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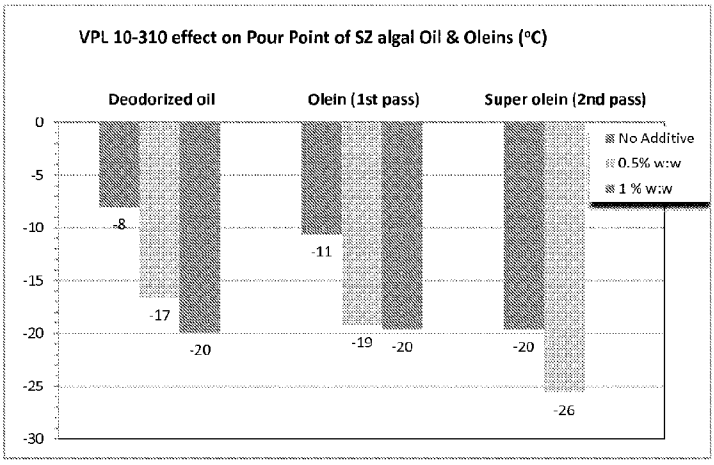

Fig. 3

(57) Abstract: Methods and compositions for the production of dielectric fluids from lipids produced by microorganisms are provided, including oil-bearing microorganisms and methods of low cost cultivation of such microorganisms. Microalgal cells containing exogenous genes encoding, for example, a sucrose transporter, a sucrose invertase, a fructokinase, a polysaccharide-degrading enzyme, a lipid pathway modification enzyme, a fatty acyl-ACP thioesterase, a desaturase, a fatty acyl-CoA/aldehyde reductase, and/or an acyl carrier protein are useful in manufacturing dielectric fluids.

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MICROBIAL OILS WITH LOWERED POUR POINTS, DIELECTRIC FLUIDS PRODUCED THEREFROM, AND RELATED METHODS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of prior U.S. provisional application no. 61/546.932, filed October 13, 2011; prior U.S. provisional application no. 61/522,231, filed August 10, 2011; prior U.S. provisional application no. 61/438,966, filed February 2, 2011; prior U.S. provisional application no. 61/409,902, filed November 3, 2010, which are all hereby incorporated by reference in their entireties.

REFERENCE TO A SEQUENCE LISTING

[0002] This application includes a sequence listing as shown in pages 1-44, appended hereto.

FIELD OF THE INVENTION

[0003] The present invention relates to the production of oils from microorganisms and methods for processing those oils to improve their pour points, as well as products derived from them, including food oil and foods comprising such oil and industrial products such as lubricants and dielectric fluids. Embodiments of the invention therefore relate to the fields of chemistry, particularly oleochemistry, food oils and their production and use, lubricants and their production, dielectric fluids, feedstocks and their production, microbiology, and molecular biology.

BACKGROUND

[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] Many industries, including plastics and chemical manufacturers, rely heavily on the availability of hydrocarbons as a feedstock for their manufacturing processes.

[0006] PCT Pub. Nos. 2008/151149 describe methods and materials for cultivating microalgae for the production of oil, extraction of microbial oil, and production of food, food oil, fuels, and other oleochemicals from oil produced by oleaginous microbes.

[0007] One important oleochemical application is the production of industrial dielectric fluids, which are used for electrical insulation and cooling or heat dissipation in transformers

and other electrical devices. These electrical devices include power and distribution transformers, circuit breakers, capacitors, switchgear, X-ray machines and insulating cables.

[0008] Bio-based oil, particularly high-oleic acid soybean oil, has been used as a dielectric fluid in sealed transformers since the 1990s (see Srivastava (2009) *Int'l J Computer Electrical Eng*, v. 1(2) pp. 212-216). Current bio-based dielectric fluids are purified, high-oleic triacylglycerols (TAGs) with incorporated additives (see US Patent No. 6,274,067 and US Patent App. Nos 20100243969 and 20080283803). For example, the primary benefits of high-oleic acid soybean oil dielectric fluid versus mineral oil-based dielectric fluid are (i) an increased fire point (2x), (ii) an increased transformer life (4-8x), and (iii) a lower cost of remediating spills due to bio-based oil's high biodegradability (>3x) and lower toxicity (see Schneider (2006) *J Sci Food Agric,* v. 86 pp:1769-1780).

[0009] The primary disadvantages of bio-based oils over mineral-based oils are the oxidative instability of bio-based oils, the increased cost of procuring bio-based oils and transitioning equipment from mineral-based oils to bio-based oils see Schneider (2006), supra). Although bio-based dielectric fluids occupy a significant portion of the dielectric fluid market, mineral-oil based dielectric fluids currently dominate the market. Another significant disadvantage is the cost of production of these soy-based oils and their diversion of an important food source into non-food applications.

SUMMARY

[0010] In certain embodiments, the present invention provides microbial oils with improved pour point, methods for making such oils, and products derived from them. Pour point is a function of relative concentrations of saturated to unsaturated fatty acids of the triglyceride oil and the the chain length of the fatty acids. In embodiments of the methods of the invention, the initial pour point of microbial oil is reduced by reducing the relative proportion of the saturated fraction, including palmitic and stearic triglycerides known as the stearin fraction. In accordance with these methods, the oil is fractionated to reduce the saturated triglycerides concentration of the oil. This can be accomplished in accordance with embodiments of the invention by dry fractionation, an illustrative process for carrying out "winterization". In one embodiment of this method, microbial (e.g., algal) oil is optionally first refined, bleached, deodorized or degummed to produce "RBD oil," which is characterized by an initial pour point. The temperature of the RBD oil is then lowered in a controlled manner until crystal nuclei are formed and then held at that first crystallization temperature (i.e., for several hours) to produce crystals. The crystals are then removed by filtration to produce two fractions: a solid phase containing some or most of the stearin

fraction, and a liquid phase containing mostly the olein fraction. This liquid phase is characterized by a second pour point that is lower than the initial pour point, e.g, the second pour point can be between about -10° C and about -40° C, and the fatty acid composition of the can be at least 50% C18:1 and less than 10% C18:2. The liquid phase can be subjected to fractionation again to a second, lower crystallization temperature to effect a further removal of stearin. In illustrative embodiments, the first crystallization temperature is between above 15° C to about 50° C, and the second crystallization temperature is between about -15° C and about 15° C.

[0011] In any event, the resulting purified liquid fraction, is equivalent to or substantially similarly to a super olein oil as commonly known in the vegetable oil industry, has better thermal properties than the native algal oil. In some embodiments, the properties are further improved by the addition of a chemical pour point depressant that reduces the pour point even further, as may be desired for specific applications. The microbial oil provided by this method can be used not only in food applications, but also in industrial applications, such as the production of lubricants, hydraulic fluids, industrical oils and dielectric fluids. For industrial applications (e.g., dielectric fluids), one or more additives that can be added to the microbial oil (in addition to, or instead of, a pour point depressant) include: an antioxidant, metal ion deactivator, corrosion inhibitor, demulsifier, anti-wear additive or anti-hydrolysis compound.

[0012] In various embodiments, the microbial oil is derived from oleaginous microbes, such as microalgal cells, having distinct lipid profiles (i.e., distinct fatty acid profiles), including recombinant cells expressing exogenous genes encoding proteins such as one or more fatty acyl-ACP thioesterases. In illustrative embodiments, the microbial oil is derived from a genetically engineered microbe engineered to express one or more exogenous genes, and the method additionally includes cultivating the microbe until the microbe has at least 10% oil by dry weight, and separating the oil from the microbe to produce a microbial oil that can then be refined, bleached, deodorized and optionally degummed, as described above. Other oleaginous microbes, including yeast, fungi, and bacteria, with similar or distinct lipid profiles can also be employed. In certain embodiments, the present invention thus provides methods of making lipids and oil-based products, including dielectric fluids, from such microalgal and/or oleaginous microbes, including yeast, fungi and bacteria.

[0013] In certain embodiments, the invention provides a product including a microbial oil, wherein the microbial oil has a pour point of between about 10° C and about -40° C, and wherein the fatty acid composition of the microbial oil is at least about 50% C18:1 and less

than about 10% C18:2. In variations of such embodiments, the product has a pour point of between -10° C and about -40° C. The microbial oil in the product can include, for example, include at least about 60%, at least about 70%, or at least about 80% C18:1. In some cases, the microbial oil can include less than about 5% C18:2 (e.g., is at least about 80% C18:1 and less than about 5% C18:2). In particular embodiments, the microbial oild in the product has an iodine value between about 25 and about 200. The microbial oil can, in certain embodiments, be produced by a genetically engineered microbe engineered to express one or more exogenous genes. Illustrative microbes for this purpose include species from the genus *Prototheca* or *Chlorella*. (*e.g., Prototheca moriformis*). Such microbes can be engineered to express, for example, one or more exogenous genes encoding sucrose invertase and/or fatty acyl-ACP thioesterase. In illustrative embodiments, a microbe is engineered to express exogenous genes encoding two or more fatty acyl-ACP thioesterases or sucrose invertase and one or more fatty acyl-ACP thioesterases.

[0014] In various embodiments, the product includes one or more additive(s), such as an antioxidant, a metal ion deactivator, a corrosion inhibitor, a demulsifier, an anti-wear additive, a pour point depressant, or an anti-hydrolysis compound. Illustrative products include a lubricant, a hydraulic fluid, an industrial oil, or a dielectric fluid. Dielectic fluids, in particular, can have one or more of the above-discussed additives.

[0015] In some cases, the microbial oil-based product is a dielectric fluid. In some embodiments, the microbial oil used in the dielectric fluid has one or more of the following attributes: (i) less than 0.4 micrograms/ml total carotenoids; (ii) less than 0.001 micrograms/ml lycopene; (iii) less than 0.02 micrograms/ml beta carotene; (iv) less than 0.02 milligrams of chlorophyll per kilogram of oil; (v) 0.40-0.60 milligrams of gamma tocopherol per 100 grams of oil; (vi) 3-9 mg campesterol per 100 grams of oil; or (vii) less than 0.5 milligrams of total tocotrienols per gram of oil. In some cases, the dielectric fluid has one or more of the following properties: viscosity at 40°C of less than about 110 cSt, e.g., in the range of 20-30 cSt; (b) viscosity at 100°C in the range of about 2 to about 15 cSt, e.g., 4-8 cSt; (c) a viscosity index (VI, a unitless number) at 40°C of at least 35, including but not limited to a VI of 35 to 80, a VI of 80 to 110, a VIT of 110 to 125, a VI of 125 to 160, and, in some embodiments a VI of greater than 160; (d) a pour point (the lowest temperature at which the liquid will flow) of -8 to 10°C or lower, including but not limited to a pour point of -20 to -25°C or lower, and, in some embodiments, a pour point of -30°C, or-40°C or lower; (e) lubricity equivalent to ASTM D2882; (f) low volatility; (g) a high flash point, including a flash point of 150°C or higher, including a flash point of 300°C or higher; (h) a fire point of

150°C or higher (e.g., above 300°C), including a flash point of 300°C or higher; (i) low reactivity, including resistance to decomposition in the presence of acids and bases, good thermal stability, low susceptibility to reaction with oxygen, and a low neutralization number (0.06 or lower, for example 0.03 or lower); (j) good miscibility, including high demulsibility; (k) a power factor at 25°C of 1% or lower, including but not limited to 0.5% or lower, 0.15% or lower, 0.1% or lower, and, in some embodiments 0.05% or lower, (l) a power factor at 100°C of 1.5% or lower, including but not limited to 1% or lower, 0.3% or lower, and, in some embodiments 0.1% or lower; (m) a high dielectric strength; (n) a low dissipation factor; (o) a low electrical conductivity; (p) high specific heat, including but not limited to a specific heat of at least 0.39 cal/gm/°C, and, in some embodiments, a specific heat of at least 0.45 cal/gm/°C or higher; and (q) is biodegradable, i.e., breaks down into carbon dioxide and water in the presence of microbes, such that at least 15% or more of the dielectric fluid degrades under standard test conditions biodegrades in 28 days, and in some embodiments, 30% or more, or 70% or more, or 100% biodegrades under these conditions.

[0016] The invention also provides an electrical component including the above-described dielectric fluid. In certain embodiments, the electrical component is a transformer.

[0017] The invention further provides a method of producing a product including a microbial oil. In certain embodiments, the product has a pour point of between about -10° C and about -40° C, and wherein the fatty acid composition of the microbial oil is at least 50% C18:1 and less than 10% C18:2. In such embodiments, the method entails cultivating a genetically engineered microbe engineered to express one or more exogenous genes until the microbe has at least 10% oil by dry weight and then separating the oil from the microbe. The microbial oil is then subjected to refining, bleaching, deodorizing and, optionally, degumming to produce RBD oil. The method can, optionally, further entail adding an antioxidant, metal ion deactivator, corrosion inhibitor, demulsifier, anti-wear additive, pour point depressant, or anti-hydrolysis compound to the RBD oil. Illustrative engineered microbes for can include species from the genus *Prototheca* or *Chlorella*. (*e.g., Prototheca moriformis*). Such microbes can be engineered to express, for example, one or more exogenous genes encoding sucrose invertase and/or fatty acyl-ACP thioesterase. In illustrative embodiments, a microbe is engineered to express exogenous genes encoding two or more fatty acyl-ACP thioesterases or sucrose invertase and one or more fatty acyl-ACP thioesterases.

[0018] In one embodiment, the present invention provides a method of making a dielectric fluid, the method comprising the steps of: (a) cultivating an oleaginous microbe to provide an

oleaginous microbe that is at least 10% lipid by dry weight, (b) separating the lipid from the oleaginous microbe, and (c) subjecting the lipid to at least one processing step selected from the group consisting of refining, bleaching, deodorizing, degumming, and fractionating by crystallizing or dry fractionation or by winterizing.

[0019] In some specific embodiments of the method, the oleaginous microbe is selected from the group consisting of microalgae, oleaginous yeast, oleaginous fungi and oleaginous bacteria. In some cases, the oleaginous microbe is an oleaginous bacteria that is *Rhodococcus opacus*. In some cases, the oleaginous microbe is an oleaginous fungi. In some cases, the oleaginous fungi is a species listed in Table 3. In some cases, the oleaginous microbe is an oleaginous yeast. In some cases, the oleaginous yeast is a species listed in Table 2. In some cases, the oleaginous microbe is a microalgae. In some cases, the microalgae is a species listed in Table 1. In some cases, the microalgae is of the genus *Prototheca*.

[0020] In some embodiments, the dielectric fluid produced by the method has one or more of the following attributes: (i) 0.05-0.244 mcg/g total carotenoids; (ii) less than 0.003 mcg/g lycopene; (iii) less than 0.003 mcg/g beta carotene; (iv) 0.045-0.268 mcg/g chlorophyll A; (v) 38.3-164 mcg/g gamma tocopherol; (vi) less than 0.25% brassicasterol, campesterol, stignasterol, or beta-sitosterol; (vii) 249.6-325.3 mcg/g total tocotrienols; (viii) 0.003-0.039 mcg/g lutein; and (ix) 60.8-261.7 mcg/g tocopherols. In some embodiments, the dielectric fluid produced by the method has a property selected from the group consisting of: (a) viscosity at 40°C of less than about 110 cSt, e.g., in the range of 20-30 cSt; (b) viscosity at 100°C in the range of about 2 to about 15 cSt, e.g., 4-8 cSt; (c) a viscosity index at 40°C of at least 35; (d) a pour point of -8 to -10°C or lower, including-15 to -25°C or lower; (e) lubricity equivalent to ASTM D2882; (f) a flash point of 150°C or higher; (g) a neutralization number of 0.06 or lower; (h) a power factor at 25°C of 1% or lower; (i) a specific heat of at least 0.39 cal/gm/°C; and (j) biodegradability such that at least 15% or more of the dielectric fluid degrades under standard test conditions in 28 days.

[0021] In some cases, the dielectric fluid is admixed with one or more of the following additives: (a) an antioxidant; (b) a deactivator of metal ions; (c) a corrosion inhibitor; (d) a demulsifier; (e) an anti-wear additive; (f) a malan styrene copolymer; (g) a pour point depressant, including but not limited to VISCOPLEX® 10-310 or 1-133 (Rohmax-Evonik Additives GmbH), or other poly(alkyl) acrylates and poly(methyl)acrylates such as INFINEUM® V-351 (Infineum UK limitied), PMA-D110 and PMA D; or (h) a carbodiimide; or (i) synthetic esters or (j) poly alfa olefins (PAO) or (k) ester of estolides.

[0022] In another embodiment, the present invention provides a dielectric fluid comprising an oleaginous microbial oil, wherein said dielectric fluid comprises less than approximately 10% C18:2. In some cases, the dielectric fluid comprises less than approximately 5% C18:2. In some cases, the dielectric fluid comprises less than approximately 2% C18:2. In some cases, the dielectric fluid further comprises at least 65% C18:1. In some cases, the dielectric fluid further comprises less than 30% C16:0.

[0023] In some embodiments, the microbial oil is blended with another oil to produce the dielectric fluid in accordance with embodiments of the invention. In some cases, the other oil is not a microbial oil. In some cases, the other oil is selected from the group consisting of 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, Camellina sativa, mustard seed, cashew nut, oats, lupine, kenaf, calendula, help, coffee, linseed (flax), hazelnut, euphorbia, pumpkin seed, coriander, camellia, sesame, safflower, rice, tung tree, cocoa, copra, opium poppy, castor beans, pecan, jojoba, macadamia, Brazil nuts, avocado, oleaginous yeast, oleaginous bacteria, petroleum, or a distillate fraction of any of the preceding oils.

[0024] In some embodiments, the content of the other oil in the dielectric fluid is less than 30%. In some cases, the content of the other oil in the dielectric fluid is less than 20%. In some cases, the content of the other oil in the dielectric fluid is less than 10%. In some embodiments, the content of the microbial oil in the dielectric fluid is less than 50%. In some cases, the content of the microbial oil in the dielectric fluid is less than 25%. In some cases, the content of the microbial oil in the dielectric fluid is less than 10%.

[0025] In another embodiment, the present invention provides a dielectric fluid comprising one or more of the following additives: (a) an antioxidant, including but not limited to BHT and other phenols; (b) a deactivator of metal ions such as Cu, Zn, and the like, including but not limited to a benzotriazole; (c) corrosion inhibitors, including but not limited to ester sulfonates and succinic acid esters; (d) demulsifiers; (e) anti-wear additives, including but not limited to zinc dithiophosphate; (f) additives to depress the pour point, including but not limited to malan styrene copolymers and poly(alkyl)acrylates, including but not limited to polymethacrylates; and (g) compounds that protect against hydrolysis, including but not limited to carbodiimides.

[0026] These and other embodiments of the invention are described in the detailed description below, and are exemplified in the examples below. Any or all of the features

discussed above and throughout this application can be combined in various embodiments of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0027] The invention may be understood by reference to the following description taken in conjunction with the accompanying drawings that illustrate certain specific embodiments of the present invention.

[0028] Figure 1. Typical cooling profile for RBD oil fractionation (Tf = filtration temperature).

[0029] Figure 2. Typical cooling profile for algal olein fractionation (Tf = filtration temperature).

[0030] Figure 3. VPL 10-310 effect on pour point of algal oil and fractionated oils. "Deodorized oil" is RBD oil; "olein" is olein # 1; "super olein" is super olein #1."

DETAILED DESCRIPTION

[0031] The present invention arises, in part, from the discovery that *Prototheca* and other oleaginous microorganisms have, in certain embodiments, unexpectedly advantageous properties for the production of dielectric fluids, among other applications, such as biodegradable lubricants, especially engine oils and hydraulic fluids, which were previously mainly based on mineral oils. Lubricants based on microbial oil can be used to replace of petroleum lubricants in chainsaw bar, drilling muds and oils, straight metalworking fluids, food industry lubricants, open gear oils, biodegradable grease, hydraulic fluids, marine oils and outboard engine lubricants, oils for water and underground pumps, rail flange lubricants, shock absorber lubricants, tractor oils, agricultural equipment lubricants, elevator oils, mould release oils, two stroke engine lubricants and other lubricants.

[0032] The present invention also arises, in part, from the discovery of processes for modifying microbial oils to reduce their pour point. Transesterification of lipids yields long-chain fatty acid esters. Other enzymatic and chemical processes can be tailored to yield fatty acids, aldehydes, alcohols, alkanes, and alkenes. In some applications, hydrocarbon compounds useful in dielectric fluids are produced.

[0033] This detailed description is divided into sections for the convenience of the reader. Section I provides definitions of terms used herein. Section II provides a description of culture conditions useful in embodiments of the methods of the invention. Section III provides a description of genetic engineering methods and materials. Section IV provides a description of genetic engineering of microbes to enable sucrose utilization, with specific

reference to microalgae, as exemplified by *Prototheca*. Section V provides a description of genetic engineering of to modify lipid biosynthesis. Section VI describes methods for making microbial oils of embodiments of the invention and products derived therefrom, such as dielectric fluids. Section VII discloses examples that illustrate the various 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] "Antioxidant" is a molecule that is capable of inhibiting the oxidation of other molecules. Antioxidants are frequently added to industrial products. A common use is as stabilizers in fuels and lubricants to prevent oxidation, and in gasolines to prevent the polymerization that leads to the formation of engine-fouling residues. They are also widely used to prevent the oxidative degradation of polymers such as rubbers, plastics and adhesives that causes a loss of strength and flexibility in these materials.

[0039] "Anti-hydrolysis compound" is a molecule that inhibits the decomposition of a chemical compound by reaction with water. Carbodiimides, for example, can be employed as anti-hydrolysis compounds. Anti-hydrolysis compounds are commercially available, e.g., from SpecialChem, among others.

[0040] "Anti-wear additive" is an additive to a fluid (e.g., a lubricating oil) that results in longer machine life due to higher wear and score resistance of the components. Anti-wear additives prevent direct metal-to-metal contact between the machine parts when the oil film is broken down. Typically, the additive reacts with the metal on the part surface and forms a film, which may slide over the friction surface. Anti-wear additives typically contain zinc and phosphorus compounds. Examples of anti-wear additives include zinc dithiophosphate (ZDP), zinc dialkyl dithio phosphate (ZDDP, also acts as a corrosion inhibitor and antioxidant), tricresyl phosphate (TCP, used for high-temperature operation), halocarbons (chlorinated paraffins, for extreme pressure operations), glycerol mono-oleate, stearic acid (which adheres to surfaces via reversible adsorption process under 150 °C, useful for mild contact conditions.

[0041] "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 (fatty acid) profiles of oils and cells described herein, "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.

[0042] "Axenic" refers to a culture of an organism free from contamination by other living organisms.

[0043] "Biodiesel" is a biologically produced fatty acid alkyl ester suitable for use as a fuel in a diesel engine.

[0044] "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.

[0045] "Bioreactor" is an enclosure or partial enclosure in which cells are cultured, optionally in suspension.

[0046] "Breakdown voltage" of of a dielectric fluid is the voltage at which the dielectric fluid loses its insulating properties.

[0047] "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.

[0048] "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.

[0049] "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.

[0050] "Cofactor" is any molecule, other than the substrate, required for an enzyme to carry out its enzymatic activity.

[0051] "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")).

[0052] "Corrosion inhibitor" is molecule that, when added to a fluid, decreases the corrosion rate of a metal or an alloy in contact with the fluid.

[0053] "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.

[0054] "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.

[0055] "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.

[0056] "Demulsifier"is a molecule that either breaks emulsions (usually liquid-liquid emulsions) or prevents them from forming. Demulsifiers are typically based on the following chemistries: acid catalysed phenol-formaldehyde resins, base catalysed phenol-formaldehyde resins, polyamines, di-epoxides, polyols. These molecules are usually ethoxylated (and/or propoxylated) to provide the desired degree of water/oil solubility. The addition of ethylene oxide increases water solubility, whereas propylene oxide decreases it. Commercially available demulsifier formulations are typically a mixture of two to four different chemistries, in carrier solvent(s) such as xylene, Heavy Aromatic Naptha (HAN), isopropanol, methanol, 2-ethylhexanol or diesel.

[0057] "Dielectric" or a "dielectric fluid" is a fluid that does not conduct, or has a very low level of conductivity of, an electric current under normal circumstances (or under the circumstances of its intended use). Dielectric fluids are used for electrical insulation, cooling and lubrication, for example, in transformers and other electrical devices. Electrical devices that utilize dielectric fluids include power and distribution transformers, circuit breakers, capacitors, switchgear, X-ray machines, and insulating cables.

[0058] "Dielectric strength" of a material *(e.g.*, insulator) is the maximum voltage required to produce a dielectric breakdown, *i.e.*, failure of its insulating properties, expressed as volts per unit thickness. The dielectric strength of a material can be determined according the standard methods, for example ASTM test methods D1816, D877, D3300, D117, D2413, D6180, D6181, or D1310.

[0059] "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.

[0060] "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, and is also referred to as a "transgene". 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 (nuclear or plasmid) or as an episomal molecule.

[0061] "Exogenously provided" refers to a molecule provided to the culture media of a cell culture.

[0062] "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.

[0063] "Fatty acid" is a carboxylic acid with a long aliphatic tail (chain). The aliphatic portion of the fatty acid can be fully saturated (no double bond(s)) or can be unsaturated at one or more various portions of the molecule. Most naturally occurring fatty acids have a chain of an even number of carbon atoms, from 4 to 28. Fatty acids can be components of triglycerides or other lipids, e.g., phospholipids, sphingolipids. Fatty acids can be characterized by "lipid numbers." Lipid numbers take the form C:D, where C is the number of carbon atoms in the fatty acid and D is the number of double bonds in the fatty acid. Accordingly, "C18:1" refers to a a fatty acid with 18 carbons and 1 double bond, whereas "C18:2" refers to a fatty acid with 18 carbons and 2 double bonds.

[0064] "Fatty acyl-ACP thioesterase" is an enzyme that catalyzes the cleavage of a fatty acid from an acyl carrier protein (ACP) during lipid synthesis.

[0065] "Fatty acyl-CoA/aldehyde reductase" is an enzyme that catalyzes the reduction of an acyl-CoA molecule to a primary alcohol.

[0066] "Fatty acyl-CoA reductase" is an enzyme that catalyzes the reduction of an acyl-CoA molecule to an aldehyde.

[0067] "Fatty aldehyde decarbonylase" is an enzyme that catalyzes the conversion of a fatty aldehyde to an alkane.

[0068] "Fatty aldehyde reductase" is an enzyme that catalyzes the reduction of an aldehyde to a primary alcohol.

[0069] Fire point of a material is the temperature at which it will continue to burn for at least 5 seconds after ignition by an open flame. The fire point can be determined according standard methods, for example ASTM test methods D92 or D1310.

[0070] "Flash point" is the lowest temperature at which a material can vaporize to form an ignitable mixture in air. At the flash point, the material may ignite, but the vapors produced upon the ignition may not be produced at a sufficient rate to sustain combustion. The flash point can be determined according standard methods, for example ASTM test methods D3278, D3828, D56, or D93.

[0071] "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.

[0072] "Heterotrophic" as it pertains to culture conditions is culturing in the substantial absence of light while utilizing or metabolizing a fixed carbon source.

[0073] "Homogenate" is biomass that has been physically disrupted.

[0074] "Hydraulic fluid" is the fluid serving as the power transmission medium in a hydraulic system.

[0075] "Hydrocarbon" is 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 (CH_4), 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.

[0076] "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 CH_4 (4:1).

[0077] "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.

[0078] "Increase lipid yield" refers to an increase in the lipid 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.

[0079] "Inducible promoter" is a promoter that mediates transcription of an operably linked gene in response to a particular stimulus.

[0080] "Industrial oil" is an oil that is useful in industry. Common industrial oils include chainsaw bar lubricant, metal working fluids, food grade lubricants, gear oils, marine oils, engine lubricants, tractor oils, agricultural equipment lubricants, elevator oils, mould release oils, and the like. "Chainsaw bar lubricant" is used for the external lubrication of the bar and chain of chainsaws. "Metal working fluids" are fluids used to cool and/or lubricate the process of shaping a piece of metal into a useful object. "Food grade lubricants" are lubricants that are acceptable for use in meat, poultry and other food processing equipment, applications and plants. "Gear oils" are oils that are useful for lubricating gears, e.g., in transmissions, transfer cases, and differentials in automobiles, trucks, and other machinery. "Marine oils" are oils that are useful for lubricating the moving parts of marine equipment. "Engine lubricants" are used for lubrication of various internal combustion engines. While the main function is to lubricate moving parts, engine lubricants can also clean, inhibit corrosion, improve sealing, and cool the engine by carrying heat away from moving parts. "Tractor oils" are oils that are useful for lubricating the moving parts on tractors. "Agricultural equipment lubricants" are lubricants that are useful for lubricating the moving parts of agricultural equipement. "Elevator oils" are oils used as hydraulic fluid in hydraulic elevators. "Mould release oils" are oils useful in the production of formed articles using a mould. Mould release oils facilitate release of the formed article from the mould and can have surface conditioning characteristics that provide a desired surface finish.

[0081] "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.

[0082] "In situ" means "in place" or "in its original position".

"Iodine value" (or "iodine number") is a measure of the degree of unsaturation of an oil. It is the mass of iodine that is consumed by the unsaturated bonds in an oil. For example, an oil with an iodine value of 50 is an oil in which 100 grams of oil would consume 50 grams of iodine. Iodine values are routinely determined in the art. Standard methods to determine iodine values include ASTM D5768-02(2006) and DIN 53241.

[0083] "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.

[0084] "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.

[0085] "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 desaturase, including a stearoyl acyl carrier protein desaturase (SAD) and a fatty acyl desaturase (FAD), and a fatty aldehyde decarbonylase.

[0086] "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.

[0087] "Lipid" or "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."

[0088] "Lubricant" is a substance capable of reducing friction, heat, and/or wear when introduced as a film between solid surfaces

[0089] "Lysate" is a solution containing the contents of lysed cells.

[0090] "Lysing" or "lysis" is disrupting the cellular membrane and optionally the cell wall of a biological organism or cell sufficient to release at least some intracellular content.

[0091] "Metal ion deactivator," also known as "metal deactivator" or "metal deactivating agent (MDA)" is a fuel and/or oil additive used to stabilize fluids by deactivating (usually by sequestering) metal ions. The metal ions may be produced by the action of naturally occurring acids in the fuel and acids generated in lubricants by oxidative processes with the metallic parts of systems.

[0092] "Microalgae" is a eukarytotic 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 refers to obligate heterotrophic microorganisms that have lost the ability to perform photosynthesis, such as certain dinoflagellate algae species and species of the genus *Prototheca*.

[0093] "Microorganism" and "microbe" are microscopic unicellular organisms.

[0094] "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.

[0095] "Oil" refers to any triacylglyceride oil, produced by organisms, including oleaginous yeast, plants, and/or animals. "Oil," as distinguished from "fat", refers, unless otherwise indicated, to lipids that are generally, but not always, liquid at ordinary room

temperatures and pressures. For example, "oil" includes vegetable or seed oils derived from plants, including without limitation, an oil derived from avocado, Brazil nuts, calendula, camelina, camelina sativa, canola, cashew nut, castor beans, cocoa butter (also known as cacao, which is a triacylglyceride oil derived from the cacao bean that is solid at typical room temperatures and pressures), coconut, coffee, copra, coriander, corn, cotton seed, cuphea, euphorbia, hazelnut, hemp, jatropha, jojoba, kenaf, linseed, lupine, macadamia, mustard seed, oats, olive, opium poppy, palm, palm kernel, peanut, pecan, pumpkin seed, rapeseed, rice, safflower, sesame, soy, sunflower, and tung oil tree, as well as combinations thereof. "Microbial oil" refers to an oil derived from a microbe.

[0096] "Oleaginous yeast" means yeast that can naturally accumulate more than 20% of their dry cell weight as lipid and are of the *Dikarya* subkingdom of fungi. Oleaginous yeast include, but are not limited to, organisms such as *Yarrowia lipolytica, Rhodotorula glutinis, Cryptococcus curvatus,* and *Lipomyces starkeyi*.

[0097] "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.

[0098] "Polysaccharide-degrading enzyme" is any enzyme capable of catalyzing the hydrolysis, or saccharification, of any polysaccharide. For example, cellulases catalyze the hydrolysis of cellulose.

[0099] "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.

[0100] "Pour point" is the lowest temperature at which a liquid will pour or flow under a specific set of conditions. Exemplary pour point standards include ASTM D97-11, D5853-11, and D5949-10, but others known to, or developed by, those of skill in the art can be employed in making pour point determinations in connection with the methods described herein.

[0101] "Pour point depressants" or "PPDs" are polymers that control wax crystal formation in oils or lubricants, resulting in lower pour point and improved low temperature flow performance.

[0102] "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.

[0103] "Recombinant" refers to 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. 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. Similarly, a "recombinant protein" is a protein made using recombinant techniques, i.e., through the expression of a recombinant nucleic acid.

[0104] "RBD oil" is an oil that has been subjected to refining, bleaching, or deodorizing.

[0105] "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.

[0106] "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.

[0107] "Sonication" is a process of disrupting biological materials, such as a cell, by use of sound wave energy.

[0108] "Species of furfural" is 2-furancarboxaldehyde or a derivative that retains the same basic structural characteristics.

[0109] "Stover" is the dried stalks and leaves of a crop remaining after a grain has been harvested.

[0110] "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.

[0111] "Transformer" is a device that transfers electrical energy from one circuit to another through inductively coupled conductors, typically the transformer's coils.

[0112] The terms "winterizing" oil or "winterization of oil" refer to a process that includes removing the higher melting point components from an oil and/or adding one or more pour point depressant(s).

II. CULTIVATION AND CULTURE CONDITIONS

[0113] In certain embodiments, the present invention generally relates to cultivation of oleaginous microbes, such as wild-type and recombinant microalgae, including *Chlorella* and *Prototheca* species and strains, and yeast, fungi, and bacteria species and strains, for the production of microbial oil (lipids). 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, as well as other microalgae, yeast, fungi, and bacteria useful in the methods described herein. Subsection 2 describes bioreactors useful for cultivation. Subsection 3 describes media for cultivation. Subsection 4 describes oil (lipid) production in accordance with illustrative cultivation methods described herein. Subsection 5 describes types of oleaginous yeast suitable for use in the methods described herein, culture conditions for generating yeast biomass, and the lipid profiles and chemical composition of the biomass prepared in accordance with illustrative methods described herein.

1. Prototheca species and strains and other oleaginous microbes

[0114] *Prototheca* is a remarkable microorganism for use in the production of lipid, because it can produce high levels of lipid, particularly lipid suitable for dielectric fluid and other lubricant production. The lipid produced by *Prototheca* has 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 for use in the methods described herein 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 species and strains for use in the methods described herein include *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.

[0115] Species of *Prototheca* for use in the methods described herein 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.

[0116] Thus, genomic DNA comparison can be used to identify suitable species of microalgae to be used in the methods described herein. 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 methods described herein. 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.

[0117] Illustrative microalgae for use in the methods described herein 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.

[0118] 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.

[0119] 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*).

[0120] Another example of an algorithm 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 TBLATN 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)).

[0121] 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.

[0122] A wide variety of oleaginous microbes in addition to Prototheca can be used in the methods described herein. For example, Chlorella, including but not limited to strains of the protothecoides species of Chlorella, is an excellent microalgae for use in the methods described herein. Considerations affecting the selection of microorganisms for use in the methods described herein in addition to production of suitable lipids or hydrocarbons for production of oils, fuels, and oleochemicals, can include one or more of the following: (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 microbial oil (i.e., lipids and fatty acids). Preferred organisms grow (and are grown) heterotrophically (on sugars in the substantial absence of light). Microalgae generally are excellent microbes for use in the methods described herein. Examples of microalgae that can be used to practice the methods include, but are not limited to the following algae listed in Table 1.

Table 1. Examples of oleaginous microalgae.

Achnanthes orientalis, Agmenellum, Amphiprora hyaline, Amphora coffeiformis, Amphora coffeiformis linea, Amphora coffeiformis punctata, Amphora coffeiformis taylori, Amphora coffeiformis tenuis, Amphora delicatissima, Amphora delicatissima capitata, Amphora sp., Anabaena, Ankistrodesmus, Ankistrodesmus falcatus, Boekelovia hooglandii, Borodinella sp., Botryococcus braunii, Botryococcus sudeticus, Carteria, Chaetoceros gracilis, Chaetoceros muelleri, Chaetoceros muelleri subsalsum, Chaetoceros sp., Chlorella anitrata, Chlorella Antarctica, Chlorella aureoviridis, Chlorella candida, Chlorella capsulate, Chlorella desiccate, Chlorella ellipsoidea, Chlorella emersonii, Chlorella fusca, Chlorella fusca var.

vacuolata, Chlorella glucotropha, Chlorella infusionum, Chlorella infusionum var. actophila, Chlorella infusionum var. auxenophila, Chlorella kessleri, Chlorella lobophora (strain SAG 37.88), Chlorella luteoviridis, Chlorella luteoviridis var. aureoviridis, Chlorella luteoviridis var. lutescens, Chlorella miniata, Chlorella minutissima, Chlorella mutabilis, Chlorella nocturna, Chlorella parva, Chlorella photophila, Chlorella pringsheimii, Chlorella protothecoides (including any of UTEX strains 1806, 411, 264, 256, 255, 250, 249, 31, 29, 25, and CCAP strains 211/17 and 211/8d), Chlorella protothecoides var. acidicola, Chlorella regularis, Chlorella regularis var. minima, Chlorella regularis var. umbricata, Chlorella reisiglii, Chlorella saccharophila, Chlorella saccharophila var. ellipsoidea, Chlorella salina, Chlorella simplex, Chlorella sorokiniana, Chlorella sp., Chlorella sphaerica, Chlorella stigmatophora, Chlorella vanniellii, Chlorella vulgaris, Chlorella vulgaris, Chlorella vulgaris f. tertia, Chlorella vulgaris var. autotrophica, Chlorella vulgaris var. viridis, Chlorella vulgaris var. vulgaris, Chlorella vulgaris var. vulgaris f. tertia, Chlorella vulgaris var. vulgaris f. viridis, Chlorella xanthella, Chlorella zofingiensis, Chlorella trebouxioides, Chlorella vulgaris, Chlorococcum infusionum, Chlorococcum sp., Chlorogonium, Chroomonas sp., Chrysosphaera sp., Cricosphaera sp., Cryptomonas sp., Cyclotella cryptica, Cyclotella meneghiniana, Cyclotella sp., Dunaliella sp., Dunaliella bardawil, Dunaliella bioculata, Dunaliella granulate, Dunaliella maritime, Dunaliella minuta, Dunaliella parva, Dunaliella peircei, Dunaliella primolecta, Dunaliella salina, Dunaliella terricola, Dunaliella tertiolecta, Dunaliella viridis, Dunaliella tertiolecta, Eremosphaera viridis, Eremosphaera sp., Ellipsoidon sp., Euglena, Franceia sp., Fragilaria crotonensis, Fragilaria sp., Gleocapsa sp., Gloeothamnion sp., Hymenomonas sp., Isochrysis aff. galbana, Isochrysis galbana, Lepocinclis, Micractinium, Micractinium (UTEX LB 2614), Monoraphidium minutum, Monoraphidium sp., Nannochloris sp., Nannochloropsis salina, Nannochloropsis sp., Navicula acceptata, Navicula biskanterae, Navicula pseudotenelloides, Navicula pelliculosa, Navicula saprophila, Navicula sp., Nephrochloris sp., Nephroselmis sp., Nitschia communis, Nitzschia alexandrina, Nitzschia communis, Nitzschia dissipata, Nitzschia frustulum, Nitzschia hantzschiana, Nitzschia inconspicua, Nitzschia intermedia, Nitzschia microcephala, Nitzschia pusilla, Nitzschia pusilla elliptica, Nitzschia pusilla monoensis, Nitzschia quadrangular, Nitzschia sp., Ochromonas sp., Oocystis parva, Oocystis pusilla, Oocystis sp., Oscillatoria limnetica, Oscillatoria sp., Oscillatoria subbrevis, Pascheria acidophila, Pavlova sp., Phagus, Phormidium, Platymonas sp., Pleurochrysis carterae, Pleurochrysis dentate, Pleurochrysis sp., Prototheca wickerhamii, Prototheca stagnora, Prototheca

portoricensis, Prototheca moriformis, Prototheca zopfii, Pyramimonas sp., Pyrobotrys, Sarcinoid chrysophyte, Scenedesmus armatus, Spirogyra, Spirulina platensis, Stichococcus sp., Synechococcus sp., Tetraedron, Tetraselmis sp., Tetraselmis suecica, Thalassiosira weissflogii, and *Viridiella fridericiana*

[0123] In addition to microalgae, oleaginous yeast can accumulate more than 20% of their dry cell weight as lipid and so are useful in the methods described herein. In one embodiment of the present invention, a microorganism producing a lipid or a microorganism from which oil can be extracted, recovered, or obtained, is an oleaginous yeast. Examples of oleaginous yeast that can be used in the methods described herein include, but are not limited to, the oleaginous yeast listed in Table 2. Illustrative methods for the cultivation of oleaginous yeast (*Yarrowia lipolytica* and *Rhodotorula graminis*) in order to achieve high oil content are provided in the examples below.

Table 2. Examples of oleaginous yeast.

Candida apicola, Candida sp., Cryptococcus curvatus, Cryptococcus terricolus, Debaromyces hansenii, Endomycopsis vernalis, Geotrichum carabidarum, Geotrichum cucujoidarum, Geotrichum histeridarum, Geotrichum silvicola, Geotrichum vulgare, Hyphopichia burtonii, Lipomyces lipofer, Lypomyces orentalis, Lipomyces starkeyi, Lipomyces tetrasporous, Pichia mexicana, Rodosporidium sphaerocarpum, Rhodosporidium toruloides Rhodotorula aurantiaca, Rhodotorula dairenensis, Rhodotorula diffluens, Rhodotorula glutinus, Rhodotorula glutinis var. glutinis, Rhodotorula gracilis, Rhodotorula graminis Rhodotorula minuta, Rhodotorula mucilaginosa,Rhodotorula mucilaginosa var. mucilaginosa, Rhodotorula terpenoidalis, Rhodotorula toruloides, Sporobolomyces alborubescens, Starmerella bombicola, Torulaspora delbruekii, Torulaspora pretoriensis, Trichosporon behrend, Trichosporon brassicae, Trichosporon domesticum, Trichosporon laibachii, Trichosporon loubieri, Trichosporon loubieri var. loubieri, Trichosporon montevideense, Trichosporon pullulans, Trichosporon sp., Wickerhamomyces Canadensis, Yarrowia lipolytica, and Zygoascus meyerae.

[0124] In one embodiment of the present invention, a microorganism producing a lipid or a microorganism from which a lipid can be extracted, recovered or obtained, is a fungus. Examples of fungi that can be used in the methods described herein include, but are not limited to, the fungi listed in Table 3.

Table 3. Examples of oleaginous fungi.

Mortierella, Mortierrla vinacea, Mortierella alpine, Pythium debaryanum, Mucor circinelloides, Aspergillus ochraceus, Aspergillus terreus, Pennicillium iilacinum, Hensenulo, Chaetomium, Cladosporium, Malbranchea, Rhizopus, and *Pythium*

[0125] Thus, in one embodiment of the present invention, the microorganism used for the production of microbial biomass for use in the methods described herein is a fungus. Examples of suitable fungi (*e.g., Mortierella alpine, Mucor circinelloides,* and *Aspergillus ochraceus*) include those that have been shown to be amenable to genetic manipulation, as described in the literature (see, for example, *Microbiology,* Jul; 153(Pt.7): 2013-25 (2007); *Mol Genet Genomics*, Jun; 271(5): 595-602 (2004); *Curr Genet*, Mar;21(3):215-23 (1992); *Current Microbiology*, 30(2):83-86 (1995); Sakuradani, NISR Research Grant, "Studies of Metabolic Engineering of Useful Lipid-producing Microorganisms" (2004); and PCT/JP2004/012021).

[0126] In other embodiments of the present invention, a microorganism producing a lipid or a microorganism from which oil can be extracted, recovered, or obtained is an oleaginous bacterium. Oleaginous bacteria are bacteria that can accumulate more than 20% of their dry cell weight as lipid. Species of oleaginous bacteria for use in the methods described herein, include species of the genus *Rhodococcus,* such as *Rhodococcus opacus* and *Rhodococcus sp.* Methods of cultivating oleaginous bacteria, such as *Rhodococcus opacus,* are known in the art (see Waltermann, et al., (2000) *Microbiology*, 146: 1143-1149). Illustrative methods for cultivating *Rhodococcus opacus* to achieve high oil content are provided in the examples below.

2. Bioreactor

[0127] Microrganisms are cultured both for purposes of conducting genetic manipulations and for production of microbial oil (e.g., hydrocarbons such as 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. Microalgae, including *Prototheca* species, as well as the other oleaginous microbes described herein, are typically cultured in the methods described herein in liquid media within a bioreactor. Typically, the bioreactor does not allow substantial amounts of light or any amount of light to enter. In some embodiments, the entire cultivation step(s) of the oleaginous microbe, including microalgae, is performed under substantial absence of light.

[0128] 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. Microalgae and other oleaginous microbes described herein are typically 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.

[0129] Bioreactors can be configured to flow culture media though 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 (or other microbial)l 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.

[0130] 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.

[0131] 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.

[0132] 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/CO_2 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.

[0133] 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

[0134] Microalgal as well as other microbial 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, $(NH_4)_2SO_4$ and NH_4OH), 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 $ZnCl_2$, H_3BO_3, $CoCl_2 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, $MnCl_2 \cdot 4H_2O$ and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$.

[0135] 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.

[0136] 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 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.

[0137] 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 $NaNO_3$, 0.17 mM $CaCl_2 \cdot 2H_2O$, 0.3 mM $MgSO_4 \cdot 7H_2O$, 0.43 mM, 1.29 mM KH_2PO_4, 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 postassium 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 described herein 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.

[0138] For cost-efficient 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. Suitable carbon sources include, for example, acetate, floridoside, fructose, galactose, glucuronic acid, glucose, glycerol, lactose, mannose, N-acetylglucosamine, rhamnose, raffinose, stachyose, sucrose, and/or xylose. Suitable feedstocks useful in accordance with the methods described herein include, for example, black liquor, corn starch, depolymerized cellulosic material, milk whey, invert sugar (glucose/fructose), molasses, potato, sorghum, sucrose, sugar beet, sugar cane, thick cane juice, rice, and wheat. Carbon sources can also be provided as a mixture, such as a mixture of sucrose and depolymerized sugar beet pulp.

[0139] The one or more carbon source(s) can be supplied at a concentration of at least about 50 μM, at least about 100 μM, at least about 500 μM, 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). Highly concentrated carbon sources as feedstock for fermentation are preferred, and in various embodiments, the carbon source is provided in a feedstock at a concentration approaching its maximum solubility (i.e., at a concentration exceeding 90% solubility, such as a concentration of 95% or higher, i.e., 99% solubility).

[0140] For example, in some embodiments glucose levels of at least 300g/L, at least 400g/L, at least 500g/L, or at least 600g/L or more are used in the feedstock in a fed batch cultivation, in which the highly concentrated fixed carbon source is fed to the cells over time as the cells grow and accumulate microbial oil (lipid). In other embodiments, sucrose levels of at least 500g/L, at least 600g/L, at least 700g/L, at least 800g/L or more are used in the feedstock in a fed batch cultivation. Non-limiting examples of highly concentrated sucrose carbon sources include thick cane juice, sugar cane juice, sugar beet juice and molasses. Carbon sources of particular interest for purposes of the methods described herein include cellulose (in a depolymerized form), glycerol, sucrose, and sorghum, each of which is discussed in more detail below.

[0141] In accordance with the methods described herein, 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.

[0142] 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), soybean meal, 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 cellulosics 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.

[0143] Cellulosic materials are treated to increase the efficiency with which the microbe can utilize the sugar(s) contained within the materials. The methods described herein can be practiced to take advantage of new 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).

[0144] Celluose 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 constitutent 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 cabilities.

[0145] The methods described herein typically involve fermentation to higher cell densities than what is achieved in ethanol fermentation. Because of the higher densities of the cultures for heterotrophic lipid 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 lignin 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 from cellulosic materials of 300 g/L, 400 g/L, 500 g/L, or higher for six-carbon sugars that may be used in large scale production applications of 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.

[0146] 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, in particular embodiments, the present invention provides methods in which strains capable of tolerating compounds such as furfurals or hydroxymethyl furfurals are used for production. In another embodiment, the present invention also provides methods for using microorganisms that are not only capable of tolerating furfurals in the fermentation media, but are actually able to metabolize these by-products during fermentation.

[0147] The explosion process also generates significant levels of salts. For example, typical conditions for explosion can result in conductivites 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. In accordance with the methods described herein, these salts can be removed so that the resulting concentrated cellulosic sugar stream(s) can be used in heterotrophic processes for producing lipid. 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.

[0148] A preferred embodiment for the process of preparing of exploded cellulosic biomass for use in heterotrophic microbial oil production using oleaginous microbes is conducted as follows. A first step 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 μS/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. A second step comprises enzymatic saccharification of the washed cellulosic biomass. In one embodiment, Accellerase (Genencor) is used. A third step 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. A fourth step comprises microfiltration to remove contaminating solids with recovery of the permeate. A fifth step 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.

[0149] 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.

[0150] 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 increase cell density rapidly, and are then fed glucose to accumulate microbial oil (lipids), can improve the efficiency with which the oil is produced. The use of the glycerol byproduct of the transesterification process provides significant economic advantages when put back into a production process for microbial oil. Other feeding methods are provided as well, such as those employing mixtures of glycerol and glucose as the fixed carbon source. Feeding such mixtures also captures similar economic benefits. In addition, in certain embodiments, the invention provides methods of feeding alternative sugars to microalgae such as sucrose in various combinations with glycerol.

[0151] In another embodiment of the methods of the invention, the carbon source is invert sugar. Invert sugar is produced by splitting the sucrose into its monosaccharide components, fructose and glucose. Production of invert sugar can be achieved through several methods that are known in the art. One such method is heating an aqueous solution of sucrose. Often, catalysts are employed to accelerate the conversion of sucrose into invert sugar. These catalysts can be biological; for example, enzymes such as invertases and sucrases can be added to the sucrose to accelerate the hydrolysis reaction to produce invert sugar. Acid is an example of a non-biological catalyst that, when paired with heat, can accelerate the hydrolysis reaction. Once the invert sugar is made, it is less prone to crystallization compared to sucrose and thus provides advantages for storage and fed batch fermentations, where, in the case of heterotrophic cultivation of microbes, including microalgae, there is a need for a concentrated carbon source. In one embodiment, the carbon source is invert sugar, preferably in a concentrated form (at least 90% of its maximum solubility in the conditions used, as described above), i.e., at least 800g/liter, at least 900 g/liter, at least 1000 g/liter or at least 1100 g/liter. The invert sugar, preferably in a concentrated form, is fed to the cells over time as the cells grow and accumulate lipid.

[0152] 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. As noted above, because of the higher densities of the cultures for

heterotrophic oil production, the fixed carbon source (e.g., sucrose, glucose, etc.) is in a concentrated form, i.e., at least 500 g/liter, at least 600 g/liter, at least 700 g/liter or at least 800 g/liter of the fixed carbon source 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. In some cases, the carbon source is sucrose in the form of thick cane juice, typically in a concentrated form, i.e., at least 60% solids or about 770 g/liter sugar, at least 70% solids or about 925 g/liter sugar, or at least 80% solids or about 1125 g/liter sugar prior to the cultivation step, which is optionally a fed batch cultivation. The concentrated thick cane juice is fed to the cells over time as the cells grow and accumulate lipid.

[0153] 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 secrectable 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 microbe used in the methods described herein 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.

[0154] 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 described herein 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

[0155] For the production of oil (lipid) in accordance with the methods described herein, 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. For example, *Prototheca* and other microalgae species can be 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.

[0156] As an example, an inoculum of lipid-producing microalgal cells is 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 fatty acids in the oil produced, and/or production of a specific fatty acid and corresponding lipid(s).

[0157] Preferably, microorganisms grown using conditions described herein and others 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 or other oleaginous microbe 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.

[0158] 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 oil (e.g., lipids and fatty acids) yield over microbial oil 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 a vitamin required by a lipid pathway enzyme, such as, for example: biotin and pantothenate. Genes encoding cofactors suitable for use in the methods described herein or that participate in the synthesis of such cofactors are well known and can be introduced into microbes (e.g., microalgae or other oleaginous microbe described herein), using contructs and techniques such as those described above.

[0159] 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.

[0160] Microalgal biomass with a high percentage of oil/lipid accumulation by dry weight has been generated (see PCT Pub. No. 2008/151149). Microalgal biomass generated by the culture methods described herein and useful in accordance with the methods described herein 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.

[0161] The microalgal oil of the biomass described herein, or extracted from the biomass for use in the methods and compositions described herein 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 containing them) can be manipulated to modify the properties or proportions of the fatty acid molecules in the microalgal oils described herein via culture conditions or via lipid pathway engineering, as described in more detail in Section V, below. Thus, specific blends of algal (or other microbial) oil can be prepared either within a single species of algae or by mixing together the biomass or algal oil from two or more species of microalgae, or by blending algal oil described herein 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, Camellina sativa, mustard seed, cashew nut, oats, lupine, kenaf, calendula, help, coffee, linseed (flax), hazelnut, euphorbia, pumpkin seed, coriander, camellia, sesame, safflower, rice, tung tree, cocoa,

copra, opium poppy, castor beans, pecan, jojoba, macadamia, Brazil nuts, avocado, petroleum, or a distillate fraction of any of the preceding oils.

[0162] As noted above, 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 consituents in the cell's glycerolipids.

[0163] Generally, *Prototheca* strains have lipid profiles with C16 and C18 fatty acids as the predominant species. Such longer chain length fatty acids, especially the monosaturated C16 and C18 fatty acids (i.e., C16:1 and C18:1) are generally preferred for production of dielectric fluids (see, for example, US Patent No. 6,274,067). For example, *Prototheca moriformis* (UTEX 1435), *Prototheca stagnora* (UTEX 327), and *Prototheca moriformis* (UTEX 1441) contain between 12% and 30% C16 fatty acids and between 50% and 58% C18:1 fatty acids. *Chlorella protothecoides* (UTEX 250) contains about 73% C18:1 fatty acids, and other *Chlorella protothecoides* strains, including, but not limited to, UTEX 25, UTEX 249, UTEX 256, UTEX 264, UTEX 411, CCAP 211/17, CCAP 221/8D and SAG 221 10d, can contain between 7% and 18% C 16 fatty acids and between 55% and 75% C18:1 fatty acids. In various embodiments, microbial oil (lipid) useful in products described herein (such as dielectric fluids) is at least about 50% C18:1, e.g., at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, and at least about 90% C18:1. In these or other embodiments, the microbial oil (lipid) is less than about 10% C18:2, e.g., less than about 7.5%, less than about 5%, less than about 2.5%, and less than about 1% C18:2. The microbial oil can have any combination of percentages of C18:1 and C18:2 that adds up to 100% or less. For example the microbial oil can have at least 50% C18:1 and less than 10% C18:2 or at least 80% C18:1 and less than 5% C18:2.

[0164] Microalgal (or other microbial) oil (lipid) 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, the species, the extraction method used to recover oil from the biomass and other factors that may affect oil composition. Non-limiting examples of such constituents include carotenoids, present at less than 0.4 micrograms/ml; lycopene, present at less than 0.001

micrograms/ml; beta carotene, present at less than 0.02 micrograms/ml; chlorophyll, present at less than 0.02 milligrams per kilogram of oil; gamma tocopherol, present from 0.40 to 0.60 milligrams per 100 grams of oil; campesterol, present from 3 to 9 milligrams per 100 grams of oil; and tocotrienols, present at less than 0.5 milligrams per gram of oil.

[0165] 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-crytoxanthin), and various organic or inorganic compounds. In some cases, the oil extracted from *Prototheca* species comprises between 0.003 to 0.039 micrograms lutein/gram of oil, less than 0.003 micrograms lycopene/gram of oil; and less than 0.003 micrograms beta carotene/gram of oil.

5. Oleaginous yeast strains and culture conditions

[0166] The present invention provides methods for producing oils/lipids from oleaginous yeast biomass. The invention arose, in part, from discoveries that yeast biomass can be prepared with a high oil content and the extracted oil can be converted into a variety of useful products, including dielectric fluids and other lubricants. Yeast oil, which can comprise a mixture of saturated and mid to longer chain fatty acids (e.g., C16 and C18 fatty acids), provides excellent starting material for the preparation of chemicals including dielectric fluids.

[0167] A variety of species of yeast that produce suitable oils and/or lipids can be used in accordance with the methods described herein, although yeast that naturally produces high levels of suitable oils or lipids are preferred.

[0168] In particular embodiments, the oleaginous yeast comprise cells that are at least 20% or more triglyceride oil by dry weight. In other embodiments, the oleaginous yeast contains at least 25-35% or more triglyceride oil by dry weight. Generally, in these embodiments, the more oil contained in the oleaginous yeast, the more oil that can be extracted from the biomass, so the oleaginous yeast can be cultured to contain at least 40%, at least 50%, or at least 60% or more triglyceride oil by dry weight are typically preferred. Not all types of lipids are desirable for use in chemicals, such as dielectric fluids, as they may have an undesirable chain length, saturation levels, or associated with undesireable contaminants. These considerations also influence the selection of oleaginous yeast (or any other microbe) for use in the methods described herein.

[0169] Suitable species of oleaginous yeast for use in the methods described herein include, but are not limited to *Candida apicola, Candida sp., Cryptococcus curvatus, Cryptococcus terricolus, Debaromyces hansenii, Endomycopsis vernalis, Geotrichum carabidarum,*

Geotrichum cucujoidarum, Geotrichum histeridarum, Geotrichum silvicola, Geotrichum vulgare, Hyphopichia burtonii, Lipomyces lipofer, Lypomyces orentalis, Lipomyces starkeyi, Lipomyces tetrasporous, Pichia mexicana, Rodosporidium sphaerocarpum, Rhodosporidium toruloides Rhodotorula aurantiaca, Rhodotorula dairenensis, Rhodotorula diffluens, Rhodotorula glutinus, Rhodotorula glutinis var. glutinis, Rhodotorula gracilis, Rhodotorula graminis Rhodotorula minuta, Rhodotorula mucilaginosa,Rhodotorula mucilaginosa var. mucilaginosa, Rhodotorula terpenoidalis, Rhodotorula toruloides, Sporobolomyces alborubescens, Starmerella bombicola, Torulaspora delbruekii, Torulaspora pretoriensis, Trichosporon behrend, Trichosporon brassicae, Trichosporon domesticum, Trichosporon laibachii, Trichosporon loubieri, Trichosporon loubieri var. loubieri, Trichosporon montevideense, Trichosporon pullulans, Trichosporon sp., Wickerhamomyces Canadensis, Yarrowia lipolytica, and Zygoascus meyerae.

[0170] Species of oleaginous yeast for use in the methods described herein can be identified by comparison of certain target regions of their genome with those same regions of species identified herein; preferred species are those that exhibit identity or at least a very high level of homology with the species identified herein and produce similar amounts, and similar types of, lipid as the strains specifically described herein. For examples, identification of a specific oleaginous yeast species or strain can be achieved through amplification and sequencing of genomic DNA using primers and methodology using appropriate regions of the genome, for example using the methods described in Kurtzman and Robnett, *Antonie van Leeuwenhoek* 73(4): 331-371 (1998), Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Well established methods of phylogenetic analysis, such as amplification and sequencing of nuclear 18S and 26S and internal transcribed spacer (ITS) regions of ribosomal RNA genes and other conserved regions can be used by those skilled in the art to identify species of oleaginous yeasts suitable for use in the methods disclosed herein.

[0171] Thus, genomic DNA comparison can be used to identify suitable species of oleaginous yeast to be used in the methods described herein. Regions of conserved genomic DNA, such as, but not limited to conserved genomic sequences between 3' regions of fungal 18S and 5' regions of fungal 26S rRNA genes can be amplified from yeast species that may be, for example, taxonomically related to the preferred oleaginous yeasts used in the methods described herein and compared to the corresponding regions of those preferred species. Species that exhibit a high level of similarity are then selected for use in the methods described herein. Example 6 describes genomic sequencing of conserved 3' regions of fungal

18S and 5' regions of fungal 26S rRNA for 48 strains of oleaginous yeast strains. Sequence comparison to determine percent nucleotide or amino acid identity can be performed using the same methods disclosed above for microalgae/microorganisms.

[0172] Oleaginous yeast are cultured in liquid media to propagate biomass in accordance with the methods described herein. In the methods described herein, oleaginous yeast species are grown in a medium containing a fixed carbon source and/or fixed nitrogen source in the absence of light (heterotrophic growth). Heterotrophic growth of oleaginous yeast usually occurs in an aerobic environment. For example, heterotrophic growth for extended periods of time such as 10 to 15 or more days under limited nitrogen conditions can result in accumulation of light lipid/oil content in cells.

[0173] Oleaginous yeast culture media typically contains components such as a fixed carbon source (discussed below), a fixed nitrogen source (such as protein, soybean meal, yeast extract, cornsteep liquor, ammonia (pure or in salt form), nitrate, or nitrate salt), trace elements, optionally a buffer for pH maintenance, and phosphate (a source of phosphorous; other phosphate salts can be used).

[0174] In a particular example, a medium suitable for culturing oleaginous yeast strains is YPD medium. This medium is suitable for axenic cultures, and a 1L volume of the medium (pH ~6.8) can be prepared by addition of 10g bacto-yeast, 20g bacto-peptone and 40g glucose into distilled water. 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. Other methods for the growth and propagation of oleaginous yeast strains to generate high lipid levels as a percentage of dry weight have been described (see for example Li et al., *Enzyme and Microbial Technology* (2007) 41:312-317 (demonstrating the culturing *Rhodosporidium toruloides* to 67.5% w/w lipid using fed batch fermentation)). High lipid/oil content in oleaginous yeast can typically be generated by increasing the length of fermentation while providing an excess of carbon source under nitrogen limitation.

[0175] 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 oleaginous yeast can be found, for example, online at www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium186.pdf

[0176] Other suitable media for use with the methods described herein can be readily identified by consulting the URL identified above, or by consulting other organizations that maintain cultures of oleaginous yeast such as Fungal Culture Collections of The World Austrian Center of Biological Resources and Applied Mycology

(www.biotec.boku.ac.at/acbr.html); The Biomedical Fungi and Yeasts Collection (bccm.belspo.be/about/ihem.php); Czech Collection of Microorganisms (sci.muni.cz/ccm/index.html); Institut Pasteur (www.pasteur.fr/ip/easysite/go/03b-000011-08h/); German Collection of Microorganisms and Cell Cultures (www.dsmz.de/); Mychoteca Univesitatis Taurinenesis (web086.unito.it/cgi-bin/bioveg/documenti.pl/Show?_id=b522); Riken Bioresource Center Japan Collection of Microorganisms (www.jcm.riken.jp/JCM/announce.shtml); The National Collection of Yeast Cultures (www.ncyc.co.uk/); ATCC (www.atcc.org/); Phaff Yeast Culture Collection (www.phaffcollection.org/).

[0177] Oleaginous yeast useful in accordance with the methods described herein 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 oil and/or lipid and/or protein from any particular species of microbe can be difficult or impossible to predict, but those of skill in the art can readily find appropriate media by routine testing in view of the disclosure herein. 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. The examples below provide exemplary methods of culturing various species of oleaginous yeast to accumulate high levels of lipid as a percentage of dry cell weight.

[0178] The fixed carbon source is a key component of the medium. Suitable fixed carbon sources for purposes of the methods described herein, include for example, glucose, fructose, sucrose, lactose, galactose, xylose, mannose, rhamnose, arabinose, N-acetylglucosamine, glycerol, glucuronic acid, raffinose, stachyose, and/or acetate. Subsection 3 (Media) above contains a more detailed discussion regarding suitable carbon sources.

[0179] Process conditions can be adjusted to increase the percentage weight of cells that is lipid (oil). For example, in certain embodiments, oleaginous yeast is cultured in the presence of a limiting concentration of one or more nutrients, such as, for example, nitrogen, phosphate, and certain metallic ions, while providing an excess of a fixed carbon source, 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 some embodiments, the nutrient concentration is cycled

between a limiting concentration and a non-limiting concentration at least twice during the total culture period.

[0180] In a steady growth state, the cells accumulate oil (lipid) but do not undergo cell division. In one embodiment of the invention, the growth state is maintained by continuing to provide all components of the original growth media to the cells with the exception of a fixed nitrogen source. Cultivating oleaginous yeast by feeding all nutrients originally provided to the cells except a fixed nitrogen source, such as through feeding the cells for an extended period of time, results in a higher percentage of lipid by dry cell weight.

[0181] In other embodiments, high lipid biomass is generated by feeding a fixed carbon source to the cells after all fixed nitrogen has been consumed for extended periods of time, such as at least one or two weeks. In some embodiments, cells are allowed to accumulate oil in the presence of a fixed carbon source and in the absence of a fixed nitrogen source for over 10, over 15, or over 20 days. Oleaginous yeast grown using conditions described herein or otherwise known in the art can comprise at least about 20% lipid by dry weight, and often comprise 35%, 45%, 55%, 65%, and even 75% or more lipid by dry weight. Percentage of dry cell weight as lipid in microbial lipid production can therefore be improved by holding cells in a growth state in which they consume carbon and accumulate oil but do not undergo cell division.

[0182] Conditions in which nitrogen is in excess tends to increase microbial protein yield over microbial oil yield in a culture in which nitrogen is not provided in excess. Suitable nitrogen sources for oleaginous yeast may come from organic nitrogen sources and/or inorganic nitrogen sources.

[0183] Non-limiting examples of organic nitrogen sources are yeast extract, peptone, corn steep liquor, and corn steep powder. Non-limiting examples of preferred inorganic nitrogen sources include, for example, and without limitation, $(NH_4)_2SO_4$ and NH_4OH. In one embodiment, the culture media for carrying out the invention contains only inorganic nitrogen sources. In another embodiment, the culture media for carrying out the invention contains only organic nitrogen sources. In yet another embodiment, the culture media for carrying out the invention contains a mixture of organic and inorganic nitrogen sources.

[0184] An example of a medium formulation used to grow oleaginous yeast includes: 7g/L KH_2PO_4; 2g/L Na_2HPO_4; 1.5g/L $MgSO_4 \cdot 7H_2O$; 1.5g/L yeast extract; 0.2g/L $CaCl_2 \cdot 6H_2O$; 0.1g/L $FeCl_3 \cdot 6H_2O$; 0.001g/L biotin and 0.001g/L $ZnSO_4 \cdot 7H_2O$ with a pH level adjusted to 5.5 with HCL and with 12g/L glucose and 30g/L NH_4Cl as a nitrogen source. Another medium that is used to grow oleaginous yeast includes: 20g/L glucose; 0.5g/L yeast extract;

5g/L $(NH_4)_2SO_4$; and 1g/L KH_2PO_4; 0.5g/L $MgSO_4 \cdot 7H_2O$. One medium formulation for the growth of oleaginous yeast in a fermentor consists of: 30g/L glucose; 20g/L xylose; 2g/L $(NH_4)_2SO_4$; 1g/L KH_2PO_4; and 0.5g/L $MgSO_4 \cdot 7H_2O$.

[0185] In the methods described herein, a bioreactor or fermentor is used to culture oleaginous yeast cells through the various phases of their physiological cycle. As an example, an inoculum of lipid-producing oleaginous yeast cells is 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 fatty acid species produced, and/or production of a specific oil.

[0186] To produce high lipid oleaginous yeast, cells are preferably fermented in large quantities in liquid, such as in suspension cultures as an example. Bioreactors such as steel fermentors (5000 liter, 10,000 liter, 80,000 liter, and larger volumes are used in various embodiments of the invention) can accommodate very large culture volumes. 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.

[0187] Bioreactors can be configured to flow culture media though the bioreactor throughout the time period during which the oleaginous yeast 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 oleaginous yeast biomass is cultured in an aqueous medium for a period of time during which the yeast 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.

[0188] Bioreactors equipped with devices such as spinning blades and impellers, rocking mechanisms, stir bars, means for pressurized gas infusion can be used to subject oleaginous yeast cultures to mixing. Mixing may be continuous or intermittent. As briefly mentioned above, bioreactors are often equipped with various ports that, for example, allow the gas content of the culture 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/CO_2 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.

[0189] 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.

[0190] 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.

[0191] Oleaginous yeast cultures generated according to the methods described above yield oleaginous yeast biomass in fermentation media. To prepare this biomass, as well as to prepare microalgal or other microbial biomass, for extraction of oil, the biomass is typically concentrated, or harvested, from the fermentation medium. At the point of harvesting the oleaginous yeast biomass from the fermentation medium, the biomass comprises predominantly intact cells suspended in an aqueous culture medium. To concentrate the biomass, a dewatering step can be performed. Dewatering or concentrating refers to the separation of the biomass from fermentation broth or other liquid medium and so is solid-liquid separation. Thus, during dewatering, the culture medium is removed from the biomass (for example, by draining the fermentation broth through a filter that retains the biomass), or the biomass is otherwise removed from the culture medium. Common processes for dewatering include centrifugation, filtration, and the use of mechanical pressure. These processes can be used individually or in any combination.

[0192] Centrifugation involves the use of centrifugal force to separate mixtures. During centrifugation, the more dense components of the mixture migrate away from the axis of the centrifuge, while the less dense components of the mixture migrate towards the axis. By increasing the effective gravitational force (*i.e.*, by increasing the centrifugation speed), more dense material, such as solids, separate from the less dense material, such as liquids, and so separate out according to density. Centrifugation of biomass and broth or other aqueous solution forms a concentrated paste comprising the oleaginous yeast cells. Centrifugation does not remove significant amounts of intracellular water. In fact, after centrifugation, there may still be a substantial amount of surface or free moisture in the biomass (*e.g.*, upwards of 70%), so centrifugation is not considered to be a drying step.

[0193] Filtration can also be used for dewatering. One example of filtration that is suitable for the methods described herein is tangential flow filtration (TFF), also known as cross-flow filtration. Tangential flow filtration is a separation technique that uses membrane systems and flow force to separate solids from liquids. For an illustrative suitable filtration method, see Geresh, Carb. Polym. 50; 183-189 (2002), which describes the use of a MaxCell A/G Technologies 0.45uM hollow fiber filter. Also see, for example, Millipore Pellicon® devices, used with 100kD, 300kD, 1000 kD (catalog number P2C01MC01), 0.1uM (catalog number P2VVPPV01), 0.22uM (catalog number P2GVPPV01), and 0.45uM membranes (catalog number P2HVMPV01). The retentate preferably does not pass through the filter at a significant level, and the product in the retentate preferably does not adhere to the filter material. TFF can also be performed using hollow fiber filtration systems. Filters with a pore size of at least about 0.1 micrometer, for example about 0.12, 0.14, 0.16, 0.18, 0.2, 0.22, 0.45, or at least about 0.65 micrometers, are suitable. Preferred pore sizes of TFF allow solutes and debris in the fermentation broth to flow through, but not microbial cells.

[0194] Dewatering can also be effected with mechanical pressure directly applied to the biomass to separate the liquid fermentation broth from the microbial biomass sufficient to dewater the biomass but not to cause predominant lysis of cells. Mechanical pressure to dewater microbial biomass can be applied using, for example, a belt filter press. A belt filter press is a dewatering device that applies mechanical pressure to a slurry (*e.g.*, microbial biomass taken directly from the fermentor or bioreactor) that is passed between the two tensioned belts through a serpentine of decreasing diameter rolls. The belt filter press can actually be divided into three zones: the gravity zone, where free draining water/liquid is drained by gravity through a porous belt; a wedge zone, where the solids are prepared for

pressure application; and a pressure zone, where adjustable pressure is applied to the gravity drained solids.

[0195] After concentration, oleaginous yeast biomass is processed, as described hereinbelow, to prepare it for oil extraction.

[0196] Oleaginous yeast biomass with a high percentage of oil/lipid accumulation by dry weight has been generated using different methods of culture, including methods known in the art. Oleaginous yeasts with a higher percentage of accumulated oil/lipid are useful in the methods described herein. *Candida 107* was shown to be able to accumulate up to 40% lipid wt/wt under nitrogen limiting conditions (Gill et al., *Appl and Environ Microbiology* (1977) pp.231-239). Li et al. demonstrated the production of *Rhodosporidium toruloids* 44 in fed-batch cultures to a lipid content of 48% w/w (Li et al., *Enzyme and Microbial Technology* (2007) 41:312-317. *Yarrowia lipolytica* has been shown to be able to produce between 0.44-0.54g of lipid per gram of biomass when using animal fat (stearin) as a carbon source (Panpanikolaou et al., *Appl Microbiol Biotechnol* (2002) 58:308-312.

[0197] Biomass generated by the culture methods described herein and useful in accordance with the methods described herein comprises at least 10% oil by dry weight. In some embodiments, the biomass comprises at least 25%, at least 50%, at least 55%, or at least 60% oil by dry weight. In some embodiments, the biomass contains from 10-90% oil, from 25-75% oil, from 40-75% oil, or from 50-70% oil by dry weight.

[0198] The oil of the biomass described herein, or extracted from the biomass for use in the methods and compositions described herein 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 oil composition, *i.e.*, the properties and proportions of the fatty acid consitutents of the glycerolipids, can be manipulated by combining biomass or oil from at least two distinct species of oleaginous yeast (or a strain of oleaginous yeast and another oil producing microbe). In some embodiments, at least two of the distinct species of microbe have different glycerolipid profiles. The distinct species of microbe can be cultured together or separately as described herein, preferably under heterotrophic conditions, to generate the respective oils. Different species of microbe can contain different percentages of distinct fatty acid constituents in the cell's glycerolipids.

[0199] *Yarrowia lipolytica* has been genetically engineered. An embodiment of the invention uses engineered strains of *Yarrowia lipolytica* containing lipid modification enzymes to make oils suitable for use as lubricants and dielectric fluids. Examples of

engineering Yarrowia are described in US Patent Nos. 7,465,565 and 7,273,746 and US Patent Application Serial Nos. 10/840579, 11/613420, 11/714377 and 11/264737.

III. GENETIC ENGINEERING METHODS AND MATERIALS

[0200] The methods described herein can be practiced using recombinant microalgae or other recombinant oleaginous microbes. This section describes methods and materials for genentically modifying oleaginous microbes, such as microalgae, specifically exemplifying *Prototheca* cells, to make recombinant host cells useful in the methods described herein, 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

[0201] 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 or other microbial 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).

[0202] 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 oleaginous microbes, such as a *Prototheca* cell.

2. Engineering methods - homologous recombination

[0203] Homologous recombination relates to the ability of complementary DNA sequences to align and exchange regions of homology. In the homologous recombination process, 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.

[0204] The ability to carry out homologous recombination in a host organism has many practical implications for what can be done at the molecular genetic level and is useful in the generation of an oleaginous microbe that can produced tailored oils (lipids). 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 can 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 a particular genome environment on gene expression.

[0205] Particularly useful genetic engineering applications using homologous recombination co-opt specific host regulatory elements such as promoters/UTRs to drive heterologous gene expression in a highly specific fashion. For example, ablation or knockout of desaturase genes/gene families with a heterologous gene encoding a selective marker can be used to increase overall percentage of saturated fatty acids produced in the host cell. Example 4 describes the homologous recombination targeting constructs and a working example of such desaturase gene ablations (knockouts) generated in *Prototheca moriformis*. Another approach to decreasing expression of an endogenous gene is to use an RNA-induced method of downregulation or silencing of gene expression including, but not limited to, an RNAi or antisense approach, as well as a dsRNA approach. Antisense, RNAi, dsRNA, and hairpin RNA approaches are well known in the art and include the introduction of an expression construct that, when expressed as mRNA, leads to the formation of a hairpin RNA or an expression construct containing a portion of the target gene that is transcribed in the antisense orientation. All of these approaches result in the decreased expression of the target gene. Example 4 also describes expression constructs and a working example of the down-regulation of an endogenous *Prototheca moriformis* delta 12 desaturase gene (FADc) by a hairpin RNA approach.

[0206] Because homologous recombination is a precise gene targeting event, it can be used to modify any nucleotide(s) within a gene or region of interest precisely, 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 to modify enzyme activities such as substrate specificity, affinity, and Km, thus effecting 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, and gene inversion, and in the exchange of gene expression regulatory elements such as promoters, enhancers and 3'UTRs.

[0207] Homologous recombination can be achieved 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.

[0208] For purposes of non-limiting illustration, regions of donor DNA sequences that are useful for homologous recombination include the KE858 region of DNA in *Prototheca moriformis*. 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. This region and examples of using this region for homologous recombination targeting has been described in PCT Application No. PCT/US2009/66142. Another useful region of donor DNA is the 6S genomic sequence.

3. Vectors and vector components

[0209] Vectors for transformation of microorganisms 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 that can be contained on vectors. Subsection B describes genes typically contained in vectors as well as codon optimization methods and genes prepared using them.

A. Control Sequences

[0210] 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.

[0211] Thus, an exemplary vector design for expression of an exogenous gene in a microalgae or other oleaginous microbe 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 the microalgae or other oleaginous microbe. 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) and other microbes.

[0212] 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.

[0213] The promoter used to express an exogenous gene can be the promoter naturally linked to that gene or can be a heterologous gene promoter. 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 promoters derived from 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. Typically, 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.

[0214] 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 described herein. Inducible promoters useful in the methods described herein 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.

[0215] Inclusion of termination region control sequence is optional, and if employed, then the choice is be primarily one of convenience, as termination regions are 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.

[0216] The methods described herein may also make use of vectors containing control sequences and recombinant genes that provide for the compartmentalized expression of a gene of interest. Organelles for targeting are chloroplasts, plastids, mitochondria, and endoplasmic reticulum. In addition, the methods described herein may also make use of control sequences and recombinant genes and vectors containing them described herein that provide for the secretion of a protein outside the cell.

[0217] 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, vectors containing such control sequences are used in the methods described herein to target expression of a protein to a *Prototheca* plastid.

[0218] 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 protothecodies* cells and are described in PCT Application No. PCT/US2009/066142.

[0219] In another embodiment, the expression of a polypeptide in *Prototheca* or another oleaginous microbe is targeted to the endoplasmic reticulum. The inclusion of an appropriate retention or sorting signal in an expression vector ensures 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.

[0220] 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 other microalgae, such as *Prototheca*, as well.

B. Genes and Codon Optimization

[0221] 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.

[0222] 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, or 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* or any other oleaginous microbe useful in the methods described herein. 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 and other oleaginous microbes to antibiotics are well known. For example, see Mol Gen Genet. 1996 Oct 16;252(5):572-9.

[0223] Other selectable markers that are not antibiotic-based can also be employed in a transgene construct useful for transforming microalgae in general, including *Prototheca* species. Genes that confers the ability to utilize certain carbon sources that were previously unable to be utilized by the microalgae can also be used as a selectable marker. By way of illustration, *Prototheca moriformis* strains typically grow poorly, if at all, on sucrose. Using a construct containing a sucrose invertase gene can confer the ability of positive transformants to grow on sucrose as a carbon substrate.

[0224] For purposes of certain embodiments of the methods described herein, the expression vector used to prepare a recombinant host cell will include at least two, and often three, genes, if one of the genes is a selectable marker. For example, a genetically engineered *Prototheca* can be made by transformation with vectors that comprise, in addition to a selectable marker, one or more exogenous genes, such as, for example, a sucrose invertase gene or an 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).

[0225] 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.

[0226] Other illustrative vectors 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* or other microalgal or microbial cell transformed with either type of vector produces 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 even 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 lipid (fatty acid) species produced relative to other lipid species, 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.

[0227] 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.

[0228] Codon-optimized nucleic acids useful for the successful expression of recombinant proteins in *Prototheca* are described herein. 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 4 below.

Table 4. 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)			
	GCC	442 (0.46)	Pro	CCG	161 (0.29)
				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)
				CGG	102 (0.18)
Phe	TTT	89 (0.29)		CGA	49 (0.08)
	TTC	216 (0.71)		CGT	51 (0.09)
				CGC	331 (0.57)
Gly	GGG	92 (0.12)			
	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)
				TCA	31 (0.06)
His	CAT	42 (0.21)		TCT	55 (0.10)
	CAC	154 (0.79)		TCC	173 (0.31)
Ile	ATA	4 (0.01)	Thr	ACG	184 (0.38)
	ATT	30 (0.08)		ACA	24 (0.05)
	ATC	338 (0.91)		ACT	21 (0.05)
				ACC	249 (0.52)
Lys	AAG	284 (0.98)			
	AAA	7 (0.02)	Val	GTG	308 (0.50)
				GTA	9 (0.01)
Leu	TTG	26 (0.04)		GTT	35 (0.06)
	TTA	3 (0.00)		GTC	262 (0.43)
	CTG	447 (0.61)			
	CTA	20 (0.03)	Trp	TGG	107 (1.00)
	CTT	45 (0.06)			
	CTC	190 (0.26)	Tyr	TAT	10 (0.05)
				TAC	180 (0.95)
Met	ATG	191 (1.00)			
			Stop	TGA/TAG/TAA	

[0229] In other embodiments, the gene in the recombinant vector has been codon-optimized with reference to a microalgal strain other than a *Prototheca* strain or another microbial strain. For example, methods of recoding genes for expression in microalgae are described in U.S. Patent No. 7,135,290. Additional information for codon optimization is available, e.g., at the codon usage database of GenBank.

[0230] While the methods and materials described herein allow for the introduction of any exogenous gene into *Prototheca* or other microalgae or other oleaginous microbes, genes relating to sucrose utilization and lipid pathway modification are of particular interest for microbes unable to utilize it naturally or for microbes that utilize it inefficiently, as discussed in the following sections.

IV. SUCROSE UTILIZATION

[0231] In embodiment, the recombinant *Prototheca* or other microalgal or other microbial cell 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* or any other microalgal or other microbial cell 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.

[0232] In one embodiment, the methods described herein are practiced with 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 useful in the methods described herein. 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.

[0233] *Prototheca* species expressing an invertase that is secreted into a media containing sucrose are a preferred microalgal species for the production of microbial oil for use as a dielectric fluid or other lubricant (for production of food oils, some consumers may prefer oil produced using non-recombinant microbes). 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 practice of the methods described herein may utilize *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. Invertase genes are useful as selectable markers in *Prototheca* and other microalgal 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 is a powerful, selectable marker for algal molecular genetics.

[0234] The successful expression of a sucrose invertase in *Prototheca* also demonstrates that heterologous (recombinant) proteins can be expressed in an algal cell and successfully transit outside of the cell and into the culture medium in a fully active and functional form. Thus, methods and reagents for expressing a wide and diverse array of heterologous proteins in microalgae and secreting them outside of the host cell are available. Such proteins include, for example, industrial enzymes such as, for example, lipases, proteases, cellulases, pectinases, amylases, esterases, oxidoreductases, transferases, lactases, isomerases, and invertases.

[0235] 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 5. 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 described herein 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 5.

Table 5. Sucrose invertases.

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

[0236] The secretion of an invertase to the culture medium by *Prototheca* enables 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, methods described herein may involve the use of a microbial culture containing a population of *Prototheca* or other microalgal 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). While the microbes exemplified here are altered such that they can utilize sucrose, the methods and reagents described herein can be applied so that feedstocks such as cellulosics are utilizable by an engineered host microbe 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

[0237] In addition to altering the ability of *Prototheca* (or other microalgal or other microbial cells) to utilize feedstocks such as sucrose-containing feedstocks, recombinant *Prototheca* (or other microalgal or other microbial cells) that have been modified to alter the properties and/or proportions of lipids produced are useful in the methods described herein. 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 cells have, relative to their untransformed counterparts, an increased or optimized lipid yield per unit volume and/or per unit time, carbon chain length (e.g., for industrial chemicals, including but not limited to dielectric fluids, and other 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 (fatty acid) or of a population of distinct lipid.

[0238] 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 also be used for amplification of the construct and a concomitant increase in the expression level of the encoded enzyme. Examples of enzymes suitable for up-regulation according to the methods described herein 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).

[0239] 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).

[0240] The methods described herein can also be practiced with recombinant *Prototheca* (or other microalgal or other microbial) 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 6), fatty acyl-CoA/aldehyde reductases (see Table 8), fatty acyl-CoA reductases, fatty aldehyde decarbonylase, fatty aldehyde reductases, desaturases (such as stearoyl-ACP desaturases and fatty acyl desaturases) 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* (or other microalgal or other microbial) 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 described herein, irrespective of whether they are or are not naturally co-expressed in a particular tissue or organism. Thus, in certain embodiments, 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.

[0241] In still other embodiments, an exogenous gene encoding a desaturase is transformed into the *Prototheca* (or other microalgal or other microbial) cell in conjunction with one or more genes encoding other lipid modification enzymes to provide modifications with respect to lipid saturation. In another embodiment, an endogenous desaturase gene is overexpressed (e.g., through the introduction of additional copies of the gene) in a *Prototheca* (or other microalgal or other microbial) cell. 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. For illustrative purposes, stearoyl-ACP desaturases (SAD) are responsible for for the synthesis of C18:1 fatty acids from C18:0 precursors. Another family of desaturases are the fatty acyl desaturases (FAD), including delta 12 fatty acid desaturases. These desaturases also provide modifications with respect to lipid saturation. For illustrative purposes, delta 12 fatty acid desaturases are responsible for the synthesis of C18:2 fatty acids from C18:1 precursors. 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. In another embodiment, if the desired fatty acid profile is an increase in monounsaturates (such as C16:1 and/or C18:1) overexpression of a SAD or expression of a heterologous SAD can be coupled with the silencing or inactivation (e.g., through mutation, RNAi, hairpin RNAs, knockout of an endogenous desaturase gene, etc.) of a fatty acyl desaturase (FAD). Example 4 below describes the targeted ablation or knockout of stearoyl-ACP desaturases and delta 12 fatty acid desaturases and also describes the use of hairpin RNA antisense constructs to decrease the expression of an endogenous desaturase gene.

[0242] 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 desaturase, a fatty aldehyde decarbonylase, or a naturally co-expressed acyl carrier protein. Suitable expression methods are described above for 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.

[0243] In some embodiments, fatty acids, glycerolipids, or the corresponding primary alcohols, aldehydes, alkanes, or alkenes, generated by the methods described herein, contain 16 or 18 carbon atoms. Preferred fatty acids for the production of dielectric fluids or the corresponding alcohols, aldehydes, alkanes and alkenes contain 16-18 carbon atoms. In certain embodiments, the above fatty acids are saturated (with no carbon-carbon double or triple bonds; mono-unsaturated (single double bond); poly-unsaturated (two or more double bonds; and can be either linear (not cyclic) or branched or a mixture of the two types. For dielectric fluids, mono-unsaturated fatty acids are preferred, especially oleic acid (C18:1). To increase production of lipids having the desired chain length and/or degree of saturation, one can engineer the microalgal cell to over-express a thioesterase with the desired chain-length specificity, to knockout production of thioesterases with shorter chain length specificity or to reduce the expression of such genes, and/or to knock-out desaturase genes responsible for the degree of saturation in the desired lipids.

[0244] Various enzymes described above typically 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* (or other microalgal or other microbial) 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 and preferably from 16 to 18 carbon atoms.

[0245] Other fatty acyl-ACP thioesterases suitable for use with the microbes and methods described herein include, without limitation, those listed in Table 6.

Table 6. Fatty acyl-ACP thioesterases and GenBank accession numbers.

Umbellularia californica fatty acyl-ACP thioesterase (GenBank #AAC49001)
Cinnamomum camphora fatty acyl-ACP thioesterase (GenBank #Q39473)
Umbellularia californica fatty acyl-ACP thioesterase (GenBank #Q41635)
Myristica fragrans fatty acyl-ACP thioesterase (GenBank #AAB71729)
Myristica fragrans fatty acyl-ACP thioesterase (GenBank #AAB71730)
Elaeis guineensis fatty acyl-ACP thioesterase (GenBank #ABD83939)
Elaeis guineensis fatty acyl-ACP thioesterase (GenBank #AAD42220)
Populus tomentosa fatty acyl-ACP thioesterase (GenBank #ABC47311)
Arabidopsis thaliana fatty acyl-ACP thioesterase (GenBank #NP_172327)
Arabidopsis thaliana fatty acyl-ACP thioesterase (GenBank #CAA85387)
Arabidopsis thaliana fatty acyl-ACP thioesterase (GenBank #CAA85388)
Gossypium hirsutum fatty acyl-ACP thioesterase (GenBank #Q9SQI3)
Cuphea lanceolata fatty acyl-ACP thioesterase (GenBank #CAA54060)
Cuphea hookeriana fatty acyl-ACP thioesterase (GenBank #AAC72882)
Cuphea calophylla subsp. mesostemon fatty acyl-ACP thioesterase (GenBank #ABB71581)
Cuphea lanceolata fatty acyl-ACP thioesterase (GenBank #CAC19933)
Elaeis guineensis fatty acyl-ACP thioesterase (GenBank #AAL15645)
Cuphea hookeriana fatty acyl-ACP thioesterase (GenBank #Q39513)
Gossypium hirsutum fatty acyl-ACP thioesterase (GenBank #AAD01982)
Vitis vinifera fatty acyl-ACP thioesterase (GenBank #CAN81819)
Garcinia mangostana fatty acyl-ACP thioesterase (GenBank #AAB51525)
Brassica juncea fatty acyl-ACP thioesterase (GenBank #ABI18986)
Madhuca longifolia fatty acyl-ACP thioesterase (GenBank #AAX51637)
Brassica napus fatty acyl-ACP thioesterase (GenBank #ABH11710)
Oryza sativa (indica cultivar-group) fatty acyl-ACP thioesterase (GenBank #EAY86877)
Oryza sativa (japonica cultivar-group) fatty acyl-ACP thioesterase (GenBank #NP_001068400)
Oryza sativa (indica cultivar-group) fatty acyl-ACP thioesterase (GenBank #EAY99617)
Cuphea hookeriana fatty acyl-ACP thioesterase (GenBank #AAC49269)
Ulmus Americana fatty acyl-ACP thioesterase (GenBank #AAB71731)
Cuphea lanceolata fatty acyl-ACP thioesterase (GenBank #CAB60830)
Cuphea palustris fatty acyl-ACP thioesterase (GenBank #AAC49180)
Iris germanica fatty acyl-ACP thioesterase (GenBank #AAG43858)
Iris germanica fatty acyl-ACP thioesterase (GenBank #AAG43858.1)
Cuphea palustris fatty acyl-ACP thioesterase (GenBank #AAC49179)
Myristica fragrans fatty acyl-ACP thioesterase (GenBank# AAB71729)
Myristica fragrans fatty acyl-ACP thioesterase (GenBank# AAB717291.1)
Cuphea hookeriana fatty acyl-ACP thioesterase (GenBank #U39834)
Umbelluaria californica fatty acyl-ACP thioesterase (GenBank # M94159)
Cinnamomum camphora fatty acyl-ACP thioesterase (GenBank #U31813)
Ricinus communis fatty-acyl ACP thioesterase (GenBank#ABS30422.1)

[0246] Bio-oil based chemicals such as dielectric fluids have fatty acid compositions of high oleic acid (C18:1) originating from natural esters (i.e., seed oils) such as from sunflower oil and canola oil. Table 7 shows the fatty acid profiles of common commercial seed oils.

All commercial seed oil data below were compiled from the US Pharmacopeias Food and Chemicals Codes, 7th Ed. 2010-2011.

Table 7. Lipid profiles of commercial seed oils.

	C8:0	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:0-diOH	C18:1-OH	C18:2	C18:3 α
R. communis (Castor oil)	0	0	0	0	0.9-1.6	1.0-1.8	3.7-6.7	0.4-1.3	83.6-89.0	0	0.2-0.6
C. nucifera (Coconut oil)	5.0-9.0	4.0-8.0	44-52	15-21	8.0-11.0	1.0-4.0	5.0-8.0	0	0	0-2.5	0
Z. mays (Corn oil)	0	0	0	< 1.0	8.0-19.0	0.5-4.0	19-50	0	0	38-65	< 2.0
G. barbadense (Cottonseed oil)	0	0	< 0.1	0.5-2.0	17-29	1.0-4.0	13-44	0	0	40-63	0.1-2.1
B. rapa, B napus, B. juncea (Canola)	0	0	< 0.1	< 0.2	< 6.0	< 2.5	> 50	0	0	< 40	< 14
O. europea (Olive)	0	0	0	< 0.1	6.5-20.0	0.5-5.0	56-85	0	0	3.5-20.0	< 1.2
A. hypogaea (Peanut)	0	0	< 0.1	< 0.2	7.0-16.0	1.3-6.5	35-72	0	0	13.0-43	< 0.6
E. guineensis (Palm kernel)	3.0-5.0	2.5-6.0	40-52	14.0-18.0	7.0-10.0	1.0-3.0	11.0-19.0	0	0	0.5-4.0	0
E. guineensis (Palm)	0	0	0	0.5-5.9	32.0-47.0	2.0-8.0	34-44	0	0	7.2-12.0	0
C. tinctorus (Safflower)	0	0	< 0.1	< 0.1	2.0-10.0	1.0-10.0	7.0-16.0	0	0	72-81	< 1.5
H. annus (Sunflower)	0	0	< 0.1	< 0.5	3.0-10.0	1.0-10.0	14-65	0	0	20-75	< 0.5
G. max (Soybean)	0	0	< 0.1	< 0.5	7.0-12.0	2.0-5.5	19-30	0	0	48-65	5.0-10.0
L. usitatissimum (Solin-Flax)	0	0	< 0.1	< 0.5	2.0-9.0	2.0-5.0	8.0-60	0	0	40-80	< 5.0
B. parkii (Sheanut)	0	0	0	0	3.8-4.1	41.2-56.8	34.0-46.9	0	0	3.7-6.5	0

[0247] Fatty acyl-CoA/aldehyde reductases suitable for use with the microbes and methods described herein include, without limitation, those listed in Table 8.

Table 8. 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

[0248] Acyl-ACP thioesterases are the terminators of higher plant (and some microalgal species) fatty acid biosynthesis, and in most plant species, this is carried out by members of the FatA gene family, whose role is to terminate elongation at the C16:0 to C18:0 stage. In species that synthesize shorter chain fatty acids (such as *Cuphea, Elaeis, Myristica,* or *Umbellularia*), a different group of acyl-ACP thioesterases encoded by FatB genes carry out this termination step.

[0249] Other suitable enzymes for use in the methods described herein include those that have at least 70% amino acid identity with one of the proteins listed in Tables 6 and 8, 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.

[0250] By selecting the desired combination of exogenous genes to be expressed (or endogenous genes to be inactivated or both), one can tailor the oil 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; (ii) optionally, a naturally co-expressed acyl carrier protein or an acyl carrier protein having affinity for the fatty acid acyl-ACP thioesterase; (iii) a mutated endogenous desaturase gene, wherein the mutation renders the desaturase gene or desaturase protein inactive, such as a desaturase

knockout; (iv) overexpression of an endogenous stearoyl acyl carrier protein desaturase or the expression of a heterologous SAD; and (v) any combination of the foregoing.

[0251] Genes encoding such enzymes, such as fatty acyl ACP thioesterases, 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. Methods for identifying genes that can alter (improve) lipid production in microalgae are described in PCT Pub. No.2008/151149, incorporated herein by reference.

[0252] Thus, in certain embodiments, the practice of the present invention may utilize a *Prototheca* or other microalgal or other microbial 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 one embodiment in which the cell expresses the lipid pathway enzyme at a lower level, the lipid pathway enzyme comprises citrate synthase.

[0253] 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. In some cases, the cell has been genetically engineered and/or selected to express a lower level of a lipid pathway enzyme, or not to express a specific lipid pathway enzyme at all (i.e., wherein a lipid pathway enzyme has been knocked out or replaced with an exogenous gene).

[0254] In other embodiments, practice of the present invention utilizes an oil-producing microbe containing one or more exogenous genes and/or one or more inactivated, endogenous genes, wherein the exogenous or endogenous 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, a

desaturase, and an acyl carrier protein. In another embodiment, an endogenous desaturase gene is overexpressed in a microbe containing one or more of the above exogenous genes. 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 or otherwise targeted to 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.*

[0255] In one embodiment, the exogenous gene or inactivated endogenous gene encodes a fatty acid acyl-ACP thioesterase. In some cases, the thioesterase encoded by the exogenous or inactivated endogenous 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 or inactivated endogenous 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 or inactivated endogenous gene catalyzes the cleavage of a 12-carbon fatty acid from an ACP. In some embodiments, the thioesterase encoded by the exogenous gene catalyzes the cleavage of a 16-18 carbon fatty acid from an ACP.

[0256] 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 or inactivated endogenous 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 or inactivated endogenous gene catalyzes the reduction of a 12-carbon fatty acyl-CoA to dodecanol.

[0257] Practice of the methods described herein may utilize a recombinant *Prototheca* (or other microalgal or microbial) cell containing two exogenous genes (or two inactivated endogenous genes), wherein a first exogenous gene or inactivated endogenous gene encodes a fatty acyl-ACP thioesterase and a second exogenous gene or inactivated endogenous 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* and other microalgal and other microbial species, and can be used as a trigger to induce oil (lipid) 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%.

[0258] The novel oils (lipids) and dielectric fluids derived from them disclosed herein are distinct from other naturally occurring oils that are high in C16 and C18 fatty acids, such as sunflower and canola oil.

[0259] 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. Additionally, in those embodiments where oils of longer chain length are desired, expression of one or more shorter chain length (i.e., below C14, such as C12, C10, and/or C8) TE and/or corresponding ACP genes is reduced (via altering its expression) or eliminated (via a knockout, for example).

[0260] In the various embodiments described above, the *Prototheca* (or other microalgal or other microbial) cell can contain at least one exogenous or at least one inactivated (or engineered to reduce expression) endogenous gene encoding a lipid pathway enzyme. In some cases, the lipid pathway enzyme is selected from the group consisting of a stearoyl-ACP desaturase, a fatty acid 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* or other 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. PRODUCTION OF MICROBIAL OIL AND PRODUCTS DERIVED THEREFROM

1. Production of microbial oil

[0261] For the production of microbial oil in accordance with the methods described herein, the raw, unprocessed oil (lipids) produced by microbial cells is harvested, or otherwise collected, by any convenient means. The oil can be isolated by whole cell extraction, for example. In this method, the cells are first disrupted, and then intracellular and cell membrane/cell wall-associated lipids and fatty acids 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 many embodiments, extracted after or during the process of lysing the microbial cells.

[0262] More specifically, after completion of culturing, the microorganisms are typically separated from the fermentation broth. Often, the separation is effected by centrifugation to generate a concentrated paste of microbial biomass. . 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.

[0263] 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%.

[0264] In particular embodiments, the microorganism is lysed after growth, for example to increase the exposure of microbial oil for extraction or further processing. If an exogenous lipase gene is being utilized, 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. A number of lysis techniques are described below. These techniques can be used individually or in combination.

[0265] 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, or 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.

[0266] 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 embodiments of 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.

[0267] 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 mN or preferably 40-160 nM. Acid lysis is preferably performed at above room temperature (e.g., at 40-160°, i.e., 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.

[0268] 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., cellulose from Trichoderma viride; Sigma Aldrich, St. Louis, MO; #C9422), and driselase (e.g., driselase from *Basidiomycetes sp.*; Sigma Aldrich, St. Louis, MO; #D9515).

[0269] 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, and/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.

[0270] 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.

[0271] 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 by 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.

[0272] 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.

[0273] In another embodiment of the present invention, the step of lysing a microorganism is performed by applying an osmotic shock (i.e., suspending the microorganism cells in a hypotonic solution).

[0274] 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 methods described herein, 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.

[0275] In another embodiment of the present invention, the step of lysing a microorganism comprises autolysis. In this embodiment, a microorganism 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.

[0276] 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 CO_2 extraction (see for example Mendes et al. 2003, Inorganica Chimica Acta 356:328-334). Miao and Wu describe a protocol of the recovery of microalgal lipid from a

culture of *Chlorella prototheocoides* 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.

[0277] Thus, lipids, lipid derivatives and hydrocarbons generated by the microorganisms described herein 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.

[0278] Other methods for extracting lipids from microorganisms are described in PCT application No. US10/031108, incorporated herein by reference.

[0279] 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. 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*.

2. Further processing of microbial oil

[0280] 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.

[0281] Once lipids are extracted, the lipids can be, in accordance with the methods described herein, put through one or more processing steps. These processing steps are distinct from refining steps performed on crude oil (e.g., petroleum and other sources) when producing fuels. These processing steps are in some aspects comparable to those performed on seed oils during production for human consumption. In some embodiments, the extracted lipids are degummed to extract lecithin and other phospholipids. In other embodiments, the extracted lipids are refined using a base or alkaline metal. In still other embodiments, the extracted lipids are passed through a bleaching clay, usually an acidic clay. In other embodiments, the extracted lipids are deodorized to eliminate or reduce volatile impurities such as aldehydes and ketones. In still other embodiments, the extracted lipids are winterized to eliminate or reduce waxes or saturated fats. The foregoing processing steps can be performed in any and all combinations on the extracted lipids, depending on the characteristics of the desired product. Extracted lipids that have been refined (e.g., with a base or alkaline metal), bleached (e.g., with a bleaching clay) and/or deodorized is usually referred to as RBD oil. RBD oil produced from extracted lipids from microalgae and/or oleaginous yeast described herein are useful in a variety of industrial applications, including the production of dielectric fluids.

[0282] In some embodiments, degumming is performed to remove contaminants such as phospholipids from the oil. In some embodiments of the invention, degumming of the extracted oil is part of the refining, bleaching and deodorizing (or RBD). The RBD process eliminates or reduces the odor, color and/or taste of the extracted oil. In some embodiments, the refining process usually consists of two steps, degumming and a neutralization step that removes the free fatty acids (FFA) in the oil through caustic stripping with sodium hydroxide. The bleaching step may involve mixing the oil with various bleaching clays to absorb color, trace metals and sulfur compounds. The deodorizing step may be a distillation process that occurs at low pressure and high temperature. In an illustrative distillation process, the oil is put under a vaccum and heated with steam to remove any leftover taste or odors and FFAs. Deodorizing can also be achieved by treatment with activated charcoal.

[0283] The above-recited steps can serve to reduce the pour point. In various embodiments, the pour point of the microbial oil (lipid) can be reduced to about -10 degrees C, about -15 degrees C, about -20 degrees C, about -25 degrees C, about 30 degrees C, about -35 degrees C, or about -40 degrees C. In addition, the pour point of the microbial oil can fall within any range bounded by any of these values, e.g., about -10 degrees C to -40 degrees C or about -15 degrees C to about -35 degrees C, etc. The reduction in pour point may occur because these steps reduce the relative proportion of the saturated fraction, which consists primarily of palmitic and stearic triglycerides, known as the stearin fraction. Fractionating the oil reduces the saturated triglycerides concentration of the oil. Fractionation may be accomplished by dry fractionation, as in the winterizing process known in the vegetable oil industry. In this process, the microbial (e.g., algal) oil is first refined, bleached and deodorized by methods similar to those used in the vegetable oil industry. This results in oil with a pour point in the range of -5 to -10 degrees C, for example -8 degrees C.

[0284] The temperature of the RBD oil may then lowered in a controlled manner until crystal nuclei are formed. The oil may then be then held at that crystallization temperature for several hours to facilitate growth of the crystals. The crystals are then removed by filtration to result in two fractions: a solid phase containing some or most of the stearin fraction, and a liquid phase containing mostly the olein fraction. This results in oil with a pour point in the range of -8 to -15 degrees C, for example -11 degrees C. The liquid phase can be subjected to fractionation again to a lower crystallization temperature to effect a further removal of stearin. The resulting purified liquid fraction, equivalent to a super olein, as commonly known in the vegetable oil industry, has better thermal properties than the native microbial oil. For example, a second fractionation can result in oil with a pour point in the range of -15 degrees to -25 degrees C, for example -20 degrees C. The resulting oil is exceptionally useful in a variety of applications, including, importantly food applications, in which the microbial oil can be used as a cheaper, and often healthier, replacement, in whole or in part, of animal and vegetable oils.

3. Products derived from microbial oils

[0285] Microbial oils described herein can also be used to produce products, such as lubricants, hydraulic fluids, industrial oils, or dielectric fluids. Common industrial oils include chainsaw bar lubricants, metal working fluids, food grade lubricants, gear oils, marine oils, engine lubricants, tractor oils, agricultural equipment lubricants, elevator oils, mould release oils, and the like. Dielectic fluids are typically used to cool and/or electrically insulate electrical components (especially in high voltage electrical power distribution

equipment), such as, for example, autoreclosers, capacitors, circuit breakers, high voltage fluid-filled transmission cables, power distribution components, switching gear (e.g., a high-voltage loadbreak switch, such as those described in USPN 6,797,909), transformers, transmission components, and voltage regulators.

[0286] Traditional dielectric fluids include the mineral oil-based lubricants. These include the Group 1, II, and II+ base oils, which are petroleum base oils that have been conventionally refined or mildly hydrotreated and have a viscosity index (VI) of less than 120. These also include the Group III base oils (including "synthetic motor oil" in the US) that are highly refined conventional oil products. The Group III base oils can be made by hydroprocessing (hydrocracking and/or hydroisomerizing) Group 1 or Group II/II+ base oils and contain less saturates, sulfur, and nitrogen than the Group I, II, or II+ base oils and have a VI greater than 120. The American Society of Testing and Materials (ASTM) establishes specifications for dielectric fluids and other hydrocarbon compositions (such as diesel fuel (ASTM D975), jet fuel (ASTM D1655), and biodiesel (ASTM D6751)) according to any of a number of factors, such as the boiling point, cetane number, cloud point, flash point, viscosity, aniline point, sulfur content, water content, ash content, copper strip corrosion, and carbon residue.

[0287] Bio-based dielectric fluids can be prepared by a variety of processes. For example, one process, starting with crude vegetable oil involves the steps of degumming, alkali refining, bleaching, deodorizing, hydrogenating, winterizing (to yield RBD vegetable oil), treating with clay to remove trace polar compounds and acidic materials (see U.S. Patent No. 6,274,067), and combining with additives to produce bio-based dielectric fluids.

[0288] Key properties of dielectric fluids include viscosity, flammability, reactivity, miscibility, electrical insulating capability, biodegradability, and cost of manufacture. While these and other properties are reviewed below, the reader can better appreciate some of the advantages of certain embodiments of the present invention by understanding some of the advantages and disadvantages of traditional bio-based dielectric fluids over mineral oil-based dielectric fluids. For viscosity, bio-based dielectric fluids generally have a higher viscosity and pour point, and thus poorer low temperature properties relative to mineral oil-based dielectric fluids. However, the viscosity of the latter may vary from lot to lot due to the inconsistency between and complexity of the compounds in various sources of mineral oil. Bio-based dielectric fluids generally have higher flash and fire points (by at least two fold) relative to mineral-oil based dielectric fluids. Bio-based dielectric fluids generally have inferior hydrolytic, thermal, and oxidative stability, and a higher acid number (by about two

fold) relative to mineral-oil based dielectric fluids. Bio-based dielectric fluids generally are more biodegradable and have lower toxicity relative to mineral-oil based dielectric fluids and are made from a renewable, as opposed to non-renewable, resource. Bio-based dielectric fluids generally cost more to produce and require more additives relative to mineral-oil based dielectric fluids.

[0289] The methods of the present invention provide new dielectric fluids that, in certain embodiments, have all of the advantages of traditional bio-based dielectric fluids with fewer, and in some embodiments, none of, the disadvantages. These and other advantages of the present methods can be better appreciated after consideration of the following discussion of the general properties of dielectric fluids.

[0290] Ideally, the viscosity of a dielectric fluid should vary as little as possible with temperature. Viscosity is a measure of the resistance of a fluid to flow or shear ("thickness") and is measured in kinematic (kv) and absolute (dynamic) (cSt or mm^2/s @ 40 and 100 °C). (ASTM D2270-04; ASTM D445; ASTM D88). Generally, the least viscous lubricant which adequately forces two moving surfaces apart is desired. Viscosity is sometimes considered the most important characteristic of a hydraulic fluid. If the viscosity is too high, then friction, pressure drop, power consumption, and heat generation increase. If the viscosity is too low, then increased internal leakage may result under higher operating temperatures. The oil film may be insufficient to prevent excessive wear or possible seizure of moving parts. Illustrative viscosities (in cSt units) of dielectric fluid derived from various sources are: mineral oil-derived: 20 at 40°C and 4 at 100°C; soybean oil-derived: 30 at 40°C and 7.6 at 100°C; sunflower oil-derived: 40 at 40°C and 8.7 at 100°C; and rapeseed (canola) oil-derived: 33 at 40°C. (Siniawski et al.; *J. Synthetic Lubrication*; 24, 101-110 (2007); Schneider; *J. Sci. Food Agric.*, 86, 1769-1780 (2006)). The methods of the present invention can, in particular embodiments, provide dielectric fluids having viscosities similar to those of dielectric fluids derived from the foregoing sources. In illustrative embodiments, the dielectric fluid has a viscosity at 40°C of less than about 110 cSt, e.g., in the range of 20-30 cSt and/or a viscosity at 100°C in the range of about 2 to about 15 cSt, e.g., 4-8 cSt.

[0291] The viscosity index (VI, a unitless number) is a measurement of the variation in viscosity with variation of temperature. For VI, one compares the kv of oil at 40°C to two reference oils (with VI's of 0 and 100), where all oils have the same kv at 100 °C (ASTM D2270). The VI value generally should be as high as possible. High VI values indicate the viscosity of the oil changes little with temperature. In general: a low VI is below 35; a

medium VI is 35 to 80; a high VI is 80 to 110; a very high VI is 110 to 125; a super VI is 125 to 160; and a super high VI is equal to or greater than 160. VIs of dielectric fluids derived from various starting materials include: mineral oil-derived: 103; soybean oil-derived: 246; and sunflower oil-derived: 206. (Siniawski et al.; *J. Synthetic Lubrication*; 24, 101-110 (2007)). The methods of the present invention can, in particular embodiments, provide dielectric fluids having VIs similar to those of dielectric fluids derived from the foregoing sources.

[0292] The pour point is the lowest temperature at which a liquid will pour or flow (°C) (ASTM D97). The pour point should be at least 10°C lower than the lowest anticipated ambient temperature at which the dielectric fluid is to be used. The pour points of dielectric fluids derived from various starting materials include: mineral oil-derived: -50°C; soybean oil-derived: -9°C; sunflower oil-derived: -12°C; and rapeseed (canola) oil-derived: -21°C. (Siniawski et al.; *J. Synthetic Lubrication*; 24, 101-110 (2007)). The methods of the present invention can, in particular embodiments, provide dielectric fluids having pour points similar to those of dielectric fluids derived from the foregoing sources. In various embodiments, the pour point of a microbial oil-based dielectric fluid can be about -10 degrees C, about -15 degrees C, about -20 degrees C, about -25 degrees C, about 30 degrees C, about -35 degrees C, or about -40 degrees C. In addition, the pour point of the microbial oil-based dielectric fluid can fall within any range bounded by any of these values, e.g., about -10 degrees C to -40 degrees C or about -15 degrees C to about -35 degrees C, etc.

[0293] For example, and as described above, RBD oil produced in accordance with the methods described herein can readily be produced with pour points of approximately -8°C or lower. This pour point can be further lowered by admixing the RBD oil with a pour point depressant to achieve oils with pour points in the range of -15 to -20°C or lower based on the amount of pour point depressant added to the oil. The olein fraction from a single fractionation readily produces oil with a pour point of about -11°C, which can be lowered by admixing the olein fraction with a pour point depressant to achieve oils with pour points in the range of -16 to -20°C or lower based on the amount of pour point depressant added to the oil. The olein fraction from a second fractionation ("super olein") readily produces oil with a pour point of approximately -20°C, which can be lowered by admixing the super olein fraction with a pour point depressant to achieve oils with pour points below -20°C, i.e., -26°C or lower based on the amount of pour point depressant added to the oil. A wide variety of pour point depressants are available commercially from Chevron, Oronite, Infineum, General

Electric, RohmMax Evonik, and others. Illustrative pour point depressants for use with the microbial oils (lipids) described herein include VISCOPLEX® 10-310 or 1-133 (Rohmax-Evonik Additives GmbH), or other poly(alkyl) acrylates and poly(methyl)acrylates such as INFINEUM® V-351 (Infineum UK limitied), PMA-D110 and PMA D.

[0294] The lubricity (anti-wear properties) of a dielectric fluid is important, as premature wear occurs when the fluid viscosity is insufficient and the fluid film does not prevent surface contact (ASTM D2882). In some embodiments, the methods of the present invention provide dielectric fluids having good lubricity (equivalent or better than ASTM D2882).

[0295] The volatility, or the tendency for an oil to vaporize (atm vapor vs. °C), is also important for a dielectric fluid. Generally, lower volatility is preferred. In some embodiments, the methods of the present invention can provide dielectric fluids having volatility as low as and even lower than mineral oil-based and traditional bio-based dielectric fluids.

[0296] The flammability of the dielectric fluid is important. Generally, lower flammability is preferred (see "Bio-Based Lubricants: A Market Opportunity Study Update" United Soybean Board, Nov. 2008, Omni Tech International, Ltd., www.soynewuses.org/downloads/reports/BioBasedLubricantsMarketStudy.pdf). The methods of the present invention can, in particular embodiments, provide dielectric fluids having flammability as low and even lower than mineral oil-based and traditional bio-based dielectric fluids.

[0297] The flash point is the lowest temperature (°C) at which an oil vaporizes to form an ignitable mixture in air. ASTM D3278, D3828, D56, and D93 describe flash point specifications suitable for dielectric fluids. To prevent ignition of the oil, the flash point should generally be as high as possible. Flash points of dielectric fluids derived from various sources include: mineral oil-derived: 147°C; and TAGs-derived (typical): 324°C. (New Safety Dielectric Coolants for Distribution and Power Transformers, www.cooperpower.com/Library/pdf/00048.pdf) In some embodiments, the methods of the present invention can provide dielectric fluids having flash points similar to those of dielectric fluids derived from the foregoing sources and equal to or higher than ASTM D1310 and ASTM D92 specifications.

[0298] The fire point is lowest temperature (°C) at which an oil will continue to burn for at least 5 seconds after ignition by an open flame. ASTM D1310 and ASTM D92 describe fire point specifications suitable for dielectric fluids. To prevent ignition of the oil, the fire point should be as high as possible. Fire points of dielectric fluids derived from various sources

include: mineral oil-derived: 165°C; and TAGs-derived (typical): 360°C. (New Safety Dielectric Coolants for Distribution and Power Transformers, www.cooperpower.com/Library/pdf/00048.pdf) In some embodiments, the methods of the present invention can provide dielectric fluids having fire points similar to those of dielectric fluids derived from the foregoing sources and equal to or higher than ASTM D1310 and ASTM D92 specifications. In some embodiments, that fire point is above 300°C, e.g., 300° C to 450° C.

[0299] The reactivity of a dielectric fluid is important; the dielectric fluid should not react (or should have a low reactivity) with acids/bases, heat, and air.

[0300] Hydrolytic reactivity refers to the susceptibility of fluid to decomposition in the presence of acids or bases. ASTM D2619 and ASTM D943 describe hydrolytic reactivity suitable for dielectric fluids. In TAGs, the susceptible functional groups are the esters and acid/base susceptible functional groups. The methods of the present invention can, in particular embodiments, provide dielectric fluids having low hydrolytic reactivity (equivalent or better than ASTM D2619 and/or ASTM D943).

[0301] Thermal stability refers to the susceptibility of a dielectric fluid to thermal decomposition. In bio-oil-derived dielectric fluids, thermal instability is typically due to the β-hydrogens on glycerol, ultimately resulting in elimination products. The methods of the present invention can, in particular embodiments, provide dielectric fluids having high thermal stability (equal to or greater than that of traditional bio-oil-derived dielectric fluids).

[0302] Oxidative susceptibility refers to the susceptibility of a dielectric fluid to reaction with oxygen to form oxidation products. ASTM D943 and ASTM D2272 describe oxidative stability suitable for dielectric fluids. Low susceptibility to oxidation is desired; higher values indicate more oxidative lubricants. In certain embodiments, the methods of the present invention can, in particular embodiments, provide dielectric fluids having low oxidative susceptibility (e.g., ASTM D943 or ASTM D2272).

[0303] The neutralization number (acid value / acid number) is a measure of the amount of acid in an oil or dielectric fluid. Acids are formed as oils (or dielectric fluids) oxidize with age and service. Acids arise in bio-based lubricants from oxidation, ester thermolysis, or acid/base hydrolysis. ASTM D947, ASTM D3487, and ASTM D6871 describe neutralization numbers suitable for dielectric fluids. Generally, the acid value should be as low as possible. The acid number for standard mineral oil is 0.03 and for bio-based oil is 0.06. (Ester Transformer Fluids, IEEE/PES Transformer Committee Meeting, October 7, 2003,

www.transformerscommittee.org/info/F03/F03-EsterFluids.pdf). The methods of the present invention can, in particular embodiments, provide dielectric fluids having low acid numbers (e.g., ASTM D947, ASTM D3487, or ASTM D6871).

[0304] Miscibility refers to the ability of a fluid to mix with other fluids. Ideally, a dielectric fluid should mix well with other lubricants, fluids, and additives but not with water. Demulsibility refers to how well a hydraulic fluid resists mixing with water. Demulsibility is optimal in a dielectric fluid. Miscibility with desired lubricants and additives is optimal in a dielectric fluid. In certain embodiments, the methods of the present invention can, in particular embodiments, provide dielectric fluids with good miscibility and demulsibility.

[0305] Dielectric fluids should have good electrical insulation properties, i.e., they should prevent dissipation of electrical current. Insulation power factor tests are conducted on transformers to measure dielectric losses (measured in %). This value reports on the condition of the transformer - wetness, dryness, deterioration of insulation, condition of the windings, barriers, tap changers, bushings and oil. The power factor values associated with a dielectric fluid should be as low as possible, typically 0.5% or less. For example, the power factor of new oil shipped from a refinery should be no more than 0.05% at 25°C and no more than 0.3% at 100°C. (IEEE Guideline C57, 106-1991 as cited in www.nttworldwide.com/tech2209.htm). For new oil in new equipment operating at or below 69 kV, the power factor should be no more than 0.15% at 25°C and no more than 1.5% at 100°C; operating at 69 kV to at or below 288 kV, the power factor should be no more than 0.10% at 25°C and no more than 1.0% at 100°C; operating at 345 kV or higher, the power factor should be no more than 0.05% at 25°C and no more than 0.3% at 100°C. New oil for circuit breakers should have a power factor of no more than 0.05% at 25°C and no more than 0.3% at 100°C. Oil used in circuit breakers should not have a power factor above 1.0% at 25°C. Certain embodiments of the methods of the present invention provide dielectric fluids with favorable power factor requirements.

[0306] The dielectric strength refers to the maximum electric field strength the dielectric fluid (electrical insulator) can resist before breaking down. The dielectric strength is measured in units of MV/m, (relative permittivity), and ASTM D877 provides specifications suitable for dielectric fluids. For use as an electrical insulator, the dielectric strength of the lubricant should be as high as possible. The methods of the present invention can, in particular embodiments, provide dielectric fluids with dielectric strengths equal or superior to those specified by ASTM D877.

[0307] The dissipation factor is a measure of electrical loss due to the dielectric fluid when used as an electrical insulator and is measured in % units at 25 °C. ASTM D924 provides specifications suitable for dielectric fluids. As an electrical insulator, the dissipation factor value should be as low as possible. In certain embodiments, the methods of the present invention provide dielectric fluids with dissipation factors equal or superior to those specified by ASTM D924.

[0308] The electrical conductivity is a measure of a dielectric fluid's ability, when used as an electrical insulator, to conduct an electrical current and is measured in units of $S \bullet m^{-1}$. ASTM D2624 provides specifications suitable for dielectric fluids. As an insulator, the electrical conductivity value of the dielectric fluid should be as low as possible. The embodiments of the methods of the present invention provide dielectric fluids with favorable electrical conductivity compared to those specified by ASTM D2624.

[0309] For use in electrical transformers and other applications, the thermal properties of the dielectric fluid should be such that heat is efficiently transferred. Specific heat refers to the thermal capacity of a substance and is measured in units of cal/gm/°C. ASTM D-2766 provides specifications suitable for dielectric fluids. Higher specific heat values enable more efficient heat transfer and cooling. Specific heat values for mineral oil-derived dielectric fluids are generally about 0.39 and for TAGs-derived dielectric fluids about 0.45. (Safety Dielectric Coolants for Distribution and Power Transformers, www.cooperpower.com/Library/pdf/00048.pdf). Methods in accordance with embodiments of the present invention may provide dielectric fluids with specific heat values equal or higher to 0.39 and/or that meet ASTM D2624 specifications.

[0310] The environmental properties of a dielectric fluid are important. Generally, one should employ dielectric fluids selected so as to mitigate the environmental effects of a spill or other accident. Biodegradability refers to the property of a dielectric fluid to decompose into carbon dioxide and water in the environment and is generally measured in units of % per 28 days. OECD 301B and ASTM D-6046 provide biodegradability specifications suitable for dielectric fluids. Readily biodegradable biodegradability values are generally ~ 100%; inherently biodegradable biodegradability values are generally 20-70%; and non-biodegradable biodegradability values are generally negligible to 0%. Mineral oil-derived dielectric fluids generally have biodegradability values in the range of 15-35%, and bio-oil-derived dielectric fluids generally have biodegradability values in the range of 70-100%. Certain embodiments of the methods of the present invention may provide dielectric fluids

with biodegradability values in the range of 70-100% (see Renewable Lubricants Manual: Biobased Oils, Fluids, & Greases www.renewablelubricants.com/ RenewableLubricantsManual_ Biodegradable.html#Introduction).

[0311] The iodine value (or iodine number) is a measure of the degree of unsaturation on an oil. More specifically, the iodine valuie is the mass of iodine that is consumed by the unsaturated bonds in an oil. Drying oils have relatively high iodine values of about 175 or higher. Soybean oils are about 130, and olive oils have iodine values of about 80. Iodine values are routinely determined in the art. Standard methods to determine iodine values include ASTM D5768-02(2006) and DIN 53241. In various embodiments, a microbial oil in a microbial oil-based product, e.g., a dielectric fluid, can have an iodine value of between about 25 and about 200, e.g., about 50, about 75, about 100, about 125, about 150, or about 175. Furthermore, the iodine value can be within any range bounded by any of these values, e.g., about 25 to about 175, about 50 to about 200, about 50 to about 175, etc.

[0312] Fatty acid unsaturation can also be altered. Increasing unsaturation decreases freezing/pour points. Monounsaturation, such as that seen in high oleic acid bio-lubricants, is currently optimal and represents a balance between pour point and oxidative reactivity. Monounsaturated oils react with air, but much more slowly than poly-unsaturated FAs or PUFAs. Examples of PUFAs include arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Di- and poly-unsaturated FAs are highly susceptible to oxidation and unsuitable for electrical applications. One problem with dielectric fluids derived from vegetable oils is the presence of polyunsaturated FAs (e.g., linoleic acid and linolenic acid). One advantage of the dielectric fluids of some embodiments of the present invention is that the microbial oil they comprise (or are derived from) contains less, and in some embodiments, no, di- and poly-unsaturated FAs than do dielectric fluids derived from other bio-oils.

[0313] The lipid profile of the dielectric fluid is usually highly similar to the lipid profile of the feedstock oil. High amounts of longer chain (C16-C18) mono-unsaturated fatty acids are preferable for use as dielectric fluids. Polyunsaturated fatty acids (such as C18:2, C18:3, ARA, EPA and DHA) are not preferred due to oxidation and the production of oxidation products. Saturated fatty acids tend to be solid or a liquid with a high freezing point, thereby making saturated fatty acids undesireable in large quantities in dielectric fluids. In various embodiments, microbial oil (lipid) useful in dielectric fluids is at least about 50% C18:1, e.g., at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, and at least about 90% C18:1. In these or other

embodiments, the microbial oil (lipid) is less than about 10% C18:2, e.g., less than about 7.5%, less than about 5%, less than about 2.5%, and less than about 1% C18:2. The microbial oil can have any combination of percentages of C18:1 and C18:2 that adds up to 100% or less. For example the microbial oil can have at least 50% C18:1 and less than 10% C18:2 or at least 80% C18:1 and less than 5% C18:2.

[0314] For illustrative purposes, provided herein are TAG oils from oleaginous microbes that contain less than 2% C18:2 (see Example 4), compared to 20-75% in sunflower oil and 48-65% in soybean oil. Also provided are TAG oils with less than 0.5% C18:3, compared to 5-10% in soybean oil.

[0315] These and other properties of a dielectric fluid can be achieved, manipulated, and/or varied in accordance with the methods described herein so as to provide a product, such as a lubricant, a hydraulic fluid, a industrial oil, or dielectric fluid, suitable for any application. For example, genetic manipulation of oleaginous microbes can be performed, as described above, to alter chain length, saturation, and/or composition of the various fatty acids in the lipid. In certain embodiments, a microbial oil useful as described herein is produced by a genetically engineered microbe that has been engineered to expess one or more exogenous genes. For example, the genetically engineered microbe can be *Prototheca* (e.g, *Prototheca* moriformis) or *Chlorella*. Illustrative exogenous genes include those encoding sucrose invertase and/or fatty acyl-ACP thioesterase.

[0316] In addition, lipid extracted from a microalgae or oleaginous yeast can be subjected to various chemical modifications to achieve a desired property in a dielectric fluid. Typical alterations include altering fatty acid (FA) chain length. Shorter-chain FAs have decreased pour points. Chemical modifications can also be used in accordance with embodiments of the methods of the invention to reduce unsaturation and include alkylation, radical addition, acylation, ene-reactions, hydroformylation, selective hydrogenation, oligomerization, hydroaminomethylation, acyloxylation, and epoxidation. In addition, or as an alternative, an additive, such as pour point depressant, can be admixed with the processed microbial oil to achieve a desired property, e.g., pour point. Illustrative additive are discussed in greated detail below.

[0317] As discussed above, in particular embodiments, the raw microbial oil extracted from an oleaginous microbe is typically "enriched" prior to incorporation into a product of the invention. For example, there can be contaminants in microbial lipids that can crystallize and/or precipitate and fall out of solution as sediment. Sediment formation is particularly a problem when a dielectric fluid is used at lower temperatures. The sediment or precipitates

may cause problems such as decreasing flow, clogging, etc. Processes are well-known in the art that specifically deal with the removal of these contaminants and sediments 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).

[0318] Winterization can be used in accordance with embodiments of the methods of the invention to enrich the microbial oil. There are several approaches to winterizing a dielectric fluid in accordance with embodiments of the present invention. One approach is to blend the fluid with other dielectric fluids. Another approach is to use additives that can lower the freeze point. Dry fractionation can also be used to reduce the relative proportion of the saturated fraction (the stearin fraction). By cooling the oil, one can crystallize the saturates and then filter out the crystals. Fractionation selectively separates a fluid into individual components or fractions, allowing for the removal or inclusion of specific fractions. Other fractionation methods include urea fractionation, solvent fractionation and thermal distillation.

[0319] Diatomaceous earth or other filtering material such as bleaching clay may then added to the cooled liquid to form a slurry, which may then filtered through a pressure leaf or other type of filter to remove the particulates. The filtered liquid may then be run through a polish filter to remove any remaining sediments and diatomaceous earth, so as to produce a final product. Alternatively, or in addition, this product, or the microbial oil produced at the end of any of the foregoing process steps, can be admixed with a pour point depressant to produce a product of the invention, such as a dielectric fluid.

[0320] In one embodiment of the present invention, a method for producing a lubricant oil or a dielectric fluid is provided that 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 the lipid composition from the lysed microorganism, and (d) enriching the isolated lipid composition, whereby a lubricant oil or dielectric fluid is produced. Typically, step (d) will include one or more refining, bleaching, and/or deodorizing steps and one or more fractionation steps to reduce the relative proportion of the saturated fraction by removing palmitic and/or stearic triglycerides. In a further embodiment, the lubricant oil or dielectric fluid resulting from step (d) is admixed with a pour point depressant.

[0321] Optionally, other additives for increasing the oxidative stability of the isolated lipids can be admixed with the microbial oil, lubricant, or dielectric fluid produced by these

methods. Examples of such additives include antioxidants such as tocopherols (vitamin E, e.g., alpha-, beta- and/or delta-tocopherol), ascorbic acid (vitamin C). Suitable anti-oxidants are commercially available. The BASF company markets a line of suitable phenol based and amine based antioxidants under the brand name IRGANOX®. IRGANOX L109, IRGANOX L64, IRGANOX L57, other IRGANOX antioxidants, and other phenol based and amine based compounds are suitable as antioxidant additives to the oils and products including dielectric fluids. Other nonlimiting examples of antioxidants include butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), mono-tertiary butyl hydro quinone (TBHQ), butylated hydroanisole, tetrahydrobutrophenone, ascorbyl palmitate, and propyl gallate. In certain embodiments, a microbial oil-based product, e.g., a dielectric fluid, additionally includes an antioxidant at 0.1% to 5% by weight, and preferably at 0.5% to 2%.

[0322] Other additives that can be optionally added to the isolated lipids for use as products such as dielectric fluids are deactivators for metal ions, corrosion inhibitors, anti-wear additives, and/or hydrolysis protectants. Some widely used additives in dielectric fluids are described in Schneider, 2006, *J Science Food and Agriculture*; 86: 1769-1780.). Metal ion deactivators have two main functions. They suppress chemical attack on the surface of the metal and they also passivate the metal surface to suppress any residues that may act as catalysts for radical (unpaired electron) formation. Metal deactivators are commercially available. For example, the BASF company provides a line of metal deactivators, including the IRGAMET® line of metal deactivators. The RTVANDERBILT company sells the CUVAN® line of metal deactivators. Other examples of metal deactivators include derivatized triazoles including 1-(di-isooctylaminomethyl)-1,2,4-triazole, 1-(2-methoxyprop-2-yl)tolyltriazole, 1-(1-cyclohexyloxypropyl)tolyltriazole, 1-(1-cyclohexyloxyheptyl)tolyltriazole, 1-(1-cyclohexyloxybutyl)tolyltriazole, 1-[bis(2-ethylhexyl)aminomethyl-4-methylbenzotriazole, derivatized borons including triethyl borate, tripropyl borate, triisopropyl borate, tributyl borate, tripentyl borate, trihexyl borate, tricyclohexyl borate, trioctyl borate, triisooctyl borate, and other derivatized hydrazine metal deactivator, e.g., 2',3-bis[[3-[3,5-di-tert-butyl-4-hydroxyphenyl]propionyl]]proponiohydrazine, and the like.). In certain embodiments, a microbial oil-based product, e.g., a dielectric fluid, additionally includes one or more metal deactivators at 0.1% to 5% by weght, and preferably at 0.5% to 2%.

[0323] Thus, dielectric fluids prepared in accordance with the methods described herein may contain a number of additives, including but not limited to one or more of the following additives: (a) an antioxidant, including but not limited to BHT and other phenols; (b) a

deactivator of metal ions such as Cu, Zn, and the like, including but not limited to a benzotriazole; (c) corrosion inhibitors, including but not limited to ester sulfonates and succinic acid esters; (d) demulsifiers; (e) anti-wear additives, including but not limited to zinc dithiophosphate; (f) additives to depress the pour point, including but not limited to malan styrene copolymers, poly(alkyl)acrylates, including but not limited to polymethacrylates; and (g) compounds that protect against hydrolysis, including but not limited to carbodiimides.

[0324] In certain embodiments, a method of the invention produces a product including a microbial oil that has a pour point of between about -10° C and about -40° C, and wherein the fatty acid composition of the microbial oil is at least 50% C18:1 and less than 10% C18:2. The method entails cultivating a genetically engineered microbe engineered to express one or more exogenous genes until the microbe has at least 10% oil by dry weight. Illustrative genetically engineered microbes include *Prototheca* (e.g, *Prototheca* moriformis) or *Chlorella*. Illustrative exogenous genes include those encoding sucrose invertase and/or fatty acyl-ACP thioesterase. In some embodiments, the genetically engineered microbe expresses at least two exogenous genes, e.g., encoding sucrose invertase and fatty acyl-ACP thioesterase, encoding two different fatty acyl-ACP thioesterases, or encoding sucrose invertase and two different fatty acyl-ACP thioesterases. Once the microbe has at least 10% oil by dry weight, the oil is separated from the microbe and subjected to refining, bleaching, deodorizing or degumming to produce RBD oil. Optionally, an antioxidant, metal ion deactivator, corrosion inhibitor, demulsifier, anti-wear additive, pour point depressant, and/or anti-hydrolysis compound can be added to the RBD oil to produce a desired product.

[0325] In particular embodiments, a fractionation method of the invention produces a microbial oil suitable for incorporation into products (e.g., a dielectic fluid) that has a pour point of between about -10° C and about -40° C, and wherein the fatty acid composition of the microbial oil is at least 50% C18:1 and less than 10% C18:2. The method entails subjecting a starting (i.e., "first") microbial oil to refining, bleaching, deodorizing or degumming to produce RBD oil, wherein the RBD oil is characterized by an initial pour point and a first temperature, lowering the temperature of the RBD oil to a second temperature, and filtering the RBD oil at the second temperature to provide a second microbial oil characterized by a second pour point that is lower than the initial pour point, wherein the second pour point is between about -10° C and about -40° C, and wherein the fatty acid composition of the second microbial oil is at least 50% C18:1 and less than 10% C18:2. An illustrative first temperature is between above 15° C to about 50° C, and an illustrative second temperature is between about -15° C and about 15° C. Optionally, an antioxidant, metal ion deactivator, corrosion

inhibitor, demulsifier, anti-wear additive, pour point depressant, and/or anti-hydrolysis compound can be added to the second microbial oil to produce a desired product. In variations of these embodiments, the first microbial oil is produced by cultivating a genetically engineered microbe engineered to express one or more exogenous genes until the microbe has at least 10% oil by dry weight and then separating the oil from the microbe to produce the first microbial oil. This method can be employed to produce, e.g., a lubricant, a hydraulic fluid, an industrial oil, or a dielectric fluid. In certain embodiments, where the product is a dielectic fluid, the fluid includes one or more of an antioxidant, a metal ion deactivator, a corrosion inhibitor, a demulsifier, an anti-wear additive, a pour point depressant, or an anti-hydrolysis compound.

[0326] In one embodiment of the invention, a dielectric fluid is produced by blending oils and/or dielectric fluids derived from oleaginous microbes with existing oils or dielectric fluids. The existing oils and dielectric fluids can be of plant or animal (or both, i.e., petroleum) in origin.

[0327] Thus, the present invention includes a variety of methods in which lipid from oleaginous microbes is undertaken to yield dielectric fluids and other products useful in a variety of industrial and other applications. Examples of processes for modifying oil produced by the methods disclosed herein include, but are not limited to, hydrolysis of the oil, hydroprocessing of the oil, and esterification of the oil. Other chemical modification of microalgal lipid include, without limitation, epoxidation, oxidation, hydrolysis, sulfations, sulfonation, ethoxylation, propoxylation, amidation, and saponification. The modification of the microalgal oil produces basic oleochemicals that can be further modified into selected derivative oleochemicals for a desired function. In a manner similar to that described above with reference to fuel producing processes, these chemical modifications can also be performed on oils generated from the microbial cultures described herein.

[0328] In certain embodiments, a dielectric fluid described herein is employed in a an electrical system, such as a transformer, including a tank housing a transformer core/coil assembly, wherein the dielectric fluid surrounds the core/coil assembly. In variations of such embodiments, the tank also includes an oxygen absorbing material that is in contact with gases in the tank, but isolated from contact with the dielectric insulating fluid. Suitable oxygen absorbing materials are those that are capable of reducing the concentration of free oxygen in the atmosphere surrounding the dielectric fluid inside the tank and that in turn reduce the presence of dissolved oxygen in the fluid itself. Such compounds can be referred to as oxygen scavenging compounds. Useful oxygen scavenging compounds include those

commonly employed in the food packaging industry. Representative of the oxygen scavenging compounds useful in the practice of the invention include the following: sodium sulfite; copper sulfate pentahydrate; a combination of carbon and activated iron powder; mixtures of hydrosulfite, calcium hydroxide, sodium bicarbonate and activated carbon; a metal halide powder coated on the surface of a metal powder; and combinations of alkali compounds, such as calcium hydroxide, with sodium carbonate or sodium bicarbonate. Mixtures and combinations of one or more of the above compositions are also considered useful. Also useful as oxygen scavenging compounds are those compositions provided according to U.S. Pat. No. 2,825,651, which is incorporated by reference, including an oxygen remover composition comprising an intermixing of a sulfite salt and an accelerator such as hydrated copper sulfate, stannous chloride, or cobaltous oxide. Another useful class of oxygen scavenging compounds includes those compositions comprising a salt of manganese, iron, cobalt or nickel, an alkali compound, and a sulfite or deliquescent compound, such as disclosed by U.S. Pat. No. 4,384,972, which also is incorporated by reference. Preferred oxygen scavenging compounds include (or include as their base component) at least one basic iron oxide, such as a ferrous iron oxide, or are made of mixtures of iron oxide materials. Useful iron oxide-containing compositions are available commercially, for example, under the "Ageless" trade name from the Mitsubishi Gas Chemical Company of Duncan, South Carolina and under the "Freshmax" trade name from Multisorb Technologies, Inc. of Buffalo, N.Y. Also useful are oxygen absorbing agents comprising a mixture of ferrous salts and an oxidation modifier and/or a metallic sulfite or sulfate compound.

[0329] 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

EXAMPLE 1: Methods for culturing *Prototheca*

[0330] *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 $CaCl_2$ $2H_2O$, 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 9.

Table 9. Percent oil by dry cell weight

Species	Strain	% Oil
Prototheca stagnora	UTEX 327	13.14
Prototheca moriformis	UTEX 1441	18.02
Prototheca moriformis	UTEX 1435	27.17

[0331] Microalgae samples from multiple strains from the genus *Prototheca* 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 ul 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).

[0332] 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'-TGTTGAAGAATGAGCCGGCGAC-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 dH_2O 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:

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

[0333] Lipid samples from a subset of the above-listed strains were analyzed for lipid profile using HPLC. Results are shown below in Table 10.

Table 10. Diversity of lipid chains in *Prototheca* species

Strain	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0	C20:1
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

[0334] Oil extracted from *Prototheca moriformis* UTEX 1435 (via solvent extraction or using an expeller press was analyzed for carotenoids, chlorophyll, tocopherols, other sterols and tocotrienols. The results are summarized below in Table 11.

Table 11. 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-Crytoxanthin	0.007	0.010
t-beta-Crytoxanthin	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
detal Tocotrienol	<0.01	<0.01
Total Tocotrienols	**0.36**	**0.36**

[0335] Oil extracted from *Prototheca moriformis*, from four separate lots, was refined and bleached using standard vegetable oil processing methods. Briefly, crude oil extracted from *Prototheca moriformis* was clarified in a horizontal decanter, where the solids were separated from the oil. The clarified oil was then transferred to a tank with citric acid and water and

left to settle for approximately 24 hours. After 24 hours, the mixture in the tank formed 2 separate layers. The bottom layer was composed of water and gums that were then removed by decantation prior to transferring the degummed oil into a bleaching tank. The oil was then heated along with another dose of citric acid. Bleaching clay was then added to the bleaching tank and the mixture was further heated under vacuum in order to evaporate off any water that was present. The mixture was then pumped through a leaf filter to remove the bleaching clay. The filtered oil was then passed through a final 5μm polishing filter and then collected for storage until use. The refined and bleached (RB) oil was then analyzed for carotenoids, chlorophyll, sterols, tocotrienols and tocopherols. The results of these analyses are summarized in Table 12 below. "Nd" denotes none detected and the sensitivity of detection is listed below:

Sensitivity of Detection
Carotenoids (mcg/g) nd = <0.003 mcg/g
Chlorophyll (mcg/g) nd = <0.03 mcg/g
Sterols (%) nd = 0.25%
Tocopherols (mcg/g); nd = 3 mcg/g

Table 12. Carotenoid, chlorophyll, sterols, tocotrienols and tocopherol analysis from refined and bleached *Prototheca moriformis* oil.

	Lot A	Lot B	Lot C	Lot D
Carotenoids (mcg/g)				
Lutein	0.025	0.003	nd	0.039
Zeaxanthin	nd	nd	nd	nd
cis-Lutein/Zeaxanthin	nd	nd	nd	nd
trans-alpha-Cryptoxanthin	nd	nd	nd	nd
trans-beta-Cryptoxanthin	nd	nd	nd	nd
trans-alpha-Carotene	nd	nd	nd	nd
cis-alpha-Carotene	nd	nd	nd	nd
trans-beta-Carotene	nd	nd	nd	nd
cis-beta-Carotene	nd	nd	nd	nd
Lycopene	nd	nd	nd	nd
Unidentified	0.219	0.066	0.050	0.026
Total Carotenoids	**0.244**	**0.069**	**0.050**	**0.065**
Chlorophyll (mcg/g)				
Chlorophyll A	0.268	0.136	0.045	0.166
Chlorophyll B	nd	nd	nd	nd
Total Chlorophyll	**0.268**	**0.136**	**0.045**	**0.166**
Sterols (%)				
Brassicasterol	nd	nd	nd	nd
Campesterol	nd	nd	nd	nd
Stigmasterol	nd	nd	nd	nd
beta-Sitosterol	nd	nd	nd	nd
Total Sterols	nd	nd	nd	nd
Tocopherols (mcg/g)				
alpha-Tocopherol	23.9	22.8	12.5	8.2
beta-Tocopherol	3.72	nd	nd	nd
gamma-Tocopherol	164	85.3	43.1	38.3
delta-Tocopherol	70.1	31.1	18.1	14.3
Total Tocopherols	**262**	**139.2**	**73.7**	**60.8**
Tocotrienols (mcg/g)				
alpha-Tocotrienol	190	225	253	239
beta-Tocotrienol	nd	nd	nd	nd
gamma-Tocotrienol	47.3	60.4	54.8	60.9
delta-Tocotrienol	12.3	16.1	17.5	15.2
Total Tocotrienols	**250**	**302**	**325**	**315**

[0336] The same four lots of *Prototheca moriformis* oil was also analyzed for trace elements and the results are summarized below in Table 13.

Table 13. Elemental analysis of refined and bleached *Prototheca moriformis* oil.

	Lot A	Lot B	Lot C	Lot D
Elemental Analysis (ppm)				
Calcium	0.08	0.07	< 0.04	0.07
Phosphorous	< 0.2	0.38	< 0.2	0.33
Sodium	< 0.5	0.55	< 0.5	< 0.5
Potassium	1.02	1.68	< 0.5	0.94
Magnesium	< 0.04	< 0.04	< 0.04	0.07
Manganese	< 0.05	< 0.05	< 0.05	< 0.05
Iron	< 0.02	< 0.02	< 0.02	< 0.02
Zinc	< 0.02	< 0.02	< 0.02	< 0.02
Copper	< 0.05	< 0.05	< 0.05	< 0.05
Sulfur	2.55	4.45	2.36	4.55
Lead	< 0.2	< 0.2	< 0.2	< 0.2
Silicon	0.37	0.41	0.26	0.26
Nickel	< 0.2	< 0.2	< 0.2	< 0.2
Organic chloride	< 1.0	< 1.0	< 1.0	2.2
Inorganic chloride	< 1.0	< 1.0	< 1.0	< 1.0
Nitrogen	4.4	7.8	4.2	6.9
Lithium	< 0.02	< 0.02	< 0.02	< 0.02
Boron	0.07	0.36	0.09	0.38
Aluminum	--	< 0.2	< 0.2	< 0.2
Vanadium	< 0.05	< 0.05	<0.05	< 0.05
Lovibond Color (°L)				
Red	5.0	4.3	3.2	5.0
Yellow	70.0	70.0	50.0	70.0
Mono & Diglycerides by HPLC (%)				
Diglycerides	1.68	2.23	1.25	1.61
Monoglycerides	0.03	0.04	0.02	0.03
Free fatty acids (FFA)	1.02	1.72	0.86	0.83
Soaps	0	0	0	
Oxidized and Polymerized Triglycerides				
Oxidized Triglycerides (%)	3.41	2.41	4.11	1.00
Polymerized Triglycerides (%)	1.19	0.45	0.66	0.31
Peroxide Value (meg/kg)	0.75	0.80	0.60	1.20
p-Anisidine value (dimensionless)	5.03	9.03	5.44	20.1
Water and Other Impurities (%)				
Karl Fisher Moisture	0.8	0.12	0.07	0.18
Total polar compounds	5.02	6.28	4.54	5.23
Unsaponificable matter	0.92	1.07	0.72	1.04
Insoluble impurities	<0.01	<0.01	0.01	< 0.01
Total oil (%)				
Neutral oil	98.8	98.2	99.0	98.9

EXAMPLE 2: General methods for biolistic transforming *Prototheca*

[0337] Seashell Gold Microcarriers 550 nanometers were prepared according to the protocol from manufacturer. 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 μl)

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 µl of cold 100% ethanol, pelleted by brief spinning in the microfuge, and resuspended with 50 µl of ice-cold ethanol. After a brief (1-2 sec) sonication, 10 µl of DNA-coated particles were immediately transferred to the carrier membrane.

[0338] *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) with 2% glucose on a gyratory shaker until it reached a cell density of $2x10^6$cells/ml. The cells were harvested, washed once with sterile distilled water, and resuspended in 50 µl of medium. 1 x 10^7 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 (1350 psi) were used, and the plates were placed 6 cm below the screen/macrocarrier 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 µl 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. Colonies were picked and spotted on selective (either antibiotic or carbon source) agar plates for a second round of selection.

EXAMPLE 3: Expression of heterologous fatty acyl acp thioesterase genes in microalgal cells

[0339] Methods for and the results of expressing heterologous thioesterase gene in microalgal cells, including *Prototheca* species, have been previously described in PCT Application No. PCT/US2009/66412, hereby incorporated by reference. This example describes results using other thioesterase gene/gene products from higher plant species.

[0340] A fatty acyl-ACP thioesterase from *Ricinus communis* was introduced into a *Prototheca moriformis* UTEX 1435 genetic background, and the codon-optimized cDNA sequence (SEQ ID NO:87) and amino acid sequences (from GenBank Accession No. ABS30422.1)(SEQ ID NO:88) are listed in the Sequence Listing. The expression construct contained 5' (SEQ ID NO:100) and 3' (SEQ ID NO:101) homologous recombination targeting sequences (flanking the construct) to the 6S genomic region for integration into the nuclear genome and a *S. cerevisiae suc2* sucrose invertase coding region under the control of *C. reinhardtii* β-tubulin promoter/5'UTR and *Chlorella vulgaris* nitrate reductase 3' UTR. This *S. cerevisiae suc2* expression cassette is listed as SEQ ID NO:78 and served as a

selection marker. The *R. communis* coding region was under the control of the *Prototheca moriformis* Amt03 promoter/5'UTR (SEQ ID NO:84) and *C. vulgaris* nitrate reductase 3'UTR (SEQ ID NO:85). The *Ricinus communis* native transit peptide was also replaced with the transit peptide from *C.protothecoides* stearoyl desaturase (SEQ ID NO:86) and the cDNA sequence of the thioesterase with the replaced transit peptide is listed as SEQ ID NO:87. The entire *Ricinus communis* expression cassette was termed pSZ1375 and transformed into a *Prototheca moriformis* genetic background. Positive clones were screened on plates with sucrose as the sole carbon source. A subset of the positive clones were selected and grown under lipid production conditions and lipid (fatty acid) profiles were determined using direct transesterification methods as described above. The fatty acid profiles of the selected clones are summarized in Table 14 below.

Table 14. Fatty acid profiles of *Ricinus communis* ACP-thioesterase transgenic *Prototheca* cells.

Strain	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2
wildtype	0.01	0.03	0.98	24.65	3.68	62.48	6.26
pSZ1375 clone A	0.01	0.03	0.91	18.34	2.55	67.93	8.35
pSZ1375 clone B	0.01	0.03	0.97	18.51	2.47	67.83	8.25
pSZ1375 clone C	0.01	0.03	0.93	18.65	2.84	67.58	7.90
pSZ1375 clone D	0.01	0.03	0.92	18.90	2.30	67.48	8.37

[0341] The results show that transformants with the *Ricinus communis* thioesterase transgene have altered levels of C16:0 fatty acids and, to a lesser extent, C18:0 fatty acids, relative to the wild-type strain. Also, there was a concomitant increase in the C18:1 fatty acid level when compared to the wild-type level.

EXAMPLE 4: Altering the levels of saturated fatty acids in the microalgae *Prototheca moriformis*

A. Decreasing stearoyl ACP desaturease and delta 12 fatty acid desaturase expression by gene knock-out approach

[0342] As part of a genomics screen using a bioinformatics based approach based on cDNAs, Illumia transcriptome and Roche 454 sequencing of genomic DNA from *Prototheca moriformis* (UTEX 1435), two specific groups of genes involved in fatty acid desaturation were identified: stearoyl ACP desaturases (SAD) and delta 12 fatty acid desaturases (Δ12

FAD). Stearoyl ACP desaturase enzymes are part of the lipid synthesis pathway and they function to introduce double bonds into the fatty acyl chains, for example, the synthesis of C18:1 fatty acids from C18:0 fatty acids. Delta 12 fatty acid desaturases are also part of the lipid synthesis pathway and they function to introduce double bonds into already unsaturated fatty acids, for example, the synthesis of C18:2 fatty acids from C18:1 fatty acids. Southern blot analysis using probes based on the two classes of fatty acid desaturase genes identified during the bioinformatics efforts indicated that each class of desaturase genes was likely comprised of multiple family members. Additionally the genes encoding stearoyl ACP desaturases fell into two distinct families. Based on these results, three gene disruption constructs were designed to disrupt multiple gene family members by targeting more highly conserved coding regions within each family of desaturase enzymes.

[0343] Three homologous recombination targeting constructs were designed using: (1) highly conserved portions of the coding sequence of delta 12 fatty acid desaturase (d12FAD) family members and (2) two constructs targeting each of the two distinct families of SAD, each with conserved regions of the coding sequences from each family. This strategy is designed to embed a selectable marker gene (the *suc*2 sucrose invertase cassette from *S. cerevisiae* conferring the ability to hydrolyze sucrose) into these highly conserved coding regions (targeting multiple family members) rather than a classic gene replacement strategy where the homologous recombination would target flanking regions of the targeted gene.

[0344] All constructs were introduced into the cells by biolistic transformation using the methods described above and constructs were linearized before being shot into the cells. Transformants were selected on sucrose containing plates/media and changes in fatty acid profile were assayed using the above-described method. Relevant sequences from each of the three targeting constructs are listed below.

Description	SEQ ID NO:
5' sequence of d12FAD targeting construct	SEQ ID NO:30
3' sequence of d12FAD targeting construct	SEQ ID NO:31
d12FAD targeting construct cDNA sequence	SEQ ID NO:32
5' sequence of SAD2A targeting construct	SEQ ID NO:33
3' sequence of SAD2A targeting construct	SEQ ID NO:34
SAD2A targeting construct cDNA sequence	SEQ ID NO:35
5' sequence of SAD2B targeting construct	SEQ ID NO:36
3' sequence of SAD2B targeting construct	SEQ ID NO:37
SAD2B targeting construct cDNA sequence	SEQ ID NO:38

[0345] Representative positive clones from transformations with each of the constructs were picked and the fatty acid profiles for these clones were determined (expressed in Area %) and summarized in Table 15 below.

Table 15. Fatty acid profiles for desaturase knockouts.

Fatty Acid	d12FAD KO	SAD2A KO	SAD2B KO	wt UTEX 1435
C8:0	0	0	0	0
C10:0	0.01	0.01	0.01	0.01
C12:0	0.03	0.03	0.03	0.03
C14:0	1.08	0.985	0.795	1.46
C16:0	24.42	25.335	23.66	29.87
C18:0	6.85	12.89	19.555	3.345
C18:1	58.35	47.865	43.115	54.09
C18:2	7.33	10.27	9.83	9.1
C18:3 alpha	0.83	0.86	1	0.89
C20:0	0.48	0.86	1.175	0.325

[0346] Each of the constructs had a measurable impact on the desired class of fatty acid, and in all three cases C18:0 levels increased markedly, particularly with the two SAD knockouts. Further comparison of multiple clones from the SAD knockouts indicated that the SAD2B knockout lines had significantly greater reductions in C18:1 fatty acids than the C18:1 fatty acid levels observed with the SAD2A knockout lines.

[0347] Additional Δ12 fatty acid desaturase (FAD) knockouts were generated in a *Prototheca moriformis* (UTEX 1435) background using the methods described above. To identify potential homologous of Δ12FADs, the following primers were used to amplify a genomic region encoding a putative FAD:

Primer 1 5'-TCACTTCATGCCGGCGGTCC-3' SEQ ID NO:74
Primer 2 5'- GCGCTCCTGCTTGGCTCGAA-3' SEQ ID NO:75

[0348] The sequences resulting from the genomic amplification of *Prototheca moriformis* genomic DNA using the above primers were highly similar, but indicated that multiple genes or alleles of Δ12FADs exist in *Prototheca moriformis*.

[0349] Based on this result, two gene disruption constructs were designed to ablate one or more Δ12FAD genes. The strategy was to embed a sucrose invertase (*suc2* from *S.*

cerevisiae) cassette, thus conferring the ability to hydrolyze sucrose as a selectable marker, into highly conserved coding regions rather than use a classic gene replacement strategy. The first construct, termed pSZ1124, contained 5' and 3' genomic targeting sequences flanking a *C. reinhardtii* β-tubulin promoter driving the expression of the *S. cerevisiae suc2* gene and a *Chlorella vulgaris* nitrate reductase 3'UTR (*S. cerevisiae suc2* cassette). The second construct, termed pSZ1125, contained 5' and 3' genomic targeting sequences flanking a *C. reinhardtii* β-tubulin promoter driving the expression of the *S. cerevisiae suc2* gene and a *Chlorella vulgaris* nitrate reductase 3'UTR. The relevant sequences of the constructs are listed in the Sequence Listing:

pSZ1124 (FAD2B) 5' genomic targeting sequence	SEQ ID NO:76
pSZ1124 (FAD2B) 3' genomic targeting sequence	SEQ ID NO:77
S. cerevisiae suc2 cassette	SEQ ID NO:78
pSZ1125 (FAD2C) 5' genomic targeting sequence	SEQ ID NO:79
pSZ1125 (FAD2C) 3' genomic targeting sequence	SEQ ID NO:80

[0350] pSZ1124 and pSZ1125 were each introduced into a *Prototheca moriformis* background and positive clones were selected based on the ability to hydrolyze sucrose. Table 16 summarizes the fatty acid profiles (in Area %, generated using methods described above) obtained in two transgenic lines in which pSZ1124 and pSZ1125 targeting vectors were utilized.

Table 16. Fatty acid profiles of Δ12 FAD knockouts.

	C10:0	C12:0	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3α
parent	0.01	0.03	1.15	26.13	1.32	4.39	57.20	8.13	0.61
FAD2B	0.02	0.03	0.80	12.84	1.92	0.86	74.74	7.08	0.33
FAD2C	0.02	0.04	1.42	25.85	1.65	2.44	66.11	1.39	0.22

[0351] The transgenic containing the FAD2B (pSZ1124) construct gave a very interesting and unexpected result in lipid profile, in that the C18:2 levels, which would be expected to decrease, only decreased by about one Area %. However, the C18:1 fatty acid levels increased significantly, almost exclusively at the expense of the C16:0 levels, which decreased significantly. The transgenic containing the FAD2C (pSZ1125) construct also gave a change in fatty acid profile: the levels of C18:2 are reduced significantly along with a corresponding increase in C18:1 levels.

B. RNA hairpin approach to down-regulation of delta 12 desaturase (FADc) in *Prototheca* cells

[0352] Vectors down-regulating FADc (delta 12 desaturase gene) gene expression by hairpin RNAs were introduced into a *Prototheca moriformis* UTEX 1435 genetic background. The *Saccharomyces cerevisiae suc2* sucrose invertase gene was utilized as a selectable marker, conferring the ability to grow on sucrose as a sole-carbon source to positive clones, and two types of constructs were used. The first type of construct utilized a portion of the first exon of the FADc coding region linked in cis to its first intron followed by a repeat unit of the first exon in reverse orientation. This type of construct was designed to form a hairpin when expressed as mRNA. Two constructs of this first type were created, one driven by the *Prototheca moriformis* Amt03 promoter (SEQ ID NO:84), termed pSZ1468, and a second driven by the *Chlamydomomas reinhardtii* β-tubulin promoter (SEQ ID NO:89), termed pSZ1469. The second type of construct utilized the large FADc exon 2 in the antisense orientation driven by either the *Prototheca moriformis* Amt03 promoter (SEQ ID NO:84), termed pSZ1470, or driven by the *Chlamydomomas reinhardtii* β-tubulin promter (SEQ ID NO:89), termed pSZ1471. All four constructs had a *S. cerevisiae suc2* sucrose invertase cassette (SEQ ID NO:78) and a 5' (SEQ ID NO:100) and 3' (SEQ ID NO:101) homologous recombination targeting sequences (flanking the construct) to the 6S genomic region for integration into the nuclear genome. Sequences of the FADc portions of each hairpin RNA construct along with the relevant portions of each construct are listed in the Sequence Listing as:

Description	SEQ ID NO:
pSZ1468 FADc hairpin RNA cassette	SEQ ID NO:90
Relevant portions of the pSZ1468 construct	SEQ ID NO:91
pSZ1469 FADc hairpin RNA cassette	SEQ ID NO:92
Relevant portions of the pSZ1469 construct	SEQ ID NO:93
pSZ1470 FADc exon 2 of a hairpin RNA cassette	SEQ ID NO:94
Relevant portions of the pSZ1470 construct	SEQ ID NO:95
pSZ1471 FADc exon 2 of a hairpin RNA cassette	SEQ ID NO:96
Relevant portions of the pSZ1471 construct	SEQ ID NO:97

[0353] Each of the four constructs was transformed into a *Prototheca moriformis* background and positive clones were screened using plates with sucrose as the sole carbon source. Positive clones were picked from each transformation and a subset were selected to

determine the impact of the hairpin and antisense cassettes contained in pSZ1468, pSZ1469, pSZ1470 and pSZ1471 on fatty acid profiles. The selected clones from each transformation were grown under lipid producing conditions and the fatty acid profiles were determined using direct transesterification methods as described above. Representative fatty acid profiles from each of the transformations are summarized below in Table 17. Wildtype 1 and 2 cells were untransformed *Prototheca moriformis* cells that were run with each of the transformants as a negative control.

Table 17. Fatty acid profiles of *Prototheca moriformis* cells containing hairpin RNA constructs to down-regulate the expression of delta 12 desaturase gene (FADc).

Strain	C10:0	C12:0	C14:0	C16:0	C18:0	C18:1	C18:2
wildtype 1	0.01	0.03	1.20	27.08	4.01	57.58	7.81
pSZ1468 clone A	0.01	0.04	1.33	25.95	3.68	65.60	1.25
pSZ1468 clone B	0.01	0.03	1.18	23.43	2.84	65.32	4.91
pSZ1468 clone C	0.01	0.04	1.34	23.18	4.27	63.65	5.17
pSZ1468 clone D	0.01	0.03	1.24	23.00	3.85	61.92	7.62
pSZ1470 clone A	0.01	0.03	1.23	24.79	4.33	58.43	8.92
pSZ1470 clone B	0.01	0.03	1.26	24.91	4.14	57.59	9.64
pSZ1470 clone C	0.01	0.03	1.21	23.35	4.75	58.52	9.70
wildtype 2	0.01	0.03	0.98	24.65	3.68	62.48	6.26
pSZ1469 clone A	0.01	0.03	1.05	21.74	2.71	71.33	1.22
pSZ1469 clone B	0.01	0.03	1.01	22.60	2.98	70.19	1.27
pSZ1469 clone C	0.01	0.03	1.03	19.82	2.38	72.95	1.82
pSZ1469 clone D	0.01	0.03	1.03	20.54	2.66	70.96	2.71
pSZ1471 clone A	0.01	0.03	1.03	18.42	2.63	66.94	8.55
pSZ1471 clone B	0.01	0.03	0.94	18.61	2.58	67.13	8.66
pSZ1471 clone C	0.01	0.03	1.00	18.31	2.46	67.41	8.71
pSZ1471 clone D	0.01	0.03	0.93	18.82	2.54	66.84	8.77

[0354] The above results show that the hairpin constructs pSZ1468 and pSZ1469 showed expected phenotypes: a reduction in C18:2 fatty acid levels and an increase in C18:1 fatty acid levels as compared to wildtypel and wildtype 2, respectively. The antisense constructs, pSZ1470 and pSZ1471 did not result in a decrease in C18:2 fatty acid levels but instead showed a slight increase when compared to wildtype 1 and wildtype 2, respectively and a slight decrease in C16:0 fatty acid levels.

C. Expression of an exogenous stearoyl-ACP desaturase

[0355] The *Olea europaea* stearoyl-ACP desaturase (GenBank Accession No. AAB67840.1) was introduced into a *Prototheca moriformis* UTEX1435 genetic background. The expression construct contained a 5' (SEQ ID NO:100) and 3' (SEQ ID NO:101) homologous recombination targeting sequences (flanking the construct) to the 6S genomic region for integration into the nuclear genome and a *S. cerevisiae suc2* sucrose invertase coding region under the control of *C. reinhardtii* β-tubulin promoter/5'UTR and *Chlorella vulgaris* nitrate reductase 3' UTR. This *S. cerevisiae suc2* expression cassette is listed as SEQ ID NO:78 and served as a selection marker. The *Olea europaea* stearoyl-ACP desaturase coding region was under the control of the *Prototheca moriformis* Amt03 promoter/5'UTR (SEQ ID NO:84) and *C. vulgaris* nitrate reductase 3'UTR, and the native transit peptide was replaced with the *Chlorella protothecoides* stearoyl-ACP desaturase transit peptide (SEQ ID NO:86). The codon-optimized cDNA sequences and amino acid sequences (with the replaced transit peptide) are listed in the Sequence Listing as SEQ ID NO:98 and SEQ ID NO:99, respectively. The entire *O. europaea* SAD expression cassette was termed pSZ1377 and transformed into a *Prototheca moriformis* genetic background. Positive clones were screened on plates with sucrose as the sole carbon source. A subset of the positive clones were selected and grown under lipid production conditions and fatty acid profiles were determined using direct transesterification methods as described above. The fatty acid profiles of the selected clones are summarized in Table 18 below.

Table 18. Fatty acid profile of *Olea europaea* stearoyl-ACP desaturase transgenic *Prototheca moriformis* cells.

Strain	C14:0	C16:0	C18:0	C18:1	C18:2
wildtype	0.88	22.82	3.78	64.43	6.54
pSZ1377 clone A	0.94	18.60	1.50	69.45	7.67
pSZ1377 clone B	0.93	18.98	1.35	69.12	7.67
pSZ1377 clone C	0.93	19.01	2.31	68.56	7.43

[0356] The above results demonstrate that the introduction of a heterologous desaturase, in this case a stearoyl-ACP desaturase from *Olea europaea*, can result in higher levels of C18:1 fatty acid and a concomitant decrease in C18:0 and C16:0 fatty acid levels.

EXAMPLE 5: Cultivation of oleaginous yeast

[0357] Oleaginous yeast strains used in this and subsequent Examples were obtained from either the Deutsche Sammlung von Mikroorganismen un Zellkulturen GmbH (DSMZ), located at Inhoffenstrabe 7B, 38124 Braunschweig, Germany, or Centraalbureau voor Schimmelscultures (CBS) Fungal Biodiversity Centre located at P.O. Box 85167, 3508 Utrecht, the Netherlands. One hundred eighty five oleaginous yeast strains were screened for growth rate and lipid production.

[0358] All strains were rendered axenic via streaking to single colonies on YPD agar (YPD medium as described below with 2% agar added) plates. Single colonies from the YPD plates of each strain were picked and grown to late log phase in YPD medium (10g bacto-yeast extract, 20g bacto-peptone and 20g glucose/1 L final volume in distilled water) on a rotary shaker at 200 rpm at 30°C.

[0359] For lipid productivity assessment, 2mL of YPD medium was added to a 50mL tared Bioreactor tube (MidSci, Inc.) and inoculated from a frozen stock of each strain. The tubes were then placed in a 30°C incubator and grown for 24 hours, shaking at 200 rpm to generate a seed culture. After 24 hours, 8 mL of Y1 medium (Yeast nitrogen base without amino acids, Difco) containing 0.1M phthalate buffer, pH 5.0 was added and mixed well by pipetting gently. The resulting culture was divided equally into a second, tared bioreactor tube. The resulting duplicate cultures of 5mL each were then placed in a 30°C incubator with 200rpm agitation for 5 days. The cells were then harvested for lipid productivity and lipid profile. 3mL of the culture was used for determination of dry cell weight and total lipid content (lipid productivity) and 1mL was used for fatty acid profile determination. In either case, the cultures were placed into tubes and centrifuged at 3500 rpm for 10 minutes in order to pellet the cells. After decanting the supernatant, 2mL of deionized water was added to each tube and used to wash the resulting cell pellet. The tubes were spun again at 3500rpm for 10 minutes to pellet the washed cells, the supernatant was then decanted and the cell pellets were placed in a -70°C freezer for 30 minutes. The tubes were then transferred into a lyophilizer overnight to dry. The following day, the weight of the conical tube plus the dried biomass resulting from the 3mL culture was recorded and the resulting cell pellet was

subjected to total lipid extraction using an Ankom Acid Hydrolysis system (according to the manufacturer's instructions) to determine total lipid content.

[0360] Of the 185 strains screened, 30 strains were chosen based on the growth rate and lipid productivity. The lipid productivity (expressed as percent lipid of dry cell weight) of these 30 strains is summarized below in Table 19.

Table 19. Lipid productivity of oleaginous yeast strains.

Species	Collection No.	% Lipid (DCW)
Rhodotorula terpenoidalis	CBS 8445	27
Rhodotorula glutinus	DSMZ 70398	53.18
Lipomyces tetrasporous	CBS 1810	51
Lipomyces tetrasporous	CBS 7656	17.63
Lipomyces tetrasporous	CBS 8724	18
Cryptococcus curvatus	CBS 5324	53
Cryptococcus curvatus	CBS 2755	48
Rhodosporidium sphaerocarpum	CBS 2371	43
Rhodotorula glutinus	CBS 4476	30.97
Lipomyces tetrasporous	CBS 1808	29
Trichosporon domesticum	CBS 8111	35.16
Trichosporon sp.	CBS 7617	40.09
Lipomyces tetrasporous	CBS 5911	27.63
Lipomyces tetrasporous	CBS 5607	12.81
Cryptococcus curvatus	CBS 570	38.64
Cryptococcus curvatus	CBS 2176	40.57
Cryptococcus curvatus	CBS 5163	35.26
Torulaspora delbruekii	CBS 2924	40.00
Rhodotorula toruloides	CBS 8761	36.52
Geotrichum histeridarum	CBS 9892	33.77
Yarrowia lipolytica	CBS 6012	29.21
Geotrichum vulgare	CBS 10073	28.04
Trichosporon montevideense	CBS 8261	25.60
Lipomyces starkeyi	CBS 7786	25.43
Trichosporon behrend	CBS 5581	23.93
Trichosporon loubieri var. loubieri	CBS 8265	22.39
Rhodosporidium toruloides	CBS 14	21.03
Trichosporon brassicae	CBS 6382	20.34
Rhodotorula aurantiaca	CBS 317	17.51
Sporoholomyces alborubescens	CBS 482	10.09

[0361] Cell pellets resulting from 1mL culture were subjected to direct transesterification and analysis by GC for fatty acid profile determination. A summary of the fatty acid profiles for 17 of the above yeast strains are summarized below in Table 20.

Table 20. Fatty acid profiles of oleaginous yeast strains.

Species	Collection No.	C12:0	C14:0	C15:0	C16:0	C16:1	C17:0	C17:1	C18:0	C18:1	C18:2	>C20
Rhodotorula terpenoidalis	CBS 8445	0.06	0.8	0.02	27.44	0.67	0.03	0.03	5.6	59.44	3.37	2.13
Rhodotorula glutinus	DSMZ 70398	0.05	1.55	0.09	27.34	0.34	0.23	0.08	10.47	44.68	11.65	2.23
Lipomyces tetrasporous	CBS 1810	nd	0.26	0.08	24.22	2.13	0.28	0.30	9.93	55.04	4.48	3.01
Lipomyces tetrasporous	CBS 76556	nd	0.293	0.212	28.14	4.24	0.37	0.66	6.61	48.48	8.33	1.178
Lipomyces tetrasporous	CBS 8724	nd	0.27	0.08	30.69	2.12	0.27	0.24	11.8	46.71	4.36	2.89
Cryptococcus curvatus	CBS 5324	nd	0.27	0.22	23.31	0.49	0.12	0.09	11.55	50.78	10.80	1.61
Cryptococcus curvatus	CBS 27556	nd	0.62	0.03	25.07	0.31	0.05	0.03	17.07	45.74	14.60	2.01
Rhodosporidium sphaerocarpum	CBS 2371	0.03	0.68	0.03	17.86	0.13	0.54	0.17	10.4	51.01	14.60	1.82
Rhodotorula glutinus	CBS 4476	0.021	0.47	0.02	24.64	0.16	0.064	0.27	13.73	42.46	16.29	1.642
Lipomyces tetrasporous	CBS 1808	0.01	0.40	0.12	26.64	3.11	0.25	0.39	7.39	54.15	3.96	2.34
Trichosporon domesticum	CBS 8111	0.066	0.486	0.10	23.19	0.11	0.37	0.033	30.65	29.75	11.66	3.414
Trichosporon sp.	CBS 7617	0.046	0.527	0.063	24.26	0.187	0.171	0.026	19.61	41.95	9.97	2.61
Lipomyces tetrasporous	CBS 5911	0.017	0.45	0.16	30.79	3.56	0.29	0.48	7.77	49.99	4.40	1.433
Lipomyces tetrasporous	CBS 5607	nd	0.35	0.17	37.56	3.0	0.328	0.40	9.31	42.36	4.28	1.376
Cryptococcus curvatus	CBS 570	0.017	0.21	0.09	12.78	0.13	0.147	0.09	19.6	53.17	8.42	4.01
Cryptococcus curvatus	CBS 2176	0.02	0.31	0.09	19.0	0.87	0.08	0.10	7.24	60.51	9.26	2.154
Cryptococcus curvatus	CBS 5163	0.019	0.34	0.06	22.7	0.70	0.13	0.10	10.65	51.36	10.34	2.24

nd denotes none detected.

[0362] Fatty acid profile analysis was performed on additional strains of oleaginous yeast and several strains were found to produce a high percentage of C16:1 fatty acid including, *Torulaspora delbruekii* CBS 2924. This oleaginous yeast strain had a lipid productivity of approximately 40% lipid as a percentage of DCW and a fatty acid profile of: C12:0 (0.36%); C14:0 (1.36%); C15:0 (0.16%); C16:0 (10.82%); C 16:1 (42.9%); C17:0 (0.11%); C18:0 (2.1%); C18:1 (35.81%); C18:2 (4.62%). This strain was found to have a particularly high percentage of C16:1 (palmitoleic acid) as part of its fatty acid profile. Four additional strains were identified as producing a high percentage 16:1: *Yarrowia lipolytica* CBS 6012 (10.10%); *Yarrowia lipolytica* CBS 6331 (14.80%), *Yarrowia lipolytica* CBS 10144 (12.90%) and *Yarrowia lipolytica* CBS 5589 (14.20%).

EXAMPLE 6: Genotyping oleaginous yeast strains

[0363] Genotyping of 48 different strains of oleaginous yeast was performed. Genomic DNA was isolated from each of the 48 different strains of oleaginous yeast 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 ul 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).

[0364] 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 SZ5434 forward primer (5' GTCCCTGCCCTTTGTACACAC -3' (SEQ ID NO:39) at 10mM stock concentration) and 0.4 µl primer SZ5435 reverse primer (5'- TTGATATGCTTAAGTTCAGCGGG -3' (SEQ ID NO:40) at 10 mM stock concentration). The primers were selected based on sequence conservation between three prime regions of 18S and five prime regions of fungal 26S rRNA genes. The forward primer is identical to nucleotides 1632-1652 of Genbank Ascension # AY550243 and the reverse primer is identical to nucleotides 464271-464293 of Genbank Ascension # NC_001144. Next, 5 µl of diluted total DNA and 3.2 µl dH_2O 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. The resulting purified PCR products were cloned and transformed into *E. coli* using ZeroBlunt PCR4Blunt-TOPO vector kit (Invitrogen) according to manufacturer's instructions. Sequencing reactions were carried out directly on ampicillin resistant colonies. Purified plasmid DNA was sequenced in both directions using M13 forward and reverse primers. 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.

[0365] A list of the 48 strains of oleaginous yeast that were genotyped is in Table 21 along with the corresponding SEQ ID NOs.

Table 21. Genotyped oleaginous yeast strains.

Strain Name	Strain Number	SEQ ID NO
Rhodotorula glutinis	DSMZ-DSM 7098	SEQ ID NO:41
Lipomyces tetrasporus	CBS 5911	SEQ ID NO:41
Rhodotorula glutinis var. glutinis	CBS 3044	SEQ ID NO:42
Lipomyces tetrasporus	CBS 8664	SEQ ID NO:42
Lipomyces tetrasporus	CBS 1808	SEQ ID NO:43
Lipomyces tetrasporus	CBS 1810	SEQ ID NO:43
Lipomyces starkeyi	CBS 1809	SEQ ID NO:44
Trichosporon montevideense	CBS 8261	SEQ ID NO:44
Yarrowia lipolytica	CBS 6331	SEQ ID NO:45
Cryptococcus curvatus	CBS 5324	SEQ ID NO:46
Rhodotorula mucilaginosa var. mucilaginosa	CBS 316	SEQ ID NO:46
Cryptococcus curvatus	CBS 570	SEQ ID NO:46
Cryptococcus curvatus	CBS 2176	SEQ ID NO:46
Cryptococcus curvatus	CBS 2744	SEQ ID NO:46
Cryptococcus curvatus	CBS 2754	SEQ ID NO:46
Cryptococcus curvatus	CBS 2829	SEQ ID NO:46
Cryptococcus curvatus	CBS 5163	SEQ ID NO:46
Cryptococcus curvatus	CBS 5358	SEQ ID NO:46
Trichosporon sp.	CBS 7617	SEQ ID NO:47
Spororobolomyces alborubescens	CBS 482	SEQ ID NO:48
Rhodotorula glutinis var. glutinis	CBS 324	SEQ ID NO:49
Rhodotorula glutinis var. glutinis	CBS 4476	SEQ ID NO:50
Trichosporon behrend	CBS 5581	SEQ ID NO:51
Geotrichum histeridarum	CBS 9892	SEQ ID NO:52
Rhodotorula aurantiaca	CBS 8411	SEQ ID NO:53
Cryptococcus curvatus	CBS 8126	SEQ ID NO:53
Trichosporon domesticum	CBS 8111	SEQ ID NO:54
Rhodotorula toruloides	CBS 8761	SEQ ID NO:55
Rhodotorula terpendoidalis	CBS 8445	SEQ ID NO:56
Yarrowia lipolytica	CBS 10144	SEQ ID NO:57

Rhodotorula glutinis var. glutinis	CBS 5805	SEQ ID NO:58
Yarrowia lipolytica	CBS 10143	SEQ ID NO:59
Lipomyces tetrasporus	CBS 5607	SEQ ID NO:60
Yarrowia lipolytica	CBS 5589	SEQ ID NO:61
Lipomyces tetrasporus	CBS 8724	SEQ ID NO:62
Rhodosporidium sphaerocarpum	CBS 2371	SEQ ID NO:63
Trichosporon brassicae	CBS 6382	SEQ ID NO:64
Cryptococcus curvatus	CBS 2755	SEQ ID NO:65
Lipomyces tetrasporus	CBS 7656	SEQ ID NO:65
Lipomyces starkeyi	CBS 7786	SEQ ID NO:66
Yarrowia lipolytica	CBS 6012	SEQ ID NO:67
Trichosporon loubieri var. loubieri	CBS 8265	SEQ ID NO:68
Geotrichum vulgare	CBS 10073	SEQ ID NO:69
Rhodosporidium toruloides	CBS 14	SEQ ID NO:70
Rhodotorula glutinis var. glutinis	CBS 6020	SEQ ID NO:71
Lipomyces orientalis	CBS 10300	SEQ ID NO:71
Rhodotorula aurantiaca	CBS 317	SEQ ID NO:72
Torulaspora delbrueckii	CBS 2924	SEQ ID NO:73

EXAMPLE 7: Cultivation of *Rhodococcus opacus* to achieve high oil content

[0366] A seed culture of *Rhodococcus opacus* PD630 (DSM 44193, Deutsche Sammlung von Mikroorganismen und Zellkuttwen GmbH) was generated using 2ml of a cryo-preserved stock inoculated into 50 ml of MSM media with 4% sucrose (see Schlegel, et al., (1961) *Arch Mikrobiol* 38, 209-22) in a 250 ml baffle flask. The seed culture was grown at 30°C with 200 rpm agitation until it reached an optical density of 1.16 at 600 nm. 10ml of the seed flask culture was used to inoculate cultures for lipid production under two different nitrogen conditions: 10mM NH_4Cl and 18.7mM NH_4Cl (each in duplicate). The growth cultures were grown at 30°C with 200 rpm agitation for 6 days. Cells grown in the 10 mM NH_4Cl condition reached a maximal 57.2% (average) lipid by DCW after 6 days of culture. Cells grown in the 18.7 mM NH_4Cl condition reached a maximal 51.8% (average) lipid by DCW after 5 days in culture.

[0367] A sample of *Rhodococcus opacus* biomass was subjected to direct transesterification and analyzed via GC/FID for a fatty acid profile. The results were: C14:0 (2.33); C15:0 (9.08); C16:0 (24.56); C16:1 (11.07); C17:0 (10.50); 2 double bond equivalent (2DBE) C17 species (19.90); C18:0 (2.49); C18:1 (17.41); C18:2 (0.05); C19:0 (0.75) and 2DBE C19 species (1.87).

EXAMPLE 8: Extraction of oil from microorganisms

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

[0368] 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 to extract oil. Other methods of extraction of oil from microalgae using an expeller press are described in PCT Application No. PCT/US2010/31108, incorporated herein by reference.

B. Extraction of oil from oleaginous yeast using an expeller press

[0369] Yeast strain *Rhodotorula glutinis* (DSMZ-DSM 70398) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganism and Cell Culture, Inhoffenstraße 7B, 38124 Braunschweig, Germany. Cryopreserved cells were thawed and added to 50 mL YPD media (described above) with 1x DAS vitamin solution (1000x: 9g/L tricine; 0.67g/L thiamine-HCl; 0.01 g/L d-biotin; 0.008 cyannocobalamin; 0.02 calcium pantothenate; and 0.04 g/L p-Aminobenzoic acid) and grown at 30°C with 200 rpm agitation for 18-24 hours until an OD reading was over 5 OD (A600). The culture was then transferred to 7-L fermentors and switched to YP1 medium (8.5 g/L Difco Yeast Nitrogen Base without Amino Acids and Ammonium Sulfate, 3 g/L Ammonium Sulfate, 4 g/L yeast extract) with 1x DAS vitamin solution. The cultures were sampled twice per day and assayed for OD (A600), dry cell weight (DCW) and lipid concentration. When the cultures reached over 50g/L DCW, the cultures were harvested. Based on dry cell weight, the yeast biomass contained approximately 50% oil. Two samples of yeast biomass were subjected to direct transesterification and analyzed via GC/FID for a fatty acid profile. The results are expressed in Area Percent, and shown in Table 22, below.

Table 22. Fatty acid profile of transesterified yeast biomass samples.

	C10:0	C12:0	C14:0	C15:0	C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C18:3α	≥C:20
Sample 1	0.03	0.21	3.36	0.25	33.26	0.76	0.20	6.88	42.68	9.28	1.33	1.1
Sample 2	0.02	0.10	2.18	0.12	29.94	0.49	0.16	8.17	48.12	7.88	0.84	1.45

[0370] The harvested yeast broth was dried using three different methods for comparison: (1) tray dried in a forced air oven at 75°C overnight; (2) dried on a drum dryer without concentration; and (3) the yeast broth was concentrated to 22% solids and the slurry was then

dried on a drum dryer. Material from each of the three different drying conditions was heat conditioned and fed through a screw press for oil extraction. The press temperature was at 150°F and the conditioned dried yeast biomass was held at about 190°F until it was ready to be fed into the press.

[0371] The moisture content of the tray dried yeast was 1.45% and the dried yeast was then conditioned in an oven at 90°C for 10 minutes. The moisture content after conditioning was 0.9%. The conditioned tray dried material was then fed into a bench-top Taby screw press (Taby Pressen Type 70 oil press with a 2.2 Hp motor and 70mm screw diameter) for oil extraction. This material did not yield any significant amount of oil and heavy footing was observed with the press.

[0372] The moisture content of the drum dried yeast broth without concentration was 5.4% and the drum dried yeast was then conditioned in an oven at 90°C for 20 minutes. The moisture content after conditioning was 1.4%. The conditioned drum dried yeast was then fed into a bench-top Taby screw press for oil extraction. This material oiled well, with minimal footing.

[0373] The moisture content of the drum dried concentrated yeast broth was 2.1% and the drum dried concentrated yeast was then conditioned in an oven at 90°C for 20 minutes. The moisture content after conditioning was 1.0%. The conditioned drum dried concentrated yeast was then fed into a bench-top Taby screw press for oil extraction. This material oiled well, with minimal footing.

C. Drying and Oil Extraction from Oleaginous Bacteria

[0374] Oleaginous bacteria strain *Rhodococcus opacus* PD630 (DSMZ-DSM 44193) was cultured according to the methods provided herein to produce oleaginous bacteria biomass with approximately 32% lipid by DCW.

[0375] The harvested *Rhodococcus opacus* broth was concentrated using centrifugation and then washed with deionized water and resuspended in 1.8L of deionized water. 50 grams of purified cellulose (PB20-Pre-co-Floc, EP Minerals, Nevada) was added to the resuspended biomass, and the total solids was adjusted with deionized water to 20%. The *Rhodococcus* biomass was then dried on a drum drier and the moisture content of the *Rhodococcus* after drum drying was approximately 3%.

[0376] The drum-dried material was then heat conditioned in a oven at 130°C for 30 minutes with a resulting moisture content of approximately 1.2%. The heat conditioned biomass was then fed through a bench top Taby press (screw press) for oil extraction. The press temperature was at 209°F and the conditioned dried yeast biomass was held at about

240°F until it was ready to be fed into the press. Oil recovery was accompanied by heavy footing.

EXAMPLE 9: Processing of extracted oil; lowering of pour point

Summary

[0377] Microbial oil prepared in accordance with the foregoing examples can be processed in accordance with the methods described herein to improve its properties for use in foods and lubricants. In addition to the microbes described in the above examples, the microalgae *Chlorella protothecoides* is an excellent producer of microbial oil. For methods of culturing *Chlorella* species and strains to obtain high oil and to extract oil therefrom, see PCT Pub. Nos. 2008/151149, 2010/120939, and 2010/138,620, incorporated herein by reference.

[0378] Pour point was reduced in oil obtained from *Chlorella protothecoides* by reducing the relative proportion of the saturated fraction, which consists primarily of palmitic and stearic triglycerides known in the trade as the stearin fraction. This was accomplished by fractionating the oil to reduce the saturated triglycerides concentration of the oil. This was done by crystallizing or dry fractionation, similar to the winterizing process known in the vegetable oil industry. The algal oil was first refined, bleached and deodorized by methods described above (methods similar to those used in the vegetable oil industry could also be employed) to produce "RBD oil".

[0379] The temperature of the RBD oil was lowered in a controlled manner until crystal nuclei were formed. The oil was then held at that crystallization temperature for several hours to faciliate growth of the crystals. The crystals were then removed by filtration to result in two fractions: a solid phase containing some or most of the stearin fraction, and a liquid phase containing mostly the olein fraction. The liquid phase was subjected to fractionation again to a lower crystallization temperature to effect a further removal of stearin. The resulting purified liquid fraction, equivalent to a super olein as commonly known in the vegetable oil industry, has better thermal properties than the native algal oil.

Materials and Methods

Materials

[0380] Algal oil (refined, bleached, and deodorized) was produced by Solazyme, Inc (South San Francisco, CA). Table 23 summarizes the properties of the oil used in the study.

Table 23. Properties of algal oil used in the study

Analysis	Value
Moisture [%]	0.01
Free fatty acid [% as oleic]	0.03
Iodine value	83.5
Fatty Acid Profile	
8:0	0.00
10:0	0.00
12:0	0.03
14:0	1.12
16:0	14.02
18:0	3.24
18:1	67.73
18:2	11.18
18:3	0.62
20:0	0.32
20:1	0.20

[0381] Poly alkyl methacrylate copolymer-based Pour Point Depressant (PPD) VISCOPLEX® 10-310 containing ~50% (w/w) of rapeseed oil carrier and VISCOPLEX® 1-133 containing refined mineral oil carrier were supplied by RohmMax Evonik (Horsham, PA).

Methods

A. Dry Fractionation: Crystallization

[0382] Around 2.5 kg of algal oil was placed in a 3-L jacketed vessel connected to a temperature-controlled circulating water bath, which served to heat and cool the product (Crystallization & Degumming, Charleroi, Belgium). The reactor was fitted with a variable-speed agitator. Cooling was controlled by monitoring the temperatures of the oil and the water circulating between the double walls of the reactor. A droplet of crystal suspension was sampled from the reactor with a stick and deposited on a coverslip to monitor crystal formation at the end of cooling. The sample was analyzed immediately under a microscope before the crystals had a chance to melt.

[0383] The overall cooling pattern is shown in Fig. 1. Agitator speed was 30 rpm during the first phase and 15 rpm up to the end of the cooling program.

B. Dry Fractionation: Filtration

[0384] At the end of crystallization, the crystal suspension was filtered using a 1-L membrane press filter (Choquenet SA, Chauny, France). Filtration was carried out in a

chamber that was kept at the final cooling temperature. The filtration time was 20 min and the filter supply pressure was 4 barg.

[0385] At the end of the separation step, the stearin and olein fractions were weighed, the fraction yields calculated, and a sample of each fraction was set aside for further analysis. Algal super olein #1 was produced by processing the olein from the first fractionation and repeating the crystallization and filtration process described above following the cooling program shown in Fig 2. Algal super olein #2 and #3 were produced by first fractionating deodorized oil and repeating the crystallization and filtration process using a cooling program similar to that shown in Fig. 2.

C. Pour point (PP)

[0386] Pour Point Depressants (0.5 and 1.0 grams) were weighed into flasks. Algal oil, olein and superolein fractions (100 grams) were added to each flask. The mixtures were mixed thoroughly. Each sample was tested according to the D 97 ASTM (The American Society for Testing and Materials) standard method. The sample was poured into a test tube and heated without stirring in a water bath where the temperature was set at 48.0 ^{0}C The sample was heated until it reached 46.0 ^{0}C. After heating, the sample was cooled to 25.0 ^{0}C (in a water bath). The sample was then placed in a metal cylinder in a methanol bath. The temperature of the methanol bath was set at -1.0 ^{0}C to -2.0 ^{0}C until the temperature of the sample reached 10.0 ^{0}C. Then, the temperature of the methanol bath was reduced to -17.0 ^{0}C until the temperature of the sample reached -7.0 ^{0}C. When the temperature of the sample was about 11.0 ^{0}C above the expected pour point, the sample was taken out of the methanol bath at every reduction by 3.0 ^{0}C, to check the pour ability. The pour point of the sample was determined as the temperature when the sample in the test tube stopped pouring when taken out of the methanol bath. To the temperature recorded, 3.0 ^{0}C was added, to give the actual pour point value of the sample.

[0387] The properties of the oil produced at each step could be further improved in accordance with the methods described herein by the addition of a chemical pour point depressant that reduced the pour point even further. The pour point depressants used for this example were VISCOPLEX® 10-310 and 1-133, both produced by Evonik, but similar results could be obtained using any standard pour point depressant. The results are shown in Table 24, below, and in Fig. 3.

Table 24. Effect of Fractionation and Pour Point[(1)] Depressants on Algal Oil (°C)

SAMPLE	No additive	VISCOPLEX® 10-310 [(2)] (% w:w)		VISCOPLEX® 1-133 [(3)] (% w:w)	
	0	0.5	1	0.5	1
Refined, Bleached, Deodorized oil	-8	-17	-20	-14	-16
Olein #1 (liquid from 1st fractionation)	-11	-19	-20	-16	-17
Super Olein #1 (liquid from olein fractionation =2nd pass)	-20	-26	NT	NT	NT
Super Olein #2 (liquid from olein fractionation =2nd pass)	-14	-20	-23	NT	NT
Super Olein #3 (liquid from olein fractionation =2nd pass)	-20	-23	-29	NT	NT

(1) Pour point ASTM D97

(2) 50:50 mix of poly(alkyl) acrylate and rapeseed oil. Rated biodegradable

(3) Mix of poly(alkyl) acrylate and refined mineral oil.

NT = Not Tested.

EXAMPLE 10: Pour points of oil produced from engineered microalgae

[0388] *Protheca moriformis* (UTEX 1435) was transformed with one of the following plasmid constructs in Table 25 using the methods of Example 2.

Table 25. Plasmid constructs used to transform *Protheca moriformis* (UTEX 1435).

Plasmid Construct	Sequence Elements
1	6SA-CrbTub_yInv_nr::CrbTub_hpFADc_nr-6SB
2	6SA-bTub-yInv-nr-6SB
3	FADc5'_btub-yInv-nr::amt03-S106SAD-CtOTE-nr-FADc3'
4	SAD2B5'-CrbTub_yInv_ef1::amt03_CWTE2_nr-SAD2B3'

[0389] Each of the constructs contained a region for integration into the nuclear genome and a *S. cerevisiae suc2* sucrose invertase coding region under the control of *C. reinhardtii* β-

tubulin promoter/5'UTR and *Chlorella vulgaris* nitrate reductase 3' UTR. This *S. cerevisiae suc2* expression cassette is listed as SEQ ID NO:78 and served as a selection marker. Relevant sequences for the targeting regions used for nuclear genome integration are shown below.

Description	SEQ ID NO:
5' sequence for the 6S genomic targeting sequence	SEQ ID NO:100
3' sequence for the 6S genomic targeting sequence	SEQ ID NO:101
5' sequence for genomic integration at the FADc locus	SEQ ID NO:102
3' sequence for genomic integration at the FADc locus	SEQ ID NO:103
5' sequence for genomic integration at the SAD2B locus	SEQ ID NO:36
3' sequence for genomic integration at the SAD2B locus	SEQ ID NO:37

[0390] In addition to the sucrose selectable marker, three of the four constructs also contained different, additional sequences for the expression of either proteins or RNA. Table 26 lists important enzymes or hairpin RNA cassettes that are encoded by the DNA sequence in the indicated construct. All protein coding regions were codon optimized to reflect the codon bias inherent in *Prototheca moriformis* UTEX 1435 (see Table 2) nuclear genes. Both amino acid sequences and the cDNA sequences for the construct used are listed in the sequence listing.

Table 26. Plasmid constructs for thioesterases or hairpin RNA expression used to transform *Protheca moriformis* (UTEX 1435).

Plasmid construct	Protein or hairpin RNA	Seq ID NO:
1	FADc hairpin	SEQ ID NO:92
3	*Carthamus tinctorius* ACP thioesterase (GenBank Accession No: AAA33019.1)	SEQ ID NO:104
4	*Cuphea wrightii* FatB2 thioesterase (GenBank Accession No. U56104)	SEQ ID NO:105

[0391] Both the *Carthamus tinctorius* ACP thioesterase (CtOTE in Construct 3) and the *Cuphea wrightii* FatB2 thioesterase (CwTE2 in Construct 4) coding regions were under the control of the *Prototheca moriformis* Amt03 promoter/5'UTR (SEQ ID NO:84) and *C. vulgaris* nitrate reductase 3'UTR (SEQ ID NO:85). The native transit peptide of the *C. tinctorius* ACP thioesterase was replaced with the *Chlorella protothecoides* stearoyl-ACP

desaturase transit peptide (SEQ ID NO:86). The codon-optimized cDNA sequences and amino acid sequences (with the replaced transit peptide) of the *C. tinctorius* ACP thioesterase are listed in the Sequence Listing as SEQ ID NO:106 and SEQ ID NO:104, respectively. The codon-optimized cDNA sequences and amino acid sequences of the *Cuphea wrightii* FatB2 thioesterase are listed in the Sequence Listing as SEQ ID NO:107 and SEQ ID NO:105, respectively. Construct 1 containing the FADc hairpin RNA is described in Example 4.

[0392] Each construct was transformed into a *Prototheca moriformis* genetic background. Positive clones were screened on plates with sucrose as the sole carbon source. A subset of the positive clones were selected and grown under lipid production conditions. Wild type UTEX 1435 was grown using glucose while all other transgenic lines were cultivated in sucrose. For each construct, transformants were grown and oil was isolated. The isolated oils were analyzed for fatty acid profiles and the pour points were determined as described herein. Pour points were determined using the ASTM D97 standard test method for pour point evaluation. The fatty acid profiles and the pour points of the oils for transgenic strains are shown in Table 27 below. Table 27 discloses the data for successful manipulation of the pour points of the oils produced by genetically engineered microalgae. The pour point of the oil transformed with Construct 3 was decreased from -10.5°C to -19.5° C.

Table 27. Fatty acid profiles and pour point temperatures of *Prototheca moriformis* cells containing different constructs.

	Wild Type	Construct 1	Construct 2	Construct 3	Construct 4
C6:0	0	0	0	0	0
C8:0	0	0	0	0	0
C10:0	0	0	0.01	0.03	0.01
C12:0	0.03	0.02	0.03	0.11	0.03
C14:0	1.12	0.68	0.75	0.90	1.08
C16:0	14.02	15.55	13.26	7.75	26.09
C18:0	3.24	3.79	5.26	1.78	12.37
C18:1	67.76	76.84	71.75	86.40	53.42
C18:2	11.49	0.91	6.44	0.12	4.38
C18:3α	0.62	0.09	0.07	0.02	0.2
Pour Point	-10.5°C	-7.6° C	-7.6° C	-19.5° C	10.4° C

EXAMPLE 11: Engineered microalgae with altered fatty acid profiles

[0393] As described above, integration of heterologous genes to knockout or knockdown specific endogenous lipid pathway enzymes in *Prototheca* species can alter fatty acid profiles. As endogenous fatty acyl-ACP thioesterases catalyze the cleavage of a fatty acid from an acyl carrier protein during lipid synthesis, they are important lipid pathway enzymes

in establishing the lipid profile of the host organism. Plasmid constructs were created to assess whether the lipid profile of a host cell can be affected as a result of a knockout or knockdown of an endogenous fatty acyl-ACP thioesterase gene, FATA1.

A. Altering fatty acid profiles by knockout of an endogenous *Prototheca moriformis* thioesterase gene

[0394] A classically mutagenized derivative of *Protheca moriformis* UTEX 1435, S1920, was transformed with one of the following plasmid constructs in Table 28 using the methods of Example 2. Each construct contained a region for integration into the nuclear genome to interrupt the endogenous FATA1 gene and a *S. cerevisiae suc2* sucrose invertase coding region under the control of *C. reinhardtii* β-tubulin promoter/5'UTR and *Chlorella vulgaris* nitrate reductase 3' UTR. This *S. cerevisiae suc2* expression cassette is listed as SEQ ID NO:78 and served as a selection marker. All protein coding regions were codon optimized to reflect the codon bias inherent in *Prototheca moriformis* UTEX 1435 (see Table 2) nuclear genes. Relevant sequences for the targeting regions for the FATA1 gene used for nuclear genome integration are shown below.

Description	SEQ ID NO:
5' sequence for integration into FATA1 locus	SEQ ID NO:108
3' sequence for integration into FATA1 locus	SEQ ID NO:109

Table 28. Plasmid constructs used to transform *Protheca moriformis* (UTEX 1435) S1920.

Plasmid Construct	Sequence Elements
pSZ1883	FATA1-CrbTub_yInv_nr-FATA1
pSZ1925	FATA1-CrbTub_yInv_nr::amt03_CwTE2_nr-FATA1

[0395] Relevant restriction sites in the construct FATA1-CrbTub_yInv_nr-FATA1 are indicated in lowercase in the sequence below, bold and underlining and are 5'-3' *BspQ* 1, *Kpn* I, *Asc* I, *Mfe* I, *Sac* I, *BspQ* I, respectively. *BspQI* sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent genomic DNA from S1920 that permit targeted integration at FATA1 locus via homologous recombination. Proceeding in the 5' to 3' direction, the *C. reinhardtii* β -tubulin promoter driving the expression of the yeast sucrose invertase gene (conferring the ability of S1920 to metabolize sucrose) is indicated by

boxed text. The initiator ATG and terminator TGA for invertase are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics. The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase underlined text followed by the S190 FATA1 genomic region indicated by bold, lowercase text.

gctcttcggagtcactgtgccactgagttcgactggtagctgaatggagtcgctgctccactaaacgaattgtcagcaccgcca gccggccgaggacccgagtcatagcgagggtagtagcgcgccatggcaccgaccagcctgcttgccagtactggcgtctcttc cgcttctctgtggtcctctgcgcgctccagcgcgtgcgcttttccggtggatcatgcggtccgtggcgcaccgcagcggccgctg cccatgcagcgccgctgcttccgaacagtggcggtcagggccgcacccgcggtagccgtccgtccggaacccgcccaagagt tttgggagcagcttgagccctgcaagatggcggaggacaagcgcatcttcctggaggagcaccggtgcgtggaggtccgggg ctgaccggccgtcgcattcaacgtaatcaatcgcatgatgatcagaggacacgaagtcttggtggcggtggccagaaacact gtccattgcaagggcatagggatgcgttccttcacctctcatttctcatttctgaatccctccctgctcactctttctcctcctcttc ccgttcacgcagcattcggggtaccctttcttgcgctatgacacttccagcaaaaggtagggcgggctgcgagacggcttcccggc gctgcatgcaacaccgatgatgcttcgaccccccgaagctccttcggggctgcatgggcgctccgatgccgctccagggcgagcgc tgtttaaatagccaggcccccgattgcaaagacattatagcgagctaccaaagccatattcaaacacctagatcactaccacttctacac aggccactcgagcttgtgatcgcactccgctaaggggggcgcctcttcctcttcgtttcagtcacaacccgcaaac**ggcgcgcc*ATG***

*ctgctgcaggccttcctgttcctgctggccggcttcgccgccaagatcagcgcctccatgacgaacgagacgtccgaccgcccct ggtgcacttcacccccaacaagggctggatgaacgaccccaacggcctgtggtacgacgagaaggacgccaagtggcacctgt acttccagtacaacccgaacgacaccgtctgggggacgcccttgttctggggccacgccacgtccgacgacctgaccaactggg aggaccagcccatcgccatcgccccgaagcgcaacgactccggcgccttctccggctccatggtggtggactacaacaacacct ccggcttcttcaacgacaccatcgacccgcgccagcgctgcgtggccatctggacctacaacaccccggagtccgaggagcagt acatctcctacagcctggacggcggctacaccttcaccgagtaccagaagaaccccgtgctggccgccaactccacccagttcc gcgacccgaaggtcttctggtacgagccctcccagaagtggatcatgaccgcggccaagtcccaggactacaagatcgagatct actcctccgacgacctgaagtcctggaagctggagtccgcgttcgccaacgagggcttcctcggctaccagtacgagtgccccgg cctgatcgaggtccccaccgagcaggaccccagcaagtcctactgggtgatgttcatctccatcaaccccggcgccccggccgg cggctccttcaaccagtacttcgtcggcagcttcaacggcacccacttcgaggccttcgacaaccagtcccgcgtggtggacttcg gcaaggactactacgccctgcagaccttcttcaacaccgacccgacctacgggagcgccctgggcatcgcgtgggcctccaact gggagtactccgccttcgtgcccaccaacccctggcgctcctccatgtccctcgtgcgcaagttctccctcaacaccgagtaccag gccaacccggagacggagctgatcaacctgaaggccgagccgatcctgaacatcagcaacgccggccccctggagccggttcg ccaccaacaccacgttgacgaaggccaacagctacaacgtcgacctgtccaacagcaccggcaccctggagttcgagctggtg tacgccgtcaacaccacccagacgatctccaagtccgtgttcgcggacctctccctctggttcaagggcctggaggaccccgagg agtacctccgcatgggcttcgaggtgtccgcgtcctccttcttcctggaccgcgggaacagcaaggtgaagttcgtgaaggagaa ccctacttcaccaaccgcatgagcgtgaacaaccagcccttcaagagcgagaacgacctgtcctactacaaggtgtacggcttg

ctggaccagaacatcctggagctgtacttcaacgacggcgacgtcgtgtccaccaacacctacttcatgaccaccgggaacgcc
*ctgggctccgtgaacatgacgacgggggtggacaacctgttctacatcgacaagttccaggtgcgcgaggtcaag**TGA***caatt*
ggcagcagcagctcggatagtatcgacacactctggacgctggtcgtgtgatggactgttgccgccacacttgctgccttgacctgtga
atatccctgccgcttttatcaaacagcctcagtgtgtttgatcttgtgtgtacgcgcttttgcgagttgctagctgcttgtgctatttgcgaata
ccacccccagcatccccttccctcgtttcatatcgcttgcatcccaaccgcaacttatctacgctgtcctgctatccctcagcgctgctcct
gctcctgctcactgcccctcgcacagccttggtttgggctccgcctgtattctcctggtactgcaacctgtaaaccagcactgcaatgctg
atgcacgggaagtagtgggatgggaacacaaatggaggatcgta**gagctc**actagtatcgatttcgaa**gacagggtggttggctgg**
atggggaaacgctggtcgcgggattcgatcctgctgcttatatcctccctggaagcacacccacgactctgaagaagaaaacg
tgcacacacacaacccaaccggccgaatatttgcttccttatcccgggtccaagagagactgcgatgcccccctcaatcagcat
cctcctccctgccgcttcaatcttccctgcttgcctgcgcccgcggtgcgccgtctgcccgcccagtcagtcactcctgcacaggc
cccttgtgcgcagtgctcctgtacccttaccgctccttccattctgcgaggcccccctattgaatgtattcgttgcctgtgtggcca
agcgggctgctgggcgcgccgccgtcgggcagtgctcggcgactttggcggaagccgattgttcttctgtaagccacgcgcttg
ctgctttgggaagagaaggggggggtactgaatggatgaggaggagaaggaggggtattggtattatctgagttgggtgaa
gagc (SEQ ID NO:111)

[0396] To introduce the *Cuphea wrightii* ACP-thioesterase 2 (CwFatB2) gene (Accession No: U56104) into S1920 at the FATA1 locus, a construct was generated to express the protein coding region of the CwFatB2 gene under the control of the *Prototheca moriformis* Amt03 promoter/5'UTR (SEQ ID NO:84) and *C. vulgaris* nitrate reductase 3'UTR (SEQ ID NO:85). The construct that has been expressed in S1920 can be written as FATA1-CrbTub_yInv_nr::amt03_CwTE2_nr-FATA1.

[0397] Relevant restriction sites in the construct FATA1-CrbTub_yInv_nr::amt03_CwTE2_nr-FATA1 are indicated in lowercase, bold and underlining in the sequence below and are 5'-3' *BspQ* 1, *Kpn* I, *Asc* I, *Mfe* I, *BamH* I, *EcoR* I, *Spe* I, *Asc* I, *Pac* I, *Sac* I, *BspQ* I, respectively. *BspQI* sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent genomic DNA from S1920 that permit targeted integration at FATA1 locus via homologous recombination. Proceeding in the 5' to 3' direction, the *C. reinhardtii* β -tubulin promoter driving the expression of the yeast sucrose invertase gene (conferring the ability of S1920 to metabolize sucrose) is indicated by boxed text. The initiator ATG and terminator TGA for invertase are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics. The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase underlined text followed by an endogenous amt03 promoter of *Prototheca moriformis,* indicated by boxed italics text. The Initiator ATG and terminator TGA codons of the *C. wrightii* ACP-thioesterase are indicated

by uppercase, bold italics, while the remainder of the ACP-thioesterase coding region is indicated by bold italics. The *C. vulgaris* nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the S1920 FATA1 genomic region indicated by bold, lowercase text. The codon-optimized cDNA sequences and amino acid sequences of the *Cuphea wrightii* FatB2 thioesterase are listed in the Sequence Listing as SEQ ID NO:107 and SEQ ID NO:105, respectively.

gctcttcggagtcactgtgccactgagttcgactggtagctgaatggagtcgctgctccactaaacgaattgtcagcaccgcca
gccggccgaggacccgagtcatagcgagggtagtagcgcgccatggcaccgaccagcctgcttgccagtactggcgtctcttc
cgcttctctgtggtcctctgcgcgctccagcgcgtgcgcttttccggtggatcatgcggtccgtggcgcaccgcagcggccgctg
cccatgcagccgccgctgcttccgaacagtggcggtcagggccgcacccgcggtagccgtccgtccggaacccgcccaagagt
tttgggagcagcttgagccctgcaagatggcggaggacaagcgcatcttcctggaggagcaccggtgcgtggaggtccgggg
ctgaccggccgtcgcattcaacgtaatcaatcgcatgatgatcagaggacacgaagtcttggtggcggtggccagaaacact
gtccattgcaagggcatagggatgcgttccttcacctctcatttctcatttctgaatccctccctgctcactctttctcctcctcttc
ccgttcacgcagcattcggggtaccctttcttgcgctatgacacttccagcaaaaggtagggcgggctgcgagacggcttcccggc
gctgcatgcaacaccgatgatgcttcgaccccccgaagctccttcggggctgcatgggcgctccgatgccgctccagggcgagcgc
tgtttaaatagccaggcccccgattgcaaagacattatagcgagctaccaaagccatattcaaacacctagatcactaccacttctacac
aggccactcgagcttgtgatcgcactccgctaagggggcgcctcttcctcttcgtttcagtcacaacccgcaaac**ggcgcgcc*ATG***
ctgctgcaggccttcctgttcctgctggccggcttcgccgccaagatcagcgcctccatgacgaacgagacgtccgaccgccccct
ggtgcacttcacccccaacaagggctggatgaacgaccccaacggcctgtggtacgacgagaaggacgccaagtggcacctgt
acttccagtacaacccgaacgacaccgtctgggggacgcccttgttctggggccacgccacgtccgacgacctgaccaactggg
aggaccagcccatcgccatcgccccgaagcgcaacgactccggcgccttctccggctccatggtggtggactacaacaacacct
ccggcttcttcaacgacaccatcgacccgcgccagcgctgcgtggccatctggacctacaacaccccggagtccgaggagcagt
acatctcctacagcctggacggcggctacaccttcaccgagtaccagaagaaccccgtgctggccgccaactccacccagttcc
gcgacccgaaggtcttctggtacgagccctcccagaagtggatcatgaccgcggccaagtcccaggactacaagatcgagatct
actcctccgacgacctgaagtcctggaagctggagtccgcgttcgccaacgagggcttcctcggctaccagtacgagtgccccgg
cctgatcgaggtccccaccgagcaggaccccagcaagtcctactgggtgatgttcatctccatcaaccccggcgccccggccgg
cggctccttcaaccagtacttcgtcggcagcttcaacggcacccacttcgaggccttcgacaaccagtcccgcgtggtggacttcg
gcaaggactactacgccctgcagaccttcttcaacaccgacccgacctacgggagcgccctgggcatcgcgtgggcctccaact
gggagtactccgccttcgtgcccaccaaccccctggcgctcctccatgtccctcgtgcgcaagttctccctcaacaccgagtaccag
gccaacccggagacggagctgatcaacctgaaggccgagccgatcctgaacatcagcaacgccggcccctggagccggttcg
ccaccaacaccacgttgacgaaggccaacagctacaacgtcgacctgtccaacagcaccggcaccctggagttcgagctggtg
tacgccgtcaacaccacccagacgatctccaagtccgtgttcgcggacctctccctctggttcaagggcctggaggaccccgagg

agtacctccgcatgggcttcgaggtgtccgcgtcctccttcttcctggaccgcgggaacagcaaggtgaagttcgtgaaggagaa
cccctacttcaccaaccgcatgagcgtgaacaaccagcccttcaagagcgagaacgacctgtcctactacaaggtgtacggcttg
ctggaccagaacatcctggagctgtacttcaacgacggcgacgtcgtgtccaccaacacctacttcatgaccaccgggaacgcc
*ctgggctccgtgaacatgacgacgggggtggacaacctgttctacatcgacaagttccaggtgcgcgaggtcaag**TGA**caatt*
ggcagcagcagctcggatagtatcgacacactctggacgctggtcgtgtgatggactgttgccgccacacttgctgccttgacctgtga
atatccctgccgcttttatcaaacagcctcagtgtgtttgatcttgtgtgtacgcgcttttgcgagttgctagctgcttgtgctatttgcgaata
ccacccccagcatccccttccctcgtttcatatcgcttgcatcccaaccgcaacttatctacgctgtcctgctatccctcagcgctgctcct
gctcctgctcactgcccctcgcacagccttggtttgggctccgcctgtattctcctggtactgcaacctgtaaaccagcactgcaatgctg
atgcacgggaagtagtgggatgggaacacaaatgga**ggatcc**cgcgtctcgaacagagcgcgcagaggaacgctgaaggtctcg
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cgattgttcttctgtaagccacgcgcttgctgctttgggaagagaaggggggggtactgaatggatgaggaggagaaggag
gggtattggtattatctgagttgggtgaagagc (SEQ ID NO:112)

[0398] Upon transformation of FATA1-CrbTub_yInv_nr-FATA1 into S1920, primary transformants were clonally purified and grown under standard lipid production conditions at pH 5.0 similar to the conditions as disclosed in Example 1. Fatty acid profiles were analyzed using standard fatty acid methyl ester gas chromatography flame ionization (FAME GC/FID) detection methods. Table 29 below provides the fatty acid profiles of several transformants.

Table 29. Fatty acid profiles of *Prototheca moriformis* cells containing a selectable marker to disrupt an endogenous FATA1 allele.

Transformation	% C14:0	% C16:0	% C18:0	% C18:1	% C18:2
Wildtype	1.23	25.68	2.83	60.54	7.52
pSZ1883 Transformant 1	0.86	16.95	1.75	68.44	9.78
pSZ1883 Transformant 2	0.85	17.33	1.71	68.57	9.31
pSZ1883 Transformant 3	0.82	17.40	1.78	68.55	9.22
pSZ1883 Transformant 4	0.84	17.43	1.78	68.25	9.53
pSZ1883 Transformant 5	0.75	17.64	2.02	69.02	8.61

[0399] These results show that ablation of the host's endogenous FATA1 allele alters the lipid profile of the engineered microalgae. The impact of targeting a selectable marker to the endogenous FATA1 allele is a clear diminution of C16:0 fatty acid production with an increase in C18:1 fatty acid production.

[0400] Upon transformation of FATA1-CrbTub_yInv_nr::amt03_CwTE2_nr-FATA1 into S1920, primary transformants were clonally purified and grown under standard lipid production conditions at pH 7.0 with different carbon sources provided to a total concentration of 40 g/L. The sucrose concentration was 40 g/L. Where only glucose was used as the carbon source, glucose was provided at 40 g/L. Where glucose and fructose was used as the carbon source, glucose was provided at 20 g/L and fructose was provided at 20 g/L. Fatty acid profiles were assessed by GC-FID. The resulting fatty acid profiles are listed in Table 30.

Table 30. Fatty acid profiles of *Prototheca moriformis* cells containing a selectable marker and an exogenous thioesterase to disrupt an endogenous FATA1 allele.

Transformant	Copy Number	Carbon source	% C10:0	% C12:0	% C14:0	% C16:0	% C18:0	% C18:1	% C18:2
Wildtype	0	Glucose	0.01	0.04	1.38	28.83	3.00	56.05	8.21
Wildtype	0	Glucose	0.01	0.04	1.50	29.38	3.00	55.29	8.23
Wildtype	0	Glucose/ Fructose	0.01	0.05	1.48	28.58	3.20	57.14	7.27
Wildtype	0	Glucose/ Fructose	0.01	0.04	1.54	29.05	3.23	56.47	7.32
pSZ1925 Transformant 1	> 2	Glucose/ Fructose	4.29	19.98	9.17	20.68	3.47	34.38	6.37
pSZ1925 Transformant 2	> 2	Glucose/ Fructose	3.11	16.17	9.91	15.97	1.57	45.72	5.81
pSZ1925 Transformant 3	> 2	Sucrose	4.84	24.22	11.56	19.48	2.67	29.56	6.02
pSZ1925 Transformant 4	> 2	Sucrose	3.24	16.67	10.39	16.34	1.43	44.41	6.00
pSZ1925 Transformant 5	1-2	Glucose/ Fructose	0.18	1.64	1.85	14.43	2.12	70.30	7.63
pSZ1925 Transformant 6	1-2	Glucose/ Fructose	0.18	1.56	1.74	13.56	2.25	71.04	7.72
pSZ1925 Transformant 7	1-2	Sucrose	0.19	1.69	1.89	13.79	3.15	69.97	7.68
pSZ1925 Transformant 8	1-2	Sucrose	0.15	1.26	1.49	13.44	2.73	71.46	7.77

[0401] Concordant with targeting a selectable marker alone to the host's FATA1 allele, integration of a selectable marker concomitant with an exogenous thioesterase alters the lipid profile of the engineered microalgae. As above, targeting an exogenous gene to the FATA1 allele results in a clear diminution of C16:0 fatty acid production. The additional expression of the CwTE2 thioesterase at the FATA1 locus also impacts mid chain fatty acids and C18:1 fatty acid production to an extent that is dependent upon the level of exogenous thioesterase activity present in the transformants analyzed. Genes bordered by repeat units such as the *C. vulgaris* nitrate reductase 3' UTR in constructs such as FATA1-CrbTub_yInv_nr::amt03_CwTE2_nr-FATA1, can be amplified upon integration in the host genome. There is good concordance between copy number of the amplified transgene at the target integration site and thioesterase levels as revealed either by impacts on fatty acid profiles or recombinant protein accumulation as assessed by western blotting.

[0402] Transgenic lines in which the *Cw*TE2 gene has undergone amplification show a marked increase in mid chain (C10:0-C14:0) fatty acids and a concurrent decrease in C18:1 fatty acids. In contrast, those transformants in which *Cw*TE2 has undergone little or no amplification (likely 1-2 copies) are consistent with lower expression of the exogenous thioesterase, resulting in a slight increase in mid chain fatty acids and a far greater impact on the increase of C18:1 fatty acids.

[0403] Collectively, these data show that ablation of the host's endogenous FATA1 allele alters the lipid profile of the engineered microalgae.

B. Altering lipid profiles by knockdown of an endogenous *Prototheca moriformis* thioesterase gene

[0404] A construct, pSZ1773, to down-regulate the *Prototheca moriformis* FATA1 gene expression by a hairpin RNA was introduced into a *Prototheca moriformis* UTEX 1435 S1920 genetic background. The *Saccharomyces cerevisiae suc2* sucrose invertase gene was utilized as a selectable marker, conferring the ability to grow on sucrose as a sole-carbon source. The portion of the construct that encodes the hairpin RNA utilized the first exon of FatA1 coding region, followed by the endogenous intron, and a repeat unit of the first exon in the reverse orientation. 5' and 3' homologous recombination targeting sequences (flanking the construct) to the 6S genomic region, listed as SEQ ID NO:100 and 101 respectively, were included for integration of the hairpin construct into the nuclear genome. This construct is designated 6S::β-Tub:suc2:nr:: β-tub:hairpinFatA:nr::6S.

[0405] Relevant restriction sites in 6S::β-Tub:suc2:nr:: β-tub:hairpin FatA:nr::6S are indicated in lowercase, bold and underlining in the sequence below and are 5'-3' *BspQ* 1, *Kpn* I, *Mfe* I, *BamH* I, *EcoR* I, *Spe* I, *Xho* I, *Sac* I, *BspQ* I, respectively. *BspQI* sites delimit the 5' and 3' ends of the transforming DNA. Bold, lowercase sequences represent genomic DNA from S1920 that permit targeted integration at 6s locus via homologous recombination. Proceeding in the 5' to 3' direction, the *C. reinhardtii* β -tubulin promoter driving the expression of the yeast sucrose invertase gene (conferring the ability of S1920 to metabolize sucrose) is indicated by boxed text. The initiator ATG and terminator TGA for invertase are indicated by uppercase, bold italics while the coding region is indicated in lowercase italics. The *Chlorella vulgaris* nitrate reductase 3' UTR is indicated by lowercase underlined text followed by the second *C. reinhardtii* β -tubulin promoter driving the expression of the Hairpin FatA1, indicated by boxed italics text. The initiator ATG codon of the FatA1 is indicated by uppercase, bold italics, while the remainder of the first exon of FatA1 coding

region is indicated by uppercase. The intron of the FatA gene is indicated as underlined uppercase, and a linker region shown in underlined uppercase, bold italics was created at the FatA1 intron/reversed first exon junction to aid in RNA splicing in these vectors. The inverted first exon of FatA1 is indicated by uppercase. The *C. vulgaris* nitrate reductase 3' UTR is again indicated by lowercase underlined text followed by the S1920 6S genomic region indicated by bold, lowercase text. The sequences of the FATA portions of this RNAi construct is listed as SEQ ID NO:110.

gctcttcgccgccgccactcctgctcgagcgcgcccgcgcgtgcgccgccagcgccttggccttttcgccgcgctcgtgcgcgtc
gctgatgtccatcaccaggtccatgaggtctgccttgcgccggctgagccactgcttcgtccgggcggccaagaggagcatga
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CACCGGTGCGTGGAGGTCCGGGGCTGACCGGCCGTCGCATTCAACGTAATCAAT
CGCATGATGATCAGAGGACACGAAGTCTTGGTGGCGGTGGCCAGAAACACTGTC
CATTGCAAGGGCATAGGGATGCGTTCCTTCACCTCTCATTTCTCATTTCTGAATCC
CTCCCTGCTCACTCTTTCTCCTCCTCCTTCCCGTTCACGCAG***CATTCGGGGCAACG***
AGGTGGGCCCGTGCTCCTCCAGGAAGATGCGCTTGTCCTCCGCCATCTTGCAGGG
CTCAAGCTGCTCCCAAAACTCTTGGGCGGGTTCCGGACGGACGGCTACCGCGGGT
GCGGCCCTGACCGCCACTGTTCGGAAGCAGCGGCGCTGCATGGGCAGCGGCCGC
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GCAGAGGACCACAGAGAAGCGGAAGAGACGCCAGTACTGGCAAGCAGGCTGGT
CGGTGCCAT**atcgat**agatctcttaaggcagcagcagctcggatagtatcgacacactctggacgctggtcgtgtgatggact
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[0406] Expression of 6S::β-Tub:suc2:nr:: β-tub:hairpin FatA:nr::6S leads to the formation of a hairpin RNA to silence the target FatA genes. Upon its transformation into S1920, primary transformants were clonally purified and grown under standard lipid production conditions at pH 5.0. The resulting profiles from representative transformant clones are listed in Table 31.

Table 31. Fatty acid profiles of *Prototheca moriformis* cells containing a hairpin RNA construct to down-regulate the expression of FATA.

Transformant	% C10:0	% C12:0	% C14:0	% C16:0	% C16:1	% C18:0	% C18:1	% C18:2
Wildtype	0.01	0.03	1.23	25.68	0.96	2.83	60.54	7.52
pSZ1773 Transformant 1	0.01	0.03	0.71	15.10	1.05	1.67	72.08	8.27
pSZ1773 Transformant 2	0.01	0.03	0.81	15.66	1.16	1.56	70.03	9.61
pSZ1773 Transformant 3	0.01	0.03	1.09	22.67	1.05	2.12	63.18	8.66
pSZ1773 Transformant 4	0.01	0.04	1.14	23.31	1.01	2.23	62.83	8.26

[0407] The above results show that the FATA hairpin construct yielded expected phenotypes: a reduction in C16 fatty acid levels and an increase in C18:1 fatty acid levels as compared to the wildtype, untransformed control.

[0408] 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.

[0409] All references cited herein, including patents, patent applications, and publications, including Genbank Accession numbers, 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: PCT Application No. PCT/US2009/066142, filed November 30, 2009, entitled "Production of Tailored Oils in Heterotrophic Microorganisms"; PCT Application No. PCT/US2009/066141, filed November 30, 2009, entitled "Production of Tailored Oils in Heterotrophic Microorganisms"; and PCT Application No. PCT/US2010/31108 filed April 14, 2010, entitled "Methods of Microbial Oil Extraction and Separation."

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[0410] The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgement or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

[0411] Comprises/comprising and grammatical variations thereof when used in this specification are to be taken to specify the presence of stated features, integers, steps or components or groups thereof, but do not preclude the presence or addition of one or more other features, integers, steps, components or groups thereof.

SEQUENCE LISTING

SEQ ID NO:1
HUP promoter from Chlorella (subsequence of GenBank accession number X55349)

gatcagacgggcctgacctgcgagataatcaagtgctcgtaggcaaccaactcagcagctgcttggtgttgggtctgcaggatagt
gttgcagggccccaaggacagcaggggaacttacaccttgtccccgacccagttttatggagtgcattgcctcaagagcctagccg
gagcgctaggctacatacttgccgcaccggtatgaggggatatagtactcgcactgcgctgtctagtgagatgggcagtgctgccc
ataaacaactggctgctcagccatttgttggcggaccattctgggggggccagcaatgcctgactttcgggtagggtgaaaactgaa
caaagactaccaaaacagaatttcttcctccttggaggtaagcgcaggccggcccgcctgcgcccacatggcgctccgaacacctc
catagctgtaagggcgcaaacatggccggactgttgtcagcactctttcatggccatacaaggtcatgtcgagattagtgctgagtaa
gacactatcaccccatgttcgattgaagccgtgacttcatgccaacctgcccctgggcgtagcagacgtatgccatcatgaccactag
ccgacatgcgctgtcttttgccaccaaaacaactggtacaccgctcgaagtcgtgccgcacacctccgggagtgagtccggcgact
cctccccggcgggccgcggccctacctgggtagggtcgccatacgcccacgaccaaacgacgcaggaggggattggggtagg
gaatcccaaccagcctaaccaagacggcacctataataataggtgggggactaacagccctatatcgcaagctttgggtgcctatc
ttgagaagcacgagttggagtggctgtgtacggtcgaccctaaggtgggtgtgccgcagcctgaaacaaagcgtctagcagctgct
tctataatgtgtcagccgttgtgtttcagttatattgtatgctattgtttgttcgtgctagggtggcgcaggcccacctactgtggcgggcc
attggttggtgcttgaattgcctcaccatctaaggtctgaacgctcactcaaacgcctttgtacaactgcagaactttccttggcgctgca
actacagtgtgcaaaccagcacatagcactcccttacatcacccagcagtacaaca

SEQ ID NO:2
Chlorella ellipsoidea nitrate reductase promoter from AY307383

cgctgcgcaccagggccgccagctcgctgatgtcgctccaaatgcggtcccccgatttttgttcttcatcttctccaccttggtggcct
tcttggccagggccttcagctgcatgcgcacagaccgttgagctcctgatcagcatcctcaggaggccctttgacaagcaagccccct
gtgcaagcccattcacggggtaccagtggtgctgaggtagatgggtttgaaaaggattgctcggtcgattgctgctcatggaattggc
atgtgcatgcatgttcacaatatgccaccaggctttggagcaagagagcatgaatgccttcaggcaggttgaaagttcctgggggtg
aagaggcagggccgaggattggaggaggaaagcatcaagtcgtcgctcatgctcatgttttcagtcagagtttgccaagctcacag
gagcagagacaagactggctgctcaggtgttgcatcgtgtgtgtggtgggggggggggggttaatacggtacgaaatgcacttgg
aattcccacctcatgccagcggacccacatgcttgaattcgaggcctgtggggtgagaaatgctcactctgccctcgttgctgaggta
cttcaggccgctgagctcaaagtcgatgccctgctcgtctatcagggcctgcacctctgggctgaccggctcagcctccttcgcggg
catggaggaggcgccggcagcgttcatgtccgggcccagggcagcgggtggtgccataaatgtcggtgatggtggggagggggg
ccgtcgccacaccattgccgttgctggctgacgcatgcacatgtggcctggctggcaccggcagcactggtctccagccagccag
caagtggctgttcaggaaagcggccatgttgttggtccctgcgcatgtaattccccagatcaaaggagggaacagcttggatttgatg
tagtgcccaaccggactgaatgtgcgatggcaggtccctttgagtctcccgaattactagcagggcactgtgacctaacgcagcatg
ccaaccgcaaaaaaatgattgacagaaaatgaagcggtgtgtcaatatttgctgtatttattcgttttaatcagcaaccaagttcgaaac
gcaactatcgtggtgatcaagtgaacctcatcagacttacctcgttcggcaaggaaacggaggcaccaaattccaatttgatattatcg
cttgccaagctagagctgatctttgggaaaccaactgccagacagtggactgtgatggagtgccccgagtggtggagcctcttcgat
tcggttagtcattactaacgtgaaccctcagtgaagggaccatcagaccagaaagaccagatctcctcctcgacaccgagagagtgt
tgcggcagtaggacgacaag

SEQ ID NO:3
Yeast sucrose invertase

MTNETSDRPLVHFTPNKGWMNDPNGLWYDEKDAKWHLYFQYNPNDTVWGTPLF
WGHATSDDLTNWEDQPIAIAPKRNDSGAFSGSMVVDYNNTSGFFNDTIDPRQRCV
AIWTYNTPESEEQYISYSLDGGYTFTEYQKNPVLAANSTQFRDPKVFWYEPSQKWI
MTAAKSQDYKIEIYSSDDLKSWKLESAFANEGFLGYQYECPGLIEVPTEQDPSKSY

WVMFISINPGAPAGGSFNQYFVGSFNGTHFEAFDNQSRVVDFGKDYYALQTFFNTD
PTYGSALGIAWASNWEYSAFVPTNPWRSSMSLVRKFSLNTEYQANPETELINLKAE
PILNISNAGPWSRFATNTTLTKANSYNVDLSNSTGTLEFELVYAVNTTQTISKSVFA
DLSLWFKGLEDPEEYLRMGFEVSASSFFLDRGNSKVKFVKENPYFTNRMSVNNQPF
KSENDLSYYKVYGLLDQNILELYFNDGDVVSTNTYFMTTGNALGSVNMTTGVDNL
FYIDKFQVREVK

SEQ ID NO:4
Yeast secretion signal

MLLQAFLFLLAGFAAKISAS

SEQ ID NO:5
Higher plants secretion signal

MANKSLLLLLLLGSLASG

SEQ ID NO:6
Consensus eukaryotic secretion signal

MARLPLAALG

SEQ ID NO:7
Combination higher plant/eukaryotic secretion signal

MANKLLLLLLLLLLPLAASG

SEQ ID NO:8
S. cerevisiae sucrose invertase NP_012104

GAATTCCCCAACATGGTGGAGCACGACACTCTCGTCTACTCCAAGAATATCAAA
GATACAGTCTCAGAAGACCAAAGGGCTATTGAGACTTTTCAACAAAGGGTAAT
ATCGGGAAACCTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTCATCAAAAG
GACAGTAGAAAAGGAAGGTGGCACCTACAAATGCCATCATTGCGATAAAGGAA
AGGCTATCGTTCAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCCCAC
CCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAA
GTGGATTGATGTGAACATGGTGGAGCACGACACTCTCGTCTACTCCAAGAATAT
CAAAGATACAGTCTCAGAAGACCAAAGGGCTATTGAGACTTTTCAACAAAGGG
TAATATCGGGAAACCTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTCATCAA
AAGGACAGTAGAAAAGGAAGGTGGCACCTACAAATGCCATCATTGCGATAAAG
GAAAGGCTATCGTTCAAGATGCCTCTGCCGACAGTGGTCCCAAAGATGGACCCC
CACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAG
CAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCAC
TATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGG
ACACGCTGAAATCACCAGTCTCTCTCTACAAATCTATCTCTGGCGCGCCATATC
AATGCTTCTTCAGGCCTTTCTTTTTCTTCTTGCTGGTTTTGCTGCCAAGATCAGCG
CCTCTATGACGAACGAAACCTCGGATAGACCACTTGTGCACTTTACACCAAACA
AGGGCTGGATGAATGACCCCAATGGACTGTGGTACGACGAAAAAGATGCCAAG
TGGCATCTGTACTTTCAATACAACCCGAACGATACTGTCTGGGGGACGCCATTG
TTTTGGGGCCACGCCACGTCCGACGACCTGACCAATTGGGAGGACCAACCAAT

AGCTATCGCTCCGAAGAGGAACGACTCCGGAGCATTCTCGGGTTCCATGGTGGT
TGACTACAACAATACTTCCGGCTTTTTCAACGATACCATTGACCCGAGACAACG
CTGCGTGGCCATATGGACTTACAACACACCGGAGTCCGAGGAGCAGTACATCTC
GTATAGCCTGGACGGTGGATACACTTTTACAGAGTATCAGAAGAACCCTGTGCT
TGCTGCAAATTCGACTCAGTTCCGAGATCCGAAGGTCTTTTGGTACGAGCCCTC
GCAGAAGTGGATCATGACAGCGGCAAAGTCACAGGACTACAAGATCGAAATTT
ACTCGTCTGACGACCTTAAATCCTGGAAGCTCGAATCCGCGTTCGCAAACGAGG
GCTTTCTCGGCTACCAATACGAATGCCCAGGCCTGATAGAGGTCCCAACAGAGC
AAGATCCCAGCAAGTCCTACTGGGTGATGTTTATTTCCATTAATCCAGGAGCAC
CGGCAGGAGGTTCTTTTAATCAGTACTTCGTCGGAAGCTTTAACGGAACTCATT
TCGAGGCATTTGATAACCAATCAAGAGTAGTTGATTTTGGAAAGGACTACTATG
CCCTGCAGACTTTCTTCAATACTGACCCGACCTATGGGAGCGCTCTTGGCATTG
CGTGGGCTTCTAACTGGGAGTATTCCGCATTCGTTCCTACAAACCCTTGGAGGT
CCTCCATGTCGCTCGTGAGGAAATTCTCTCTCAACACTGAGTACCAGGCCAACC
CGGAAACCGAACTCATAAACCTGAAAGCCGAACCGATCCTGAACATTAGCAAC
GCTGGCCCCTGGAGCCGGTTTGCAACCAACACCACGTTGACGAAAGCCAACAG
CTACAACGTCGATCTTTCGAATAGCACCGGTACACTTGAATTTGAACTGGTGTA
TGCCGTCAATACCACCCAAACGATCTCGAAGTCGGTGTTCGCGGACCTCTCCCT
CTGGTTTAAAGGCCTGGAAGACCCCGAGGAGTACCTCAGAATGGGTTTCGAGG
TTTCTGCGTCCTCCTTCTTCCTTGATCGCGGGAACAGCAAAGTAAAATTTGTTAA
GGAGAACCCATATTTTACCAACAGGATGAGCGTTAACAACCAACCATTCAAGA
GCGAAAACGACCTGTCGTACTACAAAGTGTATGGTTTGCTTGATCAAAATATCC
TGGAACTCTACTTCAACGATGGTGATGTCGTGTCCACCAACACATACTTCATGA
CAACCGGGAACGCACTGGGCTCCGTGAACATGACGACGGGTGTGGATAACCTG
TTCTACATCGACAAATTCCAGGTGAGGGAAGTCAAGTGAGATCTGTCGATCGAC
AAGCTCGAGTTTCTCCATAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAG
GGTTCCTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCTTAGTATGT
ATTTGTATTTGTAAAATACTTCTATCAATAAAATTTCTAATTCCTAAAACCAAAA
TCCAGTACTAAAATCCAGATCCCCCGAATTAA

SEQ ID NO:9

TGTTGAAGAATGAGCCGGCGAC

SEQ ID NO:10

CAGTGAGCTATTACGCACTC

SEQ ID NO:11

UTEX 329 *Prototheca kruegani*

TGTTGAAGAATGAGCCGGCGAGTTAAAAAGAGTGGCATGGTTAAAGAAAATAC
TCTGGAGCCATAGCGAAAGCAAGTTTAGTAAGCTTAGGTCATTCTTTTTAGACC
CGAAACCGAGTGATCTACCCATGATCAGGGTGAAGTGTTAGTAAAATAACATG
GAGGCCCGAACCGACTAATGTTGAAAAATTAGCGGATGAATTGTGGGTAGGGG
CGAAAAACCAATCGAACTCGGAGTTAGCTGGTTCTCCCCGAAATGCGTTTAGGC
GCAGCAGTAGCAGTACAAATAGAGGGGTAAAGCACTGTTTCTTTTGTGGGCTTC
GAAAGTTGTACCTCAAAGTGGCAAACTCTGAATACTCTATTTAGATATCTACTA

GTGAGACCTTGGGGGATAAGCTCCTTGGTCAAAAGGGAAACAGCCCAGATCAC
CAGTTAAGGCCCCAAAATGAAAATGATAGTGACTAAGGATGTGGGTATGTCAA
AACCTCCAGCAGGTTAGCTTAGAAGCAGCAATCCTTTCAAGAGTGCGTAATAGC
TCACTG

SEQ ID NO:12
UTEX 1440 *Prototheca wickerhamii*

TGTTGAAGAATGAGCCGGCGACTTAAAATAAATGGCAGGCTAAGAGATTTAAT
AACTCGAAACCTAAGCGAAAGCAAGTCTTAATAGGGCGTCAATTTAACAAAAC
TTTAAATAAATTATAAAGTCATTTATTTTAGACCCGAACCTGAGTGATCTAACC
ATGGTCAGGATGAAACTTGGGTGACACCAAGTGGAAGTCCGAACCGACCGATG
TTGAAAAATCGGCGGATGAACTGTGGTTAGTGGTGAAATACCAGTCGAACTCA
GAGCTAGCTGGTTCTCCCCGAAATGCGTTGAGGCGCAGCAATATATCTCGTCTA
TCTAGGGGTAAAGCACTGTTTCGGTGCGGGCTATGAAAATGGTACCAAATCGTG
GCAAACTCTGAATACTAGAAATGACGATATATTAGTGAGACTATGGGGGATAA
GCTCCATAGTCGAGAGGGAAACAGCCCAGACCACCAGTTAAGGCCCCAAAATG
ATAATGAAGTGGTAAAGGAGGTGAAAATGCAAATACAACCAGGAGGTTGGCTT
AGAAGCAGCCATCCTTTAAAGAGTGCGTAATAGCTCACTG

SEQ ID NO:13
UTEX 1442 *Prototheca stagnora*

TGTTGAAGAATGAGCCGGCGAGTTAAAAAAAATGGCATGGTTAAAGATATTTC
TCTGAAGCCATAGCGAAAGCAAGTTTTACAAGCTATAGTCATTTTTTTTAGACC
CGAAACCGAGTGATCTACCCATGATCAGGGTGAAGTGTTGGTCAAATAACATG
GAGGCCCGAACCGACTAATGGTGAAAAATTAGCGGATGAATTGTGGGTAGGGG
CGAAAAACCAATCGAACTCGGAGTTAGCTGGTTCTCCCCGAAATGCGTTTAGGC
GCAGCAGTAGCAACACAAATAGAGGGGTAAAGCACTGTTTCTTTTGTGGGCTTC
GAAAGTTGTACCTCAAAGTGGCAAACTCTGAATACTCTATTTAGATATCTACTA
GTGAGACCTTGGGGGATAAGCTCCTTGGTCAAAAGGGAAACAGCCCAGATCAC
CAGTTAAGGCCCCAAAATGAAAATGATAGTGACTAAGGACGTGAGTATGTCAA
AACCTCCAGCAGGTTAGCTTAGAAGCAGCAATCCTTTCAAGAGTGCGTAATAGC
TCACTG

SEQ ID NO:14
UTEX 288 *Prototheca moriformis*

TGTTGAAGAATGAGCCGGCGAGTTAAAAAGAGTGGCATGGTTAAAGATAATTC
TCTGGAGCCATAGCGAAAGCAAGTTTAACAAGCTAAAGTCACCCTTTTTAGACC
CGAAACCGAGTGATCTACCCATGATCAGGGTGAAGTGTTGGTAAAATAACATG
GAGGCCCGAACCGACTAATGGTGAAAAATTAGCGGATGAATTGTGGGTAGGGG
CGAAAAACCAATCGAACTCGGAGTTAGCTGGTTCTCCCCGAAATGCGTTTAGGC
GCAGCAGTAGCAACACAAATAGAGGGGTAAAGCACTGTTTCTTTTGTGGGCTTC
GAAAGTTGTACCTCAAAGTGGCAAACTCTGAATACTCTATTTAGATATCTACTA
GTGAGACCTTGGGGGATAAGCTCCTTGGTCAAAAGGGAAACAGCCCAGATCAC
CAGTTAAGGCCCCAAAATGAAAATGATAGTGACTAAGGATGTGGGTATGTTAA

AACCTCCAGCAGGTTAGCTTAGAAGCAGCAATCCTTTCAAGAGTGCGTAATAGC
TCACTG

SEQ ID NO:15

UTEX 1439, UTEX 1441, UTEX 1435, UTEX 1437 *Prototheca moriformis*

TGTTGAAGAATGAGCCGGCGACTTAAAATAAATGGCAGGCTAAGAGAATTAAT
AACTCGAAACCTAAGCGAAAGCAAGTCTTAATAGGGCGCTAATTTAACAAAAC
ATTAAATAAAATCTAAAGTCATTTATTTTAGACCCGAACCTGAGTGATCTAACC
ATGGTCAGGATGAAACTTGGGTGACACCAAGTGGAAGTCCGAACCGACCGATG
TTGAAAAATCGGCGGATGAACTGTGGTTAGTGGTGAAATACCAGTCGAACTCA
GAGCTAGCTGGTTCTCCCCGAAATGCGTTGAGGCGCAGCAATATATCTCGTCTA
TCTAGGGGTAAAGCACTGTTTCGGTGCGGGCTATGAAAATGGTACCAAATCGTG
GCAAACTCTGAATACTAGAAATGACGATATATTAGTGAGACTATGGGGGATAA
GCTCCATAGTCGAGAGGGAAACAGCCCAGACCACCAGTTAAGGCCCCAAAATG
ATAATGAAGTGGTAAAGGAGGTGAAAATGCAAATACAACCAGGAGGTTGGCTT
AGAAGCAGCCATCCTTTAAAGAGTGCGTAATAGCTCACTG

SEQ ID NO:16

UTEX 1533 *Prototheca wickerhamii*

TGTTGAAGAATGAGCCGTCGACTTAAAATAAATGGCAGGCTAAGAGAATTAAT
AACTCGAAACCTAAGCGAAAGCAAGTCTTAATAGGGCGCTAATTTAACAAAAC
ATTAAATAAAATCTAAAGTCATTTATTTTAGACCCGAACCTGAGTGATCTAACC
ATGGTCAGGATGAAACTTGGGTGACACCAAGTGGAAGTCCGAACCGACCGATG
TTGAAAAATCGGCGGATGAACTGTGGTTAGTGGTGAAATACCAGTCGAACTCA
GAGCTAGCTGGTTCTCCCCGAAATGCGTTGAGGCGCAGCAATATATCTCGTCTA
TCTAGGGGTAAAGCACTGTTTCGGTGCGGGCTATGAAAATGGTACCAAATCGTG
GCAAACTCTGAATACTAGAAATGACGATATATTAGTGAGACTATGGGGGATAA
GCTCCATAGTCGAGAGGGAAACAGCCCAGACCACCAGTTAAGGCCCCAAAATG
ATAATGAAGTGGTAAAGGAGGTGAAAATGCAAATACAACCAGGAGGTTGGCTT
AGAAGCAGCCATCCTTTAAAGAGTGCGTAATAGCTCACTG

SEQ ID NO:17

UTEX 1434 *Prototheca moriformis*

TGTTGAAGAATGAGCCGGCGAGTTAAAAAGAGTGGCGTGGTTAAAGAAAATTC
TCTGGAACCATAGCGAAAGCAAGTTTAACAAGCTTAAGTCACTTTTTTTAGACC
CGAAACCGAGTGATCTACCCATGATCAGGGTGAAGTGTTGGTAAAATAACATG
GAGGCCCGAACCGACTAATGGTGAAAAATTAGCGGATGAATTGTGGGTAGGGG
CGAAAAACCAATCGAACTCGGAGTTAGCTGGTTCTCCCCGAAATGCGTTTAGGC
GCAGCAGTAGCAACACAAATAGAGGGGTAAAGCACTGTTTCTTTTGTGGGCTCC
GAAAGTTGTACCTCAAAGTGGCAAACTCTGAATACTCTATTTAGATATCTACTA
GTGAGACCTTGGGGGATAAGCTCCTTGGTCGAAAGGGAAACAGCCCAGATCAC
CAGTTAAGGCCCCAAAATGAAAATGATAGTGACTAAGGATGTGAGTATGTCAA
AACCTCCAGCAGGTTAGCTTAGAAGCAGCAATCCTTTCAAGAGTGCGTAATAGC
TCACTG

SEQ ID NO:18
UTEX 1438 *Prototheca zopfii*

TGTTGAAGAATGAGCCGGCGAGTTAAAAAGAGTGGCATGGTTAAAGAAAATTC
TCTGGAGCCATAGCGAAAGCAAGTTTAACAAGCTTAAGTCACTTTTTTTAGACC
CGAAACCGAGTGATCTACCCATGATCAGGGTGAAGTGTTGGTAAAATAACATG
GAGGCCCGAACCGACTAATGGTGAAAAATTAGCGGATGAATTGTGGGTAGGGG
CGAAAAACCAATCGAACTCGGAGTTAGCTGGTTCTCCCCGAAATGCGTTTAGGC
GCAGCAGTAGCAACACAAATAGAGGGGTAAAGCACTGTTTCTTTCGTGGGCTTC
GAAAGTTGTACCTCAAAGTGGCAAACTCTGAATACTCTATTTAGATATCTACTA
GTGAGACCTTGGGGGATAAGCTCCTTGGTCAAAAGGGAAACAGCCCAGATCAC
CAGTTAAGGCCCCAAAATGAAAATGATAGTGACTAAGGATGTGAGTATGTCAA
AACCTCCAGCAGGTTAGCTTAGAAGCAGCAATCCTTTCAAGAGTGCGTAATAGC
TCACTG

SEQ ID NO:19
UTEX 1436 *Prototheca moriformis*

TGTTGAAGAATGAGCCGGCGACTTAGAAAAGGTGGCATGGTTAAGGAAATATT
CCGAAGCCGTAGCAAAAGCGAGTCTGAATAGGGCGATAAAATATATTAATATT
TAGAATCTAGTCATTTTTTCTAGACCCGAACCCGGGTGATCTAACCATGACCAG
GATGAAGCTTGGGTGATACCAAGTGAAGGTCCGAACCGACCGATGTTGAAAAA
TCGGCGGATGAGTTGTGGTTAGCGGTGAAATACCAGTCGAACCCGGAGCTAGC
TGGTTCTCCCCGAAATGCGTTGAGGCGCAGCAGTACATCTAGTCTATCTAGGGG
TAAAGCACTGTTTCGGTGCGGGCTGTGAGAACGGTACCAAATCGTGGCAAACTC
TGAATACTAGAAATGACGATGTAGTAGTGAGACTGTGGGGGATAAGCTCCATT
GTCAAGAGGGAAACAGCCCAGACCACCAGCTAAGGCCCCAAAATGGTAATGTA
GTGACAAAGGAGGTGAAAATGCAAATACAACCAGGAGGTTGGCTTAGAAGCAG
CCATCCTTTAAAGAGTGCGTAATAGCTCACTG

SEQ ID NO:20
Chicorium intybus invertase: Genbank Accession No. Y11124

MSNSSNASESLFPATSEQPYRTAFHFQPPQNWMNDPNGPMCYNGVYHLFYQYNPF
GPLWNLRMYWAHSVSHDLINWIHLDLAFAPTEPFDINGCLSGSATVLPGNKPIMLY
TGIDTENRQVQNLAVPKDLSDPYLREWVKHTGNPIISLPEEIQPDDFRDPTTTWLEE
DGTWRLLVGSQKDKTGIAFLYHSGDFVNWTKSDSPLHKVSGTGMWECVDFFPVW
VDSTNGVDTSIINPSNRVKHVLKLGIQDHGKDCYLIGKYSADKENYVPEDELTLSTL
RLDYGMYYASKSFFDPVKNRRIMTAWVNESDSEADVIARGWSGVQSFPRSLWLDK
NQKQLLQWPIEEIEMLHQNEVSFHNKKLDGGSSLEVLGITASQADVKISFKLANLEE
AEELDPSWVDPQLICSENDASKKGKFGPFGLLALASSDLREQTAIFFRVFRKNGRYV
VLMCSDQSRSSMKNGIEKRTYGAFVDIDPQQDEISLRTLIDHSIVESFGGRGKTCITT
RVYPTLAIGEQARLFAFNHGTESVEISELSAWSMKKAQMKVEEP

SEQ ID NO:21
Schizosaccharomyces pombe Invertase: Genbank Accession No. AB011433

MFLKYILASGICLVSLLSSTNAAPRHLYVKRYPVIYNASNITEVSNSTTVPPPPFVNT
TAPNGTCLGNYNEYLPSGYYNATDRPKIHFTPSSGFMNDPNGLVYTGGVYHMFFQ
YSPKTLTAGEVHWGHTVSKDLIHWENYPIAIYPDEHENGVLSLPFSGSAVVDVHNS
SGLFSNDTIPEERIVLIYTDHWTGVAERQAIAYTTDGGYTFKKYSGNPVLDINSLQF
RDPKVIWDFDANRWVMIVAMSQNYGIAFYSSYDLIHWTELSVFSTSGYLGLQYECP
GMARVPVEGTDEYKWVLFISINPGAPLGGSVVQYFVGDWNGTNFVPDDGQTRFVD
LGKDFYASALYHSSSANADVIGVGWASNWQYTNQAPTQVFRSAMTVARKFTLRD
VPQNPMTNLTSLIQTPLNVSLLRDETLFTAPVINSSSSLSGSPITLPSNTAFEFNVTLSI
NYTEGCTTGYCLGRIIIDSDDPYRLQSISVDVDFAASTLVINRAKAQMGWFNSLFTP
SFANDIYIYGNVTLYGIVDNGLLELYVNNGEKTYTNDFFFLQGATPGQISFAAFQGV
SFNNVTVTPLKTIWNC

SEQ ID NO:22
Picha anomala beta-fructofuranosidase (invertase): Genbank Accession No. X80640

MIQLSPLLLLPLFSVFNSIADASTEYLRPQIHLTPDQGWMNDPNGMFYDRKDKLWH
VYFQHNPDKKSIWATPVTWGHSTSKDLLTWDYHGNALEPENDDEGIFSGSVVVDR
NNTSGFFNDSTDPEQRIVAIYTNNAQLQTQEIAYSLDKGYSFIKYDQNPVINVNSSQ
QRDPKVLWHDESNQWIMVVAKTQEFKVQIYGSPDLKKWDLKSNFTSNGYLGFQY
ECPGLFKLPIENPLNDTVTSKWVLLLAINPGSPLGGSINEYFIGDFDGTTFHPDDGAT
RFMDIGKDFYAFQSFDNTEPEDGALGLAWASNWQYANTVPTENWRSSMSLVRNY
TLKYVDVNPENYGLTLIQKPVYDTKETRLNETLKTLETINEYEVNDLKLDKSSFVA
TDFNTERNATGVFEFDLKFTQTDLKMGYSNMTTQFGLYIHSQTVKGSQETLQLVFD
TLSTTWYIDRTTQHSFQRNSPVFTERISTYVEKIDTTDQGNVYTLYGVVDRNILELY
FNDGSIAMTNTFFFREGKIPTSFEVVCDSEKSFITIDELSVRELARK

SEQ ID NO:23
Debaryomyces occidentalis Invertase: Genbank Accession No. X17604

MVQVLSVLVIPLLTLFFGYVASSSIDLSVDTSEYNRPLIHFTPEKGWMNDPNGLFYD
KTAKLWHLYFQYNPNATAWGQPLYWGHATSNDLVHWDEHEIAIGPEHDNEGIFS
GSIVVDHNNTSGFFNSSIDPNQRIVAIYTNNIPDLQTQDIAFSLDGGYTFTKYENNPVI
DVSSNQFRDPKVFWHERFKSMDHGCSEIARVKIQIFGSANLKNWVLNSNFSSGYYG
NQYGMSRLIEVPIENSDKSKWVMFLAINPGSPLGGSINQYFVGDFDGFQFVPDDSQT
RFVDIGKDFYAFQTFSEVEHGVLGLAWASNWQYADQVPTNPWRSSTSLARNYTLR
YVIQMLKLTANIDKSVLPDSINVVDKLKKKNVKLTNKKPIKTNFKGSTGLFDFNITF
KVLNLNVSPGKTHFDILINSQELNSSVDSIKIGFDSSQSLFYIDRHIPNVEFPRKQFFT
DKLAAYLEPLDYDQDLRVFSLYGIVDKNIIELYFNDGTVAMTNTFFMGEGKYPHDI
QIVTDTEEPLFELESVIIRELNK

SEQ ID NO:24
Oryza sativa Invertase: Genbank Accession No. AF019113

MATSRLTPAYDLKNAAAAAVYTPLPEQPHSAEVEIRDRKPFKIISAIILSSLLLLALILV
AVNYQAPPSHSSGDNSQPAAVMPPSRGVSQGVSEKAFRGASGAGNGVSFAWSNL
MLSWQRTSYHFQPVKNWMNDPNGPLYYKGWYHLFYQYNPDSAVWGNITWGHA
VSTDLINWLHLPFAMVPDQWYDVNGVWTGSATILPDGRIVMLYTGDTDDYVQDQ
NLAFPANLSDPLLVDWVKYPNNPVIYPPPGIGVKDFRDPTTAGTAGMQNGQRLVTI

GSKVGKTGISLVYETTNFTTFKLLYGVLHAVPGTGMWECVDLYPVSTTGENGLDT
SVNGLGVKHVLKTSLDDDKHDYYALGTYDPVKNKWTPDNPDLDVGIGLRLDYGK
YYAARTFYDQNKQRRILWGWIGETDLEAVDLMKGWASLQAIPRTIVFDKKTGTNV
LQRPEEEVESWSSGDPITQRRIFEPGSVVPIHVSGATQLDITASFEVDETLLETTSESH
DAGYDCSNSGGAGTRGSLGPFGLLVVADEKLSELTPVYLYVAKGGDGKAKAHLC
AYQTRSSMASGVEKEVYGSAVPVLDGENYSARILIDHSIVESFAQAGRTCVRSRDY
PTKDIYGAARCFFFNNATEASVRASLKAWQMKSFIRPYPFIPDQKS

SEQ ID NO:25

Allium cepa Invertase: Genbank Accession No. AJ006067

MSSDDLESPPSSYLPIPPSDEFHDQPPPLRSWLRLLSIPLALMFLLFLATFLSNLESPPS
DSGLVSDPVTFDVNPAVVRRGKDAGVSDKTSGVDSGFVLDPVAVDANSVVVHRG
KDAGVSDKTSGVDSGLLKDSPLGPYPWTNQMLSWQRTGFHFQPVKNWMNDPNGP
LYYKGWYHFFYQYNPEGAVWGNIAWGHAVSRDLVHWTHLPLAMVPDQWYDIN
GVWTGSATILPDGQIVMLYTGATNESVQVQNLAVPADQSDTLLLRWKKSEANPIL
VPPPGIGDKDFRDPTTAWYEPSDDTWRIVIGSKDSSHSGIAIVYSTKDFINYKLIPGIL
HAVERVGMWECVDFYPVATADSSHANHGLDPSARPSPAVKHVLKASMDDDRHD
YYAIGTYDPAQNTWVPDDASVDVGIGLRYDWGKFYASKTFYDHAKKRRILWSWI
GETDSETADIAKGWASLQGVPRTVLLDVKTGSNLITWPVVEIESLRTRPRDFSGITV
DAGSTFKLDVGGAAQLDIEAEFKISSEELEAVKEADVSYNCSSSGGAAERGVLGPF
GLLVLANQDLTE
QTATYFYVSRGMDGGLNTHFCQDEKRSSKASDIVKRIVGHSVPVLDGESFALRILV
DHSIVESFAQGGRASATSRVYPTEAIYNNARVFVFNNATGAKVTAQSLKVWHMST
AINEIYDPATSVM

SEQ ID NO:26

Beta vulgaris subsp. vulgaris Invertase: Genbank Accession No. AJ278531

LFYQYNPNGVIWGPPVWGHSTSKDLVNWVPQPLTMEPEMAANINGSWSGSATILP
GNKPAILFTGLDPKYEQVQVLAYPKDTSDPNLKEWFLAPQNPVMFPTPQNQINATS
FRDPTTAWRLPDGVWRLLIGSKRGQRGLSLLFRSRDFVHWVQAKHPLYSDKLSGM
WECPDFFPVYANGDQMGVDTSIIGSHVKHVLKNSLDITKHDIYTIGDYNIKKDAYT
PDIGYMNDSSLRYDYGKYYASKTFFDDAKKERILLGWANESSSVEDDIKKGWSGIH
TIPRKIWLDKLGKQLIQWPIANIEKLRQKPVNIYRKVLKGGSQIEVSGITAAQADVEI
SFKIKDLKNVEKFDASWTSPQLLCSKKGASVKGGLGPFGLLTLASXGLEEYTAVFF
RIFKAYDNKFVVLMCSDQSRSSLNPTNDKTTYGTFVDVNPIREGLSLRVLIDHSVVE
SFGAKGKNVITARVYPTLAINEKAHLYVFNRGTSNVEITGLTAWSMKKANIA

SEQ ID NO:27

Bifidobacterium breve UCC2003 beta-fructofuranosidase (invertase): Genbank Accession No. AAT28190

MTDFTPETPVLTPIRDHAAELAKAEAGVAEMAAKRNNRWYPKYHIASNGGWINDP
NGLCFYKGRWHVFYQLHPYGTQWGPMHWGHVSSTDMLNWKREPIMFAPSLEQE
KDGVFSGSAVIDDNGDLRFYYTGHRWANGHDNTGGDWQVQMTALPDNDELTSA
TKQGMIIDCPTDKVDHHYRDPKVWKTGDTWYMTFGVSSEDKRGQMWLFSSKDM
VRWEYERVLFQHPDPDVFMLECPDFFPIKDKDGNEKWVIGFSAMGSKPSGFMNRN

VNNAGYMIGTWEPGGEFKPETEFRLWDCGHNYYAPQSFNVDGRQIVYGWMSPFV
QPIPMEDDGWCGQLTLPREITLDDDGDVVTAPVAEMEGLREDTLDHGSITLDMDG
EQVIADDAEAVEIEMTIDLAASTADRAGLKIHATEDGAYTYVAYDDQIGRVVVDR
QAMANGDHGYRAAPLTDAELASGKLDLRVFVDRGSVEVYVNGGHQVLSSYSYAS
EGPRAIKLVAEFGNLKVESLKLHHMKSIGLE

SEQ ID NO:28
Saccharomyces cerevisiae Invertase: Genbank Accession No. NP_012104

MLLQAFLFLLAGFAAKISASMTNETSDRPLVHFTPNKGWMNDPNGLWYDEKDAK
WHLYFQYNPNDTVWGTPLFWGHATSDDLTNWEDQPIAIAPKRNDSGAFSGSMVV
DYNNTSGFFNDTIDPRQRCVAIWTYNTPESEEQYISYSLDGGYTFTEYQKNPVLAA
NSTQFRDPKVFWYEPSQKWIMTAAKSQDYKIEIYSSDDLKSWKLESAFANEGFLGY
QYECPGLIEVPTEQDPSKSYWVMFISINPGAPAGGSFNQYFVGSFNGTHFEAFDNQS
RVVDFGKDYYALQTFFNTDPTYGSALGIAWASNWEYSAFVPTNPWRSSMSLVRKF
SLNTEYQANPETELINLKAEPILNISNAGPWSRFATNTTLTKANSYNVDLSNSTGTLE
FELVYAVNTTQTISKSVFADLSLWFKGLEDPEEYLRMGFEVSASSFFLDRGNSKVKF
VKENPYFTNRMSVNNQPFKSENDLSYYKVYGLLDQNILELYFNDGDVVSTNTYFM
TTGNALGSVNMTTGVDNLFYIDKFQVREVK

SEQ ID NO:29
Zymomonas mobilis Invertase A: Genbank Accession No. AY171597

MESPSYKNLIKAEDAQKKAGKRLLSSEWYPGFHVTPLTGWMNDPNGLIFFKGEYH
LFYQYYPFAPVWGPMHWGHAKSRDLVHWETLPVALAPGDLFDRDGCFSGCAVDN
NGVLTLIYTGHIVLSNDSPDAIREVQCMATSIDGIHFQKEGIVLEKAPMPQVAHFRD
PRVWKENDHWFMVVGYRTDDEKHQGIGHVALYRSENLKDWIFVKTLLGDNSQLP
LGKRAFMWECPDFFSLGNRSVLMFSPQGLKASGYKNRNLFQNGYILGKWQAPQFT
PETSFQELDYGHDFYAAQRFEAKDGRQILIAWFDMWENQKPSQRDGWAGCMTLP
RKLDLIDNKIVMTPVREMEILRQSEKIESVVTLSDAEHPFTMDSPLQEIELIFDLEKSS
AYQAGLALRCNGKGQETLLYIDRSQNRIILDRNRSGQNVKGIRSCPLPNTSKVRLHI
FLDRSSIEIFVGDDQTQGLYSISSRIFPDKDSLKGRLFAIEGYAVFDSFKRWTLQDAN
LAAFSSDAC

SEQ ID NO:30
5' donor DNA sequence of *Prototheca moriformis* delta 12 FAD knockout homologous recombination targeting construct

GCTCTTCGGGTTTGCTCACCCGCGAGGTCGACGCCCAGCATGGCTATCAAGACG
AACAGGCAGCCTGTGGAGAAGCCTCCGTTCACGATCGGGACGCTGCGCAAGGC
CATCCCCGCGCACTGTTTCGAGCGCTCGGCGCTTCGTAGCAGCATGTACCTGGC
CTTTGACATCGCGGTCATGTCCCTGCTCTACGTCGCGTCGACGTACATCGACCCT
GCGCCGGTGCCTACGTGGGTCAAGTATGGCGTCATGTGGCCGCTCTACTGGTTC
TTCCAGGTGTGTGTGAGGGTTGTGGTTGCCCGTATCGAGGTCCTGGTGGCGCGC
ATGGGGGAGAAGGCGCCTGTCCCGCTGACCCCCCCGGCTACCCTCCCGGCACCT
TCCAGGGCGCCTTCGGCACGGGTGTCTGGGTGTGCGCGCACGAGTGCGGCCACC
AGGCCTTTTCCTCCAGCCAGGCCATCAACGACGGCGTGGGCCTGGTGTTCCACA
GCCTGCTGCTGGTGCCCTACTACTCCTGGAAGCACTCGCACCGCCGCCACCACT

CCAACACGGGGTGCCTGGACAAGGACGAGGTGTTTGTGCCGCCGCACCGCGCA
GTGGCGCACGAGGGCCTGGAGTGGGAGGAGTGGCTGCCCATCCGCATGGGCAA
GGTGCTGGTCACCCTGACCCTGGGCTGGCCGCTGTACCTCATGTTCAACGTCGC
CTCGCGGCCGTACCCGCGCTTCGCCAACCACTTTGACCCGTGGTCGCCCATCTTC
AGCAAGCGCGAGGTACCCTTTCTTGCGCTATGACACTTCCAGCAAAAGGTAGGG
CGGGCTGCGAGACGGCTTCCCGGCGCTGCATGCAACACCGATGATGCTTCGACC
CCCCGAAGCTCCTTCGGGGCTGCATGGGCGCTCCGATGCCGCTCCAGGGCGAGC
GCTGTTTAAATAGCCAGGCCCCCGATTGCAAAGACATTATAGCGAGCTACCAAA
GCCATATTCAAACACCTAGATCACTACCACTTCTACACAGGCCACTCGAGCTTG
TGATCGCACTCCGCTAAGGGGGCGCCTCTTCCTCTTCGTTTCAGTCACAACCCGC
AAACGGCGCGCC

SEQ ID NO:31

3' donor DNA sequence of *Prototheca moriformis* delta 12 FAD knockout homologous recombination targeting construct

CAATTGGCAGCAGCAGCTCGGATAGTATCGACACACTCTGGACGCTGGTCGTGT
GATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCCGCTT
TTATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGCGAGTTGC
TAGCTGCTTGTGCTATTTGCGAATACCACCCCCAGCATCCCCTTCCCTCGTTTCA
TATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCTATCCCTCAGCGC
TGCTCCTGCTCCTGCTCACTGCCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGT
ATTCTCCTGGTACTGCAACCTGTAAACCAGCACTGCAATGCTGATGCACGGGAA
GTAGTGGGATGGGAACACAAATGGAGCATCGAGGTGGTCATCTCCGACCTCGC
GTTGGTGGCGGTGCTCAGCGGGCTCAGCGTGCTGGGCCGCACCATGGGCTGGG
CCTGGCTGGTCAAGACCTACGTGGTGCCCTACATGATCGTGAACATGTGGCTGG
TGCTCATCACGCTGCTCCAGCACACGCACCCGGCCCTGCCGCACTACTTCGAGA
AGGACTGGGACTGGCTACGCGGCGCCATGGCCACCGTCGACCGCTCCATGGGC
CCGCCCTTCATGGACAGCATCCTGCACCACATCTCCGACACCCACGTGCTGCAC
CACCTCTTCAGCACCATCCCGCACTACCACGCCGAGGAGGCCTCCGCCGCCATC
CGGCCCATCCTGGGCAAGTACTACCAATCCGACAGCCGCTGGGTCGGCCGCGCC
CTGTGGGAGGACTGGCGCGACTGCCGCTACGTCGTCCCCGACGCGCCCGAGGA
CGACTCCGCGCTCTGGTTCCACAAGTGAGCGCGCCTGCGCGAGGACGCAGAAC
AACGCTGCCGCCGTGTCTTTTGCACGCGCGACTCCGGCGCTTCGCTGGTGGCAC
CCCCATAAAGAAACCCTCAATTCTGTTTGTGGAAGACACGGTGTACCCCCACCC
ACCCACCTGCACCTCTATTATTGGTATTATTGACGCGGGAGTGGGCGTTGTACC
CTACAACGTAGCTTCTCTAGTTTTCAGCTGGCTCCCACCATTGTAAAGAGCCTCT
AGAGTCGACCTGCAGGCATGCAAGCTTGGCGTAATCATGGTCATAGCTGTTTCC
TGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCAT
AAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTT
GCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATG
AATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCC

SEQ ID NO:32

Prototheca moriformis delta 12 FAD knockout homologous recombination targeting construct

GCTCTTCGGGTTTGCTCACCCGCGAGGTCGACGCCCAGCATGGCTATCAAGACG
AACAGGCAGCCTGTGGAGAAGCCTCCGTTCACGATCGGGACGCTGCGCAAGGC
CATCCCCGCGCACTGTTTCGAGCGCTCGGCGCTTCGTAGCAGCATGTACCTGGC
CTTTGACATCGCGGTCATGTCCCTGCTCTACGTCGCGTCGACGTACATCGACCCT
GCGCCGGTGCCTACGTGGGTCAAGTATGGCGTCATGTGGCCGCTCTACTGGTTC
TTCCAGGTGTGTGTGAGGGTTGTGGTTGCCCGTATCGAGGTCCTGGTGGCGCGC
ATGGGGGAGAAGGCGCCTGTCCCGCTGACCCCCCCGGCTACCCTCCCGGCACCT
TCCAGGGCGCCTTCGGCACGGGTGTCTGGGTGTGCGCGCACGAGTGCGGCCACC
AGGCCTTTTCCTCCAGCCAGGCCATCAACGACGGCGTGGGCCTGGTGTTCCACA
GCCTGCTGCTGGTGCCCTACTACTCCTGGAAGCACTCGCACCGCCGCCACCACT
CCAACACGGGGTGCCTGGACAAGGACGAGGTGTTTGTGCCGCCGCACCGCGCA
GTGGCGCACGAGGGCCTGGAGTGGGAGGAGTGGCTGCCCATCCGCATGGGCAA
GGTGCTGGTCACCCTGACCCTGGGCTGGCCGCTGTACCTCATGTTCAACGTCGC
CTCGCGGCCGTACCCGCGCTTCGCCAACCACTTTGACCCGTGGTCGCCCATCTTC
AGCAAGCGCGAGGTACCCTTTCTTGCGCTATGACACTTCCAGCAAAAGGTAGGG
CGGGCTGCGAGACGGCTTCCCGGCGCTGCATGCAACACCGATGATGCTTCGACC
CCCCGAAGCTCCTTCGGGGCTGCATGGGCGCTCCGATGCCGCTCCAGGGCGAGC
GCTGTTTAAATAGCCAGGCCCCCGATTGCAAAGACATTATAGCGAGCTACCAAA
GCCATATTCAAACACCTAGATCACTACCACTTCTACACAGGCCACTCGAGCTTG
TGATCGCACTCCGCTAAGGGGGCGCCTCTTCCTCTTCGTTTCAGTCACAACCCGC
AAACGGCGCGCCATGCTGCTGCAGGCCTTCCTGTTCCTGCTGGCCGGCTTCGCC
GCCAAGATCAGCGCCTCCATGACGAACGAGACGTCCGACCGCCCCCTGGTGCA
CTTCACCCCCAACAAGGGCTGGATGAACGACCCCAACGGCCTGTGGTACGACG
AGAAGGACGCCAAGTGGCACCTGTACTTCCAGTACAACCCGAACGACACCGTC
TGGGGGACGCCCTTGTTCTGGGGCCACGCCACGTCCGACGACCTGACCAACTGG
GAGGACCAGCCCATCGCCATCGCCCCGAAGCGCAACGACTCCGGCGCCTTCTCC
GGCTCCATGGTGGTGGACTACAACAACACCTCCGGCTTCTTCAACGACACCATC
GACCCGCGCCAGCGCTGCGTGGCCATCTGGACCTACAACACCCCGGAGTCCGA
GGAGCAGTACATCTCCTACAGCCTGGACGGCGGCTACACCTTCACCGAGTACCA
GAAGAACCCCGTGCTGGCCGCCAACTCCACCCAGTTCCGCGACCCGAAGGTCTT
CTGGTACGAGCCCTCCCAGAAGTGGATCATGACCGCGGCCAAGTCCCAGGACT
ACAAGATCGAGATCTACTCCTCCGACGACCTGAAGTCCTGGAAGCTGGAGTCCG
CGTTCGCCAACGAGGGCTTCCTCGGCTACCAGTACGAGTGCCCCGGCCTGATCG
AGGTCCCCACCGAGCAGGACCCCAGCAAGTCCTACTGGGTGATGTTCATCTCCA
TCAACCCCGGCGCCCCGGCCGGCGGCTCCTTCAACCAGTACTTCGTCGGCAGCT
TCAACGGCACCCACTTCGAGGCCTTCGACAACCAGTCCCGCGTGGTGGACTTCG
GCAAGGACTACTACGCCCTGCAGACCTTCTTCAACACCGACCCGACCTACGGGA
GCGCCCTGGGCATCGCGTGGGCCTCCAACTGGGAGTACTCCGCCTTCGTGCCCA
CCAACCCCTGGCGCTCCTCCATGTCCCTCGTGCGCAAGTTCTCCCTCAACACCG
AGTACCAGGCCAACCCGGAGACGGAGCTGATCAACCTGAAGGCCGAGCCGATC
CTGAACATCAGCAACGCCGGCCCCTGGAGCCGGTTCGCCACCAACACCACGTTG
ACGAAGGCCAACAGCTACAACGTCGACCTGTCCAACAGCACCGGCACCCTGGA
GTTCGAGCTGGTGTACGCCGTCAACACCACCCAGACGATCTCCAAGTCCGTGTT
CGCGGACCTCTCCCTCTGGTTCAAGGGCCTGGAGGACCCCGAGGAGTACCTCCG
CATGGGCTTCGAGGTGTCCGCGTCCTCCTTCTTCCTGGACCGCGGGAACAGCAA
GGTGAAGTTCGTGAAGGAGAACCCCTACTTCACCAACCGCATGAGCGTGAACA
ACCAGCCCTTCAAGAGCGAGAACGACCTGTCCTACTACAAGGTGTACGGCTTGC
TGGACCAGAACATCCTGGAGCTGTACTTCAACGACGGCGACGTCGTGTCCACCA

ACACCTACTTCATGACCACCGGGAACGCCCTGGGCTCCGTGAACATGACGACG
GGGGTGGACAACCTGTTCTACATCGACAAGTTCCAGGTGCGCGAGGTCAAGTG
ACAATTGGCAGCAGCAGCTCGGATAGTATCGACACACTCTGGACGCTGGTCGTG
TGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCCGCT
TTTATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGCGAGTTG
CTAGCTGCTTGTGCTATTTGCGAATACCACCCCCAGCATCCCCTTCCCTCGTTTC
ATATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCTATCCCTCAGCG
CTGCTCCTGCTCCTGCTCACTGCCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTG
TATTCTCCTGGTACTGCAACCTGTAAACCAGCACTGCAATGCTGATGCACGGGA
AGTAGTGGGATGGGAACACAAATGGAGCATCGAGGTGGTCATCTCCGACCTCG
CGTTGGTGGCGGTGCTCAGCGGGCTCAGCGTGCTGGGCCGCACCATGGGCTGG
GCCTGGCTGGTCAAGACCTACGTGGTGCCCTACATGATCGTGAACATGTGGCTG
GTGCTCATCACGCTGCTCCAGCACACGCACCCGGCCCTGCCGCACTACTTCGAG
AAGGACTGGGACTGGCTACGCGGCGCCATGGCCACCGTCGACCGCTCCATGGG
CCCGCCCTTCATGGACAGCATCCTGCACCACATCTCCGACACCCACGTGCTGCA
CCACCTCTTCAGCACCATCCCGCACTACCACGCCGAGGAGGCCTCCGCCGCCAT
CCGGCCCATCCTGGGCAAGTACTACCAATCCGACAGCCGCTGGGTCGGCCGCGC
CCTGTGGGAGGACTGGCGCGACTGCCGCTACGTCGTCCCCGACGCGCCCGAGG
ACGACTCCGCGCTCTGGTTCCACAAGTGAGCGCGCCTGCGCGAGGACGCAGAA
CAACGCTGCCGCCGTGTCTTTTGCACGCGCGACTCCGGCGCTTCGCTGGTGGCA
CCCCCATAAAGAAACCCTCAATTCTGTTTGTGGAAGACACGGTGTACCCCCACC
CACCCACCTGCACCTCTATTATTGGTATTATTGACGCGGGAGTGGGCGTTGTAC
CCTACAACGTAGCTTCTCTAGTTTTCAGCTGGCTCCCACCATTGTAAAGAGCCTC
TAGAGTCGACCTGCAGGCATGCAAGCTTGGCGTAATCATGGTCATAGCTGTTTC
CTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCA
TAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGT
TGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAAT
GAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCC

SEQ ID NO:33

5' donor DNA sequence of *Prototheca moriformis* SAD2A knockout homologous recombination targeting construct

GCTCTTCCGCCTGGAGCTGGTGCAGAGCATGGGTCAGTTTGCGGAGGAGAGGG
TGCTCCCCGTGCTGCACCCCGTGGACAAGCTGTGGCAGCCGCAGGACTTCCTGC
CCGACCCCGAGTCGCCCGACTTCGAGGACCAGGTGGCGGAGCTGCGCGCGCGC
GCCAAGGACCTGCCCGACGAGTACTTTGTGGTGCTGGTGGGCGACATGATCACG
GAGGAGGCGCTGCCGACCTACATGGCCATGCTCAACACCTTGGACGGTGTGCG
CGACGACACGGGCGCGGCTGACCACCCGTGGGCGCGCTGGACGCGGCAGTGGG
TGGCCGAGGAGAACCGGCACGGCGACCTGCTGAACAAGTACTGTTGGCTGACG
GGGCGCGTCAACATGCGGGCCGTGGAGGTGACCATCAACAACCTGATCAAGAG
CGGCATGAACCCGCAGACGGACAACAACCCTTACTTGGGCTTCGTCTACACCTC
CTTCCAGGAGCGCGCCACCAAGTAGGTACC

SEQ ID NO:34

3' donor DNA sequence of *Prototheca moriformis* SAD2A knockout homologous recombination targeting construct

CAATTGGCAGCAGCAGCTCGGATAGTATCGACACACTCTGGACGCTGGTCGTGT
GATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCCGCTT
TTATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGCGAGTTGC
TAGCTGCTTGTGCTATTTGCGAATACCACCCCCAGCATCCCCTTCCCTCGTTTCA
TATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCTATCCCTCAGCGC
TGCTCCTGCTCCTGCTCACTGCCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGT
ATTCTCCTGGTACTGCAACCTGTAAACCAGCACTGCAATGCTGATGCACGGGAA
GTAGTGGGATGGGAACACAAATGGAAGGATCGTAGAGCTCCAGCCACGGCAAC
ACCGCGCGCCTGGCGGCCGAGCACGGCGACAAGGGCCTGAGCAAGATCTGCGG
GCTGATCGCCAGCGACGAGGGCCGGCACGAGATCGCCTACACGCGCATCGTGG
ACGAGTTCTTCCGCCTCGACCCCGAGGGCGCCGTCGCCGCCTACGCCAACATGA
TGCGCAAGCAGATCACCATGCCCGCGCACCTCATGGACGACATGGGCCACGGC
GAGGCCAACCCGGGCCGCAACCTCTTCGCCGACTTCTCCGCCGTCGCCGAGAAG
ATCGACGTCTACGACGCCGAGGACTACTGCCGCATCCTGGAGCACCTCAACGCG
CGCTGGAAGGTGGACGAGCGCCAGGTCAGCGGCCAGGCCGCCGCGGACCAGGA
GTACGTTCTGGGCCTGCCCCAGCGCTTCCGGAAACTCGCCGAGAAGACCGCCGC
CAAGCGCAAGCGCGTCGCGCGCAGGCCCGTCGCCTTCTCCTGGAGAGAAGAGC
CTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCGTAATCATGGTCATAGCTGT
TTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAA
GCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTG
CGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATT
AATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCC

SEQ ID NO:35

Prototheca moriformis SAD2A knockout homologous recombination targeting construct

GCTCTTCCGCCTGGAGCTGGTGCAGAGCATGGGTCAGTTTGCGGAGGAGAGGG
TGCTCCCCGTGCTGCACCCCGTGGACAAGCTGTGGCAGCCGCAGGACTTCCTGC
CCGACCCCGAGTCGCCCGACTTCGAGGACCAGGTGGCGGAGCTGCGCGCGCGC
GCCAAGGACCTGCCCGACGAGTACTTTGTGGTGCTGGTGGGCGACATGATCACG
GAGGAGGCGCTGCCGACCTACATGGCCATGCTCAACACCTTGGACGGTGTGCG
CGACGACACGGGCGCGGCTGACCACCCGTGGGCGCGCTGGACGCGGCAGTGGG
TGGCCGAGGAGAACCGGCACGGCGACCTGCTGAACAAGTACTGTTGGCTGACG
GGGCGCGTCAACATGCGGGCCGTGGAGGTGACCATCAACAACCTGATCAAGAG
CGGCATGAACCCGCAGACGGACAACAACCCTTACTTGGGCTTCGTCTACACCTC
CTTCCAGGAGCGCGCCACCAAGTAGGTACCCTTTCTTGCGCTATGACACTTCCA
GCAAAAGGTAGGGCGGGCTGCGAGACGGCTTCCCGGCGCTGCATGCAACACCG
ATGATGCTTCGACCCCCCGAAGCTCCTTCGGGGCTGCATGGGCGCTCCGATGCC
GCTCCAGGGCGAGCGCTGTTTAAATAGCCAGGCCCCCGATTGCAAAGACATTAT
AGCGAGCTACCAAAGCCATATTCAAACACCTAGATCACTACCACTTCTACACAG
GCCACTCGAGCTTGTGATCGCACTCCGCTAAGGGGGCGCCTCTTCCTCTTCGTTT
CAGTCACAACCCGCAAACGGCGCGCCATGCTGCTGCAGGCCTTCCTGTTCCTGC
TGGCCGGCTTCGCCGCCAAGATCAGCGCCTCCATGACGAACGAGACGTCCGAC
CGCCCCCTGGTGCACTTCACCCCCAACAAGGGCTGGATGAACGACCCCAACGG
CCTGTGGTACGACGAGAAGGACGCCAAGTGGCACCTGTACTTCCAGTACAACC
CGAACGACACCGTCTGGGGGACGCCCTTGTTCTGGGGCCACGCCACGTCCGACG
ACCTGACCAACTGGGAGGACCAGCCCATCGCCATCGCCCCGAAGCGCAACGAC
TCCGGCGCCTTCTCCGGCTCCATGGTGGTGGACTACAACAACACCTCCGGCTTC

TTCAACGACACCATCGACCCGCGCCAGCGCTGCGTGGCCATCTGGACCTACAAC
ACCCCGGAGTCCGAGGAGCAGTACATCTCCTACAGCCTGGACGGCGGCTACAC
CTTCACCGAGTACCAGAAGAACCCCGTGCTGGCCGCCAACTCCACCCAGTTCCG
CGACCCGAAGGTCTTCTGGTACGAGCCCTCCCAGAAGTGGATCATGACCGCGGC
CAAGTCCCAGGACTACAAGATCGAGATCTACTCCTCCGACGACCTGAAGTCCTG
GAAGCTGGAGTCCGCGTTCGCCAACGAGGGCTTCCTCGGCTACCAGTACGAGTG
CCCCGGCCTGATCGAGGTCCCCACCGAGCAGGACCCCAGCAAGTCCTACTGGGT
GATGTTCATCTCCATCAACCCCGGCGCCCCGGCCGGCGGCTCCTTCAACCAGTA
CTTCGTCGGCAGCTTCAACGGCACCCACTTCGAGGCCTTCGACAACCAGTCCCG
CGTGGTGGACTTCGGCAAGGACTACTACGCCCTGCAGACCTTCTTCAACACCGA
CCCGACCTACGGGAGCGCCCTGGGCATCGCGTGGGCCTCCAACTGGGAGTACTC
CGCCTTCGTGCCCACCAACCCCTGGCGCTCCTCCATGTCCCTCGTGCGCAAGTTC
TCCCTCAACACCGAGTACCAGGCCAACCCGGAGACGGAGCTGATCAACCTGAA
GGCCGAGCCGATCCTGAACATCAGCAACGCCGGCCCCTGGAGCCGGTTCGCCA
CCAACACCACGTTGACGAAGGCCAACAGCTACAACGTCGACCTGTCCAACAGC
ACCGGCACCCTGGAGTTCGAGCTGGTGTACGCCGTCAACACCACCCAGACGATC
TCCAAGTCCGTGTTCGCGGACCTCTCCCTCTGGTTCAAGGGCCTGGAGGACCCC
GAGGAGTACCTCCGCATGGGCTTCGAGGTGTCCGCGTCCTCCTTCTTCCTGGAC
CGCGGGAACAGCAAGGTGAAGTTCGTGAAGGAGAACCCCTACTTCACCAACCG
CATGAGCGTGAACAACCAGCCCTTCAAGAGCGAGAACGACCTGTCCTACTACA
AGGTGTACGGCTTGCTGGACCAGAACATCCTGGAGCTGTACTTCAACGACGGCG
ACGTCGTGTCCACCAACACCTACTTCATGACCACCGGGAACGCCCTGGGCTCCG
TGAACATGACGACGGGGGTGGACAACCTGTTCTACATCGACAAGTTCCAGGTG
CGCGAGGTCAAGTGACAATTGGCAGCAGCAGCTCGGATAGTATCGACACACTC
TGGACGCTGGTCGTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGT
GAATATCCCTGCCGCTTTTATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGTAC
GCGCTTTTGCGAGTTGCTAGCTGCTTGTGCTATTTGCGAATACCACCCCCAGCAT
CCCCTTCCCTCGTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTC
CTGCTATCCCTCAGCGCTGCTCCTGCTCCTGCTCACTGCCCCTCGCACAGCCTTG
GTTTGGGCTCCGCCTGTATTCTCCTGGTACTGCAACCTGTAAACCAGCACTGCA
ATGCTGATGCACGGGAAGTAGTGGGATGGGAACACAAATGGAAGGATCGTAGA
GCTCCAGCCACGGCAACACCGCGCGCCTGGCGGCCGAGCACGGCGACAAGGGC
CTGAGCAAGATCTGCGGGCTGATCGCCAGCGACGAGGGCCGGCACGAGATCGC
CTACACGCGCATCGTGGACGAGTTCTTCCGCCTCGACCCCGAGGGCGCCGTCGC
CGCCTACGCCAACATGATGCGCAAGCAGATCACCATGCCCGCGCACCTCATGG
ACGACATGGGCCACGGCGAGGCCAACCCGGGCCGCAACCTCTTCGCCGACTTCT
CCGCCGTCGCCGAGAAGATCGACGTCTACGACGCCGAGGACTACTGCCGCATC
CTGGAGCACCTCAACGCGCGCTGGAAGGTGGACGAGCGCCAGGTCAGCGGCCA
GGCCGCCGCGGACCAGGAGTACGTTCTGGGCCTGCCCCAGCGCTTCCGGAAACT
CGCCGAGAAGACCGCCGCCAAGCGCAAGCGCGTCGCGCGCAGGCCCGTCGCCT
TCTCCTGGAGAGAAGAGCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCGT
AATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACA
CAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGA
GCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACC
TGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTG
CGTATTGGGCGCTCTTCC

SEQ ID NO:36

5' donor DNA sequence of *Prototheca moriformis* SAD2B knockout homologous recombination targeting construct

GCTCTTCCCGCCTGGAGCTGGTGCAGAGCATGGGGCAGTTTGCGGAGGAGAGG
GTGCTCCCCGTGCTGCACCCCGTGGACAAGCTGTGGCAGCCGCAGGACTTCCTG
CCCGACCCCGAGTCGCCCGACTTCGAGGACCAGGTGGCGGAGCTGCGCGCGCG
CGCCAAGGACCTGCCCGACGAGTACTTTGTGGTGCTGGTGGGCGACATGATCAC
GGAGGAGGCGCTGCCGACCTACATGGCCATGCTCAACACCTTGGACGGTGTGC
GCGACGACACGGGCGCGGCTGACCACCCGTGGGCGCGCTGGACGCGGCAGTGG
GTGGCCGAGGAGAACCGGCACGGCGACCTGCTGAACAAGTACTGTTGGCTGAC
GGGGCGCGTCAACATGCGGGCCGTGGAGGTGACCATCAACAACCTGATCAAGA
GCGGCATGAACCCGCAGACGGACAACAACCCTTACTTGGGCTTCGTCTACACCT
CCTTCCAGGAGCGCGCCACCAAGTAGGTACC

SEQ ID NO:37

3' donor DNA sequence of *Prototheca moriformis* SAD2B knockout homologous recombination targeting construct

CAGCCACGGCAACACCGCGCGCCTTGCGGCCGAGCACGGCGACAAGAACCTGA
GCAAGATCTGCGGGCTGATCGCCAGCGACGAGGGCCGGCACGAGATCGCCTAC
ACGCGCATCGTGGACGAGTTCTTCCGCCTCGACCCCGAGGGCGCCGTCGCCGCC
TACGCCAACATGATGCGCAAGCAGATCACCATGCCCGCGCACCTCATGGACGA
CATGGGCCACGGCGAGGCCAACCCGGGCCGCAACCTCTTCGCCGACTTCTCCGC
GGTCGCCGAGAAGATCGACGTCTACGACGCCGAGGACTACTGCCGCATCCTGG
AGCACCTCAACGCGCGCTGGAAGGTGGACGAGCGCCAGGTCAGCGGCCAGGCC
GCCGCGGACCAGGAGTACGTCCTGGGCCTGCCCCAGCGCTTCCGGAAACTCGCC
GAGAAGACCGCCGCCAAGCGCAAGCGCGTCGCGCGCAGGCCCGTCGCCTTCTC
CTGGAGAAGAGCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCGTAATCA
TGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACA
TACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAA
CTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGT
GCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATT
GGGCGCTCTTCC

SEQ ID NO:38

Prototheca moriformis SAD2B knockout homologous recombination targeting construct

GCTCTTCCCGCCTGGAGCTGGTGCAGAGCATGGGGCAGTTTGCGGAGGAGAGG
GTGCTCCCCGTGCTGCACCCCGTGGACAAGCTGTGGCAGCCGCAGGACTTCCTG
CCCGACCCCGAGTCGCCCGACTTCGAGGACCAGGTGGCGGAGCTGCGCGCGCG
CGCCAAGGACCTGCCCGACGAGTACTTTGTGGTGCTGGTGGGCGACATGATCAC
GGAGGAGGCGCTGCCGACCTACATGGCCATGCTCAACACCTTGGACGGTGTGC
GCGACGACACGGGCGCGGCTGACCACCCGTGGGCGCGCTGGACGCGGCAGTGG
GTGGCCGAGGAGAACCGGCACGGCGACCTGCTGAACAAGTACTGTTGGCTGAC
GGGGCGCGTCAACATGCGGGCCGTGGAGGTGACCATCAACAACCTGATCAAGA
GCGGCATGAACCCGCAGACGGACAACAACCCTTACTTGGGCTTCGTCTACACCT
CCTTCCAGGAGCGCGCCACCAAGTAGGTACCCTTTCTTGCGCTATGACACTTCC
AGCAAAAGGTAGGGCGGGCTGCGAGACGGCTTCCCGGCGCTGCATGCAACACC

GATGATGCTTCGACCCCCCGAAGCTCCTTCGGGGCTGCATGGGCGCTCCGATGC
CGCTCCAGGGCGAGCGCTGTTTAAATAGCCAGGCCCCCGATTGCAAAGACATTA
TAGCGAGCTACCAAAGCCATATTCAAACACCTAGATCACTACCACTTCTACACA
GGCCACTCGAGCTTGTGATCGCACTCCGCTAAGGGGGCGCCTCTTCCTCTTCGTT
TCAGTCACAACCCGCAAACGGCGCGCCATGCTGCTGCAGGCCTTCCTGTTCCTG
CTGGCCGGCTTCGCCGCCAAGATCAGCGCCTCCATGACGAACGAGACGTCCGA
CCGCCCCCTGGTGCACTTCACCCCCAACAAGGGCTGGATGAACGACCCCAACG
GCCTGTGGTACGACGAGAAGGACGCCAAGTGGCACCTGTACTTCCAGTACAAC
CCGAACGACACCGTCTGGGGGACGCCCTTGTTCTGGGGCCACGCCACGTCCGAC
GACCTGACCAACTGGGAGGACCAGCCCATCGCCATCGCCCCGAAGCGCAACGA
CTCCGGCGCCTTCTCCGGCTCCATGGTGGTGGACTACAACAACACCTCCGGCTT
CTTCAACGACACCATCGACCCGCGCCAGCGCTGCGTGGCCATCTGGACCTACAA
CACCCCGGAGTCCGAGGAGCAGTACATCTCCTACAGCCTGGACGGCGGCTACA
CCTTCACCGAGTACCAGAAGAACCCCGTGCTGGCCGCCAACTCCACCCAGTTCC
GCGACCCGAAGGTCTTCTGGTACGAGCCCTCCCAGAAGTGGATCATGACCGCG
GCCAAGTCCCAGGACTACAAGATCGAGATCTACTCCTCCGACGACCTGAAGTCC
TGGAAGCTGGAGTCCGCGTTCGCCAACGAGGGCTTCCTCGGCTACCAGTACGAG
TGCCCCGGCCTGATCGAGGTCCCCACCGAGCAGGACCCCAGCAAGTCCTACTGG
GTGATGTTCATCTCCATCAACCCCGGCGCCCCGGCCGGCGGCTCCTTCAACCAG
TACTTCGTCGGCAGCTTCAACGGCACCCACTTCGAGGCCTTCGACAACCAGTCC
CGCGTGGTGGACTTCGGCAAGGACTACTACGCCCTGCAGACCTTCTTCAACACC
GACCCGACCTACGGGAGCGCCCTGGGCATCGCGTGGGCCTCCAACTGGGAGTA
CTCCGCCTTCGTGCCCACCAACCCCTGGCGCTCCTCCATGTCCCTCGTGCGCAAG
TTCTCCCTCAACACCGAGTACCAGGCCAACCCGGAGACGGAGCTGATCAACCTG
AAGGCCGAGCCGATCCTGAACATCAGCAACGCCGGCCCCTGGAGCCGGTTCGC
CACCAACACCACGTTGACGAAGGCCAACAGCTACAACGTCGACCTGTCCAACA
GCACCGGCACCCTGGAGTTCGAGCTGGTGTACGCCGTCAACACCACCCAGACG
ATCTCCAAGTCCGTGTTCGCGGACCTCTCCCTCTGGTTCAAGGGCCTGGAGGAC
CCCGAGGAGTACCTCCGCATGGGCTTCGAGGTGTCCGCGTCCTCCTTCTTCCTG
GACCGCGGGAACAGCAAGGTGAAGTTCGTGAAGGAGAACCCCTACTTCACCAA
CCGCATGAGCGTGAACAACCAGCCCTTCAAGAGCGAGAACGACCTGTCCTACT
ACAAGGTGTACGGCTTGCTGGACCAGAACATCCTGGAGCTGTACTTCAACGACG
GCGACGTCGTGTCCACCAACACCTACTTCATGACCACCGGGAACGCCCTGGGCT
CCGTGAACATGACGACGGGGGTGGACAACCTGTTCTACATCGACAAGTTCCAG
GTGCGCGAGGTCAAGTGACAATTGGCAGCAGCAGCTCGGATAGTATCGACACA
CTCTGGACGCTGGTCGTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCT
GTGAATATCCCTGCCGCTTTTATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGT
ACGCGCTTTTGCGAGTTGCTAGCTGCTTGTGCTATTTGCGAATACCACCCCCAGC
ATCCCCTTCCCTCGTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTG
TCCTGCTATCCCTCAGCGCTGCTCCTGCTCCTGCTCACTGCCCCTCGCACAGCCT
TGGTTTGGGCTCCGCCTGTATTCTCCTGGTACTGCAACCTGTAAACCAGCACTGC
AATGCTGATGCACGGGAAGTAGTGGGATGGGAACACAAATGGACAGCCACGGC
AACACCGCGCGCCTTGCGGCCGAGCACGGCGACAAGAACCTGAGCAAGATCTG
CGGGCTGATCGCCAGCGACGAGGGCCGGCACGAGATCGCCTACACGCGCATCG
TGGACGAGTTCTTCCGCCTCGACCCCGAGGGCGCCGTCGCCGCCTACGCCAACA
TGATGCGCAAGCAGATCACCATGCCCGCGCACCTCATGGACGACATGGGCCAC
GGCGAGGCCAACCCGGGCCGCAACCTCTTCGCCGACTTCTCCGCGGTCGCCGAG
AAGATCGACGTCTACGACGCCGAGGACTACTGCCGCATCCTGGAGCACCTCAA

CGCGCGCTGGAAGGTGGACGAGCGCCAGGTCAGCGGCCAGGCCGCCGCGGACC
AGGAGTACGTCCTGGGCCTGCCCCAGCGCTTCCGGAAACTCGCCGAGAAGACC
GCCGCCAAGCGCAAGCGCGTCGCGCGCAGGCCCGTCGCCTTCTCCTGGAGAAG
AGCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGCGTAATCATGGTCATAGC
TGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCG
GAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTA
ATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTG
CATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCT
TCC

SEQ ID NO:39
Forward primer SZ5434

GTCCCTGCCCTTTGTACACAC

SEQ ID NO:40
Reverse primer SZ5435

TTGATATGCTTAAGTTCAGCGGG

SEQ ID NO:41
Rhodotorula glutinis DSMZ-DSM 70398 and *Lipomyces tetrasporus* CBS 5911

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCCGGATTGGCTATT
GGGAGCTCGCGAGAGCACCTGACTGCCGAGAAGTTGTACGAACTTGGTCATTTA
GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTAGTGAATATTAGGGTGTCCAACTTAACTTGGAGCCCGACCCTCACTTTCTAA
CCCTGTGCATTTGTCTTGGGTAGTAGCTTGCGTCAGCGAGCGAATCCCATTTCAC
TTACAAACACAAAGTCTATGAATGTAACAAATTTATAACAAAACAAAACTTTCA
ACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAC
GTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCG
CTCCATGGTATTCCGTGGAGCATGCCTGTTTGAGTGTCATGAATTCTTCAACCCA
CCTCTTTCTTAGTGAATCAGGCGGTGTTTGGATTCTGAGCGCTGCTGGCTTCGCG
GCCTAGCTCGCTCGTAATGCATTAGCATCCGCAATCGAACTTCGGATTGACTCG
GCGTAATAGACTATTCGTTGAGGATTCTGGTCTCTGACTGGAGCCGGGTAAGGT
TAAAGGGAGCTACTAATCCTCATGTCTATCTTGAGATTAGACCTCAAATCAGGT
AGGACTA

SEQ ID NO:42
Rhodotorula glutinis var. glutinis CBS 3044 and *Lipomyces tetrasporus* CBS 8664

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCCGGATTGGCTATT
GGGAGCTCGCGAGAGCACCCGACTGCCGAGAAGTTGTACGAACTTGGTCATTT
AGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC
ATTAGTGAATATTAGGGCGTCCAACTTAACTTGGAGCCCGAACTCTCACTTTCT
AACCCTGTGCATCTGTTTCTGGTCAGTAGCTCTCTCGGGAGTGAACGCCATTCA
CTTAAAACACAAAGTCTATGAATGTATAAAATTTATAACAAAACAAAACTTTCA
ACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAA

GTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCG
CTCTCTGGTATTCCGGAGAGCATGCCTGTTTGAGTGTCATGAAATCTTCAACCCT
CTCTTTTCTTAATGAATCGAGAGGTGCTTGGATCCTGAGCGCTGCTGGCTTCGGC
CTAGCTCGTTCGTAATGCATTAGCATCCGCAATCGAACTTCGGATTGACTTGGC
GTAATAGACTATTCGCTGAGGATTCTGGTCTCGTACCAGAGCCGGGTTGGGTTA
AAGGAAGCTTCTAATCCTAAAAGTCTAACTTTTGATTAGATCTCAAATCAGGTA
GGACTA

SEQ ID NO:43
Lipomyces tetrasporus CBS 1808 and *Lipomyces tetrasporus* CBS 1810

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTTCGGACTGGCTCCA
GAAAATGGGAAACCATTATCAGGAGCTGGAAAGTTGGTCAAACTTGGTCATTT
AGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC
ATTACTGAGTATTTGTCTTTTAAAGACATCTCTCTATCCATAAACTCTTTTTTCTA
AAAAGACATGATTTACACAATTAGTCTGAATGATTATATAAAAATCTTCAAAAC
TTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCAAAATGCG
ATAAGTATTGTGAATTGCAGGATTTTGTGAATCATCGAATTTTTGAACGCACAT
TGCACCTTCTGGTATTCCGGAGGGTATACCTGTTTGAGCGTCATTTATATACTCA
AAACTTTGTTTTGGTGATGGGCACATATCTGGTGAGAGCTAGATTTGCCTGAAA
TATAGTGGTAGAGATTGCTACGAGTTATGCAAGTTAGCCAATGCTATTAAGTTA
ATTCGTTGGTGAAGCATGCGGAGCTTTAGCGGTCGCCTTCCTTAACTATTGGAA
TTTTTCTAATTTTGACCTCAAATCAGGCAGGAGTA

SEQ ID NO:44
Lipomyces starkeyi CBS 1809 and *Trichosporon montevideense* CBS 8261

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGACCCTCGGATTGGCGTTA
GGAAGCCGGCAACGGCATCCTTTGGCCGAGAAGTTGGTCAAACTTGGTCATTTA
GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTAGTGATTGCCTTTATAGGCTTATAACTATATCCACTTACACCTGTGAACTGTT
CTATTACTTGACGCAAGTCGAGTATTTTTACAAACAATGTGTAATGAACGTCGT
TTTATTATAACAAAATAAAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGA
TGAAGAACGCAGCGAATTGCGATAAGTAATGTGAATTGCAGAATTCAGTGAAT
CATCGAATCTTTGAACGCAGCTTGCGCTCTCTGGTATTCCGGAGAGCATGCCTG
TTTCAGTGTCATGAAATCTCAACCACTAGGGTTTCCTAATGGATTGGATTTGGG
CGTCTGCGATCTCTGATCGCTCGCCTTAAAAGAGTTAGCAAGTTTGACATTAAT
GTCTGGTGTAATAAGTTTCACTGGGTCCATTGTGTTGAAGCGTGCTTCTAATCGT
CCGCAAGGACAATTACTTTGACTCTGGCCTGAAATCAGGTAGGACTA

SEQ ID NO:45
Yarrowia lipolytica CBS 6331

CGCCCGTCGCTACTACCGATTGAATGGTTTAGTGAGACCTTGGGAGGGCGAGAT
GAGGGGGGCAACCCCTTTTGAACATCCAAACTTGGTCAAACTTGATTATTTAGA
GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATT
ATTGATTTTATCTATTTCTGTGGATTTCTGGTATATTACAGCGTCATTTTATCTCA
ATTATAACTATCAACAACGGATCTCTTGGCTCTCACATCGATGAAGAACGCAGC

GAACCGCGATATTTTTTGTGACTTGCAGATGTGAATCATCAATCTTTGAACGCA
CATTGCGCGGTATGGCATTCCGTACCGCACGGATGGAGGAGCGTGTTCCCTCTG
GGATCGCATTGCTTTCTTGAAATGGATTTTTTAAACTCTCAATTATTACGTCATT
TCACCTCCTTCATCCGAGATTA

SEQ ID NO:46

Cryptococcus curvatus CBS 5324, *Rhodotorula mucilaginosa var. mucilaginosa* CBS 316, *Cryptococcus curvatus* CBS 570, *Cryptococcus curvatus* CBS 2176, *Cryptococcus curvatus* CBS 2744, *Cryptococcus curvatus* CBS 2754, *Cryptococcus curvatus* CBS 2829, *Cryptococcus curvatus* CBS 5163, and *Cryptococcus curvatus* CBS 5358

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGATTTCTGGATTGGCGTTA
GGAAGCCGGCAACGGCATCCTTTGGCTGAGAAGTTACTCAAACTTGGTCATTTA
GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTAGTGATTTGCCTTCGGGCTAAACTATATCCATAACACCTGTGAACTGTTGATT
GACTTCGGTCAATATTTTTACAAACATTGTGTAATGAACGTCATGTTATAATAA
CAAATATAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCA
GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTT
GAACGCAACTTGCGCTCTCTGGTATTCCGGAGAGCATGCCTGTTTGAGTGTCAT
GAAATCTCAACCATTAGGGTTTCTTAATGGCTTGGATTTGGACGTTTGCCAGTC
AAATGGCTCGTCTTAAAAGAGTTAGTGAATTTAACATTTGTCTTCTGGCGTAAT
AAGTTTCGCTGGGCTGATAGTGTGAAGTTTGCTTCTAATCGTCCGCAAGGACAA
TTCTTGAACTCTGGCCTCAAATCAGGTAGGACTA

SEQ ID NO:47

Trichosporon sp. CBS 7617

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGACCCTCGGATTGGCGTTA
AGAAGCCGGCAACGGCATCTTTTGGCCGAGAAGTTGGTCAAACTTGGTCATTTA
GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTAGTGAATTGCTCTTTGAGCGTTAAACTATATCCATCTACACCTGTGAACTGTT
GATTGACTTCGGTCAATTACTTTTACAAACATTGTGTAATGAACGTCATGTTATT
ATAACAAAAATAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAA
CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA
TCTTTGAACGCAACTTGCGCTCTCTGGTATTCCGGAGAGCATGCCTGTTTGAGTA
TCATGAAATCTCAACCATTAGGGTTTCTTAATGGCTTGGATTTGGGCGCTGCCA
CTTGCCTGGCTCGCCTTAAAAGAGTTAGCGTATTAACTTGTCGATCTGGCGTAA
TAAGTTTCGCTGGTGTAGACTTGAGAAGTGCGCTTCTAATCGTCCTCGGACAAT
TCTTGAACTCTGGTCTCAAATCAGGTAGGACTA

SEQ ID NO:48

Sporobolomyces alborubescens CBS 482

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCCGGATTGGCTATT
GGGAGCTCGCGAGAGCACCCGACTGCCGAGAAGTTGTACGAACTTGGTCATTT
AGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC
ATTAGTGAATATAGGACGTCCAACTTAACTTGGAGTCCGAACTCTCACTTTCTA
ACCCTGTGCACTTGTTTGGGATAGTAACTCTCGCAAGAGAGCGAACTCCTATTC

ACTTATAAACACAAAGTCTATGAATGTATTAAATTTTATAACAAAATAAAACTT
TCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGAT
AAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTG
CGCTCCATGGTATTCCGTGGAGCATGCCTGTTTGAGTGTCATGAATACTTCAAC
CCTCCTCTTTCTTAATGATTGAAGAGGTGTTTGGTTTCTGAGCGCTGCTGGCCTT
TACGGTCTAGCTCGTTCGTAATGCATTAGCATCCGCAATCGAATTTCGGATTGA
CTTGGCGTAATAGACTATTCGCTGAGGAATTCTAGTCTTCGGATTAGAGCCGGG
TTGGGTTAAAGGAAGCTTCTAATCAGAATGTCTACATTTTAAGATTAGATCTCA
AATCAGGTAGGACTA

SEQ ID NO:49
Rhodotorula glutinis var. glutinis CBS 324

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCCGGATTGGCTATT
GGGAGCTCGCGAGAGCACCCGACTGCCGAGAAGTTGTACGAACTTGGTCATTT
AGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC
ATTAGTGAATCTAGGACGTCCAACTTAACTTGGAGTCCGAACTCTCACTTTCTA
ACCCTGTGCATCTGTTTTAAAATTGGCTAGTAGCTCTTCGGAGCGAACCACCAT
TTTTCACTTATACAAACACAAAGTCTATGAATGTAAACAAATTTATAACAAAAC
AAAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAA
ATGCGATACGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG
CACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATGAAAT
CTTCAACCCACCTCTTTCTTAGTGAATCTGGTGGTGCTTGGTTTCTGAGCGCTGC
TCTGCTTCGGCTTAGCTCGTTCGTAATGCATTAGCATCCGCAACCGAAACTTCG
GATTGACTTGGCGTAATAGACTATTCGCTGAGGATTCCAGACTTGTTCTGGAGC
CGAGTTGGGTTAAAGGAAGCTTCTAATCCTAAAGTCTATTTTTTGATTAGATCTC
AAATCAGGTAGGACTA

SEQ ID NO:50
Rhodotorula glutinis var. glutinis CBS 4476

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGGCTCCGGATTGGCTTCT
GGGAGCCGGCAACGGCACCTAGTCGCTGAGAAGTTGGACGAACTTGGTCATTT
AGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC
ATTAATGAAATGCAAGGACGCTCTTTTTAGAGGTCCGACCCAATTCATTTTCTC
ACACTGTGCACACACTACTTTTTACACCATTTTTAACACTTGAAGTCTAAGAATG
TAAACAGTCTCTTAATTGAGCATAAAATTTAAACAAAACTTTCAGCAACGGATC
TCTTGGCTCTCCCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAAT
TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCACTCTTTGGTATT
CCGAAGAGTATGTCTGTTTGAGTGTCATGAAACTCTCAACCCCCCTGTTTTGTAA
TGAACCAGGCGTGGGCTTGGATTATGGCTGCTGCCGGCGTAATTGTCGACTCGG
CTGAAATACACGAGCTACCCATTTCATAAGAAATAGACGGTTTGACTCGGCGTA
ATAACATATTTCGCTGAGGACGTCACATTCTTTACCTAGTGGTGCTTCTAATGCG
ACATCTAAACTTTAAGCTTTAGACCTCAAATCAGTCAGGACTA

SEQ ID NO:51
Trichosporon behrend CBS 5581

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGACCCTCGGATTGGCGTTA
GGAAGCCGGCAACGGCATCCTTTGGCCGAGAAGTTGGTCAAACTTGGTCATTTA
GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTAGTGATTGCCTTCATAGGCTTAAACTATATCCACATACACCTGTGAACTGTTC
CACCACTTGACGCAAGTCGAGTGTTTTTACAAACAATGTGTAATGAACGTCGTT
TTATTATAACAAAATAAAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGAT
GAAGAACGCAGCGAATTGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC
ATCGAATCTTTGAACGCAGCTTGCGCTCTCTGGTATTCCGGAGAGCATGCCTGT
TTCAGTGTCATGAAATCTCAACCACTAGGGTTTCCTAATGGATTGGATTTGGGC
GTCTGCGATCTCTGATCGCTCGCCTTAAAAGAGTTAGCAAGTTTGACATTAATG
TCTGGTGTAATAAGTTTCACTGGGTCCATTGTGTTGAAGCGTGCTTCTAATCGTC
CGCAAGGACAATTACTTTGACTCTGGCCTGAAATCAGGTAGGACTA

SEQ ID NO:52
Geotrichum histeridarum CBS 9892

CGCCCGTCGCTACTACCGATCGAATGGCTTAGTGAGGCTTCCGGATTGATTTGG
GAGAGAGGGCGACTTTTTTCCTGGAACGAGAAGCTAGTCAAACTTGGTCATTTA
GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTAGAAAAATGCGATATTAGTGGTTTATTTTGCTCGCCGAAAGGCAAACTTTTA
ACATACCTACCTTTTTTTAACTATAAAAACTTTTAACAACGGATCTCTTGGTTCT
CGCATCGATGAAGAACGCAGCGAATTGCGATACGTTTTGTGAATTGCAGAAGT
GAATCATCAATCTTTGAACGCACATTGCGCCTGGTGGTATTCCGCCAGGCATAC
CTGTTTGAGCGTTGTTCTCTCTGGGATTGTCTACTTTCCTCAAAGAAATTAAACA
AACAAGTTTGACACAACACCTCAACCTCAGATCAGGTAGGACTA

SEQ ID NO:53
Rhodotorula aurantiaca CBS 8411 and *Cryptococcus curvatus* CBS 8126

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTTCGGATTGGCTTCT
GGGAGCCGGCAACGGCACCTAGTCGCTGAGAAGTTTGACGAACTTGGTCATTTA
GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTAATGAATTTTAGGACGTTCTTTTTAGAAGTCCGACCCTTTCATTTTCTTACAC
TGTGCACACACTTCTTTTTTACACACACTTTTAACACCTTAGTATAAGAATGTAA
TAGTCTCTTAATTGAGCATAAATAAAAACAAAACTTTCAGCAACGGATCTCTTG
GCTCTCGCATCGATGAAGAACGCAGCGAATTGCGATAAGTAATGTGAATTGCA
GAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCACTCTTTGGTATTCCGA
AGAGTATGTCTGTTTGAGTGTCATGAAACTCTCAACCCCCCTATTTTGTAATGAG
ATGGGTGTGGGCTTGGATTATGGTTGTCTGTCGGCGTAATTGCCGGCTCAACTG
AAATACACGAGCAACCCTATTGAAATAAACGGTTTGACTTGGCGTAATAATTAT
TTCGCTAAGGACGCTTTCTTCAAATATAAGAGGTGCTTCTAATTCGCTTCTAATA
GCATTTAAGCTTTAGACCTCAAATCAGTCAGGACTA

SEQ ID NO:54
Trichosporon domesticum CBS 8111

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGACCTCCGGATTGGCGTTG
AGAAGCCGGCAACGGCATCTCTTGGCTGAGAAGTTGGTCAAACTTGGTCATTTA

GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTAGTGATTGCCTTAATTGGCTTAAACTATATCCATCTACACCTGTGAACTGTTT
GATTGAATCTTCGGATTCGATTTTATACAAACATTGTGTAATGAACGTCATTATA
TTATAACAAAAAAAAAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGA
AGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCAT
CGAATCTTTGAACGCAACTTGCGCTCTCTGGTATTCCGGAGAGCATGCCTGTTT
GAGTGTCATGAAATCTCAACCATTAGGGTTTCTTAATGGCTTGGATTTGGAGGT
TTGCCAGTCTGACTGGCTCCTCTTAAAAGAGTTAGCAAGTTGAACTATTGCTAT
CTGGCGTAATAAGTTTCGCTGGAATGGTATTGTGAAGCGTGCTTCTAATCGTCTT
CGGACAATTTTTTGACTCTGGCCTCAAATCAGGTAGGACTA

SEQ ID NO:55
Rhodotorula toruloides CBS 8761

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCCGGATTGGCTATC
GGGAGCTCGCGAGAGCACCTGACTGCCGAGAAGTTGTACGAACTTGGTCATTTA
GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTAGTGAATATTAGGGTGTCCAACTTAACTTGGAGCCCGACCCTCACTTTCTAA
CCCTGTGCATTTGTCTTGGGTAGTAGCTCGTGTCAGCGAGCGAATCCCATTTCAC
TTACAAACACAAAGTCTATGAATGTAACAAATTTATAACAAACAAAACTTTCAA
CAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATACGT
AATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCT
CCATGGTATTCCGTGGAGCATGCCTGTTTGAGTGTCATGAATTCTTCAACCCACC
TCTTTCTTAGTGAATCAGGCGGTGTTTGGATTCTGAGCGTTGCTGGCTTCGCGGC
CTAGCTCGCTCGTAATGCATTAGCATCCGCAATCGAACTTCGGATTGACTCGGC
GTAATAGACTATTCGCTGAGGATTCTGGTCTCTGACTGGAGCCGGGTAAGATTA
AAGGAAGCTACTAATCCTCATGTCTATCTTTTGAGATTAGACCTCAAATCAGGT
AGGACTA

SEQ ID NO:56
Rhodotourula terpendoidalis CBS 8445

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCCGGACTGGCTATT
GGGATCTCGCGAGAGAACCTGACTGCTGGGAAGTTGTACGAACTTGGTCATTTA
GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTAATGAATATTAGGGTGCTCTTTTCATCAAAGAGGCCTGACCTTCATTCTTCTA
CCCTGTGCACTATTCAAACATTCGGCAGTTGGTAATTTGGCTTGTAAAAGAGCC
AGACGACTCTGCTGAATTCACTCTTAAACTCTAAAGTATAAGAATGTTACAAAT
AAAACAAATAAAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAA
CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA
TCTTTGAACGCACCTTGCGCTCGCTGGTATTCCGGCGAGCATGCCTGTTTGAGTG
TCATGAAAACCTCAACCCTTCAATTCCTTGTTGAATTGTAAGGTGTTTGGATTCT
GAATGTTTGCTGGCTTGAAGGGCCCTTGGCTACTTCAAAAGCGAAGCTCATTCG
TAATACATTAGCATCTCAATTTCGAATATTCGGATTGACTCGGCGTAATAGACT
TTATTCGCTGAGGACACCTTCACAAGGTGGCCGAATTTCGAGGTAGAAGCTTCC
AATTCGATCAAAAGTCACTCTTAGTTTAGACCTCAGATCAGGCAGGACTA

SEQ ID NO:57

Yarrowia lipolytica CBS 10144

CGCCCGTCGCTACTACCGATTGAATGGTTTAGTGAGACCTTGGGAGGGCGAGAT
GAGGGGGGCAACCCCTTTTGAACATCCAAACTTGGTCAAACTTGATTATTTAGA
GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATT
ATTGATTTTATCTATTTCTGTGGATTTCTGGTATATTACAGCGTCATTTTATCTCA
ATTATAACTATCAACAACGGATCTCTTGGCTCTCACATCGATGAAGAACGCAGC
GAACCGCGATATTTTTTGTGACTTGCAGATGTGAATCATCAATCTTTGAACGCA
CATTGCGCGGTATGGTATTCCGTACCGCACGGATGGAGGAGCGTGTTCCCTCTG
GGATCGCATTGCTTTCTTGAAATGGATTTTTTAAACTCTCAATTATTACGTCATT
TCACCTCCTTCATCCGAGATTA

SEQ ID NO:58
Rhodotorula glutinis var. glutinis CBS 5805

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCCGGATTGGCTATT
GGGAGCTCGCGAGAGCACCTGACTGCCGAGAAGTTGTACGAACTTGGTCATTTA
GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTAGTGAATATTAGGGTGTCCAACTTAACTTGGAACCCGACCCTCACTTTCTAA
CCCTGTGCATTTGTCTTGGGTAGTAGCTTGCGTCGGCGAGCGAATCCCATTTCAC
TTACAAACACAAAGTCTATGAATGTAACAAATTTATAACAAACAAAACTTTCAA
CAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATACGT
AATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCT
CCATGGTATTCCGTGGAGCATGCCTGTTTGAGTGTCATGAATTCTTCAACCCACC
TATTTCTTAGTGAATCAGGCGGTGTTTGGATTCTGAGCGCTGCTGGCCTCACGG
CCTAGCTCGCTCGTAATGCATTAGCATCCGCAATCGAACTTCGGATTGACTCGG
CGTAATAGACTATTCGCTGAGGATTCTGGTCTCTGACTGGAGCCGGGTGAGATT
AAAGGAAGCTACTAATCCTCATGTCTATCTTGAGATTAGACCTCAAATCAGGTA
GGACTA

SEQ ID NO:59
Yarrowia lipolytica CBS 10143

GTCCCTGCCCTTTGTACACACCGCCCGTCGCTACTACCGATTGAATGGTTTAGTG
AGACCTTGGGAGGGCGAGATGAGGGGGGCAACCCCTTCTGAACATCCAAACTT
GGTCAAACTTGATTATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGT
GAACCTGCGGAAGGATCATTATTGATTTTATCTATTTCTGTGGATTTCTATTCTA
TTACAGCGTCATTTTATCTCAATTATAACTATCAACAACGGATCTCTTGGCTCTC
ACATCGATGAAGAACGCAGCGAACCGCGATATTTTTTGTGACTTGCAGATGTGA
ATCATCAATCTTTGAACGCACATTGCGCGGTATGGCATTCCGTACCGCACGGAT
GGAGGAGCGTGTTCCCTCTGGGATCGCATTGCTTTCTTGAAATGGATTTTTTAAA
CTCTCAATTATTACGTCATTTCACCTCCTTCATCCGAGATTACCCGCTGAACTTA
AGCATATCAA

SEQ ID NO:60
Lipomyces tetrasporus CBS 5607

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCCGGATTGGCTATT
GGGAGCTCGCGAGAGCACCTGACTGCTGAGAAGTTGTACGAACTTGGTCATTTA
GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTAGTGAATCTAGGACGTCCAACTTAACTTGGAGTCCGAAATCTCACTTTCTAA
CCCTGTGCATCTGTTAATTGGAATAGTAGCTCTTCGGAGTGAACCACCATTCAC
TTATAAAACACAAAGTCTATGAATGTATACAAATTTATAACAAAACAAAACTTT
CAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGAT
ACGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTG
CGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATGAAATCTTCAAC
CCACCTCTTTCTTAGTGAATCTGGTGGTGCTTGGTTTCTGAGCGCTGCTCTGCTT
CGGCTTAGCTCGTTCGTAATGCATTAGCATCCGCAACCGAACTTCGGATTGACT
TGGCGTAATAGACTATTCGCTGAGGATTCTAGTTTACTAGAGCCGAGTTGGGTT
AAAGGAAGCTCCTAATCCTAAAGTCTATTTTTTGATTAGATCTCAAATCAGGTA
GGACTA

SEQ ID NO:61
Yarrowia lipolytica CBS 5589

CGCCCGTCGCTACTACCGATTGAATGGTTTAGTGAGACCTTGGGAGGGCGAGAT
GAGGGGGGCAACCCCTTCTGAACATCCAAACTTGGTCAAACTTGATTATTTAGA
GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATT
ATTGATTTTATCTATTTCTGTGGATTTCTATTCTATTACAGCGTCATTTTATCTCA
ATTATAACTATCAACAACGGATCTCTTGGCTCTCACATCGATGAAGAACGCAGC
GAACCGCGATATTTTTTGTGACTTGCAGATGTGAATCATCAATCTTTGAACGCA
CATTGCGCGGTATGGCATTCCGTACCGCACGGATGGAGGAGCGTGTTCCCTCTG
GGATCGCATTGCTTTCTTGAAATGGATTTTTTTAAACTCTCAATTATTACGTCAT
TTCACCTCCTTCATCCGAGATTA

SEQ ID NO:62
Lipomyces tetrasporus CBS 8724

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTTCGGACTGGCTCCA
GAAAATGGGAAACCATTATCAGGAGCTGGAAAGTTGGTCAAACTTGGTCATTT
AGAGGAAGTAAAAGTCGTAACAAGGTTTCCTTCCGTAGCACTTACTGAAGCTTT
AGCAGCCCGAAAAGGCGAATGCTAGCGACTATAAATAAATATGGCGTTCTTAA
ATGCTAGTCTCTGATTAGAGGCGACATTGCCAAATTGCGGGGACATCCTAAAGA
TCTTGATACCAAGCTGGTAGTCGAAAGACGCCAGTGGCCGAGCTAACAGCCCT
GGGTATGGTAATAATTCAAGATATGGAACAATGGGTAATCCGCAGCCAAGTCC
TAAACTACGCAAGTAGCATGGATGCAGTTCACAGGCCAAATGGTGATGGGTAG
ATTACTAAATCTGCTTAAGATATGGTCGGTCCCGCTGTGAGAGCAGATGGGAAG
CTACAAAGCAGACTCGTGAGTTTGCGCAAACGTAACTAAAAACGTTCCGTAGGT
GAACCTGCGGAAGGATCATTACTGAGTATTTGTCTTTTAAAGACATCTCTCTATC
CATAAACTCTTTTTTCTAAAAAGACATGATTTACACAATTAGTCTGAATGATTAT
ATAAAAATCTTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAA
GAACGCAGCAAAATGCGATAAGTATTGTGAATTGCAGGATTTTGTGAATCATCG
AATTTTTGAACGCACATTGCACCTTCTGGTATTCCGGAGGGTATACCTGTTTGAG
CGTCATTTATATACTCAAAACTTCGTTTTGGTGATGGGCACATATCTGGTGAGA
GCTAGATTTGCCTGAAATATAGTGGTAGAGATTGCTACGAGTTATGCAAGTTAG

CCAATGCTATTAAGTTAATTCGTTGGTGAAGCATGCGGAGCTTTAGTGATCGCC
TTCCTTAACTATTGGAATTTTTCTAATTTTGACCTCAAATCAGGCAGGAGTA

SEQ ID NO:63
Rhodosporidium sphaerocarpum CBS 2371

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCCGGACCGGCTATT
GGGAGCTCGCGAGAGCACCCGACTGCTGGGAAGTTGTACGAACTTGGTCATTTA
GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTAGTGAATATAGGACGTCCAACTTAACTTGGAGTCCGAACTCTCACTTTCTAA
CCCTGTGCATTTGTTTGGGATAGTAGCCTCTCGGGGTGAACTCCTATTCACTCAT
AAACACAAAGTCTATGAATGTATTTAATTTATAACAAAATAAAACTTTCAACAA
CGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAAT
GTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCA
TGGTATTCCGTGGAGCATGCCTGTTTGAGTGTCATGAATACTTCAACCCTCCTCT
TTTCTAGTGAAAGAGAAGGTGCTTGGTTTCTGAGCGTTTTGCTGGCCTCACGGT
CGAGCTCGCTCGTAATGCATTAGCATCCGCAATCGAACTTCGGATTGACTTGGC
GTAATAGACTATTCGCTGAGGAATTCTAATCTTCGGATTAGAGCCGGGTTGGGT
TAAAGGAAGCTTCTAATCCTAATGTCTATATTTTTAGATTAGATCTCAAATCAG
GTAGGACTA

SEQ ID NO:64
Trichosporon brassicae CBS 6382

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGACCTCCGGATTGGCGTTG
AGAAGCCGGCAACGGCATCTCTTGGCCGAGAAGTTGGTCAAACTTGGTCATTTA
GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTAGTGATTGCCTTAATTGGCTTAAACTATATCCAACTACACCTGTGAACTGTTC
GATTGAATCTTCGATTCAATTTTACAAACATTGTGTAAAGAACGTCATTAGATC
ATAACAAAAAAAAAACTTTTAACAACGGATCTCTTGGCTCTCGCATCGATGAAGA
ACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGA
ATCTTTGAACGCAACTTGCGCTCTCTGGTATTCCGGAGAGCATGCCTGTTTGAGT
GTCATGAAATCTCACACATCAAGGTTTCTTGATGAAGTGGATTTGGAGGTTGCC
AGTCTAACTGGCTCCTCTTAAAGGAGTTAGCATATTTGATTATTGCTGTCTGGCG
TAATAAGTTTCGCTAGTTTGGCATTTTGAAGTGTGCTTCTAATCGTCTTCGGACA
ATTTTTTGACTCTGGCCTCAAATCAGGTAGGACTA

SEQ ID NO:65
Cryptococcus curvatus CBS 2755 and *Lipomyces tetrasporus* CBS 7656

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGATTTCCGGATTGGCGTTA
GGAAGCCGGCAACGGCATCCTTTGGCTGAGAAGCTACTCAAACTTGGTCATTTA
GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTAGTGATTTGCCTTCGGGCTAACTATATCCATAACACCTGTGAACTGTTGATTG
ACTTCGGTCAATATTTTTACAAACATTGTGTAATGAACGTCATGTTATAATAAC
AAATATAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAG
CGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTG
AACGCAACTTGCGCTCTCTGGTATTCCGGAGAGCATGCCTGTTTGAGTGTCATG

AAATCTCAACCATTAGGGTTTCTTAATGGCTTGGATTTGGACGTTTGCCAGTCA
AATGGCTCGTCTTAAAAGAGTTAGTGAATTTAACATTTGTCTTCTGGCGTAATA
AGTTTCGCTGGGCTGATAGTGTGAAGTTTGCTTCTAATCGTCCGCAAGGACAAT
TCTTGAACTCTGGCCTCAAATCAGGTAGGACTA

SEQ ID NO:66
Lipomyces starkeyi CBS 7786

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTTCGGACTGGCTCCA
GAAAATGGGAAACCATTATCAGGAGCTGGAAAGTTGGTCAAACTTGGTCATTT
AGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC
ATTACTGAGTATTTGTCTTTTCAAGACATCTCTCTATCCATAAACTCTTTTTTTA
AAAAGACATGATTTATAACAATTAGTCTGAATGATTATTTTTAAATCTTCAAAA
CTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCAAATTGC
GATAAGTAATGTGAATTGCAGGATTTTGTGAATCATCGAATTTTTGAACGCACA
TTGCACCTTCTGGTATTCCGGAGGGTATACCTGTTTGAGCGTCATTTATATACTC
AAAACTTACGTTTTGGTGATGGGCACGTATCTGGCTTCTAAGTTAGATTTGCCTG
AAATATAGCGGTAGAGGTCGCTAGAAGCGATGCAAGTTAGCCAATGCTATTAA
AGTTAATTCGTTGGTGACGCATGTTGAGCTTTTGGTGAAGTCTTCCTTAATTATT
GGAATTTTTTTCTAATTTTGACCTCAAATCAGGCAGGAGTA

SEQ ID NO:67
Yarrowia lipolytica lipoyltica CBS 6012

CGCCCGTCGCTACTACCGATTGAATGGTTTAGTGAGACCTTGGGAGGGCGAGAT
GAGGGGGGCAACCCCTTTTGAACATCCAAACTTGGTCAAACTTGATTATTTAGA
GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATT
ATTGATTTTATCTATTTCTGTGGATTTCTATTCTATTACAGCGTCATTTTATCTCA
ATTATAACTATCAACAACGGATCTCTTGGCTCTCACATCGATGAAGAACGCAGC
GAACCGCGATATTTTTTGTGACTTGCAGATGTGAATCATCAATCTTTGAACGCA
CATTGCGCGGTATGGCATTCCGTACCGCACGGATGGAGGAGCGTGTTCCCTCTG
GGATCGCATTGCTTTCTTGAAATGGATTTTTTTAAACTCTCAATTATTACGTCAT
TTCACCTCCTTCATCCGAGATTA

SEQ ID NO:68
Trichosporon loubieri var. loubieri CBS 8265

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGACCTCCGGATTGGCGTTG
AGAAGCCGGCAACGGCATCTCTTGGCCGAGAAGTTGGTCAAACTTGGTCATTTA
GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTAGTGATTGCCATCTTGGCTTAAACTATATCCATCTACACCTGTGAACCGTTTG
ATTGAATCTTCTGATTCAATTTTACAAACATTGTGTAATGAACGTCATTAGATCA
TAATAAGAAAAAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAA
CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAA
TCTTTGAACGCAACTTGCGCTCTCTGGTATTCCGGAGAGCATGCCTGTTTGAGTG
TCATGAAATCTCAACCATTAGGGTTTCTTAATGGCTTGGATTTGGAGGTTGCCAT
TCTAAATGGCTCCTCTTAAAAGAGTTAGCGAGTTTAACTATTGCTATCTGGCGT

AATAAGTTTCGCTGGAATGGTATTGTGAAGCGCGCTTCTAATCGTCTTCGGACA
ATTTTTTGACTCTGGCCTCAAATCAGGTAGGACTA

SEQ ID NO:69
Geotrichum vulgare CBS 10073

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCTTCCGGATTGATTAGT
TGGAGAGGGAGACTTTTCTGACTGAACGAGAAGCTAGTCAAACTTGGTCATTTA
GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTAAAGATTTAATATTAATTGTGAAATTAAAACGATATTAACAAAAAATCATAC
AATCAATTATAAAAAAAATCAAAACTTTTAACAATGGATCTCTTGGTTCTCGTA
TCGATGAAGAACGCAGCGAAACGCGATATTTCTTGTGAATTGCAGAAGTGAAT
CATCAGTTTTTGAACGCACATTGCACTTTGGGGTATCCCCCAAAGTATACTTGTT
TGAGCGTTGTTTCTCTCTTGGAATTGCATTGCTTTTCTAAAAAATCGAATCAAAT
TCGTTTGAAACATCCATTCTTCAACCTCAGATCAAGTAGGATTA

SEQ ID NO:70
Rhodosporidium toruloides CBS 14

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCCGGATTGGCTATT
GGGAGCTCGCGAGAGCACCTGACTGCCGAGAAGTTGTACGAACTTGGTCATTTA
GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTAGTGAATATTAGGGTGTCCAACTTAACTTGGAGCCCGACCCTCACTTTCTAA
CCCTGTGCATTTGTCTTGGGTAGTAGCTTGCGTCAGCGAGCGAATCCCATTTCAC
TTACAAACACAAAGTCTATGAATGTAACAAATTTATAACAAAACAAAACTTTCA
ACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAC
GTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCG
CTCCATGGTATTCCGTGGAGCATGCCTGTTTGAGTGTCATGAATTCTTCAACCCA
CCTCTTTCTTAGTGAATCAGGCGGTGTTTGGATTCTGAGCGCTGCTGGCTTCGCG
GCCTAGCTCGCTCGTAATGCATTAGCATCCGCAATCGAACTTCGGATTGACTCG
GCGTAATAGACTATTCGCTGAGGATTCTGGTCTCTGACTGGAGCCGGGTAAGGT
TAAAGGGAGCTACTAATCCTCATGTCTATCTTGAGATTAGACCTCAAATCAGGT
AGGACTA

SEQ ID NO:71
Rhodotorula glutinis var. glutinis CBS 6020 and *Lipomyces orientalis* CBS 10300

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGCCTCCGGATTGGCTATT
GGGAGCTCGCGAGAGCACCTGACTGCTGAGAAGTTGTACGAACTTGGTCATTTA
GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTAGTGAATCTAGGACGTCCAACTTAACTTGGAGTCCGAACTCTCACTTTCTAA
CCCTGTGCATCTGTTAATTGGAATAGTAGCTCTTCGGAGTGAACCACCATTCAC
TTATAAAACACAAAGTCTATGAATGTATACAAATTTATAACAAAACAAAACTTT
CAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGAT
ACGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTG
CGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATGAAATCTTCAAC
CCACCTCTTTCTTAGTGAATCTGGTGGTGCTTGGTTTCTGAGCGCTGCTCTGCTT
CGGCTTAGCTCGTTCGTAATGCATTAGCATCCGCAACCGAACTTCGGATTGACT

TGGCGTAATAGACTATTCGCTGAGGATTCTAGTTTACTAGAGCCGAGTTGGGTT
AAAGGAAGCTCCTAATCCTAAAGTCTATTTTTTGATTAGATCTCAAATCAGGTA
GGACTA

SEQ ID NO:72
Rhodotorula aurantiaca CBS 317

CGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGATTTCCGGATTGGCGTTA
GGAAGCCGGCAACGGCATCCTTTGGCTGAGAAGCTACTCAAACTTGGTCATTTA
AAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA
TTAGTGATTTGCCTTCGGGCTAACTATATCCATAACACCTGTGAACTGTTGATTG
ACTTCGGTCAATATTTTTACAAACATTGTGTAATGAACGTCATGTTATAATAAC
AAATATAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAG
CGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTG
AACGCAACTTGCGCTCTCTGGTATTCCGGAGAGCATGCCTGTTTGAGTGTCATG
AAATCTCAACCATTAGGGTTTCTTAATGGCTTGGATTTGGACGTTTGCCAGTCA
AATGGCTCGTCTTAAAAGAGTTAGTGAATTTAACATTTGTCTTCTGGCGTAATA
AGTTTCGCTGGGCTGATAGTGTGAAGTTTGCTTCTAATCGTCCGCAAGGACAAT
TCTTGAACTCTGGCCTCAAATCAGGTAGGACTA

SEQ ID NO:73
Torulaspora delbrueckii CBS 2924

CGCCCGTCGCTAGTACCGATTGAATGGCTTAGTGAGGCCTCAGGATCTGCTTAG
AGAAGGGGGCAACTCCATCTCAGAGCGGAGAATCTGGTCAAACTTGGTCATTT
AGAGGAACTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC
ATTAGAGAAATCTATATGAATGAAGTTAGAGGACGTCTAAAGATACTGTAAGA
GAGGATCAGGTTCAAGACCAGCGCTTAATTGCGCGGTTGCGGCTTGGTTCGCCT
TTTGCGGAACATGTCTTTTCTCGTTGTTAACTCTACTTCAACTTCTACAACACTG
TGGAGTTTTCTACACAACTTTTCTTCTTTGGGAAGATACGTCTTGTGCGTGCTTC
CCAGAGGTGACAAACACAAACAACTTTTTATTATTATAAACCAGTCAAAACCAA
TTTCGTTATGAAATTAAAAATATTTAAAACTTTCAACAACGGATCTCTTGGTTCT
CGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAGAATT
CCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGGTATTCCAGGGGG
CATGCCTGTTTGAGCGTCATTTCCTTCTCAAACAATCATGTTTGGTAGTGAGTGA
TACTCTGTCAAGGGTTAACTTGAAATTGCTAGCCTGTTATTTGGTTGTGATTTTG
CTGGCTTGGATGACTTTGTCCAGTCTAGCTAATACCGAATTGTCGTATTAGGTTT
TACCAACTTCGGCAGACTGTGTGTTGGCTCGGGCGCTTTAAAGACTTTGTCGTA
AACGATTTATCGTTTGTTTGAGCTTTTCGCATACGCAATCCGGGCGAACAATAC
TCTCAAAGTTTGACCTCAAATCAGGTAGGAATA

SEQ ID NO:74
5' primer Δ12 FAD genomic amplification from *Prototheca moriformis* (UTEX 1435)

5'-TCACTTCATGCCGGCGGTCC-3'

SEQ ID NO:75
3' primer Δ12 FAD genomic amplification from *Prototheca moriformis* (UTEX 1435)

5'- GCGCTCCTGCTTGGCTCGAA-3'

SEQ ID NO:76
pSZ1124 5' gene targeting sequence (FAD2B)

gctcttcgagacgtggtctgaatcctccaggcgggtttccccgagaaagaaagggtgccgatttcaaagcagagccatgtgccggg
ccctgtggcctgtgttggcgcctatgtagtcacccccccctcacccaattgtcgccagtttgcgcaatccataaactcaaaactgcagct
tctgagctgcgctgttcaagaacacctctggggtttgctcacccgcgaggtcgacgcccagcatggctatcaagacgaacaggcag
cctgtggagaagcctccgttcacgatcgggacgctgcgcaaggccatccccgcgcactgtttcgagcgctcggcgcttcgtagca
gcatgtacctggcctttgacatcgcggtcatgtccctgctctacgtcgcgtcgacgtacatcgaccctgcgccggtgcctacgtgggt
caagtatggcgtcatgtggccgctctactggttcttccaggtgtgtgtgagggttgtggttgcccgtatcgaggtcctggtggcgcgc
atgggggagaaggcgcctgtcccgctgaccccccggctaccctcccggcaccttccagggcgccttcggcacgggtgtctggg
tgtgcgcgcacgagtgcggccaccaggccttttcctccagccaggccatcaacgacggcgtgggcctggtgttccacagcctgct
gctggtgccctactactcctggaagcactcgcaccgggtacc

SEQ ID NO:77
pSZ1124 3' gene targeting sequence (FAD2B)

ccgccaccactccaacacggggtgcctggacaaggacgaggtgtttgtgccgccgcaccgcgcagtggcgcacgagggcctgg
agtgggaggagtggctgcccatccgcatgggcaaggtgctggtcaccctgaccctgggctggccgctgtacctcatgttcaacgtc
gcctcgcggccgtacccgcgcttcgccaaccactttgacccgtggtcgcccatcttcagcaagcgcgagcgcatcgaggtggtcat
ctccgacctggcgctggtggcggtgctcagcgggctcagcgtgctgggccgcaccatgggctgggcctggctggtcaagaccta
cgtggtgccctacctgatcgtgaacatgtggctcgtgctcatcacgctgctccagcacacgcacccggcgctgccgcactacttcga
gaaggactgggactggctgcgcggcgccatggccaccgtggaccgctccatgggcccgcccttcatggacaacatcctgcacca
catctccgacacccacgtgctgcaccacctcttcagcaccatcccgcactaccacgccgaggaggcctccgccgccatcaggccc
atcctgggcaagtactaccagtccgacagccgctgggtcggccgcgccctgtgggaggactggcgcgactgccgctacgtcgtc
ccggacgcgcccgaggacgactccgcgctctggttccacaagtgagtgagtgagaagagc

SEQ ID NO:78
S. cerevisiae suc2 casscttc

ctttcttgcgctatgacacttccagcaaaaggtagggcgggctgcgagacggcttcccggcgctgcatgcaacaccgatgatgcttc
gaccccccgaagctccttcggggctgcatgggcgctccgatgccgctccagggcgagcgctgtttaaatagccaggcccccgatt
gcaaagacattatagcgagctaccaaagccatattcaaacacctagatcactaccacttctacacaggccactcgagcttgtgatcgc
actccgctaagggggcgcctcttcctcttcgtttcagtcacaacccgcaaacggcgcgccATGctgctgcaggccttcctgttcct
gctggccggcttcgccgccaagatcagcgcctccatgacgaacgagacgtccgaccgccccctggtgcacttcacccccaacaa
gggctggatgaacgaccccaacggcctgtggtacgacgagaaggacgccaagtggcacctgtacttccagtacaacccgaacga
caccgtctgggggacgcccttgttctggggccacgccacgtccgacgacctgaccaactgggaggaccagcccatcgccatcgc
cccgaagcgcaacgactccggcgccttctccggctccatggtggtggactacaacaacacctccggcttcttcaacgacaccatcg
acccgcgccagcgctgcgtggccatctggacctacaacaccccggagtccgaggagcagtacatctcctacagcctggacggcg
gctacaccttcaccgagtaccagaagaaccccgtgctggccgccaactccacccagttccgcgacccgaaggtcttctggtacgag
ccctcccagaagtggatcatgaccgcggccaagtcccaggactacaagatcgagatctactcctccgacgacctgaagtcctggaa
gctggagtccgcgttcgccaacgagggcttcctcggctaccagtacgagtgccccggcctgatcgaggtccccaccgagcagga
ccccagcaagtcctactgggtgatgttcatctccatcaaccccggcgccccggccggcggctccttcaaccagtacttcgtcggcag
cttcaacggcacccacttcgaggccttcgacaaccagtcccgcgtggtggacttcggcaaggactactacgccctgcagaccttctt
caacaccgacccgacctacgggagcgccctgggcatcgcgtgggcctccaactgggagtactccgccttcgtgcccaccaaccc
ctggcgctcctccatgtccctcgtgcgcaagttctccctcaacaccgagtaccaggccaacccggagacggagctgatcaacctga

aggccgagccgatcctgaacatcagcaacgccggcccctggagccggttcgccaccaacaccacgttgacgaaggccaacagc
tacaacgtcgacctgtccaacagcaccggcaccctggagttcgagctggtgtacgccgtcaacaccacccagacgatctccaagtc
cgtgttcgcggacctctccctctggttcaagggcctggaggaccccgaggagtacctccgcatgggcttcgaggtgtccgcgtcct
ccttcttcctggaccgcgggaacagcaaggtgaagttcgtgaaggagaaccccctacttcaccaaccgcatgagcgtgaacaacca
gcccttcaagagcgagaacgacctgtcctactacaaggtgtacggcttgctggaccagaacatcctggagctgtacttcaacgacg
gcgacgtcgtgtccaccaacacctacttcatgaccaccgggaacgccctgggctccgtgaacatgacgacgggggtggacaacct
gttctacatcgacaagttccaggtgcgcgaggtcaagTGAcaattggcagcagcagctcggatagtatcgacacactctggacg
ctggtcgtgtgatggactgttgccgccacacttgctgccttgacctgtgaatatccctgccgcttttatcaaacagcctcagtgtgtttga
tcttgtgtgtacgcgcttttgcgagttgctagctgcttgtgctatttgcgaataccacccccagcatccccttccctcgtttcatatcgcttg
catcccaaccgcaacttatctacgctgtcctgctatccctcagcgctgctcctgctcctgctcactgcccctcgcacagccttggtttgg
gctccgcctgtattctcctggtactgcaacctgtaaaccagcactgcaatgctgatgcacgggaagtagtgggatgggaacacaaat
gga

SEQ ID NO:79
pSZ1125 5' gene targeting sequence (FAD2C)

gctcttcgaggggctggtctgaatccttcaggcgggtgttacccgagaaagaaagggtgccgatttcaaagcagacccatgtgccg
ggccctgtggcctgtgttggcgcctatgtagtcacccccctcacccaattgtcgccagtttgcgcactccataaactcaaaacagca
gcttctgagctgcgctgttcaagaacacctctggggtttgctcacccgcgaggtcgacgcccagcatggctatcaagacgaacagg
cagcctgtggagaagcctccgttcacgatcgggacgctgcgcaaggccatccccgcgcactgtttcgagcgctcggcgcttcgta
gcagcatgtacctggcctttgacatcgcggtcatgtccctgctctacgtcgcgtcgacgtacatcgaccctgcaccggtgcctacgtg
ggtcaagtacggcatcatgtggccgctctactggttcttccaggtgtgtttgagggttttggttgcccgtattgaggtcctggtggcgcg
catggaggagaaggcgcctgtcccgctgacccccccggctaccctcccggcaccttccagggcgccttcggcacgggtgtctgg
gtgtgcgcgcacgagtgcggccaccaggccttttcctccagccaggccatcaacgacggcgtgggcctggtgttccacagcctgc
tgctggtgccctactactcctggaagcactcgcaccgggtacc

SEQ ID NO:80
pSZ1125 3' gene targeting sequence (FAD2C)

ccgccaccactccaacacggggtgcctggacaaggacgaggtgtttgtgccgccgcaccgcgcagtggcgcacgagggcctgg
agtgggaggagtggctgcccatccgcatgggcaaggtgctggtcaccctgaccctgggctggccgctgtacctcatgttcaacgtc
gcctcgcggccgtacccgcgcttcgccaaccactttgacccgtggtcgcccatcttcagcaagcgcgagcgcatcgaggtggtcat
ctccgacctggcgctggtggcggtgctcagcgggctcagcgtgctgggccgcaccatgggctgggcctggctggtcaagaccta
cgtggtgccctacctgatcgtgaacatgtggctcgtgctcatcacgctgctccagcacacgcacccggcgctgccgcactacttcga
gaaggactgggactggctgcgcggcgccatggccaccgtggaccgctccatgggcccgcccttcatggacaacatcctgcacca
catctccgacacccacgtgctgcaccacctcttcagcaccatcccgcactaccacgccgaggaggcctccgccgccatcaggccc
atcctgggcaagtactaccagtccgacagccgctgggtcggccgcgccctgtgggaggactggcgcgactgccgctacgtcgtc
ccggacgcgcccgaggacgactccgcgctctggttccacaagtgagtgagtgagaagagc

SEQ ID NO:81
5' 6S genomic donor sequence

GCTCTTCGCCGCCGCCACTCCTGCTCGAGCGCGCCCGCGCGTGCGCCGCCAGCG
CCTTGGCCTTTTCGCCGCGCTCGTGCGCGTCGCTGATGTCCATCACCAGGTCCAT
GAGGTCTGCCTTGCGCCGGCTGAGCCACTGCTTCGTCCGGGCGGCCAAGAGGA
GCATGAGGGAGGACTCCTGGTCCAGGGTCCTGACGTGGTCGCGGCTCTGGGAG
CGGGCCAGCATCATCTGGCTCTGCCGCACCGAGGCCGCCTCCAACTGGTCCTCC
AGCAGCCGCAGTCGCCGCCGACCCTGGCAGAGGAAGACAGGTGAGGGGGGTAT

GAATTGTACAGAACAACCACGAGCCTTGTCTAGGCAGAATCCCTACCAGTCATG
GCTTTACCTGGATGACGGCCTGCGAACAGCTGTCCAGCGACCCTCGCTGCCGCC
GCTTCTCCCGCACGCTTCTTTCCAGCACCGTGATGGCGCGAGCCAGCGCCGCAC
GCTGGCGCTGCGCTTCGCCGATCTGAGGACAGTCGGGGAACTCTGATCAGTCTA
AACCCCCTTGCGCGTTAGTGTTGCCATCCTTTGCAGACCGGTGAGAGCCGACTT
GTTGTGCGCCACCCCCCACACCACCTCCTCCCAGACCAATTCTGTCACCTTTTTG
GCGAAGGCATCGGCCTCGGCCTGCAGAGAGGACAGCAGTGCCCAGCCGCTGGG
GGTTGGCGGATGCACGCTCAGGTACC

SEQ ID NO:82
Relevant expression construct for *Cinnamomum camphora* thioesterase
(βtub::neo::nitred::βtub::*C.camphora* TE::nitred)

CTTTCTTGCGCTATGACACTTCCAGCAAAAGGTAGGGCGGGCTGCGAGACGGCT
TCCCGGCGCTGCATGCAACACCGATGATGCTTCGACCCCCCGAAGCTCCTTCGG
GGCTGCATGGGCGCTCCGATGCCGCTCCAGGGCGAGCGCTGTTTAAATAGCCAG
GCCCCCGATTGCAAAGACATTATAGCGAGCTACCAAAGCCATATTCAAACACCT
AGATCACTACCACTTCTACACAGGCCACTCGAGCTTGTGATCGCACTCCGCTAA
GGGGGCGCCTCTTCCTCTTCGTTTCAGTCACAACCCGCAAACTCTAGAATATCA
ATGATCGAGCAGGACGGCCTCCACGCCGGCTCCCCCGCCGCCTGGGTGGAGCG
CCTGTTCGGCTACGACTGGGCCCAGCAGACCATCGGCTGCTCCGACGCCGCCGT
GTTCCGCCTGTCCGCCCAGGGCCGCCCCGTGCTGTTCGTGAAGACCGACCTGTC
CGGCGCCCTGAACGAGCTGCAGGACGAGGCCGCCCGCCTGTCCTGGCTGGCCA
CCACCGGCGTGCCCTGCGCCGCCGTGCTGGACGTGGTGACCGAGGCCGGCCGC
GACTGGCTGCTGCTGGGCGAGGTGCCCGGCCAGGACCTGCTGTCCTCCCACCTG
GCCCCCGCCGAGAAGGTGTCCATCATGGCCGACGCCATGCGCCGCCTGCACACC
CTGGACCCCGCCACCTGCCCCTTCGACCACCAGGCCAAGCACCGCATCGAGCGC
GCCCGCACCCGCATGGAGGCCGGCCTGGTGGACCAGGACGACCTGGACGAGGA
GCACCAGGGCCTGGCCCCCGCCGAGCTGTTCGCCCGCCTGAAGGCCCGCATGCC
CGACGGCGAGGACCTGGTGGTGACCCACGGCGACGCCTGCCTGCCCAACATCA
TGGTGGAGAACGGCCGCTTCTCCGGCTTCATCGACTGCGGCCGCCTGGGCGTGG
CCGACCGCTACCAGGACATCGCCCTGGCCACCCGCGACATCGCCGAGGAGCTG
GGCGGCGAGTGGGCCGACCGCTTCCTGGTGCTGTACGGCATCGCCGCCCCCGAC
TCCCAGCGCATCGCCTTCTACCGCCTGCTGGACGAGTTCTTCTGACAATTGGCA
GCAGCAGCTCGGATAGTATCGACACACTCTGGACGCTGGTCGTGTGATGGACTG
TTGCCGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCCGCTTTTATCAAAC
AGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGCGAGTTGCTAGCTGCTT
GTGCTATTTGCGAATACCACCCCCAGCATCCCCTTCCCTCGTTTCATATCGCTTG
CATCCCAACCGCAACTTATCTACGCTGTCCTGCTATCCCTCAGCGCTGCTCCTGC
TCCTGCTCACTGCCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCTCCTG
GTACTGCAACCTGTAAACCAGCACTGCAATGCTGATGCACGGGAAGTAGTGGG
ATGGGAACACAAATGGAGGATCCCGCGTCTCGAACAGAGCGCGCAGAGGAACG
CTGAAGGTCTCGCCTCTGTCGCACCTCAGCGCGGCATACACCACAATAACCACC
TGACGAATGCGCTTGGTTCTTCGTCCATTAGCGAAGCGTCCGGTTCACACACGT
GCCACGTTGGCGAGGTGGCAGGTGACAATGATCGGTGGAGCTGATGGTCGAAA
CGTTCACAGCCTAGGGATATCGAATTCCTTTCTTGCGCTATGACACTTCCAGCA
AAAGGTAGGGCGGGCTGCGAGACGGCTTCCCGGCGCTGCATGCAACACCGATG

ATGCTTCGACCCCCCGAAGCTCCTTCGGGGCTGCATGGGCGCTCCGATGCCGCT
CCAGGGCGAGCGCTGTTTAAATAGCCAGGCCCCCGATTGCAAAGACATTATAG
CGAGCTACCAAAGCCATATTCAAACACCTAGATCACTACCACTTCTACACAGGC
CACTCGAGCTTGTGATCGCACTCCGCTAAGGGGGCGCCTCTTCCTCTTCGTTTCA
GTCACAACCCGCAAACACTAGTATGGCCACCGCATCCACTTTCTCGGCGTTCAA
TGCCCGCTGCGGCGACCTGCGTCGCTCGGCGGGCTCCGGGCCCCGGCGCCCAGC
GAGGCCCCTCCCCGTGCGCGGGCGCGCCCCCGACTGGTCCATGCTGTTCGCCGT
GATCACCACCATCTTCTCCGCCGCCGAGAAGCAGTGGACCAACCTGGAGTGGA
AGCCCAAGCCCAACCCCCCCCCAGCTGCTGGACGACCACTTCGGCCCCCACGGCC
TGGTGTTCCGCCGCACCTTCGCCATCCGCAGCTACGAGGTGGGCCCCGACCGCT
CCACCAGCATCGTGGCCGTGATGAACCACCTGCAGGAGGCCGCCCTGAACCAC
GCCAAGTCCGTGGGCATCCTGGGCGACGGCTTCGGCACCACCCTGGAGATGTCC
AAGCGCGACCTGATCTGGGTGGTGAAGCGCACCCACGTGGCCGTGGAGCGCTA
CCCCGCCTGGGGCGACACCGTGGAGGTGGAGTGCTGGGTGGGCGCCTCCGGCA
ACAACGGCCGCCGCCACGACTTCCTGGTGCGCGACTGCAAGACCGGCGAGATC
CTGACCCGCTGCACCTCCCTGAGCGTGATGATGAACACCCGCACCCGCCGCCTG
AGCAAGATCCCCGAGGAGGTGCGCGGCGAGATCGGCCCCGCCTTCATCGACAA
CGTGGCCGTGAAGGACGAGGAGATCAAGAAGCCCCAGAAGCTGAACGACTCCA
CCGCCGACTACATCCAGGGCGGCCTGACCCCCCGCTGGAACGACCTGGACATC
AACCAGCACGTGAACAACATCAAGTACGTGGACTGGATCCTGGAGACCGTGCC
CGACAGCATCTTCGAGAGCCACCACATCTCCTCCTTCACCATCGAGTACCGCCG
CGAGTGCACCATGGACAGCGTGCTGCAGTCCCTGACCACCGTGAGCGGCGGCT
CCTCCGAGGCCGGCCTGGTGTGCGAGCACCTGCTGCAGCTGGAGGGCGGCAGC
GAGGTGCTGCGCGCCAAGACCGAGTGGCGCCCCAAGCTGACCGACTCCTTCCG
CGGCATCAGCGTGATCCCCGCCGAGTCCAGCGTGATGGACTACAAGGACCACG
ACGGCGACTACAAGGACCACGACATCGACTACAAGGACGACGACGACAAGTGA
CTCGAGGCAGCAGCAGCTCGGATAGTATCGACACACTCTGGACGCTGGTCGTGT
GATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCCGCTT
TTATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGCGAGTTGC
TAGCTGCTTGTGCTATTTGCGAATACCACCCCCAGCATCCCCTTCCCTCGTTTCA
TATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCTATCCCTCAGCGC
TGCTCCTGCTCCTGCTCACTGCCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGT
ATTCTCCTGGTACTGCAACCTGTAAACCAGCACTGCAATGCTGATGCACGGGAA
GTAGTGGGATGGGAACACAAATGGAAAGCTT

SEQ ID NO:83

Prototheca moriformis SugT Sugar tranporter promoter/5"UTR

CCAGGCAGGCGGTAGGGTTGCCGATTGCTTGAGCGAATTGGAAGATATAATTTT
TTGTGGTGTCCCTGGACGCTGTTTGTGGCGCTCCTTTTTGGAGAAGATTGCGTGG
GGGAGCTTTCCATGTACCACGCTTCCTTCTGAAAGGATTCTGGCCGAGTCCTGA
TGAGCCCAAAGAAAACACCTGCCTTTCAGTGCTGGCACTCTGAAAACGTCAACA
GATGATTATACATGTCACAAAAGGCAGCCGATTAGGAACGGGAGCTCTGGCCG
TTCGTTTGGCTGCCTGGGCTGATTGAAGTGATCCACCCTGTTCGAATGAAGGCG
GTCGAGTCGAATTATCGACCGGAGCTGTCGGGAAGGCGTCCGGGGCAGAGTGA
GGTGCTGCGGCCTGGTTGTCGTTCAAAAAGACCCCGGTAGCCCAACAATCACGA
ACGAAAGGAATATAATTGCTTGCATACTATACATTCAGTTTCTATGTGGCGGGT
AGACAAGTCTCATGGGCTTCTAAAGGCTGTCCCTTGAAGGCTACTTATAAAAAC

TTGCTGCGCCATGGCACGGATCGCGCTTGCGCAGGCTGCAACCCTGCGCGCAAG
GTCAAATACACAGCAAAAGATACTAACAGAATTTCTAAAAACATTTAAATATTT
GTTTCGACCAGCCAATTGTGGTCGTAGGCACGCAAAAGACTTTGTTTTGCGCCC
ACCGAGCATCCACGCTGGCAGTCAAGCCAGTCCGATGTGCATTGCGTGGCAGC
ATCGAGGAGCATCAAAAACCTCGTGCACGCTTTTCTGTCAATCATCATCAACCA
CTCCACCATGTATACCCGATGCATCGCGGTGCGCAGCGCGCCACGCGTCCCAGA
CCCGCCCAAAAACCCAGCAGCGGCGAAAGCAAATCTTCACTTGCCCGAAACCC
CGAGCAGCGGCATTCACACGTGGGCGAAAACCCCACTTGCCCTAACAGGCGTA
TGTCTGCTGTCACGATGCCTGACAACGGTATTATAGATATACACTGATTAATGT
TTGAGTGTGTGCGAGTCGCGAATCAGGAATGAATTGCTAGTAGGCACTCCGACC
GGGCGGGGGCCGAGGGACCA

SEQ ID NO:84
amt03 promoter/UTR sequence

GGCCGACAGGACGCGCGTCAAAGGTGCTGGTCGTGTATGCCCTGGCCGGCAGG
TCGTTGCTGCTGCTGGTTAGTGATTCCGCAACCCTGATTTTGGCGTCTTATTTTG
GCGTGGCAAACGCTGGCGCCCGCGAGCCGGGCCGGCGGCGATGCGGTGCCCCA
CGGCTGCCGGAATCCAAGGGAGGCAAGAGCGCCCGGGTCAGTTGAAGGGCTTT
ACGCGCAAGGTACAGCCGCTCCTGCAAGGCTGCGTGGTGGAATTGGACGTGCA
GGTCCTGCTGAAGTTCCTCCACCGCCTCACCAGCGGACAAAGCACCGGTGTATC
AGGTCCGTGTCATCCACTCTAAAGAGCTCGACTACGACCTACTGATGGCCCTAG
ATTCTTCATCAAAAACGCCTGAGACACTTGCCCAGGATTGAAACTCCCTGAAGG
GACCACCAGGGGCCCTGAGTTGTTCCTTCCCCCCGTGGCGAGCTGCCAGCCAGG
CTGTACCTGTGATCGAGGCTGGCGGGAAAATAGGCTTCGTGTGCTCAGGTCATG
GGAGGTGCAGGACAGCTCATGAAACGCCAACAATCGCACAATTCATGTCAAGC
TAATCAGCTATTTCCTCTTCACGAGCTGTAATTGTCCCAAAATTCTGGTCTACCG
GGGGTGATCCTTCGTGTACGGGCCCTTCCCTCAACCCTAGGTATGCGCGCATGC
GGTCGCCGCGCAACTCGCGCGAGGGCCGAGGGTTTGGGACGGGCCGTCCCGAA
ATGCAGTTGCACCCGGATGCGTGGCACCTTTTTTGCGATAATTTATGCAATGGA
CTGCTCTGCAAAATTCTGGCTCTGTCGCCAACCCTAGGATCAGCGGCGTAGGAT
TTCGTAATCATTCGTCCTGATGGGGAGCTACCGACTACCCTAATATCAGCCCGA
CTGCCTGACGCCAGCGTCCACTTTTGTGCACACATTCCATTCGTGCCCAAGACA
TTTCATTGTGGTGCGAAGCGTCCCCAGTTACGCTCACCTGTTTCCCGACCTCCTT
ACTGTTCTGTCGACAGAGCGGGCCCACAGGCCGGTCGCAGCC

SEQ ID NO:85
Chlorella vulgaris nitrate reductase 3'UTR

GCAGCAGCAGCTCGGATAGTATCGACACACTCTGGACGCTGGTCGTGTGATGG
ACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCCGCTTTTATC
AAACAGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGCGAGTTGCTAGCT
GCTTGTGCTATTTGCGAATACCACCCCCAGCATCCCCTTCCCTCGTTTCATATCG
CTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCTATCCCTCAGCGCTGCTC
CTGCTCCTGCTCACTGCCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCT
CCTGGTACTGCAACCTGTAAACCAGCACTGCAATGCTGATGCACGGGAAGTAGT
GGGATGGGAACACAAATGGAGGATCC

SEQ ID NO:86

Codon optimized *Chlorella protothecoides* (UTEX 250) stearoyl ACP desaturase transit peptide cDNA sequence.

ACTAGTATGGCCACCGCATCCACTTTCTCGGCGTTCAATGCCCGCTGCGGCGAC
CTGCGTCGCTCGGCGGGCTCCGGGCCCCGGCGCCCAGCGAGGCCCCTCCCCGTG
CGCGGGCGCGCC

SEQ ID NO:87

Codon-optimized coding sequence of *Ricinus communis* ACP-thioesterase with *Chlorella protothecoides* UTEX250 stearoyl-ACP desaturase transit peptide from pSZ1375

ACTAGTATGGCCACCGCATCCACTTTCTCGGCGTTCAATGCCCGCTGCGGCGAC
CTGCGTCGCTCGGCGGGCTCCGGGCCCCGGCGCCCAGCGAGGCCCCTCCCCGTG
CGCGGGCGCGCCCCGCTCCTACGAGGTGGGCATCAACAAGACCGCCACCGTGGA
GACCATCGCCAACCTGCTGCAGGAGGTGGGCTGCAACCACGCCCAGTCCGTGG
GCTTCTCCACCGACGGCTTCGCCACCACCACCTCCATGCGCAAGATGCACCTGA
TCTGGGTGACCGCCCGCATGCACATCGAGATCTACAAGTACCCCGCCTGGTCCG
ACGTGGTGGAGGTGGAGACCTGGTGCCAGTCCGAGGGCCGCATCGGCACCCGC
CGCGACTGGATCCTGACCGACTACGCCACCGGCCAGATCATCGGCCGCGCCACC
TCCAAGTGGGTGATGATGAACCAGGACACCCGCCGCCTGCAGAAGGTGACCGA
CGACGTGCGCGAGGAGTACCTGGTGTTCTGCCCCCGCGAGCTGCGCCTGGCCTT
CCCCGAGGAGAACAACCGCTCCTCCAAGAAGATCTCCAAGCTGGAGGACCCCG
CCCAGTACTCCAAGCTGGGCCTGGTGCCCCGCCGCGCCGACCTGGACATGAACC
AGCACGTGAACAACGTGACCTACATCGGCTGGGTGCTGGAGTCCATCCCCCAG
GAGATCATCGACACCCACGAGCTGCAGACCATCACCCTGGACTACCGCCGCGA
GTGCCAGCACGACGACATCGTGGACTCCCTGACCTCCGTGGAGCCCTCCGAGAA
CCTGGAGGCCGTGTCCGAGCTGCGCGGCACCAACGGCTCCGCCACCACCACCG
CCGGCGACGAGGACTGCCGCAACTTCCTGCACCTGCTGCGCCTGTCCGGCGACG
GCCTGGAGATCAACCGCGGCCGCACCGAGTGGCGCAAGAAGTCCGCCCGCATG
GACTACAAGGACCACGACGGCGACTACAAGGACCACGACATCGACTACAAGGA
CGACGACGACAAGTGAATCGAT

SEQ ID NO:88

Amino acid sequence of *Ricinus communis* ACP-thioesterase (Accession No. ABS30422.1)

MLKVPCCNATDPIQSLSSQCRFLTHFNNRPYFTRRPSIPTFFSSKNSSASLQAVVSDIS
SVESAACDSLANRLRLGKLTEDGFSYKEKFIVRSYEVGINKTATVETIANLLQEVGC
NHAQSVGFSTDGFATTTSMRKMHLIWVTARMHIEIYKYPAWSDVVEVETWCQSEG
RIGTRRDWILTDYATGQIIGRATSKWVMMNQDTRRLQKVTDDVREEYLVFCPREL
RLAFPEENNRSSKKISKLEDPAQYSKLGLVPRRADLDMNQHVNNVTYIGWVLESIP
QEIIDTHELQTITLDYRRECQHDDIVDSLTSVEPSENLEAVSELRGTNGSATTTAGDE
DCRNFLHLLRLSGDGLEINRGRTEWRKKSAR

SEQ ID NO:89

Chlamydomonas reinhardtii TUB2 promoter/5' UTR

CTTTCTTGCGCTATGACACTTCCAGCAAAAGGTAGGGCGGGCTGCGAGACGGCT
TCCCGGCGCTGCATGCAACACCGATGATGCTTCGACCCCCCGAAGCTCCTTCGG
GGCTGCATGGGCGCTCCGATGCCGCTCCAGGGCGAGCGCTGTTTAAATAGCCAG
GCCCCCGATTGCAAAGACATTATAGCGAGCTACCAAAGCCATATTCAAACACCT
AGATCACTACCACTTCTACACAGGCCACTCGAGCTTGTGATCGCACTCCGCTAA
GGGGGCGCCTCTTCCTCTTCGTTTCAGTCACAACCCGCAAAC

SEQ ID NO:90

FADc portion of the hairpin RNA expression cassette from pSZ1468

ACTAGTATGGCTATCAAGACGAACAGGCAGCCTGTGGAGAAGCCTCCGTTCAC
GATCGGGACGCTGCGCAAGGCCATCCCCGCGCACTGTTTCGAGCGCTCGGCGCT
TCGTAGCAGCATGTACCTGGCCTTTGACATCGCGGTCATGTCCCTGCTCTACGTC
GCGTCGACGTACATCGACCCTGCACCGGTGCCTACGTGGGTCAAGTACGGCATC
ATGTGGCCGCTCTACTGGTTCTTCCAGGTGTGTTTGAGGGTTTTGGTTGCCCGTA
TTGAGGTCCTGGTGGCGCGCATGGAGGAGAAGGCGCCTGTCCCGCTGACCCCCC
CGGCTACCCTCCCGGCACCTTCCAGGGCGCGTACGGGAAGAACCAGTAGAGCG
GCCACATGATGCCGTACTTGACCCACGTAGGCACCGGTGCAGGGTCGATGTACG
TCGACGCGACGTAGAGCAGGGACATGACCGCGATGTCAAAGGCCAGGTACATG
CTGCTACGAAGCGCCGAGCGCTCGAAACAGTGCGCGGGGATGGCCTTGCGCAG
CGTCCCGATCGTGAACGGAGGCTTCTCCACAGGCTGCCTGTTCGTCTTGATAGC
CAT

SEQ ID NO:91

Relevant portion of the FADc hairpin RNA expression cassette from pSZ1468

GCTCTTCGCCGCCGCCACTCCTGCTCGAGCGCGCCCGCGCGTGCGCCGCCAGCG
CCTTGGCCTTTTCGCCGCGCTCGTGCGCGTCGCTGATGTCCATCACCAGGTCCAT
GAGGTCTGCCTTGCGCCGGCTGAGCCACTGCTTCGTCCGGGCGGCCAAGAGGA
GCATGAGGGAGGACTCCTGGTCCAGGGTCCTGACGTGGTCGCGGCTCTGGGAG
CGGGCCAGCATCATCTGGCTCTGCCGCACCGAGGCCGCCTCCAACTGGTCCTCC
AGCAGCCGCAGTCGCCGCCGACCCTGGCAGAGGAAGACAGGTGAGGGGGGTAT
GAATTGTACAGAACAACCACGAGCCTTGTCTAGGCAGAATCCCTACCAGTCATG
GCTTTACCTGGATGACGGCCTGCGAACAGCTGTCCAGCGACCCTCGCTGCCGCC
GCTTCTCCCGCACGCTTCTTTCCAGCACCGTGATGGCGCGAGCCAGCGCCGCAC
GCTGGCGCTGCGCTTCGCCGATCTGAGGACAGTCGGGGAACTCTGATCAGTCTA
AACCCCCTTGCGCGTTAGTGTTGCCATCCTTTGCAGACCGGTGAGAGCCGACTT
GTTGTGCGCCACCCCCCACACCACCTCCTCCCAGACCAATTCTGTCACCTTTTTG
GCGAAGGCATCGGCCTCGGCCTGCAGAGAGGACAGCAGTGCCCAGCCGCTGGG
GGTTGGCGGATGCACGCTCAGGTACCCTTTCTTGCGCTATGACACTTCCAGCAA
AAGGTAGGGCGGGCTGCGAGACGGCTTCCCGGCGCTGCATGCAACACCGATGA
TGCTTCGACCCCCCGAAGCTCCTTCGGGGCTGCATGGGCGCTCCGATGCCGCTC
CAGGGCGAGCGCTGTTTAAATAGCCAGGCCCCCGATTGCAAAGACATTATAGC
GAGCTACCAAAGCCATATTCAAACACCTAGATCACTACCACTTCTACACAGGCC
ACTCGAGCTTGTGATCGCACTCCGCTAAGGGGGCGCCTCTTCCTCTTCGTTTCAG
TCACAACCCGCAAACGGCGCGCCATGCTGCTGCAGGCCTTCCTGTTCCTGCTGG
CCGGCTTCGCCGCCAAGATCAGCGCCTCCATGACGAACGAGACGTCCGACCGC

CCCCTGGTGCACTTCACCCCCAACAAGGGCTGGATGAACGACCCCAACGGCCTG
TGGTACGACGAGAAGGACGCCAAGTGGCACCTGTACTTCCAGTACAACCCGAA
CGACACCGTCTGGGGGACGCCCTTGTTCTGGGGCCACGCCACGTCCGACGACCT
GACCAACTGGGAGGACCAGCCCATCGCCATCGCCCCGAAGCGCAACGACTCCG
GCGCCTTCTCCGGCTCCATGGTGGTGGACTACAACAACACCTCCGGCTTCTTCA
ACGACACCATCGACCCGCGCCAGCGCTGCGTGGCCATCTGGACCTACAACACCC
CGGAGTCCGAGGAGCAGTACATCTCCTACAGCCTGGACGGCGGCTACACCTTCA
CCGAGTACCAGAAGAACCCCGTGCTGGCCGCCAACTCCACCCAGTTCCGCGACC
CGAAGGTCTTCTGGTACGAGCCCTCCCAGAAGTGGATCATGACCGCGGCCAAGT
CCCAGGACTACAAGATCGAGATCTACTCCTCCGACGACCTGAAGTCCTGGAAGC
TGGAGTCCGCGTTCGCCAACGAGGGCTTCCTCGGCTACCAGTACGAGTGCCCCG
GCCTGATCGAGGTCCCCACCGAGCAGGACCCCAGCAAGTCCTACTGGGTGATGT
TCATCTCCATCAACCCCGGCGCCCCGGCCGGCGGCTCCTTCAACCAGTACTTCG
TCGGCAGCTTCAACGGCACCCACTTCGAGGCCTTCGACAACCAGTCCCGCGTGG
TGGACTTCGGCAAGGACTACTACGCCCTGCAGACCTTCTTCAACACCGACCCGA
CCTACGGGAGCGCCCTGGGCATCGCGTGGGCCTCCAACTGGGAGTACTCCGCCT
TCGTGCCCACCAACCCCTGGCGCTCCTCCATGTCCCTCGTGCGCAAGTTCTCCCT
CAACACCGAGTACCAGGCCAACCCGGAGACGGAGCTGATCAACCTGAAGGCCG
AGCCGATCCTGAACATCAGCAACGCCGGCCCCTGGAGCCGGTTCGCCACCAAC
ACCACGTTGACGAAGGCCAACAGCTACAACGTCGACCTGTCCAACAGCACCGG
CACCCTGGAGTTCGAGCTGGTGTACGCCGTCAACACCACCCAGACGATCTCCAA
GTCCGTGTTCGCGGACCTCTCCCTCTGGTTCAAGGGCCTGGAGGACCCCGAGGA
GTACCTCCGCATGGGCTTCGAGGTGTCCGCGTCCTCCTTCTTCCTGGACCGCGG
GAACAGCAAGGTGAAGTTCGTGAAGGAGAACCCCTACTTCACCAACCGCATGA
GCGTGAACAACCAGCCCTTCAAGAGCGAGAACGACCTGTCCTACTACAAGGTG
TACGGCTTGCTGGACCAGAACATCCTGGAGCTGTACTTCAACGACGGCGACGTC
GTGTCCACCAACACCTACTTCATGACCACCGGGAACGCCCTGGGCTCCGTGAAC
ATGACGACGGGGGTGGACAACCTGTTCTACATCGACAAGTTCCAGGTGCGCGA
GGTCAAGTGACAATTGGCAGCAGCAGCTCGGATAGTATCGACACACTCTGGAC
GCTGGTCGTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATA
TCCCTGCCGCTTTTATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCT
TTTGCGAGTTGCTAGCTGCTTGTGCTATTTGCGAATACCACCCCCAGCATCCCCT
TCCCTCGTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCT
ATCCCTCAGCGCTGCTCCTGCTCCTGCTCACTGCCCCTCGCACAGCCTTGGTTTG
GGCTCCGCCTGTATTCTCCTGGTACTGCAACCTGTAAACCAGCACTGCAATGCT
GATGCACGGGAAGTAGTGGGATGGGAACACAAATGGAGGATCCCGCGTCTCGA
ACAGAGCGCGCAGAGGAACGCTGAAGGTCTCGCCTCTGTCGCACCTCAGCGCG
GCATACACCACAATAACCACCTGACGAATGCGCTTGGTTCTTCGTCCATTAGCG
AAGCGTCCGGTTCACACACGTGCCACGTTGGCGAGGTGGCAGGTGACAATGAT
CGGTGGAGCTGATGGTCGAAACGTTCACAGCCTAGGGATATCGAATTCGGCCG
ACAGGACGCGCGTCAAAGGTGCTGGTCGTGTATGCCCTGGCCGGCAGGTCGTTG
CTGCTGCTGGTTAGTGATTCCGCAACCCTGATTTTGGCGTCTTATTTTGGCGTGG
CAAACGCTGGCGCCCGCGAGCCGGGCCGGCGGCGATGCGGTGCCCCACGGCTG
CCGGAATCCAAGGGAGGCAAGAGCGCCCGGGTCAGTTGAAGGGCTTTACGCGC
AAGGTACAGCCGCTCCTGCAAGGCTGCGTGGTGGAATTGGACGTGCAGGTCCT
GCTGAAGTTCCTCCACCGCCTCACCAGCGGACAAAGCACCGGTGTATCAGGTCC
GTGTCATCCACTCTAAAGAGCTCGACTACGACCTACTGATGGCCCTAGATTCTT
CATCAAAAACGCCTGAGACACTTGCCCAGGATTGAAACTCCCTGAAGGGACCA

CCAGGGGCCCTGAGTTGTTCCTTCCCCCCGTGGCGAGCTGCCAGCCAGGCTGTA
CCTGTGATCGAGGCTGGCGGGAAAATAGGCTTCGTGTGCTCAGGTCATGGGAG
GTGCAGGACAGCTCATGAAACGCCAACAATCGCACAATTCATGTCAAGCTAAT
CAGCTATTTCCTCTTCACGAGCTGTAATTGTCCCAAAATTCTGGTCTACCGGGGG
TGATCCTTCGTGTACGGGCCCTTCCCTCAACCCTAGGTATGCGCGCATGCGGTC
GCCGCGCAACTCGCGCGAGGGCCGAGGGTTTGGGACGGGCCGTCCCGAAATGC
AGTTGCACCCGGATGCGTGGCACCTTTTTTGCGATAATTTATGCAATGGACTGC
TCTGCAAAATTCTGGCTCTGTCGCCAACCCTAGGATCAGCGGCGTAGGATTTCG
TAATCATTCGTCCTGATGGGGAGCTACCGACTACCCTAATATCAGCCCGACTGC
CTGACGCCAGCGTCCACTTTTGTGCACACATTCCATTCGTGCCCAAGACATTTCA
TTGTGGTGCGAAGCGTCCCCAGTTACGCTCACCTGTTTCCCGACCTCCTTACTGT
TCTGTCGACAGAGCGGGCCCACAGGCCGGTCGCAGCCACTAGTATGGCTATCA
AGACGAACAGGCAGCCTGTGGAGAAGCCTCCGTTCACGATCGGGACGCTGCGC
AAGGCCATCCCCGCGCACTGTTTCGAGCGCTCGGCGCTTCGTAGCAGCATGTAC
CTGGCCTTTGACATCGCGGTCATGTCCCTGCTCTACGTCGCGTCGACGTACATCG
ACCCTGCACCGGTGCCTACGTGGGTCAAGTACGGCATCATGTGGCCGCTCTACT
GGTTCTTCCAGGTGTGTTTGAGGGTTTTGGTTGCCCGTATTGAGGTCCTGGTGGC
GCGCATGGAGGAGAAGGCGCCTGTCCCGCTGACCCCCCCGGCTACCCTCCCGGC
ACCTTCCAGGGCGCGTACGGGAAGAACCAGTAGAGCGGCCACATGATGCCGTA
CTTGACCCACGTAGGCACCGGTGCAGGGTCGATGTACGTCGACGCGACGTAGA
GCAGGGACATGACCGCGATGTCAAAGGCCAGGTACATGCTGCTACGAAGCGCC
GAGCGCTCGAAACAGTGCGCGGGGATGGCCTTGCGCAGCGTCCCGATCGTGAA
CGGAGGCTTCTCCACAGGCTGCCTGTTCGTCTTGATAGCCATCTCGAGGCAGCA
GCAGCTCGGATAGTATCGACACACTCTGGACGCTGGTCGTGTGATGGACTGTTG
CCGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCCGCTTTTATCAAACAGC
CTCAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGCGAGTTGCTAGCTGCTTGTG
CTATTTGCGAATACCACCCCCAGCATCCCCTTCCCTCGTTTCATATCGCTTGCAT
CCCAACCGCAACTTATCTACGCTGTCCTGCTATCCCTCAGCGCTGCTCCTGCTCC
TGCTCACTGCCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCTCCTGGTA
CTGCAACCTGTAAACCAGCACTGCAATGCTGATGCACGGGAAGTAGTGGGATG
GGAACACAAATGGAAAGCTGTAGAGCTCTTGTTTTCCAGAAGGAGTTGCTCCTT
GAGCCTTTCATTCTCAGCCTCGATAACCTCCAAAGCCGCTCTAATTGTGGAGGG
GGTTCGAATTTAAAAGCTTGGAATGTTGGTTCGTGCGTCTGGAACAAGCCCAGA
CTTGTTGCTCACTGGGAAAAGGACCATCAGCTCCAAAAAACTTGCCGCTCAAAC
CGCGTACCTCTGCTTTCGCGCAATCTGCCCTGTTGAAATCGCCACCACATTCATA
TTGTGACGCTTGAGCAGTCTGTAATTGCCTCAGAATGTGGAATCATCTGCCCCC
TGTGCGAGCCCATGCCAGGCATGTCGCGGGCGAGGACACCCGCCACTCGTACA
GCAGACCATTATGCTACCTCACAATAGTTCATAACAGTGACCATATTTCTCGAA
GCTCCCCAACGAGCACCTCCATGCTCTGAGTGGCCACCCCCCGGCCCTGGTGCT
TGCGGAGGGCAGGTCAACCGGCATGGGGCTACCGAAATCCCCGACCGGATCCC
ACCACCCCCGCGATGGGAAGAATCTCTCCCCGGGATGTGGGCCCACCACCAGC
ACAACCTGCTGGCCCAGGCGAGCGTCAAACCATACCACACAAATATCCTTGGC
ATCGGCCCTGAATTCCTTCTGCCGCTCTGCTACCCGGTGCTTCTGTCCGAAGCAG
GGGTTGCTAGGGATCGCTCCGAGTCCGCAAACCCTTGTCGCGTGGCGGGGCTTG
TTCGAGCTTGAAGAGC

SEQ ID NO:92

FADc portion of the hairpin RNA expression cassette from pSZ1469

ACTAGTATGGCTATCAAGACGAACAGGCAGCCTGTGGAGAAGCCTCCGTTCAC
GATCGGGACGCTGCGCAAGGCCATCCCCGCGCACTGTTTCGAGCGCTCGGCGCT
TCGTAGCAGCATGTACCTGGCCTTTGACATCGCGGTCATGTCCCTGCTCTACGTC
GCGTCGACGTACATCGACCCTGCACCGGTGCCTACGTGGGTCAAGTACGGCATC
ATGTGGCCGCTCTACTGGTTCTTCCAGGTGTGTTTGAGGGTTTTGGTTGCCCGTA
TTGAGGTCCTGGTGGCGCGCATGGAGGAGAAGGCGCCTGTCCCGCTGACCCCCC
CGGCTACCCTCCCGGCACCTTCCAGGGCGCGTACGGGAAGAACCAGTAGAGCG
GCCACATGATGCCGTACTTGACCCACGTAGGCACCGGTGCAGGGTCGATGTACG
TCGACGCGACGTAGAGCAGGGACATGACCGCGATGTCAAAGGCCAGGTACATG
CTGCTACGAAGCGCCGAGCGCTCGAAACAGTGCGCGGGGATGGCCTTGCGCAG
CGTCCCGATCGTGAACGGAGGCTTCTCCACAGGCTGCCTGTTCGTCTTGATAGC
CAT

SEQ ID NO:93
Relevant portion of the FADc hairpin RNA expression cassette from pSZ1469

GCTCTTCGCCGCCGCCACTCCTGCTCGAGCGCGCCCGCGCGTGCGCCGCCAGCG
CCTTGGCCTTTTCGCCGCGCTCGTGCGCGTCGCTGATGTCCATCACCAGGTCCAT
GAGGTCTGCCTTGCGCCGGCTGAGCCACTGCTTCGTCCGGGCGGCCAAGAGGA
GCATGAGGGAGGACTCCTGGTCCAGGGTCCTGACGTGGTCGCGGCTCTGGGAG
CGGGCCAGCATCATCTGGCTCTGCCGCACCGAGGCCGCCTCCAACTGGTCCTCC
AGCAGCCGCAGTCGCCGCCGACCCTGGCAGAGGAAGACAGGTGAGGGGGGTAT
GAATTGTACAGAACAACCACGAGCCTTGTCTAGGCAGAATCCCTACCAGTCATG
GCTTTACCTGGATGACGGCCTGCGAACAGCTGTCCAGCGACCCTCGCTGCCGCC
GCTTCTCCCGCACGCTTCTTTCCAGCACCGTGATGGCGCGAGCCAGCGCCGCAC
GCTGGCGCTGCGCTTCGCCGATCTGAGGACAGTCGGGGAACTCTGATCAGTCTA
AACCCCCTTGCGCGTTAGTGTTGCCATCCTTTGCAGACCGGTGAGAGCCGACTT
GTTGTGCGCCACCCCCCACACCACCTCCTCCCAGACCAATTCTGTCACCTTTTTG
GCGAAGGCATCGGCCTCGGCCTGCAGAGAGGACAGCAGTGCCCAGCCGCTGGG
GGTTGGCGGATGCACGCTCAGGTACCCTTTCTTGCGCTATGACACTTCCAGCAA
AAGGTAGGGCGGGCTGCGAGACGGCTTCCCGGCGCTGCATGCAACACCGATGA
TGCTTCGACCCCCCGAAGCTCCTTCGGGGCTGCATGGGCGCTCCGATGCCGCTC
CAGGGCGAGCGCTGTTTAAATAGCCAGGCCCCCGATTGCAAAGACATTATAGC
GAGCTACCAAAGCCATATTCAAACACCTAGATCACTACCACTTCTACACAGGCC
ACTCGAGCTTGTGATCGCACTCCGCTAAGGGGGCGCCTCTTCCTCTTCGTTTCAG
TCACAACCCGCAAACGGCGCGCCATGCTGCTGCAGGCCTTCCTGTTCCTGCTGG
CCGGCTTCGCCGCCAAGATCAGCGCCTCCATGACGAACGAGACGTCCGACCGC
CCCCTGGTGCACTTCACCCCCAACAAGGGCTGGATGAACGACCCCAACGGCCTG
TGGTACGACGAGAAGGACGCCAAGTGGCACCTGTACTTCCAGTACAACCCGAA
CGACACCGTCTGGGGGACGCCCTTGTTCTGGGGCCACGCCACGTCCGACGACCT
GACCAACTGGGAGGACCAGCCCATCGCCATCGCCCCGAAGCGCAACGACTCCG
GCGCCTTCTCCGGCTCCATGGTGGTGGACTACAACAACACCTCCGGCTTCTTCA
ACGACACCATCGACCCGCGCCAGCGCTGCGTGGCCATCTGGACCTACAACACCC
CGGAGTCCGAGGAGCAGTACATCTCCTACAGCCTGGACGGCGGCTACACCTTCA
CCGAGTACCAGAAGAACCCCGTGCTGGCCGCCAACTCCACCCAGTTCCGCGACC
CGAAGGTCTTCTGGTACGAGCCCTCCCAGAAGTGGATCATGACCGCGGCCAAGT
CCCAGGACTACAAGATCGAGATCTACTCCTCCGACGACCTGAAGTCCTGGAAGC

TGGAGTCCGCGTTCGCCAACGAGGGCTTCCTCGGCTACCAGTACGAGTGCCCCG
GCCTGATCGAGGTCCCCACCGAGCAGGACCCCAGCAAGTCCTACTGGGTGATGT
TCATCTCCATCAACCCCGGCGCCCCGGCCGGCGGCTCCTTCAACCAGTACTTCG
TCGGCAGCTTCAACGGCACCCACTTCGAGGCCTTCGACAACCAGTCCCGCGTGG
TGGACTTCGGCAAGGACTACTACGCCCTGCAGACCTTCTTCAACACCGACCCGA
CCTACGGGAGCGCCCTGGGCATCGCGTGGGCCTCCAACTGGGAGTACTCCGCCT
TCGTGCCCACCAACCCCTGGCGCTCCTCCATGTCCCTCGTGCGCAAGTTCTCCCT
CAACACCGAGTACCAGGCCAACCCGGAGACGGAGCTGATCAACCTGAAGGCCG
AGCCGATCCTGAACATCAGCAACGCCGGCCCCTGGAGCCGGTTCGCCACCAAC
ACCACGTTGACGAAGGCCAACAGCTACAACGTCGACCTGTCCAACAGCACCGG
CACCCTGGAGTTCGAGCTGGTGTACGCCGTCAACACCACCCAGACGATCTCCAA
GTCCGTGTTCGCGGACCTCTCCCTCTGGTTCAAGGGCCTGGAGGACCCCGAGGA
GTACCTCCGCATGGGCTTCGAGGTGTCCGCGTCCTCCTTCTTCCTGGACCGCGG
GAACAGCAAGGTGAAGTTCGTGAAGGAGAACCCCTACTTCACCAACCGCATGA
GCGTGAACAACCAGCCCTTCAAGAGCGAGAACGACCTGTCCTACTACAAGGTG
TACGGCTTGCTGGACCAGAACATCCTGGAGCTGTACTTCAACGACGGCGACGTC
GTGTCCACCAACACCTACTTCATGACCACCGGGAACGCCCTGGGCTCCGTGAAC
ATGACGACGGGGGTGGACAACCTGTTCTACATCGACAAGTTCCAGGTGCGCGA
GGTCAAGTGACAATTGGCAGCAGCAGCTCGGATAGTATCGACACACTCTGGAC
GCTGGTCGTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATA
TCCCTGCCGCTTTTATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCT
TTTGCGAGTTGCTAGCTGCTTGTGCTATTTGCGAATACCACCCCCAGCATCCCCT
TCCCTCGTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCT
ATCCCTCAGCGCTGCTCCTGCTCCTGCTCACTGCCCCTCGCACAGCCTTGGTTTG
GGCTCCGCCTGTATTCTCCTGGTACTGCAACCTGTAAACCAGCACTGCAATGCT
GATGCACGGGAAGTAGTGGGATGGGAACACAAATGGAGGATCCCGCGTCTCGA
ACAGAGCGCGCAGAGGAACGCTGAAGGTCTCGCCTCTGTCGCACCTCAGCGCG
GCATACACCACAATAACCACCTGACGAATGCGCTTGGTTCTTCGTCCATTAGCG
AAGCGTCCGGTTCACACACGTGCCACGTTGGCGAGGTGGCAGGTGACAATGAT
CGGTGGAGCTGATGGTCGAAACGTTCACAGCCTAGGGATATCGAATTCCTTTCT
TGCGCTATGACACTTCCAGCAAAAGGTAGGGCGGGCTGCGAGACGGCTTCCCG
GCGCTGCATGCAACACCGATGATGCTTCGACCCCCCGAAGCTCCTTCGGGGCTG
CATGGGCGCTCCGATGCCGCTCCAGGGCGAGCGCTGTTTAAATAGCCAGGCCCC
CGATTGCAAAGACATTATAGCGAGCTACCAAAGCCATATTCAAACACCTAGATC
ACTACCACTTCTACACAGGCCACTCGAGCTTGTGATCGCACTCCGCTAAGGGGG
CGCCTCTTCCTCTTCGTTTCAGTCACAACCCGCAAACACTAGTATGGCTATCAAG
ACGAACAGGCAGCCTGTGGAGAAGCCTCCGTTCACGATCGGGACGCTGCGCAA
GGCCATCCCCGCGCACTGTTTCGAGCGCTCGGCGCTTCGTAGCAGCATGTACCT
GGCCTTTGACATCGCGGTCATGTCCCTGCTCTACGTCGCGTCGACGTACATCGA
CCCTGCACCGGTGCCTACGTGGGTCAAGTACGGCATCATGTGGCCGCTCTACTG
GTTCTTCCAGGTGTGTTTGAGGGTTTTGGTTGCCCGTATTGAGGTCCTGGTGGCG
CGCATGGAGGAGAAGGCGCCTGTCCCGCTGACCCCCCCGGCTACCCTCCCGGCA
CCTTCCAGGGCGCGTACGGGAAGAACCAGTAGAGCGGCCACATGATGCCGTAC
TTGACCCACGTAGGCACCGGTGCAGGGTCGATGTACGTCGACGCGACGTAGAG
CAGGGACATGACCGCGATGTCAAAGGCCAGGTACATGCTGCTACGAAGCGCCG
AGCGCTCGAAACAGTGCGCGGGGATGGCCTTGCGCAGCGTCCCGATCGTGAAC
GGAGGCTTCTCCACAGGCTGCCTGTTCGTCTTGATAGCCATCTCGAGGCAGCAG
CAGCTCGGATAGTATCGACACACTCTGGACGCTGGTCGTGTGATGGACTGTTGC

CGCCACACTTGCTGCCTTGACCTGTGAATATCCCTGCCGCTTTTATCAAACAGCC
TCAGTGTGTTTGATCTTGTGTGTACGCGCTTTTGCGAGTTGCTAGCTGCTTGTGC
TATTTGCGAATACCACCCCCAGCATCCCCTTCCCTCGTTTCATATCGCTTGCATC
CCAACCGCAACTTATCTACGCTGTCCTGCTATCCCTCAGCGCTGCTCCTGCTCCT
GCTCACTGCCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCTCCTGGTAC
TGCAACCTGTAAACCAGCACTGCAATGCTGATGCACGGGAAGTAGTGGGATGG
GAACACAAATGGAAAGCTGTAGAGCTCTTGTTTTCCAGAAGGAGTTGCTCCTTG
AGCCTTTCATTCTCAGCCTCGATAACCTCCAAAGCCGCTCTAATTGTGGAGGGG
GTTCGAATTTAAAAGCTTGGAATGTTGGTTCGTGCGTCTGGAACAAGCCCAGAC
TTGTTGCTCACTGGGAAAAGGACCATCAGCTCCAAAAAACTTGCCGCTCAAACC
GCGTACCTCTGCTTTCGCGCAATCTGCCCTGTTGAAATCGCCACCACATTCATAT
TGTGACGCTTGAGCAGTCTGTAATTGCCTCAGAATGTGGAATCATCTGCCCCCT
GTGCGAGCCCATGCCAGGCATGTCGCGGGCGAGGACACCCGCCACTCGTACAG
CAGACCATTATGCTACCTCACAATAGTTCATAACAGTGACCATATTTCTCGAAG
CTCCCCAACGAGCACCTCCATGCTCTGAGTGGCCACCCCCCGGCCCTGGTGCTT
GCGGAGGGCAGGTCAACCGGCATGGGGCTACCGAAATCCCCGACCGGATCCCA
CCACCCCCGCGATGGGAAGAATCTCTCCCCGGGATGTGGGCCCACCACCAGCA
CAACCTGCTGGCCCAGGCGAGCGTCAAACCATACCACACAAATATCCTTGGCAT
CGGCCCTGAATTCCTTCTGCCGCTCTGCTACCCGGTGCTTCTGTCCGAAGCAGG
GGTTGCTAGGGATCGCTCCGAGTCCGCAAACCCTTGTCGCGTGGCGGGGCTTGT
TCGAGCTTGAAGAGC

SEQ ID NO:94

FADc portion of the hairpin RNA expression cassette from pSZ1470

ACTAGTTCACTTGTGGAACCAGAGCGCGGAGTCGTCCTCGGGCGCGTCCGGGAC
GACGTAGCGGCAGTCGCGCCAGTCCTCCCACAGGGCGCGGCCGACCCAGCGGC
TGTCGGACTGGTAGTACTTGCCCAGGATGGGCCTGATGGCGGCGGAGGCCTCCT
CGGCGTGGTAGTGCGGGATGGTGCTGAAGAGGTGGTGCAGCACGTGGGTGTCG
GAGATGTGGTGCAGGATGTTGTCCATGAAGGGCGGGCCCATGGAGCGGTCCAC
GGTGGCCATGGCGCCGCGCAGCCAGTCCCAGTCCTTCTCGAAGTAGTGCGGCAG
CGCCGGGTGCGTGTGCTGGAGCAGCGTGATGAGCACGAGCCACATGTTCACGA
TCAGGTAGGGCACCACGTAGGTCTTGACCAGCCAGGCCCAGCCCATGGTGCGG
CCCAGCACGCTGAGCCCGCTGAGCACCGCCACCAGCGCCAGGTCGGAGATGAC
CACCTCGATGCGCTCGCGCTTGCTGAAGATGGGCGACCACGGGTCAAAGTGGTT
GGCGAAGCGCGGGTACGGCCGCGAGGCGACGTTGAACATGAGGTACAGCGGCC
AGCCCAGGGTCAGGGTGACCAGCACCTTGCCCATGCGGATGGGCAGCCACTCC
TCCCACTCCAGGCCCTCGTGCGCCACTGCGCGGTGCGGCGGCACAAACACCTCG
TCCTTGTCCAGGCACCCCGTGTTGGAGTGGTGGCGGCGGTGCGAGTGCTTCCAG
GAGTAGTAGGGCACCAGCAGCAGGCTGTGGAACACCAGGCCCACGCCGTCGTT
GATGGCCTGGCTGGAGGAAAAGGCCTGGTGGCCGCACTCGTGCGCGCACACCC
AGACACCCGTGCCGAAGGCGCCCTGGAAGGTGCCGGGAGGGTAGCCGGGGGG
GTCAGCGGGACAGGCGCCTTCTCCTCCATGCGCGCCACCAGGACCTCAATACGG
GCAACCAAAACCCTCAAACACACCTGGAAGAACCAGTAGAGCGGCCACATGAT
GCCGTACTTGACCCACGTAGGCACCGGTGCAGGGTCGATGTACGTCGACGCGA
CGTAGAGCAGGGACATGACCGCGATGTCAAAGGCCAGGTACATGCTGCTACGA
AGCGCCGAGCGCTCGAAACAGTGCGCGGGGATGGCCTTGCGCAGCGTCCCGAT
CGTGAACGGAGGCTTCTCCACAGGCTGCCTGTTCGTCTTGATAGCCAT

SEQ ID NO:95

Relevant portion of the FADc hairpin RNA expression cassette from pSZ1470

GCTCTTCGCCGCCGCCACTCCTGCTCGAGCGCGCCCGCGCGTGCGCCGCCAGCG
CCTTGGCCTTTTCGCCGCGCTCGTGCGCGTCGCTGATGTCCATCACCAGGTCCAT
GAGGTCTGCCTTGCGCCGGCTGAGCCACTGCTTCGTCCGGGCGGCCAAGAGGA
GCATGAGGGAGGACTCCTGGTCCAGGGTCCTGACGTGGTCGCGGCTCTGGGAG
CGGGCCAGCATCATCTGGCTCTGCCGCACCGAGGCCGCCTCCAACTGGTCCTCC
AGCAGCCGCAGTCGCCGCCGACCCTGGCAGAGGAAGACAGGTGAGGGGGGTAT
GAATTGTACAGAACAACCACGAGCCTTGTCTAGGCAGAATCCCTACCAGTCATG
GCTTTACCTGGATGACGGCCTGCGAACAGCTGTCCAGCGACCCTCGCTGCCGCC
GCTTCTCCCGCACGCTTCTTTCCAGCACCGTGATGGCGCGAGCCAGCGCCGCAC
GCTGGCGCTGCGCTTCGCCGATCTGAGGACAGTCGGGGAACTCTGATCAGTCTA
AACCCCCTTGCGCGTTAGTGTTGCCATCCTTTGCAGACCGGTGAGAGCCGACTT
GTTGTGCGCCACCCCCCACACCACCTCCTCCCAGACCAATTCTGTCACCTTTTTG
GCGAAGGCATCGGCCTCGGCCTGCAGAGAGGACAGCAGTGCCCAGCCGCTGGG
GGTTGGCGGATGCACGCTCAGGTACCCTTTCTTGCGCTATGACACTTCCAGCAA
AAGGTAGGGCGGGCTGCGAGACGGCTTCCCGGCGCTGCATGCAACACCGATGA
TGCTTCGACCCCCCGAAGCTCCTTCGGGGCTGCATGGGCGCTCCGATGCCGCTC
CAGGGCGAGCGCTGTTTAAATAGCCAGGCCCCCGATTGCAAAGACATTATAGC
GAGCTACCAAAGCCATATTCAAACACCTAGATCACTACCACTTCTACACAGGCC
ACTCGAGCTTGTGATCGCACTCCGCTAAGGGGGCGCCTCTTCCTCTTCGTTTCAG
TCACAACCCGCAAACGGCGCGCCATGCTGCTGCAGGCCTTCCTGTTCCTGCTGG
CCGGCTTCGCCGCCAAGATCAGCGCCTCCATGACGAACGAGACGTCCGACCGC
CCCCTGGTGCACTTCACCCCCAACAAGGGCTGGATGAACGACCCCAACGGCCTG
TGGTACGACGAGAAGGACGCCAAGTGGCACCTGTACTTCCAGTACAACCCGAA
CGACACCGTCTGGGGGACGCCCTTGTTCTGGGGCCACGCCACGTCCGACGACCT
GACCAACTGGGAGGACCAGCCCATCGCCATCGCCCCGAAGCGCAACGACTCCG
GCGCCTTCTCCGGCTCCATGGTGGTGGACTACAACAACACCTCCGGCTTCTTCA
ACGACACCATCGACCCGCGCCAGCGCTGCGTGGCCATCTGGACCTACAACACCC
CGGAGTCCGAGGAGCAGTACATCTCCTACAGCCTGGACGGCGGCTACACCTTCA
CCGAGTACCAGAAGAACCCCGTGCTGGCCGCCAACTCCACCCAGTTCCGCGACC
CGAAGGTCTTCTGGTACGAGCCCTCCCAGAAGTGGATCATGACCGCGGCCAAGT
CCCAGGACTACAAGATCGAGATCTACTCCTCCGACGACCTGAAGTCCTGGAAGC
TGGAGTCCGCGTTCGCCAACGAGGGCTTCCTCGGCTACCAGTACGAGTGCCCCG
GCCTGATCGAGGTCCCCACCGAGCAGGACCCCAGCAAGTCCTACTGGGTGATGT
TCATCTCCATCAACCCCGGCGCCCCGGCCGGCGGCTCCTTCAACCAGTACTTCG
TCGGCAGCTTCAACGGCACCCACTTCGAGGCCTTCGACAACCAGTCCCGCGTGG
TGGACTTCGGCAAGGACTACTACGCCCTGCAGACCTTCTTCAACACCGACCCGA
CCTACGGGAGCGCCCTGGGCATCGCGTGGGCCTCCAACTGGGAGTACTCCGCCT
TCGTGCCCACCAACCCCTGGCGCTCCTCCATGTCCCTCGTGCGCAAGTTCTCCCT
CAACACCGAGTACCAGGCCAACCCGGAGACGGAGCTGATCAACCTGAAGGCCG
AGCCGATCCTGAACATCAGCAACGCCGGCCCCTGGAGCCGGTTCGCCACCAAC
ACCACGTTGACGAAGGCCAACAGCTACAACGTCGACCTGTCCAACAGCACCGG
CACCCTGGAGTTCGAGCTGGTGTACGCCGTCAACACCACCCAGACGATCTCCAA
GTCCGTGTTCGCGGACCTCTCCCTCTGGTTCAAGGGCCTGGAGGACCCCGAGGA
GTACCTCCGCATGGGCTTCGAGGTGTCCGCGTCCTCCTTCTTCCTGGACCGCGG

GAACAGCAAGGTGAAGTTCGTGAAGGAGAACCCCTACTTCACCAACCGCATGA
GCGTGAACAACCAGCCCTTCAAGAGCGAGAACGACCTGTCCTACTACAAGGTG
TACGGCTTGCTGGACCAGAACATCCTGGAGCTGTACTTCAACGACGGCGACGTC
GTGTCCACCAACACCTACTTCATGACCACCGGGAACGCCCTGGGCTCCGTGAAC
ATGACGACGGGGGTGGACAACCTGTTCTACATCGACAAGTTCCAGGTGCGCGA
GGTCAAGTGACAATTGGCAGCAGCAGCTCGGATAGTATCGACACACTCTGGAC
GCTGGTCGTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATA
TCCCTGCCGCTTTTATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCT
TTTGCGAGTTGCTAGCTGCTTGTGCTATTTGCGAATACCACCCCCAGCATCCCCT
TCCCTCGTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCT
ATCCCTCAGCGCTGCTCCTGCTCCTGCTCACTGCCCCTCGCACAGCCTTGGTTTG
GGCTCCGCCTGTATTCTCCTGGTACTGCAACCTGTAAACCAGCACTGCAATGCT
GATGCACGGGAAGTAGTGGGATGGGAACACAAATGGAGGATCCCGCGTCTCGA
ACAGAGCGCGCAGAGGAACGCTGAAGGTCTCGCCTCTGTCGCACCTCAGCGCG
GCATACACCACAATAACCACCTGACGAATGCGCTTGGTTCTTCGTCCATTAGCG
AAGCGTCCGGTTCACACACGTGCCACGTTGGCGAGGTGGCAGGTGACAATGAT
CGGTGGAGCTGATGGTCGAAACGTTCACAGCCTAGGGATATCGAATTCGGCCG
ACAGGACGCGCGTCAAAGGTGCTGGTCGTGTATGCCCTGGCCGGCAGGTCGTTG
CTGCTGCTGGTTAGTGATTCCGCAACCCTGATTTTGGCGTCTTATTTTGGCGTGG
CAAACGCTGGCGCCCGCGAGCCGGGCCGGCGGCGATGCGGTGCCCCACGGCTG
CCGGAATCCAAGGGAGGCAAGAGCGCCCGGGTCAGTTGAAGGGCTTTACGCGC
AAGGTACAGCCGCTCCTGCAAGGCTGCGTGGTGGAATTGGACGTGCAGGTCCT
GCTGAAGTTCCTCCACCGCCTCACCAGCGGACAAAGCACCGGTGTATCAGGTCC
GTGTCATCCACTCTAAAGAGCTCGACTACGACCTACTGATGGCCCTAGATTCTT
CATCAAAAACGCCTGAGACACTTGCCCAGGATTGAAACTCCCTGAAGGGACCA
CCAGGGGCCCTGAGTTGTTCCTTCCCCCCGTGGCGAGCTGCCAGCCAGGCTGTA
CCTGTGATCGAGGCTGGCGGGAAAATAGGCTTCGTGTGCTCAGGTCATGGGAG
GTGCAGGACAGCTCATGAAACGCCAACAATCGCACAATTCATGTCAAGCTAAT
CAGCTATTTCCTCTTCACGAGCTGTAATTGTCCCAAAATTCTGGTCTACCGGGGG
TGATCCTTCGTGTACGGGCCCTTCCCTCAACCCTAGGTATGCGCGCATGCGGTC
GCCGCGCAACTCGCGCGAGGGCCGAGGGTTTGGGACGGGCCGTCCCGAAATGC
AGTTGCACCCGGATGCGTGGCACCTTTTTTGCGATAATTTATGCAATGGACTGC
TCTGCAAAATTCTGGCTCTGTCGCCAACCCTAGGATCAGCGGCGTAGGATTTCG
TAATCATTCGTCCTGATGGGGAGCTACCGACTACCCTAATATCAGCCCGACTGC
CTGACGCCAGCGTCCACTTTTGTGCACACATTCCATTCGTGCCCAAGACATTTCA
TTGTGGTGCGAAGCGTCCCCAGTTACGCTCACCTGTTTCCCGACCTCCTTACTGT
TCTGTCGACAGAGCGGGCCCACAGGCCGGTCGCAGCCACTAGTTCACTTGTGGA
ACCAGAGCGCGGAGTCGTCCTCGGGCGCGTCCGGGACGACGTAGCGGCAGTCG
CGCCAGTCCTCCCACAGGGCGCGGCCGACCCAGCGGCTGTCGGACTGGTAGTA
CTTGCCCAGGATGGGCCTGATGGCGGCGGAGGCCTCCTCGGCGTGGTAGTGCG
GGATGGTGCTGAAGAGGTGGTGCAGCACGTGGGTGTCGGAGATGTGGTGCAGG
ATGTTGTCCATGAAGGGCGGGCCCATGGAGCGGTCCACGGTGGCCATGGCGCC
GCGCAGCCAGTCCCAGTCCTTCTCGAAGTAGTGCGGCAGCGCCGGGTGCGTGTG
CTGGAGCAGCGTGATGAGCACGAGCCACATGTTCACGATCAGGTAGGGCACCA
CGTAGGTCTTGACCAGCCAGGCCCAGCCCATGGTGCGGCCCAGCACGCTGAGC
CCGCTGAGCACCGCCACCAGCGCCAGGTCGGAGATGACCACCTCGATGCGCTC
GCGCTTGCTGAAGATGGGCGACCACGGGTCAAAGTGGTTGGCGAAGCGCGGGT
ACGGCCGCGAGGCGACGTTGAACATGAGGTACAGCGGCCAGCCCAGGGTCAGG

GTGACCAGCACCTTGCCCATGCGGATGGGCAGCCACTCCTCCCACTCCAGGCCC
TCGTGCGCCACTGCGCGGTGCGGCGGCACAAACACCTCGTCCTTGTCCAGGCAC
CCCGTGTTGGAGTGGTGGCGGCGGTGCGAGTGCTTCCAGGAGTAGTAGGGCAC
CAGCAGCAGGCTGTGGAACACCAGGCCCACGCCGTCGTTGATGGCCTGGCTGG
AGGAAAAGGCCTGGTGGCCGCACTCGTGCGCGCACACCCAGACACCCGTGCCG
AAGGCGCCCTGGAAGGTGCCGGGAGGGTAGCCGGGGGGGTCAGCGGGACAGG
CGCCTTCTCCTCCATGCGCGCCACCAGGACCTCAATACGGGCAACCAAAACCCT
CAAACACACCTGGAAGAACCAGTAGAGCGGCCACATGATGCCGTACTTGACCC
ACGTAGGCACCGGTGCAGGGTCGATGTACGTCGACGCGACGTAGAGCAGGGAC
ATGACCGCGATGTCAAAGGCCAGGTACATGCTGCTACGAAGCGCCGAGCGCTC
GAAACAGTGCGCGGGGATGGCCTTGCGCAGCGTCCCGATCGTGAACGGAGGCT
TCTCCACAGGCTGCCTGTTCGTCTTGATAGCCATCTCGAGGCAGCAGCAGCTCG
GATAGTATCGACACACTCTGGACGCTGGTCGTGTGATGGACTGTTGCCGCCACA
CTTGCTGCCTTGACCTGTGAATATCCCTGCCGCTTTTATCAAACAGCCTCAGTGT
GTTTGATCTTGTGTGTACGCGCTTTTGCGAGTTGCTAGCTGCTTGTGCTATTTGC
GAATACCACCCCCAGCATCCCCTTCCCTCGTTTCATATCGCTTGCATCCCAACCG
CAACTTATCTACGCTGTCCTGCTATCCCTCAGCGCTGCTCCTGCTCCTGCTCACT
GCCCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCTCCTGGTACTGCAACC
TGTAAACCAGCACTGCAATGCTGATGCACGGGAAGTAGTGGGATGGGAACACA
AATGGAAAGCTGTAGAGCTCTTGTTTTCCAGAAGGAGTTGCTCCTTGAGCCTTT
CATTCTCAGCCTCGATAACCTCCAAAGCCGCTCTAATTGTGGAGGGGGTTCGAA
TTTAAAAGCTTGGAATGTTGGTTCGTGCGTCTGGAACAAGCCCAGACTTGTTGC
TCACTGGGAAAAGGACCATCAGCTCCAAAAAACTTGCCGCTCAAACCGCGTAC
CTCTGCTTTCGCGCAATCTGCCCTGTTGAAATCGCCACCACATTCATATTGTGAC
GCTTGAGCAGTCTGTAATTGCCTCAGAATGTGGAATCATCTGCCCCCTGTGCGA
GCCCATGCCAGGCATGTCGCGGGCGAGGACACCCGCCACTCGTACAGCAGACC
ATTATGCTACCTCACAATAGTTCATAACAGTGACCATATTTCTCGAAGCTCCCC
AACGAGCACCTCCATGCTCTGAGTGGCCACCCCCCGGCCCTGGTGCTTGCGGAG
GGCAGGTCAACCGGCATGGGGCTACCGAAATCCCCGACCGGATCCCACCACCC
CCGCGATGGGAAGAATCTCTCCCCGGGATGTGGGCCCACCACCAGCACAACCT
GCTGGCCCAGGCGAGCGTCAAACCATACCACACAAATATCCTTGGCATCGGCCC
TGAATTCCTTCTGCCGCTCTGCTACCCGGTGCTTCTGTCCGAAGCAGGGGTTGCT
AGGGATCGCTCCGAGTCCGCAAACCCTTGTCGCGTGGCGGGGCTTGTTCGAGCT
TGAAGAGC

SEQ ID NO:96

FADc portion of the hairpin RNA expression cassette from pSZ1471

ACTAGTTCACTTGTGGAACCAGAGCGCGGAGTCGTCCTCGGGCGCGTCCGGGAC
GACGTAGCGGCAGTCGCGCCAGTCCTCCCACAGGGCGCGGCCGACCCAGCGGC
TGTCGGACTGGTAGTACTTGCCCAGGATGGGCCTGATGGCGGCGGAGGCCTCCT
CGGCGTGGTAGTGCGGGATGGTGCTGAAGAGGTGGTGCAGCACGTGGGTGTCG
GAGATGTGGTGCAGGATGTTGTCCATGAAGGGCGGGCCCATGGAGCGGTCCAC
GGTGGCCATGGCGCCGCGCAGCCAGTCCCAGTCCTTCTCGAAGTAGTGCGGCAG
CGCCGGGTGCGTGTGCTGGAGCAGCGTGATGAGCACGAGCCACATGTTCACGA
TCAGGTAGGGCACCACGTAGGTCTTGACCAGCCAGGCCCAGCCCATGGTGCGG
CCCAGCACGCTGAGCCCGCTGAGCACCGCCACCAGCGCCAGGTCGGAGATGAC
CACCTCGATGCGCTCGCGCTTGCTGAAGATGGGCGACCACGGGTCAAAGTGGTT

GGCGAAGCGCGGGTACGGCCGCGAGGCGACGTTGAACATGAGGTACAGCGGCC
AGCCCAGGGTCAGGGTGACCAGCACCTTGCCCATGCGGATGGGCAGCCACTCC
TCCCACTCCAGGCCCTCGTGCGCCACTGCGCGGTGCGGCGGCACAAACACCTCG
TCCTTGTCCAGGCACCCCGTGTTGGAGTGGTGGCGGCGGTGCGAGTGCTTCCAG
GAGTAGTAGGGCACCAGCAGCAGGCTGTGGAACACCAGGCCCACGCCGTCGTT
GATGGCCTGGCTGGAGGAAAAGGCCTGGTGGCCGCACTCGTGCGCGCACACCC
AGACACCCGTGCCGAAGGCGCCCTGGAAGGTGCCGGGAGGGTAGCCGGGGGG
GTCAGCGGGACAGGCGCCTTCTCCTCCATGCGCGCCACCAGGACCTCAATACGG
GCAACCAAAACCCTCAAACACACCTGGAAGAACCAGTAGAGCGGCCACATGAT
GCCGTACTTGACCCACGTAGGCACCGGTGCAGGGTCGATGTACGTCGACGCGA
CGTAGAGCAGGGACATGACCGCGATGTCAAAGGCCAGGTACATGCTGCTACGA
AGCGCCGAGCGCTCGAAACAGTGCGCGGGGATGGCCTTGCGCAGCGTCCCGAT
CGTGAACGGAGGCTTCTCCACAGGCTGCCTGTTCGTCTTGATAGCCAT

SEQ ID NO:97
Relevant portion of the FADc hairpin RNA expression cassette from pSZ1471

GCTCTTCGCCGCCGCCACTCCTGCTCGAGCGCGCCCGCGCGTGCGCCGCCAGCG
CCTTGGCCTTTTCGCCGCGCTCGTGCGCGTCGCTGATGTCCATCACCAGGTCCAT
GAGGTCTGCCTTGCGCCGGCTGAGCCACTGCTTCGTCCGGGCGGCCAAGAGGA
GCATGAGGGAGGACTCCTGGTCCAGGGTCCTGACGTGGTCGCGGCTCTGGGAG
CGGGCCAGCATCATCTGGCTCTGCCGCACCGAGGCCGCCTCCAACTGGTCCTCC
AGCAGCCGCAGTCGCCGCCGACCCTGGCAGAGGAAGACAGGTGAGGGGGGTAT
GAATTGTACAGAACAACCACGAGCCTTGTCTAGGCAGAATCCCTACCAGTCATG
GCTTTACCTGGATGACGGCCTGCGAACAGCTGTCCAGCGACCCTCGCTGCCGCC
GCTTCTCCCGCACGCTTCTTTCCAGCACCGTGATGGCGCGAGCCAGCGCCGCAC
GCTGGCGCTGCGCTTCGCCGATCTGAGGACAGTCGGGGAACTCTGATCAGTCTA
AACCCCCTTGCGCGTTAGTGTTGCCATCCTTTGCAGACCGGTGAGAGCCGACTT
GTTGTGCGCCACCCCCCACACCACCTCCTCCCAGACCAATTCTGTCACCTTTTTG
GCGAAGGCATCGGCCTCGGCCTGCAGAGAGGACAGCAGTGCCCAGCCGCTGGG
GGTTGGCGGATGCACGCTCAGGTACCCTTTCTTGCGCTATGACACTTCCAGCAA
AAGGTAGGGCGGGCTGCGAGACGGCTTCCCGGCGCTGCATGCAACACCGATGA
TGCTTCGACCCCCCGAAGCTCCTTCGGGGCTGCATGGGCGCTCCGATGCCGCTC
CAGGGCGAGCGCTGTTTAAATAGCCAGGCCCCCGATTGCAAAGACATTATAGC
GAGCTACCAAAGCCATATTCAAACACCTAGATCACTACCACTTCTACACAGGCC
ACTCGAGCTTGTGATCGCACTCCGCTAAGGGGGCGCCTCTTCCTCTTCGTTTCAG
TCACAACCCGCAAACGGCGCGCCATGCTGCTGCAGGCCTTCCTGTTCCTGCTGG
CCGGCTTCGCCGCCAAGATCAGCGCCTCCATGACGAACGAGACGTCCGACCGC
CCCCTGGTGCACTTCACCCCCAACAAGGGCTGGATGAACGACCCCAACGGCCTG
TGGTACGACGAGAAGGACGCCAAGTGGCACCTGTACTTCCAGTACAACCCGAA
CGACACCGTCTGGGGGACGCCCTTGTTCTGGGGCCACGCCACGTCCGACGACCT
GACCAACTGGGAGGACCAGCCCATCGCCATCGCCCCGAAGCGCAACGACTCCG
GCGCCTTCTCCGGCTCCATGGTGGTGGACTACAACAACACCTCCGGCTTCTTCA
ACGACACCATCGACCCGCGCCAGCGCTGCGTGGCCATCTGGACCTACAACACCC
CGGAGTCCGAGGAGCAGTACATCTCCTACAGCCTGGACGGCGGCTACACCTTCA
CCGAGTACCAGAAGAACCCCGTGCTGGCCGCCAACTCCACCCAGTTCCGCGACC
CGAAGGTCTTCTGGTACGAGCCCTCCCAGAAGTGGATCATGACCGCGGCCAAGT
CCCAGGACTACAAGATCGAGATCTACTCCTCCGACGACCTGAAGTCCTGGAAGC

TGGAGTCCGCGTTCGCCAACGAGGGCTTCCTCGGCTACCAGTACGAGTGCCCCG
GCCTGATCGAGGTCCCCACCGAGCAGGACCCCAGCAAGTCCTACTGGGTGATGT
TCATCTCCATCAACCCCGGCGCCCCGGCCGGCGGCTCCTTCAACCAGTACTTCG
TCGGCAGCTTCAACGGCACCCACTTCGAGGCCTTCGACAACCAGTCCCGCGTGG
TGGACTTCGGCAAGGACTACTACGCCCTGCAGACCTTCTTCAACACCGACCCGA
CCTACGGGAGCGCCCTGGGCATCGCGTGGGCCTCCAACTGGGAGTACTCCGCCT
TCGTGCCCACCAACCCCTGGCGCTCCTCCATGTCCCTCGTGCGCAAGTTCTCCCT
CAACACCGAGTACCAGGCCAACCCGGAGACGGAGCTGATCAACCTGAAGGCCG
AGCCGATCCTGAACATCAGCAACGCCGGCCCCTGGAGCCGGTTCGCCACCAAC
ACCACGTTGACGAAGGCCAACAGCTACAACGTCGACCTGTCCAACAGCACCGG
CACCCTGGAGTTCGAGCTGGTGTACGCCGTCAACACCACCCAGACGATCTCCAA
GTCCGTGTTCGCGGACCTCTCCCTCTGGTTCAAGGGCCTGGAGGACCCCGAGGA
GTACCTCCGCATGGGCTTCGAGGTGTCCGCGTCCTCCTTCTTCCTGGACCGCGG
GAACAGCAAGGTGAAGTTCGTGAAGGAGAACCCCTACTTCACCAACCGCATGA
GCGTGAACAACCAGCCCTTCAAGAGCGAGAACGACCTGTCCTACTACAAGGTG
TACGGCTTGCTGGACCAGAACATCCTGGAGCTGTACTTCAACGACGGCGACGTC
GTGTCCACCAACACCTACTTCATGACCACCGGGAACGCCCTGGGCTCCGTGAAC
ATGACGACGGGGGTGGACAACCTGTTCTACATCGACAAGTTCCAGGTGCGCGA
GGTCAAGTGACAATTGGCAGCAGCAGCTCGGATAGTATCGACACACTCTGGAC
GCTGGTCGTGTGATGGACTGTTGCCGCCACACTTGCTGCCTTGACCTGTGAATA
TCCCTGCCGCTTTTATCAAACAGCCTCAGTGTGTTTGATCTTGTGTGTACGCGCT
TTTGCGAGTTGCTAGCTGCTTGTGCTATTTGCGAATACCACCCCCAGCATCCCCT
TCCCTCGTTTCATATCGCTTGCATCCCAACCGCAACTTATCTACGCTGTCCTGCT
ATCCCTCAGCGCTGCTCCTGCTCCTGCTCACTGCCCCTCGCACAGCCTTGGTTTG
GGCTCCGCCTGTATTCTCCTGGTACTGCAACCTGTAAACCAGCACTGCAATGCT
GATGCACGGGAAGTAGTGGGATGGGAACACAAATGGAGGATCCCGCGTCTCGA
ACAGAGCGCGCAGAGGAACGCTGAAGGTCTCGCCTCTGTCGCACCTCAGCGCG
GCATACACCACAATAACCACCTGACGAATGCGCTTGGTTCTTCGTCCATTAGCG
AAGCGTCCGGTTCACACACGTGCCACGTTGGCGAGGTGGCAGGTGACAATGAT
CGGTGGAGCTGATGGTCGAAACGTTCACAGCCTAGGGATATCGAATTCCTTTCT
TGCGCTATGACACTTCCAGCAAAAGGTAGGGCGGGCTGCGAGACGGCTTCCCG
GCGCTGCATGCAACACCGATGATGCTTCGACCCCCCGAAGCTCCTTCGGGGCTG
CATGGGCGCTCCGATGCCGCTCCAGGGCGAGCGCTGTTTAAATAGCCAGGCCCC
CGATTGCAAAGACATTATAGCGAGCTACCAAAGCCATATTCAAACACCTAGATC
ACTACCACTTCTACACAGGCCACTCGAGCTTGTGATCGCACTCCGCTAAGGGGG
CGCCTCTTCCTCTTCGTTTCAGTCACAACCCGCAAACACTAGTTCACTTGTGGAA
CCAGAGCGCGGAGTCGTCCTCGGGCGCGTCCGGGACGACGTAGCGGCAGTCGC
GCCAGTCCTCCCACAGGGCGCGGCCGACCCAGCGGCTGTCGGACTGGTAGTACT
TGCCCAGGATGGGCCTGATGGCGGCGGAGGCCTCCTCGGCGTGGTAGTGCGGG
ATGGTGCTGAAGAGGTGGTGCAGCACGTGGGTGTCGGAGATGTGGTGCAGGAT
GTTGTCCATGAAGGGCGGGCCCATGGAGCGGTCCACGGTGGCCATGGCGCCGC
GCAGCCAGTCCCAGTCCTTCTCGAAGTAGTGCGGCAGCGCCGGGTGCGTGTGCT
GGAGCAGCGTGATGAGCACGAGCCACATGTTCACGATCAGGTAGGGCACCACG
TAGGTCTTGACCAGCCAGGCCCAGCCCATGGTGCGGCCCAGCACGCTGAGCCC
GCTGAGCACCGCCACCAGCGCCAGGTCGGAGATGACCACCTCGATGCGCTCGC
GCTTGCTGAAGATGGGCGACCACGGGTCAAAGTGGTTGGCGAAGCGCGGGTAC
GGCCGCGAGGCGACGTTGAACATGAGGTACAGCGGCCAGCCCAGGGTCAGGGT
GACCAGCACCTTGCCCATGCGGATGGGCAGCCACTCCTCCCACTCCAGGCCCTC

GTGCGCCACTGCGCGGTGCGGCGGCACAAACACCTCGTCCTTGTCCAGGCACCC
CGTGTTGGAGTGGTGGCGGCGGTGCGAGTGCTTCCAGGAGTAGTAGGGCACCA
GCAGCAGGCTGTGGAACACCAGGCCCACGCCGTCGTTGATGGCCTGGCTGGAG
GAAAAGGCCTGGTGGCCGCACTCGTGCGCGCACACCCAGACACCCGTGCCGAA
GGCGCCCTGGAAGGTGCCGGGAGGGTAGCCGGGGGGGTCAGCGGGACAGGCG
CCTTCTCCTCCATGCGCGCCACCAGGACCTCAATACGGGCAACCAAAACCCTCA
AACACACCTGGAAGAACCAGTAGAGCGGCCACATGATGCCGTACTTGACCCAC
GTAGGCACCGGTGCAGGGTCGATGTACGTCGACGCGACGTAGAGCAGGGACAT
GACCGCGATGTCAAAGGCCAGGTACATGCTGCTACGAAGCGCCGAGCGCTCGA
AACAGTGCGCGGGGATGGCCTTGCGCAGCGTCCCGATCGTGAACGGAGGCTTC
TCCACAGGCTGCCTGTTCGTCTTGATAGCCATCTCGAGGCAGCAGCAGCTCGGA
TAGTATCGACACACTCTGGACGCTGGTCGTGTGATGGACTGTTGCCGCCACACT
TGCTGCCTTGACCTGTGAATATCCCTGCCGCTTTTATCAAACAGCCTCAGTGTGT
TTGATCTTGTGTGTACGCGCTTTTGCGAGTTGCTAGCTGCTTGTGCTATTTGCGA
ATACCACCCCCAGCATCCCCTTCCCTCGTTTCATATCGCTTGCATCCCAACCGCA
ACTTATCTACGCTGTCCTGCTATCCCTCAGCGCTGCTCCTGCTCCTGCTCACTGC
CCCTCGCACAGCCTTGGTTTGGGCTCCGCCTGTATTCTCCTGGTACTGCAACCTG
TAAACCAGCACTGCAATGCTGATGCACGGGAAGTAGTGGGATGGGAACACAAA
TGGAAAGCTGTAGAGCTCTTGTTTTCCAGAAGGAGTTGCTCCTTGAGCCTTTCAT
TCTCAGCCTCGATAACCTCCAAAGCCGCTCTAATTGTGGAGGGGGTTCGAATTT
AAAAGCTTGGAATGTTGGTTCGTGCGTCTGGAACAAGCCCAGACTTGTTGCTCA
CTGGGAAAAGGACCATCAGCTCCAAAAAACTTGCCGCTCAAACCGCGTACCTCT
GCTTTCGCGCAATCTGCCCTGTTGAAATCGCCACCACATTCATATTGTGACGCTT
GAGCAGTCTGTAATTGCCTCAGAATGTGGAATCATCTGCCCCCTGTGCGAGCCC
ATGCCAGGCATGTCGCGGGCGAGGACACCCGCCACTCGTACAGCAGACCATTA
TGCTACCTCACAATAGTTCATAACAGTGACCATATTTCTCGAAGCTCCCCAACG
AGCACCTCCATGCTCTGAGTGGCCACCCCCCGGCCCTGGTGCTTGCGGAGGGCA
GGTCAACCGGCATGGGGCTACCGAAATCCCCGACCGGATCCCACCACCCCCGC
GATGGGAAGAATCTCTCCCCGGGATGTGGGCCCACCACCAGCACAACCTGCTG
GCCCAGGCGAGCGTCAAACCATACCACACAAATATCCTTGGCATCGGCCCTGA
ATTCCTTCTGCCGCTCTGCTACCCGGTGCTTCTGTCCGAAGCAGGGGTTGCTAGG
GATCGCTCCGAGTCCGCAAACCCTTGTCGCGTGGCGGGGCTTGTTCGAGCTTGA
AGAGC

SEQ ID NO:98

Codon-optimized coding sequence of *Olea europaea* stearoyl-ACP desaturase with *Chorella protothecoides* UTEX250 stearoyl-ACP transit peptide from pSZ1377

ACTAGTATGGCCACCGCATCCACTTTCTCGGCGTTCAATGCCCGCTGCGGCGAC
CTGCGTCGCTCGGCGGGCTCCGGGCCCCGGCGCCCAGCGAGGCCCCTCCCCGTG
CGCGGGCGCGCCGAGGTGCACGTGCAGGTGACCCACTCCCTGGCCCCCGAGAA
GCGCGAGATCTTCAACTCCCTGAACAACTGGGCCCAGGAGAACATCCTGGTGCT
GCTGAAGGACGTGGACAAGTGCTGGCAGCCCTCCGACTTCCTGCCCGACTCCGC
CTCCGAGGGCTTCGACGAGCAGGTGATGGAGCTGCGCAAGCGCTGCAAGGAGA
TCCCCGACGACTACTTCATCGTGCTGGTGGGCGACATGATCACCGAGGAGGCCC
TGCCCACCTACCAGACCATGCTGAACACCCTGGACGGCGTGCGCGACGAGACC
GGCGCCTCCCTGACCCCCTGGGCCATCTGGACCCGCGCCTGGACCGCCGAGGAG
AACCGCCACGGCGACCTGCTGAACAAGTACCTGTACCTGTCCGGCCGCGTGGAC

ATGAAGCAGATCGAGAAGACCATCCAGTACCTGATCGGCTCCGGCATGGACCC
CCGCACCGAGAACAACCCCTACCTGGGCTTCATCTACACCTCCTTCCAGGAGCG
CGCCACCTTCATCTCCCACGGCAACACCGCCCGCCTGGCCAAGGAGCACGGCG
ACCTGAAGCTGGCCCAGATCTGCGGCATCATCGCCGCCGACGAGAAGCGCCAC
GAGACCGCCTACACCAAGATCGTGGAGAAGCTGTTCGAGATCGACCCCGACGG
CACCGTGCTGGCCCTGGCCGACATGATGCGCAAGAAGGTGTCCATGCCCGCCCA
CCTGATGTACGACGGCCAGGACGACAACCTGTTCGAGAACTTCTCCTCCGTGGC
CCAGCGCCTGGGCGTGTACACCGCCAAGGACTACGCCGACATCCTGGAGTTCCT
GGTGGGCCGCTGGGACATCGAGAAGCTGACCGGCCTGTCCGGCGAGGGCCGCA
AGGCCCAGGACTACGTGTGCACCCTGCCCCCCCGCATCCGCCGCCTGGAGGAGC
GCGCCCAGTCCCGCGTGAAGAAGGCCTCCGCCACCCCCTTCTCCTGGATCTTCG
GCCGCGAGATCAACCTGATGGACTACAAGGACCACGACGGCGACTACAAGGAC
CACGACATCGACTACAAGGACGACGACGACAAGTGAATCGAT

SEQ ID NO:99
Amino acid sequence of *Olea europaea* stearoyl-ACP desaturase (Accession No. AAB67840.1)

MALKLCFPPHKMPSFPDARIRSHRVFMASTIHSPSMEVGKVKKPFTPPREVHVQVT
HSLAPEKREIFNSLNNWAQENILVLLKDVDKCWQPSDFLPDSASEGFDEQVMELRK
RCKEIPDDYFIVLVGDMITEEALPTYQTMLNTLDGVRDETGASLTPWAIWTRAWTA
EENRHGDLLNKYLYLSGRVDMKQIEKTIQYLIGSGMDPRTENNPYLGFIYTSFQERA
TFISHGNTARLAKEHGDLKLAQICGIIAADEKRHETAYTKIVEKLFEIDPDGTVLALA
DMMRKKVSMPAHLMYDGQDDNLFENFSSVAQRLGVYTAKDYADILEFLVGRWDI
EKLTGLSGEGRKAQDYVCTLPPRIRRLEERAQSRVKKASATPFSWIFGREINL

SEQ ID NO:100
5' 6S genomic donor sequence of *Prototheca moriformis*

GCTCTTCGCCGCCGCCACTCCTGCTCGAGCGCGCCCGCGCGTGCGCCGCCAGCG
CCTTGGCCTTTTCGCCGCGCTCGTGCGCGTCGCTGATGTCCATCACCAGGTCCAT
GAGGTCTGCCTTGCGCCGGCTGAGCCACTGCTTCGTCCGGGCGGCCAAGAGGA
GCATGAGGGAGGACTCCTGGTCCAGGGTCCTGACGTGGTCGCGGCTCTGGGAG
CGGGCCAGCATCATCTGGCTCTGCCGCACCGAGGCCGCCTCCAACTGGTCCTCC
AGCAGCCGCAGTCGCCGCCGACCCTGGCAGAGGAAGACAGGTGAGGGGGGTAT
GAATTGTACAGAACAACCACGAGCCTTGTCTAGGCAGAATCCCTACCAGTCATG
GCTTTACCTGGATGACGGCCTGCGAACAGCTGTCCAGCGACCCTCGCTGCCGCC
GCTTCTCCCGCACGCTTCTTTCCAGCACCGTGATGGCGCGAGCCAGCGCCGCAC
GCTGGCGCTGCGCTTCGCCGATCTGAGGACAGTCGGGGAACTCTGATCAGTCTA
AACCCCCTTGCGCGTTAGTGTTGCCATCCTTTGCAGACCGGTGAGAGCCGACTT
GTTGTGCGCCACCCCCCACACCACCTCCTCCCAGACCAATTCTGTCACCTTTTTG
GCGAAGGCATCGGCCTCGGCCTGCAGAGAGGACAGCAGTGCCCAGCCGCTGGG
GGTTGGCGGATGCACGCTCAGGTACC

SEQ ID NO:101
3'6S genomic donor sequence of *Prototheca moriformis*

GAGCTCCTTGTTTTCCAGAAGGAGTTGCTCCTTGAGCCTTTCATTCTCAGCCTCG
ATAACCTCCAAAGCCGCTCTAATTGTGGAGGGGGTTCGAATTTAAAAGCTTGGA
ATGTTGGTTCGTGCGTCTGGAACAAGCCCAGACTTGTTGCTCACTGGGAAAAGG
ACCATCAGCTCCAAAAAACTTGCCGCTCAAACCGCGTACCTCTGCTTTCGCGCA
ATCTGCCCTGTTGAAATCGCCACCACATTCATATTGTGACGCTTGAGCAGTCTGT
AATTGCCTCAGAATGTGGAATCATCTGCCCCCTGTGCGAGCCCATGCCAGGCAT
GTCGCGGGCGAGGACACCCGCCACTCGTACAGCAGACCATTATGCTACCTCACA
ATAGTTCATAACAGTGACCATATTTCTCGAAGCTCCCCAACGAGCACCTCCATG
CTCTGAGTGGCCACCCCCCGGCCCTGGTGCTTGCGGAGGGCAGGTCAACCGGCA
TGGGGCTACCGAAATCCCCGACCGGATCCCACCACCCCCGCGATGGGAAGAAT
CTCTCCCCGGGATGTGGGCCCACCACCAGCACAACCTGCTGGCCCAGGCGAGC
GTCAAACCATACCACACAAATATCCTTGGCATCGGCCCTGAATTCCTTCTGCCG
CTCTGCTACCCGGTGCTTCTGTCCGAAGCAGGGGTTGCTAGGGATCGCTCCGAG
TCCGCAAACCCTTGTCGCGTGGCGGGGCTTGTTCGAGCTTGAAGAGC

SEQ ID NO:102

5' donor DNA sequence of *Prototheca moriformis* FADc knockout homologous recombination targeting construct

GGGCTGGTCTGAATCCTTCAGGCGGGTGTTACCCGAGAAAGAAAGGGTGCCGA
TTTCAAAGCAGACCCATGTGCCGGGCCCTGTGGCCTGTGTTGGCGCCTATGTAG
TCACCCCCCCTCACCCAATTGTCGCCAGTTTGCGCACTCCATAAACTCAAAACA
GCAGCTTCTGAGCTGCGCTGTTCAAGAACACCTCTGGGGTTTGCTCACCCGCGA
GGTCGACGCCCAGCATGGCTATCAAGACGAACAGGCAGCCTGTGGAGAAGCCT
CCGTTCACGATCGGGACGCTGCGCAAGGCCATCCCCGCGCACTGTTTCGAGCGC
TCGGCGCTTCGTAGCAGCATGTACCTGGCCTTTGACATCGCGGTCATGTCCCTG
CTCTACGTCGCGTCGACGTACATCGACCCTGCACCGGTGCCTACGTGGGTCAAG
TACGGCATCATGTGGCCGCTCTACTGGTTCTTCCAGGTGTGTTTGAGGGTTTTGG
TTGCCCGTATTGAGGTCCTGGTGGCGCGCATGGAGGAGAAGGCGCCTGTCCCGC
TGACCCCCCCGGCTACCCTCCCGGCACCTTCCAGGGCGCCTTCGGCACGGGTGT
CTGGGTGTGCGCGCACGAGTGCGGCCACCAGGCCTTTTCCTCCAGCCAGGCCAT
CAACGACGGCGTGGGCCTGGTGTTCCACAGCCTGCTGCTGGTGCCCTACTACTC
CTGGAAGCACTCGCACCG

SEQ ID NO:103

3' donor DNA sequence of *Prototheca moriformis* FADc knockout homologous recombination targeting construct

CCGCCACCACTCCAACACGGGGTGCCTGGACAAGGACGAGGTGTTTGTGCCGC
CGCACCGCGCAGTGGCGCACGAGGGCCTGGAGTGGGAGGAGTGGCTGCCCATC
CGCATGGGCAAGGTGCTGGTCACCCTGACCCTGGGCTGGCCGCTGTACCTCATG
TTCAACGTCGCCTCGCGGCCGTACCCGCGCTTCGCCAACCACTTTGACCCGTGG
TCGCCCATCTTCAGCAAGCGCGAGCGCATCGAGGTGGTCATCTCCGACCTGGCG
CTGGTGGCGGTGCTCAGCGGGCTCAGCGTGCTGGGCCGCACCATGGGCTGGGC
CTGGCTGGTCAAGACCTACGTGGTGCCCTACCTGATCGTGAACATGTGGCTCGT
GCTCATCACGCTGCTCCAGCACACGCACCCGGCGCTGCCGCACTACTTCGAGAA
GGACTGGGACTGGCTGCGCGGCGCCATGGCCACCGTGGACCGCTCCATGGGCC
CGCCCTTCATGGACAACATCCTGCACCACATCTCCGACACCCACGTGCTGCACC

ACCTCTTCAGCACCATCCCGCACTACCACGCCGAGGAGGCCTCCGCCGCCATCA
GGCCCATCCTGGGCAAGTACTACCAGTCCGACAGCCGCTGGGTCGGCCGCGCCC
TGTGGGAGGACTGGCGCGACTGCCGCTACGTCGTCCCGGACGCGCCCGAGGAC
GACTCCGCGCTCTGGTTCCACAAGTGAGTGAGTGA

SEQ ID NO:104

Carthamus tinctorius oleoyl-acyl carrier protein (CtOTE) thioesterase with *Chlorella protothecoides* (UTEX 250) stearoyl ACP desaturase transit peptide (39 residues, underlined) and 3xFLAG (bold)

MATASTFSAFNARCGDLRRSAGSGPRRPARPLPVRGRAATGEQPSGVASLREADKE
KSLGNRLRLGSLTEDGLSYKEKFVIRCYEVGINKTATIETIANLLQEVGGNHAQGVG
FSTDGFATTTTMRKLHLIWVTARMHIEIYRYPAWSDVIEIETWVQGEGKVGTRRD
WILKDYANGEVIGRATSKWVMMNEDTRRLQKVSDDVREEYLVFCPRTLRLAFPEE
NNNSMKKIPKLEDPAEYSRLGLVPRRSDLDMNKHVNNVTYIGWALESIPPEIIDTHE
LQAITLDYRRECQRDDIVDSLTSREPLGNAAGVKFKEINGSVSPKKDEQDLSRFMHL
LRSAGSGLEINRCRTEWRKKPAKR**MDYKDHDGDYKDHDIDYKDDDDK**

SEQ ID NO:105

Protein sequence for *Cuphea wrightii* FatB2 (CwTE2) thioesterase; GenBank Accession No. U56104

MVVAAAASSAFFPVPAPRPTPKPGKFGNWPSSLSQPFKPKSNPNGRFQVKANVSPH
PKANGSAVSLKSGSLNTLEDPPSSPPPRTFLNQLPDWSRLRTAITTVFVAAEKQFTRL
DRKSKRPDMLVDWFGSETIVQDGLVFRERFSIRSYEIGADRTASIETLMNHLQDTSL
NHCKSVGLLNDGFGRTPEMCTRDLIWVLTKMQIVVNRYPTWGDTVEINSWFSQSG
KIGMGREWLISDCNTGEILVRATSAWAMMNQKTRRFSKLPCEVRQEIAPHFVDAPP
VIEDNDRKLHKFDVKTGDSICKGLTPGWNDFDVNQHVSNVKYIGWILESMPTEVLE
TQELCSLTLEYRRECGRESVVESVTSMNPSKVGDRSQYQHLLRLEDGADIMKGRTE
WRPKNAGTNRAIST

SEQ ID NO:106

Codon optimized sequence for *Carthamus tinctorius* oleoyl-acyl carrier protein (CtOTE) thioesterase with *Chlorella protothecoides* (UTEX 250) stearoyl ACP desaturase transit peptide and 3xFLAG tag

ATGGCCACCGCATCCACTTTCTCGGCGTTCAATGCCCGCTGCGGCGACCTGCGT
CGCTCGGCGGGCTCCGGGCCCCGGCGCCCAGCGAGGCCCCTCCCCGTGCGCGG
GCGCGCCGCCACCGGCGAGCAGCCCTCCGGCGTGGCCTCCCTGCGCGAGGCCG
ACAAGGAGAAGTCCCTGGGCAACCGCCTGCGCCTGGGCTCCCTGACCGAGGAC
GGCCTGTCCTACAAGGAGAAGTTCGTGATCCGCTGCTACGAGGTGGGCATCAAC
AAGACCGCCACCATCGAGACCATCGCCAACCTGCTGCAGGAGGTGGGCGGCAA
CCACGCCCAGGGCGTGGGCTTCTCCACCGACGGCTTCGCCACCACCACCACCAT
GCGCAAGCTGCACCTGATCTGGGTGACCGCCCGCATGCACATCGAGATCTACCG
CTACCCCGCCTGGTCCGACGTGATCGAGATCGAGACCTGGGTGCAGGGCGAGG
GCAAGGTGGGCACCCGCCGCGACTGGATCCTGAAGGACTACGCCAACGGCGAG
GTGATCGGCCGCGCCACCTCCAAGTGGGTGATGATGAACGAGGACACCCGCCG
CCTGCAGAAGGTGTCCGACGACGTGCGCGAGGAGTACCTGGTGTTCTGCCCCCG

CACCCTGCGCCTGGCCTTCCCCGAGGAGAACAACAACTCCATGAAGAAGATCC
CCAAGCTGGAGGACCCCGCCGAGTACTCCCGCCTGGGCCTGGTGCCCCGCCGCT
CCGACCTGGACATGAACAAGCACGTGAACAACGTGACCTACATCGGCTGGGCC
CTGGAGTCCATCCCCCCCGAGATCATCGACACCCACGAGCTGCAGGCCATCACC
CTGGACTACCGCCGCGAGTGCCAGCGCGACGACATCGTGGACTCCCTGACCTCC
CGCGAGCCCCTGGGCAACGCCGCCGGCGTGAAGTTCAAGGAGATCAACGGCTC
CGTGTCCCCCAAGAAGGACGAGCAGGACCTGTCCCGCTTCATGCACCTGCTGCG
CTCCGCCGGCTCCGGCCTGGAGATCAACCGCTGCCGCACCGAGTGGCGCAAGA
AGCCCGCCAAGCGCATGGACTACAAGGACCACGACGGCGACTACAAGGACCAC
GACATCGACTACAAGGACGACGACGACAAGTGA

SEQ ID NO:107

Codon optimized sequence for for *Cuphea wrightii* FatB2 (CwTE2) thioesterase

ATGGTGGTGGCCGCCGCCGCCAGCAGCGCCTTCTTCCCCGTGCCCGCCCC
CCGCCCCACCCCCAAGCCCGGCAAGTTCGGCAACTGGCCCAGCAGCCTGA
GCCAGCCCTTCAAGCCCAAGAGCAACCCCAACGGCCGCTTCCAGGTGAAG
GCCAACGTGAGCCCCCACGGGCGCGCCCCCAAGGCCAACGGCAGCGCCGT
GAGCCTGAAGTCCGGCAGCCTGAACACCCTGGAGGACCCCCCCCAGCAGCC
CCCCCCCCCCGCACCTTCCTGAACCAGCTGCCCGACTGGAGCCGCCTGCGC
ACCGCCATCACCACCGTGTTCGTGGCCGCCGAGAAGCAGTTCACCCGCCT
GGACCGCAAGAGCAAGCGCCCCGACATGCTGGTGGACTGGTTCGGCAGCG
AGACCATCGTGCAGGACGGCCTGGTGTTCCGCGAGCGCTTCAGCATCCGC
AGCTACGAGATCGGCGCCGACCGCACCGCCAGCATCGAGACCCTGATGAA
CCACCTGCAGGACACCAGCCTGAACCACTGCAAGAGCGTGGGCCTGCTGA
ACGACGGCTTCGGCCGCACCCCCGAGATGTGCACCCGCGACCTGATCTGG
GTGCTGACCAAGATGCAGATCGTGGTGAACCGCTACCCCACCTGGGGCGA
CACCGTGGAGATCAACAGCTGGTTCAGCCAGAGCGGCAAGATCGGCATGG
GCCGCGAGTGGCTGATCAGCGACTGCAACACCGGCGAGATCCTGGTGCGC
GCCACCAGCGCCTGGGCCATGATGAACCAGAAGACCCGCCGCTTCAGCAA
GCTGCCCTGCGAGGTGCGCCAGGAGATCGCCCCCCACTTCGTGGACGCCC
CCCCCGTGATCGAGGACAACGACCGCAAGCTGCACAAGTTCGACGTGAAG
ACCGGCGACAGCATCTGCAAGGGCCTGACCCCCGGCTGGAACGACTTCGA
CGTGAACCAGCACGTGAGCAACGTGAAGTACATCGGCTGGATTCTGGAGA
GCATGCCCACCGAGGTGCTGGAGACCCAGGAGCTGTGCAGCCTGACCCTG
GAGTACCGCCGCGAGTGCGGCCGCGAGAGCGTGGTGGAGAGCGTGACCAG
CATGAACCCCAGCAAGGTGGGCGACCGCAGCCAGTACCAGCACCTGCTGC
GCCTGGAGGACGGCGCCGACATCATGAAGGGCCGCACCGAGTGGCGCCCC
AAGAACGCCGGCACCAACCGCGCCATCAGCACCTGA

SEQ ID NO:108

5' donor DNA sequence of *Prototheca moriformis* FATA1 knockout homologous recombination targeting construct

GCTCTTCGGAGTCACTGTGCCACTGAGTTCGACTGGTAGCTGAATGGAGTCGCT
GCTCCACTAAACGAATTGTCAGCACCGCCAGCCGGCCGAGGACCCGAGTCATA
GCGAGGGTAGTAGCGCGCCATGGCACCGACCAGCCTGCTTGCCAGTACTGGCG
TCTCTTCCGCTTCTCTGTGGTCCTCTGCGCGCTCCAGCGCGTGCGCTTTTCCGGT
GGATCATGCGGTCCGTGGCGCACCGCAGCGGCCGCTGCCCATGCAGCGCCGCT
GCTTCCGAACAGTGGCGGTCAGGGCCGCACCCGCGGTAGCCGTCCGTCCGGAA
CCCGCCCAAGAGTTTTGGGAGCAGCTTGAGCCCTGCAAGATGGCGGAGGACAA
GCGCATCTTCCTGGAGGAGCACCGGTGCGTGGAGGTCCGGGGCTGACCGGCCG
TCGCATTCAACGTAATCAATCGCATGATGATCAGAGGACACGAAGTCTTGGTGG
CGGTGGCCAGAAACACTGTCCATTGCAAGGGCATAGGGATGCGTTCCTTCACCT
CTCATTTCTCATTTCTGAATCCCTCCCTGCTCACTCTTTCTCCTCCTCCTTCCCGT
TCACGCAGCATTCGGGGTACC

SEQ ID NO:109

3' donor DNA sequence of *Prototheca moriformis* FATA1 knockout homologous recombination targeting construct

GACAGGGTGGTTGGCTGGATGGGGAAACGCTGGTCGCGGGATTCGATCCTGCT
GCTTATATCCTCCCTGGAAGCACACCCACGACTCTGAAGAAGAAAACGTGCAC
ACACACAACCCAACCGGCCGAATATTTGCTTCCTTATCCCGGGTCCAAGAGAGA
CTGCGATGCCCCCCTCAATCAGCATCCTCCTCCCTGCCGCTTCAATCTTCCCTGC
TTGCCTGCGCCCGCGGTGCGCCGTCTGCCCGCCCAGTCAGTCACTCCTGCACAG
GCCCCTTGTGCGCAGTGCTCCTGTACCCTTTACCGCTCCTTCCATTCTGCGAGGC
CCCCTATTGAATGTATTCGTTGCCTGTGTGGCCAAGCGGGCTGCTGGGCGCGCC
GCCGTCGGGCAGTGCTCGGCGACTTTGGCGGAAGCCGATTGTTCTTCTGTAAGC
CACGCGCTTGCTGCTTTGGGAAGAGAAGGGGGGGGGTACTGAATGGATGAGGA
GGAGAAGGAGGGGTATTGGTATTATCTGAGTTGGGTGAAGAGC

SEQ ID NO:110

FATA portion of the hairpin RNA expression cassette

ATGGCACCGACCAGCCTGCTTGCCAGTACTGGCGTCTCTTCCGCTTCTCTGTGGT
CCTCTGCGCGCTCCAGCGCGTGCGCTTTTCCGGTGGATCATGCGGTCCGTGGCG
CACCGCAGCGGCCGCTGCCCATGCAGCGCCGCTGCTTCCGAACAGTGGCGGTCA

GGGCCGCACCCGCGGTAGCCGTCCGTCCGGAACCCGCCCAAGAGTTTTGGGAG
CAGCTTGAGCCCTGCAAGATGGCGGAGGACAAGCGCATCTTCCTGGAGGAGCA
CCGGTGCGTGGAGGTCCGGGGCTGACCGGCCGTCGCATTCAACGTAATCAATCG
CATGATGATCAGAGGACACGAAGTCTTGGTGGCGGTGGCCAGAAACACTGTCC
ATTGCAAGGGCATAGGGATGCGTTCCTTCACCTCTCATTTCTCATTTCTGAATCC
CTCCCTGCTCACTCTTTCTCCTCCTCCTTCCCGTTCACGCAGCATTCGGGGCAAC
GAGGTGGGCCCGTGCTCCTCCAGGAAGATGCGCTTGTCCTCCGCCATCTTGCAG
GGCTCAAGCTGCTCCCAAAACTCTTGGGCGGGTTCCGGACGGACGGCTACCGCG
GGTGCGGCCCTGACCGCCACTGTTCGGAAGCAGCGGCGCTGCATGGGCAGCGG
CCGCTGCGGTGCGCCACGGACCGCATGATCCACCGGAAAAGCGCACGCGCTGG
AGCGCGCAGAGGACCACAGAGAAGCGGAAGAGACGCCAGTACTGGCAAGCAG
GCTGGTCGGTGCCAT

SEQ ID NO:111

Portion of construct FATA1-CrbTub_yInv_nr-FATA1, containing a region for integration into the nuclear genome to interrupt the endogenous FATA1 gene and a *S. cerevisiae suc2* sucrose invertase coding region under the control of *C. reinhardtii* β-tubulin promoter/5'UTR and *Chlorella vulgaris* nitrate reductase 3' UTR

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SEQ ID NO:112

Portion of construct FATA1-CrbTub_yInv_nr::amt03_CwTE2_nr-FATA1, containing a region for integration into the nuclear genome to interrupt the endogenous FATA1 gene and a *S. cerevisiae suc2* sucrose invertase coding region under the control of *C. reinhardtii* β-tubulin promoter/5'UTR and *Chlorella vulgaris* nitrate reductase 3' UTR

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SEQ ID NO:113

Portion of construct 6S::β-Tub:suc2:nr:: β-tub:hairpin FatA:nr::6S, containing the first exon of FatA1 coding region, followed by the endogenous intron, and a repeat unit of the first exon in the reverse orientation

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WHAT IS CLAIMED IS:

1. A dielectric fluid comprising microbial oil, wherein the oil has a pour point of less than about -5° C, and wherein the fatty acid composition of the microbial oil is at least 50% C18:1 and less than 2% C18:2.

2. The dielectric fluid of claim 1, which has a pour point of between about -10° C and about -40° C.

3. The dielectric fluid of any preceding claim, wherein the fatty acid composition of the microbial oil is at least 60%, at least 70% or at least 80% C18:1.

4. The dielectric fluid of any preceding claim, wherein the fatty acid composition of the microbial oil is less than 1% C18:2.

5. The dielectric fluid of any preceding claim, which has a flash point of 250° C to 375° C, a fire point of 300° C to 450° C, a breakdown voltage of 20kV to 75kV, or a dielectric strength of 20 MV/m (RMS) to 75 MV/m (RMS).

6. The dielectric fluid of any preceding claim, wherein the iodine value of the microbial oil is between 25 and 200.

7. The dielectric fluid of any preceding claim, wherein the dielectric fluid further comprises an antioxidant, a metal ion deactivator, a corrosion inhibitor, a demulsifier, an anti-wear additive, a pour point depressant, or an anti-hydrolysis compound.

8. The dielectric fluid of any preceding claim, wherein the microbial oil is produced by a genetically engineered *Prototheca* microbe engineered to express one or more exogenous genes.

9. The dielectric fluid of claim 8, wherein the genetically engineered microbe is *Prototheca moriformis*.

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10. The dielectric fluid of any of claims 8-9, wherein the one or more exogenous genes encode(s) sucrose invertase and/or one or more fatty acyl-ACP thioesterase(s).

11. An electrical component comprising the dielectric fluid of any of claims 1-10.

12. The electrical component of claim 11 wherein the electrical component is a transformer.

13. A method of producing an RBD microbial oil, wherein the microbial oil has a pour point of less than about -5° C, and wherein the fatty acid composition of the microbial oil is at least 50% C18:1 and less than 2% C18:2, the method comprising:

a. cultivating a genetically engineered *Prototheca* microbe engineered to express one or more exogenous genes until the microbe has at least 10% oil by dry weight;

b. separating the oil from the microbe; and

c. subjecting the oil to refining, bleaching, deodorizing or degumming to produce RBD microbial oil.

14. A method of producing a dielectric fluid comprising microbial oil, wherein the dielectric fluid has a pour point of less than about -5° C, and wherein the fatty acid composition of the microbial oil is at least 50% C18:1 and less than 2% C18:2, the method comprising:

a. subjecting a first microbial oil to refining, bleaching, deodorizing or degumming to produce RBD oil, wherein the RBD oil is characterized by an initial pour point and a first temperature;

b. lowering the temperature of the RBD oil to a second temperature, which is lower than the first temperature;

c. holding the RBD oil at the second temperature; and

d. filtering the RBD oil at the second temperature to provide a second microbial oil characterized by a second pour point that is lower than the initial pour point, wherein the second pour point is less than about -5° C.

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15. The method of claim 14, wherein the method additionally comprises producing the first microbial oil by:

a. cultivating a genetically engineered *Prototheca* microbe engineered to express one or more exogenous genes until the microbe has at least 10% oil by dry weight; and

b. separating the oil from the microbe to produce the first microbial oil.

16. The method of any one of claims 13-15, wherein the microbial oil has a pour point of between about -10° C and about -40° C.

17. The method of any one of claims 13-16, wherein the method additionally comprises adding an antioxidant, metal ion deactivator, corrosion inhibitor, demulsifier, anti-wear additive, pour point depressant, or anti-hydrolysis compound to the RBD oil or the second microbial oil.

18. The method of claim 16, wherein the genetically engineered microbe is *Prototheca moriformis*.

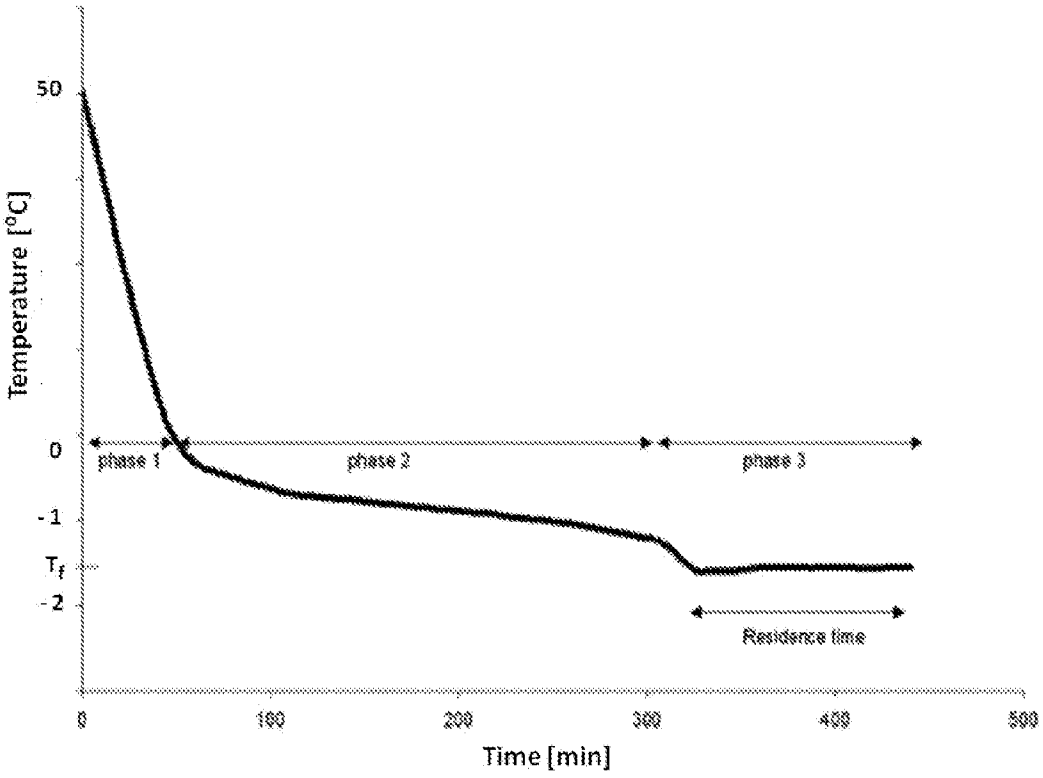
19. The method of any one of claims 13, 15 or 18, wherein the one or more exogenous genes encode(s) sucrose invertase and/or one or more fatty acyl-ACP thioesterase(s).

20. The method of claim 14, wherein the first temperature is between about 15° C to about 50° C, and the second temperature is between about -15° C and about 15° C.

SOLAZYME, INC.

WATERMARK PATENT AND TRADE MARKS ATTORNEYS

P38585AU00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-1
T_f
-2
Temperature [°C]
phase 1
phase 2
phase 3
Residence time
0
100
200
300
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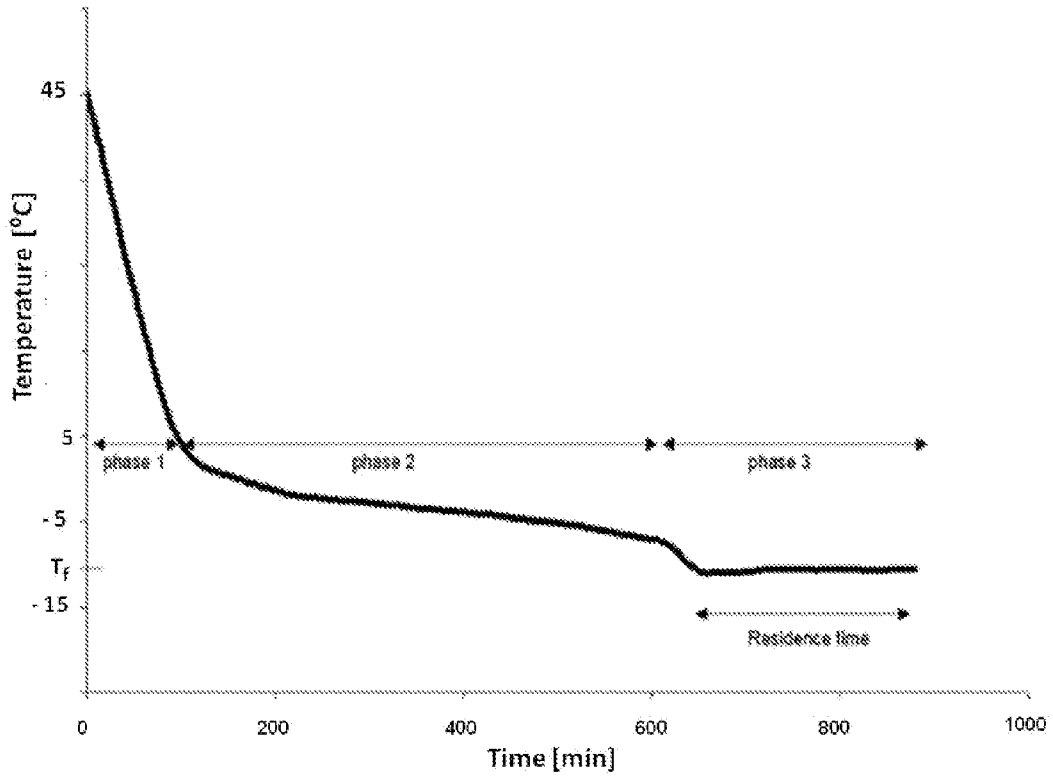
Time [min]

Fig. 1


45
5
- 5
T_f
- 15
Temperature [°C]
phase 1
phase 2
phase 3
Residence time
0
200
400
600
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1000
Time [min]

Fig. 2

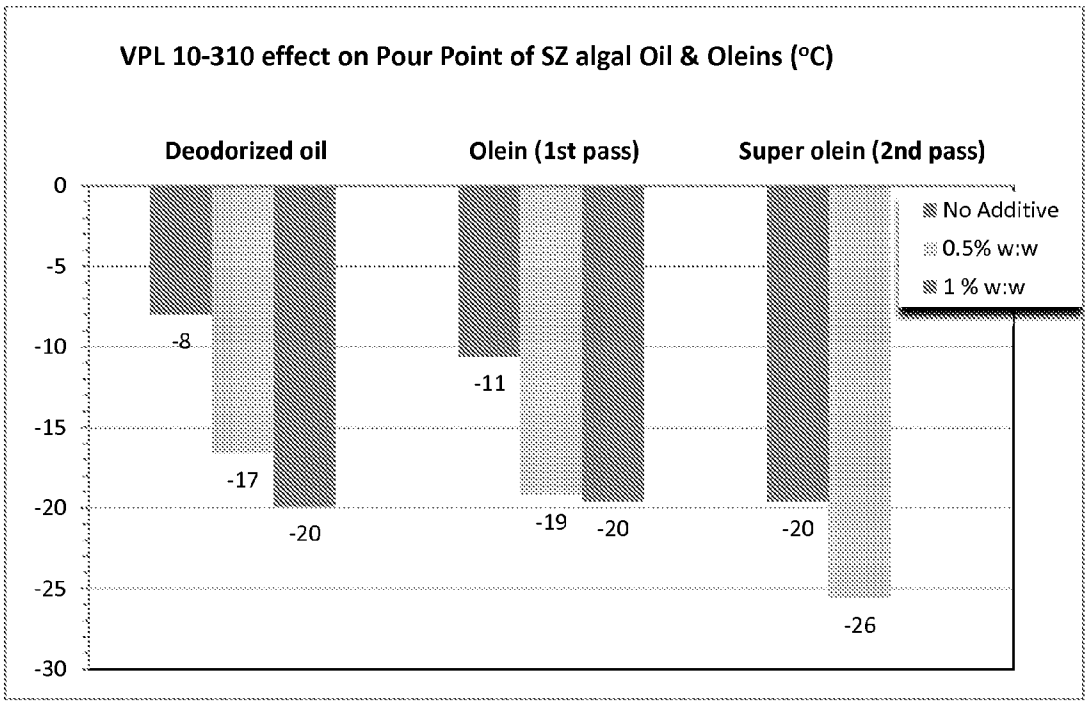
VPL 10-310 effect on Pour Point of SZ algal Oil & Oleins (°C)
Deodorized oil
Olein (1st pass)
Super olein (2nd pass)
0
-5
-10
-15
-20
-25
-30
-8
-17
-20
-11
-19
-20
-20
-26
No Additive
0.5% w:w
1 % w:w

Fig. 3