GENETICALLY ENGINEERED MICROBIAL STRAINS INCLUDING *PROTOTHECA* LIPID PATHWAY GENES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. 119(e) of US Provisional Patent Application No. 61/564,247, filed November 28, 2011, US Provisional Patent Application No. 61/581,538, filed December 29, 2011, and US Provisional Patent Application No. 61/674,251, filed July 20, 2012. Each of these applications is incorporated herein by reference in its entirety for all purposes.

REFERENCE TO A SEQUENCE LISTING

[0002] This application includes a sequence listing.

FIELD OF THE INVENTION

[0003] In certain embodiments genetically engineered microbes, e.g., *Prototheca* strains are provided that show improved oil production and so find uses in the fields of chemistry, food production, fuel and industrial chemicals production, microbiology, and molecular biology.

DESCRIPTION OF RELATED DISCLOSURES

[0004] Microalgae, including genetically engineered microalgae, have been identified as important new sources of oil for use in food and fuels. See PCT Pub. Nos. WO 2008/151149, WO 2009/126843, and WO 2010/045368, each of which is incorporated herein by reference in its entirety for all purposes. While *Chlorella* strains have been the focus of much effort in developing microbial oil production methods, more recently strains of the genus *Prototheca* have been identified as also having promise as a new source of microbial oils, including tailored oils for specific applications. See PCT Pub. Nos. WO 2010/063031, WO 2010/063032, and WO 2011/150410and WO 2012/106560, each of which is incorporated herein by reference in its entirety for all purposes.

SUMMARY

[0005] In one aspect, the present invention refers to a recombinant nucleic acid comprising a coding sequence that encodes a *Prototheca* lipid droplet protein 1 having at least 95% sequence identity with SEQ ID NO:120, wherein the coding sequence is operably linked to an exogenous nucleic acid sequence. In another aspect, the present invention refers to an expression cassette comprising a recombinant nucleic acid of the present invention. In still another aspect, the present invention refers to a genetically engineered microbial cell transformed with a recombinant nucleic acid of the present invention. In another aspect, the present invention refers to method for obtaining microbial oil comprising culturing a genetically engineered *Prototheca* cell of the present invention under conditions such that oil is produced. In certain ~~embodiments~~examples, the ~~invention~~ disclosure also provides a recombinant (e.g., isolated) nucleic acid comprising a coding

sequence that encodes a *Prototheca* lipid biosynthesis protein or portion thereof. In some examplesembodiments, provided is a recombinant nucleic acid comprising a coding sequence that encodes a *Prototheca* lipid biosynthesis protein, provided that the protein is not ketoacyl acyl carrier protein synthase II, stearoyl acyl carrier protein desaturase, or delta 12 fatty acid desaturase. In some examplesembodiments the

protein is not a ketoacyl acyl carrier protein synthase II protein of SEQ ID NO: 270, a stearol acyl carrier protein desaturase of SEQ ID NO: 271, or a delta 12 fatty acid desaturase of SEQ ID NOs: 272 or 273. In some examples~~embodiments~~, the coding sequence is in operable linkage with a promoter, an untranslated control element, and/or a targeting sequence, such as a plastidial targeting sequence and mitochondrial targeting sequence. The recombinant nucleic acid may be, e.g., a DNA molecule. In certain examples~~embodiments~~, recombinant nucleic acid is an expression vector. The recombinant nucleic acid can, for example, include an expression cassette that encodes an mRNA that encodes a functional *Prototheca* lipid biosynthesis enzyme. Alternatively or in addition, the recombinant nucleic acid can include an expression cassette that encodes an inhibitory RNA that suppresses expression of a *Prototheca* lipid biosynthesis gene. In some examples~~embodiments~~, the lipid biosynthesis protein is a protein in Table 1. In some examples~~embodiments~~, the protein has at least 50%, 60%, 70%, 80%, 85%, 90% or 95% sequence identity to a protein provided herein or a protein encoded by a gene provided herein. In some examples~~embodiments~~ the protein or the gene encoding the protein is listed in Table 1.

[0006] In some examples~~embodiments~~, the protein encoded by the coding sequence in the nucleic acid contains one or more point mutations, deletions, substitutions, or combinations thereof. In other examples~~embodiments~~, the protein has at least one point mutation in comparison to a protein in Table 1. In some examples~~embodiments~~, the protein encoded by the coding sequence is a functional protein. In some examples~~embodiments~~, the protein is diacylglycerol diacyltransferase (DGAT) having at least one point mutation. In other examples~~embodiments~~, the recombinant nucleic acid further encodes sucrose invertase.

[0007] In certain examples~~embodiments~~, also provided is a genetically engineered microbial cell transformed with a recombinant nucleic acid provided herein. In some examples~~embodiments~~, the cell is a microbial, plant, or yeast cell. In particular examples~~embodiments~~, provided is a cell comprising one or more exogenous gene(s), wherein the exogenous gene is a *Prototheca* lipid biosynthesis gene selected from the genes listed in Table 1. The genetically engineered microbial cell can, for example, be a cell of the genus *Prototheca* or *Chlorella.* In particular examples~~embodiments~~, the cell comprises both an endogenous lipid biosynthesis gene and one or more exogenous *Prototheca* lipid biosynthesis gene(s) selected from the genes listed in Table 1. In certain examples~~embodiments~~, the exogenous gene can encode a lipid biosynthesis protein, wherein the amino acid sequence of the lipid biosynthesis protein is identical to the endogenous lipid biosynthesis protein. For example, the exogenous gene can include a nucleotide sequence in which the codons of the nucleotide sequence

encoding the amino acids of the lipid biosynthesis protein have been altered, as compared to the codons in the native nucleic acid. In various examples~~embodiments~~, the exogenous gene can encode a protein with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% amino acid identity to the native *Prototheca* protein. The exogenous gene can, in some examples~~embodiments~~, be in operable linkage with a promoter element that is not the native *Prototheca* promoter, an untranslated control element that is not the native *Prototheca* untranslated control element, and/or a nucleotide sequence encoding a transit peptide that is not the native *Prototheca* transit peptide. The transit peptide can, for example, be a plastidial targeting sequence or a mitochondrial targeting sequence. In certain examples~~embodiments~~, the cell has a 23S rRNA sequence with at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% nucleotide identity to SEQ ID NO: 5. In particular examples~~embodiments~~, the cell is a *Prototheca* cell, wherein the cell has a fatty acid profile that is at least 10% C8-C14.

[0008] In certain examples~~embodiments~~, ~~another aspect of the invention is~~it is disclosed a method for obtaining microbial oil comprising culturing a genetically engineered cell, e.g., a *Prototheca* cell, described above under conditions such that oil is produced. In certain examples~~embodiments~~, the microbial oil thus produced has a fatty acid profile that is at least 10% C8-C14. The ~~invention~~ disclosure also includes a microbial oil produced by this method.

[0009] In particular examples~~embodiments~~, the ~~invention~~ disclosure provides genetically engineered cell, e.g., of the genus *Prototheca,* wherein the activity of one or more endogenous lipid biosynthesis gene, selected from the genes listed in Table 1, has been attenuated. In various examples~~embodiments~~, the activity of the endogenous gene has been attenuated through chromosomal gene deletion, chromosomal gene insertion, frameshift mutation, point mutation, and/or inhibitory RNA. The genetically engineered cell can, in certain embodiments, further comprise an exogenous *Prototheca* lipid biosynthesis pathway gene selected from the genes listed in Table 1. In particular examples~~embodiments~~, one or more allele(s) of an endogenous lipid biosynthesis gene in the genetically engineered cell is attenuated.

[0010] In certain examples~~embodiments~~, one allele of the endogenous lipid biosynthesis gene is replaced, in the genetically engineered cell, with a polynucleotide encoding, e.g., an exogenous *Prototheca* lipid biosynthesis pathway gene selected from Table 1 and a selectable marker. In a variation of this example~~embodiment~~, two or more alleles of the endogenous lipid biosynthesis gene are each replaced with a polynucleotide encoding an exogenous *Prototheca* lipid biosynthesis pathway gene selected from Table 1 and a

selectable marker. In certain examples~~embodiments~~, the genetically engineered cell has a 23S rRNA sequence with at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% nucleotide identity to SEQ ID NO: 5. In particular examples~~embodiments~~, the genetically engineered cell is a *Prototheca* cell, wherein the cell has a fatty acid profile that is at least 10% C8-C14. In some examples~~embodiments~~, the cell has a fatty acid profile that is at least at least 50%, 60%, or 70% C12:0. In some examples~~embodiments~~, the cell has a fatty acid profile that is at least at least 70%, 75%, 80%, 85%, or 90% C18:1. In certain examples~~embodiments~~, the ~~invention~~ disclosure also provides a method for obtaining microbial oil comprising culturing this genetically engineered cell, which may be, e.g., a *Prototheca* cell, under conditions such that oil is produced. In certain examples~~embodiments~~, the microbial oil thus produced has a fatty acid profile that is at least 10% C8-C14. The ~~invention~~ disclosure also includes a microbial oil produced by this method.

[0011] In another ~~aspect~~example, ~~the present invention provides~~it is also disclosed a genetically engineered microbial cell, e.g., *Prototheca* cell, in one or more lipid biosynthesis genes have been modified to increase or decrease expression of such one or more genes such that the fatty acid profile of the genetically engineered strain differs from that of the strain from which it was derived. In one example~~embodiment~~, at least two genes have been modified. In various examples~~embodiments~~, the genetic modifications include one or more of the following modifications: (i) attenuation of a gene or its enzymatic product; and (ii) increased expression of a gene or its enzymatic product; (iii) altered activity of a gene or its enzymatic product.

[0012] In various examples~~embodiments~~, the genetically engineered cell has one or more attenuated genes, wherein the genes attenuated have been attenuated by a means selected from the group consisting of a homologous recombination event and introduction of an exogenous gene that codes for an interfering RNA. In various examples~~embodiments~~, one or more alleles of a gene are attenuated.

[0013] In various examples~~embodiments~~, the genetically engineered cell has one or more over-expressed genes, wherein the genes over-expressed have been up-regulated by a means selected from the group consisting of introduction of additional copies of said gene into said cell; introduction of new expression control elements for said gene; and alteration of the protein-coding sequence of the gene. In various examples~~embodiments~~, one or more alleles of a gene are over-expressed.

[0014] In various examples~~embodiments~~, the modified genes of the genetically engineered cell are selected from the group consisting of *Prototheca* lipid biosynthesis genes presented in Table 1. In various examples~~embodiments~~, the genetically engineered cell comprises an exogenous gene selected from the group consisting of *Prototheca* lipid biosynthesis genes presented in Table 1. In various examples~~embodiments~~, the genetically engineered cell comprises one or more over-expressed alleles of a gene, the gene selected from the group consisting of *Prototheca* lipid biosynthesis genes presented in Table 1. In various examples~~embodiments~~, the genetically engineered cell has an attenuated gene selected from the group consisting of *Prototheca* lipid biosynthesis genes presented in Table 1. In various examples~~embodiments~~, the genetically engineered cell has one more attenuated alleles of a gene, the gene selected from the group consisting of *Prototheca* lipid biosynthesis genes presented in Table 1.

[0015] In various examples~~embodiments~~, the genetically engineered cell has a fatty acid profile selected from the group consisting of: 3% to 60% C8:0, 3% to 60% C10:0, 3% to 70% C12:0, 3% to 95% C14:0, 3% to 95% C16:0, 3% to 95% C18:0, 3% to 95% C18:1, 0% to 60% C18:2, 0% to 60% C18:3 or combinations thereof. In various examples~~embodiments~~, the ratio of C10:0 to C12:0 is at least 3:1. In some cases, the ratio of C10:0 to C14:0 is at least 10:1. In various examples~~embodiments~~, the ratio of C12:0 to C14:0 is at least 3:1. In various examples~~embodiments~~, the genetically engineered cell has a fatty acid profile of at least 40% saturated fatty acids, of at least 60% saturated fatty acids, or at least 85% saturated fatty acids.

[0016] In another example~~aspect~~, the present ~~invention~~ disclosure provides methods for obtaining microbial oil comprising culturing a genetically engineered *Prototheca* cell of the invention under conditions such that oil is produced. In various examples~~embodiments~~, the microbial oil has a fatty acid profile selected from the group consisting of: 3% to 40% C8:0, 3% to 60% C10:0, 3% to 70% C12:0, 3% to 95% C14:0, 3% to 95% C16:0, 3% to 95% C18:0, 3% to 95% C18:1, 0% to 60% C18:2, 0% to 60% C18:3 or combinations thereof. In various examples~~embodiments~~, the ratio of C10:0 to C12:0 is at least 3:1. In some cases, the ratio of C10:0 to C14:0 is at least 10:1. In various examples~~embodiments~~, the ratio of C12:0 to C14:0 is at least 3:1. In various examples~~embodiments~~, the genetically engineered cell has a fatty acid profile of at least 40% saturated fatty acids, of at least 60% saturated fatty acids, or at least 85% saturated fatty acids.

[0017] In an additional example~~aspect~~, the present ~~invention~~ disclosure provides microbial oils and foods, fuels, and chemicals containing said oil or a chemical derived therefrom.

[0018] In another ~~aspect~~example, the present ~~invention~~ disclosure provides recombinant nucleic acids useful in methods for making genetically modified *Prototheca* and other cells. The nucleic acids of ~~the invention~~ described herein comprise all or some portion of a *Prototheca* lipid biosynthesis gene.

[0019] In various examples~~embodiments~~, these nucleic acids include expression cassettes, which consist of a coding sequence and control sequences that regulate expression of the coding sequence, which may code for an mRNA that encodes a lipid biosynthesis protein, enzyme, or for an RNAi that acts to suppress expression of a lipid biosynthesis gene.

[0020] In other examples~~embodiments~~, these nucleic acids are expression vectors that include one or more expression cassettes and stably replicate in a *Prototheca* or other host cell, either by integration into chromosomal DNA of the host cell or as freely replicating vectors.

[0021] In other examples~~embodiments~~, these nucleic acids comprise only a portion of a *Prototheca* lipid biosynthesis gene, which portion may be a portion of a coding sequence, an exon, or a control element. Such nucleic acids are useful in the construction of expression cassettes for *Prototheca* and non-*Prototheca* host cells, for integration of exogenous DNA into *Prototheca* host cells, and for construction of nucleic acids useful for attenuating *Prototheca* lipid biosynthetic genes by homologous recombination.

[0022] In some examples~~embodiments~~, provided are sequences, compositions, host cells, and methods for overexpression of a lipid biosynthesis gene. In some examples~~aspects~~, the overexpressed lipid biosynthesis gene is one or more of LEC2, DGAT, ATP:citrate lyase (ACL), malic enzyme, lipase, fatty acyl-CoA reductase, Acyl-CoA Binding Proteins (ACBPs), or Lipoic Acid Synthase (LS1).

[0023] These and other aspects and embodiments of the invention are described in the accompanying drawings, a brief description of which immediately follows, the detailed description of the invention below, and are exemplified in the examples below. Any or all of the features discussed above and throughout the application can be combined in various embodiments of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] Figure 1 shows PstI restriction maps of *Prototheca moriformis* FADc alleles with and without a targeted gene disruption, as described in Example 37.

[0025] Figure 2 shows the results of the Southern blot described in Example 37.

WHAT IS CLAIMED IS:

1. A recombinant nucleic acid comprising a coding sequence that encodes a *Prototheca* lipid droplet protein 1 having at least 95% sequence identity with SEQ ID NO:120, wherein the coding sequence is operably linked to an exogenous nucleic acid sequence.

2. The recombinant nucleic acid of claim 1, wherein the protein has at least one amino acid substitution in comparison to SEQ ID NO:120.

3. The recombinant nucleic acid of claim 1, wherein the exogenous nucleic acid sequence comprises a promoter.

4. The recombinant nucleic acid of claim 1, wherein the exogenous nucleic acid sequence comprises an untranslated control element.

5. The recombinant nucleic acid of claim 1, wherein the exogenous nucleic acid sequence encodes a targeting sequence.

6. The recombinant nucleic acid of claim 5, wherein the targeting sequence is a transit peptide selected from the group of plastidial targeting sequence and mitochondrial targeting sequence.

7. The recombinant nucleic acid of claim 1, wherein the nucleic acid is a DNA molecule.

8. The recombinant nucleic acid of claim 1 that encodes a functional *Prototheca* LPD1.

9. The recombinant nucleic acid of claim 1 that further encodes a sucrose invertase.

10. The recombinant nucleic acid of claim 1 that further encodes an inhibitory RNA that suppresses expression of a *Prototheca* lipid biosynthesis gene.

11. An expression cassette comprising a recombinant nucleic acid of claim 1.

12. A genetically engineered microbial cell transformed with a recombinant nucleic acid of claim 1.

13. A method for obtaining microbial oil comprising culturing a genetically engineered *Prototheca* cell of claim 12 under conditions such that oil is produced.

WHAT IS CLAIMED IS:

1. A recombinant nucleic acid comprising a coding sequence that encodes a *Prototheca* lipid biosynthesis protein, provided that the protein is not ketoacyl acyl carrier protein synthase II, stearoyl acyl carrier protein desaturase, or delta 12 fatty acid desaturase.
2. The recombinant nucleic acid of claim 1 wherein the lipid biosynthesis protein is selected from a protein in Table 1.
3. The recombinant nucleic acid of claim 2 wherein the protein has at least one point mutation in comparison to a protein in Table 1.
4. The recombinant nucleic acid of claim 1 wherein the protein has at least 50%, 60%, 70%, 80%, 85%, 90% or 95% sequence identity to a protein in Table 1.
5. The recombinant nucleic acid of any one of claims 1 to 4, wherein the coding sequence is in operable linkage with a promoter.
6. The recombinant nucleic acid of any one of claims 1 to 5, wherein the coding sequence is in operable linkage with an untranslated control element.
7. The recombinant nucleic acid of any one of claims 1 to 6, wherein the coding sequence is in operable linkage with a targeting sequence.
8. The recombinant nucleic acid of claim 7, wherein the targeting sequence is a transit peptide selected from the group of plastidial targeting sequence and mitochondrial targeting sequence.
9. The recombinant nucleic acid of any one of claims 1 to 8, wherein the nucleic acid is a DNA molecule.
10. The recombinant nucleic acid of any one of claims 1 to 9 that encodes a functional *Prototheca* lipid biosynthesis protein.
11. The recombinant nucleic acid of claim 3 wherein the protein is diacylglycerol diacyltransferase (DGAT) having at least one point mutation.
12. The recombinant nucleic acid of any one of claims 1 to 11 that further encodes a sucrose invertase.
13. The recombinant nucleic acid of any of one of claims 1 to 12 that further encodes an inhibitory RNA that suppresses expression of a *Prototheca* lipid biosynthesis gene.
14. An expression cassette comprising a recombinant nucleic acid of any one of claims 1 to 13.

15. A genetically engineered microbial cell transformed with a recombinant nucleic acid of any one of claims 1 to 13.

16. The genetically engineered microbial cell of claim 15, wherein the cell is a cell of the genus *Prototheca* or *Chlorella.*

17. The genetically engineered microbial cell of claim 16, wherein the cell is a cell of the genus *Prototheca.*

18. The cell of claim 17, wherein said cell comprises both an endogenous lipid biosynthesis gene and one or more exogenous *Prototheca* lipid biosynthesis gene selected from the genes listed in Table 1.

19. The cell of claim 18, wherein said exogenous gene encodes a lipid biosynthesis protein and wherein the amino acid sequence of the lipid biosynthesis protein is identical to the endogenous lipid biosynthesis protein.

20. The cell of claim 19,wherein said exogenous gene comprises a nucleotide sequence in which the codons of the nucleotide sequence encoding the amino acids of the lipid biosynthesis protein have been altered.

21. The cell of any one of claims 15 to 20, wherein said exogenous gene encodes a protein with at least 50% amino acid identity to a native *Prototheca* protein.

22. The cell of any of claims 15 to 21, wherein said exogenous gene is in operable linkage with a promoter element that is not a native *Prototheca* promoter.

23. The cell of any of claims 15 to 22, wherein said exogenous gene is in operable linkage with an untranslated control element that is not a native *Prototheca* untranslated control element.

24. The cell of any of claims 15 to 23 wherein said exogenous gene is in operable linkage with a nucleotide sequence encoding a transit peptide that is not a native *Prototheca* transit peptide.

25. The cell of claim 24, wherein the transit peptide is a plastidial targeting sequence or a mitochondrial targeting sequence.

26. The cell of any of claims 15 to 25, wherein the cell has a 23S rRNA sequence with at least 75% nucleotide identity to SEQ ID NO: 5.

27. The *Prototheca* cell of any one of claims 15 to 26, wherein the cell has a fatty acid profile that is at least 10% C8-C14.

28. The *Prototheca* cell of any one of claims 15 to 26, wherein the cell has a fatty acid profile that is at least 50% C12.

29. The *Prototheca* cell of any one of claims 15 to 26, wherein the cell has a fatty acid profile that is at least 70% C18:1.

30. A method for obtaining microbial oil comprising culturing a genetically engineered *Prototheca* cell of any one of claims 15 to 29 under conditions such that oil is produced.

31. A microbial oil produced by the method of claim 30.

32. A genetically engineered cell of the genus *Prototheca* wherein the activity of one or more endogenous lipid biosynthesis gene selected from the genes listed in Table 1 has been attenuated.

33. The cell of claim 32, wherein the activity of the endogenous gene has been attenuated through chromosomal gene deletion, chromosomal gene insertion, frameshift mutation, point mutation, or inhibitory RNA.

34. The genetically engineered cell of claims 32 or 33, further comprising an exogenous *Prototheca* lipid biosynthesis pathway gene selected from the genes listed in Table 1.

35. The genetically engineered cell of any one of claims 32 to 34, wherein one or more allele of an endogenous lipid biosynthesis gene is attenuated.

36. The genetically engineered cell of any one of claims 32 to 35, wherein one allele of the endogenous lipid biosynthesis gene is replaced with an exogenous *Prototheca* lipid biosynthesis pathway gene selected from Table 1 and a selectable marker.

37. The genetically engineered cell of any one of claims 32 to 36 wherein two or more alleles of the endogenous lipid biosynthesis gene are replaced with an exogenous *Prototheca* lipid biosynthesis pathway gene selected from Table 1 and a selectable marker.

38. The genetically engineered cell of any one of claims 15 to 37 wherein the cell has a 23S rRNA sequence with at least 75% nucleotide identity to SEQ ID NO:5.

39. The *Prototheca* cell of any of one of claims 32 to 35 wherein the cell has a fatty acid profile that is at least 10% C8-C14.

40. A method for obtaining microbial oil comprising culturing a genetically engineered *Prototheca* cell of any one of claims 32 to 39 under conditions such that oil is produced.

41. A microbial oil produced by the method of claim 40.