysis activity towards one or more fatty acyl-ACP substrates, the recombinant nucleic acid further comprising a plastid targeting sequence from microalgae that is in-frame with the codon-optimized fatty acyl-ACP thioesterase coding sequence.
2. The codon-optimized recombinant nucleic acid of claim 1, wherein the plastid targeting sequence is a sequence selected from the group consisting of SEQ ID NOs: 127-130.
3. A codon-optimized recombinant nucleic acid comprising a fatty acyl-ACP thioesterase coding sequence codon-optimized for expression in *Prototheca*, wherein the coding sequence contains the most or second most preferred codon of Table 1 for at least 60% of the codons of the coding sequence, such that the codon-optimized sequence is more efficiently translated in *Prototheca* than a native sequence encoding the fatty acyl-ACP thioesterase, the fatty acyl-ACP thioesterase having hydrolysis activity towards one or more fatty acyl-ACP substrates, the recombinant nucleic acid further comprising a plastid targeting sequence in-frame with the codon-optimized fatty acyl-ACP thioesterase coding sequence, the recombinant nucleic acid further comprising first and second flanking nucleic acid sequences endogenous to the nuclear genome of a cell of the genus *Prototheca*, wherein the first and second flanking sequences are each at least 200 nucleotides long and the first and second flanking sequences flank the codon-optimized recombinant nucleic acid for integration of the recombinant nucleic acid into the nuclear genome by homologous recombination with a corresponding genomic sequence.
4. The codon-optimized recombinant nucleic acid of claim 3, wherein the first and second flanking sequences are found on the same *Prototheca* chromosome and are located no more than 20 kB apart from each other.
5. A codon-optimized recombinant nucleic acid comprising a fatty acyl-ACP thioesterase coding sequence codon-optimized for expression in *Prototheca*, wherein the coding sequence

contains the most or second most preferred codon of Table 1 for at least 60% of the codons of the coding sequence, such that the codon-optimized sequence is more efficiently translated in *Prototheca* than a native sequence encoding the fatty acyl-ACP thioesterase, the fatty acyl-ACP thioesterase having hydrolysis activity towards one or more fatty acyl-ACP substrates, the recombinant nucleic acid further comprising a plastid targeting sequence from microalgae that is in-frame with the codon-optimized fatty acyl-ACP thioesterase coding sequence, and wherein the amino acid sequence of the fatty acyl-ACP thioesterase comprises an amino acid sequence of a fatty acyl-ACP thioesterase from an organism selected from the group consisting of *Cuphea hookeriana*, *Umbellularia californica*, *Cinnamomum camphora*, *Cuphea palustris* and *Ulmus americana*.

6. A codon-optimized recombinant nucleic acid comprising a fatty acyl-ACP thioesterase coding sequence and a sucrose invertase coding sequence, each coding sequence codon-optimized for expression in *Prototheca*, wherein each coding sequence contains the most or second most preferred codon of Table 1 for at least 60% of the codons of the coding sequence, such that each codon-optimized sequence is more efficiently translated in *Prototheca* than a native sequence encoding the fatty acyl-ACP thioesterase or the native sequence encoding the sucrose invertase, the fatty acyl-ACP thioesterase having hydrolysis activity towards one or more fatty acyl-ACP substrates, the recombinant nucleic acid further comprising a plastid targeting sequence in-frame with the codon-optimized fatty acyl-ACP thioesterase coding sequence.

7. A host cell of the genus *Prototheca* comprising a recombinant nucleic acid comprising a fatty acyl-ACP thioesterase coding sequence codon-optimized for expression in *Prototheca*, wherein the coding sequence contains the most or second most preferred codon of Table 1 for at least 60% of the codons of the coding sequence, such that the codon-optimized sequence is more efficiently translated in *Prototheca* than a native sequence encoding the fatty acyl-ACP thioesterase, and wherein a plastid targeting sequence is in-frame with the codon-optimized fatty acyl-ACP thioesterase coding sequence and the fatty acyl-ACP thioesterase has hydrolysis activity towards one or more fatty acyl-ACP substrates.

8. A host cell of the genus *Prototheca* comprising a codon optimized recombinant nucleic acid comprising a fatty acyl-ACP thioesterase coding sequence codon-optimized for expression in *Prototheca*, wherein the coding sequence contains the most or second most preferred codon of Table 1 for at least 60% of the codons of the coding sequence, such that

the codon-optimized sequence is more efficiently translated in *Prototheca* than a native sequence encoding the fatty acyl-ACP thioesterase, the fatty acyl-ACP thioesterase having hydrolysis activity towards a fatty acyl-ACP substrate of chain length C8, C10, C12 or C14, the recombinant nucleic acid further comprising a plastid targeting sequence that is in-frame with the codon-optimized fatty acyl-ACP thioesterase coding sequence.

9. A host cell of the genus *Prototheca* comprising the recombinant nucleic acid of claim 1.
10. A host cell of the genus *Prototheca* comprising the recombinant nucleic acid of claim 2.
11. A host cell of the genus *Prototheca* comprising the recombinant nucleic acid of claim 3.
12. A host cell of the genus *Prototheca* comprising the recombinant nucleic acid of claim 4.
13. A host cell of the genus *Prototheca* comprising the recombinant nucleic acid of claim 5.
14. A host cell of the genus *Prototheca* comprising the recombinant nucleic acid of claim 6.
15. A codon-optimized recombinant nucleic acid comprising a fatty acyl-ACP thioesterase coding sequence codon-optimized for expression in *Prototheca*, wherein the coding sequence contains the most or second most preferred codon of Table 1 for at least 60% of the codons of the coding sequence, such that the codon-optimized sequence is more efficiently translated in *Prototheca* than a native sequence encoding the fatty acyl-ACP thioesterase, the fatty acyl-ACP thioesterase having hydrolysis activity towards one or more fatty acyl-ACP substrates, further comprising a promoter comprising the nucleotide sequence of any of SEQ ID NOS: 91-102.
16. The codon-optimized recombinant nucleic acid of claim 6, wherein each coding sequence contains the most preferred codon of Table 1 for at least 60% of the codons of each coding sequence.
17. The codon-optimized recombinant nucleic acid of claim 6, wherein each coding sequence contains the most preferred codon of Table 1 for at least 80% of the codons of each coding sequence.

MANUFACTURING OF TAILORED OILS IN RECOMBINANT HETEROTROPHIC MICROORGANISMS

Abstract

5

Methods and compositions for the production of oil, fuels, oleochemicals, and other compounds in *Prototheca* are provided, including oil-bearing microorganisms and methods of low cost cultivation of such microorganisms. *Prototheca* cells containing exogenous genes encoding, for example, a lipase, a sucrose transporter, a sucrose invertase, a fructokinase, a polysaccharide-degrading enzyme, a fatty acyl-ACP thioesterase, a fatty acyl-CoA/aldehyde reductase, a fatty acyl-CoA reductase, a fatty aldehyde reductase, a fatty aldehyde decarbonylase, and/or an acyl carrier protein are useful in manufacturing transportation fuels such as renewable diesel, biodiesel, and renewable jet fuel.

15 Figure 12