PRODUCTION OF OIL IN MICROORGANISMS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. provisional patent application Nos.: 60/941,581, filed June 1, 2007; 60/959,174, filed July 10, 2007; 60/968,291, filed August 27, 2007; and 61/024,069, filed January 28, 2008, the disclosures of which are incorporated herein by reference in their entirety for all purposes.

FIELD OF THE INVENTION

[0002] This disclosure relates to the production of oils, fuels, and oleochemicals made from microorganisms. In particular, the disclosure relates to oil-bearing microorganisms, including microalgae, yeast and fungi, and to methods of cultivating such microorganisms for the production of useful compounds, including lipids, fatty acid esters, fatty acids, aldehydes, alcohols, and alkanes, for use in industry or as an energy or food source. The microorganisms of the invention can be selected or genetically engineered for use in the methods or other aspects of the invention described herein.

BACKGROUND OF THE INVENTION

[0003] 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.

[0004] In common dialogue, fossil fuel, also known as mineral fuel, is used synonymously with other hydrocarbon-containing natural resources such as coal, oil and natural gas. The utilization of fossil fuels has enabled large-scale industrial development and largely supplanted water driven mills, as well as the combustion of wood or peat for heat. Fossil fuels are a finite, non-renewable resource.

[0005] When generating electricity, energy from the combustion of fossil fuels is often used to power a turbine. Older generations often used steam generated by the burning of the fuel to turn the turbine, but in newer power plants, the gases produced by burning of the fuel turn a gas turbine directly. With global modernization in the 20th and 21st centuries, the thirst for energy from fossil fuels, especially gasoline derived from oil, is one of the causes of major regional and global conflicts.

[0006] The burning of fossil fuels by humans is the largest source of emissions of carbon dioxide, which is one of the greenhouse gases that allows radiative forcing and contributes to global warming. In the United States, more than 90% of greenhouse gas emissions come from the combustion of fossil fuels. In addition, other air pollutants, such as nitrogen oxides, sulfur dioxide, volatile organic compounds (VOCs), and heavy metals are produced.

[0007] Human activity raises levels of greenhouse gases primarily by releasing carbon dioxide from fossil fuel combustion, but other gases, e.g., methane, are not negligible. The concentrations of several greenhouse gases have increased over time due to human activities, such as burning of fossil fuels and deforestation leading to higher carbon dioxide concentrations. According to the global warming hypothesis, greenhouse gases from industry and agriculture have played a major role in the recently observed global warming.

[0008] Increased demand for energy by the global economy has also placed increasing pressure on the cost of hydrocarbons. Aside from energy, many industries, including plastics and chemical manufacturers, rely heavily on the availability of hydrocarbons as a feedstock for their manufacturing processes. Cost-effective alternatives to current sources of supply could help mitigate the upward pressure on energy and these raw material costs.

SUMMARY OF THE INVENTION

[0008a] In a first aspect, the present invention refers to a method of producing fuel, comprising:

(a) culturing a population of microalgae in the presence of a fixed carbon source, wherein:

(i) the microalgae express an exogenous sucrose invertase;

(ii) the microalgae accumulate at least 10% of their dry cell weight as lipid; and

(ii) the fixed carbon source is selected from the group consisting of sugar cane juice, sugar cane, sugar beet, molasses, or other sucrose-containing feedstock;

(b) isolating lipid components from the cultured microalgae; and

(c) subjecting the isolated lipid components to one or more chemical reactions to generate fatty acid esters and/or alkanes,

whereby fuel is produced.

[0008b] In a second aspect, the present invention refers to a composition of alkanes or fatty acid esters made according to the method of the present invention.

[0008c] In a third aspect, the present invention refers to a microalgal cell containing at least 10% of its dry cell weight as lipid, and comprising an exogenous gene encoding a sucrose invertase enzyme.

[0008d] In a fourth aspect, the present invention refers to a lipid produced by a microalgal cell of the present invention.

[0008e] In a fifth aspect, the present invention refers to a method of producing a lipid, comprising:

(a) culturing a population of microalgae comprising an exogenous gene encoding a sucrose invertase in a culture medium comprising a fixed carbon source until the microalgae accumulate 10% or more of their dry cell weight as lipid; and

(b) separating the lipid from the microalgae.

[0008f] In a sixth aspect, the present invention refers to a lipid produced by the method of the present invention.

[0009] In one example, the present disclosure is directed to a microbe, which in various examples can comprise a microalgae cell, an oleaginous yeast, or a fungus containing an exogenous gene that encodes a protein selected from the group consisting of a lipase, sucrose transporter, sucrose invertase, fructokinase, polysaccharide-degrading enzyme, a fatty acyl-ACP thioesterase, a fatty acyl-CoA/aldehyde reductase, a fatty acyl-CoA reductase, a fatty aldehyde reductase, a fatty aldehyde decarbonylase, and an acyl carrier protein (ACP). The microbe (e.g., microalgae cell) can, for example, be selected from Table 1. In particular examples, the cell is a species of the genus *Chlorella*, such as, e.g., *Chlorella fusca, Chlorella protothecoides, Chlorella pyrenoidosa, Chlorella kessleri, Chlorella vulgaris, Chlorella saccharophila, Chlorella sorokiniana* or *Chlorella ellipsoidea*. In other examples, the microbe is an oleaginous yeast selected from the group consisting of *Cryptococcus curvatus, Cryptococcus terricolus, Candida sp., Lipomyces starkeyi, Lipomyces lipofer, Endomycopsis vernalis, Rhodotorula glutinis, Rhodotorula gracilis,* and *Yarrowia lipolytica*. In still other examples, the microbe is a fungus selected from the group consisting of a species of the genus *Mortierella, Mortierrla vinacea, Mortierella*

alpine, Pythium debaryanum, Mucor circinelloides, Aspergillus ochraceus, Aspergillus terreus, Pennicillium iilacinum, a species of the genus *Hensenulo*, a species of the genus *Chaetomium*, a species of the genus *Cladosporium*, a species of the genus *Malbranchea*, a species of the genus *Rhizopus*, and a species of the genus *Pythium*. In other examples, the disclosure includes expression of hydrocarbon modification enzymes in bacterial hosts such as *E. Coli* and *Bacill*a method of producing renewable diesel. In one example, the method comprises (a) culturing a population of microorganisms in the presence of a fixed carbon source, wherein (i) the microorganisms accumulate at least 10% of their dry cell weight as lipid, and (ii) the fixed carbon source is selected from the group consisting of glycerol, depolymerized cellulosic material, sucrose, molasses, glucose, arabinose, galactose, xylose, fructose, arabinose, mannose, acetate, and any combination of the foregoing, (b) isolating lipid components from the cultured microorganisms, and (c) subjecting the isolated lipid components to one or more chemical reactions to generate straight chain alkanes, whereby renewable diesel is produced.

[0010] In another example, the present disclosure is directed to a composition of liquid hydrocarbons made according to the method described directly above, wherein the composition conforms to the specifications of ASTM D975.

[0011] In another example, the present disclosure is directed to a method of producing jet fuel. In one example, the method comprises (a) culturing a population of microorganisms in the presence of a fixed carbon source, wherein (i) the microorganisms accumulate at least 10% of their dry cell weight as lipid, and (ii) the fixed carbon source is selected from the group consisting of glycerol, depolymerized cellulosic material, sucrose, glucose, arabinose, galactose, xylose, fructose, arabinose, mannose, acetate, and any combination of the foregoing, (b) isolating lipid components from the cultured microorganisms, (c) subjecting the isolated lipid components to one or more chemical reactions to generate straight chain alkanes, (d) cracking the straight chain alkanes, whereby jet fuel is produced.

[0012] In another example, the present disclosure is directed to a composition of liquid hydrocarbons produced according to the method described directly above, wherein the composition conforms to the specifications of ASTM D1655.

[0013] In another example, the present disclosure is directed to a microalgae or yeast cell that has been genetically engineered and/or selected to express a lipid pathway enzyme at an altered level compared to a wild-type cell of the same species. In some cases, the cell

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

[0014] In some examples, the microalgae or yeast cell described above 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.

[0015] In another example, the present disclosure is directed to an oil-producing microbe containing one or more exogenous genes, wherein the exogenous genes encode protein(s) selected from the group consisting of a fatty acyl-ACP thioesterase, a fatty acyl-CoA reductase, a fatty aldehyde reductase, a fatty acyl-CoA/aldehyde reductase, a fatty aldehyde decarbonylase, and an acyl carrier protein. In some cases, the microbe is *Chlorella protothecoides, Chlorella minutissima, Chlorella emersonii, Chlorella sorokiniana, Chlorella ellipsoidea,* or *Chlorella sp.* In other cases, the microbe is another species as described herein. In one example, 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 light. In some cases, the exogenous gene is expressed in a cellular compartment. In some examples, the cellular compartment is selected from the group consisting of a chloroplast and a mitochondrion.

[0016] In one example, the exogenous gene encodes a fatty acid acyl-ACP thioesterase. In some cases, the thioesterase encoded by the exogenous gene catalyzes the cleavage of an 8 to 18-carbon fatty acid from an acyl carrier protein (ACP). In some cases, the thioesterase encoded by the exogenous gene catalyzes the cleavage of a 10 to 14-carbon fatty acid from an

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

[0017] In one example, the exogenous gene encodes a fatty acyl-CoA/aldehyde reductase. In some cases, the reductase encoded by the exogenous gene catalyzes the reduction of a 20 to 30-carbon fatty acyl-CoA to a corresponding primary alcohol. In some cases, the reductase encoded by the exogenous gene catalyzes the reduction of an 8 to 18-carbon fatty acyl-CoA to a corresponding primary alcohol. In some cases, the reductase encoded by the exogenous gene catalyzes the reduction of a 10 to 14-carbon fatty acyl-CoA to a corresponding primary alcohol. In one example, the reductase encoded by the exogenous gene catalyzes the reduction of a 12-carbon fatty acyl-CoA to dodecanol.

[0018] In one example, the exogenous gene encodes a fatty acyl-CoA 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 aldehyde. In one example, the reductase encoded by the exogenous gene catalyzes the reduction of a 12-carbon fatty acyl-CoA to dodecanal.

[0019] In at least one example, the microbe of the disclosure further contains one or more exogenous sucrose utilization genes.

[0020] In another example, the present disclosure is directed to a microbe containing two exogenous genes, wherein a first exogenous gene encodes a fatty acyl-ACP thioesterase and a second exogenous gene encodes a protein selected from the group consisting of a fatty acyl-CoA reductase, a fatty acyl-CoA/aldehyde reductase, and an acyl carrier protein. In some cases, the microbe is *Chlorella minutissima, Chlorella emersonii, Chlorella sorokiniana, Chlorella ellipsoidea, Chlorella sp.*, or *Chlorella protothecoides*. In other cases, the microbe is another species as described herein. 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.

[0021] In one example, the thioesterase encoded by the first exogenous gene catalyzes the cleavage of an 8 to 18-carbon fatty acid from an ACP. In some examples, the second exogenous gene encodes a fatty acyl-CoA/aldehyde reductase which catalyzes the reduction of an 8 to 18-carbon fatty acyl-CoA to a corresponding primary alcohol. In some cases, the thioesterase encoded by the first exogenous gene catalyzes the cleavage of a 10 to 14-carbon fatty acid from an ACP, and the reductase encoded by the second exogenous gene catalyzes

the reduction of a 10 to 14-carbon fatty acyl-CoA to the corresponding primary alcohol, wherein the thioesterase and the reductase act on the same carbon chain length. In one example, the thioesterase encoded by the first exogenous gene catalyzes the cleavage of a 12-carbon fatty acid from an ACP, and the reductase encoded by the second exogenous gene catalyzes the reduction of a 12-carbon fatty acyl-CoA to dodecanol. In some examples, the the second exogenous gene encodes a fatty acyl-CoA reductase which catalyzes the reduction of an 8 to 18-carbon fatty acyl-CoA to a corresponding aldehyde.

[0022] In some examples, the second exogenous gene encodes a fatty acyl-CoA reductase, and the microbe further contains a third exogenous gene encoding a fatty aldehyde decarbonylase. In some cases, the thioesterase encoded by the first exogenous gene catalyzes the cleavage of an 8 to 18-carbon fatty acid from an ACP, the reductase encoded by the second exogenous gene catalyzes the reduction of an 8 to 18-carbon fatty acyl-CoA to a corresponding fatty aldehyde, and the decarbonylase encoded by the third exogenous gene catalyzes the conversion of an 8 to 18-carbon fatty aldehyde to a corresponding alkane, wherein the thioesterase, the reductase, and the decarbonylase act on the same carbon chain length.

[0023] In some examples, the second exogenous gene encodes an acyl carrier protein that is naturally co-expressed with the fatty acyl-ACP thioesterase.

[0024] In some examples, the second exogenous gene encodes an acyl carrier protein, and the microbe further contains a third exogenous gene encoding a protein selected from the group consisting of a fatty acyl-CoA reductase and a fatty acyl-CoA/aldehyde reductase. In some cases, the third exogenous gene encodes a fatty acyl-CoA reductase, and the microbe further contains a fourth exogenous gene encoding a fatty aldehyde decarbonylase.

[0025] In another example, the present disclosure is directed to a method of producing a molecule in a microbe population. In one example, the method comprises culturing a population of microbes in a culture medium, wherein the microbes contain (i) a first exogenous gene encoding a fatty acyl-ACP thioesterase, and (ii) a second exogenous gene encoding a fatty acyl-CoA/aldehyde reductase, and the microbes synthesize a fatty acid linked to an acyl carrier protein (ACP), the fatty acyl-ACP thioesterase catalyzes the cleavage of the fatty acid from the ACP to yield, through further processing, a fatty acyl-CoA, and the fatty acyl-CoA/aldehyde reductase catalyzes the reduction of the acyl-CoA to an alcohol.

[0026] In one example of the method of producing a molecule in a microbe population, the microbe is *Chlorella minutissima, Chlorella emersonii, Chlorella sorokiniana, Chlorella ellipsoidea, Chlorella sp.* or *Chlorella protothecoides*. In other cases, the microbe is another species of microorganism as described herein. In some cases, the culture medium contains glycerol. In one example, the glycerol is a byproduct of a transesterification process. In some cases, the culture medium contains glycerol and at least one other fixed carbon source. In one example, the at least one other fixed carbon source is sucrose. In some cases, all of the glycerol and all of the at least one other fixed carbon source are provided to the microbes at the beginning of fermentation. 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. In some culture methods of the disclosure, glycerol is provided to the microbes in the absence of the at least one other fixed carbon source for a first period of time, the at least one other fixed carbon source is provided at the end of the first period of time, and the microbes are cultured for a second period of time in the presence of the at least one other fixed carbon source.

[0027] In some examples, the exogenous genes are in operable linkage with a promoter that is inducible in response to a first stimulus. In some cases, the method further comprises providing the first stimulus, and incubating the population of microbes for a first period of time in the presence of the first stimulus to produce an alcohol. In some cases, the method further comprises extracting the alcohol from aqueous biomass comprising the culture medium and the microbes.

[0028] In some examples, the thioesterase encoded by the first exogenous gene catalyzes the cleavage of an 8 to 18-carbon fatty acid from the ACP, and the reductase encoded by the second exogenous gene catalyzes the reduction of an 8 to 18-carbon fatty acyl-CoA to a corresponding primary alcohol, wherein the thioesterase and the reductase act on the same carbon chain length. In some cases, the thioesterase encoded by the first exogenous gene catalyzes the cleavage of an 10 to 14-carbon fatty acid from the ACP, and the reductase encoded by the second exogenous gene catalyzes the reduction of an 10 to 14-carbon fatty acyl-CoA to a corresponding primary alcohol, wherein the thioesterase and the reductase act on the same carbon chain length. In one example, the thioesterase encoded by the first exogenous gene catalyzes the cleavage of a 12-carbon fatty acid from the ACP, and the reductase encoded by the second exogenous gene catalyzes the reduction of a 12-carbon fatty acyl-CoA to dodecanol. In some cases, the microbes further contain a third

exogenous gene encoding an acyl carrier protein. In some examples, the third exogenous gene encodes an acyl carrier protein that is naturally co-expressed with the fatty acyl-ACP thioesterase.

[0029] In another example, the present disclosure is directed to a method of producing a lipid molecule in a microbe population. In one example, the method comprises culturing a population of microbes in a culture medium, wherein the microbes contain (i) a first exogenous gene encoding a fatty acyl-ACP thioesterase, and (ii) a second exogenous gene encoding a fatty acyl-CoA reductase, and wherein the microbes synthesize a fatty acid linked to an acyl carrier protein (ACP), the fatty acyl-ACP thioesterase catalyzes the cleavage of the fatty acid from the ACP to yield, through further processing, a fatty acyl-CoA, and the fatty acyl-CoA reductase catalyzes the reduction of the acyl-CoA to an aldehyde. In some cases, the microbe is *Chlorella minutissima, Chlorella emersonii, Chlorella sorokiniana, Chlorella ellipsoidea, Chlorella sp.*, or *Chlorella protothecoides*. In other cases, the microbe is another species of microorganism as described herein.

[0030] In some examples, the exogenous genes are in operable linkage with a promoter that is inducible in response to a first stimulus, and the method further comprises providing the first stimulus, and incubating the population of microbes for a first period of time in the presence of the first stimulus to produce an aldehyde. In one example, the method further comprises extracting the aldehyde from aqueous biomass comprising the culture medium and the population of microbes.

[0031] In some examples, the thioesterase encoded by the first exogenous gene catalyzes the cleavage of an 8 to 18-carbon fatty acid from the ACP, and the reductase encoded by the second exogenous gene catalyzes the reduction of an 8 to 18-carbon fatty acyl-CoA to a corresponding aldehyde, wherein the thioesterase and the reductase act on the same carbon chain length. In some cases, the microbes further contain a third exogenous gene encoding a fatty aldehyde decarbonylase that catalyzes the conversion of the aldehyde to an alkane.

[0032] In some cases, the exogenous genes are in operable linkage with a promoter that is inducible in response to a first stimulus, and the method further comprises providing the first stimulus, and incubating the population of microbes for a first period of time in the presence of the first stimulus to produce an alkane. In some cases, the method further comprises

extracting the alkane from aqueous biomass comprising culture medium and the microbe population.

[0033] In some cases, the thioesterase encoded by the first exogenous gene catalyzes the cleavage of an 8 to 18-carbon fatty acid from the ACP, the reductase encoded by the second exogenous gene catalyzes the reduction of an 8 to 18-carbon fatty acyl-CoA to a corresponding aldehyde, and the decarbonylase encoded by the third exogenous gene catalyzes the conversion of an 8 to 18-carbon aldehyde to a corresponding alkane, wherein the thioesterase, the reductase, and the decarbonylase act on the same carbon chain length. In some examples, the microbes further contain a third exogenous gene encoding an acyl carrier protein. In some cases, the third exogenous gene encodes an acyl carrier protein that is naturally co-expressed with the fatty acyl-ACP thioesterase. In some cases, the microbes further contain a fourth exogenous gene encoding a fatty aldehyde decarbonylase that catalyzes the conversion of the aldehyde to an alkane.

[0034] In some methods, the culture medium contains glycerol. In one example, the glycerol is a byproduct of a transesterification process. In some cases, the culture medium contains glycerol and at least one other fixed carbon source. In one example, the at least one other fixed carbon source is sucrose. In some cases, all of the glycerol and all of the at least one other fixed carbon source are provided to the microbes at the beginning of fermentation. 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. In one example, glycerol is provided to the microbes in the absence of the at least one other fixed carbon source for a first period of time, the at least one other fixed carbon source is provided at the end of the first period of time, and the microbes are cultured for a second period of time in the presence of the at least one other fixed carbon source.

[0035] In another example, the present disclosure is directed to a method of producing a fatty acid molecule having a specified carbon chain length in a microbe population. In one example, the method comprises culturing a population of lipid-producing microbes in a culture medium, wherein the microbes contain an exogenous gene encoding a fatty acyl-ACP thioesterase having an activity specific to a carbon chain length, and wherein the microbes synthesize a fatty acid linked to an acyl carrier protein (ACP) and the thioesterase catalyzes the cleavage of the fatty acid from the ACP when the fatty acid has been synthesized to the specific carbon chain length. In some cases, the microbe is *Chlorella minutissima, Chlorella*

emersonii, *Chlorella sorokiniana*, *Chlorella ellipsoidea*, *Chlorella sp.*, or *Chlorella protothecoides*. In other cases, the microbe is another species of microorganism as described herein.

[0036] In some examples, the exogenous gene is in operable linkage with a promoter that is inducible in response to a first stimulus, and the method further comprises providing the first stimulus, and incubating the population of microbes for a period of time in the presence of the first stimulus. In some cases, the method further comprises extracting the fatty acid from aqueous biomass comprising culture medium and the microbe population.

[0037] In some cases, the microbes further contain a second exogenous gene encoding an acyl carrier protein. In some examples, the second exogenous gene encodes an acyl carrier protein that is naturally co-expressed with the fatty acyl-ACP thioesterase. In one example, the acyl-ACP thioesterase catalyzes the cleavage of an 8 to 18-carbon fatty acid from the ACP.

[0038] In some cases, the culture medium contains glycerol. In one example, the glycerol is a byproduct of a transesterification process. In some examples, the culture medium contains glycerol and at least one other fixed carbon source. In one example, the at least one other carbon source is sucrose. In some cases, all of the glycerol and all of the at least one other fixed carbon source are provided to the microbes at the beginning of fermentation. 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. In one example, glycerol is provided to the microbes in the absence of the at least one other fixed carbon source for a first period of time, the at least one other fixed carbon source is provided at the end of the first period of time, and the microbes are cultured for a second period of time in the presence of the at least one other fixed carbon source.

[0039] In another example, the present disclosure is directed to a microalgae cell containing an exogenous gene, wherein the exogenous gene encodes a protein selected from the group consisting of a lipase, a sucrose transporter, a sucrose invertase, a fructokinase, or a polysaccharide-degrading enzyme. In some cases, the cell is *Chlorella minutissima*, *Chlorella emersonii*, *Chlorella sorokiniana*, *Chlorella ellipsoidea*, *Chlorella sp.*, or *Chlorella protothecoides*. In other cases, the cell is another species of microalgae as described herein.

[0040] In some cases, the exogenous gene is in operable linkage with a promoter. In some cases, the promoter is inducible or repressible in response to a stimulus. In various

examples, the stimulus is selected from the group consisting of an exogenously provided small molecule, heat, cold, and light. In some cases, the exogenous gene is expressed in a cellular compartment. In some examples, the cellular compartment is selected from the group consisting of a chloroplast and a mitochondrion.

[0041] In some cases, the gene encodes a lipase that has at least 70% amino acid identity with a lipase selected from Table 9. In one example, the lipase is novozym-435. In one example, the polysaccharide-degrading enzyme is endogenous to a *Chlorella* virus.

[0042] In another example, the present disclosure is directed to a microalgae cell containing two exogenous genes, wherein a first exogenous gene encodes a lipase and a second exogenous gene encodes a polysaccharide-degrading enzyme. In some cases, the exogenous genes are each in operable linkage with a promoter. In some cases, the exogenous genes are each in operable linkage with promoters that are inducible in response to a stimulus. In some cases, the exogenous genes are each in operable linkage with promoters that are inducible in response to the same stimulus. In some cases, the exogenous genes are each in operable linkage with a promoter that is inducible in response to at least one stimulus that does not induce the other promoter.

[0043] In another example, the present disclosure is directed to a method of manufacturing a lipid molecule in a microbe. In one example, the method comprises (a) culturing the microbe for a first period of time sufficient to increase the cell density, wherein the microbe contains (i) an exogenous gene encoding a lipase, and/or (ii) an exogenous gene encoding a polysaccharide-degrading enzyme,wherein the exogenous gene(s) are in operable linkage with a promoter that is inducible in response to a stimulus, (b) providing the stimulus, and (c) incubating the microbe for a second period of time in the presence of the stimulus.

[0044] In another example, the present disclosure is directed to a method of manufacturing a lipid molecule in a microbe. In one example, the method comprises (a) culturing a lipid-producing microbe for a first period of time sufficient to increase the cell density, (b) providing a virus capable of infecting and lysing the microbe when in direct contact with the microbe, and (c) incubating the microbe for a second period of time to produce lysed aqueous biomass. In one example, the method further compries extracting lipid molecules from the lysed aqueous biomass.

[0045] In another example, the present disclosure is directed to a microalgae cell containing an exogenous gene, wherein the exogenous gene encodes a cofactor for a lipid pathway enzyme or encodes a protein that participates in the synthesis of the cofactor.

[0046] In another example, the present disclosure is directed to a method of culturing a lipid-producing microbe. In one example, the method comprises culturing the microbe in the presence of a sufficient amount of one or more cofactor(s) for a lipid pathway enzyme to increase microbial lipid yield over microbial lipid yield in the absence of said one or more cofactors. In some cases, the one or more cofactors is a vitamin required by one or more lipid pathway enzymes. In one example, the one or more cofactors is biotin. In some cases, the one or more cofactors is/are provided by including in the culture a microbe that has been genetically engineered to produce the one or more cofactors.

[0047] In another example, the present disclosure is directed to a method of fermenting a microorganism, which comprises providing a mixture comprising glucose and xylose as an energy source to the microorganism. In one example, the mixture further comprises lignin. In one example, the mixture further comprises at least one species of furfural. In some cases, the mixture is depolymerized cellulosic material. In some cases, the mixture further comprises as least one sucrose utilization enzyme. In one example, the mixture comprises a sucrose invertase.

[0048] In some cases, the microorganism is selected from the group consisting of *Bracteococcus minor, Chlorella ellipsoidea, Chlorella kessleri, Chlorella luteoviridis, Bracteococcus medionucleatus, Chlorella minutissima, Chlorella ovalis, Chlorella protothecoides, Chlorella saccharophila, Chlorella sorokiniana, Chlorella sp., Chlorella vulgaris, Parachlorella kessleri, Prototheca moriformis,* and *Pseudochlorella aquatica.* In other cases, the microorganism is another species of microorganism as described herein. In some cases, the microorganism has been genetically engineered to express an exogenous gene encoding at least one lipid modification enzyme, hydrocarbon modification enzyme, or sucrose utilization enzyme.

[0049] In another example, the present disclosure is directed to a method of culturing a microalgae, which comprises culturing the microalgae in a culture medium including a feedstock comprising at least one carbon substrate selected from the group consisting of a cellulosic material, a 5-carbon sugar, a 6-carbon sugar, and acetate. In some cases, the carbon substrate is glucose and the microalgae is of a genus selected from the group

consisting of *Chlorella, Parachlorella, Pseudochlorella, Bracteococcus, Prototheca* and *Scenedesmus*. In some cases, the carbon substrate is xylose and the microalgae is of a genus selected from the group consisting of *Chlorella, Pseudochlorella,* and *Prototheca*. In some cases, the carbon substrate is sucrose and the microalgae is of a genus selected from the group consisting of *Chlorella,* and *Bracteococcus*. In some cases, the carbon substrate is fructose and the microalgae is of a genus selected from the group consisting of *Chlorella, Parachlorella, Prototheca,* and *Scenedesmus*. In some cases, the carbon substrate is arabinose and the microalgae is *Chlorella sp.* In some cases, the carbon substrate is mannose and the microalgae is of a genus selected from the group consisting of *Chlorella, Parachlorella, Bracteococcus, Prototheca,* and *Scenedesmus*. In some cases, the carbon substrate is galactose and the microalgae is of a genus selected from the group consisting of *Bracteococcus, Parachlorella, , Chlorella, Pseudochlorella, Bracteococcus,* and *Prototheca*. In some cases, the carbon substrate is acetate and the microalgae is of a genus selected from the group consisting of *Chlorella, Parachlorella,* and *Prototheca*.

[0050] In one example, the culture medium further includes at least one sucrose utilization enzyme. In some cases, the microalgae has been genetically engineered to express an exogenous gene encoding at least one lipid modification enzyme, hydrocarbon modification enzyme, or sucrose utilization enzyme. In some cases, the culture medium includes a sucrose invertase.

[0051] In another example, the present disclosure is directed to a method of culturing microalgae comprising placing a plurality of microalgae cells in the presence of depolymerized cellulosic material. In some cases, the microalgae are cultured in the presence of an additional fixed carbon source selected from the group consisting of glycerol, sucrose, glucose, arabinose, galactose, xylose, fructose, arabinose, mannose, acetate, and any combination of the foregoing. In one example, the microalgae are cultured in the presence of at least one sucrose utilization enzyme.

[0052] In some cases, the microalgae is selected from a species of the genus *Bracteococcus,* a species of the genus *Chlorella,* a species of the genus *Parachlorella,* a species of the genus *Prototheca,* or a species of the genus *Pseudochlorella*. In some cases, the microalgae is selected from *Bracteococcus minor, Chlorella ellipsoidea, Chlorella kessleri, Chlorella luteoviridis, Bracteococcus medionucleatus, Chlorella minutissima, Chlorella ovalis, Chlorella protothecoides, Chlorella saccharophila, Chlorella sorokiniana,*

Chlorella sp., Chlorella vulgaris, Parachlorella kessleri, Prototheca moriformis, and *Pseudochlorella aquatica*. In other cases, the microalgae is another species of microalgae as described herein.

[0053] In some examples, the microalgae has been genetically engineered to express an exogenous gene encoding at least one lipid modification enzyme, hydrocarbon modification enzyme, or sucrose utilization enzyme. In one example, the at least one sucrose utilization enzyme is a sucrose invertase. In some cases, the at least one lipid modification enzyme is selected from a stearoyl-ACP desaturase, a glycerolipid desaturase, a pyruvate dehydrogenase, an acetyl-CoA carboxylase, and a glycerol-3 phosphate acyltransferase. In some cases, the at least one hydrocarbon modification enzyme is selected from a fatty acyl-ACP thioesterase, a fatty acyl-CoA reductase, a fatty aldehyde reductase, a fatty acyl-CoA/aldehyde reductase, a fatty aldehyde decarbonylase, and an acyl carrier protein.

[0054] In another example, the present disclosure is directed to a method of culturing a lipid-producing microbe, the method comprising culturing the microbe in the presence of acetic acid and the absence of a fixed nitrogen source. In some cases, the microbe is cultured in the presence of a sufficient amount of acetic acid to increase microbial lipid yield over microbial lipid yield in the absence of acetic acid, wherein culture conditions are otherwise the same between the two cultures.

[0055] In another example, the present disclosure is directed to a microbial culture containing a population of microorganisms and a culture medium comprising glucose, xylose, and a molecule selected from the group consisting of lignin and a species of furfural. In some cases, the microorganisms are selected from *Bracteococcus minor, Chlorella ellipsoidea, Chlorella kessleri, Chlorella luteoviridis, Bracteococcus medionucleatus, Chlorella minutissima, Chlorella ovalis, Chlorella protothecoides, Chlorella saccharophila, Chlorella sorokiniana, Chlorella sp., Chlorella vulgaris, Parachlorella kessleri, Prototheca moriformis,* and *Pseudochlorella aquatica*. In other cases, the microorganisms are another species of microorganism as described herein.

[0056] In another example, the present disclosure is directed to a method of cultivating microalgae. In one example, the method comprises (a) providing a microalgae cell capable of performing heterotrophic growth, (b) placing the microalgae cell in growth media, wherein the growth media comprises depolymerized cellulosic material, and (c) incubating the microalgae for a period of time sufficient to allow the cell to grow.

[0057] In another example, the present disclosure is directed to a method of biodiesel manufacturing. In one example, the method comprises (a) culturing a lipid-producing microorganism in a first microbial culture, (b) recovering lipid from the biomass produced by the first microbial culture, (c) subjecting the lipid to transesterification to produce fatty acid ester(s) and glycerol, and (d) adding the glycerol to a second microbial culture. In some cases, the first and second microbial cultures are cultures of the same species of microorganism. In some cases, the second microbial culture comprises microorganisms selected from the group consisting of *Parachlorella kessleri, Chlorella protothecoides, Bracteococcus medionucleatus, Prototheca moriformis, Chlorella minutissima, Chlorella sp.,* and *Chlorella sorokiniana*. In other cases, the second microbial culture comprises another species of microorganism as described herein.

[0058] In another example, the present disclosure is directed to a method of fermentation comprising culturing a microorganism in the presence of glycerol and at least one other fixed carbon source. 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, 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, all of the glycerol and the at least one other fixed carbon source are fed to the microorganism at a predetermined rate over the course of the fermentation. In one example of the method, glycerol is provided to the microorganism in the absence of the at least one other fixed carbon source for a first period of time, the at least one other fixed carbon source is provided at the end of the first period of time, and the microorganism is cultured for a second period of time in the presence of the at least one other fixed carbon source. In one example, the at least one other fixed carbon source is fed to the microorganism at a predetermined rate during the second period of time. In some cases, all of the at least one other fixed carbon source is provided to the microorganism at the end of the first period of time. In one example of the method, the at least one other fixed carbon source is provided to the microorganism in the absence of glycerol for a first period of time, glycerol is provided at the end of the first period of time, and the microorganism is cultured for a second period of time in the presence of glycerol. In one example, the glycerol is a byproduct of a transesterification process. In one example, the glycerol is acidulated. In another example, the glycerol is non-acidulated. In some cases, the at least one other fixed carbon source is glucose. In some

cases, the at least one other fixed carbon source is depolymerized cellulosic material. In one example, the at least one other fixed carbon source is sucrose.

[0059] In another example, the present disclosure is directed to a fermentor comprising a population of microorganisms, glycerol, and at least one sugar selected from the group consisting of xylose, glucose, and sucrose. In one example, the glycerol is a byproduct of a lipid transesterification process. In some cases, the microorganisms are selected from the group consisting of *Parachlorella kessleri, Chlorella protothecoides, Bracteococcus medionucleatus, Prototheca moriformis, Chlorella minutissima, Chlorella sp.,* and *Chlorella sorokiniana*. In other cases, the microorganisms are another species as described herein.

[0060] In another example, the present disclosure is directed to a method of fermenting a microorganism. In one example, the method comprises providing byproduct glycerol from a transesterification process as a sole source of fixed carbon energy. In one example, no light energy is provided to the microorganism. In another example, light energy is provided to the microorganism. In some cases, the microorganism is selected from *Parachlorella kessleri, Chlorella protothecoides, Bracteococcus medionucleatus, Prototheca moriformis, Chlorella minutissima, Chlorella sp.,* and *Chlorella sorokiniana*. In other cases, the microorganism is another species as described herein.

[0061] In another example, the present disclosure is directed to a microorganism containing an exogenous sucrose utilization gene. In one example, the gene encodes a sucrose transporter. In one example, the gene encodes a sucrose invertase. In one example, the gene encodes a fructokinase. In some cases, the microorganism is a species selected from the group consisting of *Chlorella minutissima, Chlorella emersonii, Chlorella sorokiniana, Chlorella ellipsoidea, Chlorella sp.*, or *Chlorella protothecoides*. In other cases, the microorganism is another species as described herein.

[0062] In another example, the present disclosure is directed to a cell of the species *Chlorella protothecoides, Chlorella emersonii, or Chlorella minutissima* wherein the cell contains an exogenous gene. In some cases, the exogenous gene encodes a protein selected from the group consisting of a sucrose transporter, a sucrose invertase, a lipid modification enzyme, a hydrocarbon modification enzyme and a fructokinase. In some examples, the protein is a sucrose invertase secreted into the extracellular space. In some examples, the protein is a sucrose invertase targeted to the cytoplasm.

[0063] In another example, the present disclosure is directed to a microbial culture containing a population of microorganisms, and a culture medium comprising (i) sucrose and (ii) a sucrose invertase enzyme.

[0064] In another example, the present disclosure is directed to a microbial culture containing a population of microorganisms, and a culture medium comprising (i) molasses and (ii) a sucrose invertase enzyme.

[0065] In another example, the present disclosure is directed to a microbial culture containing a population of microorganisms, and a culture medium comprising (i) sucrose, (ii) lignin, and (iii) a sucrose invertase enzyme.

[0066] In the various microbial cultures described above, the microorganisms contain at least one exogenous sucrose utilization gene. In some examples, the sucrose utilization gene encodes a sucrose transporter, a sucrose invertase, a hexokinase, a glucokinase, or a fructokinase. In one example, the sucrose invertase enzyme is a secrectable sucrose invertase enzyme encoded by an exogenous sucrose invertase gene expressed by the population of microorganisms. In some cases, the microorganisms contain at least one exogenous gene encoding a lipid pathway enzyme or a hydrocarbon modification enzyme.

[0067] In another example, the present disclosure is directed to a nucleic acid comprising a cDNA encoding a sucrose utilization gene, and a cDNA encoding a protein conferring resistance to the antibiotic hygromycin or the antibiotic G418.

[0068] In examples of the various methods, compositions, cells, microorganisms, microbes, microbial cultures, fermentors, and the like, described above, the microorganism or microbe can be a microalgae, an oleaginous yeast, a fungus, or a bacterium, unless otherwise specified. In some cases, the microorganism is selected from the group consisting of the microalgae listed in Table 1. In some cases, the microorganism is a species of the genus *Chlorella*. In some cases, the microorganism is selected from the group consisting of *Chlorella anitrata, Chlorella antarctica, Chlorella aureoviridis, Chlorella candida , Chlorella capsulata, Chlorella desiccata, 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 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, 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. airidis, Chlorella vulgaris var. vulgaris, Chlorella vulgaris var. vulgaris f. tertia, Chlorella vulgaris var. vulgaris f. viridis, Chlorella xanthella, and *Chlorella zofingiensis*. In some cases, the microorganism is an oleaginous yeast selected from the group consisting of *Cryptococcus curvatus, Cryptococcus terricolus, Candida sp., Lipomyces starkeyi, Lipomyces lipofer, Endomycopsis vernalis, Rhodotorula glutinis, Rhodotorula gracilis,* and *Yarrowia lipolytica.* In some cases, the microorganism is a fungus selected from the group consisting of a species of the genus *Mortierella, Mortierrla vinacea, Mortierella alpine, Pythium debaryanum, Mucor circinelloides, Aspergillus ochraceus, Aspergillus terreus, Pennicillium iilacinum,* a species of the genus *Hensenulo*, a species of the genus *Chaetomium*, a species of the genus *Cladosporium*, a species of the genus *Malbranchea*, a species of the genus *Rhizopus*, and a species of the genus *Pythium*.

[0069] In the various examples described above, the microorganism can contain at least one exogenous sucrose utilization gene. In some cases, the sucrose utilization gene encodes a sucrose transporter, a sucrose invertase, a hexokinase, a glucokinase, or a fructokinase.

[0070] In the various examples described above, the microorganism can contain at least one exogenous gene encoding a lipid pathway enzyme. In some cases, the lipid pathway enzyme is selected from the group consisting of a stearoyl-ACP desaturase, a glycerolipid desaturase, a pyruvate dehydrogenase, an acetyl-CoA carboxylase, an acyl carrier protein, and a glycerol-3 phosphate acyltransferase.

[0071] In the various examples described above, the microorganism can contain at least one exogenous gene encoding a hydrocarbon modification enzyme. In some cases, the hydrocarbon modification enzyme is 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.

[0072] Any two or more of the various examples described above can be combined together to produce additional examples encompassed within the scope of the present disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

[0073] Figure 1 shows dry cell weight per liter of multiple species and strains of *Chlorella* when cultured in the presence of various types of glycerol with and without additional glucose.

[0074] Figure 2 shows dry cell weight per liter of multiple species and strains of *Chlorella* when cultured in the presence of various types of glycerol with additional glucose.

[0075] Figure 3 shows relative lipid concentration of cultures of multiple species and strains of *Chlorella* when cultured in the presence of various types of glycerol with additional glucose.

[0076] Figure 4 shows lipid concentration of cultures of multiple species and strains of *Chlorella* when cultured in the presence of various types of glycerol with and without additional glucose.

[0077] Figure 5 shows lipid as a percent of dry cell weight of two species and strains of *Chlorella* when cultured in the presence of various types of glycerol with additional glucose, wherein glycerol is added sequentially after glucose.

[0078] Figure 6 shows lipid as a percent of dry cell weight of two species and strains of *Chlorella* when cultured in the presence of various types of glycerol with additional glucose.

[0079] Figure 7 shows relative lipid concentration of cultures of multiple species and strains of *Chlorella* when cultured in the presence of 2% glucose and 1% glucose + 1% reagent grade glycerol.

[0080] Figure 8 shows lipid as a percent of dry cell weight of multiple species and strains of *Chlorella* when cultured in the presence of glucose with and without reagent grade glycerol, wherein glycerol is added sequentially or in combination with glucose.

[0081] Figure 9 shows relative lipid concentration of cultures of multiple species and strains of *Chlorella* when cultured in the presence of various types of glycerol with additional glucose, wherein glycerol is added sequentially or in combination with glucose.

[0082] Figure 10 shows dry cell weight per liter of multiple species and strains of *Chlorella* when cultured in the presence of various types of glycerol with additional glucose, wherein glycerol is added sequentially or in combination with glucose.

[0083] Figure 11(a) shows lipid as a percent of dry cell weight of *Spirulina platensis* when cultured in the presence of glucose, reagent grade glycerol, non-acidulated biodiesel byproduct glycerol, and a combination of glycerol and glucose.

[0084] Figure 11(b) shows lipid as a percent of dry cell weight of *Navicula pelliculosa* when cultured in the presence of various types of glycerol and in the presence of combinations of glycerol and glucose.

[0085] Figure 12(a) shows lipid as a percent of dry cell weight of *Scenedesmus armatus* when cultured in the presence of various types of glycerol and in the presence of a combination of glycerol and glucose.

[0086] Figure 12(b) shows dry cell weight per liter of *Scenedesmus armatus* when cultured in the presence of various types of glycerol and in the presence of a combination of biodiesel byproduct glycerol and glucose.

[0087] Figure 13 shows dry cell weight per liter of *Navicula pelliculosa* when cultured in the presence of various types of glycerol and in the presence of a combination of non-acidulated biodiesel byproduct glycerol and glucose.

[0088] Figure 14 shows dry cell weight per liter of *Scenedesmus armatus* and *Navicula pelliculosa* when cultured in the presence of acidulated and non-acidulated biodiesel byproduct glycerol with additional glucose, wherein glycerol is added sequentially or in combination with glucose.

[0089] Figure 15 shows a synergistic effect of a combination of xylose and glucose on growth of *Chlorella* compared to xylose or glucose alone.

[0090] Figure 16 shows genotyping of *Chlorella protothecoides* transformants containing an exogenous gene.

[0091] Figure 17 shows codon usage of *Chlorella protothecoides*.

[0092] Figure 18 shows codon usage of *D. salina* and *Chlorella pyrenoidosa*.

[0093] Figure 19 shows (a) reagent grade glycerol; (b) non-acidulated biodiesel byproduct glycerol; and (c) acidulated biodiesel byproduct glycerol, all of which were used in experiments described in the Examples.

[0094] Figure 20 shows growth of *Chlorella protothecoides* on glucose and fructose.

[0095] Figure 21 shows growth of *Chlorella fusca* on 1% sucrose.

[0096] Figure 22 shows growth of *Chlorella kessleri* on 1% sucrose.

[0097] Figure 23 shows dry cell weight per liter of *Chlorella protothecoides* when cultured in the presence of glucose, sucrose, or one of several molasses samples (designated BS1, BS2 and HTM) in the presence or absence of a sucrose invertase.

[0098] Figure 24 shows growth of *Chlorella protothecoides* when cultured in the presence of glucose, sucrose, or one of several molasses samples (designated BS1, BS2 and HTM) in the presence or absence of a sucrose invertase as measured by relative cell density.

[0099] Figure 25 shows an illustration of various plasmid constructs of yeast invertase (SUC2) with three different promoters (designated CMV, CV and HUP1) as well as restriction sites useful for subcloning.

[0100] Figure 26 shows genotyping of *Chlorella protothecoides* transformants selected on sucrose in the dark containing an exogenous sucrose invertase gene.

[0101] Figure 27 shows genotyping of *Chlorella protothecoides* cells transformed with a gene encoding a secreted sucrose invertase from *S. cerevisiae.*

[0102] Figure 28 shows genotyping of *Chlorella minutissima* and *Chlorella emersonii* cells transformed with a gene encoding a secreted sucrose invertase from *S. cerevisiae.*

[0103] Figure 29 illustrates a gas chromatograph generated by analysis of a renewable diesel product produced in accordance with the methods of the present invention, and described in Example 27.

[0104] Figure 30 illustrates a boiling point distribution plot for a renewable diesel product produced in accordance with the methods of the presence invention, and described in Example 27.

DETAILED DESCRIPTION OF THE INVENTION

I. DEFINITIONS

[0105] 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.

[0106] As used with reference to a nucleic acid, "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. Examples of promoters active in microalgae are promoters endogenous to certain algae species and promoters found in plant viruses.

[0107] An "acyl carrier protein" or "ACP" is a protein which 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. The phrase "naturally co-expressed" with reference to an acyl carrier protein in conjunction with a fatty acyl-ACP thioesterase means that the ACP and the thioesterase are co-expressed naturally in a tissue or organism from which they are derived, e.g., because the genes encoding the two enzymes are under the control of a common regulatory sequence or because they are expressed in response to the same stimulus.

[0108] An "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.

[0109] "Axenic" means a culture of an organism that is free from contamination by other living organisms.

[0110] "Biodiesel" is a biologically produced fatty acid alkyl ester suitable for use as a fuel in a diesel engine.

[0111] The term "biomass" refers to material produced by growth and/or propagation of cells. Biomass may contain cells and/or intracellular contents as well as extracellular material. Extracellular material includes, but is not limited to, compounds secreted by a cell.

[0112] "Bioreactor" means an enclosure or partial enclosure in which cells are cultured, optionally in suspension.

[0113] As used herein, a "catalyst" refers to 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 thus 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 metal catalysts used in fossil oil refining processes.

[0114] "Cellulosic material" means the products 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 caner bagasses, sugar beet pulp, corn stover, wood chips, sawdust and switchgrass.

[0115] The term "co-culture", and variants thereof such as "co-cultivate", 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.

[0116] The term "cofactor" is used herein to refer to any molecule, other than the substrate, that is required for an enzyme to carry out its enzymatic activity.

[0117] As used herein, "complementary DNA" ("cDNA") is a DNA representation of mRNA, usually obtained by reverse transcription of messenger RNA (mRNA) or amplification (e.g., via polymerase chain reaction ("PCR")).

[0118] The term "cultivated", and variants thereof, 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 intended culture conditions. The combination of both growth and propagation may be termed proliferation. The one or more cells may be those of a microorganism, such as microalgae. Examples of intended 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. The term does not refer to the growth or propagation of

microorganisms in nature or otherwise without direct human intervention, such as natural growth of an organism that ultimately becomes fossilized to produce geological crude oil.

[0119] As used herein, the term "cytolysis" refers to 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.

[0120] As used herein, the terms "expression vector" or "expression construct" refer to a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription 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.

[0121] "Exogenous gene" refers to a nucleic acid transformed into a cell. A transformed cell may be referred to as a recombinant cell, into which additional exogenous gene(s) may be introduced. The exogenous gene may be from a different species (and so heterologous), or from the same species (and so homologous) relative to the cell being transformed. In the case of a homologous gene, it occupies a different location in the genome of the cell relative to the endogenous copy of the gene. The exogenous gene may be present in more than one copy in the cell. The exogenous gene may be maintained in a cell as an insertion into the genome or as an episomal molecule.

[0122] "Exogenously provided" describes a molecule provided to the culture media of a cell culture.

[0123] As used herein, a "fatty acyl-ACP thioesterase" is an enzyme that catalyzes the cleavage of a fatty acid from an acyl carrier protein (ACP) during lipid synthesis.

[0124] As used herein, a "fatty acyl-CoA/aldehyde reductase" is an enzyme that catalyzes the reduction of an acyl-CoA molecule to a primary alcohol.

[0125] As used herein, a "fatty acyl-CoA reductase" is an enzyme that catalyzes the reduction of an acyl-CoA molecule to an aldehyde.

[0126] As used herein, a "fatty aldehyde decarbonylase" is an enzyme that catalyzes the conversion of a fatty aldehyde to an alkane.

[0127] As used herein, a "fatty aldehyde reductase" is an enzyme that catalyzes the reduction of an aldehyde to a primary alcohol.

[0128] "Fixed carbon source" means molecule(s) containing carbon, preferably organic, that are present at ambient temperature and pressure in solid or liquid form.

[0129] "Fungus," as used herein, means heterotrophic organisms characterized by a chitinous cell wall from the kingdom of fungi.

[0130] "Homogenate" means biomass that has been physically disrupted.

[0131] As used herein, "hydrocarbon" refers to: (a) a molecule containing only hydrogen and carbon atoms wherein the carbon atoms are covalently linked to form a linear, branched, cyclic, or partially cyclic backbone to which the hydrogen atoms are attached; or (b) a molecule that only primarily contains hydrogen and carbon atoms and that can be converted to contain only hydrogen and carbon atoms by one to four chemical reactions. Nonlimiting examples of the latter include hydrocarbons containing an oxygen atom between one carbon and one hydrogen atom to form an alcohol molecule, as well as aldehydes containing a single oxygen atom. Methods for the reduction of alcohols to hydrocarbons containing only carbon and hydrogen atoms are well known. Another example of a hydrocarbon is an ester, in which an organic group replaces a hydrogen atom (or more than one) in an oxygen acid. 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, triolein, and squalene.

[0132] "Hydrocarbon modification enzyme" refers to an enzyme that alters the covalent structure of a hydrocarbon. Examples of hydrocarbon modification enzymes include a lipase, a fatty acyl-ACP thioesterase, a fatty acyl-CoA/aldehyde reductase, a fatty acyl-CoA reductase, a fatty aldehyde reductase, and a fatty aldehyde decarbonylase. Compounds produced by the enzymatic activity of hydrocarbon modification enzymes, including fatty acids, alcohols, aldehydes, alkanes, or other compounds derived therefrom are referred to herein interchangeably as hydrocarbons or lipids.

[0133] The term "hydrogen:carbon ratio" refers to 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).

[0134] "Hydrophobic fraction" refers to 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.

[0135] As used herein, the phrase "increase lipid yield" refers to an increase in the productivity of a microbial culture by, for example, increasing dry weight of cells per liter of culture, increasing the percentage of cells that constitute lipid, or increasing the overall amount of lipid per liter of culture volume per unit time.

[0136] An "inducible promoter" is one that mediates transcription of an operably linked gene in response to a particular stimulus.

[0137] As used herein, the phrase "in operable linkage" refers to a functional linkage between two sequences, such a control sequence (typically a promoter) and the linked sequence. A promoter is in operable linkage with an exogenous gene if it can mediate transcription of the gene.

[0138] The term "in situ" means "in place" or "in its original position". For example, a culture may contain a first microalgae secreting a catalyst and a second microorganism secreting a substrate, wherein the first and second cell types produce the components necessary for a particular chemical reaction to occur in situ in the co-culture without requiring further separation or processing of the materials.

[0139] A "limiting concentration of a nutrient" is a concentration 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.

[0140] As used herein, a "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.

[0141] As used herein, a "lipid pathway enzyme" is any enzyme that plays a role in lipid metabolism, i.e., either lipid synthesis, modification, or degradation. This term encompasses proteins that chemically modify lipids, as well as carrier proteins.

[0142] "Lipids" are a class of hydrocarbon 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."

[0143] As used herein, the term "lysate" refers to a solution containing the contents of lysed cells.

[0144] As used herein, the term "lysis" refers to the breakage of the plasma membrane and optionally the cell wall of a biological organism sufficient to release at least some intracellular content, often by mechanical, viral or osmotic mechanisms that compromise its integrity.

[0145] As used herein, the term "lysing" refers to disrupting the cellular membrane and optionally the cell wall of a biological organism or cell sufficient to release at least some intracellular content.

[0146] "Microalgae" means a eukarytotic microbial organism that contains a chloroplast, 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 can refer to unicellular organisms that separate from sister cells shortly after cell division, such as

Chlamydomonas, and can also refer to microbes such as, for example, *Volvox*, which is a simple multicellular photosynthetic microbe of two distinct cell types. "Microalgae" can also refer to cells such as *Chlorella* and *Dunaliella*. "Microalgae" also includes other microbial photosynthetic organisms that exhibit cell-cell adhesion, such as *Agmenellum, Anabaena*, and *Pyrobotrys*. "Microalgae" also includes obligate heterotrophic microorganisms that have lost the ability to perform photosynthesis, such as certain dinoflagellate algae species.

[0147] The terms "microorganism" and "microbe" are used interchangeably herein to refer to microscopic unicellular organisms.

[0148] "Oleaginous yeast," as used herein, means yeast that can naturally accumulate more than 10% of its dry cell weight as lipid or can do so as a result of genetic engineering. Oleaginous yeast includes organisms such as *Yarrowia lipolytica*, as well as engineered strains of yeast such as *Saccharomyces cerevisiae* that have been engineered to accumulate more than 10% of their dry cell weight as lipid.

[0149] As used herein, the term "osmotic shock" refers to 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.

[0150] "Photobioreactor" refers to a container, at least part of which is at least partially transparent or partially open, thereby allowing light to pass through, in which one or more microalgae cells are cultured. Photobioreactors may be closed, as in the instance of a polyethylene bag or Erlenmeyer flask, or may be open to the environment, as in the instance of an outdoor pond.

[0151] As used herein, a "polysaccharide-degrading enzyme" refers to any enzyme capable of catalyzing the hydrolysis, or depolymerization, of any polysaccharide. For example, cellulases catalyze the hydrolysis of cellulose.

[0152] "Polysaccharides" (also called "glycans") are carbohydrates made up of monosaccharides joined together by glycosidic linkages. Cellulose is an example of 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.

[0153] "Port", in the context of a bioreactor, refers to an opening in the bioreactor that allows influx or efflux of materials such as gases, liquids, and cells. Ports are usually connected to tubing leading from the photobioreactor.

[0154] A "promoter" is defined as an array of nucleic acid control sequences that direct 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.

[0155] As used herein, the term "recombinant" when used with reference, e.g., to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of an exogenous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, e.g., recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all. By the term "recombinant nucleic acid" herein is meant nucleic acid, originally formed in vitro, in general, by the manipulation of nucleic acid, e.g., using polymerases and endonucleases, in a form not normally found in nature. In this manner, operably linkage of different sequences is achieved. Thus an isolated nucleic acid, in a linear form, or an expression vector formed in vitro by ligating DNA molecules that are not normally joined, are both considered recombinant for the purposes of this invention. It is understood that once a recombinant nucleic acid is made and reintroduced into a host cell or organism, it will replicate non-recombinantly, i.e., using the in vivo cellular machinery of the host cell rather than in vitro manipulations; however, such nucleic acids, once produced recombinantly, although subsequently replicated non-recombinantly, are still considered recombinant for the purposes of the invention. Similarly, a "recombinant protein" is a protein made using recombinant techniques, i.e., through the expression of a recombinant nucleic acid as depicted above.

[0156] As used herein, the term "renewable diesel" refers to alkanes (such as C:10:0, C12:0, C:14:0, C16:0 and C18:0) produced through hydrogenation and deoxygenation of lipids.

[0157] As used herein, the term "sonication" refers to a process of disrupting biological materials, such as a cell, by use of sound wave energy.

[0158] "Species of furfural" refers to 2-Furancarboxaldehyde or a derivative thereof which retains the same basic structural characteristics.

[0159] As used herein, "stover" refers to the dried stalks and leaves of a crop remaining after a grain has been harvested.

[0160] A "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.

[0161] "Wastewater" is watery waste which typically contains washing water, laundry waste, faeces, urine and other liquid or semi-liquid wastes. It includes some forms of municipal waste as well as secondarily treated sewage.

[0162] 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.

[0163] 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*).

[0164] Another example algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul *et al., J. Mol. Biol.* 215:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (at the web address

www.ncbi.nlm.nih.gov). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al., supra.*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. For identifying whether a nucleic acid or polypeptide is within the scope of the invention, the default parameters of the BLAST programs are suitable. The BLASTN program (for nucleotide sequences) uses as defaults a word length (W) of 11, an expectation (E) of 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a word length (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix. The 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)).

[0165] 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.

II. GENERAL

[0166] The invention is premised in part on the insight that certain microorganisms can be used to produce oils, fuels, and other hydrocarbon or lipid compositions economically and in large quantities for use in the transportation fuel, petrochemical industry, and/or food and cosmetic industries, among other applications. Suitable microorganisms include microalgae, oleaginous yeast, and fungi. A preferred genus of microalgae for use in the invention is the lipid-producing microalgae *Chlorella*. Transesterification of lipids yields long-chain fatty acid esters useful as biodiesel. Other enzymatic and chemical processes can be tailored to yield fatty acids, aldehydes, alcohols, alkanes, and alkenes. The present application describes methods for genetic modification of multiple species and strains of microorganisms, including *Chlorella* and similar microbes to provide organisms having characteristics that facilitate the production of lipid suitable for conversion into oils, fuels, and oleochemicals. In some applications, renewable diesel, jet fuel, or other hydrocarbon compounds are produced. The present application also describes methods of cultivating microalgae for increased productivity and increased lipid yield, and/or for more cost-effective production of the compositions described herein.

[0167] In particular embodiments, the present application describes genetically engineering strains of microalgae with one or more exogenous genes. For example, microalgae that produce high levels of triacylglycerides (TAGs) suitable for biodiesel can be engineered to express a lipase, which can facilitate transesterification of microalgal TAGs. The lipase can optionally be expressed using an inducible promoter, so that the cells can first be grown to a desirable density in a fermentor and then harvested, followed by induction of the promoter to express the lipase, optionally in the presence of sufficient alcohol to drive conversion of TAGs to fatty acid esters.

[0168] Some microalgal lipid is sequestered in cell membranes and other non-aqueous parts of the cell. Therefore, to increase the yield of the transesterification reaction, it can be beneficial to lyse the cells to increase the accessibility of the lipase to the lipid. Cell disruption can be performed, for example, mechanically, through addition of pressurized steam, or by employing a virus that lyses the microalgae cells, expressing a gene to produce a lytic protein in the cell, or treating the culture with an agent that lyses microalgae cells. Steam treatment of microalgae for cell disruption is described, for example, in U.S. Patent 6,750,048.

[0169] Also disclosed herein is the genetic engineering of microalgae that produce high levels of TAGs to express a gene that lyses microalgae cells, such as for example, a gene from a lytic virus. This gene can be expressed using an inducible promoter, so that the cells can first be grown to a desirable density in a fermentor and then harvested, followed by induction of the promoter to express the gene to lyse the cells. A gene encoding a polysaccharide-degrading enzyme, for example, can be expressed to lyse the cells.

[0170] Optionally, the lipase can be expressed in an intracellular compartment, where it remains separate from the majority of the microalgal lipid until transesterification. Generally, it is preferable to carry out transesterification after water has been substantially removed from the preparation and/or an excess of alcohol has been added. Lipases can use water, as well as alcohol, as a substrate in transesterification. With water, the lipid is conjugated to a hydroxyl moiety to produce a polar fatty acid, rather than an ester. With an alcohol, such as methanol, the lipid is conjugated to a methyl group, producing a non-polar fatty acid ester, which is typically preferable for a transportation fuel. To limit exposure of the lipase to microalgal lipid until conditions are suitable for transesterification to produce fatty acid esters, the lipase can be expressed, for example, in the chloroplast, mitochondria, or other cellular organelle. This compartmentalized expression results in sequestration of the lipase from the majority of the cellular lipid until after the cells have been disrupted.

[0171] In other particular embodiments, the present application describes genetically engineering strains of microalgae, oleaginous yeast, bacteria, or fungi with one or more exogenous genes to produce various hydrocarbon compounds. For example, microalgae that would naturally, or through genetic modification, produce high levels of lipids can be engineered (or further engineered) to express an exogenous fatty acyl-ACP thioesterase, which can facilitate the cleavage of fatty acids from the acyl carrier protein (ACP) during lipid synthesis. These fatty acids can be recovered or, through further enzymatic processing within the cell, yield other hydrocarbon compounds. Optionally, the fatty acyl-ACP thioesterase can be expressed from a gene operably linked to an inducible promoter, and/or can be expressed in an intracellular compartment.

[0172] The fatty acyl-ACP thioesterase can be chosen based on its specificity for a growing (during fatty acid synthesis) fatty acid having a particular carbon chain length. For example, the fatty acyl-ACP thioesterase can have a specificity for a carbon chain length ranging from

8 to 34 carbon atoms, preferably from 8 to 18 carbon atoms, and more preferably from 10 to 14 carbon atoms. A specificity for a fatty acid with 12 carbon atoms is most preferred.

[0173] Further, the invention provides genetically engineered strains of microalgae to express two or more exogenous genes, such as, for example, a lipase and a lytic gene, e.g., one encoding a polysaccharide-degrading enzyme. 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. The invention also provides vectors and methods for engineering lipid-producing microbes to metabolize sucrose, which is an advantageous trait because it allows the engineered cells to convert sugar cane or other feedstocks into lipids appropriate for production of oils, fuels, oleochemicals and the like.

[0174] In other embodiments, the invention provides genetically engineered strains of microbes (e.g., microalgae, oleaginous yeast, bacteria, or fungi) that express two or more exogenous genes, such as, for example, a fatty acyl-ACP thioesterase and a fatty acyl-CoA/aldehyde reductase, the combined action of which yields an alcohol product. The invention further provides other combinations of exogenous genes, including without limitation, a fatty acyl-ACP thioesterase and a naturally co-expressed acyl carrier protein to generate length-specific fatty acids, or a fatty acyl-ACP thioesterase and a fatty acyl-CoA reductase to generate aldehydes. The invention also provides for the combination of a fatty acyl-ACP thioesterase, a fatty acyl-CoA reductase, and a fatty aldehyde decarbonylase to generate alkanes or alkenes. One or more of the exogenous genes can be expressed using an inducible promoter.

[0175] The invention provides further modifications of microalgae, for example to provide microalgae with desired growth characteristics and/or to enhance the amount and/or quality of lipids produced. For example, microalgae can be engineered to increase carbon flux into the lipid pathway and/or modify the lipid pathway to beneficially alter the proportions or properties of lipid produced by the cells.

[0176] This application discloses genetically engineering strains of microalgae to express two or more exogenous genes, one encoding a transporter of a fixed carbon source (such as sucrose) and a second encoding a sucrose invertase enzyme. The resulting fermentable organisms produce hydrocarbons at lower manufacturing cost than what has been obtainable by previously known methods of biological hydrocarbon production. The insertion of the

two exogenous genes described above can be combined with the disruption of polysaccharide biosynthesis through directed and/or random mutagenesis, which steers ever greater carbon flux into hydrocarbon production. Individually and in combination, trophic conversion, engineering to alter hydrocarbon production and treatment with exogenous enzymes alter the hydrocarbon composition produced by a microorganism. The alteration can be a change in the amount of hydrocarbons produced, the amount of one or more hydrocarbon species produced relative to other hydrocarbons, and/or the types of hydrocarbon species produced in the microorganism. For example, microalgae can be engineered to produce a higher amount and/or percentage of TAGs.

III. OIL- OR LIPID-PRODUCING MICROORGANISMS

[0177] Any species of organism that produces suitable lipid or hydrocarbon can be used, although microorganisms that naturally produce high levels of suitable lipid or hydrocarbon are preferred. Production of hydrocarbons by microorganisms is reviewed by Metzger et al. Appl Microbiol Biotechnol (2005) 66: 486–496 and A Look Back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from Algae, NREL/TP-580-24190, John Sheehan, Terri Dunahay, John Benemann and Paul Roessler (1998).

[0178] Considerations affecting the selection of microorganisms for use in the invention include, in addition to production of suitable lipids or hydrocarbons for production of oils, fuels, and oleochemicals: (1) high lipid content as a percentage of cell weight; (2) ease of growth; (3) ease of genetic engineering; and (4) ease of biomass processing. In particular embodiments, the wild-type or genetically engineered microorganism yields cells that are at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, or at least 70% or more lipid. Preferred organisms grow heterotrophically (on sugars in the absence of light) or can be engineered to do so using, for example, methods disclosed herein. The ease of transformation and availability of selectable markers and promoters, constitutive and/or inducible, that are functional in the microorganism affect the ease of genetic engineering. Processing considerations can include, for example, the availability of effective means for lysing the cells.

A. Algae

[0179] In one embodiment of the present invention, the microorganism is a microalgae. Nonlimiting examples of microalgae that can be used in accordance with the present invention can be found in Table 1.

[0180] Table 1. Examples of 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, Bracteococcus minor, Bracteococcus medionucleatus, 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 ovalis, Chlorella parva, Chlorella photophila, Chlorella pringsheimii, Chlorella protothecoides (including any of UTEX strains 1806, 411, 264, 256, 255, 250, 249, 31, 29, 25), 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 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., Crypthecodinium cohnii, 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, Parachlorella kessleri, Pascheria acidophila, Pavlova sp., Phagus, Phormidium, Platymonas sp., Pleurochrysis carterae, Pleurochrysis dentate, Pleurochrysis sp., Prototheca wickerhamii, Prototheca stagnora, Prototheca portoricensis, Prototheca moriformis, Prototheca zopfii, Pseudochlorella aquatica, Pyramimonas sp., Pyrobotrys, Rhodococcus opacus, Sarcinoid chrysophyte, Scenedesmus armatus, Schizochytrium, Spirogyra, Spirulina platensis, Stichococcus sp., Synechococcus sp., Tetraedron, Tetraselmis sp., Tetraselmis suecica, Thalassiosira weissflogii, and *Viridiella fridericiana*

1. *Chlorella*

[0181] In a preferred embodiment of the present invention, the microorganism is of the genus *Chlorella,* preferably, *Chlorella protothecoides*, *Chlorella ellipsoidea*, *Chlorella minutissima*, or *Chlorella emersonii.*

[0182] *Chlorella* is a genus of single-celled green algae, belonging to the phylum Chlorophyta. It is spherical in shape, about 2 to 10 μm in diameter, and is without flagella. Some species of *Chlorella* are naturally heterotrophic.

[0183] *Chlorella*, particularly *Chlorella protothecoides*, is a preferred microorganism for use in the invention because of its high composition of lipid, particularly long-chain lipid suitable for biodiesel. In addition, this microalgae grows heterotrophically and can be genetically engineered as demonstrated in the Examples herein.

[0184] In a preferred embodiment of the present invention, the microorganism used for expression of a transgene is of the genus *Chlorella,* preferably, *Chlorella protothecoides*, *Chlorella minutissima*, or *Chlorella emersonii.* Examples of expression of transgenes in, e.g., *Chlorella*, can be found in the literature (see for example *Current Microbiology* Vol. 35 (1997), pp. 356–362; Sheng Wu Gong Cheng Xue Bao. 2000 Jul;16(4):443-6; *Current*

Microbiology Vol. 38 (1999), pp. 335–341; *Appl Microbiol Biotechnol* (2006) 72: 197–205; *Marine Biotechnology* 4, 63–73, 2002; *Current Genetics* 39:5, 365-370 (2001); *Plant Cell Reports* 18:9, 778-780, (1999); *Biologia Plantarium* 42(2): 209-216, (1999); *Plant Pathol. J* 21(1): 13-20, (2005)). Also see Examples herein. Other lipid-producing microalgae can be engineered as well, including prokaryotic Microalgae (see Kalscheuer st al., Applied Microbiology and Biotechnology, Volume 52, Number 4 / October, 1999).

2. Identification of *Chlorella* Species

[0185] Species of *Chlorella* for use in the invention can be identified by amplification of certain target regions of the genome. For example, identification of a specific *Chlorella* 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), 18S rRNA, and other conserved genomic regions can be used by those skilled in the art to identify species of not only *Chlorella*, but other hydrocarbon and lipid producing organisms capable of using the methods disclosed herein. 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.

B. Oleaginous Yeast

[0186] In one embodiment of the present invention, the microorganism is an oleaginous yeast. Nonlimiting examples of oleaginous yeast that can be used in accordance with the present invention can be found in Table 2.

[0187] Table 2. Examples of oleaginous yeast.

Cryptococcus curvatus, Cryptococcus terricolus, Candida sp., Lipomyces starkeyi, Lipomyces lipofer, Endomycopsis vernalis, Rhodotorula glutinis, Rhodotorula gracilis, and *Yarrowia lipolytica*

C. Other Fungi

[0188] In one embodiment of the present invention, the microorganism is a fungus. Nonlimiting examples of fungi that can be used in accordance with the present invention can be found in Table 3.

[0189] Table 3. Examples of fungi.

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

D. Bacteria

[0190] In one embodiment of the present invention, the microorganism is a bacterium.

[0191] Examples of expression of exogenous genes in bacteria, such as *E. coli,* are well known; see for example *Molecular Cloning: A Laboratory Manual,* Sambrook *et al.* (3d edition, 2001, Cold Spring Harbor Press).

IV. METHODS OF CULTURING MICROORGANISMS

[0192] Microrganisms are cultured both for purposes of conducting genetic manipulations and for subsequent production of hydrocarbons (e.g., lipids, fatty acids, aldehydes, alcohols, and alkanes). The former type of culture is conducted on a small scale and initially, at least, under conditions in which the starting microorganism can grow. For example, if the starting microorganism is a photoautotroph the initial culture is conducted in the presence of light. The culture conditions can be changed if the microorganism is evolved or engineered to grow independently of light. Culture for purposes of hydrocarbon production is usually conducted on a large scale. Preferably a fixed carbon source is present. The culture can also be exposed to light some or all of the time.

[0193] Microalgae can be cultured in liquid media. The culture can be contained within a bioreactor. Optionally, the bioreactor does not allow light to enter. Alternatively, microalgae can also be cultured in photobioreactors that contain a fixed carbon source and allow light to strike the cells. Exposure of microalgae cells to light, even in the presence of a fixed carbon source that the cells transport and utilize (i.e., mixotrophic growth), nonetheless accelerates growth compared to culturing cells in the dark. Culture condition parameters can be manipulated to optimize total hydrocarbon production, the combination of hydrocarbon

species produced, and/or production of a hydrocarbon species. In some instances it is preferable to culture cells in the dark, such as, for example, when using extremely large (40,000 liter and higher) fermentors that do not allow light to strike the culture.

[0194] Microalgal culture media typically contains components such as a fixed nitrogen source, trace elements, optionally a buffer for pH maintenance, and phosphate. Other components can include a fixed carbon source such as acetate or glucose, and salts such as sodium chloride, particularly for seawater microalgae. 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 $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$.

[0195] For organisms able to grow on a fixed carbon source, the fixed carbon source can be, for example, glucose, fructose, sucrose, galactose, xylose, mannose, rhamnose, N-acetylglucosamine, glycerol, floridoside, and/or glucuronic acid. 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). Some microalgae species can grow by utilizing a fixed carbon source such as glucose or acetate in the absence of light. Such growth is known as heterotrophic growth. For *Chlorella protothecoides*, for example, heterotrophic growth results in high production of biomass and accumulation of high lipid content in cells.

[0196] Some microorganisms naturally grow on or can be engineered to grow on a fixed carbon source that is a heterogeneous source of compounds such as municipal waste, secondarily treated sewage, wastewater, and other sources of fixed carbon and other nutrients such as sulfates, phosphates, and nitrates. The sewage component serves as a nutrient source in the production of hydrocarbons, and the culture provides an inexpensive source of hydrocarbons.

[0197] Other culture parameters can also be manipulated, such as the pH of the culture media, the identity and concentration of trace elements and other media constituents.

A. Photosynthetic Growth

[0198] Microalgae can be grown in the presence of light. The number of photons striking a culture of microalgae cells can be manipulated, as well as other parameters such as the wavelength spectrum and ratio of dark:light hours per day. Microalgae can also be cultured

in natural light, as well as simultaneous and/or alternating combinations of natural light and artificial light. For example, microalgae of the genus *Chlorella* can be cultured under natural light during daylight hours and under artificial light during night hours.

[0199] The gas content of a photobioreactor to grow microorganisms like microalgae can be manipulated. Part of the volume of a photobioreactor can contain gas rather than liquid. Gas inlets can be used to pump gases into the photobioreactor. Any gas can be pumped into a photobioreactor, including air, air/CO_2 mixtures, noble gases such as argon and others. The rate of entry of gas into a photobioreactor can also be manipulated. Increasing gas flow into a photobioreactor increases the turbidity of a culture of microalgae. Placement of ports conveying gases into a photobioreactor can also affect the turbidity of a culture at a given gas flow rate. Air/CO_2 mixtures can be modulated to generate optimal amounts of CO_2 for maximal growth by a particular organism. Microalgae grow significantly faster in the light under, for example, 3% CO_2/97% air than in 100% air. 3% CO_2/97% air is approximately 100-fold more CO_2 than found in air. For example, air:CO_2 mixtures of about 99.75% air:0.25% CO_2, about 99.5% air:0.5% CO_2, about 99.0% air:1.00% CO_2, about 98.0% air:2.0% CO_2, about 97.0% air:3.0% CO_2, about 96.0% air:4.0% CO_2, and about 95.00% air:5.0% CO_2 can be infused into a bioreactor or photobioreactor.

[0200] Microalgae cultures can also be subjected to mixing using devices such as spinning blades and impellers, rocking of a culture, stir bars, infusion of pressurized gas, and other instruments.

[0201] Photobioreactors can have ports allowing entry of gases, solids, semisolids and liquids into the chamber containing the microalgae. Ports are usually attached to tubing or other means of conveying substances. Gas ports, for example, convey gases into the culture. Pumping gases into a photobioreactor can serve to both feed cells CO_2 and other gases and to aerate the culture and therefore generate turbidity. The amount of turbidity of a culture varies as the number and position of gas ports is altered. For example, gas ports can be placed along the bottom of a cylindrical polyethylene bag. Microalgae grow faster when CO_2 is added to air and bubbled into a photobioreactor. For example, a 5% CO_2:95% air mixture is infused into a photobioreactor containing *Botryococcus* cells (see for example J Agric Food Chem. 2006 Jun 28;54(13):4593-9; J Biosci Bioeng. 1999;87(6):811-5; and J Nat Prod. 2003 Jun;66(6):772-8).

[0202] Photobioreactors can be exposed to one or more light sources to provide microalgae with light as an energy source via light directed to a surface of the photobioreactor. Preferably the light source provides an intensity that is sufficient for the cells to grow, but not so intense as to cause oxidative damage or cause a photoinhibitive response. In some instances a light source has a wavelength range that mimics or approximately mimics the range of the sun. In other instances a different wavelength range is used. Photobioreactors can be placed outdoors or in a greenhouse or other facility that allows sunlight to strike the surface. Preferred photon intensities for species of the genus *Botryococcus* are between 25 and 500 $\mu E\ m^{-2}\ s^{-1}$ (see for example Photosynth Res. 2005 Jun;84(1-3):21-7).

[0203] Photobioreactors preferably have one or more ports that allow media entry. 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 photobioreactor and then later can be used for sampling, gas entry, gas exit, or other purposes. In some instances a photobioreactor is filled with culture media at the beginning of a culture and no more growth media is infused after the culture is inoculated. In other words, the microalgal biomass is cultured in an aqueous medium for a period of time during which the microalgae reproduce and increase in number; however quantities of aqueous culture medium are not flowed through the photobioreactor throughout the time period. Thus in some embodiments, aqueous culture medium is not flowed through the photobioreactor after inoculation.

[0204] In other instances culture media can be flowed though the photobioreactor throughout the time period during which the microalgae reproduce and increase in number. In some embodiments media is infused into the photobioreactor after inoculation but before the cells reach a desired density. In other words, 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.

[0205] Photobioreactors preferably have one or more ports that allow gas entry. Gas can serve to both provide nutrients such as CO_2 as well as to provide turbulence in the culture media. Turbulence can be achieved by placing a gas entry port below the level of the aqueous culture media so that gas entering the photobioreactor bubbles to the surface of the culture. One or more gas exit ports allow gas to escape, thereby preventing pressure buildup in the photobioreactor. Preferably a gas exit port leads to a "one-way" valve that prevents contaminating microorganisms from entering the photobioreactor. In some instances cells are

cultured in a photobioreactor for a period of time during which the microalgae reproduce and increase in number, however a turbulent flow regime with turbulent eddies predominantly throughout the culture media caused by gas entry is not maintained for all of the period of time. In other instances a turbulent flow regime with turbulent eddies predominantly throughout the culture media caused by gas entry can be maintained for all of the period of time during which the microalgae reproduce and increase in number. In some instances a predetermined range of ratios between the scale of the photobioreactor and the scale of eddies is not maintained for the period of time during which the microalgae reproduce and increase in number. In other instances such a range can be maintained.

[0206] Photobioreactors preferably have at least one port that can be used for sampling the culture. 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. Alternatively a sampling port can allow continuous sampling. Photobioreactors preferably have at least one port that allows inoculation of a culture. Such a port can also be used for other purposes such as media or gas entry.

B. Heterotrophic Growth

[0207] As an alternative to photosynthetic growth of microorganisms, as described above, some microorganisms can be cultured under heterotrophic growth conditions in which a fixed carbon source provides energy for growth and lipid accumulation.

[0208] In one heterotrophic culture method in accordance with the invention, the cost of biodiesel production, crude, partially purified, or purified glycerol produced as a byproduct of lipid transesterification can be employed as a feedstock for fermenting, for example, lipid-producing microbial cultures. Thus, the invention encompasses culturing a microbe (e.g., a microalgae) in a first microbial culture; recovering microbial lipid from the culture; subjecting the microbial lipid to transesterification to produce fatty acid ester(s) and glycerol, as described above; and adding the glycerol to a second microbial culture as a feedstock. The first and second microbial cultures can, but need not, be cultures of the same microbe. If desired, a continuous system can be devised whereby glycerol produced from the lipid recovered from a culture can be fed back into the same culture.

[0209] The invention provides significantly improved culture parameters incorporating the use of glycerol for fermentation of multiple genera of both eukaryotic and prokaryotic

microbes, including microbes of the genera *Chlorella, Navicula, Scenedesmus, and Spirulina*. As the Examples demonstrate, microbes of extremely divergent evolutionary lineages, including *Chlorella, Navicula, Scenedesmus, and Spirulina* as well as cultures of multiple distinct *Chlorella* species and strains grow very well on not only purified reagent-grade glycerol, but also on acidulated and non-acidulated glycerol byproduct from biodiesel transesterification. In some instances microalgae, such as *Chlorella* strains, undergo cell division faster in the presence of glycerol than in the presence of glucose. In these instances, two-stage growth processes in which cells are first fed glycerol to rapidly increase cell density, and are then fed glucose to accumulate lipids can improve the efficiency with which lipids are produced. The use of the glycerol byproduct of the transesterification process provides significant economic advantages when put back into the production process. Other feeding methods are provided as well, such as mixtures of glycerol and glucose. Feeding such mixtures also captures the same economic benefits. In addition, the invention provides methods of feeding alternative sugars to microalgae such as sucrose in various combinations with glycerol. These benefits provided by the invention have been demonstrated herein on microbes from extremely divergent evolutionary lineages, including both prokaryotes and eukaryotes, demonstrating the utility of the invention for microbial fermentation.

[0210] Standard methods for the growth and propagation of *Chlorella protothecoides* are known (see for example Miao and Wu, *J. Biotechnology*, 2004, 11:85-93 and Miao and Wu, *Biosource Technology* (2006) 97:841-846). The invention also provides novel growth conditions for *Chlorella*. For example, multiple species of *Chlorella* and multiple strains within a species can be grown in the presence of glycerol, including glycerol byproduct from biodiesel transesterification.

[0211] For hydrocarbon production, cells, including recombinant cells of the invention described herein, are preferably cultured or fermented in large quantities. The culturing may be in large liquid volumes, such as in suspension cultures as an example. Other examples include starting with a small culture of cells which expand into a large biomass in combination with cell growth and propagation as well as hydrocarbon production. Bioreactors or steel fermentors can be used to accommodate large culture volumes. A fermentor similar those used in the production of beer and/or wine is suitable, as are extremely large fermentors used in the production of ethanol.

[0212] Appropriate nutrient sources for culture in a fermentor are provided. These include raw materials such as one or more of the following: a fixed carbon source such as glucose, corn starch, depolymerized cellulosic material, sucrose, sugar cane, sugar beet, lactose, milk whey, or molasses; a fat source, such as fats or vegetable oils; a nitrogen source, such as protein, soybean meal, cornsteep liquor, ammonia (pure or in salt form), nitrate or nitrate salt, or molecular nitrogen; and a phosphorus source, such as phosphate salts. Additionally, a fermentor allows for the control of culture conditions such as temperature, pH, oxygen tension, and carbon dioxide levels. Optionally, gaseous components, like oxygen or nitrogen, can be bubbled through a liquid culture. Other Starch (glucose) sources such as wheat, potato, rice, and sorghum. Other carbon sources include process streams such as technical grade glycerol, black liquor, organic acids such as acetate, and molasses. Carbon sources can also be provided as a mixture, such as a mixture of sucrose and depolymerized sugar beet pulp.

[0213] A fermentor can be used to allow cells to undergo the various phases of their growth cycle. As an example, an inoculum of hydrocarbon-producing cells can be introduced into a medium followed by a lag period (lag phase) before the cells begin growth. Following the lag period, the growth rate increases steadily and enters the log, or exponential, phase. The exponential phase is in turn followed by a slowing of growth due to decreases in nutrients and/or increases in toxic substances. After this slowing, growth stops, and the cells enter a stationary phase or steady state, depending on the particular environment provided to the cells.

[0214] Hydrocarbon production by cells disclosed herein can occur during the log phase or thereafter, including the stationary phase wherein nutrients are supplied, or still available, to allow the continuation of hydrocarbon production in the absence of cell division.

[0215] Preferably, microorganisms grown using conditions described herein and known in the art comprise at least about 20% by weight of lipid, preferably at least about 40% by weight, more preferably at least about 50% by weight, and most preferably at least about 60% by weight.

[0216] A surprising discovery is that multiple species, and multiple strains within a species of *Chlorella* perform better in the presence of glycerol byproduct from transesterification than in an equivalent amount of reagent grade glycerol. Glycerol byproduct from transesterification usually contains residual methanol and other contaminants in addition to

glycerol. For example, Figures 1-6 demonstrate that strains of *Chlorella protothecoides* and *Chlorella kessleri* exhibit better productivity on acidulated and non-acidulated glycerol byproduct from lipid transesterification reactions than when grown on pure reagent grade glycerol. Other microbes, such as *Scenedesmus* and *Navicula* microalgae can also perform better in the presence of glycerol byproduct from transesterification than in an equivalent amount of reagent grade glycerol.

[0217] Dry Cell Weight per Liter: Figure 1 demonstrates that dry cell weight was higher on biodiesel glycerol byproduct than on pure glycerol, and this trend held true when the cells were grown in glycerol by itself or in combination with glucose. Figure 2 shows the same trends with additional strains of *Chlorella*. Figure 12(b) demonstrates that dry cell weight per liter of *Scenedesmus armatus* is higher on acidulated and non-acidulated biodiesel byproducts glycerol than on pure reagent grade glycerol. Figure 13 demonstrates that dry cell weight per liter of *Navicula pelliculosa* is higher on non-acidulated biodiesel byproduct glycerol than on pure reagent grade glycerol.

[0218] Lipid Content per liter: Figures 3 and 4 demonstrates that with multiple species of *Chlorella* and multiple strains within a species of *Chlorella*, lipid levels per liter are higher when the cells are cultured in the presence of biodiesel glycerol byproduct than when cultured in the presence of equivalent concentrations of pure reagent grade glycerol.

[0219] Lipid as a Percentage of Cell Weight: Figures 5 and 6 demonstrate that multiple species of *Chlorella* and multiple strains within a species of *Chlorella* accumulate a higher percentage of dry cell weight as lipid when cultured in the presence of biodiesel glycerol byproduct than when cultured in the presence of equivalent concentrations of pure reagent grade glycerol. Figure 11 demonstrates that both *Spirulina platensis* and *Navicula pelliculosa* can accumulate a higher percentage of dry cell weight as lipid when cultured in the presence of biodiesel glycerol byproduct than when cultured in the presence of equivalent concentrations of pure reagent grade glycerol. Figure 12(a) demonstrates that *Scenedesmus armatus* can accumulate a higher percentage of dry cell weight as lipid when cultured in the presence of biodiesel glycerol byproduct than when cultured in the presence of equivalent concentrations of pure reagent grade glycerol.

[0220] Another surprising result is that multiple species of microbes, including microalgae such as *Chlorella* and multiple strains within a species of *Chlorella*, and other microalgae such as *Scenedesmus*, *Navicula*, and *Spirulina* exhibit better characteristics as biodiesel

producers in the presence of mixtures of glycerol and glucose than in the presence of only glucose.

[0221] Lipid Content per liter: Figure 7 demonstrates that *Chlorella* can accumulate higher levels of lipid per liter of culture in the presence of 1% glycerol/1% glucose than in the presence of 2% glucose.

[0222] Dry Cell Weight per Liter: Figure 12(b) demonstrates that dry cell weight per liter of *Scenedesmus armatus* is higher when cultured in the presence of 1% biodiesel byproduct glycerol/1% glucose than in the presence of 2% glucose. Figure 13 demonstrates that dry cell weight per liter of *Navicula pelliculosa* is higher when cultured in the presence of 1% biodiesel byproduct glycerol/1% glucose than in the presence of 2% glucose.

[0223] Lipid as a Percentage of Cell Weight: Figure 8 demonstrates that *Chlorella* can accumulate a higher percentage of dry cell weight as lipid when cultured in the presence of an equal concentration (weight percent) mixture of glycerol and glucose than when cultured in the presence of only glucose. Figure 11(a) demonstrates that *Spirulina platensis* can accumulate a higher percentage of dry cell weight as lipid when cultured in the presence of an equal concentration (weight percent) mixture of biodiesel byproduct glycerol and glucose than when cultured in the presence of only glucose. Figure 11(b) demonstrates that *Navicula pelliculosa* can accumulate a higher percentage of dry cell weight as lipid when cultured in the presence of an equal concentration (weight percent) mixture of reagent grade glycerol and glucose, as well as biodiesel byproduct glycerol and glucose, than when cultured in the presence of only glucose. Figure 12(b) demonstrates that *Scenedesmus armatus* can accumulate a higher percentage of dry cell weight as lipid when cultured in the presence of an equal concentration (weight percent) mixture of biodiesel byproduct glycerol and glucose than when cultured in the presence of only glucose.

[0224] An additional and unexpected discovery is that adding glycerol and glucose to microbes, including microalgae such as *Chlorella, Scenedesmus*, and *Navicula* sequentially rather than as a single batch mixture of glycerol and glucose can generate additional yield gains. This attribute of multiple species of *Chlorella* and multiple strains within a species of *Chlorella* was tested in the presence of both biodiesel glycerol byproduct and reagent grade glycerol.

[0225] Lipid as a Percentage of Cell Weight: Figure 8 demonstrates that *Chlorella* can accumulate a higher percentage of dry cell weight as lipid when glycerol is added to a culture

for a first period of time, followed by addition of glucose and continued culturing for a second period of time, than when the same quantities of glycerol and glucose are added together at the beginning of the experiment.

[0226] Lipid Content per liter: Figure 9 shows *Chlorella* exhibiting higher levels of lipid per liter of culture in when glycerol and glucose are added sequentially than when the same quantities of glycerol and glucose are added together at the beginning of the experiment. This trend was observed when acidulated biodiesel byproduct glycerol, non-acidulated biodiesel byproduct glycerol, or reagent grade glycerol was used.

[0227] Dry Cell Weight per Liter: Figure 10 demonstrates four different strains of *Chlorella* of two different species accumulating higher dry cell weight per liter of culture when glycerol and glucose are added sequentially than when the same quantities of glycerol and glucose are added together at the beginning of the experiment. This trend was observed when acidulated biodiesel byproduct glycerol, non-acidulated biodiesel byproduct glycerol, or reagent grade glycerol was used. Figure 14(a) and (b) demonstrates that both *Scenedesmus armatus* and *Navicula pelliculosa* can exhibit increases in dry cell weight per liter when biodiesel byproduct glycerol only is added to a culture for a first period of time, followed later by addition of glucose, compared to adding identical amounts of glycerol and glucose at the beginning of the fermentation.

[0228] Three different markers of productivity (dry cell weight per liter, grams per liter of lipid, and percentage of dry cell weight as lipid) in microbial lipid production are improved by the use of biodiesel byproduct and temporal separation of carbon sources. The invention therefore provides novel methods of generating higher quantities of lipid per unit time in multiple species of microbes from highly divergent areas of the evolutionary tree, including both prokaryotes and eukaryotes. The methods of manufacturing lipids and hydrocarbons disclosed herein using glycerol are not limited to microalgae, but can be used with any microbe capable of utilizing glycerol as an energy source.

[0229] In an alternate heterotrophic growth method in accordance with the present invention, microorganisms can be cultured using depolymerized cellulosic biomass as a feedstock. Cellulosic biomass (e.g., stover, such as corn stover) is inexpensive and readily available; however, attempts to use this material as a feedstock for yeast have failed. In particular, such feedstock 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. Accordingly, the invention provides a method of culturing a microalgae in the presence of a cellulosic material and/or a 5-carbon sugar. Cellulosic materials generally include:

Component	Percent Dry Weight
Cellulose	40-60%
Hemicellulose	20-40%
Lignin	10-30%

[0230] Suitable cellulosic materials include residues from herbaceous and woody energy crops, as well as agricultural crops, i.e., the plant parts, primarily stalks and leaves, not removed from the fields with the primary food or fiber product. Examples include agricultural wastes such as sugarcane bagasse, rice hulls, corn fiber (including stalks, leaves, husks, and cobs), wheat straw, rice straw, sugar beet pulp, citrus pulp, citrus peels; forestry wastes such as hardwood and softwood thinnings, and hardwood and softwood residues from timber operations; wood wastes such as saw mill wastes (wood chips, sawdust) and pulp mill waste; urban wastes such as paper fractions of municipal solid waste, urban wood waste and urban green waste such as municipal grass clippings; and wood construction waste. Additional 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.

[0231] Surprisingly, some species of *Chlorella* have been shown herein to exhibit higher levels of productivity when cultured on a combination of glucose and xylose than when cultured on either glucose or xylose alone. This synergistic effect provides a significant advantage in that it allows cultivation of *Chlorella* on combinations of xylose and glucose, such as cellulosic material, and is shown in Figure 15.

[0232] In still another alternative heterotrophic growth method in accordance with the present invention, which itself may optionally be used in combination with the methods described above, sucrose, produced by example from sugar cane or sugar beet, is used as a feedstock. As described in greater detail in the section entitled "Microbe Engineering" below, lipid production can be facilitated or made more efficient through the engineering of microbes such as *Chlorella*, to utilize sucrose as a carbon source. For example, expression of a sucrose transporter and a sucrose invertase allows *Chlorella* 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 sucrose invertases are Genbank accession numbers CAB95010, NP_012104 and CAA06839. Examples of suitable fructokinases are Genbank accession numbers P26984, P26420 and CAA43322. Vectors for transformation of microalgae, including *Chlorella*, encoding one or more of such genes can be designed as described herein.

[0233] Secretion of a sucrose invertase can obviate 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 disclosed herein. For example, expression of a sucrose invertase (such as SEQ ID NO:14) with a secretion signal (such as that of SEQ ID NO:15 (from yeast), SEQ ID NO:16 (from higher plants), SEQ ID NO:17 (eukaryotic consensus secretion signal), and SEQ ID NO:18 (combination of signal sequence from higher plants and eukaryotic consensus) generates invertase activity outside the cell. See Hawkins et al., Current Microbiology Vol. 38 (1999), pp. 335–341 for examples of secretion signals active in *Chlorella*. 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. In cells such as *Chlorella protothecoides*, *Chlorella minutissima*, and *Chlorella emersonii* which as demonstrated herein can use both extracellular fructose and extracellular glucose as an energy source, secretion of an invertase can provide the sole catalytic activity necessary for use of sucrose as an efficient, inexpensive energy source.

[0234] For example, as shown in Figure 26, *Chlorella protothecoides* can be engineered with a sucrose invertase gene under the regulatory control of one of three promoters (Cauliflower mosaic virus 35S promoter (CMV), Chlorella virus promoter (CV), or Chlorella HUP1 promoter (HUP1)). The sucrose invertase gene used in this example comprises a modification to the *S. cerevisiae* SUC2 gene to optimize for *C. protothecoides* codon usage. The cDNA and amino acid sequences of the optimized gene correspond to SEQ ID NO:8 and SEQ ID NO:19, respectively. An illustration of the plasmid constructs used in the transformation is shown in Figure 25. Expression of a secretable sucrose invertase, such as

that described herein, permits the use of molasses, sugar cane juice, and other sucrose-containing feedstocks for cell fermentation.

[0235] Similarly, Figures 27 and 28 show the results of transformation of *Chlorella protothecoides*, and *Chlorella minutissima* and *Chlorella emersonii*, respectively, with the sucrose invertase gene from *S. cerevisiae* under the control of the CMV promoter.

[0236] The growth potential of microorganisms expressing an exogenous secretable sucrose invertase is illustrated by the addition of an invertase to the culture medium of *Chlorella protothecoides*, as described in further detail in the Examples. Figures 23 and 24 illustrate the surprising result that *Chlorella* cells 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 hydrocarbons and other oils. Molasses contains lignin and other cellulosic waste products that poison many microorganisms and retard their growth, however it was discovered that *Chlorella* cells thrive in the presence of such poisons. Figures 23-24 show the growth of cells on three unique sources of molasses (designated BS1, BS2 and HTM), as compared to growth on glucose or sucrose in the presence or absence of an extracellular sucrose invertase.

[0237] Alternatively, a sucrose invertase can also be expressed intracellularly in cells that express a sucrose transporter, as well as in cells that express any carbohydrate transporter that allows sucrose to enter the cell.

[0238] A foreign gene was transformed into and expressed in *Chlorella protothecoides*, as described in Example 12. Expression of sucrose utilization genes can be accomplished using the same or similar methodology and vector design.

[0239] Bioreactors can be employed for use in heterotrophic growth methods. As will be appreciated, provisions made to make light available to the cells in photosynthetic growth methods are unnecessary when using a fixed-carbon source in the heterotrophic growth methods described herein.

[0240] The specific examples of process conditions and heterotrophic growth methods described herein can be combined in any suitable manner to improve efficiencies of microbial growth and lipid production. In addition, the invention includes the selection and/or genetic engineering of microbes, such as microalgae, to produce microbes that are even more suitable for use in the above-described methods. For example, the microbes having a greater ability

to utilize any of the above-described feedstocks for increased proliferation and/or lipid (e.g., fatty acid) production are within the scope of the invention.

C. Mixotrophic Growth

[0241] Mixotrophic growth is the use of both light and fixed carbon source(s) as energy sources for cells to grow and produce hydrocarbons. Mixotrophic growth can be conducted in a photobioreactor. Microalgae can be grown and maintained in closed photobioreactors made of different types of transparent or semitransparent material. Such material can include Plexiglas® enclosures, glass enclosures, bags made from substances such as polyethylene, transparent or semitransparent pipes, and other materials. Microalgae can be grown and maintained in open photobioreactors such as raceway ponds, settling ponds, and other non-enclosed containers.

D. Growth Media

[0242] 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.

[0243] Solid and liquid growth media are generally available from a wide variety of sources, and instructions for the preparation of particular media that is suitable for a wide variety of strains of microorganisms can be found, for example, online at http://www.utex.org/, a site maintained by the University of Texas at Austin for its culture collection of algae (UTEX). For example, various fresh water and salt water media include those shown in Table 4, below.

[0244] Table 4. Exemplary Algal Media.

Fresh Water Media	Salt Water Media
1/2 CHEV Diatom Medium	1% F/2
1/3 CHEV Diatom Medium	1/2 Enriched Seawater Medium
1/5 CHEV Diatom Medium	1/2 Erdschreiber Medium
1:1 DYIII/PEA + Gr+	1/2 Soil+Seawater Medium
2/3 CHEV Diatom Medium	1/3 Soil+Seawater Medium

2X CHEV Diatom Medium	1/4 ERD
Ag Diatom Medium	1/4 Soil+Seawater Medium
Allen Medium	1/5 Soil+Seawater Medium
BG11-1 Medium	2/3 Enriched Sewater Medium
Bold 1NV Medium	20% Allen + 80 % ERD
Bold 3N Medium	2X Erdschreiber's Medium
Botryococcus Medium	2X Soil+Seawater Medium
Bristol Medium	5% F/2 Medium
CHEV Diatom Medium	5/3 Soil+Seawater Agar Medium
Chu's Medium	Artificial Seawater Medium
CR1 Diatom Medium	BG11-1 + .36% NaCl Medium
CR1+ Diatom Medium	BG11-1 + 1% NaCl Medium
CR1-S Diatom Medium	Bold 1NV:Erdshreiber (1:1)
Cyanidium Medium	Bold 1NV:Erdshreiber (4:1)
Cyanophycean Medium	Bristol-NaCl Medium
Desmid Medium	Dasycladales Seawater Medium
DYIII Medium	Enriched Seawater Medium
Euglena Medium	Erdschreiber's Medium
HEPES Medium	ES/10 Enriched Seawater Medium
J Medium	ES/2 Enriched Seawater Medium
Malt Medium	ES/4 Enriched Seawater Medium
MES Medium	F/2 Medium
Modified Bold 3N Medium	F/2+NH4
Modified COMBO Medium	LDM Medium
N/20 Medium	Modified 2 X CHEV
Ochromonas Medium	Modified 2 X CHEV + Soil
P49 Medium	Modified Artificial Seawater Medium
Polytomella Medium	Modified CHEV
Proteose Medium	Porphridium Medium
Snow Algae Media	Soil+Seawater Medium
Soil Extract Medium	SS Diatom Medium
Soilwater: BAR Medium	
Soilwater: GR- Medium	
Soilwater: GR-/NH4 Medium	
Soilwater: GR+ Medium	
Soilwater: GR+/NH4 Medium	
Soilwater: PEA Medium	
Soilwater: Peat Medium	
Soilwater: VT Medium	
Spirulina Medium	
Tap Medium	
Trebouxia Medium	
Volvocacean Medium	
Volvocacean-3N Medium	
Volvox Medium	
Volvox-Dextrose Medium	
Waris Medium	
Waris+Soil Extract Medium	

[0245] In a particular example, a medium suitable for culturing *Chlorella protothecoides* (UTEX 31) comprises Proteose Medium. This 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.

[0246] Other suitable media for use with the methods of the invention can be readily identified by consulting the URL identified above, or by consulting other organizations that maintain cultures of microorganisms, such as SAG, CCAP, or CCALA. SAG refers to the Culture Collection of Algae at the University of Göttingen (Göttingen, Germany), CCAP refers to the culture collection of algae and protozoa managed by the Scottish Association for Marine Science (Scotland, United Kingdom), and CCALA refers to the culture collection of algal laboratory at the Institute of Botany (Třeboň, Czech Republic).

E. Increasing Yield of Lipids

[0247] 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 microbe (e.g., a microalgae) is cultured in the presence of a limiting concentration of one or more nutrients, such as, for example, carbon and/or 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%, 20%, 30%, 40%, 50%, 75%, 100%, 200%, 300%, 400%, 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.

[0248] To increase lipid yield, acetic acid can be employed in the feedstock for a lipid-producing microbe (e.g., a microalgae). Acetic acid feeds directly into the point of metabolism that initiates fatty acid synthesis (i.e., acetyl-CoA); thus providing acetic acid in the culture can increase fatty acid production. Generally, the microbe is cultured in the presence of a sufficient amount of acetic acid to increase microbial lipid yield, and/or microbial fatty acid yield, specifically, over microbial lipid (e.g., fatty acid) yield in the absence of acetic acid.

[0249] In another embodiment, lipid yield is increased by culturing a lipid-producing microbe (e.g., microalgae) in the presence of one or more cofactor(s) for a lipid pathway

enzyme (e.g., a fatty acid synthetic enzyme). Generally, the concentration of the cofactor(s) is sufficient to increase microbial lipid (e.g., fatty acid) yield over microbial lipid yield in the absence of the cofactor(s). In a particular embodiment, the cofactor(s) are provided to the culture by including in the culture a microbe (e.g., microalgae) containing an exogenous gene encoding the cofactor(s). Alternatively, cofactor(s) may be provided to a culture by including a microbe (e.g., microalgae) containing an exogenous gene that encodes a protein that participates in the synthesis of the cofactor. In certain embodiments, suitable cofactors include any vitamin required by a lipid pathway enzyme, such as, for example: biotin, pantothenate. Genes encoding cofactors suitable for use in the invention or that participate in the synthesis of such cofactors are well known and can be introduced into microbes (e.g., microalgae), using contructs and techniques such as those described above.

V. LIPID PATHWAY ENGINEERING

[0250] In some embodiments of the present invention, microorganisms of the present invention are modified to alter the properties and/or proportions of lipids produced and/or to increase carbon flux into lipids. The pathway can further, or alternatively, be modified to alter the properties and/or proportions of various hydrocarbon molecules produced through enzymatic processing of lipids.

A. Alteration of Properties or Proportions of Lipids or Hydrocarbons Produced

[0251] In the case of microalgae, some wild-type cells already have good growth characteristics but do not produce the desired types or quantities of lipids. Examples include *Pyrobotrys, Phormidium, Agmenellum, Carteria, Lepocinclis, Pyrobotrys, Nitzschia, Lepocinclis, Anabaena, Euglena, Spirogyra, Chlorococcum, Tetraedron, Oscillatoria, Phagus*, and *Chlorogonium,* which have the desirable growth characteristic of growing in municipal sewage or wastewater. Such cells, as well as species of *Chlorella* and other microbes, can be engineered to have improved lipid production characteristics. Desired characteristics include optimizing lipid yield per unit volume and/or per unit time, carbon chain length (e.g., for biodiesel production or for industrial applications requiring hydrocarbon feedstock), reducing the number of double or triple bonds, optionally to zero, removing or eliminating rings and cyclic structures, and increasing the hydrogen:carbon ratio of a particular species of lipid or of a population of distinct lipid. In addition, microalgae that

produce appropriate hydrocarbons can also be engineered to have even more desirable hydrocarbon outputs. Examples of such microalgae include species of the genus *Chlorella*.

1. Regulation of Enzymes that Control Branch Points in Fatty Acid Synthesis

[0252] In particular embodiments, one or more key enzymes that control branch points in metabolism to fatty acid synthesis can be up-regulated or down-regulated to improve lipid production. Up-regulation can be achieved, for example, by transforming cells with expression constructs in which a gene encoding the enzyme of interest is expressed, e.g., using a strong promoter and/or enhancer elements that increase transcription. Such constructs can include a selectable marker such that the transformants can be subjected to selection, which can result in amplification of the construct and an increase in the expression level of the encoded enzyme. Examples of enzymes suitable for up-regulation according to the methods of the invention include pyruvate dehydrogenase, which plays a role in converting pyruvate to acetyl-CoA (examples, some from microalgae, include Genbank accession numbers NP_415392; AAA53047; Q1XDM1; and CAF05587). Up-regulation of pyruvate dehydrogenase can increase production of acetyl-CoA, and thereby increase fatty acid synthesis. Acetyl-CoA carboxylase catalyzes the initial step in fatty acid synthesis. Accordingly, this enzyme can be up-regulated to increase production of fatty acids (examples, some from microalgae, include Genbank accession numbers BAA94752; AAA75528; AAA81471; YP_537052; YP_536879; NP_045833; and BAA57908). Fatty acid production can also be increased by up-regulation of acyl carrier protein (ACP), which carries the growing acyl chains during fatty acid synthesis (examples, some from microalgae, include Genbank accession numbers A0T0F8; P51280; NP_849041; YP_874433) . Glycerol-3-phosphate acyltransferase catalyzes the rate-limiting step of fatty acid synthesis. Up-regulation of this enzyme can increase fatty acid production (examples, some from microalgae, include Genbank accession numbers AAA74319; AAA33122; AAA37647; P44857; and ABO94442). The preceding proteins are candidates for expression in microalge, including species of the genus *Chlorella*.

[0253] Down-regulation of an enzyme of interest can achieved using, e.g., antisense, catalytic RNA/DNA, RNA interference (RNA_i), "knock-out," "knock-down," or other mutagenesis techniques. Enzyme expression/function can also be inhibited using intrabodies. Examples of enzymes suitable for down-regulation according to the methods of the invention include citrate synthase, which consumes acetyl-CoA as part of the tricarboxylic acid (TCA)

cycle. Down-regulation of citrate synthase can force more acetyl-CoA into the fatty acid synthetic pathway.

2. Modulation of Global Regulators of Fatty Acid Synthetic Genes

[0254] Global regulators modulate 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). Global regulators can be up- or down-regulated, for example, as described above with respect to regulation of control point enzymes.

3. Regulation of Hydrocarbon Modification Enzymes

[0255] The methods of the invention also include transforming cells with one or more genes encoding hydrocarbon modification enzymes such as, for example, a fatty acyl-ACP thioesterase (see examples in Table 5 with accession numbers), a fatty acyl-CoA/aldehyde reductase (see examples in Table 6 with accession numbers), a fatty acyl-CoA reductase (see examples in Table 7 with accession numbers), a fatty aldehyde decarbonylase (see examples in Table 8 with accession numbers), a fatty aldehyde reductase, or a squalene synthase gene (see GenBank Accession number AF205791). In some embodiments, genes encoding a fatty acyl-ACP thioesterase and a naturally co-expressed acyl carrier protein may be transformed into a cell, optionally with one or more genes encoding other hydrocarbon modification enzymes. In other embodiments, the ACP and the fatty acyl-ACP thioesterase may have an affinity for one another that imparts an advantage when the two are used together in the microbes and methods of the present invention, irrespective of whether they are or are not naturally co-expressed in a particular tissue or organism. Thus, the present invention contemplates both naturally co-expressed pairs of these enzymes as well as those that share an affinity for interacting with one another to facilitate cleavage of a length-specific carbon chain from the ACP.

[0256] In still other embodiments, an exogenous gene encoding a desaturase can be transformed into the cell in conjunction with one or more genes encoding other hydrocarbon modification enzymes in order to provide modifications with respect to hydrocarbon saturation. Stearoyl-ACP desaturase (see, e.g., GenBank Accession numbers AAF15308;

ABM45911; and AAY86086), for example, catalyzes the conversion of stearoyl-ACP to oleoyl-ACP. Up-regulation of this gene can increase the proportion of monounsaturated fatty acids produced by a cell; whereas down-regulation can reduce the proportion of monounsaturates. Similarly, the expression of one or more glycerolipid desaturases can be controlled to alter the ratio of unsaturated to saturated fatty acids such as ω-6 fatty acid desaturase, ω-3 fatty acid desaturase, or ω-6-oleate desaturase. In some embodiments, the desaturase can be selected with reference to a desired carbon chain length, such that the desaturase is capable of making location specific modifications within a specified carbon-length substrate, or substrates having a carbon-length within a specified range.

[0257] In particular embodiments, microbes of the present invention are genetically engineered to express one or more exogenous genes selected from a fatty acyl-ACP thioesterase, a fatty acyl-CoA/aldehyde reductase, a fatty acyl-CoA reductase, a fatty aldehyde reductase, a fatty aldehyde decarbonylase, or a naturally co-expressed acyl carrier protein. Suitable expression methods are described above with respect to the expression of a lipase gene, including, among other methods, inducible expression and compartmentalized expression.

[0258] Without intending to be bound by any particular theory or cellular mechanism, 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.

[0259] The enzymes described directly above have a specificity for acting on a substrate which includes a specific number of carbon atoms. For example, a fatty acyl-ACP thioesterase may have a specificity 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 microbe can

contain an exogenous gene that encodes a protein with specificity for catalyzing an enzymatic activity (e.g., cleavage of a fatty acid from an ACP, reduction of an acyl-CoA to an aldehyde or an alcohol, or conversion of an aldehyde to an alkane) with regard to the number of carbon atoms contained in the substrate. The enzymatic specificity can, in various embodiments, be for a substrate having from 8 to 34 carbon atoms, preferably from 8 to 18 carbon atoms, and more preferably from 10 to 14 carbon atoms. The most preferred specificity is for a substrate having 12 carbon atoms. In other embodiments the specificity can be for 20 to 30 carbon atoms.

[0260] Fatty acyl-ACP thioesterases suitable for use with the microbes and methods of the invention include, without limitation, those listed in Table 5.

[0261] Table 5. 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)

[0262] Fatty acyl-CoA/aldehyde reductases suitable for use with the microbes and methods of the invention include, without limitation, those listed in Table 6.

[0263] Table 6. 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

[0264] Fatty acyl-CoA reductases suitable for use with the microbes and methods of the invention include, without limitation, those listed in Table 7.

[0265] Table 7. Fatty acyl-CoA reductases listed by GenBank accession numbers.

NP_187805, ABO14927, NP_001049083, CAN83375, NP_191229, EAZ42242, EAZ06453, CAD30696, BAD31814, NP_190040, AAD38039, CAD30692, CAN81280,

NP_197642, NP_190041, AAL15288, and NP_190042

[0266] Fatty aldehyde decarbonylases suitable for use with the microbes and methods of the invention include, without limitation, those listed in Table 8.

[0267] Table 8. Fatty aldehyde decarbonylases listed by GenBank accession numbers.

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

[0268] Combinations of naturally co-expressed fatty acyl-ACP thioesterases and acyl carrier proteins are suitable for use with the microbes and methods of the invention.

[0269] Additional examples of hydrocarbon modification enzymes include amino acid sequences contained in, referenced in, or encoded by nucleic acid sequences contained or referenced in, any of the following US patents: 6,610,527; 6,451,576; 6,429,014; 6,342,380; 6,265,639; 6,194,185; 6,114,160; 6,083,731; 6,043,072 ; 5,994,114; 5,891,697; 5,871,988; 6,265,639, and further described in GenBank Accession numbers: AAO18435; ZP_00513891; Q38710; AAK60613; AAK60610; AAK60611; NP_113747; CAB75874; AAK60612; AAF20201; BAA11024; AF205791; and CAA03710.

[0270] Other suitable enzymes for use with the microbes and the methods of the invention include those that have at least 70% amino acid identity with one of the proteins listed in Tables 5-8, 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 as if fully set forth.

[0271] The hydrocarbon modification enzymes described above are useful in the production of various hydrocarbons from a microbe (e.g., a microalgae, an oleaginous yeast, or a fungus) or population of microbes, whereby 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.

[0272] The hydrocarbon modification enzymes have a specificity for acting on a substrate which includes a specific number of carbon atoms. For example, a fatty acyl-ACP thioesterase may have a specificity for cleaving a fatty acid having 12 carbon atoms from the ACP. Therefore, in various embodiments, the microbe can contain an exogenous gene that encodes a protein with specificity for catalyzing an enzymatic activity (e.g., cleavage of a fatty acid from an ACP, reduction of an acyl-CoA to an aldehyde or an alcohol, or conversion of an aldehyde to an alkane) with regard to the number of carbon atoms contained in the substrate. The enzymatic specificity can, in various embodiments, be for a substrate having from 8 to 34 carbon atoms, preferably from 8 to 18 carbon atoms, and more preferably from 10 to 14 carbon atoms. The most preferred specificity is for a substrate having 12 carbon atoms. In other embodiments the specificity can be for 20 to 30 carbon atoms.

[0273] In some embodiments, fatty acids, or the corresponding primary alcohols, aldehydes, alkanes or alkenes, generated by the methods described herein, contain at least about 8, at least about 10, at least about 12, at least about 14, at least about 16, at least about 18, at least about 20, at least about 22, at least about 24, at least about 26, at least about 28, at least about 30, at least about 32, or at least about 34 carbon atoms or more. Preferred fatty acids for the produciton of biodiesel, renewable diesel, or jet fuel, or the corresponding primary alcohols, aldehydes, alkanes and alkenes, for industrial applications contain at least about 8 carbon atoms or more. In certain embodiments, the above fatty acids, as well as the other corresponding hydrocarbon molecules, are saturated (with no carbon-carbon double or triple bonds); mono unsaturated (single double bond); poly unsturated (two or more double bonds); are linear (not cyclic); and/or have little or no branching in their structures.

[0274] By selecting the desired combination of exogenous genes to be expressed, one can tailor the product generated by the microbe, which may then be extracted from the aqueous biomass. For example, the microbe can contain: (i) an exogenous gene encoding a fatty

acyl-ACP thioesterase; and, optionally, (ii) a naturally co-expressed acyl carrier protein or an acyl carrier protein otherwise having affinity for the fatty acyl-ACP thioesterase (or conversely); and, optionally, (iii) an exogenous gene encoding a fatty acyl-CoA/aldehyde reductase or a fatty acyl-CoA reductase; and, optionally, (iv) an exogenous gene encoding a fatty aldehyde reductase or a fatty aldehyde decarbonylase. The microbe, when cultured as described below, synthesizes a fatty acid linked to an ACP and the fatty acyl-ACP thioesterase catalyzes the cleavage of the fatty acid from the ACP to yield, through further enzymatic processing, a fatty acyl-CoA molecule. When present, the fatty acyl-CoA/aldehyde reducatase catalyzes the reduction of the acyl-CoA to an alcohol. Similarly, the fatty acyl-CoA reductase, when present, catalyzes the reduction of the acyl-CoA to an aldehyde. In those embodiments in which an exogenous gene encoding a fatty acyl-CoA reductase is present and expressed to yield an aldehyde product, a fatty aldehyde reductase, encoded by the third exogenous gene, catalyzes the reduction of the aldehyde to an alcohol. Similarly, a fatty aldehyde decarbonylase catalyzes the conversion of the aldehyde to an alkane or an alkene, when present.

[0275] Genes encoding such enzymes can be obtained from cells already known to exhibit significant lipid production such as *Chlorella protothecoides*. Genes already known to have a role in lipid production, e.g., a gene encoding an enzyme that saturates double bonds, can be transformed individually into recipient cells. However, to practice the invention it is not necessary to make *a priori* assumptions as to which genes are required. A library of DNA containing different genes, such as cDNAs from a good lipid-production organism, can be transformed into recipient cells. The cDNA is preferably in operable linkage with a promoter active in microalgae. Different recipient microalgae cells transformed by a library receive different genes from the library. Transformants having improved lipid production are identified though screening methods known in the art, such as, for example, HPLC, gas chromatography, and mass spectrometry methods of hydrocarbon analysis (for examples of such analysis, see Biomass and Bioenergy Vol. 6. No. 4. pp. 269-274 (1994); Experientia 38; 47-49 (1982); and Phytochemistry 65 (2004) 3159–3165). These transformants are then subjected to further transformation with the original library and/or optionally interbred to generate a further round of organisms having improved lipid production. General procedures for evolving whole organisms to acquire a desired property are described in, e.g., US 6,716,631. Such methods entail, e.g., introducing a library of DNA fragments into a plurality of cells, whereby at least one of the fragments undergoes recombination with a segment in the

genome or an episome of the cells to produce modified cells. The modified cells are then screened for modified cells that have evolved toward acquisition of the desired function. Vectors and methods for transformation are analogous to those discussed in connection with expression of lipase genes.

[0276] Furthermore, subtractive libraries can be used to identify genes whose transcription is induced under different conditions, especially conditions employed in culturing microorganisms for biodiesel production, or for the production of hydrocarbons useful as a feedstock for industrial applications. Subtractive libraries contain nucleotide sequences reflecting the differences between two different samples. Such libraries are prepared by procedures that include the steps of denaturing and hybridizing populations of polynucleotides (e.g., mRNA, cDNA, amplified sequences) from each sample. Sequences common to both samples hybridize and are removed, leaving the sequences that differ between the samples. In this manner, sequences that are induced under particular conditions can be identified. This technique can be employed, for example, to identify genes useful for increasing lipid (e.g., fatty acid) production and, in particular, lipid production under any desired culture conditions. The subtractive hybridization technique can also be employed to identify promoters, e.g., inducible promoters, useful in expression constructs according to the invention.

[0277] Thus, for example, subtractive libraries can be prepared from microorganism cultures grown autotrophically (in the light without a fixed carbon source) or heterotrophically (in the dark in the presence of a fixed carbon source). In particular, heterotrophic genes may be induced during dark growth in the presence of a fixed carbon source and may therefore be present in a library generated by subtracting sequences from autotrophic cells from sequences from dark heterotrophic cells. Subtractive libraries can also be prepared from cultures to which a particular carbon substrate, such as glucose, has been added to identify genes that play a role in metabolizing the substrate. Subtractive libraries prepared from cultures grown in the presence of excess versus limited nitrogen can be used to identify genes that control cell division as opposed to hydrocarbon accumulation production. The preparation of a subtractive library from a culture to which lipids (e.g., fatty acids) have been added can help identify genes whose overexpression increases fatty acid production. More specifically, the addition of fatty acids to a culture of cells that can use the added fatty acids will lead to the down-regulation of fatty acid synthetic genes to down-regulate fatty acid production. The overexpression of one or more such genes will have the opposite effect.

B. Increased Carbon Flux into Lipid Pathway

[0278] Some microalgae produce significant quantities of non-lipid metabolites, such as, for example, polysaccharides. Because polysaccharide biosynthesis can use a significant proportion of the total metabolic energy available to cells, mutagenesis of lipid-producing cells followed by screening for reduced or eliminated polysaccharide production generates novel strains that are capable of producing higher yields of lipids.

[0279] The phenol: sulfuric acid assay detects carbohydrates (see Hellebust, Handbook of Phycological Methods, Cambridge University Press, 1978; and Cuesta G., et al., J Microbiol Methods. 2003 Jan;52(1):69-73). The 1,6 dimethylmethylene blue assay detects anionic polysaccharides. (see for example Braz J Med Biol Res. 1999 May;32(5):545-50; Clin Chem. 1986 Nov;32(11):2073-6).

[0280] Polysaccharides can also be analyzed through methods such as HPLC, size exclusion chromatography, and anion exchange chromatography (see for example Prosky L, Asp N, Schweizer TF, DeVries JW & Furda I (1988) Determination of insoluble, soluble and total dietary fiber in food and food products: Interlaboratory study. Journal of the Association of Official Analytical Chemists 71, 1017±1023; Int J Biol Macromol. 2003 Nov;33(1-3):9-18). Polysaccharides can also be detected using gel electrophoresis (see for example Anal Biochem. 2003 Oct 15;321(2):174-82; Anal Biochem. 2002 Jan 1;300(1):53-68).

VI. METHODS OF RECOVERING LIPIDS AND HYDROCARBONS

[0281] Hydrocarbons (e.g., lipids, fatty acids, aldehydes, alcohols, and alkanes) produced by cells of the invention can be harvested, or otherwise collected, by any convenient means. For example, hydrocarbons secreted from cells can be centrifuged to separate the hydrocarbons in a hydrophobic layer from contaminants in an aqueous layer and optionally from any solid materials as a precipitate in after centrifugation. Material containing cell or cell fractions can be treated with proteases to degrade contaminating proteins before or after centrifugation. In some instances the contaminating proteins are associated, possibly covalently, to hydrocarbons or hydrocarbon precursors which form hydrocarbons upon removal of the protein. In other instances the hydrocarbon molecules are in a preparation that also contains proteins. Proteases can be added to hydrocarbon preparations containing proteins to degrade proteins (for example, the protease from *Streptomyces griseus* can be used (SigmaAldrich catalog number P5147). After digestion, the hydrocarbons are preferably purified from residual proteins, peptide fragments, and amino acids. This

purification can be accomplished, for example, by methods listed above such as centrifugation and filtration.

[0282] Extracellular hydrocarbons can also be extracted *in vivo* from living microalgae cells which are then returned to a bioreactor by exposure of the cells, in an otherwise sterile environment, to a non-toxic extraction solvent, followed by separation of the living cells and the hydrophobic fraction of extraction solvent and hydrocarbons, wherein the separated living cells are then returned to a culture container such as a stainless steel fermentor or photobioreactor (see Biotechnol Bioeng. 2004 Dec 5;88(5):593-600 and Biotechnol Bioeng. 2004 Mar 5;85(5):475-81).

[0283] Hydrocarbons can also be isolated by whole cell extraction. The cells are first disrupted, as described in the section entitled "Lysing Cells", and then intracellular and cell membrane/cell wall-associated hydrocarbons as well as extracellular hydrocarbons can be collected from the whole cell mass, such as by use of centrifugation as described above.

[0284] Various methods are available for separating hydrocarbons and lipids from cellular lysates produced by the above methods. For example, hydrocarbons can be extracted with a hydrophobic solvent such as hexane (see Frenz et al. 1989, Enzyme Microb. Technol., 11:717). Hydrocarbons 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).

[0285] 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 mortor and then extracted with *n*-hexane. Miao and Wu, Biosource Technology (2006) 97:841-846.

A. Lysing Cells

[0286] Intracellular lipids and hydrocarbons produced in microorganisms are, in some embodiments, extracted after lysing the cells of the microorganism. Once extracted, the lipids and/or hydrocarbons can be further refined to produce oils, fuels, or oleochemicals.

[0287] After completion of culturing, the microorganisms can be separated from the fermentation broth. Optionally, the separation is effected by centrifugation to generate a concentrated paste. Centrifugation does not remove significant amounts of intracellular water from the microorganisms and is not a drying step. The biomass can then 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, luyophilized, 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.

[0288] Microorganisms containing a lipid and/or hydrocarbon 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.

[0289] 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%.

[0290] In particular embodiments, the microorganism is lysed after growth, for example to increase the exposure of cellular lipid and/or hydrocarbon for extraction or further processing. The timing of lipase expression (e.g., via an inducible promoter) or cell lysis can be adjusted to optimize the yield of lipids and/or hydrocarbons. Below are described a number of lysis techniques. These techniques can be used individually or in combination.

1. Heat-Induced Lysis

[0291] In a preferred embodiment of the present invention, the step of lysing a microorganism comprises heating of a cellular suspension containing the microorganism. In this embodiment, the fermentation broth containing the microorganisms (or a suspension of microorganisms isolated from the fermentation broth) is heated until the microorganisms, i.e.,

the cell walls and membranes of microorganisms degrade or breakdown. Typically, temperatures applied are at least 50°C. Higher temperatures, such as, at least 30°C at least 60°C, at least 70°C, at least 80°C, at least 90°C, at least 100°C, at least 110°C, at least 120°C, at least 130°C or higher are used for more efficient cell lysis.

[0292] 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.

[0293] 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.

2. Lysis Using A Base

[0294] In another preferred embodiment of the present invention, the step of lysing a microorganism comprises adding a base to a cellular suspension containing the microorganism.

[0295] The base should be strong enough to hydrolyze at least a portion of the proteinaceous compounds of the microorganisms used. Bases which are useful for solubilizing proteins are known in the art of chemistry. Exemplary bases which are useful in the methods of the present invention include, but are not limited to, hydroxides, carbonates and bicarbonates of lithium, sodium, potassium, calcium, and mixtures thereof. A preferred base is KOH. Base treatment of microalgae for cell disruption is described, for example, in U.S. Patent No. 6,750,048.

3. Acidic Lysis

[0296] In another preferred 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°, and preferably a temperature of 50-130°. For moderate temperatures (e.g., room temperature to 100°C and particularly room temperature to 65°, acid treatment can usefully be combined with sonication or other cell disruption methods.

4. Lysing Cells Using Enzymes

[0297] In another preferred 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.

a) Cellulases

[0298] In a preferred embodiment of the present invention, a cellulase for lysing a microorganism is a polysaccharide-degrading enzyme, optionally from *Chlorella* or a *Chlorella* virus.

b) Proteases

[0299] Proteases 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), and other proteases can be used to lyse microorganisms. Other proteases that can be used include Alcalase 2.4 FG (Novozymes) and Flavourzyme 100 L (Novozymes).

c) Combinations

[0300] 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.

5. Lysing Cells Using Ultrasound

[0301] In another preferred 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.

6. Mechanical Lysis

[0302] In another preferred 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.

[0303] 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.

7. Lysing Cells By Osmotic Shock (Cytolysis)

[0304] In another preferred embodiment of the present invention, the step of lysing a microorganism is performed by applying an osmotic shock.

8. Infection With A Lytic Virus

[0305] In a preferred embodiment of the present invention, the step of lysing a microorganism comprises infection of the microorganism with a lytic virus. A wide variety of viruses are known to lyse microorganisms suitable for use in the present invention, and the selection and use of a particular lytic virus for a particular microorganism is within the level of skill in the art.

[0306] 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.

9. Autolysis (Expression of a Lytic Gene)

[0307] In another preferred embodiment of the present invention, the step of lysing a microorganism comprises autolysis. In this embodiment, a microorganism according to the invention is genetically engineered to produce a lytic protein that will lyse the microorganism. This lytic gene can be expressed using an inducible promoter so that the cells can first be grown to a desirable density in a fermentor, followed by induction of the promoter to express the lytic gene to lyse the cells. In one embodiment, the lytic gene encodes a polysaccharide-degrading enzyme.

[0308] 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 of the genus *Chlorella*, such as *C. protothecoides*.

[0309] Suitable expression methods are described herein with respect to the expression of a lipase gene. 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. Lytic genes from chlorella viruses are known. For example, see *Virology* 260, 308-315 (1999); *FEMS Microbiology Letters* 180 (1999) 45-53; *Virology* 263, 376-387 (1999); and *Virology* 230, 361–368 (1997).

B. Extraction of Lipids and Hydrocarbons

[0310] Lipids and hydrocarbons generated by the microorganisms of the present invention can be recovered by extraction with an organic solvent. In some cases, the preferred organic solvent is hexane. Typically, the organic solvent is added directly to the lysate without prior separation of the lysate components. In one embodiment, the lysate generated by one or more of the methods described above is contacted with an organic solvent for a period of time sufficient to allow the lipid and/or hydrocarbon components to form a solution with the organic solvent. In some cases, the solution can then be further refined to recover specific desired lipid or hydrocarbon components. Hexane extraction methods are well known in the art.

VII. METHODS OF PROCESSING LIPIDS AND HYDROCARBONS

A. Enzymatic Modification

[0311] Hydrocarbons (e.g., lipids, fatty acids, aldehydes, alcohols, and alkanes) produced by cells as described herein can be modified by the use of one or more enzymes, including a lipase, as described above. When the hydrocarbons are in the extracellular environment of the cells, the one or more enzymes can be added to that environment under conditions in which the enzyme modifies the hydrocarbon or completes its synthesis from a hydrocarbon precursor. Alternatively, the hydrocarbons can be partially, or completely, isolated from the cellular material before addition of one or more catalysts such as enzymes. Such catalysts are exogenously added, and their activity occurs outside the cell or *in vitro*.

B. Thermal and Other Catalytic Modification

[0312] 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.

[0313] Thermal methods involve the use of elevated temperature and pressure to reduce hydrocarbon size. An elevated temperature of about 800°C and pressure of about 700kPa can be used. These conditions generate "light," a term that is sometimes used to refer to hydrogen-rich hydrocarbon molecules (as distinguished from photon flux), while also generating, by condensation, heavier hydrocarbon molecules which are relatively depleted of hydrogen. The methodology provides homolytic, or symmetrical, breakage and produces alkenes, which may be optionally enzymatically saturated as described above.

[0314] Catalytic and thermal methods are standard in plants for hydrocarbon processing and oil refining. Thus hydrocarbons produced by cells as described herein can be collected and processed or refined via conventional means. See Hillen et al. (Biotechnology and Bioengineering, Vol. XXIV:193-205 (1982)) for a report on hydrocracking of microalgae-produced hydrocarbons. In alternative embodiments, the fraction is treated with another catalyst, such as an organic compound, heat, and/or an inorganic compound. For processing of lipids into biodiesel, a transesterification process is used as described in Section IV herein.

[0315] Hydrocarbons produced via methods of the present invention are useful in a variety of industrial applications. For example, the production of linear alkylbenzene sulfonate (LAS), an anionic surfactant used in nearly all types of detergents and cleaning preparations, utilizes hydrocarbons generally comprising a chain of 10-14 carbon atoms. See, for example, US Patent Nos.: 6,946,430; 5,506,201; 6,692,730; 6,268,517; 6,020,509; 6,140,302; 5,080,848; and 5,567,359. Surfactants, such as LAS, can be used in the manfacture of personal care compositions and detergents, such as those described in US Patent Nos.: 5,942,479; 6,086,903; 5,833,999; 6,468,955; and 6,407,044.

VIII. METHODS OF PRODUCING FUELS SUITABLE FOR USE IN DIESEL VEHICLES AND JET ENGINES

[0316] Increasing interest is directed to the use of hydrocarbon components of biological origin in fuels, such as biodiesel, renewable diesel, and jet fuel, since renewable biological starting materials that may replace starting materials derived from fossil fuels are available, and the use thereof is desirable. There is an urgent need for methods for producing hydrocarbon components from biological materials. The present invention fulfills this need by providing methods for production of biodiesel, renewable diesel, and jet fuel using the lipids generated by the methods described herein as a biological material to produce biodiesel, renewable diesel, and jet fuel.

[0317] Traditional diesel fuels are petroleum distillates rich in paraffinic hydrocarbons. They have boiling ranges as broad as 370° to 780°F, which are suitable for combustion in a compression ignition engine, such as a diesel engine vehicle. The American Society of Testing and Materials (ASTM) establishes the grade of diesel according to the boiling range, along with allowable ranges of other fuel properties, such as cetane number, cloud point, flash point, viscosity, aniline point, sulfur content, water content, ash content, copper strip corrosion, and carbon residue. Technically, any hydrocarbon distillate material derived from

biomass or otherwise that meets the appropriate ASTM specification can be defined as diesel fuel (ASTM D975), jet fuel (ASTM D1655), or as biodiesel (ASTM D6751).

[0318] After extraction, lipid and/or hydrocarbon components recovered from the microbial biomass described herein can be subjected to chemical treatment to manufacture a fuel for use in diesel vehicles and jet engines.

A. Biodiesel

[0319] Biodiesel is a liquid which varies in color - between golden and dark brown - depending on the production feedstock. It is practically immiscible with water, has a high boiling point and low vapor pressure. Biodiesel refers to a diesel-equivalent processed fuel for use in diesel-engine vehicles. Biodiesel is biodegradable and non-toxic. An additional benefit of biodiesel over conventional diesel fuel is lower engine wear.

[0320] Typically, biodiesel comprises C14-C18 alkyl esters. Various processes convert biomass or a lipid produced and isolated as described herein to diesel fuels. A preferred method to produce biodiesel is by transesterification of a lipid as described herein. A preferred alkyl ester for use as biodiesel is a methyl ester or ethyl ester.

[0321] Biodiesel produced by a method described herein can be used alone or blended with conventional diesel fuel at any concentration in most modern diesel-engine vehicles. When blended with conventional diesel fuel (petroleum diesel), biodiesel may be present from about 0.1% to about 99.9%. Much of the world uses a system known as the "B" factor to state the amount of biodiesel in any fuel mix. For example, fuel containing 20% biodiesel is labeled B20. Pure biodiesel is referred to as B100.

[0322] Biodiesel can also be used as a heating fuel in domestic and commercial boilers. Existing oil boilers may contain rubber parts and may require conversion to run on biodiesel. The conversion process is usually relatively simple, involving the exchange of rubber parts for synthetic parts due to biodiesel being a strong solvent. Due to its strong solvent power, burning biodiesel will increase the efficiency of boilers.

[0323] Biodiesel can be used as an additive in formulations of diesel to increase the lubricity of pure Ultra-Low Sulfur Diesel (ULSD) fuel, which is advantageous because it has virtually no sulfur content.

[0324] Biodiesel is a better solvent than petrodiesel and can be used to break down deposits of residues in the fuel lines of vehicles that have previously been run on petrodiesel.

1. Production Of Biodiesel

[0325] Biodiesel can be produced by transesterification of triglycerides contained in oil-rich biomass. Thus, in another aspect of the present invention a method for producing biodiesel is provided. In a preferred embodiment, the method for producing biodiesel comprises the steps of (a) cultivating a lipid-containing microorganism using methods disclosed herein (b) lysing a lipid-containing microorganism to produce a lysate, (c) isolating lipid from the lysed microorganism, and (d) transesterifying the lipid composition, whereby biodiesel is produced.

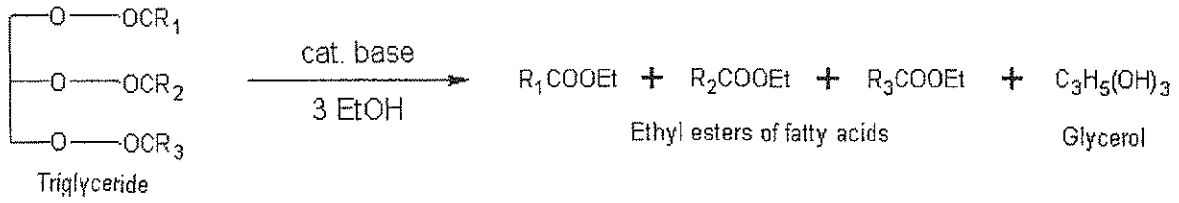
[0326] Methods for growth of a microorganism, lysing a microorganism to produce a lysate, treating the lysate in a medium comprising an organic solvent to form a heterogeneous mixture and separating the treated lysate into a lipid composition have been described above and can also be used in the method of producing biodiesel.

[0327] Lipid compositions can be subjected to transesterification to yield long-chain fatty acid esters useful as biodiesel. Preferred transesterification reactions are outlined below and include base catalyzed transesterification and transesterification using recombinant lipases.

[0328] In a base-catalyzed transesterification process, the triacylglycerides are reacted with an alcohol, such as methanol or ethanol, in the presence of an alkaline catalyst, typically potassium hydroxide. This reaction forms methyl or ethyl esters and glycerin (glycerol) as a byproduct.

a). General Chemical Process

[0329] Animal and plant oils are typically made of triglycerides which are esters of free fatty acids with the trihydric alcohol, glycerol. In transesterification, the glycerol in a triacylglyceride (TAG) is replaced with a short-chain alcohol such as methanol or ethanol. A typical reaction scheme is as follows:



[0330] In this scheme, the alcohol is deprotonated with a base to make it a stronger nucleophile. Commonly, ethanol or methanol is used in vast excess (up to 50-fold). Normally, this reaction will proceed either exceedingly slowly or not at all. Heat, as well as an acid or base can be used to help the reaction proceed more quickly. The acid or base are not consumed by the transesterification reaction, thus they are not reactants but catalysts. Almost all biodiesel has been produced using the base-catalyzed technique as it requires only low temperatures and pressures and produces over 98% conversion yield (provided the starting oil is low in moisture and free fatty acids).

b). Using Recombinant Lipases

[0331] Transesterification has also been carried out experimentally using an enzyme, such as a lipase instead of a base. Lipase-catalyzed transesterification can be carried out, for example, at a temperature between the room temperature and 80° C, and a mole ratio of the TAG to the lower alcohol of greater than 1:1, preferably about 3:1.

[0332] Lipases suitable for use in transesterification include, but are not limited to, those listed in Table 9. Other examples of lipases useful for transesterification are found in, e.g. U.S. Patent Nos. 4,798,793; 4,940,845 5,156,963; 5,342,768; 5,776,741 and WO89/01032.

[0333] Table 9. Lipases suitable for use in transesterification.

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

[0334] One challenge to using a lipase for the production of fatty acid esters suitable for biodiesel is that the price of lipase is much higher than the price of sodium hydroxide (NaOH) used by the strong base process. This challenge has been addressed by using an immobilized lipase, which can be recycled. However, the activity of the immobilized lipase

must be maintained after being recycled for a minimum number of cycles to allow a lipase-based process to compete with the strong base process in terms of the production cost. Immobilized lipases are subject to poisoning by the lower alcohols typically used in transesterification. U.S. Patent No. 6,398,707 (issued June 4, 2002 to Wu et al.) describes methods for enhancing the activity of immobilized lipases and regenerating immobilized lipases having reduced activity.

[0335] In particular embodiments, a recombinant lipase is expressed in the same microorganisms that produce the lipid on which the lipase acts. Suitable recombinant lipases include those listed above in Table 9 and/or having GenBank Accession numbers listed above in Table 9, or a polypeptide that has at least 70% amino acid identity with one of the lipases listed above in Table 9 and that exhibits lipase activity. In additional embodiments, the enzymatic activity is present in a sequence that has at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% identity with one of the above described sequences, all of which are hereby incorporated by reference as if fully set forth. DNA encoding the lipase and selectable marker is preferably codon-optimized cDNA. Methods of recoding genes for expression in microalgae are described in US Patent 7,135,290.

2. Standards

[0336] The common international standard for biodiesel is EN 14214. ASTM D6751 is the most common biodiesel standard referenced in the United States and Canada. Germany uses DIN EN 14214 and the UK requires compliance with BS EN 14214.

[0337] Basic industrial tests to determine whether the products conform to these standards typically include gas chromatography, HPLC, and others. Biodiesel meeting the quality standards is very non-toxic, with a toxicity rating (LD_{50}) of greater than 50 mL/kg.

B. Renewable Diesel

[0338] Renewable diesel comprises alkanes, such as C16:0 and C18:0 and thus, are distinguishable from biodiesel. High quality renewable diesel conforms to the ASTM D975 standard.

[0339] The lipids produced by the methods of the present invention can serve as feedstock to produce renewable diesel. Thus, in another aspect of the present invention, a method for producing renewable diesel is provided. Renewable diesel can be produced by at least three

processes: hydrothermal processing (hydrotreating); hydroprocessing; and indirect liquefaction. These processes yield non-ester distillates. During these processes, triacylglycerides produced and isolated as described herein, are converted to alkanes.

[0340] In a preferred embodiment, the method for producing renewable diesel comprises (a) cultivating a lipid-containing microorganism using methods disclosed herein (b) lysing the microorganism to produce a lysate, (c) isolating lipid from the lysed microorganism, and (d) deoxygenating and hydrotreating the lipid to produce an alkane, whereby renewable diesel is produced. Lipids suitable for manufacturing renewable diesel can be obtained via extraction from microbial biomass using an organic solvent such as hexane, or via other methods, such as those described in US Patent 5,928,696.

[0341] In some methods, the microbial lipid is first cracked in conjunction with hydrotreating to reduce carbon chain length and saturate double bonds, respectively. The material is then isomerized, also in conjunction with hydrotreating. The naptha fraction can then be removed through distillation, followed by additional distillation to vaporize and distill components desired in the diesel fuel to meet a D975 standard while leaving components that are heavier than desired for meeting a D 975 standard. Hydrotreating, hydrocracking, deoxygenation and isomerization methods of chemically modifying oils, including triglyceride oils, are well known in the art. See for example European patent applications EP1741768 (A1); EP1741767 (A1); EP1682466 (A1); EP1640437 (A1); EP1681337 (A1); EP1795576 (A1); and U.S. Patents 7,238,277; 6,630,066; 6,596,155; 6,977,322; 7,041,866; 6,217,746; 5,885,440; 6,881,873.

1. Hydrotreating

[0342] In a preferred embodiment of the method for producing renewable diesel, treating the lipid to produce an alkane is performed by hydrotreating of the lipid composition. In hydrothermal processing, typically, biomass is reacted in water at an elevated temperature and pressure to form oils and residual solids. Conversion temperatures are typically 300° to 660°F, with pressure sufficient to keep the water primarily as a liquid, 100 to 170 standard atmosphere (atm). Reaction times are on the order of 15 to 30 minutes. After the reaction is completed, the organics are separated from the water. Thereby a distillate suitable for diesel is produced.

2. Hydroprocessing

[0343] A renewable diesel, referred to as "green diesel," can be produced from fatty acids by traditional hydroprocessing technology. The triglyceride-containing oils can be hydroprocessed either as co-feed with petroleum or as a dedicated feed. The product is a diesel fuel that conforms with the ASTM D975 specification. Thus, in another preferred embodiment of the method for producing renewable diesel, treating the lipid composition to produce an alkane is performed by hydroprocessing of the lipid composition.

[0344] In some methods of making renewable diesel, the first step of treating a triglyceride is hydroprocessing to saturate double bonds, followed by deoxygenation at elevated temperature in the presence of hydrogen and a catalyst. In some methods, hydrogenation and deoxygenation occur in the same reaction. In other methods deoxygenation occurs before hydrogenation. Isomerization is then optionally performed, also in the presence of hydrogen and a catalyst. Naphtha components are preferably removed through distillation. For examples, see U.S. Patents 5,475,160 (hydrogenation of triglycerides); 5,091,116 (deoxygenation, hydrogenation and gas removal); 6,391,815 (hydrogenation); and 5,888,947 (isomerization).

[0345] Petroleum refiners use hydroprocessing to remove impurities by treating feeds with hydrogen. Hydroprocessing conversion temperatures are typically 300° to 700°F. Pressures are typically 40 to 100 atm. The reaction times are typically on the order of 10 to 60 minutes.

[0346] Solid catalysts are employed to increase certain reaction rates, improve selectivity for certain products, and optimize hydrogen consumption.

[0347] Hydrotreating and hydroprocessing ultimately lead to a reduction in the molecular weight of the feed. In the case of triglyceride-containing oils, the triglyceride molecule is reduced to four hydrocarbon molecules under hydroprocessing conditions: a propane molecule and three heavier hydrocarbon molecules, typically in the C8 to C18 range.

3. Indirect Liquefaction

[0348] A traditional ultra-low sulfur diesel can be produced from any form of biomass by a two-step process. First, the biomass is converted to a syngas, a gaseous mixture rich in hydrogen and carbon monoxide. Then, the syngas is catalytically converted to liquids. Typically, the production of liquids is accomplished using Fischer-Tropsch (FT) synthesis. This technology applies to coal, natural gas, and heavy oils. Thus, in yet another preferred

embodiment of the method for producing renewable diesel, treating the lipid composition to produce an alkane is performed by indirect liquefaction of the lipid composition.

C. Jet Fuel

[0349] The annual U.S. usage of jet fuel in 2006 was about 21 billion gallons (about 80 billion liters). Aeroplane fuel is clear to straw colored. The most common fuel is an unleaded/paraffin oil-based fuel classified as Aeroplane A-1, which is produced to an internationally standardized set of specifications. Aeroplane fuel is a mixture of a large number of different hydrocarbons, possibly as many as a thousand or more. The range of their sizes (molecular weights or carbon numbers) is restricted by the requirements for the product, for example, freezing point or smoke point. Kerosone-type Aeroplane fuel (including Jet A and Jet A-1) has a carbon number distribution between about 8 and 16 carbon numbers. Wide-cut or naphta-type Aeroplane fuel (including Jet B) typically has a carbon number distribution between about 5 and 15 carbons.

[0350] Both Aeroplanes (Jet A and jet B) may contain a number of additives. Useful additives include, but are not limited to, antioxidants, antistatic agents, corrosion inhibitors, and fuel system icing inhibitor (FSII) agents. Antioxidants prevent gumming and usually, are based on alkylated phenols, for example, AO-30, AO-31, or AO-37. Antistatic agents dissipate static electricity and prevent sparking. Stadis 450 with dinonylnaphthylsulfonic acid (DINNSA) as the active ingredient, is an example. Corrosion inhibitors, e.g., DCI-4A is used for civilian and military fuels and DCI-6A is used for military fuels. FSII agents, include, e.g., Di-EGME.

[0351] A solution is blending algae fuels with existing jet fuel. The present invention provides such a solution. The lipids produced by the methods of the present invention can serve as feedstock to produce jet fuel. Thus, in another aspect of the present invention, a method for producing jet fuel is provided. Herewith two methods for producing jet fuel from the lipids produced by the methods of the present invention are provided: fluid catalytic cracking (FCC); and hydrodeoxygenation (HDO).

1. Fluid Catalytic Cracking

[0352] Fluid Catalytic Cracking (FCC) is one method which is used to produce olefins, especially propylene from heavy crude fractions. There are reports in the literature that vegetable oils such as canola oil could be processed using FCC to give a hydrocarbon stream useful as a gasoline fuel.

[0353] The lipids produced by the method of the present invention can be converted to olefins. The process involves flowing the lipids produced through an FCC zone and collecting a product stream comprised of olefins, which is useful as a jet fuel. The lipids produced are contacted with a cracking catalyst at cracking conditions to provide a product stream comprising olefins and hydrocarbons useful as jet fuel.

[0354] In a preferred embodiment, the method for producing jet fuel comprises (a) cultivating a lipid-containing microorganism using methods disclosed herein, (b) lysing the lipid-containing microorganism to produce a lysate, (c) isolating lipid from the lysate, and (d) treating the lipid composition, whereby jet fuel is produced.

[0355] In a preferred embodiment of the method for producing a jet fuel, the lipid composition can be flowed through a fluid catalytic cracking zone, which, in one embodiment, may comprise contacting the lipid composition with a cracking catalyst at cracking conditions to provide a product stream comprising C_2-C_5 olefins.

[0356] In certain embodiments of this method it may be desirable to remove any contaminants that may be present in the lipid composition. Thus, prior to flowing the lipid composition through a fluid catalytic cracking zone, the lipid composition is pretreated. Pretreatment may involve contacting the lipid composition with an ion-exchange resin. The ion exchange resin is an acidic ion exchange resin, such as Amberlyst™-15 and can be used as a bed in a reactor through which the lipid composition is flowed, either upflow or downflow. Other pretreatments may include mild acid washes by contacting the lipid composition with an acid, such as sulfuric, acetic, nitric, or hydrochloric acid. Contacting is done with a dilute acid solution usually at ambient temperature and atmospheric pressure.

[0357] The lipid composition, optionally pretreated, is flowed to an FCC zone where the hydrocarbonaceous components are cracked to olefins. Catalytic cracking is accomplished by contacting the lipid composition in a reaction zone with a catalyst composed of finely divided particulate material. The reaction is catalytic cracking, as opposed to hydrocracking, and is carried out in the absence of added hydrogen or the consumption of hydrogen. As the cracking reaction proceeds, substantial amounts of coke are deposited on the catalyst. The catalyst is regenerated at high temperatures by burning coke from the catalyst in a regeneration zone. Coke-containing catalyst, referred to herein as "coked catalyst", is continually transported from the reaction zone to the regeneration zone to be regenerated and replaced by essentially coke-free regenerated catalyst from the regeneration zone.

Fluidization of the catalyst particles by various gaseous streams allows the transport of catalyst between the reaction zone and regeneration zone. Methods for cracking hydrocarbons, such as those of the lipid composition described herein, in a fluidized stream of catalyst, transporting catalyst between reaction and regeneration zones, and combusting coke in the regenerator are well known by those skilled in the art of FCC processes. Exemplary FCC applications and catalysts useful for cracking the lipid composition to produce C_2-C_5 olefins are described in U.S. Pat. Nos. 6,538,169, 7,288,685, which are incorporated in their entirety by reference.

[0358] In one embodiment, cracking the lipid composition of the present invention, takes place in the riser section or, alternatively, the lift section, of the FCC zone. The lipid composition is introduced into the riser by a nozzle resulting in the rapid vaporization of the lipid composition. Before contacting the catalyst, the lipid composition will ordinarily have a temperature of about 149°C to about 316°C (300°F to 600°F). The catalyst is flowed from a blending vessel to the riser where it contacts the lipid composition for a time of abort 2 seconds or less.

[0359] The blended catalyst and reacted lipid composition vapors are then discharged from the top of the riser through an outlet and separated into a cracked product vapor stream including olefins and a collection of catalyst particles covered with substantial quantities of coke and generally referred to as "coked catalyst." In an effort to minimize the contact time of the lipid composition and the catalyst which may promote further conversion of desired products to undesirable other products, any arrangement of separators such as a swirl arm arrangement can be used to remove coked catalyst from the product stream quickly. The separator, e.g. swirl arm separator, is located in an upper portion of a chamber with a stripping zone situated in the lower portion of the chamber. Catalyst separated by the swirl arm arrangement drops down into the stripping zone. The cracked product vapor stream comprising cracked hydrocarbons including light olefins and some catalyst exit the chamber via a conduit which is in communication with cyclones. The cyclones remove remaining catalyst particles from the product vapor stream to reduce particle concentrations to very low levels. The product vapor stream then exits the top of the separating vessel. Catalyst separated by the cyclones is returned to the separating vessel and then to the stripping zone. The stripping zone removes adsorbed hydrocarbons from the surface of the catalyst by counter-current contact with steam.

[0360] Low hydrocarbon partial pressure operates to favor the production of light olefins. Accordingly, the riser pressure is set at about 172 to 241 kPa (25 to 35 psia) with a hydrocarbon partial pressure of about 35 to 172 kPa (5 to 25 psia), with a preferred hydrocarbon partial pressure of about 69 to 138 kPa (10 to 20 psia). This relatively low partial pressure for hydrocarbon is achieved by using steam as a diluent to the extent that the diluent is 10 to 55 wt-% of lipid composition and preferably about 15 wt-% of lipid composition. Other diluents such as dry gas can be used to reach equivalent hydrocarbon partial pressures.

[0361] The temperature of the cracked stream at the riser outlet will be about 510°C to 621°C (950°F to 1150°F). However, riser outlet temperatures above 566°C (1050°F) make more dry gas and more olefins. Whereas, riser outlet temperatures below 566°C (1050°F) make less ethylene and propylene. Accordingly, it is preferred to run the FCC process at a preferred temperature of about 566°C to about 630°C, preferred pressure of about 138 kPa to about 240 kPa (20 to 35 psia). Another condition for the process is the catalyst to lipid composition ratio which can vary from about 5 to about 20 and preferably from about 10 to about 15.

[0362] In one embodiment of the method for producing a jet fuel, the lipid composition is introduced into the lift section of an FCC reactor. The temperature in the lift section will be very hot and range from about 700°C (1292°F) to about 760°C (1400°F) with a catalyst to lipid composition ratio of about 100 to about 150. It is anticipated that introducing the lipid composition into the lift section will produce considerable amounts of propylene and ethylene.

[0363] Gas and liquid hydrocarbon products produced can be analyzed by gas chromatography, HPLC, etc.

2. Hydrodeoxygenation

[0364] In another embodiment of the method for producing a jet fuel using the lipid composition or the lipids produced as described herein, the structure of the lipid composition or the lipids is broken by a process referred to as hydrodeoxygenation (HDO).

[0365] HDO means removal of oxygen by means of hydrogen, that is, oxygen is removed while breaking the structure of the material. Olefinic double bonds are hydrogenated and any sulphur and nitrogen compounds are removed. Sulphur removal is called

hydrodesulphurization (HDS). Pretreatment and purity of the raw materials (lipid composition or the lipids) contribute to the service life of the catalyst.

[0366] Generally in the HDO/HDS step, hydrogen is mixed with the feed stock (lipid composition or the lipids) and then the mixture is passed through a catalyst bed as a co-current flow, either as a single phase or a two phase feed stock. After the HDO/MDS step, the product fraction is separated and passed to a separate isomerzation reactor. An isomerization reactor for biological starting material is described in the literature (FI 100 248) as a co-current reactor.

[0367] The process for producing a fuel by hydrogenating a hydrocarbon feed, e.g., the lipid composition or the lipids herein, can also be performed by passing the lipid composition or the lipids as a co-current flow with hydrogen gas through a first hydrogenation zone, and thereafter the hydrocarbon effluent is further hydrogenated in a second hydrogenation zone by passing hydrogen gas to the second hydrogenation zone as a counter-current flow relative to the hydrocarbon effluent. Exemplary HDO applications and catalysts useful for cracking the lipid composition to produce C_2-C_5 olefins are described in U.S. Pat. No. 7,232,935, which is incorporated in its entirety by reference.

[0368] Typically, in the hydrodeoxygenation step, the structure of the biological component, such as the lipid composition or lipids herein, is decomposed, oxygen, nitrogen, phosphorus and sulphur compounds, and light hydrocarbons as gas are removed, and the olefinic bonds are hydrogenated. In the second step of the process, i.e. in the so-called isomerization step, isomerzation is carried out for branching the hydrocarbon chain and improving the performance of the paraffin at low temperatures.

[0369] In the first step i.e. HDO step of the cracking process, hydrogen gas and the lipid composition or lipids herein which are to be hydrogenated are passed to a HDO catalyst bed system either as co-current or counter-current flows, said catalyst bed system comprising one or more catalyst bed(s), preferably 1-3 catalyst beds. The HDO step is typically operated in a co-current manner. In case of a HDO catalyst bed system comprising two or more catalyst beds, one or more of the beds may be operated using the counter-current flow principle.

[0370] In the HDO step, the pressure varies between 20 and 150 bar, preferably between 50 and 100 bar, and the temperature varies between 200 and 500°C, preferably in the range of 300-400°C.

[0371] In the HDO step, known hydrogenation catalysts containing metals from Group VII and/or VIB of the Periodic System may be used. Preferably, the hydrogenation catalysts are supported Pd, Pt, Ni, NiMo or a CoMo catalysts, the support being alumina and/or silica. Typically, $NiMo/Al_2O_3$ and $CoMo/Al_2O_3$ catalysts are used.

[0372] Prior to the HDO step, the lipid composition or lipids herein may optionally be treated by prehydrogenation under milder conditions thus avoiding side reactions of the double bonds. Such prehydrogenation is carried out in the presence of a prehydrogenation catalyst at temperatures of 50 400°C and at hydrogen pressures of 1 200 bar, preferably at a temperature between 150 and 250°C and at a hydrogen pressure between 10 and 100 bar. The catalyst may contain metals from Group VIII and/or VIB of the Periodic System. Preferably, the prehydrogenation catalyst is a supported Pd, Pt, Ni, NiMo or a CoMo catalyst, the support being alumina and/or silica.

[0373] A gaseous stream from the HDO step containing hydrogen is cooled and then carbon monoxide, carbon dioxide, nitrogen, phosphorus and sulphur compounds, gaseous light hydrocarbons and other impurities are removed therefrom. After compressing, the purified hydrogen or recycled hydrogen is returned back to the first catalyst bed and/or between the catalyst beds to make up for the withdrawn gas stream. Water is removed from the condensed liquid. The liquid is passed to the first catalyst bed or between the catalyst beds.

[0374] After the HDO step, the product is subjected to an isomerization step. It is substantial for the process that the impurities are removed as completely as possible before the hydrocarbons are contacted with the isomerization catalyst. The isomerization step comprises an optional stripping step, wherein the reaction product from the HDO step may be purified by stripping with water vapour or a suitable gas such as light hydrocarbon, nitrogen or hydrogen. The optional stripping step is carried out in counter-current manner in a unit upstream of the isomerization catalyst, wherein the gas and liquid are contacted with each other, or before the actual isomerization reactor in a separate stripping unit utilizing counter-current principle.

[0375] After the stripping step the hydrogen gas and the hydrogenated lipid composition or lipids herein, and optionally an n-paraffin mixture, are passed to a reactive isomerization unit comprising one or several catalyst bed(s). The catalyst beds of the isomerization step may operate either in co-current or counter-current manner.

[0376] It is important for the process that the counter-current flow principle is applied in the isomerization step. In the isomerization step this is done by carrying out either the optional stripping step or the isomerization reaction step or both in counter-current manner.

[0377] The isomerization step and the HDO step may be carried out in the same pressure vessel or in separate pressure vessels. Optional prehydrogenation may be carried out in a separate pressure vessel or in the same pressure vessel as the HDO and isomerization steps.

[0378] In the isomerzation step, the pressure varies in the range of 20 150 bar, preferably in the range of 20 100 bar, the temperature being between 200 and 500°C, preferably between 300 and 400°C.

[0379] In the isomerization step, isomerization catalysts known in the art may be used. Suitable isomerization catalysts contain molecular sieve and/or a metal from Group VII and/or a carrier. Preferably, the isomerization catalyst contains SAPO-11 or SAPO41 or ZSM-22 or ZSM-23 or ferrierite and Pt, Pd or Ni and Al_2O_3 or SiO_2. Typical isomerization catalysts are, for example, Pt/SAPO-11/Al_2O_3, Pt/ZSM-22/Al_2O_3, Pt/ZSM-23/Al_2O_3 and Pt/SAPO-11/SiO_2.

[0380] As the product, a high quality hydrocarbon component of biological origin, useful as a diesel fuel or a component thereof, is obtained, the density, cetane number and performance at low temperate of said hydrocarbon component being excellent.

IX. MICROBE ENGINEERING

[0381] As noted above, in certain embodiments of the present invention it is desirable to genentically modify a microorganism to enhance lipid production, modify the properties or proportions of components generated by the microorganism, or to improve or provide de novo growth characteristics on a variety of feedstock materials. *Chlorella*, particularly *Chlorella protothecoides*, *Chlorella minutissima, Chlorella sorokiniana, Chlorella ellipsoidea*, *Chlorella sp.*, and *Chlorella emersonii* are preferred microorganisms for use in the genetic engineering methods described herein, although other *Chlorella* species as well as other varieties of microorganisms can be used.

[0382] Promoters, cDNAs, and 3'UTRs, as well as other elements of the vectors, can be generated through cloning techniques using fragments isolated from native sources (see for example Molecular Cloning: A Laboratory Manual, Sambrook et al. (3d edition, 2001, Cold

Spring Harbor Press; and U.S. Patent 4,683,202). Alternatively, elements can be generated synthetically using known methods (see for example Gene. 1995 Oct 16;164(1):49-53).

A. Codon-Optimization For Expression

[0383] DNA encoding a polypeptide to be expressed in a microorganism, e.g., a lipase and selectable marker are preferably codon-optimized Cdna. Methods of recoding genes for expression in microalgae are described in U.S. Patent 7,135,290. Additional information for codon optimization is available, e.g., at the codon usage database of GenBank. As non-limiting examples, codon usage in *Chlorella pyrenoidosa*, *Dunaliella salina*, and *Chlorella protothecoides* are shown in Tables 10, 11, and 12, respectively.

[0384] Table 10. Codon usage in *Chlorella pyrenoidosa*.

Phe	UUU	39 (0.82)	Ser	UCU	50 (1.04)
	UUC	56 (1.18)		UCC	60 (1.25)
Leu	UUA	10 (0.20)		UCA	6 (0.96)
	UUG	46 (0.91)		UCG	43 (0.89)
Tyr	UAU	15 (0.59)	Cys	UGU	46 (0.77)
	UAC	36 (1.41)		UGC	73 (1.23)
ter	UAA	9 (0.00)	ter	UGA	43 (0.00)
ter	UAG	15 (0.00)	Trp	UGG	69 (1.00)
Leu	CUU	49 (0.97)	Pro	CCU	80 (0.98)
	CUC	73 (1.45)		CCC	88 (1.08)
	CUA	22 (0.44)		CCA	93 (1.14)
	CUG	103 (2.04)		CCG	65 (0.80)
His	CAU	50 (0.88)	Arg	CGU	39 (0.76)
	CAC	3 (1.12)		CGC	63 (1.23)
Gln	CAA	59 (0.84)		CGA	46 (0.90)
	CAG	2 (1.16)		CGG	47 (0.92)
Ile	AUU	24 (0.69)	Thr	ACU	32 (0.67)
	AUC	61 (1.76)		ACC	76 (1.60)
	AUA	19 (0.55)		ACA	41 (0.86)
Met	AUG	42 (1.00)		ACG	41 (0.86)
Asn	AAU	26 (0.75)	Ser	AGU	23 (0.48)
	AAC	3 (1.25)		AGC	67 (1.39)
Lys	AAA	32 (0.54)	Arg	AGA	51 (1.00)
	AAG	86 (1.46)		AGG	61 (1.19)
Val	GUU	36 (0.75)	Ala	GCU	57 (0.79)
	GUC	54 (1.13)		GCC	97 (1.34)
	GUA	30 (0.63)		GCA	89 (1.23)
	GUG	71 (1.49)		GCG	47 (0.65)
Asp	GAU	60 (0.95)	Gly	GGU	35 (0.60)
	GAC	66 (1.05)		GGC	78 (1.33)
Glu	GAA	41 (0.68)		GGA	54 (0.92)
	GAG	80 (1.32)		GGG	67 (1.15)

[0385] Table 11. Preferred codon usage in *Dunaliella salina*.

TTC (Phe)	TAC (Tyr)	TGC (Cys)	TAA (Stop)
TGG (Trp)	CCC (Pro)	CAC (His)	CGC (Arg)
CTG (Leu)	CAG (Gln)	ATC (Ile)	ACC (Thr)
AAC (Asn)	AGC (Ser)	ATG (Met)	AAG (Lys)
GCC (Ala)	GAC (Asp)	GGC (Gly)	GTG (Val)
GAG (Glu)			

[0386] Table 12. Preferred codon usage in *Chlorella protothecoides*.

TTC (Phe)	TAC (Tyr)	TGC (Cys)	TGA (Stop)
TGG (Trp)	CCC (Pro)	CAC (His)	CGC (Arg)
CTG (Leu)	CAG (Gln)	ATC (Ile)	ACC (Thr)
GAC (Asp)	TCC (Ser)	ATG (Met)	AAG (Lys)
GCC (Ala)	AAC (Asn)	GGC (Gly)	GTG (Val)
GAG (Glu)			

B. Promoters

[0387] 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). Exogenous and/or endogenous promoters that are active in microalgae, and antibiotic resistance genes functional in microalgae are described by e.g., Curr Microbiol. 1997 Dec;35(6):356-62 (Chlorella vulgaris); Mar Biotechnol (NY). 2002 Jan;4(1):63-73 (Chlorella ellipsoidea); Mol Gen Genet. 1996 Oct 16;252(5):572-9 (Phaeodactylum tricornutum); Plant Mol Biol. 1996 Apr;31(1):1-12 (Volvox carteri); Proc Natl Acad Sci U S A. 1994 Nov 22;91(24):11562-6 (Volvox carteri); Falciatore A, Casotti R, Leblanc C, Abrescia C, Bowler C, PMID: 10383998, 1999 May;1(3):239-251 (Laboratory of Molecular Plant Biology, Stazione Zoologica, Villa Comunale, I-80121 Naples, Italy) (Phaeodactylum tricornutum and Thalassiosira weissflogii); Plant Physiol. 2002 May;129(1):7-12. (Porphyridium sp.); Proc Natl Acad Sci U S A. 2003 Jan 21;100(2):438-42. (Chlamydomonas reinhardtii); Proc Natl Acad Sci U S A. 1990 Feb;87(3):1228-32. (Chlamydomonas reinhardtii); Nucleic Acids Res. 1992 Jun 25;20(12):2959-65; Mar Biotechnol (NY). 2002 Jan;4(1):63-73 (Chlorella); Biochem Mol Biol Int. 1995 Aug;36(5):1025-35 (Chlamydomonas reinhardtii); J Microbiol. 2005 Aug;43(4):361-5 (Dunaliella); Yi Chuan Xue Bao. 2005 Apr;32(4):424-33 (Dunaliella); Mar Biotechnol (NY). 1999 May;1(3):239-251. (Thalassiosira and Phaedactylum); Koksharova,

Appl Microbiol Biotechnol 2002 Feb;58(2):123-37 (various species); Mol Genet Genomics. 2004 Feb;271(1):50-9 (Thermosynechococcus elongates); J. Bacteriol. (2000), 182, 211-215; FEMS Microbiol Lett. 2003 Apr 25;221(2):155-9; Plant Physiol. 1994 Jun;105(2):635-41; Plant Mol Biol. 1995 Dec;29(5):897-907 (Synechococcus PCC 7942); Mar Pollut Bull. 2002;45(1-12):163-7 (Anabaena PCC 7120); Proc Natl Acad Sci U S A. 1984 Mar;81(5):1561-5 (Anabaena (various strains)); Proc Natl Acad Sci U S A. 2001 Mar 27;98(7):4243-8 (Synechocystis); Wirth, Mol Gen Genet 1989 Mar;216(1):175-7 (various species); Mol Microbiol, 2002 Jun;44(6):1517-31 and Plasmid, 1993 Sep;30(2):90-105 (Fremyella diplosiphon); Hall et al. (1993) Gene 124: 75-81 (Chlamydomonas reinhardtii); Gruber et al. (1991). Current Micro. 22: 15-20; Jarvis et al. (1991) Current Genet. 19: 317-322 (Chlorella); for additional promoters see also table 1 from US Patent 6,027,900).

[0388] The promoter used to express an exogenous gene can be the promoter naturally linked to that gene or can be a heterologous gene. Some promoters are active in more than one species of microalgae. Other promoters are species-specific. Preferred promoters include promoters such as RBCS2 from *Chlamydomonas reinhardtii* and viral promoters, such as cauliflower mosaic virus (CMV) and chlorella virus, which have been shown to be active in multiple species of microalgae (see for example Plant Cell Rep. 2005 Mar;23(10-11):727-35; J Microbiol. 2005 Aug;43(4):361-5; Mar Biotechnol (NY). 2002 Jan;4(1):63-73). In other embodiments, the *Botryococcus* malate dehydrogenase promoter, such a nucleic acid comprising any part of SEQ ID NO:3, or the *Chlamydomonas reinhardtii* RBCS2 promoter (SEQ ID NO:4) can be used. Optionally, at least 10, 20, 30, 40, 50, or 60 nucleotides or more of these sequences containing a promoter are used. Preferred promoters endogenous to species of the genus *Chlorella* are SEQ ID NO:1 and SEQ ID NO:2.

[0389] Preferred promoters useful for expression of exogenous genes in *Chlorella* 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 *Chlorella*, such as SEQ ID NOs: 1-7 of U.S. Patent 6,395,965. Additional promoters active in *Chlorella* 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.

C. Selectable Markers

[0390] Any of a wide variety of selectable markers can be employed in a transgene construct useful for transforming *Chlorella*. Examples of suitable selectable markers include the nitrate reductase gene, the hygromycin phosphotransferase gene (HPT), the neomycin phosphotransferase gene, and the ble gene, which confers resistance to phleomycin. Methods of determining sensitivity of microalgae to antibiotics are well known. For example, Mol Gen Genet. 1996 Oct 16;252(5):572-9.

[0391] More specifically, Dawson et al. (1997), Current Microbiology 35:356-362 (incorporated by reference herein in its entirety), described the use of the nitrate reductase (NR) gene from *Chlorella vulgaris* as a selectable marker for NR-deficient *Chlorella sorokiniana* mutants. Kim et al. (2002), Mar. Biotechnol. 4:63-73 (incorporated by reference herein in its entirety), disclosed the use of the HPT gene as a selectable marker for transforming *Chorella ellipsoidea*. Huang et al. (2007), Appl. Microbiol. Biotechnol. 72:197-205 (incorporated by reference herein in its entirety), reported on the use of Sh ble as a selectable marker for *Chlorella sp. DT*.

D. Inducible Expression

[0392] The present invention also provides for the use of an inducible promoter to express a gene of interest. In particular, the use of an inducible promoter to express a lipase gene permits production of the lipase after growth of the microorganism when conditions have been adjusted, if necessary, to enhance transesterification, for example, after disruption of the cells, reduction of the water content of the reaction mixture, and/or addition sufficient alcohol to drive conversion of TAGs to fatty acid esters.

[0393] Inducible promoters useful in the invention include those that mediate transcription of an operably linked gene in response to a stimulus, such as an exogenously provided small molecule (e.g, glucose, as in SEQ ID NO:1), temperature (heat or cold), light, 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. In the latter case, the level of transcription of the lipase preferably does not significantly interfere with the growth of the microorganism in which it is expressed.

[0394] Expression of transgenes in *Chlorella* can be performed inducibly through promoters such as the promoter that drives the *Chlorella* hexose transporter gene (SEQ ID NO:1). This promoter is strongly activated by the presence of glucose in the culture media.

E. Expression of Two or More Exogenous Genes

[0395] Further, a genetically engineered microorganism, such as a microalgae, may comprise and express two or more exogenous genes, such as, for example, a lipase and a lytic gene, e.g., one encoding a polysaccharide-degrading enzyme. 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 a different inducible 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). Provided herein are vectors and methods for engineering lipid-producing microbes to metabolize sucrose, which is an advantageous trait because it allows the engineered cells to convert sugar cane feedstocks into lipids.

[0396] Also provided herein are genetically engineered strains of microbes (e.g., microalgae, oleaginous yeast, bacteria, or fungi) that express two or more exogenous genes, such as, for example, 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 addition, this application provides for the combination of a fatty acyl-ACP thioesterase, a fatty acyl-CoA reductase, and a fatty aldehyde decarbonylase to generate alkanes. One or more of the exogenous genes can be expressed using an inducible promoter.

[0397] Examples of further modifications suitable for use in the present invention are include genetically engineering strains of microalgae to express two or more exogenous genes, one encoding a transporter of a fixed carbon source (such as sucrose) and a second encoding a sucrose invertase enzyme. The resulting fermentable organisms produce hydrocarbons at lower manufacturing cost than what has been obtainable by previously known methods of biological hydrocarbon production. Insertion of the two exogenous genes described above can be combined with the disruption of polysaccharide biosynthesis through directed and/or random mutagenesis, which steers ever greater carbon flux into hydrocarbon production.

Individually and in combination, trophic conversion, engineering to alter hydrocarbon production and treatment with exogenous enzymes alter the hydrocarbon composition produced by a microorganism. The alteration can be a change in the amount of hydrocarbons produced, the amount of one or more hydrocarbon species produced relative to other hydrocarbons, and/or the types of hydrocarbon species produced in the microorganism. For example, microalgae can be engineered to produce a higher amount and/or percentage of TAGs.

F. Compartmentalized Expression

[0398] The present invention also provides for compartmentalized expression of a gene of interest. In particular, it can be advantageous, in particular embodiments, to target expression of the lipase to one or more cellular compartments, where it is sequestered from the majority of cellular lipids until initiation of the transesterification reaction. Preferred organelles for targeting are chloroplasts, mitochondria, and endoplasmic reticulum.

1. Expression in Chloroplasts

[0399] In one embodiment of the present invention, the expression of a polypeptide in a microorganism is targeted to chloroplasts. Methods for targeting expression of a heterologous gene to the chloroplast are known and can be employed in the present invention. Methods for targeting foreign gene products into chloroplasts are described in Shrier et al., EMBO J. (1985) 4:25 32. See also Tomai et al. Gen. Biol. Chem. (1988) 263:15104 15109 and U.S. Pat. No. 4,940,835 for the use of transit peptides for translocating nuclear gene products into the chloroplast. Methods for directing the transport of proteins to the chloroplast are also reviewed in Kenauf TIBTECH (1987) 5:40 47. Chloroplast targeting sequences endogenous to *Chlorella* are known, such as genes in the *Chlorella* nuclear genome that encode proteins that are targeted to the chloroplast; see for example GenBank Accession numbers AY646197 and AF499684.

[0400] Wageningen UR- Plant Research International sells an IMPACTVECTOR1.4 vector, which uses the secretion signal of the Chrysanthemum morifolium small subunit protein to deliver a heterologous protein into the chloroplast stroma (cytoplasmic) environment, shuttling across a double membrane system. The protein is fused to the first 11 amino acids of the mature rubisco protein in order to allow proper processing of the signal peptide (Wong et al., Plant Molecular Biology 20: 81-93 (1992)). The signal peptide contains a natural intron from the RbcS gene.

[0401] In another approach, the chloroplast genome is genetically engineered to express the heterologous protein. Stable transformation of chloroplasts of *Chlamydomonas reinhardtii* (a green alga) using bombardment of recipient cells with high-velocity tungsten microprojectiles coated with foreign DNA has been described. See, for example, Boynton et al., Science (1988) 240: 1534 1538; Blowers et al. Plant Cell (1989) 1:123 132 and Debuchy et al., EMBO J. (1989) 8: 2803 2809. The transformation technique, using tungsten microprojectiles, is described by Klein et al., Nature (London) (1987) 7:70 73. Other methods of chloroplast transformation for both plants and microalgae are known. See for example U.S. Patents 5,693,507; 6,680,426; and Plant Physiol. 2002 May;129(1):7-12; and Plant Biotechnol J. 2007 May;5(3):402-12.

[0402] As described in U.S. Patent No. 6,320,101 (issued November 20, 2001 to Kaplan et al.; which is incorporated herein by reference), cells can be chemically treated so as to reduce the number of chloroplasts per cell to about one. Then, the heterologous nucleic acid can be introduced into the cells via particle bombardment with the aim of introducing at least one heterologous nucleic acid molecule into the chloroplasts. The heterologous nucleic acid is selected such that it is integratable into the chloroplast's genome via homologous recombination which is readily effected by enzymes inherent to the chloroplast. To this end, the heterologous nucleic acid includes, in addition to a gene of interest, at least one nucleic acid sequence that is derived from the chloroplast's genome. In addition, the heterologous nucleic acid typically includes a selectable marker. Further details relating to this technique are found in U.S. Patent. Nos. 4,945,050 and 5,693,507 which are incorporated herein by reference. A polypeptide can thus be produced by the protein expression system of the chloroplast.

[0403] U.S. Patent No. 7,135,620 (issued November 14, 2006 to Daniell et al.; incorporated herein by reference) describes chloroplast expression vectors and related methods. Expression cassettes are DNA constructs including a coding sequence and appropriate control sequences to provide for proper expression of the coding sequence in the chloroplast. Typical expression cassettes include the following components: the 5' untranslated region from a microorganism gene or chloroplast gene such as psbA which will provide for transcription and translation of a DNA sequence encoding a polypeptide of interest in the chloroplast; a DNA sequence encoding a polypeptide of interest; and a translational and transcriptional termination region, such as a 3' inverted repeat region of a chloroplast gene

that can stabilize RNA of introduced genes, thereby enhancing foreign gene expression. The cassette can optionally include an antibiotic resistance gene.

[0404] Typically, the expression cassette is flanked by convenient restriction sites for insertion into an appropriate genome. The expression cassette can be flanked by DNA sequences from chloroplast DNA to facilitate stable integration of the expression cassette into the chloroplast genome, particularly by homologous recombination. Alternatively, the expression cassette may remain unintegrated, in which case, the expression cassette typically includes a chloroplast origin of replication, which is capable of providing for replication of the heterologous DNA in the chloroplast.

[0405] The expression cassette generally includes a promoter region from a gene capable of expression in the chloroplast. The promoter region may include promoters obtainable from chloroplast genes, such as the psbA gene from spinach or pea, or the rbcL and atpB promoter region from maize and Rrna promoters. Examples of promoters are described in Hanley-Bowdoin and Chua, TIBS (1987) 12:67 70; Mullet et al., Plant Molec Biol. (1985) 4: 39 54; Hanley-Bowdoin (1986) PhD. Dissertation, the Rockefeller University; Krebbers et al., Nucleic Acids Res. (1982) 10: 4985 5002; Zurawaki et al., Nucleic Acids Res. (1981) 9:3251 3270; and Zurawski et al., Proc. Nat'l Acad Sci. U.S.A. (1982) 79: 7699 7703. Other promoters can be identified and the relative strength of promoters so identified evaluated, by placing a promoter of interest 5' to a promoterless marker gene and observing its effectiveness relative to transcription obtained from, for example, the promoter from the psbA gene, a relatively strong chloroplast promoter. The efficiency of heterologus gene expression additionally can be enhanced by any of a variety of techniques. These include the use of multiple promoters inserted in tandem 5' to the heterologous gente, for example a double psbA promoter, the addition of enhancer sequences and the like.

[0406] Numerous promoters active in the *Chlorella* chloroplast can be used for expression of exogenous genes in the *Chlorella* chloroplast, such as those found in GenBank accession number NC_001865 (*Chlorella vulgaris* chloroplast, complete genome),

[0407] Where it is desired to provide for inducible expression of the heterologous gene, an inducible promoter and/or a 5' untranslated region containing sequences which provide for regulation at the level of transcription and/or translation (at the 3' end) may be included in the expression cassette. For example, the 5' untranslated region can be from a gene wherein expression is regulatable by light. Similarly, 3' inverted repeat regions could be used to

stabilize RNA of heterologous genes. Inducible genes may be identified by enhanced expression in response to a particular stimulus of interest and low or absent expression in the absence of the stimulus. For example, a light-inducible gene can be identified where enhanced expression occurs during irradiation with light, while substantially reduced expression or no expression occurs in low or no light. Light regulated promoters from green microalgae are known (see for example Mol Genet Genomics. 2005 Dec;274(6):625-36).

[0408] The termination region which is employed will be primarily one of convenience, since the termination region appears to be relatively interchangeable among chloroplasts and bacteria. The termination region may be native to the transcriptional initiation region, 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.

[0409] The expression cassettes may be transformed into a plant cell of interest by any of a number of methods. These methods include, for example, 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 chloroplast.

[0410] Additional descriptions of chloroplast expression vectors suitable for use in microorganisms such as microalgae are found in U.S. Patent Nos. 7,081,567 (issued July 25, 2006 to Xue et al.); 6,680,426 (issued January 20, 2004 to Daniell et al.); and 5,693,507 (issued December 2, 1997 to Daniell et al.).

[0411] Proteins expressed in the nuclear genome of *Chlorella* can be targeted to the chloroplast using chloroplast targeting signals. Chloroplast targeting sequences endogenous to *Chlorella* are known, such as genes in the Chlorella nuclear genome that encode proteins that are targeted to the chloroplast; see for example GenBank Accession numbers AY646197 and AF499684. Proteins can also be expressed in the *Chlorella* chloroplast by insertion of genes directly into the chloroplast genome. Chloroplast transformation typically occurs through homologous recombination, and can be performed if chloroplast genome sequences are known for creation of targeting vectors (see for example the complete genome sequence of a *Chlorella* chloroplast; Genbank accession number NC_001865). See previous sections herein for details of chloroplast transformation.

2. Expression in Mitochondria

[0412] In another embodiment of the present invention, the expression of a polypeptide in a microorganism is targeted to mitochondria. Methods for targeting foreign gene products into mitochnodria (Boutry et al. Nature (London) (1987) 328:340 342) have been described, including in green microalgae (see for example Mol Gen Genet. 1993 Jan;236(2-3):235-44).

[0413] For example, an expression vector encoding a suitable secretion signal can target a heterologus protein to the mitochondrion. The IMPACTVECTOR1.5 vector, from Wageningen UR- Plant Research International, uses the yeast CoxIV secretion signal, which was shown to deliver proteins in the mitochondrial matrix. The protein is fused to the first 4 amino acids of the yeast CoxIV protein in order to allow proper processing of the signal peptide (Kohler et al. Plant J 11: 613-621 (1997)). Other mitochondrial targeting sequences are known, including those functional in green microalgae. For example, see FEBS Lett. 1990 Jan 29;260(2):165-8; and J Biol Chem. 2002 Feb 22;277(8):6051-8.

[0414] Proteins expressed in the nuclear genome of *Chlorella* can be targeted to the mitochondria using mitochondrial targeting signals. See previous sections herein for details of mitochondrial protein targeting and transformation.

3. Expression in Endoplasmic Reticulum

[0415] In another embodiment of the present invention, the expression of a polypeptide in a microorganism is targeted to the endoplasmic reticulum. The inclusion of an appropriate retention or sorting signal in an expression vector ensure that proteins are retained in the endoplasmic reticulum (ER) and do not go downstream into Golgi. For example, the IMPACTVECTOR1.3 vector, from Wageningen UR- Plant Research International, includes the well known KDEL retention or sorting signal. With this vector, ER retention has a practical advantage in that it has been reported to improve expression levels 5-fold or more. The main reason for this appears to be that the ER contains lower concentrations and/or different proteases responsible for post-translational degradation of expressed proteins than are present in the cytoplasm. ER retention signals functional in green microalgae are known. For example, see Proc Natl Acad Sci U S A. 2005 Apr 26;102(17):6225-30.

G. Transformation

[0416] Cells can be transformed by any suitable technique including, e.g., biolistics, electroporation, glass bead transformation and silicon carbide whisker transformation. See, e.g., Examples herein.

[0417] Any convenient technique for introducing a transgene into *Chorella* can be employed in the present invention. Dawson et al. (1997) (supra) described the use of micro-projectile bombardment to introduce the nitrate reductase (NR) gene from *Chlorella vulgaris* into NR-deficient *Chlorella sorokiniana* mutants, resulting in stable transformants. Briefly, 0.4 micron tungsten beads were coated with plasmid; 3 X 10^7 *C. sorokiniana* cells were spread in the center third of a non-selective agar plate and bombarded with the PDS-1000/He Biolistic Particle Delivery® system (Bio-Rad).

[0418] A preferred method for introducing a transgene into *Chlorella* is the method described by Kim et al. (2002), *Mar. Biotechnol.* 4:63-73. Kim reports the transformation of *Chorella ellipsoidea* protoplasts using $CaCl_2$ and polyethylene glycol (PEG). In particular, protoplasts were prepared by growing *C. ellipsoidea* cells to a density of 1-2 X 10^8/Ml. Cells were recovered and washed by centrifugation for 5 minutes at 1600 g and resuspended in 5 Ml of phosphate buffer (Ph 6.0) containing 0.6 M sorbitol, 0.6 M mannitol, 4% (weight/volume) cellulose (Calbiochem), 2% (weight/volume) macerase (Calbiochem), and 50 units pectinase (Sigma). The cell suspension was incubated at 25°C for 16 hours in the dark with gentle shaking. The resultant protoplasts were recovered by centrifugation at 400 g for 5 minutes. The pellet was gently resuspended in 5 Ml of f/2 medium containing 0.6 M sorbitol and 0.6 M mannitol and centrifuged at 400 g for 5 minutes. This pellet was resuspended in 1 Ml of 0.6 M sorbitol/mannitol solution containing 50 mM$CaCl_2$. Then, 5 mg of transgene DNA was added, along with 25 μg calf thymus DNA (Sigma), to 10^7-10^8 protoplasts in 0.4 Ml. After 15 minutes at room temperature, 200 μL of PNC (40% polyethylene glycol 4000, 0.8 M NaCl, 50 Mm $CaCl_2$) was added and mixed gently for 30 minutes at room temperature. After this, 0.6 Ml of f/2 medium supplemented with 0.6 M sorbitol/mannitol solution, 1% yeast extract and 1% glucose was added, and the transformed cells were incubated at 25°C for 12 hours in the dark for cell wall regeneration. A similar method was used by Huang et al. (2007) (supra) to introduce a transgene encoding mercuric reductase into *Chlorella sp. DT.*

[0419] Electorporation has also been employed to transform *Chorella.* As reported by Maruyama et al. (2004), Biotechnology Techniques 8:821-826 (incorporated by reference herein in its entirety), this technique was used to introduce a transgene into protoplasts of *Chlorella saccharophila* c-211-1a prepared from the cells in the stationary phase. Transient expression of the introduced plasmid was observed under a field strength of between 600 and

900 V/cm, and a pulse duration of around 400 ms, where high membrane permeability to 70-kDa FITC-dextran was ascertained.

[0420] Examples of expression of transgenes in *Chlorella* can be found in the literature (see for example Current Microbiology Vol. 35 (1997), pp. 356–362; Sheng Wu Gong Cheng Xue Bao. 2000 Jul;16(4):443-6; Current Microbiology Vol. 38 (1999), pp. 335–341; Appl Microbiol Biotechnol (2006) 72: 197–205; Marine Biotechnology 4, 63–73, 2002; Current Genetics 39:5, 365-370 (2001); Plant Cell Reports 18:9, 778-780, (1999); Biologia Plantarium 42(2): 209-216, (1999); Plant Pathol. J 21(1): 13-20, (2005)). Also see Examples herein.

[0421] Examples of expression of transgenes in oleaginous yeast (e.g., *Yarrowia lipolytica*) can be found in the literature (see, for example, Bordes et al., J Microbiol Methods, Jun 27 (2007)). Examples of expression of transgenes in fungi (e.g., *Mortierella alpine*, *Mucor circinelloides*, and *Aspergillus ochraceus*) can also be found 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). Examples of expression of exogenous genes in bacteria such as *E. coli* are well known; see for example Molecular Cloning: A Laboratory Manual, Sambrook et al. (3d edition, 2001, Cold Spring Harbor Press.

[0422] Vectors for transformation of microorganisms in accordance with the present invention can be prepared by known techniques familiar to those skilled in the art. The nucleotide sequence of the construct used for transformation of multiple *Chlorella* species corresponds to SEQ ID NO:25. In one embodiment, an exemplary vector design for expression of a lipase gene in a microorganism such as a microalgae contains a gene encoding a lipase in operable linkage with a promoter active in microalgae. Alternatively, if the vector does not contain a promoter in operable linkage with the gene of interest, the gene 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). The vector can also contain a second gene that encodes a protein that, e.g., imparts resistance to an antibiotic or herbicide, i.e., a selectable marker. Optionally, one or both gene(s) is/are

followed by a 3' untranslated sequence containing a polyadenylation signal. Expression cassettes encoding the two genes can be physically linked in the vector or on separate vectors. Co-transformation of microalgae can also be used, in which distinct vector molecules are simultaneously used to transform cells (see for example Protist 2004 Dec;155(4):381-93). The transformed cells can be optionally selected based upon the ability to grow in the presence of the antibiotic or other selectable marker under conditions in which cells lacking the resistance cassette would not grow.

[0423] All references cited herein, including patents, patent applications, and publications, are hereby incorporated by reference in their entireties, whether previously specifically incorporated or not. The publications mentioned herein are cited for the purpose of describing and disclosing reagents, methodologies and concepts that may be used in connection with the present invention. Nothing herein is to be construed as an admission that these references are prior art in relation to the inventions described herein.

[0424] 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.

X. EXAMPLES

[0425] The following examples are offered to illustrate, but not to limit, the claimed invention.

EXAMPLE 1

[0426] *Chlorella* strains from the University of Texas culture collection were tested for growth on glycerol and glucose. The following *Chlorella* species and strains were cultured: *Chlorella kessleri* (strains 263, 397, 398, 2228); *Chlorella sorokiniana* (strains 1663, 1665, 1669, 1671, 1810); *Chlorella saccharophila* (2911; 2469); *Chlorella protothecoides* (31, 249, 250, 264). Each strain was inoculated from solid media into 25 ml liquid base media (2g/L yeast extract, 2.94mM $NaNO_3$, 0.17mM $CaCl_2 \cdot 2H_2O$, 0.3mM $MgSO_4 \cdot 7H_2O$, 0.4mM K_2HPO_4, 1.28mM KH_2PO_4, 0.43mM NaCl) and grown shaking at 27°C for 72 hours under a light intensity of 75$\mu Em^{-2}s^{-1}$. These cultures were used to inoculate each strain to a final density of $1x10^5$ cells per ml into 24-well plates containing 2ml of (a) base media only; (b)

base media plus 0.1% glucose; and (c) base media plus 0.5% reagent grade glycerol (EM Science, catalog #GX0185-6). Plates were placed in the dark and grown for 72 hours shaking at 27C. Samples of each strain grown in the three conditions were diluted 1.9:1 in distilled H2O and absorbance was read at 600nm in a Molecular Devices SpectraMax 340PC. All strains exhibited growth in the presence of glucose and glycerol compared to only base media.

EXAMPLE 2

[0427] Strains and Media: *Chlorella protothecoides* #1 (STRAIN 250), #2 (STRAIN 264) and *Chlorella kessleri* #1 (STRAIN 398) were obtained from the Culture Collection of Algae at the University of Texas (Austin, TX, USA). The stock cultures were maintained on modified Proteose medium. Modified Proteose medium consisted (g/L) of 0.25 g $NaNO_3$, 0.09g K_2HPO_4, 0.175 g KH_2PO_4 0.025 g, 0.025 g $CaCl_2 \cdot 2H_2O$, 0.075 g $MgSO_4 \cdot 7H_2O$, and 2 g yeast extract per liter. Glycerol wastes from biodiesel production (acidulated glycerol (AG) and non-acidulated glycerol (NAG)) were obtained from Imperial Western Products (Selma, CA, USA). "Pure" or "reagent grade" glycerol was from EM Science (a division of Merck KGA), catalog #GX0185-6.

[0428] Experimental design and Growth Measurement: For each strain, 1 ml of following different media was prepared in 24-well plates.

1. Proteose + 1 % pure glycerol
2. Proteose + 1% acidulated glycerol
3. Proteose + 1% non-acidulated glycerol
4. Proteose + 1 % pure glycerol + 1 % glucose (added after 72 hr)
5. Proteose + 1% acidulated glycerol + 1 % glucose (added after 72 hr)
6. Proteose + 1% non-acidulated glycerol + 1 % glucose (added after 72 hr)

[0429] Each strain was inoculated to different media to 5 x 10^5 cells/ml concentration. The cultures were kept in dark and were agitated by orbital shaker from Labnet (Berkshire, UK) at 430 rpm. After 72 hr of initial growth, 1% (w/v) glucose was added to samples #4, 5, and 6 and cultured another 24 hr. To measure dry cell weight, 1 ml of each culture was pelleted by centrifugation at 5,000 rpm for 5 min in an Eppendorf 5415C centrifuge. After removing supernatant, cell pellets were frozen at -80 °C and lyophilized in a lab scale freeze dryer (Labconco, MO, USA). Results are shown in Figure 1.

EXAMPLE 3

[0430] Strains and Media: *Chlorella protothecoides* #1 (STRAIN 250), #3 (STRAIN 249) and *Chlorella kessleri* #2 (strain 397) were obtained from the Culture Collection of Algae at the University of Texas (Austin, TX, USA). The stock cultures were maintained on modified Proteose medium (see EXAMPLE 2).

[0431] Experimental design and Growth Measurement: For each strain, 1 ml of following different media was prepared in 24-well plates.

1. Proteose + 1 % pure glycerol +1 % glucose
2. Proteose + 1% acidulated glycerol +1 % glucose
3. Proteose + 1% non-acidulated glycerol +1 % glucose

[0432] Each strain was inoculated to different media to 5 x 10^5 cells/ml concentration. The cultures were kept in dark and agitated by orbital shaker from Labnet (Berkshire, UK) at 430 rpm. After 96 hr, cell growth was measured for dry cell weight (see EXAMPLE 2). Results are shown in Figure 2.

EXAMPLE 4

[0433] Strains and Media: *Chlorella protothecoides* #3 (STRAIN 249), #4 (STRAIN 31), and *Chlorella kessleri* #2 (STRAIN 397) were obtained from the Culture Collection of Alga at the University of Texas (Austin, TX, USA). The stock cultures were maintained on modified Proteose medium (see EXAMPLE 2)

[0434] Experimental design and lipid assay: For each strain, 1 ml of following different media was prepared in 24-well plates.

1. Proteose + 1 % pure glycerol +1 % glucose
2. Proteose + 1% acidulated glycerol +1 % glucose
3. Proteose + 1% non-acidulated glycerol +1 % glucose

[0435] Each strain was inoculated to media containing different glycerols (pure, acidulated, or non-acidulated) to 5 x 10^5 cells/ml concentration. The cultures were kept in dark and agitated by orbital shaker from Labnet (Berkshire, UK) at 430 rpm. After 96 hr, lipid contents were measured. To measure the amount of lipid content in cells, 100 µl of cultures were collected and washed once with same volume of media. To each tube, 5 µl of washed cells and 200 µl of sulfuric acid 18 M were added. The tubes were incubated at 90°C in a water bath for 30 min, and 1 ml of phosphoric acid–vanillin reagent was added to the tubes and incubated at 37°C for 15 min. To prepare the phosphoric acid–vanillin reagent, 0.12 g of

vanillin was added to 20 ml of water, and the volume adjusted to 100 ml with 85% phosphoric acid. The optical density at 530 nm was read in a glass cuvette against a reference tube with 5 µl water as sample. Results are shown in Figure 3.

EXAMPLE 5

[0436] Strains and Media: *Chlorella protothecoides* #2 (STRAIN 264) and *Chlorella kessleri* #1 (STRAIN 398) were obtained from the Culture Collection of Alga at the University of Texas (Austin, TX, USA). The stock cultures were maintained on modified Proteose medium (see EXAMPLE 2).

[0437] Experimental design and lipid assay: For each strain, 1 ml of the following different media was prepared in 24-well plates.

1. Proteose + 1 % pure glycerol
2. Proteose + 1 % non-acidulated glycerol
3. Proteose + 1 % pure glycerol + 1 % glucose (added after 72 hr)
4. Proteose + 1 % non-acidulated glycerol + 1 % glucose (added after 72 hr)

[0438] Each strain was inoculated to media containing different glycerols (pure or non-acidulated) to 5 x 10^5 cells/ml concentration. The cultures were kept in dark and agitated by orbital shaker from Labnet (Berkshire, UK) at 430 rpm. After 72 hr of initial growth, 1% glucose was added to sample #3 and #4 and cultured another 24 hr. Lipid contents were measured in all samples (see EXAMPLE 4). The optical density at 600 nm was also measured to check for non-specific absorbance and subtracted from O.D. 530 nm to calculate the amount of lipid. The reference curve is composed of Triolein dissolved in chloroform ranging from 1 to 10 µg. Results are shown in Figure 4.

EXAMPLE 6

[0439] Strains and Media: *Chlorella protothecoides* #3 (STRAIN 249) and *Chlorella kessleri* #2 (STRAIN 397) were obtained from the Culture Collection of Alga at the University of Texas (Austin, TX, USA). The stock cultures were maintained on modified Proteose medium (see EXAMPLE 2).

[0440] Experimental design and lipid assay: For each strain, 1 ml of following different media was prepared in 24-well plates.

1. Proteose + 1 % pure glycerol + 1 % glucose (added after 72 hr)
2. Proteose + 1 % acidulated glycerol + 1 % glucose (added after 72 hr)
3. Proteose + 1 % non-acidulated glycerol + 1 % glucose (added after 72 hr)

[0441] Each strain was inoculated to media containing different glycerols (pure, acidulated, or non-acidulated) to 5 x 10^5 cells/ml concentration. The cultures were kept in dark and agitated by orbital shaker from Labnet (Berkshire, UK) at 430 rpm. After 72 hr of initial growth, 1% glucose was added and cultured another 24 hr. Dried cell-weight and lipid content were measured in all samples (see EXAMPLES 2 and 5). The lipid percentage was calculated from total lipid amount divided by dried cell weight. Results are shown in Figure 5.

EXAMPLE 7

[0442] Strains and Media: *Chlorella protothecoides* #2 (STRAIN 264) and *Chlorella kessleri* #1 (STRAIN 398) were obtained from the Culture Collection of Alga at the University of Texas (Austin, TX, USA). The stock cultures were maintained on modified Proteose medium (see EXAMPLE 2).

[0443] Experimental design and lipid assay: For each strain, 1 ml of following different media was prepared in 24-well plates.

1. Proteose + 1 % pure glycerol + 1 % glucose (added after 72 hr)
2. Proteose + 1 % non-acidulated glycerol + 1 % glucose (added after 72 hr)

[0444] Each strain was inoculated media containing either 1 % pure or 1 % non-acidulated glycerol to 5 x 10^5 cells/ml concentration. The cultures were kept in dark and agitated by orbital shaker from Labnet (Berkshire, UK) at 430 rpm. After 72 hr of initial growth, 1% glucose was added and cultured another 24 hr. Dried cell-weight and lipid content were measured in all samples (see EXAMPLE 1 and 4). The lipid percentage was calculated from total lipid amount divided by dried cell weight. Results are shown in Figure 6.

EXAMPLE 8

[0445] Strains and Media: *Chlorella protothecoides* #1 (STRAIN 250), #4 (STRAIN 31) and *Chlorella kessleri* #2 (STRAIN 397) were obtained from the Culture Collection of Alga at the University of Texas (Austin, TX, USA). The stock cultures were maintained on modified Proteose medium (see EXAMPLE 2)

[0446] Experimental design and lipid assay: For each strain, 1 ml of following different media was prepared in 24-well plates.

1. Proteose + 2 % glucose
2. Proteose + 1 % glycerol + 1 % glucose

[0447] Each strain was inoculated to different media to 5 x 10^5 cells/ml concentration. The cultures were kept in dark and agitated by orbital shaker from Labnet (Berkshire, UK) at 430 rpm. After 96 hr of initial growth, lipid contents were measured (see EXAMPLE 5). Results are shown in Figure 7.

EXAMPLE 9

[0448] Strains and Media: *Chlorella protothecoides* #3 (STRAIN 249), #4 (STRAIN 31) and *Chlorella kessleri* #1 (STRAIN 398) were obtained from the Culture Collection of Alga at the University of Texas (Austin, TX, USA). The stock cultures were maintained on modified Proteose medium (see EXAMPLE 2).

[0449] Experimental design and lipid assay: For each strain, 1 ml of following different media was prepared in 24-well plates.

1. Proteose + 2% glucose
2. Proteose + 1% glycerol + 1% glucose
3. Proteose + 1% glycerol + 1% glucose (added after 72 hr)

[0450] Each strain was inoculated to different media to 5 x 10^5 cells/ml concentration. The cultures were kept in dark and agitated by orbital shaker from Labnet (Berkshire, UK) at 430 rpm. After 72 hr of initial growth, 1% (w/v) glucose was added to #3 media and cultured another 24 hr. Dried cell-weight and lipid contents were measured in all samples (see EXAMPLES 2 and 5). The lipid percentage was calculated from total lipid amount divided by dried cell weight. Results are shown in Figure 8.

EXAMPLE 10

[0451] Strains and Media: *Chlorella protothecoides* #1 (STRAIN 250), #3 (STRAIN 249), and *Chlorella kessleri* #2 (STRAIN 397) were obtained from the Culture Collection of Alga at the University of Texas (Austin, TX, USA). The stock cultures were maintained on modified Proteose medium (see EXAMPLE 2).

[0452] Experimental design and lipid assay: For each strain, 1 ml of following different media was prepared in 24-well plates.

1. Proteose + 1% pure glycerol + 1% glucose
2. Proteose + 1% pure glycerol + 1% glucose (added after 72 hr)
3. Proteose + 1% acidulated glycerol + 1% glucose
4. Proteose + 1% acidulated glycerol + 1% glucose (added after 72 hr)
5. Proteose + 1% non-acidulated glycerol + 1% glucose

6. Proteose + 1% non-acidulated glycerol + 1% glucose (added after 72 hr)

[0453] Each strain was inoculated to different media to 5 x 10^5 cells/ml concentration. The cultures were kept in dark and agitated by orbital shaker from Labnet (Berkshire, UK) at 430 rpm. After 72 hr of initial growth, 1% (w/v) glucose was added to #2, #4, and #6 media and cultured another 24 hr. Lipid contents were measured in all samples (see EXAMPLE 4). Results are shown in Figure 9.

EXAMPLE 11

[0454] Strains and Media: *Chlorella protothecoides* #1 (STRAIN 250), #3 (STRAIN 249), #4 (STRAIN 31) and *Chlorella kessleri* #2 (STRAIN 397) were obtained from the Culture Collection of Alga at the University of Texas (Austin, TX, USA). The stock cultures were maintained on modified Proteose medium (see EXAMPLE 2).

[0455] Experimental design and lipid assay: For each strain, 1 ml of following different media was prepared in 24-well plates.

1. Proteose + 1% pure glycerol + 1% glucose
2. Proteose + 1% pure glycerol + 1% glucose (added after 72 hr)
3. Proteose + 1% acidulated glycerol + 1% glucose
4. Proteose + 1% acidulated glycerol + 1% glucose (added after 72 hr)
5. Proteose + 1% non acidulated glycerol + 1% glucose
6. Proteose + 1% non acidulated glycerol + 1% glucose (added after 72 hr)

[0456] Each strain was inoculated to different media to 5 x 10^5 cells/ml concentration. The cultures were kept in dark and agitated by orbital shaker from Labnet (Berkshire, UK) at 430 rpm. After 72 hr of initial growth, 1% (w/v) glucose was added to #2, #4, and #6 media and cultured another 24 hr. Dried cell-weight was measured in all samples (see EXAMPLE 2). Results are shown in Figure 10.

EXAMPLE 12

Vector construction

[0457] A BamHI-SacII fragment containing the CMV promoter, a hygromycin resistance cDNA, and a CMV 3' UTR (SEQ ID NO:5, a subsequence of the pCAMBIA1380 vector, Cambia, Canberra, Australia) was cloned into the BamHI and SacII sites of pBluescript and is referred to herein as pHyg.

Biolistic transformation of Chlorella

[0458] S550d gold carriers from Seashell Technology were prepared according to the protocol from manufacturer. Linearized pHyg 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.

[0459] *Chlorella protothecoides* culture (Univeristy of Texas Culture Collection 250) was grown in proteose medium (2g/L yeast extract, 2.94mM NaNO3, 0.17mM CaCl2•2H2O, 0.3mM MgSO4•7H2O, 0.4mM K2HPO4, 1.28mM KH2PO4, 0.43mM NaCl) on a gyratory shaker under continuous light at 75 μmol photons m^{-2} sec^{-1} 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 (1100 and 1350 psi) were used, and the plates were placed 9 and 12 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 hygromycin contained plates (200 μg/ml). After 7-10 days of incubation at 25°C, colonies representing transformed cells were visible on the plates from 1100 and 1350 psi rupture discs and from 9 and 12 cm distances. Colonies were picked and spotted on selective agar plates for a second round of selection.

Transformation of *Chlorella* by Electroporation

[0460] *Chlorella protothecoides* culture was grown in proteose medium on a gyratory shaker under continuous light at 75 μmol photons m^{-2} sec^{-1} until it reached a cell density of of $2x10^6$cells/ml. The cells were harvested, washed once with sterile distilled water, and resuspended in a tris-phosphate buffer (20m M Tris-HCl, pH 7.0; 1 mM potassium phosphate) containing 50 mM sucrose to a density of $4x10^8$cells/ml. About 250 μl cell suspension ($1x10^8$cells) was placed in a disposable electroporation cuvette of 4 mm gap. To the cell suspension, 5 μg of linearized pHyg plasmid DNA and 200 μg of carrier DNA

(sheared salmon sperm DNA) was added. The electroporation cuvette was then incubated in a water bath at 16°C for 10 minutes. An electrical pulse (1100 V/cm) was then applied to the cuvette at a capacitance of 25 μF (no shunt resistor was used for the electroporation) using a Gene Pulser II (Bio-Rad Labs, Hercules, CA) electroporation apparatus. The cuvette was then incubated at room temperature for 5 minutes, following which the cell suspension was transferred to 50 ml of proteose media, and shaken on a gyratory shaker for 2 days. Following recovery, the cells were harvested by centrifugation at low speed, resuspended in proteose media, and plated at low density on plates supplemented with 200 μg/ ml hygromycin. The plates were incubated under continuous light at 75 μmol photons m^{-2} sec^{-1}. Transformants appeared as colonies in 1 – 2 weeks. Colonies were picked and spotted on selective agar plates for a second round of selection.

Genotyping

[0461] A subset of colonies that survived a second round of selection were cultured in small volume and harvested. Pellets of approximately 5-10 uL volume were resuspended in 50 uL of 10mM NaEDTA by vortexing and then incubated at 100°C for 10. The tubes were then vortexed briefly and sonicated for 10 seconds, then centifuged at 12,000 x g for 1 minute. 2 uL of supernatant as template was used in a 50 uL PCR reaction. Primers used for genotyping were SEQ ID NO:6 and SEQ ID NO:7. PCR conditions were as follows: 95°C 5 min x 1 cycle; 95°C 30 sec - 58°C 30 sec - 72°C 1 min 30 sec x 35 cycles; 72°C 10 min x 1 cycle. The expected 992 bp fragment was found in 6 of 10 colonies from the biolistic method and from a single electroporation colony. A lower sized, nonspecific band was present in all lanes. Results are shown in Figure 16. To confirm the identity of the amplified 992bp fragment, two biolistic bands and the electroporation band were excised from the gel and individually sequenced. The sequence of all three bands corresponded to the expected 992 bp fragment. (DNA ladder: Bionexus® All Purpose Hi-Lo® DNA ladder catalog # BN2050).

EXAMPLE 13

[0462] Strains and Media: (a) *Spirulina platensis* (UTEX 2340) and (b) *Navicula pelliculosa* (UTEX 667) were obtained from the Culture Collection of Algae at the University of Texas (Austin, TX, USA). The stock culture of *Spirulina* was maintained in Spirulina medium and *Navicula* was maintained in soil extract medium (SEM). Spirulina medium consisted of 162 mM $NaHCO_3$, 38 mM Na_2CO_3, 1.9 mM K_2HPO_4, 29 mM $NaNO_3$, 5.75 mM K_2SO_4, 17.1 mM NaCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 0.25 mM $CaCl_2 \cdot 2H_2O$, 2 mM Na_2EDTA, 0.36 mM $FeCl_3 \cdot 6H_2O$, 0.21 mM $MnCl_2 \cdot 4H_2O$, 0.037 mM $ZnCl_2$, 0.0085 mM $CoCl_2 \cdot 6H_2O$, 0.017

mM $NaMoO_4 \cdot 2H_2O$, 0.78 μM $CuSO_4 \cdot 5H_2O$, 0.15 μM $ZnSO_4 \cdot 7H_2O$, 10 μM H_3BO_3, and 0.001 mM Vitamin B_{12}. Soil extract medium consisted of 2.94 mM $NaNO_3$, 0.17 mM$CaCl_2 \cdot 2H_2O$, 0.3 mM $MgSO_4 \cdot 7H_2O$, 0.43 mM K_2HPO_4, 1.29 mM KH_2PO_4, 0.43 mM NaCl, and soil extract. Glycerol wastes from biodiesel production (acidulated glycerol (AG) and non-acidulated glycerol (NAG)) were obtained from Imperial Western Products (Selma, CA, USA).

[0463] Experimental Design and Growth Measurement: For each strain, 1 ml of following different media was prepared in 24-well plates.

(a)

7. Spirulina medium + 2% glucose
8. Spirulina medium + 2% reagent grade glycerol
9. Spirulina medium + 2% non-acidulated glycerol
10. Spirulina medium + 1% non-acidulated glycerol + 1 % glucose

(b)

1. SEM + 2% glucose
2. SEM + 2% reagent grade glycerol
3. SEM + 1% reagent grade glycerol + 1 % glucose
4. SEM + 2% acidulated glycerol
5. SEM + 1% acidulated glycerol + 1 % glucose
6. SEM + 2% non-acidulated glycerol
7. SEM + 1% non-acidulated glycerol + 1 % glucose

[0464] Each strain was inoculated to different media to 5 x 10^5 cells/ml concentration. The cultures were kept in dark and agitated by orbital shaker from Labnet (Berkshire, UK) at 430 rpm. After 96 hr, lipid contents were measured. To measure the amount of lipid content in cells, 100 μl of cultures were collected and washed once with same volume of media. To each tube, 5 μl of washed cells and 200 μl of sulfuric acid 18 M were added. The tubes were incubated at 90 °C water bath for 30 min, and 1 ml of phosphoric acid–vanillin reagent were added to the tubes and incubated at 37 °C for 15 min. To prepare the phosphoric acid–vanillin reagent, 0.12 g of vanillin was added to 20 ml of water, and the volume adjusted to 100 ml with 85% phosphoric acid. The optical density at 530 nm was read in a glass cuvette against a reference tube with 5 μl water as sample. The reference curve is composed of Triolein dissolved in chloroform ranging from 1 to 10 μg.

[0465] To measure dried cell-weight, 0.5 ml of each culture was pelleted by centrifugation at 5000 rpm for 5 min. After removing supernatant, cell pellets were frozen at -80 °C and dried overnight in a Freeze Dry system (Labconco, MO, USA). The lipid percentage was calculated from total lipid amount divided by dried cell weight. Results are shown in Figure 11.

EXAMPLE 14

[0466] Strains and Media: *Scenedesmus armatus* (UTEX 2552) was obtained from the Culture Collection of Algae at the University of Texas (Austin, TX, USA). The stock cultures were maintained on modified Proteose medium. Modified Proteose medium consisted (g/L) of 0.25 g $NaNO_3$, 0.09g K_2HPO_4, 0.175 g KH_2PO_4 0.025 g, 0.025 g $CaCl_2 \cdot 2H_2O$, 0.075 g $MgSO_4 \cdot 7H_2O$, and 2 g yeast extract per liter.

[0467] Experimental design and Growth and lipid measurement: For each growth condition, 1 ml of following different media was prepared in 24-well plates.

(a), (b)

1. Proteose + 2% glucose
2. Proteose + 2% glycerol
3. Proteose + 2% acidulated glycerol
4. Proteose + 2% non-acidulated glycerol
5. Proteose + 1% non-acidulated glycerol + 1 % glucose

[0468] *Scenedesmus armatus* (UTEX 2552) was inoculated to different media to 5×10^5 cells/ml concentration. The cultures were kept in dark and agitated by orbital shaker from Labnet (Berkshire, UK) at 430 rpm. After 96 hr, cell growth was measured by dried cell-weight, and lipid content was measured by phosphor-vanillin assay. (see EXAMPLE 13). The lipid percentage was calculated from total lipid amount divided by dried cell weight. Results are shown in Figure 12.

EXAMPLE 15

[0469] Strains and Media: *Navicula pelliculosa* (UTEX 667) was obtained from the Culture Collection of Algae at the University of Texas (Austin, TX, USA). The stock cultures were maintained on soil extract medium (see EXAMPLE 13)

[0470] Experimental design and growth measurement: For each growth condition, 1 ml of following different media was prepared in 24-well plates.

1. SEM + 2% glucose
2. SEM + 2% glycerol
3. SEM + 2% acidulated glycerol
4. SEM + 1% acidulated glycerol + 1 % glucose
5. SEM + 2% non-acidulated glycerol
6. SEM + 1% non-acidulated glycerol + 1 % glucose

Navicula pelliculosa (UTEX 667) was inoculated to media containing glucose or different glycerols (pure, acidulated, or non-acidulated) to 5 x 10^5 cells/ml concentration. The cultures were kept in dark and agitated by orbital shaker from Labnet (Berkshire, UK) at 430 rpm. After 96 hr, cell growth was measured by dried cell-weight (see EXAMPLE 13). Results are shown in figure 13.

EXAMPLE 16

[0471] Strains and Media: *Scenedesmus armatus* (UTEX 2552) and *Navicula pelliculosa* (UTEX 667) were obtained from the Culture Collection of Algae at the University of Texas (Austin, TX, USA). The stock cultures were maintained on modified Proteose medium for *Scenedesmus armatus* and soil extract medium for *Navicula pelliculosa* (see EXAMPLE 1).

[0472] Experimental design and growth measurement: For each strain, 1 ml of following different media was prepared in 24-well plates.

Scenedesmus armatus

5. Proteose + 1 % acidulated glycerol + 1 % glucose
6. Proteose + 1 % acidulated glycerol + 1 % glucose (added after 72 hr)

Navicula pelliculosa

1. SEM + 1 % acidulated glycerol + 1 % glucose
2. SEM + 1 % acidulated glycerol + 1 % glucose (added after 72 hr)

[0473] Each strain was inoculated to media to 5 x 10^5 cells/ml concentration. The cultures were kept in dark and agitated by orbital shaker from Labnet (Berkshire, UK) at 430 rpm. After 72 hr of initial growth, 1% glucose was added to sample #2 and cultured another 24 hr. Cell growth was measured by dried cell-weight (see EXAMPLE 13). Results are shown in figure 14.

EXAMPLE 17

[0474] Strains and Media: *Chlorella protothecoides* (UTEX 31) was obtained from the Culture Collection of Algae at the University of Texas (Austin, TX, USA). The stock cultures were maintained on modified Proteose medium (see EXAMPLE 1)

[0475] Experimental design: For each condition, 1 ml of following different media was prepared in 24-well plates.

4. Proteose
5. Proteose + 0.5 % glucose
6. Proteose + 0.5 % xylose
7. Proteose + 0.25 % glucose + 0.25 % xylose

[0476] *Chlorella protothecoides #4* (UTEX 31) was inoculated to media containing different sugars (glucose, or xylose) to 3 x 10^5 cells/ml concentration. The cultures were kept in dark and agitated by orbital shaker from Labnet (Berkshire, UK) at 430 rpm. After 72 hr of growth, cell growth was measured by counting cell numbers of each culture. Results are shown in figure 15.

EXAMPLE 18

[0477] *Chlorella protothecoides* strains #1, #3, and #4 were obtained from the Culture Collection of Algae at the University of Texas (Austin, TX, USA). The stock cultures were maintained on modified Proteose medium (see EXAMPLE 1). For each condition, 1 ml of following different media was prepared in 24-well plates.

1. Proteose
2. Proteose + 1 % glucose
3. Proteose + 1 % fructose

[0478] Each strain was inoculated to media containing different sugars (glucose, or fructose) to 1 x 10^6 cells/ml concentration. The cultures were kept in dark and agitated by orbital shaker from Labnet (Berkshire, UK) at 430 rpm. After 96 hr of growth, cell density was measured by counting cell numbers of each culture. Results are shown in figure 20.

EXAMPLE 19

Chlorella on Sucrose

[0479] Materials and Methods: *Chlorella protothecoides* (UTEX 249) was inoculated into three 50ml flasks of Proteose media with 1% sucrose (2.94mM $NaNO_3$, 0.428mM K_2HPO_4, 1.28mM KH_2PO_4, 0.427mM NaCl, 0.17mM $CaCl_2$-$2H_2O$, 0.3mM $MgSO_4$-$7H_2O$, proteose peptone 1g/L) to a final cell density of 4x10^5 cells per ml. Invertase (Sigma #I4504) was

added to two of the cultures at 0.01U/ml and 0.05U/ml. All three cultures were grown in the dark for ~60hrs shaking at 150rpm.

[0480] Results: Final cell counts were performed on all three cultures after ~60hrs of shaking in the dark. The control flask reached $4.4x10^5$ cells per ml while the 0.01U/ml and 0.05U/ml flasks reached cell densities of $1x10^8$ and $3x10^8$ respectively. Each flask was checked for contamination at the end of the experiment by microscopic analysis and all were clean.

EXAMPLE 20

Chlorella strains growing on sucrose

[0481] Cultures of *Chlorella kessleri* ((a) UTEX 397 and (b) UTEX 398) and *Chlorella fusca* ((a) UTEX 251 and (b) UTEX 1801) were inoculated from autotrophic liquid cultures into 10ml of Proteose + 1% sucrose media in 50 ml flasks at 1 x 10^6 cells/ml. Control cultures were also inoculated at the same density with only Proteose media. Cultures were grown at 28°C in the dark shaking at 250 rpm for 7 days, at which point cell density was measured by hemocytometer. As shown in Figures 21-22, all four strains grew on sucrose compared to the initial cell density and the proteose-only control.

EXAMPLE 21

Chlorella protothecoides Growth on Molasses with a Sucrose Invertase

[0482] Preparation of *Chlorella* cells for Inoculation: A 10 ml liquid culture of *Chlorella* was started taking the inoculum from a solid Proteose plate. The cultures were grown in light for approximately 2 days at 26°C. Growth was measured using an optical densitomer (OD) at 750 nm and by determining dry cell weights.

[0483] Preparation of Molasses and Sugar Stock Solutions: A 5% stock solution was prepared with glucose, sucrose and three different molasses samples (labeled BS1, BS2 and HTM) obtainted from the commercial processing of sugarcane into sugar, as shown in the following Table 13. The pH of all stocks was verified to be in the range of 6-6.6, and the stocks were then autoclaved.

[0484] Table 13. Molasses and sugar solutions.

Molasses	% Sugar	5% sugar dil.in 100mls
		grams or mls
HTM	78.72	6.4
BS1 (FL)	44.25	11.3

BS2 (AU)	51.55	9.7
Sucrose	100	5
Glucose	100	5

[0485] Preparation of Invertase Solution: A 40 units/ml stock solution of invertase was prepared by reconstituting 1 mg of a 400 unit/mg Invertase (Sigma) in 10 milliliters of distilled water.

[0486] Experimental Conditions and Setup: 10 ml cultures were prepared, each consisting of 1% final molasses/sugar concentration, 0.05 units/ml Invertase, and 1.0x 10^6 cells per ml of *Chlorella protothecoides* in a base Protease media. The cultures were numbered as follows: (1) media only control; (2) 1% HTM; (3) 1% BS1; (4) 1% BS2; (5) 1% glucose; and (6) 1% sucrose. A similar control set was also prepared without the addition of Invertase. The cultures were grown in darkness for five days shaking at 250 rpm at 28°C.

[0487] Results: Growth of the *Chlorella protothecoides* cells was evaluated following the five days of incubation on the respective feedstock in darkness. As shown in Figures 23-24, the cells can be grown on molasses in the presence of a sucrose invertase with yields comparable to that of growth on pure reagent-grade glucose.

EXAMPLE 22

Genetic Engineering of *Chlorella protothecoides* to Express an Exogenous Sucrose Invertase

[0488] Strains and Media: *Chlorella protothecoides* (UTEX 250) was obtained from the Culture Collection of Alga at the University of Texas (Austin, TX, USA). The stock cultures were maintained on modified Proteose medium. Modified Proteose medium consists of 0.25 g $NaNO_3$, 0.09 g K_2HPO_4, 0.175 g KH_2PO_4 0.025 g, 0.025 g $CaCl_2 \cdot 2H_2O$, 0.075 g $MgSO_4 \cdot 7H_2O$, and 2 g yeast extract per liter (g/L).

[0489] Plasmid Construction: To express the secreted form of invertase in *Chlorella protothecoides*, a *Saccharomyces cerevisiae SUC2* gene was placed under the control of three different promoters: Cauliflower mosaic virus 35S promoter (CMV), Chlorella virus promoter (NC-1A), and Chlorella HUP1 promoter. A yeast SUC2 gene was synthesized to accomodate codon usage optimized for *C. protothecoides* and includes a signal sequence required for directing extracellular secretion of invertase. Each construct was built in pBluescript KS+, and EcoRI/AscI, AscI/XhoI, and XhoI/BamHI sites were introduced to each promoter, invertase gene, and CMV 3'UTR, respectively, by PCR ampilication using

specific primers. Purified PCR products were cloned sequentially. An illustration of the final constructs is shown in Figure 25.

[0490] Transformation of *Chlorella protothecoides*: A *Chlorella protothecoides* culture was grown in modified Proteose medium on a gyratory shaker under continuous light at 75 μmol photons m^{-2} sec^{-1} till it reached a cell density of of $6x10^6$cells/ml.

[0491] For biolistic transformation, S550d gold carriers from Seashell Technology were prepared according to the protocol from the manufacturer. Briefly, a linearized construct (20 μg) by BsaI was mixed with 50 μl of binding buffer and 60 μl (3 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 mild vortexing, DNA-coated particles were pelleted by spinning at 10,000 rpm in an Eppendorf 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. The cells were harvested, washed once with sterile distilled water, resuspended in 50 μl of medium (1×10^7 cells), and were spread in the center third of a non-selective Proteous plate. The cells were bombarded with the PDS-1000/He Biolistic Particle Delivery system (Bio-Rad). Rupture disks (1100 and 1350 psi) were used, and the plates were placed 9-12 cm below the screen/macrocarrier assembly. The cells were allowed to recover at 25°C for 12–24 hours. Upon recovery, the cells were scraped from the plates with a rubber spatula, mixed with 100 μl of medium and spread on modified Proteose plates with 1% sucrose. After 7-10 days of incubation at 25°C in the dark, colonies representing transformed cells were visible on the plates.

[0492] For transformation with electroporation, cells were harvested, washed once with sterile distilled water, and resuspended in a Tris- phosphate buffer (20m M Tris-HCl, pH 7.0; 1 mM potassium phosphate) containing 50 mM sucrose to a density of $4x10^8$cells/ml. About 250 μl cell suspension ($1x10^8$cells) was placed in a disposable electroporation cuvette of 4 mm gap. To the cell suspension, 5 μg of linearized plasmid DNA and 200 μg of carrier DNA (sheared salmon sperm DNA) were added. The electroporation cuvette was then incubated in an ice water bath at 16 °C for 10 min. An electrical pulse (1100 V/cm) was then applied to the cuvette at a capacitance of 25 μF (no shunt resistor was used for the electroporation) using a Gene Pulser II (Bio-Rad Labs, Hercules, CA) electroporation apparatus. The cuvette

was then incubated at room temperature for 5 minutes, following which the cell suspension was transferred to 50 ml of modified Proteose media, and shaken on a gyratory shaker for 2 days. Following recovery, the cells were harvested at low speed (4000 rpm), resuspended in modified Proteose media, and plated out at low density on modified Proteose plates with 1% sucrose. After 7-10 days of incubation at 25°C in the dark, colonies representing transformed cells were visible on the plates.

[0493] Screening Transformants and Genotyping: The colonies were picked from dark grown-modified Proteose plates with 1% sucrose, and approximately the same amount of cells were transferred to 24 well-plates containing 1 ml of modified Proteose liquid media with 1% sucrose. The cultures were kept in dark and agitated by orbital shaker from Labnet (Berkshire, UK) at 430 rpm for 5 days.

[0494] To verify the presence of the invertase gene introduced in Chlorella transformants, DNA of each transformant was isolated and amplified with a set of gene-specific primers (CMV construct: forward primer (CAACCACGTCTTCAAAGCAA) (SEQ ID NO:6)/ reverse primer (TCCGGTGTGTTGTAAGTCCA) (SEQ ID NO:9), CV constructs: forward primer (TTGTCGGAATGTCATATCAA) (SEQ ID NO:10)/ reverse primer (TCCGGTGTGTTGTAAGTCCA) (SEQ ID NO:11), and HUP1 construct: forward primer (AACGCCTTTGTACAACTGCA) (SEQ ID NO:12)/ reverse primer (TCCGGTGTGTTGTAAGTCCA) (SEQ ID NO:13)). For quick DNA isolation, a volume of cells (approximately 5-10 uL in size) were resuspended in 50 uL of 10 mM Na-EDTA. The cell suspension was incubated at 100°C for 10 min and sonicated for 10 sec. After centrifugation at 12000g for 1 min, 3 uL of supernatant was used for the PCR reaction. PCR amplification was performed in the DNA thermal cycler (Perkin-Elmer GeneAmp 9600). The reaction mixture (50 uL) contained 3 uL extracted DNA, 100 pmol each of the respective primers described above, 200 uM dNTP, 0.5 units of Taq DNA polymerase (NEB), and Taq DNA polymerase buffer according to the manufacturer's instructions. Denaturation of DNA was carried out at 95°C for 5 min for the first cycle, and then for 30 sec. Primer annealing and extension reactions were carried out at 58°C for 30 sec and 72°C for 1 min respectively. The PCR products were then visualized on 1 % agarose gels stained with ethidium bromide. Figure 26 shows the PCR genotype results of *C. protothecoides* transformants using the gene-specific primers identified above. Arrows show the expected size of the PCR product, and stars represent DNA samples from each transformant showing the PCR product matched to the expected size (V: Vector only, WT: wild-type).

[0495] Growth in Liquid Culture: After five days growth in darkness, the genotype-positive transformants showed growth on minimal liquid Proteose media + 1% sucrose in darkness, while wild-type cells showed no growth in the same media in darkness.

EXAMPLE 23

Transformation of algal strains with a secreted invertase derived from *S. cerevisiae*

[0496] Secreted Invertase: A gene encoding a secreted sucrose invertase (Gen Bank Accession no. NP_012104 from *Saccharomyces cerevisiae*) was synthesized de-novo as a 1599 bp Asc I-Xho fragment that was subsequently sub-cloned into a pUC19 derivative possessing the Cauliflower Mosaic Virus 35s promoter and 3' UTR as EcoR I/Asc I and Xho/Sac I cassettes, respectively.

[0497] Growth of Algal Cells: Media used in these experiments was liquid base media (see Example 1) and solid base media (+ 1.5% agarose) containing fixed carbon in the form of sucrose or glucose (as designated) at 1% final concentration. The strains used in this experiment did not grow in the dark on base media in the absence of an additional fixed carbon source. Species were struck out on plates, and grown in the dark at 28°C. Single colonies were picked and used to inoculate 500 mL of liquid base media containing 1 % glucose and allowed to grow in the dark until mid-log phase, measuring cell counts each day. Each of the following strains had been previously tested for growth on sucrose in the dark as a sole carbon source and exhibited no growth, and were thus chosen for transformation with a secreted invertase: (1) *Chlorella protothecoides* (UTEX 31); (2) *Chlorella minutissima* (UTEX 2341); and (3) *Chlorella emersonii* (CCAP 211/15).

[0498] Transformation of Algal Cells via Particle Bombardment: Sufficient culture was centrifuged to give approximately 1-5 x 10^8 total cells. The resulting pellet was washed with base media with no added fixed carbon source. Cells were centrifuged again and the pellet was resuspended in a volume of base media sufficient to give 5 x 10^7 to 2 x 10^8 cells/ml. 250-1000 µl of cells were then plated on solid base media supplemented with 1% sucrose and allowed to dry onto the plate in a sterile hood. Plasmid DNA was precipitated onto gold particles according to the manufacturer's recommendations (Seashell Technology, La Jolla, CA). Transformations were carried out using a BioRad PDS He-1000 particle delivery system using 1350 psi rupture disks with the macrocarrier assembly set at 9cm from the rupture disk holder. Following transformations, plates were incubated in the dark at 28°C.

All strains generated multiple transformant colonies. Control plates transformed with no invertase insert, but otherwise prepared in an identical fashion, contained no colonies.

[0499] Analysis of *Chlorella protothecoides* transformants: Genomic DNA was extracted from *Chlorella protothecoides* wild type cells and transformant colonies as follows: Cells were resuspended in 100 ul extraction buffer (87.5 mM Tris Cl, pH 8.0, 50 mM NaCl, 5 mM EDTA, pH 8.0, 0.25% SDS) and incubated at 60°C, with occasional mixing via inversion, for 30 minutes. For PCR, samples were diluted 1:100 in 20 mM Tris Cl, pH 8.0.

[0500] Genotyping was done on genomic DNA extracted from WT, the transformants and plasmid DNA. The samples were genotyped for the marker gene. Primers 2383 (5' CTGACCCGACCTATGGGAGCGCTCTTGGC 3') (SEQ ID NO:20) and 2279 (5' CTTGACTTCCCTCACCTGGAATTTGTCG 3') (SEQ ID NO:21) were used in this genotyping PCR. The PCR profile used was as follows: 94°C denaturation for 5 min; 35 cycles of 94°C -30 sec, 60°C - 30 sec, 72°C -3 min; 72°C -5 min. A band of identical size was amplified from the positive controls (plasmid) and two transformants of *Chlorella protothecoides* (UTEX 31), as shown in Figure 27.

[0501] Analysis of *Chlorella minutissima* and *Chlorella emersonii* transformants: Genomic DNA was extracted from *Chlorella* WT and the tranformants as follows: Cells were resuspended in 100 ul extraction buffer (87.5 mM Tris Cl, pH 8.0, 50 mM NaCl, 5 mM EDTA, pH 8.0, 0.25% SDS) and incubated at 60°C, with occasional mixing via inversion, for 30 minutes. For PCR, samples were diluted 1:100 in 20 mM Tris Cl, pH 8.0. Genotyping was done on genomic DNA extracted from WT, the transformants and plasmid DNA. The samples were genotyped for the marker gene. Primers 2336 (5' GTGGCCATATGGACTTACAA 3') (SEQ ID NO:22) and 2279 (5' CTTGACTTCCCTCACCTGGAATTTGTCG 3') (SEQ ID NO:21) were designated primer set 2 (1215 bp expected product), while primers 2465 (5' CAAGGGCTGGATGAATGACCCCAATGGACTGTGGTACGACG 3') (SEQ ID NO:23) and 2470 (5' CACCCGTCGTCATGTTCACGGAGCCCAGTGCG 3') (SEQ ID NO:24) were designated primer set 4 (1442 bp expected product). The PCR profile used was as follows: 94°C denaturation for 2 min; 29 cycles of 94°C -30 sec, 60°C - 30 sec, 72°C - 1 min, 30 sec; 72°C -5 min. A plasmid control containing the secreted invertase was used as a PCR control. Figure 28 shows the transformation of the *Chlorella minutissima* (UTEX 2341)

and *Chlorella emersonii* (CCAP 211/15) species of microalgae with the gene encoding a secreted invertase.

[0502] The sequence of the invertase construct corresponds to SEQ ID NO:25.

EXAMPLE 24

Growth of Algal Strains compared to *S. cerevisiae* on a Cellulosic Feedstock prepared with Celluclast

[0503] Strains and Culture Conditions: Algal strains used in this study are listed in Table 14 below, and were grown in Proteose media with exogenously provided cellulosic material and in some cases additional fixed carbon in the form of glucose. Twenty four algal strains were used in this study, including five different genera encompassing eleven different species of *Chlorella*, two of *Parachlorella* and *Prototheca*, and one each of *Bracteococcus* and *Pseudochlorella*. *Saccharomyces cerevisiae* (strain PJ-69-4A) was grown in YPD media (per liter, 10g Bacto-yeast extract, 20g Bacto peptone and 20g glucose). Both algae and yeast were grown at 28°C in the dark. Growth of these strains on Proteose media in the dark in the absence of cellulosic material or other additional fixed carbon either did not occur or was extremely minimal.

[0504] Liberation of Glucose from Cellulosic Material via Enzymatic Depolymerization Treatment: Wet, exploded corn stover material was prepared by the National Renewable Energy Laboratory (Golden, CO) by cooking corn stover in a 1.4% sulfuric acid solution and dewatering the resultant slurry. Using a Mettler Toledo Moisture analyzer, the dry solids in the wet corn stover were determined to be 24%. A 100 g wet sample was resuspended in deionized water to a final volume of 420 ml and the pH was adjusted to 4.8 using 10 N NaOH. Celluclast™ (Novozymes) (a cellulase) was added to a final concentration of 4% and the resultant slurry incubated with shaking at 50°C for 72 hours. The pH of this material was then adjusted to 7.5 with NaOH (negligible volume change), filter sterilized through a 0.22um filter and stored at -20°C. A sample was reserved for determination of glucose concentration using a hexokinase based kit from Sigma, as described below.

[0505] Determination of Glucose Concentration Liberated by Celluclast Treatment of Wet Corn Stover: Glucose concentrations were determined using Sigma Glucose Assay Reagent #G3293. Samples, treated as outlined above, were diluted 400 fold and 40µl was added to the

reaction. The corn stover cellulosic preparation was determined to contain approximately 23 g/L glucose.

[0506] Table 14. Algal strains grown on cellulosic feedstock.

Genus/Species	Source/Designation
Bracteococcus minor	UTEX 66
Chlorella ellipsoidea	SAG 2141
Chlorella kessleri	UTEX 1808
Chlorella kessleri	UTEX 397
Chlorella emersonii	CCAP 211/15
Chlorella luteoviridis	SAG 2133
Chlorella luteoviridis	SAG 2198
Chlorella luteoviridis	SAG 2214
Chlorella luteoviridis	UTEX 22
Bracteococcus medionucleatus	UTEX 1244
Chlorella minutissima	CCALA 20024
Chlorella minutissima	UTEX 2341
Chlorella ovalis	CCAP 211/21A
Chlorella protothecoides	CCAP 211/8d
Chlorella protothecoides	UTEX 250
Chlorella saccharophila	UTEX 2911
Chlorella sorokiniana	UTEX 1230
Chlorella sp.	SAG 241.80
Chlorella vulgaris	CCAP 211/11C
Parachlorella kessleri	SAG 12.80
Parachlorella kessleri	SAG 27.87
Prototheca moriformis	UTEX 1441
Prototheca moriformis	UTEX 1434
Pseudochlorella aquatica	SAG 2149

[0507] In Table 14, and as used herein, UTEX refers to the culture collection of algae at the University of Texas (Austin, Texas, USA), 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).

[0508] Determination of Growth on Cellulosic Material: After enzymatic treatment and saccharification of cellulose to glucose, xylose, and other monosaccharide sugars, the material prepared above was evaluated as a feedstock for the growth of 24 algal strains or *S. cerevisiae* in Proteose media or YPD media respectively. Proteose media was made up to a final glucose concentration of 23 g/L (the final concentration of glucose generated via

cellulolytic treatment of corn stover), as was YPD for growth of *S. cerevisiae*, by adding varying amounts of pure glucose and/or depolymerized cellulosic material. Varying concentrations of cellulosic material were included, providing 0, 12.5, 25, 50 and 100% of the 23 g/L glucose in each media, the components of which are shown in Table 15 below. One ml of the appropriate media was added to wells of a 24 well plate. *S. cerevisiae* grown heterotrophically at 28°C in YPD served as inoculum (20 ul) for the yeast wells. Twenty microliters of inoculum for the 24 algal strains was furnished by alga cells grown mixotrophically in Proteose media containing 20 g/L glucose.

[0509] Table 15. Cellulosic feedstock preparations.

Vol. of YPD 23.1 g/L Glucose (ml)	Vol. of Proteose Media 23.1 g/L Glucose (ml)	Vol. of 100% Cellulosics Made up to Proteose Media (ml)	Vol. of 100% Cellulosics Made up to YPD (ml)	Final Volume (ml)	Percent Cellulosics
0	1	0	0	1	0
0	0.875	0.125	0	1	12.5
0	0.75	0.25	0	1	25
0	0.5	0.5	0	1	50
0	0	1	0	1	100
1	0	0	0	1	0
0.875	0	0	0.125	1	12.5
0.75	0	0	0.25	1	25
0.5	0	0	0.5	1	50
0	0	0	1	1	100

[0510] Table 15 shows the volumes of depolymerized corn stover modified to contain Proteose or YPD media components that were added to Proteose or YPD media respectively to yield medias containing the indicated percentage of cellulosics. Medias were prepared in order to obtain a final glucose concentration of 23 g/L in all cases. Volume of media prior to the addition of either 20 µl of mid-log phase grown yeast or alga cells was 1 ml. Cells were incubated two days in the dark on the varying concentrations of cellulosic feedstocks at 28°C with shaking (300 rpm). Growth was assessed by measurement of absorbance at 750nm in a UV spectrophotometer. Surprisingly, all strains grew on the cellulosic material prepared with Celluclast, including media conditions in which 100% of fermentable sugar was cellulosic-derived.

EXAMPLE 25

Growth of 24 Algal Strains and *S. cerevisiae* on various Cellulosic Feedstocks prepared with Accellerase 1000™ and Celluclast™

[0511] Strains and Culture Conditions: Algal strains used in this Example are listed in Table 14 (above) and were grown in Proteose media plus additional fixed carbon in the form of depolymerized cellulosic material and/or pure glucose. *Saccharomyces cerevisiae* (strain pJ69-4a) was grown in YPD media plus additional fixed carbon in the form of depolymerized cellulosic material and/or pure glucose. Both algae and yeast were grown at 28°C in the dark.

[0512] Liberation of Glucose from Cellulosic Material via Enzymatic Depolymerization Treatment: Wet, exploded corn stover material was prepared by the National Renewable Energy Laboratory (Golden, CO) by cooking corn stover in a 1.4% sulfuric acid solution and dewatering the resultant slurry. Switchgrass was also prepared by The National Renewable Energy Laboratory (Golden, CO) utilizing the same method as for corn stover. Sugar beet pulp, generated via pectinase treatment, was supplied by Atlantic Biomass, Inc. of Frederick, MD. Using a Mettler Toledo Moisture analyzer, the dry solids were 24% in the wet corn stover, 26% in switch grass and 3.5% in sugar beet pulp. A 100 g wet sample of corn stover or switchgrass was resuspended in deionized water to a final volume of 420 ml and the pH adjusted to 4.8 using 10 N NaOH. For beet pulp, 8.8 grams dry solids were brought to 350 ml with deionized water and pH adjusted to 4.8 with 10N NaOH. For all feed stocks, Accellerase 1000™ (Genencor) (a cellulase enzyme complex) was used at a ratio of 0.25 ml enzyme per gram dry biomass. Samples were incubated with agitation (110 rpm) at 50°C for 72 hours. The pH of this material was then adjusted to 7.5 with NaOH (negligible volume change), filter sterilized through a 0.22um filter and used in growth experiments outlined below. A sample was reserved for determination of glucose concentration using a hexokinase-based kit from Sigma, as described below. The same set of cellulosic feedstocks were also prepared using Celluclast™ (Novozymes) (a cellulase) as described in the previous example.

[0513] Determination of Glucose Concentrations in Various Cellulosic Feedstocks Treated with Accellerase 1000: Glucose concentrations were determined using Sigma Glucose Assay Reagent #G3293. Samples, treated as outlined above, were diluted 400 fold and 40ul was added to the reaction. The cellulosic preparations from corn stover, switch grass and beet pulp were determined to contain approximately 23.6, 17.1 and 13.7 g/L glucose, respectively.

[0514] Determination of Growth on Cellulosic Material: After enzymatic depolymerization of cellulosic sources to glucose, xylose, and other monosaccharides, the materials prepared above were evaluated as feedstocks for the growth of the 24 algal strains listed in the Table

14 and *S. cerevisiae* in Proteose or YPD medias respectively. The medias were designed to contain a consistent concentration of glucose while varying the amount of cellulosic material derived from corn stover, switchgrass or beet pulp. A first set of Proteose and YPD media contained 23.6 g/L pure glucose, while a second set of media contained depolymerized corn stover, switchgrass and beet pulp, each of which contained 23.6 g/L glucose. The switchgrass and beet pulp medias were supplemented with 6.5 and 9.9 g/L pure glucose to normalize glucose in all cellulosic medias at 23.6 g/L. One ml of the appropriate media was added to wells of a 24 well plate. *S. cerevisiae* grown heterotrophically at 28°C in YPD served as inoculum (20 ul) for the yeast wells. Twenty microliters of inoculum for the 24 algal strains was furnished by alga cells grown mixotrophically in Proteose media containing 20 g/L glucose. all cells were incubated two days in the dark on the varying concentrations of cellulosic feedstocks at 28°C with shaking (300 rpm). Growth was assessed by measurement of absorbance at 750nm in a UV spectrophotometer. Surprisingly, all algae strains grew on the corn stover, switchgrass and beet pulp material prepared with Accellerase 1000™ or Celluclast™, including media conditions in which 100% of fermentable sugar was cellulosic-derived. Under no combination of cellulosic feedstock and depolymerization enzyme did *S. cerevisiae* outperform growth on an equivalent amount of pure glucose, indicating that inhibitors to yeast growth in the cellulosic material made a major impact on the productivity of the fermentation. Combinations of algae strains, depolymerization enzymes and feedstocks with 100% cellulosic-derived monosaccharides that outperformed 100% pure glucose are shown in Table 16.

[0515] Table 16. Combinations of algae, enzymes, and feedstocks.

Feedstock and Depolymerization Enzyme	Genus/Species	Source/Designation
corn stover/celluclast™	*Bracteococcus minor*	UTEX 66
beet pulp/accellerase™	*Chlorella ellipsoidea*	SAG 2141
switchgrass/accellerase™	*Chlorella kessleri*	UTEX 252
beet pulp/accellerase™	*Chlorella kessleri*	UTEX 397
switchgrass/accellerase™	*Chlorella luteoviridis*	SAG 2133
beet pulp/accellerase™	*Chlorella luteoviridis*	SAG 2133
switchgrass/accellerase™	*Chlorella luteoviridis*	UTEX 22
beet pulp/accellerase™	*Chlorella luteoviridis*	UTEX 22
corn stover/accellerase™	*Chlorella luteoviridis*	UTEX 22
beet pulp/accellerase™	*Chlorella protothecoides*	UTEX 250
beet pulp/accellerase™	*Chlorella sp.*	SAG 241.80
beet pulp/accellerase™	*Parachlorella kessleri*	SAG 12.80
switchgrass/accellerase™	*Prototheca moriformis*	UTEX 1441

beet pulp/accellerase™	*Prototheca moriformis*	UTEX 1441
corn stover/accellerase™	*Prototheca moriformis*	UTEX 1441
corn stover/celluclast™	*Prototheca moriformis*	UTEX 1441
corn stover/accellerase™	*Prototheca moriformis*	UTEX 1434
beet pulp/accellerase™	*Pseudochlorella aquatica*	SAG 2149

EXAMPLE 26

Carbon Utilization Screens

[0516] Strains and Culture Conditions: Seed cultures of the various strains of microalgae identified below were started as 1 ml liquid cultures in 24 well plates and were grown autotrophically for 48 hours in light, agitating at ~350 rpm. Plates were setup with 1.5% agarose-based solid Proteose media containing 1% of glucose, glycerol, xylose, sucrose, fructose, arabinose, mannose, galactose, or acetate as the sole fixed carbon source. For each strain, 5µl of culture from the autotrophic 24 well plate was spotted onto the solid media. Plates were incubated for 7 days in the dark at 28°C and examined for growth compared to a control plate containing no additional fixed carbon. Growth was observed for each of the species tested with each respective feedstock, as shown in Table 17 below. Growth of these strains on Proteose media in the dark in the absence of additional fixed carbon either did not occur or was extremely minimal.

[0517] Table 17. Algal species grown on various fixed-carbon feedstocks.

Fixed Carbon Source	Genus/Species	Source/Designation
Glucose	*Chlorella protothecoides*	UTEX 250
Glucose	*Chlorella kessleri*	UTEX 397
Glucose	*Chlorella sorokiniana*	UTEX 2805
Glucose	*Parachlorella kessleri*	SAG 12.80
Glucose	*Pseudochlorella aquatica*	SAG 2149
Glucose	*Chlorella reisiglii*	CCAP 11/8
Glucose	*Bracteococcus medionucleatus*	UTEX 1244
Glucose	*Prototheca stagnora*	UTEX 1442
Glucose	*Prototheca moriformis*	UTEX 1434
Glucose	*Prototheca moriformis*	UTEX 1435
Glucose	*Scenedesmus rubescens*	CCAP 232/1
Glycerol	*Parachlorella kessleri*	SAG 12.80
Glycerol	*Chlorella protothecoides*	CCAP 211/8d
Glycerol	*Bracteococcus medionucleatus*	UTEX 1244
Glycerol	*Prototheca moriformis*	UTEX 288
Glycerol	*Prototheca moriformis*	UTEX 1435
Glycerol	*Chlorella minutissima*	UTEX 2341
Glycerol	*Chlorella sp.*	CCAP 211/61
Glycerol	*Chlorella sorokiniana*	UTEX 1663

Xylose	*Chlorella luteoviridis*	SAG 2133
Xylose	*Chlorella ellipsoidea*	SAG 2141
Xylose	*Pseudochlorella aquatica*	SAG 2149
Xylose	*Chlorella sp.*	CCAP 211/75
Xylose	*Prototheca moriformis*	UTEX 1441
Xylose	*Prototheca moriformis*	UTEX 1435
Sucrose	*Chlorella saccharophila*	UTEX 2469
Sucrose	*Chlorella luteoviridis*	UTEX 22
Sucrose	*Chlorella sp.*	UTEX EE102
Sucrose	*Chlorella luteoviridis*	SAG 2198
Sucrose	*Bracteococcus medionucleatus*	UTEX 1244
Sucrose	*Chlorella minutissima*	CCALA 20024
Fructose	*Chlorella kessleri*	UTEX 398
Fructose	*Chlorella trebouxiodes*	SAG 3.95
Fructose	*Parachlorella kessleri*	SAG 27.87
Fructose	*Chlorella luteoviridis*	SAG 2214
Fructose	*Chlorella protothecoides*	UTEX 31
Fructose	*Chlorella protothecoides*	UTEX 250
Fructose	*Chlorella reisiglii*	CCAP 11/8
Fructose	*Chlorella protothecoides*	CCAP 211/8d
Fructose	*Prototheca moriformis*	UTEX 1435
Fructose	*Scenedesmus rubescens*	CCAP 232/1
Arabinose	*Chlorella sp.*	CCAP 211/75
Mannose	*Chlorella kessleri*	UTEX 263
Mannose	*Chlorella saccharophila*	UTEX 2911
Mannose	*Parachlorella kessleri*	SAG 12.80
Mannose	*Chlorella sp.*	SAG 241.80
Mannose	*Chlorella angustoellipsoidea*	SAG 265
Mannose	*Chlorella ellipsoidea*	SAG 2141
Mannose	*Chlorella protothecoides*	UTEX 250
Mannose	*Chlorella emersonii*	CCAP 211/15
Mannose	*Bracteococcus minor*	UTEX 66
Mannose	*Prototheca stagnora*	UTEX 1442
Mannose	*Prototheca moriformis*	UTEX 1439
Mannose	*Chlorella cf. minutissima*	CCALA 20024
Mannose	*Scenedesmus rubescens*	CCAP 232/1
Galactose	*Bracteococcus minor*	UTEX 66
Galactose	*Parachlorella kessleri*	SAG 14.82
Galactose	*Parachlorella beijerinckii*	SAG 2046
Galactose	*Chlorella protothecoides*	UTEX 25
Galactose	*Chlorella sorokiniana*	UTEX 1602
Galactose	*Parachlorella kessleri*	SAG 12.80
Galactose	*Pseudochlorella aquatica*	SAG 2149
Galactose	*Chlorella luteoviridis*	SAG 2214
Galactose	*Chlorella ellipsoidea*	CCAP 211/42
Galactose	*Chlorella ellipsoidea*	CCAP 211/50
Galactose	*Chlorella protothecoides*	UTEX 250
Galactose	*Chlorella protothecoides*	UTEX 264

Galactose	*Bracteococcus medionucleatus*	UTEX 1244
Galactose	*Prototheca moriformis*	UTEX 1439
Galactose	*Prototheca moriformis*	UTEX 1441
Galactose	*Chlorella kessleri*	CCALA 252
Acetate	*Chlorella sorokiniana*	UTEX 1230
Acetate	*Chlorella sorokiniana*	UTEX 1810
Acetate	*Chlorella luteoviridis*	UTEX 22
Acetate	*Parachlorella kessleri*	SAG 12.80
Acetate	*Parachlorella kessleri*	SAG 27.87
Acetate	*Chlorella sp.*	SAG 241.80
Acetate	*Chlorella luteoviridis*	SAG 2214
Acetate	*Chlorella protothecoides*	UTEX 31
Acetate	*Chlorella protothecoides*	UTEX 411
Acetate	*Chlorella ellipsoidea*	CCAP 211/42
Acetate	*Chlorella ovalis*	CCAP 211/21A
Acetate	*Chlorella protothecoides*	CCAP 211/8d
Acetate	*Prototheca stagnora*	UTEX 1442
Acetate	*Chlorella protothecoides*	UTEX 250
Acetate	*Chlorella sorokiniana*	CCALA 260
Acetate	*Chlorella vulgaris*	CCAP 211/79
Acetate	*Parachlorella kessleri*	SAG 14.82

EXAMPLE 27

Production of Renewable Diesel

[0518] Cell Production: An F-Tank batch of *Chlorella protothecoides* (UTEX 250) (about 1,200 gallons) was used to generate biomass for extraction processes. The batch (#ZA07126) was allowed to run for 100 hours, while controlling the glucose levels at 16 g/L, after which time the corn syrup feed was terminated. Residual glucose levels dropped to <0 g/L two hours later. This resulted in a final age of 102 hours. The final broth volume was 1,120 gallons. Both in-process contamination checks and a thorough analysis of a final broth sample failed to show any signs of contamination. The fermentation broth was centrifuged and drum dried. Drum dried cells were resuspended in hexane and homogenized at approximately 1000 bar. Hexane extraction was then performed using standard methods, and the resulting algal triglyceride oil was determined to be free of residual hexane.

[0519] Production of Renewable Diesel: The algal triglyceride oil had a lipid profile of approximately 3% C18:0, 71% C18:1, 15% C18:2, 1% C18:3, 8% C16:0, and 2% other components. The oil was first subjected to hydrocracking, resulting in an approximate 20% yield loss to water and gases. Hydroisomerization was then performed, with an approximate 10% loss in yield to gases. A first distillation was then performed to remove the naptha

fraction, leaving the desired product. Approximately 20% of the material was lost to naptha in this first distillation. A second distillation was then performed at a temperature sufficient to remove fractions necessary to meet the ASTM D975 specification but leave a bottom fraction that did not meet the 90% point for a D975 distillation. Approximately 30% of the material was left in the bottom fraction in the second distillation. The resulting material was then tested for all ASTM D975 specifications.

[0520] Figures 29 and 30 illustrate a gas chromatograph and a boiling point distribution plot, respectively, of the final renewable diesel product produced by the method of the invention. Table 18 shows the boiling point distribution of the resulting renewable diesel product, and Table 19 shows the results of an analysis of the final product for compliance with the ASTM D975 specifications.

[0521] Table 18. Boiling point distribution of renewable diesel product.

Recovered mass%	BP °C	Recovered mass%	BP °C	Recovered mass%	BP °C	Recovered mass%	BP °C
IBP	150.4	26.0	261.8	52.0	303.8	78.0	315.8
1.0	163.6	27.0	264.6	53.0	304.4	79.0	316.4
2.0	173.4	28.0	266.2	54.0	304.8	80.0	317.0
3.0	175.2	29.0	268.2	55.0	305.2	81.0	317.6
4.0	188.0	30.0	271.0	56.0	305.6	82.0	318.4
5.0	194.8	31.0	272.4	57.0	306.0	83.0	319.0
6.0	196.6	32.0	273.4	58.0	306.4	84.0	319.6
7.0	197.8	33.0	276.2	59.0	306.6	85.0	320.2
8.0	207.4	34.0	280.0	60.0	307.0	86.0	320.8
9.0	210.0	35.0	282.4	61.0	307.4	87.0	321.2
10.0	214.4	36.0	285.2	62.0	307.6	88.0	321.8
11.0	216.6	37.0	287.8	63.0	308.0	89.0	322.2
12.0	217.6	38.0	289.6	64.0	308.4	90.0	322.4
13.0	221.4	39.0	291.8	65.0	308.8	91.0	322.8
14.0	227.6	40.0	294.2	66.0	309.2	92.0	323.2
15.0	229.8	41.0	295.8	67.0	309.6	93.0	324.4
16.0	233.2	42.0	296.8	68.0	310.2	94.0	326.8
17.0	235.8	43.0	297.6	69.0	310.6	95.0	329.4
18.0	236.8	44.0	298.4	70.0	311.2	96.0	333.6
19.0	240.2	45.0	299.2	71.0	311.6	97.0	339.4
20.0	245.6	46.0	299.8	72.0	312.2	98.0	346.2
21.0	248.0	47.0	300.6	73.0	313.0	99.0	362.8
22.0	250.2	48.0	301.2	74.0	313.6	FBP	401.4
23.0	253.6	49.0	302.0	75.0	314.2		
24.0	255.2	50.0	302.6	76.0	314.8		
25.0	256.8	51.0	303.2	77.0	315.4		

[0522] Table 19. Analytical report for renewable diesel product using D975 specifications.

Method Number	Test Description	Results	Units
D93A	Flash Point (PMCC)	70	°C
D2709	Water and Sediment	0	Vol %
D86	Distillation 90% (Recovered)	301.0 / 573.9	°C / °F
D445	Kinematic Viscosity @ 40.0°C (104.0°F)	2.868	mm2/sec
D482	Ash	< 0.001	Wt %
D5453	Sulfur	2.4	ppm
D130	Copper Corrosion 3hours @ 50°C	1b	
D613	Cetane Number ***	>65	
D976	Calculated Cetane Index	71.2	
D2500	Cloud Point	-3	°C
D524	Ramsbottom 10% Carbon Residue	0.02	Wt %
D97	Pour Point	-3	°C
D2274	Total Insolubles (Oxidation Stability) 40Hour Test ***	4.0	mg/100mL
D4052	Density @ 15.0°C (59.0°F)	793.8	kg/m³
D4176-1	Appearance by Visual Inspection (Lab)	Clear and Bright- Pass	Visual
D4176-1	Appearance by Visual Inspection (Lab)	Free Water- Pass	Visual
D4176-1	Appearance by Visual Inspection (Lab)	Particulates- Pass	Visual
D1500	ASTM Color	L 0.5	
D664	Acid Number	< 0.10	mg KOH/g
D6079	Lubricity (Wear Scar)	405	µm

SEQUENCES

SEQ ID NO:1

HUP promoter from Chlorella (subsequence of GenBank accession number X55349):

gatcagacgggcctgacctgcgagataatcaagtgctcgtaggcaaccaactcagcagctgcttggtgttgggtctgcaggatagtgtt
gcagggccccaaggacagcaggggaacttacaccttgtccccgacccagttttatggagtgcattgcctcaagagcctagccggagc
gctaggctacatacttgccgcaccggtatgaggggatatagtactcgcactgcgctgtctagtgagatgggcagtgctgcccataaac
aactggctgctcagccatttgttggcggaccattctgggggggccagcaatgcctgactttcgggtagggtgaaaactgaacaaagac
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aagggcgcaaacatggccggactgttgtcagcactctttcatggccatacaaggtcatgtcgagattagtgctgagtaagacactatca
ccccatgttcgattgaagccgtgacttcatgccaacctgcccctgggcgtagcagacgtatgccatcatgaccactagccgacatgcg
ctgtcttttgccaccaaaacaactggtacaccgctcgaagtcgtgccgcacacctccgggagtgagtccggcgactcctccccggcg
ggccgcggccctacctgggtagggtcgccatacgcccacgaccaaacgacgcaggaggggattggggtagggaatcccaaccag
cctaaccaagacggcacctataataataggtgggggggactaacagccctatatcgcaagctttgggtgcctatcttgagaagcacgag
ttggagtggctgtgtacggtcgaccctaaggtgggtgtgccgcagcctgaaacaaagcgtctagcagctgcttctataatgtgtcagcc
gttgtgtttcagttatattgtatgctattgtttgttcgtgctagggtggcgcaggcccacctactgtggcgggccattggttggtgcttgaatt
gcctcaccatctaaggtctgaacgctcactcaaacgcctttgtacaactgcagaactttccttggcgctgcaactacagtgtgcaaacca
gcacatagcactcccttacatcacccagcagtacaaca

SEQ ID NO:2

Chlorella ellipsoidea nitrate reductase promoter from AY307383:

cgctgcgcaccagggccgccagctcgctgatgtcgctccaaatgcggtcccccgatttttgttcttcatcttctccaccttggtggccttc
ttggccagggccttcagctgcatgcgcacagaccgttgagctcctgatcagcatcctcaggaggccctttgacaagcaagcccctgtg
caagcccattcacggggtaccagtggtgctgaggtagatgggtttgaaaaggattgctcggtcgattgctgctcatggaattggcatgt
gcatgcatgttcacaatatgccaccaggctttggagcaagagagcatgaatgccttcaggcaggttgaaagttcctgggggtgaagag
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SEQ ID NO:3

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SEQ ID NO:4

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SEQ ID NO:5

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SEQ ID NO:7

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SEQ ID NO:8

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CLAIMS

1. A method of producing fuel, comprising:

(a) culturing a population of microalgae in the presence of a fixed carbon source, wherein:

(i) the microalgae express an exogenous sucrose invertase;

(ii) the microalgae accumulate at least 10% of their dry cell weight as lipid; and

(ii) the fixed carbon source is selected from the group consisting of sugar cane juice, sugar cane, sugar beet, molasses, or other sucrose-containing feedstock;

(b) isolating lipid components from the cultured microalgae; and

(c) subjecting the isolated lipid components to one or more chemical reactions to generate fatty acid esters and/or alkanes,

whereby fuel is produced.

2. The method of claim 1, wherein the one or more chemical reactions is selected from the group consisting of transesterification, hydrotreating, hydrocracking, deoxygenation and isomerization.

3. The method of claim 1 or 2, wherein the microalgae are of the genus *Chlorella* or *Prototheca.*

4. The method of claim 3, wherein the microalgae are *Chlorella protothecoides* or *Prototheca moriformis.*

5. A composition of alkanes or fatty acid esters made according to the method of any one of claims 1 to 4.

6. A microalgal cell containing at least 10% of its dry cell weight as lipid, and comprising an exogenous gene encoding a sucrose invertase enzyme.

7. The microalgal cell of claim 6, wherein the sucrose invertase enzyme is a secretable sucrose invertase enzyme.

8. The microalgal cell of claim 6 or 7, wherein the microalga is selected from the group consisting of *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, Bracteococcus minor, Bracteococcus medionucleatus, 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 ovalis, Chlorella parva, Chlorella photophila, Chlorella pringsheimii, Chlorella protothecoides (including any of UTEX strains 1806, 411, 264, 256, 255, 250, 249, 31, 29, 25), 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 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., Crypthecodinium cohnii, 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, Parachlorella kessleri, Pascheria acidophila, Pavlova sp., Phagus, Phormidium, Platymonas sp., Pleurochrysis carterae, Pleurochrysis dentate, Pleurochrysis sp., Prototheca wickerhamii, Prototheca stagnora, Prototheca portoricensis, Prototheca moriformis, Prototheca zopfii, Pseudochlorella aquatica, Pyramimonas sp., Pyrobotrys, Rhodococcus opacus, Sarcinoid chrysophyte, Scenedesmus armatus, Schizochytrium, Spirogyra, Spirulina platensis, Stichococcus sp., Synechococcus sp., Tetraedron, Tetraselmis sp., Tetraselmis suecica, Thalassiosira weissflogii, and *Viridiella fridericiana*.

9. The microalgal cell of claim 8, wherein the microalga is selected from the group consisting of *Bracteococcus, Chlorella, Parachlorella, Prototheca, Pseudochlorella,* and *Scenedesmus*.

10. The microalgal cell of claim 9, wherein the microalga is *Chlorella protothecoides* or *Prototheca moriformis.*

11. A lipid produced by a microalgal cell of any one of claims 6 to 10.

12. A method of producing a lipid, comprising:

(a) culturing a population of microalgae comprising an exogenous gene encoding a sucrose invertase in a culture medium comprising a fixed carbon source until the microalgae accumulate 10% or more of their dry cell weight as lipid; and

(b) separating the lipid from the microalgae.

13. The method of claim 12, wherein the sucrose invertase gene encodes a secretable sucrose invertase enzyme.

14. The method of claim 12 or 13, wherein the fixed carbon source is selected from the group consisting of depolymerized cellulosic material, sugar cane juice, sugar beet and molasses.

15. The method of any one of claims 12 to 14, wherein the culture medium comprises sucrose provided from a source selected from the group consisting of sugar cane

juice, sugar beet juice and molasses, wherein the sugar cane juice, sugar beet juice or molasses has a sucrose concentration of 50 μM or more.

16. The method of claim 15, wherein the sugar cane juice, sugar beet juice or molasses has a sucrose concentration of 500 μM or more.

17. The method of claim 16, wherein the sugar cane juice, sugar beet juice or molasses has a sucrose concentration of 5 mM or more.

18. The method of claim 17, wherein the sugar cane juice, sugar beet juice or molasses has a sucrose concentration of 50 mM or more.

19. The method of claim 18, wherein the sugar cane juice, sugar beet juice or molasses has a sucrose concentration of 500 mM or more.

20. The method of any one of claims 12 to 19, wherein separating the lipid from the microalgae comprises lysing the microalgae by mechanical means.

21. The method of any one of claim 12 to 20, wherein the culture medium comprises limited nitrogen.

22. The method of any one of claims 12 to 21, wherein the microalgae accumulate at least 60% of their dry cell weight as lipid.

23. The method of any one of claims 12 to 22, further comprising the additional step of forming a food product from the lipid obtained from the microalgae.

24. The method of any one of claims 12 to 23, further comprising (c) subjecting the lipid to one or more chemical reactions selected from the group consisting of ester hydrolysis, transesterification, hydrotreating, hydrocracking, deoxygenation, and/or isomerization.

25. The method of claim 24, wherein the lipid is subjected to transesterification to produce fatty acid esters and glycerol, and the glycerol is added to a second microalgal culture for use as a carbon source.

26. The method of claim 25, wherein the microalgae used to produce the lipid and the microalgae of the second microalgal culture are the same species.

27. The method of any one of claims 12 to 26, wherein the microalgae are selected from the group consisting of *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, Bracteococcus minor, Bracteococcus medionucleatus, 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 ovalis, Chlorella parva, Chlorella photophila, Chlorella pringsheimii, Chlorella protothecoides (including any of UTEX strains 1806, 411, 264, 256, 255, 250, 249, 31, 29, 25), 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 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., Crypthecodinium cohnii, 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, Parachlorella kessleri, Pascheria acidophila, Pavlova sp., Phagus, Phormidium, Platymonas sp., Pleurochrysis carterae, Pleurochrysis dentate, Pleurochrysis sp., Prototheca wickerhamii, Prototheca stagnora, Prototheca portoricensis, Prototheca moriformis, Prototheca zopfii, Pseudochlorella aquatica, Pyramimonas sp., Pyrobotrys, Rhodococcus opacus, Sarcinoid chrysophyte, Scenedesmus armatus, Schizochytrium, Spirogyra, Spirulina platensis, Stichococcus sp., Synechococcus sp., Tetraedron, Tetraselmis sp., Tetraselmis suecica, Thalassiosira weissflogii, and *Viridiella fridericiana*.

28. The method of claim 27, wherein the microalgae are selected from the group consisting of *Bracteococcus, Chlorella, Parachlorella, Prototheca, Pseudochlorella,* and *Scenedesmus*.

29. The method of claim 28, wherein the microalgae are *Chlorella protothecoides* or *Prototheca moriformis*.

30. A lipid produced by the method of any one of claims 12 to 23.

PRODUCTION OF OIL IN MICROORGANISMS

ABSTRACT

The invention provides methods and compositions useful for the production of oil, fuels, oleochemicals, and other compounds in microorgansims. In particular, the invention provides oil-bearing microorganisms and methods of low cost cultivation of such microorganisms. The invention also provides microbial cells containing exogenous genes encoding, for example, a lipase, sucrose transporter, sucrose invertase, fructokinase, a polysaccharide-degrading enzyme, fatty acyl-ACP thioesterase, fatty acyl-CoA/aldehyde reductase, fatty acyl-CoA reductase, fatty aldehyde reductase, fatty aldehyde decarbonylase, and/or an acyl carrier protein. The invention also includes methods of manufacturing transportation fuels such as renewable diesel, biodiesel, and renewable jet fuel.

FIG. 1