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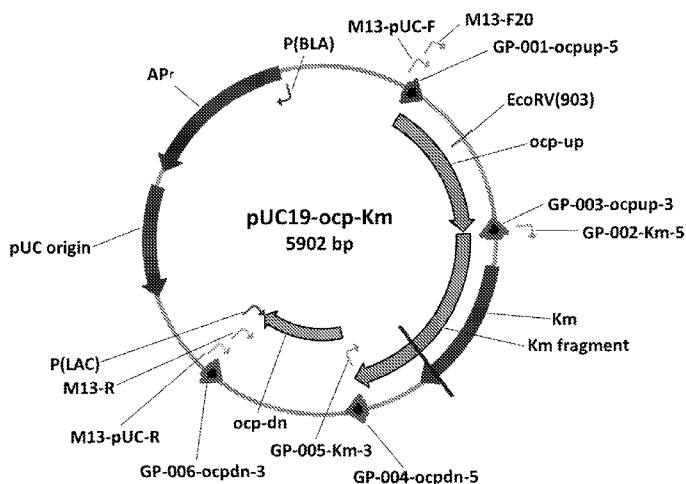


FIGURE 1

(57) **Abstract:** The invention provides a method of producing biomass or at least one biomolecule comprising culturing a photosynthetic microorganism that comprises a disrupted Non-Photochemical Quenching (NPQ) process, and isolating biomass or at least one biomolecule from the culture.

**ENHANCEMENT OF BIOMASS PRODUCTION BY DISRUPTION OF LIGHT  
ENERGY DISSIPATION PATHWAYS**

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of priority to United States provisional patent application 61/428,876 filed December 31, 2010 entitled "Enhancement of Biomass Production by Disruption of Light Energy Dissipation Pathways which is hereby incorporated by reference in its entirety.

REFERENCE TO A SEQUENCE LISTING

[0002] This application contains references to amino acid sequences and/or nucleic acid sequences which have been submitted concurrently herewith as the sequence listing text file entitled "2010EM389 (PM0004) sequences\_ST25.txt", file size 62.5 KiloBytes (KB), created on December 20, 2011. The aforementioned sequence listing is hereby incorporated by reference in its entirety pursuant to 37 C.F.R. §1.52(e)(5).

TECHNICAL FIELD

[0003] The present invention relates, in some embodiments, to methods for culturing photosynthetic microorganisms for making various products. In some aspects, the invention relates to methods for producing biomass or biomolecules that include culturing a photosynthetic microorganism that comprises a disrupted Non-Photochemical Quenching (NPQ) process.

BACKGROUND

[0004] Photosynthesis is the conversion of light energy to chemical energy by biological systems. The first step of photosynthesis is the absorption of light by pigment-protein complexes. These complexes channel light energy to the photosynthetic reaction center, where light energy excites electrons that are transferred from pigment molecules through an electron transport chain that harvests the energy for biochemical reactions.

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[0005] Microalgae, such as cyanobacteria, can be cultured photosynthetically for the production of various products, including proteins, peptides, amino acids, carbohydrates, isotopically labeled compounds, terpenoids, carotenoids, pigments, vitamins, and lipids, where light provides the energy for growth and biosynthesis of the algal products. Microalgal production systems can utilize open ponds (Ben-Amotz (1995) *J. Appl Phycol* 7: 65-68; Olaizola (2000) *J. Appl Phycol* 12: 499-506) or photobioreactors (Olaizola (2000) *J. Appl Phycol* 12: 499-506; Xu et al. (2009) *Eng. Life Sci* 9: 178-189; Lehr and Posten (2009) *Curr Opinion Biotechnol* 20: 280-285; US2009/0011492; WO2011/143619) where the energy for growth and production may be provided by natural or artificial light. In order to minimize production costs and maximize volumetric yield of photosynthetic microorganisms, it is desirable for the photosynthetic microorganisms to be grown in large volumes that reach high cell density. However, light penetration of an algal culture declines dramatically as culture depth and cell density increase. Active mixing of algal cultures propagated in a pond or photobioreactor allows the cultured algal cells to be exposed to higher levels of light intermittently when they are in proximity to the surface or light-facing boundary of a pond or photobioreactor. Actively mixed cultures cells experience some time periods of sub-optimal light, as well as some periods when the cells are at or close to the surface or perimeter of a pond or bioreactor where light may be super-saturating.

[0006] Algae typically use only a percentage of the solar radiation incident on a pond surface, and photosynthesis can be inhibited by excess solar radiation. When photosynthetic microorganisms are exposed to light of an intensity that is greater than the capacity for photosynthetic utilization, as may occur at the upper level of a pond or the periphery of a photobioreactor culture, the photosynthetic microorganisms may engage mechanisms for light energy dissipation to limit damage to the photosynthetic apparatus that might otherwise be caused by absorption of excess light energy.

[0007] Light energy can be lost from the pigment-protein complexes through mechanisms including fluorescence or by cell-regulated processes such as Non-Photochemical Quenching (NPQ). The qE component of NPQ is a protective mechanism that quenches singlet-excited chlorophylls (Chl) and harmlessly dissipates

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excess excitation energy as heat. These NPQ processes help to regulate and protect the photosynthetic apparatus from damage in environments in which light energy absorption exceeds the capacity for light utilization. In the absence of intrinsic NPQ mechanisms, such as energy dissipation mediated by carotenoids, photosynthetic organisms can incur photooxidative damage under water or nutrient limitation, low temperatures, and/or high light intensity (Demmig-Adams et al. (1996) *FASEB J.* 10: 403-412). In many cyanobacterial species, the Orange Carotenoid Protein binds the carotenoids zeaxanthin, echinenone, and/or hydroxyechinenone, and serves a photoprotective function in these species. *Synechocystis* cells having a mutant OCP gene had a greater decrease in photosynthetic activity than corresponding wild type cells in response to high light intensity (Wilson et al. *The Plant Cell* (2006) 18: 992-1007, and cyanobacterial species lacking an OCP gene were more photosynthetically impaired under high light conditions than species that have an OCP gene (Boulay et al. (2008) *Biochimica et Biophysica Acta* 1777: 1344-1354).

#### SUMMARY OF THE INVENTION

[0008] The invention provides a method of producing biomass or least one biomolecule comprising culturing a photosynthetic microorganism that comprises a disrupted Non-Photochemical Quenching (NPQ) process under conditions sufficient for the microorganism to proliferate in the culture, and isolating biomass or at least one biomolecule from the culture. In some embodiments, the amount of biomass or a biomolecule produced by the culture is at least 10% greater than the amount of biomass or a biomolecule produced by an identical culture of a microorganism identical in all respects except that it does not have a disrupted NPQ process. Additionally or alternately, the photosynthetic microorganism can be cultured phototrophically and/or under intermittent light conditions, optionally including natural light. The photosynthetic microorganism can be cultured in a culture system that includes active mixing during at least a portion of the time the culture is exposed to light (the light period). For example, the photosynthetic microorganism can be cultured in a pond or photobioreactor, such as a pond having a depth of at least 3 centimeters (cm), at least 5 cm, or at least 10 cm, or a photobioreactor having a light path of at least 3 cm, at least 5 cm, or at least 10 cm, where the culture is actively mixed. For example, the culture can

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be mixed using one or more powered paddle wheels, propellers, agitating devices, pumps, spargers, or injectors. Further additionally or alternately, the photosynthetic microorganism can be cultured in a volume of at least 20 liters of culture medium and/or can produce at least 0.1 g, for example at least 0.2 g or at least 0.3 g, of ash-free dry weight biomass per liter of culture.

[0009] A disrupted NPQ process in preferred embodiments comprises disrupted or reduced production of at least one carotenoid or at least one carotenoid binding protein in the photosynthetic microorganism, in which the microorganisms with a disrupted NPQ process exhibits less NPQ than does a control photosynthetic microorganism in which synthesis of a carotenoid or expression of a carotenoid binding protein is not disrupted. In some embodiments, disrupting the NPQ process can comprise disrupting the production of at least one carotenoid such as echinenone and/or hydroxyechinenone and/or disrupting the production of at least one carotenoid binding protein such as orange carotenoid protein (OCP). In such embodiments, disrupting the NPQ process can comprise reducing or inhibiting the expression of the OCP in the microorganism, *e.g.*, by removing all or a portion of an OCP gene in the microorganism, by disrupting an OCP gene of a photosynthetic microorganism by insertional mutagenesis, and/or by reducing expression of an OCP gene in the microorganism, for example, by expression of antisense or ribozyme constructs.

[0010] The photosynthetic microorganism used in the methods provided herein can be any photosynthetic microorganism that includes an NPQ process, such as an NPQ process mediated by a carotenoid binding protein. Additionally or alternately, the photosynthetic microorganism can be a cyanobacterium. In some embodiments, the photosynthetic microorganism can be a cyanobacterium that includes a gene encoding an Orange Carotenoid Protein. As nonlimiting examples, the cyanobacterium can be a species of an *Agmenellum*, *Anabaena*, *Anabaenopsis*, *Anacystis*, *Aphanizomenon*, *Arthrospira*, *Asterocapsa*, *Borzia*, *Calothrix*, *Chamaesiphon*, *Chlorogloeopsis*, *Chroococciopsis*, *Chroococcus*, *Crinalium*, *Crocospaera*, *Cyanobacterium*, *Cyanobium*, *Cyanocystis*, *Cyanospira*, *Cyanothece*, *Cylindrospermopsis*, *Cylindrospermum*, *Dactylococcopsis*, *Dermocarpella*, *Fischerella*, *Fremyella*,

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*Geitleria*, *Geitlerinema*, *Gloeobacter*, *Gloeocapsa*, *Gloeotheca*, *Halospirulina*, *Iyengariella*, *Leptolyngbya*, *Limnothrix*, *Lyngbya*, *Microcoleus*, *Microcystis*, *Myxosarcina*, *Nodularia*, *Nostoc*, *Nostochopsis*, *Oscillatoria*, *Phormidium*, *Planktothrix*, *Pleurocapsa*, *Prochlorococcus*, *Prochloron*, *Prochlorothrix*, *Pseudanabaena*, *Rivularia*, *Schizothrix*, *Scytonema*, *Spirulina*, *Stanieria*, *Starria*, *Stigonema*, *Symploca*, *Synechococcus*, *Synechocystis*, *Thermosynechococcus*, *Tolypothrix*, *Trichodesmium*, *Tychonema*, or *Xenococcus* genus. For example, the genetically engineered cyanobacterium can be a species of cyanobacteria that includes an endogenous gene encoding a protein that recruits to pfam PF09150 (the "Carot N" protein family) with a bit score of less than 25.0, and preferably with an e value of less than 0.01 or a protein having at least 40%, for example at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95%, amino acid sequence identity to SEQ ID NO:2. In some examples, the photosynthetic microorganism is a species of *Synechocystis*, *Arthrospira*, *Microcystis*, *Lyngbya*, *Nostoc*, *Anabaena*, *Synechococcus*, *Gloeobacter*, *Crocospaera*, or *Thermosynechococcus*.

[0011] For example, the photosynthetic microorganism having a disrupted NPQ process can have attenuated expression of a carotenoid binding protein having at least 40%, for example at least 45%, at least 50%, for example at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95%, identity to SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, or SEQ ID NO:24. The photosynthetic microorganism that has a disrupted NPQ process (*e.g.*, that has attenuated expression of an OCP gene) can produce at least one biomolecule such as a lipid, protein, peptide, amino acid, carbohydrate, isotopically labeled compound, vitamin, nucleotide, pigment, terpenoid, carotenoid, etc., or any combination thereof. For example, the microorganism can produce a lipid, such as a free fatty acid and/or a fatty acid derivative. The free fatty acid derivative can be, for example, a fatty aldehyde, a fatty alcohol, a fatty acid ester, a wax ester, an alkene,

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and/or an alkane. In some embodiments, the amount of free fatty acid or fatty acid derivative produced can be greater than the amount of free fatty acid or fatty acid derivative produced by a strain of photosynthetic microorganism identical in all respects except that it does not have a disrupted NPQ process that is grown under identical conditions.

[0012] In further aspects, the invention includes methods of producing a biomolecule, in which the methods comprise culturing a photosynthetic microorganism that comprises a disrupted Non-Photochemical Quenching (NPQ) process in which the photosynthetic microorganism includes at least one recombinant nucleic acid molecule that participates in or directs the biosynthesis the biomolecule under conditions in which the recombinant nucleic acid molecule is expressed, to produce the biomolecule. The microorganism can be cultured in a suitable culture medium, which in some examples can be a culture medium that does not included a substantial amount of a reduced carbon source, such that the cells are cultured photoautotrophically. Additionally, the culture medium can include inorganic carbon as substantially the sole source of carbon for production of the biomolecule. The photosynthetic microorganism can be cultured in pond or photobioreactor, for example a pond having a depth of at least 3 cm, or a photobioreactor having a light path of at least 3 cm, where preferably the culture undergoes active mixing. Additionally, the method can further include isolating the biomolecule from the microorganism, the culture medium, or both. The biomolecule can be any biomolecule, including, for example, a lipid, protein, carbohydrate, vitamin, peptide, amino acid, nucleotide, pigment, isotopically labeled compound, etc., or any combination thereof.

[0013] The invention also includes a photosynthetic microorganism having a disrupted NPQ process that includes at least one non-native gene for the production of a biomolecule such as a protein, lipid, pigment, terpenoid, carotenoid, vitamin, peptide, amino acid, or nucleotide. For example, a photosynthetic microorganism having a disrupted NPQ process can in some embodiments comprise one or more recombinant nucleic acid molecules that encode one or more proteins that participate in the biosynthesis of one or more lipids, such as, but not limited to, one or more free fatty acids and/or fatty acid derivatives. For example, the photosynthetic microorganism

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having a disrupted NPQ process can in some embodiments comprise one or more recombinant nucleic acid molecules that encode a thioesterase and/or a polypeptide having lipolytic activity, for example, a recombinant or exogenous nucleic acid molecule encoding one or more of the following: an acyl-ACP thioesterase; an acyl-CoA thioesterase; a hydroxybenzoyl-CoA thioesterase; a lipase that is a member of a pfam belonging to the AB Hydrolase pfam clan (CL0028); a lipase that includes a LipA domain identified as conserved protein domain COG1075, or is included in the protein family Pfam PF01674; s a lipase that includes a Lipase 3 domain identified as conserved protein domain COG3675, or is included in the protein family Pfam PF01764; a lipase that includes a Lipase 3 domain identified as conserved protein domain COG3675, or is included in the protein family Pfam PF01764; a lipase that is included in the protein family Pfam PF07819; a lipase that is included in the protein family Pfam PF03583; a lipase that is included in the protein family Pfam PF00151; or a polypeptide having lipolytic activity that recruits to Pfam PF00561, Pfam PF02230, Pfam PF07859, Pfam PF08386, Pfam PF12695, Pfam PF12697, Pfam PF12715, or Pfam PF04083, or Pfam PF01425.

[0014] Additionally or alternately, a photosynthetic microorganism having a disrupted NPQ process can in some embodiments comprise one or more recombinant nucleic acid molecules encoding a protein that participates in or directs the synthesis of a lipid, such as but not limited to an acetyl-CoA carboxylase, a malonyl CoA: ACP transacylase, a beta-ketoacyl-ACP synthase, or a combination thereof. Further additionally or alternately, a photosynthetic microorganism having a disrupted NPQ process can include one or more recombinant nucleic acid molecules encoding a protein that participates in the production of a fatty acid derivative, for example, one or more recombinant nucleic acid molecules encoding any combination of an acyl-CoA reductase, a carboxylic acid reductase, an acyl-ACP reductase, a fatty aldehyde reductase, a wax synthase, a fatty acid decarboxylase, a fatty aldehyde decarbonylase, and/or an acyl-CoA synthetase.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0015] Figure 1 shows a construct for knock-out of an OCP gene in *Synechocystis*.

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[0016] Figure 2 graphically shows the output of Chlorophyll-a Pulse Amplitude Modulated Fluorometry on *Synechocystis* PCC6803, showing that the application of high blue light (+) induces NPQ in WT (A) but not in  $\Delta$ OCP(B) NPQ can be determined by the reduction in fluorescence yield of a saturating flash of light (shown as spikes). A lower spike after the + indicates higher NPQ.

[0017] Figure 3 shows daily biomass production of *Synechocystis* cultures grown over 4 days.

#### DETAILED DESCRIPTION

[0018] As described herein, the present invention provides a method of producing biomass or at least one biomolecule comprising culturing a photosynthetic microorganism that comprises a disrupted Non-Photochemical Quenching (NPQ) process. The microorganism can be cultured photoautotrophically, for example, in a pond or photobioreactor, and can be cultured under intermittent light conditions, for example, under conditions in which the culture is actively mixed, such as by the use of one or more pumps, gas or liquid injectors, spargers, paddlewheels, propellers, or other mechanical devices for mixing, that can optionally be used in ponds or bioreactors that include one or more stationary structures that promote mixing or turbulence, and combinations thereof.

[0019] A biomolecule produced using the methods can be, without limitation, a protein, polymer, pigment, vitamin, peptide, amino acid, terpenoid, and/or lipid (*e.g.*, monoacylglyceride, diacylglyceride, triacylglyceride, fatty acid, fatty acid derivative, or the like). Advantageously, a biomolecule can be recovered from the culture, such as from the culture medium, the microorganism, or a combination thereof. A "biomolecule" refers to any organic molecule that is produced by a living organism, including large polymeric molecules. Of particular relevance to the present invention are biomolecules that are useful, for example, as fuels, fuel additives, or fuel precursors, including fuel feedstocks, as well as biomolecules that are useful as chemical, lubricants, surfactants, and/or detergents. In some preferred embodiments, a biomolecule produced using the methods of the invention can be a monoglyceride, diglyceride, triglyceride, free fatty acid, fatty acid derivative, or combination thereof.

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[0020] Alternatively or in addition, biomass can be recovered from the culture, and can optionally be used, for example, to extract, isolate, or purify one or more biomolecules or biomass components, or biomass itself can be a product of the culture where the recovered biomass can be used in further processes for example, for producing heat, energy, nutrients, syngas, one or more alcohols, etc. or can be used as a food supplement or animal feed or supplement. "Biomass" refers to organic matter stored from plants and other living things and can be regarded as an energy source, including, but not limited to, an energy source that can be converted to fuel or fuel feedstocks. Photosynthetic organisms perform photosynthesis, absorb carbon dioxide (CO<sub>2</sub>), and convert it to biomass. When that biomass is burned, it is possible to recover heat energy. Alternatively, biomass can be added to food or animal feed, or can be converted to organic molecules (*e.g.*, alcohols, through fermentation) that can be fuel or chemical feedstocks.

[0021] In some embodiments, the amount of biomass or of a biomolecule produced by the culture can be at least 10%, for example at least 15%, at least 20%, or at least 25%, greater than the amount of a biomolecule produced by an identical culture of a microorganism identical in all respects except that it does not have a disrupted NPQ process. Additionally or alternately, the photosynthetic microorganism can be cultured phototrophically and/or under intermittent light conditions, *e.g.*, in an actively mixed culture, optionally under natural light.

[0022] Further additionally or alternately, the photosynthetic microorganism can be cultured in a volume of at least 20 liters, for example at least 50 liters, at least 100 liters, at least 200 liters, or at least 400 liters, of culture medium, and/or the photosynthetic microorganism can produce at least 0.1 g, for example at least 0.2 g or at least 0.3 g, of ash-free dry weight biomass per liter of culture.

[0023] A "disrupted NPQ process" preferably comprises disrupted production of at least one carotenoid and/or reduced expression of at least one carotenoid binding protein in the photosynthetic microorganism, in which the microorganisms with a disrupted NPQ process can advantageously exhibit less NPQ than does a control photosynthetic microorganism in which synthesis of a carotenoid and/or expression of a

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carotenoid binding protein is not disrupted. In some embodiments, disrupting the NPQ process can comprise disrupting the production of at least one carotenoid such as echinenone and/or hydroxyechinenone and/or disrupting the production of at least one carotenoid binding protein such as OCP, *e.g.*, by reducing or inhibiting the expression of the OCP in the microorganism, such as by removing all or a portion of an OCP gene in the microorganism, by disrupting (such as, *e.g.*, by insertional mutagenesis) an OCP gene of a photosynthetic microorganism, by the use of antisense constructs, and/or by otherwise reducing expression of an OCP gene in the microorganism.

### Microorganisms

[0024] The genetically engineered microorganism in the present invention can be any photosynthetic microorganism, including without limitation, a cyanobacterium, alga, or the like. Photosynthetic microorganisms useful as host organisms can include, but are not limited to, any cyanobacteria that include an endogenous gene encoding an orange carotenoid protein (OCP). The microorganisms according to some embodiments of the present invention can include, but not limited to, the following genera of cyanobacteria: *Agmenellum*, *Anabaena*, *Anabaenopsis*, *Anacystis*, *Aphanizomenon*, *Arthrospira*, *Asterocapsa*, *Borzia*, *Calothrix*, *Chamaesiphon*, *Chlorogloeopsis*, *Chroococciopsis*, *Chroococcus*, *Crinalium*, *Crocospaera*, *Cyanobacterium*, *Cyanobium*, *Cyanocystis*, *Cyanospira*, *Cyanothece*, *Cylindrospermopsis*, *Cylindrospermum*, *Dactylococcopsis*, *Dermocarpella*, *Fischerella*, *Fremyella*, *Geitleria*, *Geitlerinema*, *Gloeobacter*, *Gloeocapsa*, *Gloeothece*, *Halospirulina*, *Iyengariella*, *Leptolyngbya*, *Limnothrix*, *Lyngbya*, *Microcoleus*, *Microcystis*, *Myxosarcina*, *Nodularia*, *Nostoc*, *Nostochopsis*, *Oscillatoria*, *Phormidium*, *Planktothrix*, *Pleurocapsa*, *Prochlorococcus*, *Prochloron*, *Prochlorothrix*, *Pseudanabaena*, *Rivularia*, *Schizothrix*, *Scytonema*, *Spirulina*, *Stanieria*, *Starria*, *Stigonema*, *Symploca*, *Synechococcus*, *Synechocystis*, *Thermosynechococcus*, *Tolypothrix*, *Trichodesmium*, *Tychonema*, and *Xenococcus*. Cyanobacterial species that include one or more carotenoid binding proteins that participate in NPQ, such as for example a carotenoid binding protein that binds echinenone or hydroxyechinenone may be used in the methods provided herein, including but not limited to cyanobacterial species having an endogenous gene encoding a protein that recruits to pfam PF09150

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(the “Carot N” protein family) with a bit score of less than 25.0, and preferably with an e value of less than 0.01 or that has at least 40%, for example at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95%, amino acid sequence identity to SEQ ID NO:2. The microorganism can be, for example, a species of *Synechocystis*, *Arthrospira*, *Microcystis*, *Lyngbya*, *Nostoc*, *Anabaena*, *Synechococcus*, or *Gloeobacter*.

[0025] A number of cyanobacterial species are known and have been manipulated using molecular biological techniques, including the unicellular cyanobacteria *Synechocystis* sp. PCC6803 and *Synechococcus elongatus* PCC7942, whose genomes have been completely sequenced.

[0026] In some embodiments, the cyanobacterial strain used in the methods of the invention can be a strain having a disrupted NPQ process and at least one recombinant nucleic acid molecule that encodes a protein that participates in and/or directs the production of at least one lipid, such as a fatty acid or fatty acid derivative (*e.g.*, a fatty aldehyde, fatty alcohol, wax ester, alkane, and/or alkene).

#### Disruption of Non-Photochemical Quenching (NPQ) in Microorganisms

[0027] “NonPhotochemical Quenching” or “NPQ” is used herein to refer to an *in vivo* chlorophyll fluorescence quenching process that does not involve photochemistry (in which the energy of captured photons is transferred to chemical reactants, for example, components of the photosynthetic electron transport chain). NPQ can be observed by obtaining by measuring the maximal fluorescence ( $F_m$ ) from dark adapted cells or chloroplasts stimulated with saturating light, and, after a series of light flashes observing a later maximal fluorescence ( $F_m'$ ) in response to a light flash, where the reduction in fluorescence from  $F_m$  to  $F_m'$  is due to NPQ.

[0028] In some photosynthetic microorganisms, NPQ can be induced from the activation of a carotenoid, such as echinenone and/or hydroxyechinenone, and/or the activity of a carotenoid binding protein, such as Orange Carotenoid Protein (OCP). As disclosed in the examples herein, disrupting the production of one or more carotenoids and/or a carotenoid binding protein, such as OCP, in a microorganism can increase the biomass production of the microorganism.

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[0029] “Orange Carotenoid Protein” or “OCP” is a protein that binds the carotenoid hydroxyechinenone (*e.g.*, 3'-hydroxyechinenone) and/or echinenone and contributes to nonphotochemical quenching. Nonlimiting examples of OCP include proteins that recruit to the protein family (Pfam) PF09150 with a bit score higher than the gathering cutoff for the family of 25.0, and preferable with an *e* value of less than 0.01, and proteins having the following Genbank Accession numbers and Gene Identifiers: ZP\_03274607, GI:209526075 from *Arthrospira maxima* (SEQ ID NO:4); YP\_001656905, GI:166364632 from *Microcystis aeruginosa maxima* (SEQ ID NO:5); ZP\_01624422, GI:119493856 from *Lyngbya* sp. PCC 8106 (SEQ ID NO:6); YP\_002379699, GI:218441370 from *Cyanothece* sp. PCC 7424 (SEQ ID NO:7); ZP\_01726229, GI:126654695 from *Cyanothece* sp. CCY 0110 (SEQ ID NO:8); YP\_001803065, GI:172036564 from *Cyanothece* sp. ATCC51142 (SEQ ID NO:9); YP\_001868419, GI:186685223 from *Nostoc punctiforme* PCC73102 (SEQ ID NO:10); NP\_487189, GI:17230641 from *Nostoc* sp. PCC7120 (SEQ ID NO:11); YP\_324343, GI:75910047 from *Anabaena variabilis* ATCC29413 (SEQ ID NO:12); ZP\_07112967, GI:300868341 from *Oscillatoria* sp. PCC 6506 (SEQ ID NO:13); ZP\_05028361, GI:254414596 from *Microcoleus chthonoplastes* PCC 7420 (SEQ ID NO:14); NP\_926881, GI:37523504 (SEQ ID NO:15); ZP\_01632514, GI:119513491 from *Nodularia spumigena* CCY9414 (SEQ ID NO:16); ZP\_06309354, GI:282901429 from *Cylindrospermopsis raciborskii* CS-505 (SEQ ID NO:17); ZP\_01080542, GI:87124694 from *Synechococcus* sp. RS9917 (SEQ ID NO:18); YP\_001736034, GI:170079396 from *Synechococcus* sp. PCC 7002 (SEQ ID NO:19); ZP\_06304225, GI:282896202 from *Raphidiopsis brookii* D9 (SEQ ID NO:20); ZP\_01123774, GI:88808264 from *Synechococcus* sp. WH 7805 (SEQ ID NO:21); ZP\_01468054, GI:116070785 from *Synechococcus* sp. BL107 (SEQ ID NO:22); YP\_001228248, GI:148243091 from *Synechococcus* sp. RCC307 (SEQ ID NO:23); and YP\_376983, GI:78184548 from *Synechococcus* sp. CC9902 (SEQ ID NO:24). OCPs can additionally or alternately include proteins having amino acid sequences that are at least 50%, for example at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95%, identical to the aforementioned proteins, where the

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reducing or disrupting expression of the protein in a photosynthetic microorganism reduces a nonphotochemical quenching process.

[0030] The invention provides methods of producing biomass or at least one biomolecule that can include culturing a cyanobacterium having a disrupted NPQ process in a culture volume of at least 20 liters, for example at least 50 liters, at least 100 liters, at least 200 liters, or at least 400 liters. NPQ can be reduced in the photosynthetic microorganism used in the methods by at least 5%, for example by at least 10%, at least 15%, or at least 20%, with respect to a photosynthetic microorganism identical in all respects except that it does not have a disrupted NPQ process. As provided in the Examples herein, photosynthetic microorganisms having a disrupted NPQ process can unexpectedly exhibit higher productivity than corresponding microorganisms having an intact (nondisrupted) NPQ process. Without limiting the invention to any particular mechanism, it is contemplated that in a production system in which cultures are mixed for optimal exposure to light, CO<sub>2</sub>, and nutrients, the light environment for individual cells can change quickly as mixing occurs. Thus, although only a small portion of the time might be spent in conditions where light-intensity dependent damage might occur, NPQ may be activated and extend into periods of time when the cells are not experiencing excess light. The excessive duration of NPQ activation may reduce effectiveness of overall photosynthesis by preventing a cell from utilizing available light energy for photochemistry. Thus, although NPQ processes may be photoprotective in the natural state, in a mixed culture production system they may be disadvantageous.

[0031] Disruption of an NPQ process can be by disruption of the production of one or more carotenoids such as one or more ketocarotenoids, for example, echinenone and/or hydroxyechinenone, which can occur, *e.g.*, by attenuating the expression of a gene on the biosynthetic pathway for these carotenoids. For example, production of a ketocarotenoid such as echinenone and/or hydroxyechinenone can be reduced and/or eliminated by mutation, downregulation, and/or insertional inactivation of a gene that encodes an enzyme that participates in the biosynthesis of beta-carotene, or that encodes an enzyme that participates in the conversion of beta-carotene to echinenone

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and/or hydroxyechinenone, such as for example, beta carotene hydroxylase (encoded by the crtR gene) and/or beta-carotene monoketolase (encoded by the crtO gene).

[0032] Preferably, disruption of the NPQ process does not include attenuating the expression of one or more proteins that may serve as a light absorbing antenna for photosynthetic light harvesting. For example, preferably disruption of the NPQ process does not include attenuating the expression of a chlorophyll-binding protein, such as, for example, one or more light harvesting chlorophyll binding proteins (LHCPs) that serve as an antenna for absorbing light energy for transfer to a photosynthetic reaction center.

[0033] Alternately or in addition, the expression of OCP can be disrupted by inhibiting or reducing expression of the OCP gene, and can include, for example, disrupting or deleting the OCP gene such that the OCP is not synthesized. For example, all or a portion of the OCP gene (and/or a gene encoding another carotenoid binding protein that participates in NPQ) can be deleted. In particular embodiments, when the OCP protein gene expression in a cyanobacterium is abolished or reduced, the biomass production of the cell can increase compared to a photosynthetic microorganism identical in all respects except that it does not have a disrupted OCP gene.

[0034] A gene encoding an OCP can be disrupted by replacement of all or a portion of the protein coding sequence or any part of the gene regulatory sequence of the OCP in the organism. Still further additionally or alternately, a gene can be attenuated by insertion of a sequence into the protein coding region and/or regulatory region of an OCP gene. Disruption by any such means can be performed, for example, by homologous recombination, which is well-established in cyanobacteria, among other organisms, or by using site-specific recombination in combination with homologous recombination. In preferred embodiments, gene disruption can result in a reduction in the amount of OCP produced by the engineered microorganism by at least 20%, for example by at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 97%, at least 99%, or about 100%, with respect to the same microorganism that does not include a disrupted OCP gene. In some

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preferred embodiments, the genetically engineered photosynthetic microorganism that has a disrupted OCP gene can have no detectable expression of the OCP.

[0035] In other examples, the expression of a gene encoding a polypeptide that functions in NPQ, such as an OCP gene, can be reduced or eliminated by expression of an antisense construct introduced into the photosynthetic microorganism. As used herein, an antisense construct refers particularly to a nucleic acid molecule that includes a sequence that encodes an antisense molecule, *i.e.*, a ribonucleotide sequence having homology to at least a portion of the non-coding strand of a double stranded DNA molecule of a gene that encodes a protein (for example, at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, or about 100% identical to at least a portion of the non-coding strand of a gene). Thus, an antisense molecule or “antisense RNA” can be complementary to at least a portion of the sequence of the coding strand of a double stranded DNA molecule that encodes a polypeptide. In the context of the present invention, an antisense RNA can be complementary to at least a portion of the sequence of the coding strand of a double stranded DNA molecule that encodes a polypeptide that functions in NPQ, *e.g.*, an OCP gene. The antisense sequence may be complementary to protein-coding sequences of the targeted gene, or alternatively or in addition, the antisense sequence may be complementary wholly or in part to noncoding sequences specified on the transcribed strand of a DNA molecule encoding a protein, for example, a 5' untranslated region (UTR) and/or an intron. Antisense sequences are preferably at least 85% complementary, and more preferably at least 90% or at least 95% complementary to the target nucleic acid (gene) sequence. Expression of an antisense construct results in the production of an antisense RNA that has substantial or complete identity to at least a portion of a target gene. The sequence of the antisense RNA can correspond to the full length target gene (*e.g.*, an OCP gene), or to a subsequence thereof. An antisense construct can include an antisense sequence of at least about twenty nucleotides, for example, at least about 25, at least about 30, at least about 40, at least about 50, at least about 100 or at least about 200 nucleotides having at least 85%, at least 90%, or preferably at least 95% identity to a sequence complementary to a transcribed portion of a gene.

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[0036] An antisense construct can additionally include one or more gene regulatory sequences operably linked to the antisense sequence, such as a promoter, where the promoter can be a constitutive or regulatable promoter. For example, a promoter that regulates expression of an antisense sequence can be an inducible promoter. Alternatively or in addition, the antisense construct can be integrated into the host microorganism's genome such that the antisense sequence is juxtaposed with, and operably linked to, an endogenous promoter of the host. In particular examples, the promoter can be an endogenous promoter that is active under the same conditions as the promoter that regulates expression of the endogenous gene encoding a polypeptide that functions in NPQ. In further examples, an antisense construct can include a copy of the same promoter that regulates the expression of the target gene in the host microorganism. Catalytic RNA molecules or ribozymes can also be used to inhibit expression of an OCP gene. For example, one or more ribozymes can be designed to specifically pair with the transcribed OCP-encoding RNA and cleave the phosphodiester backbone at a specific location to functionally inactivate the target RNA.

#### Further Modified Microorganisms

[0037] The present invention cultures of recombinant photosynthetic microorganisms having attenuated expression of a gene that encodes an enzyme for producing echinenone and/or hydroxyechinenone and/or having attenuated expression of a gene that encodes an OCP, can have reduced NPQ and/or can exhibit enhanced biomass production or biomolecule production with respect to a control microorganism. The recombinant photosynthetic microorganism with attenuated expression of a gene that functions in NPQ can, in some embodiments, also include other genetic modifications. Additional genetic modifications can include, without limitation, modifications that enhance the productivity or robustness of the strain. For example, one or more recombinant nucleic acid molecules can be introduced into the strain for directing the production of particular biomolecules, and/or for increasing or decreasing expression of endogenous genes that can directly or indirectly enhance the production of biomass or particular biomolecules in the modified strain.

[0038] For example, for the production of lipids, including fatty acids and/or fatty acid derivatives, the genetically engineered strain having reduced NPQ can be transformed with recombinant or heterologous thioesterase and/or lipase genes capable of producing free fatty acids from membrane lipids or storage lipids, *e.g.*, phospholipids, triacylglycerol, diacylglycerol, monoacylglycerol, or the like, or combinations thereof.

[0039] Lipids are a class of molecules that are typically 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 hydrocarbon tails which are hydrophobic in nature. Examples of lipids include fatty acids (saturated and unsaturated); glycerides or glycerolipids (such as monoglycerides (monoacylglycerides), diglycerides (diacylglycerides), triglycerides (triacylglycerides) or neutral fats, phosphoglycerides or glycerophospholipids, or the like, or combinations thereof); nonglycerides (such as sphingolipids, sterol lipids including cholesterol and steroid hormones, prenol lipids including terpenoids, fatty alcohols, waxes, polyketides, or the like, or combinations thereof); and complex lipid derivatives (such as sugar-linked lipids, or glycolipids, protein-linked lipids, or the like, or a combination thereof). Fats are a subgroup of lipids and can include triacylglycerides.

[0040] Lipases are enzymes that catalyze the hydrolysis of ester bonds in glycerolipids, including, but not limited to, mono-, di-, and tri-acyl glycerols, as well as combinations thereof, to release free fatty acids and alcohols.

[0041] In some embodiments, the present invention relates to recombinant microorganisms transformed with at least one expression system including at least one lipase gene that operates to liberate fatty acids from one or more glycerolipids. In some embodiments of the present invention, the exogenous nucleic acid molecule encoding a thioesterase can include, without limitation, an acyl-ACP thioesterase, an acyl-CoA thioesterase, a hydroxylbenzoyl-CoA thioesterase, or a combination thereof.

[0042] The term “gene” is used broadly to refer to any segment of nucleic acid (typically DNA, but optionally RNA) associated with expression of a given RNA or protein. Thus, genes include sequences encoding expressed RNA (which can include

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polypeptide coding sequences) and, often, the regulatory sequences required for their expression. Genes can be obtained from a variety of sources, including cloning from a source of interest or synthesizing from known or predicted sequence information and may include sequences designed to have desired parameters.

[0043] "Pfam" is a large collection of protein domains and protein families maintained by the Pfam Consortium and available at several sponsored world wide web sites, including: [pfam.sanger.ac.uk/](http://pfam.sanger.ac.uk/) (Wellcome Trust, Sanger Institute); [pfam.sbc.su.se/](http://pfam.sbc.su.se/) (Stockholm Bioinformatics Center); [pfam.janelia.org/](http://pfam.janelia.org/) (Janelia Farm, Howard Hughes Medical Institute); [pfam.jouy.inra.fr/](http://pfam.jouy.inra.fr/) (Institut national de la Recherche Agronomique); and [pfam.cccb.re.kr/](http://pfam.cccb.re.kr/). The latest release of Pfam is Pfam 26.0 (November 2011, 13672 families) based on the UniProt protein database release 15.6, a composite of Swiss-Prot release 57.6 and TrEMBL release 40.6. Pfam domains and families are identified using multiple sequence alignments and hidden Markov models (HMMs). Pfam-A families, which are based on high quality assignments, are generated by a curated seed alignment using representative members of a protein family and profile hidden Markov models based on the seed alignment, whereas Pfam-B families are generated automatically from the non-redundant clusters of the latest release of the Automated Domain Decomposition algorithm (ADDA; Heger A, Holm L (2003) *J Mol Biol* 328(3):749-67). All identified sequences belonging to the family are then used to automatically generate a full alignment for the family (Sonnhammer et al. (1998) *Nucleic Acids Research* 26: 320-322; Bateman et al. (2000) *Nucleic Acids Research* 26: 263-266; Bateman et al. (2004) *Nucleic Acids Research* 32, Database Issue: D138-D141; Finn et al. (2006) *Nucleic Acids Research* Database Issue 34: D247-251; Finn et al. (2010) *Nucleic Acids Research* Database Issue 38: D211-222). By accessing the pfam database, for example, using any of the above-reference websites, protein sequences can be queried against the HMMs using HMMER homology search software (e.g., HMMER3, [hmmer.janelia.org/](http://hmmer.janelia.org/)). Significant matches that identify a queried protein as being in a pfam family (or as having a particular pfam domain) are those in which the bit score is greater than or equal to the gathering threshold for the Pfam domain. The gathering threshold for the pfam Acyl-ACP thioesterase family (PF01643) is 20.3. Expectation values (e values) can also be used as a criterion for inclusion of a queried

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protein in a pfam or for determining whether a queried protein has a particular pfam domain, where low *e* values (much less than 1.0, for example less than 0.1 or less than or equal to 0.01) represent low probabilities that a match is due to chance.

[0044] The phrase “conservative amino acid substitution” or “conservative mutation” refers to the replacement of one amino acid by another amino acid with a common property. A functional way to define common properties between individual amino acids is to analyze the normalized frequencies of amino acid changes between corresponding proteins of homologous organisms (Schulz, G. E. *et al.*, (1979) Principles of Protein Structure, Springer-Verlag). According to such analyses, groups of amino acids can be defined where amino acids within a group exchange preferentially with each other, and therefore resemble each other most in their impact on the overall protein structure (Schulz, G. E. *et al.*, (1979) Principles of Protein Structure, Springer-Verlag). Examples of amino acid groups defined in this manner can include: a “charged/polar group,” including Glu, Asp, Asn, Gln, Lys, Arg, and His; an “aromatic or cyclic group,” including Pro, Phe, Tyr, and Trp; and an “aliphatic group” including Gly, Ala, Val, Leu, Ile, Met, Ser, Thr, and Cys. Within each group, subgroups can also be identified. For example, the group of charged/polar amino acids can be sub-divided into sub-groups including: the “positively-charged sub-group,” comprising Lys, Arg and His; the “negatively-charged sub-group,” comprising Glu and Asp; and the “polar sub-group” comprising Asn and Gln. In another example, the aromatic or cyclic group can be sub-divided into sub-groups including: the “nitrogen ring sub-group,” comprising Pro, His, and Trp; and the “phenyl sub-group” comprising Phe and Tyr. In another further example, the aliphatic group can be sub-divided into sub-groups including: the “large aliphatic non-polar sub-group,” comprising Val, Leu, and Ile; the “aliphatic slightly-polar sub-group,” comprising Met, Ser, Thr, and Cys; and the “small-residue sub-group,” comprising Gly and Ala. Examples of conservative mutations include amino acid substitutions of amino acids within the sub-groups above, such as, but not limited to: Lys for Arg or vice versa, such that a positive charge can be maintained; Glu for Asp or vice versa, such that a negative charge can be maintained; Ser for Thr or vice versa, such that a free -OH can be maintained; and Gln for Asn such that a free -NH<sub>2</sub> can be maintained.

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[0045] A “conservative variant” of a polypeptide is a polypeptide having one or more conservative amino acid substitutions with respect to the reference polypeptide, in which the activity, substrate affinity, binding affinity of the polypeptide does not substantially differ from that of the reference polypeptide. A substitution, insertion, or deletion can be said to adversely affect the protein when the altered sequence substantially inhibits a biological function associated with the protein.

[0046] Percent identity or homology with respect to such sequences is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the known peptides, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology and not considering any conservative substitutions as part of the sequence identity. N-terminal, C-terminal, and/or internal deletions and/or insertions into the peptide sequence shall not be construed as affecting homology.

[0047] Homology or identity at the nucleotide or amino acid sequence level can be determined by BLAST (Basic Local Alignment Search Tool) analysis using the algorithm employed by the programs blastp, blastn, blastx, tblastn, and tblastx (Altschul *et al.* (1997), *Nucleic Acids Res.* 25, 3389-3402, and Karlin *et al.* (1990), *Proc. Natl. Acad. Sci. USA* 87, 2264-2268, both fully incorporated by reference), which are tailored for sequence similarity searching. The approach used by the BLAST program is to first consider similar segments, with and without gaps, between a query sequence and a database sequence, then to evaluate the statistical significance of all matches that are identified, and finally to summarize only those matches which satisfy a preselected threshold of significance. For a discussion of basic issues in similarity searching of sequence databases, see Altschul *et al.* (1994), *Nature Genetics* 6, 119-129, which is fully incorporated by reference. The search parameters for histogram, descriptions, alignments, expect (*i.e.*, the statistical significance threshold for reporting matches against database sequences), cutoff, matrix, and filter (low complexity) can be at the default settings. The default scoring matrix used by blastp, blastx, tblastn, and tblastx is the BLOSUM62 matrix (Henikoff *et al.* (1992), *Proc. Natl. Acad. Sci. USA* 89, 10915-10919, fully incorporated by reference), recommended for query sequences over 85 in length (nucleotide bases or amino acids).

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[0048] For blastn, designed for comparing nucleotide sequences, the scoring matrix is set by the ratios of M (*i.e.*, the reward score for a pair of matching residues) to N (*i.e.*, the penalty score for mismatching residues), wherein the default values for M and N can be +5 and -4, respectively. Four blastn parameters can be adjusted as follows: Q=10 (gap creation penalty); R=10 (gap extension penalty); wink=1 (generates word hits at every winkth position along the query); and gapw=16 (sets the window width within which gapped alignments are generated). The equivalent Blastp parameter settings for comparison of amino acid sequences can be: Q=9; R=2; wink=1; and gapw=32. A Bestfit comparison between sequences, available in the GCG package version 10.0, can use DNA parameters GAP=50 (gap creation penalty) and LEN=3 (gap extension penalty), and the equivalent settings in protein comparisons can be GAP=8 and LEN=2.

[0049] Another indication that two nucleic acid sequences have substantial homology is that the two molecules hybridize specifically to each other under stringent conditions. The phrase "hybridize specifically to" refers to the binding, duplexing, and/or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions, including when that sequence is present in a complex mixture (*e.g.*, total cellular) of DNA and/or RNA. "Binds substantially" refers to complementary hybridization between a probe nucleic acid and a target nucleic acid and embraces minor mismatches that can be substantially accommodated by reducing the stringency of the hybridization media to achieve the desired detection of the target polynucleotide sequence. "Stringent hybridization conditions" and "stringent hybridization wash conditions", in the context of nucleic acid hybridization experiments such as Southern and northern hybridizations, are sequence dependent and are different under different environmental parameters.

[0050] Longer sequences can tend to hybridize specifically at higher temperatures. Generally, highly stringent hybridization and wash conditions can be selected to be about 5°C lower than the thermal melting point (T<sub>m</sub>) for the specific sequence at a defined ionic strength and pH. Typically, under "stringent conditions" a probe will generally hybridize to its target subsequence, but not to unrelated sequences.

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[0051] The  $T_m$  is defined herein as the temperature (under defined ionic strength and pH) at which approximately 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions can be selected to be equal to the  $T_m$  for a particular probe. An example of stringent hybridization conditions for hybridization of complementary nucleic acids that have more than 100 complementary residues on a filter in a Southern or northern blot is about 50% formamide with about 1 mg of heparin at about 42°C, with the hybridization being carried out overnight (for about 6-16 hours). An example of highly stringent wash conditions includes about 0.15M NaCl at about 72°C for about 15 minutes. An example of stringent wash conditions is a  $\sim 0.2 \times$  SSC wash at about 65°C for about 15 minutes (see Sambrook, *Molecular Cloning - A Laboratory Manual* (2001), Cold Spring Harbor Laboratory Press). Often, a high stringency wash can be preceded by a low stringency wash to remove background probe signal. An example of a medium stringency wash for a duplex of, *e.g.*, more than 100 nucleotides, is  $\sim 1 \times$  SSC at about 45°C for about 15 minutes. An example of a low stringency wash for a duplex of, *e.g.*, more than 100 nucleotides, is  $\sim 4-6 \times$  SSC at about 40°C for about 15 minutes. For short probes (*e.g.*, about 10 to 50 nucleotides), stringent conditions can typically involve salt concentrations of less than about 1.0 M  $\text{Na}^+$  ion, typically from about 0.01 to about 1.0 M  $\text{Na}^+$  ion, concentration (or other salts) at a pH of about 7.0 to about 8.3, with typical temperatures of at least about 30°C. Stringent conditions can additionally or alternately be achieved with the addition of destabilizing agents such as formamide. In general, a signal to noise ratio of about 2 $\times$  (or higher) than that observed for an unrelated probe in the particular hybridization assay can indicate detection of a specific hybridization.

[0052] “Exogenous nucleic acid molecule” or “exogenous gene” refers to a nucleic acid molecule or gene that has been introduced (“transformed”) into a cell. A transformed cell may be referred to as a recombinant cell, into which additional exogenous gene(s) may be introduced. (A descendent of a cell that was transformed with a nucleic acid molecule is also referred to as “transformed” if it has inherited the exogenous nucleic acid molecule). 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. An “endogenous” nucleic acid molecule, gene, or protein is

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the organism's own nucleic acid molecule, gene, or protein as it occurs in, or is naturally produced by, the organism.

[0053] When referring to a gene regulatory sequence or to an auxiliary nucleic acid sequence used for maintaining or manipulating a gene sequence (*e.g.*, a 5' untranslated region, 3' untranslated region, poly A addition sequence, intron sequence, splice site, ribosome binding site, internal ribosome entry sequence, genome homology region, recombination site, etc.), "heterologous" means that the regulatory sequence or auxiliary sequence is from a different source than the gene with which the regulatory or auxiliary nucleic acid sequence is juxtaposed in a construct, genome, chromosome, or episome. Thus, a promoter operably linked to a gene to which it is not operably linked to in its natural state (*i.e.*, in the genome of a non-genetically engineered organism) is referred to herein as a "heterologous promoter", even though the promoter may be derived from the same species (or, in some cases, the same organism) as the gene to which it is linked.

[0054] The term "native" is used herein to refer to nucleic acid sequences or amino acid sequences as they naturally occur in the host. The term "non-native" is used herein to refer to nucleic acid sequences or amino acid sequences that do not occur naturally in the host. A nucleic acid sequence or amino acid sequence that has been removed from a cell, subjected to laboratory manipulation, and introduced or reintroduced into a host cell is considered "non-native." Synthetic or partially synthetic genes introduced into a host cell are "non-native." Non-native genes further include genes endogenous to the host microorganism operably linked to one or more heterologous regulatory sequences that have been recombined into the host genome.

[0055] A "recombinant" or "engineered" nucleic acid molecule is a nucleic acid molecule that has been altered through human manipulation. As non-limiting examples, a recombinant nucleic acid molecule includes any nucleic acid molecule that: 1) has been partially or fully synthesized or modified *in vitro*, for example, using chemical or enzymatic techniques (*e.g.*, by use of chemical nucleic acid synthesis, or by use of enzymes for the replication, polymerization, digestion (exonucleolytic or endonucleolytic), ligation, reverse transcription, transcription, base modification

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(including, *e.g.*, methylation), integration or recombination (including homologous and site-specific recombination) of nucleic acid molecules); 2) includes conjoined nucleotide sequences that are not conjoined in nature, 3) has been engineered using molecular cloning techniques such that it lacks one or more nucleotides with respect to the naturally occurring nucleic acid molecule sequence, and/or 4) has been manipulated using molecular cloning techniques such that it has one or more sequence changes or rearrangements with respect to the naturally occurring nucleic acid sequence. As non-limiting examples, a cDNA is a recombinant DNA molecule, as is any nucleic acid molecule that has been generated by in vitro polymerase reaction(s), or to which linkers have been attached, or that has been integrated into a vector, such as a cloning vector or expression vector.

[0056] When applied to organisms, the term recombinant, engineered, or genetically engineered refers to organisms that have been manipulated by introduction of an exogenous or recombinant nucleic acid sequence into the organism, and includes organisms having gene knockouts, targeted mutations and gene replacement, promoter replacement, deletion, or insertion, as well as organisms having exogenous genes that have been introduced into the organism. An exogenous or recombinant nucleic acid molecule can be integrated into the recombinant/genetically engineered organism's genome or in other instances may not be integrated into the recombinant/genetically engineered organism's genome.

[0057] The term "recombinant protein" as used herein refers to a protein produced by genetic engineering.

[0058] An "expression cassette", as used herein, refers to a gene encoding a protein or functional RNA (*e.g.*, a tRNA, a microRNAs, a ribosomal RNA, *etc.*) operably linked to expression control sequences, such as a promoter, and optionally, any or a combination of other nucleic acid sequences that affect the transcription or translation of the gene, such as, but not limited to, a transcriptional terminator, a ribosome binding site, a splice site or splicing recognition sequence, an intron, an enhancer, a polyadenylation signal, an internal ribosome entry site, *etc.*

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[0059] When referring to a gene regulatory sequence or to an auxiliary nucleic acid sequence used for maintaining or manipulating a gene sequence (*e.g.*, a 5' untranslated region, 3' untranslated region, poly A addition sequence, intron sequence, splice site, ribosome binding site, internal ribosome entry sequence, genome homology region, recombination site, etc.), "heterologous" means that the regulatory sequence or auxiliary sequence is from a different source than the gene with which the regulatory or auxiliary nucleic acid sequence is juxtaposed in a construct, genome, chromosome, or episome. Thus, a promoter operably linked to a gene to which it is not operably linked to in its natural state (*i.e.*, in the genome of a non-genetically engineered organism) is referred to herein as a "heterologous promoter", even though the promoter may be derived from the same species (or, in some cases, the same organism) as the gene to which it is linked.

[0060] As used herein "attenuated" means reduced in amount, degree, intensity, or strength. Attenuated gene expression may refer to a significantly reduced amount and/or rate of transcription of the gene in question, or of translation, folding, or assembly of the encoded protein.

[0061] A photosynthetic microorganism having a disrupted NPQ process that includes a recombinant gene encoding a protein that participates in the production of fatty acids, such as, for example, a recombinant thioesterase and/or lipase gene, can produce at least one free fatty acid, such as one or more of a C<sub>6</sub>, C<sub>8</sub>, C<sub>10</sub>, C<sub>12</sub>, C<sub>14</sub>, C<sub>16</sub>, C<sub>18</sub>, C<sub>20</sub>, C<sub>22</sub>, and C<sub>24</sub> free fatty acid. In some embodiments, the microorganism can produce at least one free fatty acid during the growth of the culture, and/or can produce at least one free fatty acid in the absence of disruption or lysis of the cells. Recombinant thioesterase and/or lipase genes can optionally additionally be introduced into such photosynthetic microorganisms with a disrupted NPQ process, *e.g.*, for the production of fatty acid derivatives.

[0062] Thioesterases useful in various aspects of the invention are enzymes that catalyze the cleavage of a fatty acid thioester. For example, "acyl-ACP thioesterase" is an enzyme that catalyzes the cleavage of a fatty acid from an acyl carrier protein (ACP). In some embodiments of the present invention, the exogenous nucleic acid

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molecule encoding a thioesterase can include, without limitation, an acyl-ACP thioesterase, an acyl-CoA thioesterase, and a hydroxybenzoyl-CoA thioesterase.

[0063] For example, a microorganism for the production of free fatty acids in some embodiments can be transformed with a gene encoding an exogenous acyl-ACP thioesterase, such as a gene encoding a polypeptide that when queried against the pfam database, provides a match with Pfam PF01643 having a bit score of less than or equal to 20.3 (the gathering cut-off for PF01643). The exogenous acyl-ACP thioesterase gene can encode an acyl-ACP thioesterase from a higher plant species. Genes encoding acyl-ACP thioesterases derived from higher plants can include, without limitation, genes encoding acyl-ACP thioesterases from *Cuphea* species (e.g., *Cuphea carthagenensis*, *Cuphea wrightii* (e.g., AAC49784.1 GI:1336008), *Cuphea lanceolata* (e.g., CAA54060, GI495227), *Cuphea palustris*, (e.g., AAC49783.1 GI:1336006; AAC49179.1 GI:1215718); *Cuphea hookeriana* (e.g., AAC72882.1 GI:3859830; AAC49269.1 GI:1292906; AAC72881.1 GI:3859828; AAC72883.1 GI:3859832), *Cuphea calophylla* (e.g., ABB71580.1 GI:81361963)) or genes from other higher plant species. For example, a microorganism used in the methods and cultures disclosed herein can include a gene encoding an acyl-ACP thioesterase from species such as but not limited to, *Arabidopsis* (XP\_002885681.1 GI:297835598; NP\_172327.1 GI:15223236); *Arachis hypogaea* (e.g., ABO38556.1 GI:133754634); *Brassica* species (e.g., CAA52069.1 GI:435011), *Camellia oleifera* ((e.g., ACQ57189.1 GI:229358082); *Cinnamomum camphorum* (e.g., AAC49151.1 GI:1143156); *Cocos nucifera*; *Glycine max* (e.g., ABD91726.1 GI:90192131); *Garcinia mangostana* (e.g., AAB51525.1 GI:1930081); *Gossypium hirsutum* (e.g., AAD01982.1 GI:4104242); *Helianthus annuus* (e.g., AAQ08226 GI:33325244); *Jatropha curcas* (e.g., ABU96744.1 GI:156900676); *Macadamia tetraphylla* (e.g., ADA79524.1 GI:282160399); *Elaeis oleifera* (e.g., AAM09524.1 GI:20067070); *Oryza sativa* (e.g., BAA83582.1 GI:5803272); *Populus tomentosa* (e.g., ABC47311.1 GI:83778888); *Umbellularia californica* (e.g., AAC49001.1 GI:595955); *Ulmus Americana* (e.g., AAB71731.1 GI:2459533); and *Zea mays* (ACG41291.1 GI:195643646), or any of those disclosed in U.S. Patent No. 5,455,167; U.S. Patent No. 5,654,495; and U.S. Patent No. 5,455,167; all incorporated by reference herein in their entireties. Further

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included are acyl-ACP thioesterases from mosses (Bryophyta), such as, for example, *Physcomitrella patens*, (e.g., XP\_001770108.1 GI:168035219). These examples are not limiting with regard to the types or specific examples of acyl-ACP thioesterase genes that can be used. Further included are acyl-ACP thioesterase genes from additional organisms, including, for example, prokaryotic organisms. Illustrative examples of prokaryotic acyl-ACP thioesterases that may be expressed by a microorganism useful in the methods and cultures provided herein include, but are not limited to acyl-ACP thioesterases from *Desulfovibrio desulfuricans* (e.g., Q312L1 GI:123552742); *Elusimicrobium minutum* (e.g., ACC98705 GI:186971720); *Carboxydotherrmus hydrogenoformans* (e.g., YP\_359670 GI:78042959); *Clostridium thermocellum* (e.g., YP\_001039461 GI:125975551); *Moorella thermoacetica* (e.g., YP\_431036 GI:83591027); *Geobacter metallireducens* (e.g., YP\_384688 GI:78222941); *Salinibacter ruber* (e.g., YP\_444210 GI:83814393); *Microscilla marina* (e.g., EAY28464 123988858); *Parabacteroides distasonis* (e.g., YP\_001303423 GI:150008680); *Enterococcus faecalis* (e.g., ZP\_03949391 GI:227519342); *Lactobacillus plantarum* (e.g., YP\_003062170 GI:254555753); *Leuconostoc mesenteroides* (e.g., YP\_817783 GI:116617412); *Oenococcus oeni* (e.g., ZP\_01544069 GI:118586629); *Mycobacterium smegmatis* (e.g., ABK74560 GI:118173664); *Mycobacterium vanbaalenii* (e.g., ABM11638 GI:119954633); *Rhodococcus erythropolis* (e.g., ZP\_04385507 GI:229491686); *Rhodococcus opacus* (e.g., YP\_002778825 GI:226361047), or any of those disclosed in the co-pending, commonly-assigned United State patent application 61/426,555 entitled "Prokaryotic Acyl-ACP Thioesterases for Producing Fatty Acids in Genetically Engineered Microorganisms", filed on December 23, 2010, and which is incorporated herein by reference in its entirety.

[0064] A gene encoding an acyl-CoA thioesterase can optionally additionally be introduced into a host microorganism to generate free fatty acids. An acyl-CoA thioesterase gene transformed into a microorganism for the production of free fatty acids can be from a plant, animal, or microbial source. For example, a gene encoding the TesA or TesB thioesterase of *E. coli*, or a variant thereof, for example, an acyl-CoA thioesterase such as not limited to a variant as disclosed in PCT Publication No.

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WO 2010/075483, incorporated by reference herein in its entirety, can be introduced into a microorganism. Also included are genes encoding proteins that when queried against the Pfam database of protein families are identified as members of Pfam PF02551 (acyl-CoA thioesterase), where the bit score is equal to or greater than the gathering cut off (20.7).

[0065] Alternately or in addition, the microorganism can include one or more genes encoding an exogenous hydroxybenzoyl-CoA thioesterase, for example an exogenous 4-hydroxybenzoate thioesterase or 4-chlorobenzoate thioesterase. Genes encoding hydroxybenzoyl thioesterases that may be useful in a microorganism for producing free fatty acids can include, for example, those disclosed in the co-pending, commonly-assigned United States patent application 61/426,568 entitled "Genetically Engineered Microorganisms Comprising 4-Hydroxybenzoyl-CoA Thioesterases and Methods of Using Same for Producing Free Fatty Acids and Fatty Acid Derivatives", filed on December 23, 2010, and which is incorporated herein by reference in its entirety, 4-hydroxybenzoate thioesterases from *Bacillus* species and *Geobacillus* species, as well as 4-hydroxybenzoate thioesterases of *Acidiphilium*, *Bartonella*, *Rhodopseudomonas*, *Magnetospirillum*, *Burkholderia*, *Granulibacter*, *Rhizobium*, and *Labrenzia* species, or the like, or combinations thereof.

[0066] Further additionally or alternately, the recombinant microorganism can include those genetically engineered with exogenous or endogenous genes that encode polypeptides having lipolytic activity, such as, for example, lipases or esterases capable of producing free fatty acids from membrane lipids or storage lipids, *e.g.*, phospholipids, glycolipids, triacylglycerols, diacylglycerols, monoacylglycerols, or the like, or combinations thereof. Lipases are enzymes that catalyze the hydrolysis of ester bonds in glycerolipids, including, but not limited to, mono-, di-, and tri-acyl glycerols, as well as combinations thereof, to release free fatty acids and alcohols.

[0067] The use of lipase genes in microorganisms used in the production of free fatty acids is disclosed in the co-pending, commonly-assigned United States patent application 61/426,624 entitled "Lipase-Mediated Production of Free Fatty Acids by Recombinant Microorganisms", filed on December 23, 2010, and which is incorporated

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herein by reference in its entirety. The lipase gene can be a gene encoding any lipase, *e.g.*, that liberates a fatty acid from a glycerolipid (including a monoglyceride, a diglyceride, a triglyceride, a phospholipid, a galactolipid, etc.). For example, a lipase gene can encode a polypeptide having lipase activity that is a member of the Pfam AB Hydrolase clan, CL0028, such as but not limited to, a lipase that is a member of Pfam 01674, Pfam 01764, Pfam 07819, Pfam 03583, and/or Pfam 00151. In some embodiments, an exogenous lipase gene introduced into a microorganism can encode a protein with an amino acid sequence having an E-value parameter of 0.01 or less when queried using the Pfam Profile HMM for any of Pfam PF01674, Pfam PF 01764, Pfam PF07819, Pfam PF03583, and/or Pfam PF00151. Additionally or alternately contemplated are recombinant microorganisms that are engineered to include gene regulatory sequences that induce or increase expression of an endogenous lipase gene. For example, a microorganism can be engineered such that a heterologous promoter is inserted upstream of a coding region of an endogenous lipase gene. The heterologous promoter can replace an endogenous promoter and/or can be inserted upstream or downstream of the endogenous promoter that regulates expression of the endogenous lipase gene, for example using homologous recombination or site-specific recombination. The heterologous promoter can be a constitutive promoter or an inducible promoter that increases expression of the endogenous lipase gene.

[0068] Still further additionally or alternately, the microorganism can include nucleic acid molecules encoding variants of the above-listed acyl-ACP thioesterases, acyl-CoA thioesterases, hydroxybenzoyl-CoA thioesterases, or lipases, in which the variants have at least 80%, for example at least 85%, at least 90%, or at least 95%, identity to the amino acid sequences accessed by the provided or referenced Genbank Accession Numbers, in which the variants have at least the level of activity (*e.g.*, thioesterase or lipase activity) as the reference sequence.

[0069] In some embodiments, the present invention relates to a recombinant microorganism having a disrupted NPQ process, for example, a recombinant photosynthetic microorganism that has reduced expression of a gene on the biosynthetic pathway or a gene having reduced expression of OCP, that exhibits

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disrupted NPQ, that includes at least one recombinant expression system for at least one thioesterase gene and/or at least one lipase gene that operates to liberate and/or release fatty acids. A “free fatty acid”, as used herein, is meant to refer to a non-esterified acyl moiety that is substantially unassociated, *e.g.*, with an enzyme and/or protein, within or outside an organism (*e.g.*, globular and/or micellar storage within an organism, without esterification, can still qualify as a free fatty acid). Thus, a free fatty acid according to the present invention need not necessarily be a strict acid or be structurally “free”, but a free fatty acid specifically does not include an acyl moiety whose carboxylate oxygen is covalently linked to any other moiety other than a hydrogen atom (meaning that fatty acid esters are specifically not included in free fatty acids. However, a free fatty acid can advantageously include an acyl moiety containing at least four carbons (preferably at least 6 carbons, for example at least 8 carbons), in which the acyl moiety (i) is covalently linked to a hydrogen atom, (ii) has an ionic charge, to which a counterion can be associated (even if loosely and/or solvent-separated), and/or (iii) is associated, but not covalently bonded to another moiety that is relatively easily transformable into the corresponding acid form or the corresponding ionic form (*e.g.*, through hydrogen-bonding or the like). Nonlimiting examples of counterions can include metals salts (such as calcium, magnesium, sodium, potassium, aluminum, iron, and the like, and combinations thereof), other inorganic ions (such as ammonium, mono-, di-, tri-, and tetra- alkylammonium, sulfonium, phosphonium, and the like, and combinations thereof), organic ions (such as carbocations), and the like, and combinations thereof.

#### Other Modifications for Producing Free Fatty Acids and/or Fatty Acid Derivatives

[0070] Additionally or alternately to providing an expression system for one or more exogenous genes, such as thioesterase and lipase genes, further modifications in the microorganism may be made. For example, in addition to having an exogenous thioesterase gene and/or a recombinant lipase gene, a microorganism used in the methods herein can additionally or alternately include microorganisms having at least one additional exogenous nucleic acid molecule that encodes a polypeptide that participates in the synthesis of a fatty acid. For example, a transgenic microorganism for the production of one or more fatty acids can include an exogenous gene encoding

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an acetyl-CoA carboxylase, a malonyl CoA: ACP transacylase, or a beta-ketoacyl-ACP synthase.

[0071] The present invention also provides recombinant microorganisms that further include at least one endogenous gene that is attenuated or disrupted. Such an endogenous gene that can be attenuated or disrupted in the recombinant microorganism includes, but not limited to, acyl-CoA synthetase, acyl-ACP synthetase, acyl CoA dehydrogenase, glycerol-3-phosphate dehydrogenase, acetaldehyde CoA dehydrogenase, pyruvate dehydrogenase, acetate kinase, and the like, and combinations thereof.

[0072] Further additionally or alternately, the microorganism can be modified such that one or more genes that encode beta-oxidation pathway enzymes have been inactivated and/or downregulated, and/or such that the enzymes themselves that are operative on such beta-oxidation pathways may be inhibited. This could prevent the degradation of fatty acids released from acyl-ACPs, thus enhancing the yield of secreted fatty acids. In cases where the desired products are medium-chain fatty acids, the inactivation and/or downregulation of genes that encode acyl-CoA synthetase and/or acyl-CoA oxidase enzymes that preferentially use these chain lengths as substrates could be beneficial. Mutations in the genes encoding medium-chain-specific acyl-CoA synthetase and/or medium-chain-specific acyl-CoA oxidase enzymes, such that the activity of the enzymes could be diminished, may additionally or alternately be effective in increasing the yield of produced and/or released fatty acids. An additional modification can inactivate and/or downregulate the acyl-ACP synthetase gene and/or can inactivate and/or inhibit the encoded protein. Mutations in the genes can be introduced either by recombinant or non-recombinant methods. These enzymes and their genes are known and may be targeted specifically by disruption, deletion, generation of antisense sequences, generation of ribozymes, and/or other recombinant approaches known to the practitioner. Inactivation of the genes can additionally or alternately be accomplished by random mutation techniques such as exposure to UV and/or chemical mutagens, and the resulting cells can be screened for successful mutants. The proteins themselves can be inhibited by intracellular

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generation of appropriate antibodies, intracellular generation of peptide inhibitors, or the like, or some combination thereof.

[0073] Still further additionally or alternately, the photosynthetic microorganism can be modified such that one or more genes that encode storage carbohydrate and/or polyhydroxyalkanoate (PHA) biosynthesis pathway enzymes can be inactivated or downregulated, and/or such that the enzymes themselves that are operative on such pathways are inhibited. Examples include, but not limited to, enzymes involved in glycogen, starch, or chrysolaminarin synthesis, including glucan synthases and branching enzymes. Other examples include enzymes involved in PHA biosynthesis such as acetoacetyl-CoA synthase and PHA synthase.

#### Further Modifications for Producing Fatty Acid Derivatives

[0074] Additionally or alternately to providing an expression system for one or more appropriate recombinant genes, such as lipase genes, further modifications in the microorganism may be made. For example, in some embodiments, the genetically engineered photosynthetic microorganism having a disrupted NPQ process can produce a fatty aldehyde and can include one or more nucleic acid molecules encoding an exogenous acyl-CoA reductase, carboxylic acid reductase, and/or acyl-ACP reductase. Additionally or alternately, the genetically engineered photosynthetic microorganism can produce a fatty alcohol and can include at least one nucleic acid molecule encoding an exogenous acyl-CoA reductase, carboxylic acid reductase, or acyl-ACP reductase or at least one exogenous fatty aldehyde reductase. Alternatively or in addition, the genetically engineered photosynthetic microorganism of the described invention can produce a wax ester and can include one or more nucleic acid molecules encoding an exogenous acyl-CoA reductase, carboxylic acid reductase, or acyl-ACP reductase, and an exogenous wax synthase. Wax esters include an A chain and a B chain linked through an ester bond, one or both of which can be derived from a fatty acid generated by the exogenous 4-hydroxybenzoyl-CoA thioesterase. Wax esters produced by a photosynthetic microorganism that includes a nucleic acid molecule encoding an exogenous 4-hydroxybenzoyl-CoA thioesterase therefore can have A+B chain lengths of, for example, 16 to 36 carbons, 16 to 32 carbons, or 24 to 32 carbons.

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[0075] In some embodiments, the photosynthetic microorganism having a disrupted NPQ process can produce an alkane or alkene and can include at least one nucleic acid molecule encoding an exogenous fatty acid decarboxylase or an exogenous fatty aldehyde decarbonylase, or additionally can include at least one exogenous nucleic acid molecule encoding an exogenous acyl-CoA reductase, carboxylic acid reductase, or acyl-ACP reductase. Alkanes and alkenes produced by a photosynthetic microorganism that includes a nucleic acid molecule encoding an exogenous 4-hydroxybenzoyl-CoA thioesterase can, for example, have chain lengths of 7, 9, 11, 13, 15, 17, 19, 21, and/or 23 carbons, for example, chain lengths of 7, 9, 11, 13, 15, and/or 17 carbons, or chain lengths of 7, 9, 11, 13, and/or 15 carbons, or chain lengths of 11, 13, and/or 15 carbons.

[0076] Additionally, a genetically engineered photosynthetic microorganism that produces a fatty alcohol, fatty aldehyde, wax ester, alkane, or alkene may optionally include a nucleic acid molecule encoding an acyl-CoA synthetase.

[0077] Additionally or alternatively to the embodiments provided hereinabove, the present invention also provides recombinant microorganisms having a disrupted NPQ process that further include at least one endogenous gene that is attenuated or disrupted to enhance production of a biomolecule such as a fatty acid or fatty acid derivative. Such an endogenous gene that can be attenuated or disrupted in the recombinant microorganism includes, but not limited to, acyl-CoA synthetase, acyl-ACP synthetase, acyl CoA dehydrogenase, glycerol-3-phosphate dehydrogenase, acetaldehyde CoA dehydrogenase, pyruvate dehydrogenase, acetate kinase, and the like, and combinations thereof.

[0078] Further additionally or alternately, the microorganism can be modified such that one or more genes that encode beta-oxidation pathway enzymes have been inactivated or downregulated, and/or such that the enzymes themselves that are operative on such beta-oxidation pathways may be inhibited. This would prevent the degradation of fatty acids released from acyl-ACPs, thus enhancing the yield of secreted fatty acids. Mutations in a gene encoding acyl-CoA synthetase and/or acyl-CoA oxidase enzyme, such that the activity of one or more of these enzymes could be

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diminished, would additionally or alternately be effective in increasing the yield of produced and/or released fatty acids. An additional or alternative modification can inactivate or downregulate the acyl-ACP synthetase gene and/or can inactivate or inhibit the encoded protein. Mutations in a gene can be introduced either by recombinant or non-recombinant methods. These enzymes and their genes are known and may be targeted specifically by disruption, deletion, generation of antisense sequences, generation of ribozymes, RNAi, and/or other recombinant approaches known to the practitioner. Inactivation of the genes can additionally or alternately be accomplished by random mutation techniques such as exposure to UV and/or chemical mutagens, and the resulting cells can be screened for successful mutants. The proteins themselves can be inhibited by intracellular generation of appropriate antibodies, intracellular generation of peptide inhibitors, or the like, or some combination thereof.

[0079] Again still further additionally or alternately, the photosynthetic microorganism having a disrupted NPQ process can be modified such that one or more genes that encode storage carbohydrate and/or polyhydroxyalkanoate (PHA) biosynthesis pathway enzymes can be inactivated or downregulated, and/or such that the enzymes themselves that are operative on such pathways are inhibited. Examples include, but not limited to, enzymes involved in glycogen, starch, or chrysolaminarin synthesis, including glucan synthases and branching enzymes. Other examples include enzymes involved in PHA biosynthesis such as acetoacetyl-CoA synthase and PHA synthase.

#### Expression Systems

[0080] The recombinant microorganisms of the present invention, in some embodiments, are transformed with exogenous genes by the introduction of appropriate expression vectors.

[0081] “Expression vector” or “expression construct” refers to a nucleic acid that has been generated via human intervention, including by recombinant means and/or direct chemical synthesis, with a series of specified nucleic acid elements that permit transcription and/or translation of a particular nucleic acid in a host cell. The expression vector can be a plasmid, a part of a plasmid, a viral construct, a nucleic acid

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fragment, or the like, or a combination thereof. Typically, the expression vector can include a nucleic acid to be transcribed operably linked to a promoter in an “expression cassette”. Moreover, “inducible promoter” refers a promoter that mediates transcription of an operably linked gene in response to a particular stimulus. “Operable linkage” is a functional linkage between two nucleic acid sequences, such as a control sequence (typically a promoter) and the linked sequence (typically a sequence that encodes a protein and/or other biomolecule, also called a coding sequence). A promoter is in operable linkage with an exogenous gene if it can mediate transcription of the gene.

[0082] Vectors can be introduced into prokaryotic and eukaryotic cells via conventional transformation and/or transfection techniques. The terms “transformation” and “transfection”, conjugation and transduction, as used in the present context, are intended to comprise a multiplicity of methods known to those skilled in the art for the introduction of foreign nucleic acid (for example, exogenous DNA) into a host cell, including calcium phosphate and/or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, natural competence, chemically mediated transfer, electroporation, particle bombardment, or the like, or combinations thereof. Examples of suitable methods for the transformation and/or transfection of host cells, *e.g.*, can be found in *Molecular Cloning - A Laboratory Manual* (2001), Cold Spring Harbor Laboratory Press.

[0083] For example, algae and photosynthetic bacteria can be transformed by any suitable methods, including, as non-limiting examples, natural DNA uptake (Chung *et al.* (1998) *FEMS Microbiol. Lett.* 164: 353-361; Frigaard *et al.* (2004) *Methods Mol. Biol.* 274: 325-40; Zang *et al.* (2007) *J. Microbiol.* 45: 241-245), conjugation, transduction, glass bead transformation (Kindle *et al.* (1989) *J. Cell Biol.* 109: 2589-601; Feng *et al.* (2009) *Mol. Biol. Rep.* 36: 1433-9; U.S. Patent No. 5,661,017), silicon carbide whisker transformation (Dunahay *et al.* (1997) *Methods Mol. Biol.* 62: 503-9), biolistics (Dawson *et al.* (1997) *Curr. Microbiol.* 35: 356-62; Hallmann *et al.* (1997) 94: 7469-7474; Jakobiak *et al.* (2004) *Protist* 155:381-93; Tan *et al.* (2005) *J. Microbiol.* 43: 361-365; Steinbrenner *et al.* (2006) *Appl Environ. Microbiol.* 72: 7477-7484; Kroth (2007) *Methods Mol. Biol.* 390: 257-267; U.S. Patent No. 5,661,017),

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electroporation (Kjaerulff *et al.* (1994) *Photosynth. Res.* 41: 277-283; Iwai *et al.* (2004) *Plant Cell Physiol.* 45: 171-5; Ravindran *et al.* (2006) *J. Microbiol. Methods* 66: 174-6; Sun *et al.* (2006) *Gene* 377: 140-149; Wang *et al.* (2007) *Appl. Microbiol. Biotechnol.* 76: 651-657; Chaurasia *et al.* (2008) *J. Microbiol. Methods* 73: 133-141; Ludwig *et al.* (2008) *Appl. Microbiol. Biotechnol.* 78: 729-35), laser-mediated transformation, incubation with DNA in the presence of or after pre-treatment with any of poly(amidoamine) dendrimers (Pasupathy *et al.* (2008) *Biotechnol. J.* 3: 1078-82), polyethylene glycol (Ohnuma *et al.* (2008) *Plant Cell Physiol.* 49: 117-120), cationic lipids (Muradawa *et al.* (2008) *J. Biosci. Bioeng.* 105: 77-80), dextran, calcium phosphate, and/or calcium chloride (Mendez-Alvarez *et al.* (1994) *J. Bacteriol.* 176: 7395-7397), optionally after treatment of the cells with cell wall-degrading enzymes (Perrone *et al.* (1998) *Mol. Biol. Cell* 9: 3351-3365), or the like, or combinations thereof. Agrobacterium-mediated transformation can additionally or alternately be performed on algal cells, for example after removing or wounding the algal cell wall (*e.g.*, PCT Publication No. WO 2000/62601; Kumar *et al.* (2004) *Plant Sci.* 166: 731-738). Biolistic methods are particularly successful for transformation of the chloroplasts of plant and eukaryotic algal species (see, for example, Ramesh *et al.* (2004) *Methods Mol. Biol.* 274: 355-307; Doestch *et al.* (2001) *Curr. Genet.* 39: 49-60; U.S. Patent No. 7,294,506; PCT Publication No. WO 2003/091413; PCT Publication No. WO 2005/005643; and PCT Publication No. WO 2007/133558, all incorporated herein by reference in their entireties).

[0084] For optimal expression of a recombinant protein, in many instances it can be beneficial to employ coding sequences that produce mRNA with codons preferentially used by the host cell to be transformed. Thus, for an enhanced expression of transgenes, the codon usage of the transgene can be matched with the specific codon bias of the organism in which the transgene is desired to be expressed. For example, methods of recoding genes for expression in microalgae are described in U.S. Patent No. 7,135,290. The precise mechanisms underlying this effect are believed to be many, but can include the proper balancing of available aminoacylated tRNA pools with proteins being synthesized in the cell, coupled with more efficient translation of the transgenic messenger RNA (mRNA) when this need is met. In some

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embodiments, only a portion of the codons can be changed to reflect a preferred codon usage of a host microorganism, and in some embodiments, one or more codons can be changed to codons that are not necessarily the most preferred codon of the host microorganism encoding a particular amino acid. Additional information for codon optimization is available, *e.g.*, at the codon usage database of GenBank. Accordingly, the present invention also provides, in some embodiments, for recombinant microorganisms transformed with an isolated nucleic acid molecule including a nucleic acid sequence that is codon-optimized for expression in the recombinant microorganism.

[0085] In some embodiments, the present invention additionally or alternately provides recombinant microorganisms transformed with an isolated nucleic acid molecule including a nucleic acid sequence operably linked to one or more expression control elements. For example, in some preferred embodiments of the invention, a gene (such as a gene as disclosed herein), can be cloned into an expression vector for transformation into a fungus, an alga, or a photosynthetic or non-photosynthetic bacterium. The vector can include sequences that promote expression of the transgene of interest (*e.g.*, an exogenous lipase gene), such as a promoter, and may optionally include, for expression in eukaryotic cells, an intron sequence, a sequence having a polyadenylation signal, or the like, or combinations thereof. 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 by homologous recombination, site specific integration, and/or vector integration.

[0086] Additionally or alternately, the vector introduced in to a microorganism can include a promoter or transcriptional enhancer sequence not in operable linkage with a gene of interest, where the promoter or enhancer is positioned next to one or more sequences for directing the promoter to the chromosomal locus of a gene for producing fatty acids (*e.g.*, an endogenous lipase gene). For example, sequences for homologous recombination or site-specific recombination can be engineered to flank a transcriptional regulatory sequence in a transformation vector, such that following transformation into the cells the regulatory sequence integrates into the host

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chromosome becomes operably linked to an endogenous gene by homologous recombination, site specific integration, and/or vector integration.

[0087] Vectors designed for expression of a gene in microalgae can alternatively or in addition include a promoter active in microalgae operably linked to the exogenous gene being introduced. A variety of gene promoters and terminators that function in green algae can be utilized in expression vectors, including, but not limited to, promoters and/or terminators from *Chlamydomonas* and other algae (see, for example, Abe *et al.* (2008) *Plant Cell Physiol*, 49: 625-632), promoters and/or terminators from viruses, synthetic promoters and/or terminators, or the like, or combinations thereof.

[0088] For transformation of diatoms, a variety of gene promoters that function in diatoms can be utilized in these expression vectors, including, but not limited to: 1) promoters from *Thalassiosira* and other heterokont algae, promoters from viruses, synthetic promoters, or the like, or combinations thereof. Promoters from *Thalassiosira pseudonana* and/or *Phaeodactylum tricorutum* that could be suitable for use in expression vectors can include an alpha-tubulin promoter, a beta-tubulin promoter, an actin promoter, or a combination thereof. The terminators associated with these genes, other diatom genes, and/or particular heterologous genes can be used to stop transcription and/or provide the appropriate signal, *e.g.*, for polyadenylation.

[0089] In some instances, it can be advantageous to express an antisense molecule, or a gene encoding an exogenous and/or heterologous enzyme, such as but not limited to a lipase, at a certain point during the growth of the transgenic host, *e.g.*, to minimize any deleterious effects on the growth of the transgenic organism and/or to maximize production of the fatty acid product of interest. In such instances, one or more exogenous genes introduced into the transgenic organism can be operably linked to an inducible promoter. The promoter can be, for example, a lac promoter, a tet promoter (*e.g.*, U.S. Patent No. 5,851,796), a hybrid promoter that includes either or both of portions of a tet or lac promoter, a hormone-responsive promoter (*e.g.*, an ecdysone-responsive promoter, such as described in U.S. Patent No. 6,379,945), a metallothionien promoter (*e.g.*, U.S. Patent No. 6,410,828), a pathogenesis-related (PR) promoter that can be responsive to a chemical such as, for example, salicylic acid,

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ethylene, thiamine, and/or BTH (U.S. Patent No. 5,689,044), or the like, or some combination thereof. An inducible promoter can also be responsive to light or dark (U.S. Patent No. 5,750,385, U.S. Patent No. 5,639,952) or temperature (U.S. Patent No. 5,447,858; Abe et al. (2008) *Plant Cell Physiol.* 49: 625-632; Shroda et al. (2000) *Plant J.* 21: 121-131). The foregoing list is exemplary and not limiting. The promoter sequences can be from any organism, provided that they are functional in the host organism. Inducible promoters, as used in the constructs of the present invention, can use one or more portions or domains of the aforementioned promoters and/or other inducible promoters fused to at least a portion of a different promoter that can operate in the host organism, e.g., to confer inducibility on a promoter that operates in the host species.

[0090] For transformation of cyanobacteria, a variety of promoters that function in cyanobacteria can be utilized, including, but not limited to, the lac, tac, and trc promoters, as well as derivatives that are also inducible by the addition of isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) such as the trcY or trcE promoter. Other promoters that may find use in the invention include promoters that are naturally associated with transposon- or bacterial chromosome-borne antibiotic resistance genes (e.g., neomycin phosphotransferase, chloramphenicol acetyltransferase, spectinomycin adenyltransferase, or the like, or combinations thereof), promoters associated with various heterologous bacterial and native cyanobacterial genes, promoters from viruses and phages, synthetic promoters, or the like, or combinations thereof. Promoters isolated from cyanobacteria that can be used can include but are not limited to the following: nrs (nickel-inducible), secA (secretion; controlled by the redox state of the cell), rbc (Rubisco operon), psaAB (PS I reaction center proteins; light regulated), psbA (D1 protein of PSII; light- inducible), and the like, and combinations thereof. In some embodiments, the promoters are regulated by nitrogen compounds, such as, for example, nar, ntc, nir or nrt promoters. In some embodiments, the promoters are regulated by phosphate (e.g., pho or pst promoters) or metals (e.g., the nrs promoter (Liu and Curtis (2009) *Proc Natl Acad Sciences USA* 106: 21550-21554), or the petE promoter (Buikema and Haselkorn (2001) *Proc Natl Acad Sciences USA* 98: 2729-2734)). Inducible promoters, as used in the constructs of the present invention, can use

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one or more portions or domains of the aforementioned promoters and/or other inducible promoters fused to at least a portion of a different promoter that can operate in the host organism, e.g., to confer inducibility on a promoter that operates in the host species.

[0091] Likewise, a wide variety of transcriptional terminators can be used for expression vector construction. Examples of possible terminators can include, but are not limited to, *psbA*, *psaAB*, *rbc*, *secA*, T7 coat protein, and the like, and combinations thereof.

[0092] Transformation vectors can additionally or alternately include a selectable marker, such as but not limited to a drug resistance gene, an herbicide resistance gene, a metabolic enzyme and/or factor required for survival of the host (for example, an auxotrophic marker), or the like, or a combination thereof. Transformed cells can be optionally selected based upon the ability to grow in the presence of the antibiotic and/or other selectable marker under conditions in which cells lacking the resistance cassette or auxotrophic marker could not grow. Further additionally or alternately, a non-selectable marker may be present on a vector, such as a gene encoding a fluorescent protein or enzyme that generates a detectable reaction product.

[0093] Expression vectors can be introduced into the cyanobacterial strains by standard methods, including, but not limited to, natural DNA uptake, conjugation, electroporation, particle bombardment, abrasion with glass beads, SiC fibers, or other particles, or the like, or combinations thereof. The vectors can be: (1) targeted for integration into the cyanobacterial chromosome, e.g., by including flanking sequences that enable homologous recombination into the chromosome; (2) targeted for integration into endogenous cyanobacterial plasmids, e.g., by including flanking sequences that enable homologous recombination into the endogenous plasmids; and/or (3) designed such that the expression vectors replicate within the chosen host.

[0094] According to some preferable embodiments, the present invention can involve recombinant microorganisms transformed with an isolated nucleic acid molecule including a nucleic acid sequence that is under control of a heterologous promoter. In such embodiments, the heterologous promoter can be an inducible

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promoter, such as an isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG)-inducible promoter, for example, a lac, tac, and/or trc promoter, such as *trcE* and/or *trcY*.

#### Methods of Producing Biomass or One or More Biomolecules

[0095] The invention encompasses methods of producing biomass or at least one biomolecule by culturing the photosynthetic microorganisms described herein and isolating biomass or at least one biomolecule from the culture. The photosynthetic microorganisms used for production of biomass or a biomolecule have a disrupted NPQ process, for example, the production microorganism may have reduced production of a carotenoid or a carotenoid-binding protein. The microorganism used in the methods may be, in certain examples, a cyanobacterium with attenuated expression of an OCP. The photosynthetic microorganism can be cultured as an actively mixed culture, for example in a pond or photobioreactor. For example, the photosynthetic microorganism having a disrupted NPQ process can be cultured in a pond having a depth of at least 3 cm, at least 5 cm, or at least 10 cm, or a photobioreactor having a light path of at least 3 cm, at least 5 cm, or at least 10 cm, and the pond or bioreactor can include at least one active mixing device, such as a paddlewheel, pump, propeller, fluid injection system, sparger, or any combination thereof, optionally in combination with at least one passive mixing device.

[0096] The amount of biomass or a biomolecule produced by the culture according to some embodiments of the present invention can at least about 10%, for example at least about 20%, at least about 25%, or at least about 30% more than the amount of biomass or of a biomolecule produced by an identical culture of a microorganism identical in all respects except that it does not have a disrupted NPQ process.

[0097] In some embodiments of the present invention, the photosynthetic microorganisms can be cultured in an open pond where the culture is actively mixed, for example, by means of a paddle wheel, drag board, one or more pumps, including mechanical and air lift pumps, one or more propellers, paddles, or blades, gas spargers, or one or more streams or jets of liquid or gas and may additionally include one or more stationary structures within the pond or along one or more of its walls that promotes mixing or turbulence. As used herein "pond" means any open body of water,

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whether naturally-occurring or man-made, including ponds, canals, trenches, lagoons, channels, or raceways. The open pond can have a depth of from about 3 cm to about 500 cm, and will typically have a depth of from about 5 cm to about 100 cm, such as from about 8 cm to about 50 cm, or from about 10 cm to about 40 cm.

[0098] Cells can additionally or alternately be cultured in a photobioreactor equipped with an artificial light source and/or can having one or more walls that is transparent enough to light, including sunlight, to enable, facilitate, and/or maintain acceptable microorganism growth. The photobioreactor includes an enclosure or partial enclosure in which cells are cultured, optionally in suspension and, when suspended, preferably in an aqueous liquid. Photosynthetic microorganisms can be cultured in a photobioreactor where the culture is actively mixed, for example, during at least a portion of the light period, where active mixing is mixing that requires a power source for moving a liquid (e.g., culture media), gas, or physical structure (e.g., paddlewheel) to effect mixing of cells within the culture medium. For example, active mixing can be by means of a one or more propellers, paddles, or blades, by pumps, including mechanical and air lift pumps, by gas spargers (including gas bubbling tubes) or by means of one or more streams or jets of liquid or gas, and can further include one or more stationary structures (passive mixing devices or structures) within the photobioreactor or along one or more of its walls that promotes turbulence to effect mixing within the culture. The photobioreactor can be, as non-limiting examples, a flexible bioreactor (for example, a flexible floating bioreactor, a hanging bag reactor), a tubular bioreactor, a flat plate bioreactor, an airlift bioreactor, a bubble column bioreactor, a cascade bioreactor, or a bioreactor including elements of any of these types, or other types of bioreactors. A photobioreactor can have a light path (corresponding to the width (depth) or diameter of the bioreactor chamber measured from a boundary of the photobioreactor that is exposed to light) of from about 3 cm to about 500 cm, and in some examples can have a depth of from about 5 cm to about 200 cm. A photobioreactor can use natural light (sunlight) as a light source, where the bioreactor includes at least one surface that transmits light. Alternatively or in addition, a bioreactor can include an artificial light source that can be positioned to direct light into the bioreactor (such as through a transparent wall or cover of the bioreactor) or,

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alternatively, can be positioned within the bioreactor. A bioreactor can in some examples include one or more structures for directing light, for example, sunlight, to the cells within the bioreactor. Bioreactors can also typically allow for the control of one or more culture conditions such as temperature, pH, oxygen tension, carbon dioxide levels, and the like, as well as combinations thereof. Bioreactors can typically be configurable, for example, using ports attached to tubing, to allow gaseous components, such as CO<sub>2</sub>, CO<sub>2</sub>-enriched air, oxygen, and/or nitrogen, to be contacted with (*e.g.*, bubbled through) a liquid culture. Other culture parameters, such as the pH of the culture media, the identity and/or concentration of trace elements and/or nutrients, the identity and/or concentration of other media constituents, or the like, or combinations thereof, can typically be more readily manipulated using a bioreactor.

[0099] Culturing refers to the intentional fostering of growth (*e.g.*, increases in cell size, cellular contents, and/or cellular activity, *e.g.*, biomolecule synthesis) and/or propagation (*e.g.*, increases in cell numbers via mitosis) of one or more cells by use of selected and/or controlled conditions. The combination of both growth and propagation may be termed proliferation. Non-limiting examples of selected and/or controlled conditions can include the use of a defined medium (with known characteristics such as pH, ionic strength, and/or carbon source), specified temperature, oxygen tension, carbon dioxide levels, growth in a bioreactor, mixing of the culture, or the like, or combinations thereof. In some embodiments, the microorganism can be grown heterotrophically or mixotrophically, using both light and a reduced carbon source. The microorganism can preferably be cultured phototrophically. When growing or propagating phototrophically, the microorganism can advantageously use light as an energy source. An inorganic carbon source, such as CO<sub>2</sub> or bicarbonate, can be used for synthesis of biomolecules by the microorganism. "Inorganic carbon", as used herein, includes carbon-containing compounds or molecules that cannot be used as a sustainable energy source by an organism. Typically "inorganic carbon" can be in the form of CO<sub>2</sub> (carbon dioxide), carbonic acid, bicarbonate salts, carbonate salts, hydrogen carbonate salts, or the like, or combinations thereof, which cannot be further oxidized for sustainable energy nor used as a source of reducing power by organisms. If an organic carbon molecule or compound is provided in the culture medium of a

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microorganism grown phototrophically, it generally cannot be taken up and/or metabolized by the cell for energy and/or typically is not present in an amount sufficient to provide sustainable energy for the growth of the cell culture.

[00100] A source of inorganic carbon (such as, but not limited to, CO<sub>2</sub>, bicarbonate, carbonate salts, and the like), including, but not limited to, air, CO<sub>2</sub>-enriched air, flue gas, or the like, or combinations thereof, can be supplied to the culture. When supplying flue gas and/or other sources of inorganic that may contain CO in addition to CO<sub>2</sub>, it may be necessary to pre-treat such sources such that the CO level introduced into the (photo)bioreactor do not constitute a dangerous and/or lethal dose vis-à-vis the growth and/or survival of the microorganisms.

[00101] The growth medium can be any that supports growth of the photosynthetic microorganism. Non-limiting examples of growth media include those generally available from a wide variety of sources, where instructions for the preparation of particular media suitable for a wide variety of strains of microorganisms can be obtained from textbooks, the scientific literature, culture collections, and websites. For example, various fresh water and salt water media can include those described in Barsanti, L. and Gualtieri, P. (2005) *Algae: Anatomy, Biochemistry, and Biotechnology*, CRC Press, Taylor & Francis Group, Boca Raton, FL, USA, which is incorporated herein by reference for media and methods for culturing algae. Algal media recipes can also be found at the websites of various algal culture collections, including, as nonlimiting examples, the UTEX Culture Collection of Algae ([sbs.utexas.edu/utex/media.aspx](http://sbs.utexas.edu/utex/media.aspx)); Culture Collection of Algae and Protozoa ([ccap.ac.uk/media/pdfrecipes](http://ccap.ac.uk/media/pdfrecipes)); and Katedra Botaniky ([/botany.natur.cuni.cz/algo/caup-media.html](http://botany.natur.cuni.cz/algo/caup-media.html)). Recipes for growth media can be developed or optimized, for example for particular strains or growth conditions. Microorganisms that can be useful in accordance with the methods of the present invention can be found in various locations and environments throughout the world. Without being bound by theory, it is observed that, perhaps as a consequence of their isolation from other species and/or their evolutionary divergence, the particular growth medium for optimal growth and generation of lipid and/or hydrocarbon constituents can vary. In some cases, certain

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strains of microorganisms may be unable to grow in 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.

[00102] In some embodiments, a microorganism that produces one or more free fatty acids can be cultured in a medium that includes an increased concentration of a metal (typically provided as a salt and/or in an ionic form) such as, for example, sodium, potassium, magnesium, calcium, iron, or the like, or combinations thereof (particularly multivalent metals, such as magnesium, calcium, and/or iron), with respect to a standard medium formulation, such as, for example, standard BG-11 medium (ATCC Medium 616, Table 2), or a modified medium such as ATCC Medium 854 (BG-11 modified to contain vitamin B12) or ATCC Medium 617 (BG-11 modified for marine cyanobacteria, containing additional NaCl and vitamin B12). For example, a medium used for growing microorganisms that produce and release into the culture medium one or more free fatty acids can include at least 2-fold, for example at least 3-fold, at least 4-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold, at least 10-fold, between 2-fold and 10-fold, and/or between 10-fold and 100-fold the amount of metal (*e.g.*, calcium) as compared to a standard medium. The medium used for growing microorganisms that can produce free fatty acids can include, for example, at least about 0.5 mM, between about 0.5 mM and about 1 mM, between about 1 mM and about 2 mM, between about 2 mM and about 5 mM, between about 5 mM and about 10 mM, between about 10 mM and about 25 mM, and greater than 25 mM metal (*e.g.*, calcium) in the formulation. Addition of metal (*e.g.*, calcium) in the medium can additionally or alternately increase the tolerance of microorganism in media with a relatively high concentration of free fatty acids. Additionally or alternately, fatty acid-producing strains can advantageously be more robust with excess metal (*e.g.*, calcium) content. Other details regarding the use of a soap-forming ion source in the algal medium are described in the co-pending, commonly-assigned United States patent application 61/426,602, filed December 23, 2010, and entitled "Culturing a Microorganism in a Medium with an Elevated Level of a Carboxylate Counterion Source", which is incorporated herein by reference.

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[00103] Culturing of photosynthetic microorganisms can be performed under various conditions, such as under a light/dark cycle, and/or under natural light. In some embodiments, light/dark cycle refers to providing and removing (e.g., switching on and off) the light over a predetermined period, for example, a light dark cycle can be 12 hours of light followed by 12 hours of darkness or 14 hours of light followed by 10 hours of darkness. Alternatively or in addition, the light/dark cycle can be a natural light/dark cycle based on day-length, where the sun is the light source. "Natural light" refers to light not generated artificially, i.e. by lamps, etc., for example, sunlight and reflected sun light. Natural light can optionally be supplemented by artificial light. In some culture systems, the light period of a culture grown under natural light can be extended by the inclusion of one or more artificial light sources.

[00104] The methods of the invention include culturing a photosynthetic microorganism in a culture that undergoes mixing, such as active mixing. In general, in a mixed pond or photobioreactor, algae experience a continuous cycle of intermittent light exposure during the light period where the algae spend some of the light period time in the light and some of the light period time in darkness or near-darkness, where the cycle time and the approximate duration times the photosynthetic microorganisms are in light-exposed and dark regions of the pond or bioreactor will depend on the type and speed of mixing, the incident light intensity, the species or strain of algae in the pond, and the culture density. Mixing can be achieved by introducing turbulence into the pond so that the algae continuously rise and fall relative to the surface of the pond (or move toward and away from the light-facing perimeter of the photobioreactor).

[00105] Closed photobioreactors typically use combinations of pumps, injectors, and spargers to induce high Reynolds numbers (a measure of turbulence) within the photobioreactor. This results in mixing such that the exposure of algae to incident light (which may be in excess of photosynthetic capacity), is controlled, and prevents extended periods of time when cells are light-limited. By contrast, a conventional open pond typically generates turbulence and potential energy through the lifting action of a paddle wheel (or similar device). Frictional losses resulting from gradual dissipation of the turbulent eddies and circulation currents within the pond can be mitigated by adding

one or more additional mixing devices to a pond. Added mixing devices can enhance the turbulence within the pond and facilitate maintaining favorable conditions for capture of incident solar radiation by the algae. Static or passive devices can be used, such as static mixers, cavitation devices, baffles or the like. The passive devices are designed to maintain flow in the pond in the turbulent region over a portion of the pond volume by introducing eddies into the flow. Preferably, the passive devices allow a turbulent flow to be maintained within the pond for as large a pond volume as possible. The passive devices may be incorporated into the floor and/or the walls of the pond structure. Preferably, the passive structures are located underneath the surface of the pond.

[00106] Alternatively or in addition, one or more active mixing devices can be included at one or more locations in the pond. For example, high efficiency, slow turning in-water propellers can be added to effect or enhance mixing. A slow turning propeller refers here to propellers that turn slowly enough to mitigate or avoid damage to the algae within the pond. The speed of such device will be dependent on the species of algae and its resistance to lysing by shear forces. These active mixing devices are added to the extent needed to generate a desired amount of turbulence throughout the pond. Such mixing devices can also be employed in a photobioreactor.

[00107] The operation of active mixing devices can be optimized in relation to growth and harvesting cycles that occur over a course of a day, or over the algae life cycle. For example, in night time conditions, the additional active mixing devices in a pond or photobioreactor that uses natural light can be shut off. Since there is no incident sunlight, the light intensity experienced by the algae is not affected by the presence or absence of sufficient turbulence. In this situation, a paddle wheel (for a pond) may provide sufficient turbulence in a non-photosynthetic environment. In another example, algae in a pond may go first through a cell division / cell growth stage. A change in nutrients, pH, or another trigger is then be used to cause the algae to enter an oil production / secretion phase. During the first phase, additional in-water propellers are activated to improve the growth rate of the algae. In the second phase for

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oil production, the water propellers are stopped, resulting in reduced mixing so that oil can float to the surface and separate more easily.

[00108] The depth of a pond or light path of a photobioreactor also plays a factor in the amount of mixing that is needed to achieve a desired level of turbulence. For example, the depth of the growth pond can have a substantial impact on the Reynolds number for the pond. As an illustrative example, if the depth of the pond is reduced from 30 cm to 10 cm, the Reynolds number of such a pond can increase from about 1000 to about 3000.

[00109] Where the methods of the invention comprise production of a biomolecule, the culture methods can include inducing expression of a gene encoding a polypeptide for the production of the biomolecule. For example, for the production of fatty acids or fatty acid derivatives, the culture methods can include inducing expression of a thioesterase gene, a lipase gene, or another gene for the production of free fatty acids or fatty acid derivatives. Inducing expression can include adding a nutrient or compound to the culture, removing one or more components from the culture medium, increasing or decreasing light and/or temperature, and/or other manipulations that promote expression of the thioesterase, lipase, or other gene. Such manipulations can largely depend on the nature of the (heterologous) promoter operably linked to the thioesterase, lipase (or other) gene.

[00110] For production of certain products, such as, but not limited to, lipids such as fatty acids or fatty acid derivatives, photosynthetic microorganisms can additionally or alternately be cultured in shake flasks, test tubes, vials, microtiter dishes, petri dishes, or the like, or combinations thereof, where the cultures are subjected to intermittent light.

[00111] Biomass of the microorganism culture can be recovered by harvesting the microorganism from the medium, for example, by filtering, settling, centrifugation, or combinations thereof. In biomass production embodiments according to the invention, the amount of the biomass produced and/or recovered by the method described herein, measured as ash free dry weight (AFDW) can advantageously be at least about 0.05 g

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per liter of culture, for example at least about 0.1 g, at least about 0.2 g, at least about 0.3 g, at least about 0.4 g, at least about 0.5 g, at least about 0.6 g, at least about 0.7 g per liter of culture, at least about 1 g per liter of culture, at least about 1.5 g per liter of culture, at least about 2 g per liter of culture, at least about 2.5 g per liter of culture, or at least about 5 g per liter of culture. Although many times the goal can be to produce and/or recover as much biomass as possible, in some instances the amount of the biomass produced and/or recovered by the method described herein, measured as ash free dry weigh (AFDW) can be limited to about 15 g or less per liter of culture, for example about 12 g or less per liter of culture, about 10 g or less per liter of culture, about 5 g or less per liter of culture, about 2 g or less per liter of culture, about 1 g or less per liter of culture, or about 0.5 g or less per liter of culture.

[00112] Biomass can be used in any of a number of ways, for example, it can be processed for use as a biofuel by generating syngas from the biomass, can be supplied to an anaerobic digester for production of one or more alcohols, or the biomass can be extracted to provide algal lipids, such as but not limited to monoglycerides, diglycerides, or triglycerides, fatty acid alkyl esters, fatty acids, and/or fatty acid derivatives.

[00113] In some embodiments, fatty acids and fatty acid derivatives can be recovered from culture by recovery means known to those of ordinary skill in the art, such as by whole culture extraction, for example, using organic solvents. In some cases, recovery of fatty acids or fatty acid derivatives (such as fatty acid esters) can be enhanced by homogenization of the cells, as provided in the examples herein. When fatty acids are sufficiently released from the microorganisms into the culture medium, the recovery method can be adapted to efficiently recover only the released fatty acids, only the fatty acids produced and stored within the microorganisms, or both the produced and released fatty acids.

[00114] In further embodiments, products such as but not limited to free fatty acids and fatty acid derivatives that are secreted/released into the culture medium by the recombinant microorganisms described above can be recovered in a variety of ways. A straightforward isolation method, *e.g.*, by partition using immiscible solvents, may be

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employed. Additionally or alternately, particulate adsorbents can be employed. These can include lipophilic particulates and/or ion exchange resins, depending on the design of the recovery method. They may be circulating in the separated medium and then collected, and/or the medium may be passed over a fixed bed column, for example a chromatographic column, containing these particulates. The fatty acids can then be eluted from the particulate adsorbents, *e.g.*, by the use of an appropriate solvent. In such circumstances, one isolation method can include carrying out evaporation of the solvent, followed by further processing of the isolated fatty acids and lipids, to yield chemicals and/or fuels that can be used for a variety of commercial purposes.

[00115] The microorganisms according to some embodiments of the present invention produces free fatty acids and fatty acid derivatives in an amount greater than the amount of free fatty acids and fatty acid derivatives produced by a strain having an intact (nondisrupted) NPQ process grown under identical conditions.

[00116] Additionally or alternately, the present invention can include one or more of the following embodiments.

[00117] Embodiment 1. A method of producing biomass or at least one biomolecule comprising culturing a photosynthetic microorganism that comprises a disrupted Non-Photochemical Quenching (NPQ) process in a pond or bioreactor that includes at least one active mixing device under conditions sufficient for the microorganism to proliferate in the culture, and isolating biomass or at least one biomolecule from the culture.

[00118] Embodiment 2. The method of embodiment 1, wherein the amount of biomass or a biomolecule produced by the culture is at least 10% greater, for example at least 15% greater, at least 20% greater, or at least 25% greater, than the amount of biomass or a biomolecule produced by an identical culture of a microorganism identical in all respects except that it does not have a disrupted NPQ process.

[00119] Embodiment 3. The method of embodiment 1 or embodiment 2, wherein the culture volume is at least 20 liters, for example at least 50 liters, at least 100 liters, at least 200 liters, or at least 400 liters.

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[00120] Embodiment 4. The method of any one of the previous embodiments, wherein the photosynthetic microorganism is cultured phototrophically, such as under a light/dark cycle and/or under natural light.

[00121] Embodiment 5. The method of any one of the previous embodiments, wherein disrupting the NPQ process comprises disrupting the production of at least one carotenoid (*e.g.*, comprising echinenone and/or hydroxyechinenone) and/or reducing the expression of at least one carotenoid binding protein (*e.g.*, comprising OCP) in the microorganism.

[00122] Embodiment 6. The method of embodiment 5, wherein disrupting the NPQ process comprises inhibiting the expression of an OCP in the microorganism, optionally wherein one or more of the following are satisfied: the OCP is mutated to an inactive form, the OCP gene in the microorganism is disrupted by insertional mutagenesis, all or a portion of the OCP gene in the microorganism is removed, the microorganism expresses an antisense molecule that targets the OCP gene, the microorganism expresses a ribozyme that targets the OCP gene.

[00123] Embodiment 7. The method of any one of the previous embodiments, wherein the microorganism is a cyanobacterium, such as selected from a group of genera consisting of *Agmenellum*, *Anabaena*, *Anabaenopsis*, *Anacystis*, *Aphanizomenon*, *Arthrospira*, *Asterocapsa*, *Borzia*, *Calothrix*, *Chamaesiphon*, *Chlorogloeopsis*, *Chroococciopsis*, *Chroococcus*, *Crinalium*, *Crocospaera*, *Cyanobacterium*, *Cyanobium*, *Cyanocystis*, *Cyanospira*, *Cyanothece*, *Cylindrospermopsis*, *Cylindrospermum*, *Dactylococcopsis*, *Dermocarpella*, *Fischerella*, *Fremyella*, *Geitleria*, *Geitlerinema*, *Gloeobacter*, *Gloeocapsa*, *Gloeothece*, *Halospirulina*, *Iyengariella*, *Leptolyngbya*, *Limnothrix*, *Lyngbya*, *Microcoleus*, *Microcystis*, *Myxosarcina*, *Nodularia*, *Nostoc*, *Nostochopsis*, *Oscillatoria*, *Phormidium*, *Planktothrix*, *Pleurocapsa*, *Prochlorococcus*, *Prochloron*, *Prochlorothrix*, *Pseudanabaena*, *Rivularia*, *Schizothrix*, *Scytonema*, *Spirulina*, *Stanieria*, *Starria*, *Stigonema*, *Symploca*, *Synechococcus*, *Synechocystis*, *Thermosynechococcus*, *Tolypothrix*, *Trichodesmium*, *Tychonema*, and *Xenococcus*.

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[00124] Embodiment 8. The method of Embodiment 7, wherein the photosynthetic microorganism is a species of *Synechocystis*, *Arthrospira*, *Microcystis*, *Lyngbya*, *Nostoc*, *Anabaena*, *Synechococcus*, *Gloeobacter*, *Crocospaera*, or *Thermosynechococcus*.

[00125] Embodiment 9. The method of any of embodiments 5-8, wherein the OCP has at least 40%, at least 45%, at least 50%, for example at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95%, identity to SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, or SEQ ID NO:24.

[00126] Embodiment 10. The method of any one of the previous embodiments, wherein the microorganism produces at least 0.3 g/L of biomass.

[00127] Embodiment 11. The method of any one of the previous embodiments, wherein the microorganism produces a free fatty acid (*e.g.*, including at least one C<sub>12</sub> to C<sub>24</sub> fatty acid) or a fatty acid derivative (*e.g.*, including at least one fatty acid derivative comprising an acyl chain length from 12 to 24 carbons and/or comprising a fatty aldehyde, a fatty alcohol, a wax ester, an alkene, and/or an alkane).

[00128] Embodiment 12. The method of embodiment 11, wherein the amount of free fatty acid or fatty acid derivative produced is greater than the amount of free fatty acid or fatty acid derivative produced by an identical culture of a microorganism identical in all respects except that it does not have a disrupted NPQ process that is grown under identical conditions.

[00129] Embodiment 13. The method of embodiment 11 or 12, wherein the microorganism comprises an exogenous nucleic acid molecule encoding a thioesterase (*e.g.*, comprising an acyl-ACP thioesterase, an acyl-CoA thioesterase, a hydroxylbenzoyl-CoA thioesterase, or a combination thereof) and/or lipase (*e.g.*, a

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member of Pfam PF01674, Pfam PF01764, Pfam PF07819, Pfam PF03583, or Pfam PF00151, such as a member of Pfam PF01674, Pfam PF01764, or Pfam PF07819).

[00130] Further additionally or alternately, there can be a method according to any one of the preceding method embodiments, wherein the medium used for culturing the fatty acid-producing organism can include an increased concentration of a soap-forming ion source (*e.g.*, an inorganic soap-forming ion source, a metal ion source, a multivalent metal ion source, a divalent metal ion source, or some combination thereof, such as sodium, potassium, magnesium, calcium, iron, or combinations thereof, particularly multivalent metals, such as magnesium, calcium, and/or iron), with respect to a standard medium formulation (*e.g.*, standard BG-11 medium) or a modified medium (*e.g.*, ATCC Medium 854 or ATCC Medium 617), which increased concentration can optionally be at least about 0.5 mM (*e.g.*, between about 0.5 mM and about 1 mM, between about 1 mM and about 2 mM, between about 2 mM and about 5 mM, between about 5 mM and about 10 mM, between about 10 mM and about 25 mM, and/or greater than 25 mM) and/or can optionally but preferably be at least 2-fold (*e.g.*, at least 3-fold, at least 4-fold, at least 5-fold, at least 6-fold, at least 7-fold, at least 8-fold, at least 9-fold, at least 10-fold, between 2-fold and 10-fold, and/or between 10-fold and 100-fold) as compared to said standard/modified medium.

[00131] Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the present invention and practice the claimed methods. The following working examples, therefore, specifically point out representative embodiments of the present invention, some preferred, and are not to be construed as limiting in any way the remainder of the disclosure.

#### EXAMPLES

[00132] The following Examples have been included to provide guidance to one of ordinary skill in the art for practicing representative embodiments of the presently disclosed subject matter. In light of the present disclosure and the general level of skill in the art, those of skill can appreciate that the following Examples are intended to be

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exemplary only and that numerous changes, modifications, and/or alterations can be employed without departing from the scope of the presently disclosed subject matter.

Example 1: Absence of NPQ

[00133] A puc 19-based construct was designed for interrupting the OCP gene of *Synechocystis* sp. PCC 6803, in which a kanamycin resistance gene was positioned between *Synechocystis* sequences that occur upstream and downstream of the gene encoding Orange Carotenoid Protein in the *Synechocystis* genome (Figure 1). The sequences of the construct is provided as SEQ ID NO:3. The construct was designed such that recombination of the [OCP upstream sequences – kanamycin resistance gene – OCP downstream sequences] into the *Synechocystis* genome results in replacement of the OCP gene with the kanamycin resistances gene. Thus, kanamycin resistant transformants lack an OCP gene.

[00134] The construct having the knock-out gene integration organization as provided in Figure 1 was transformed into *Synechocystis* using standard transformation methods essentially as disclosed in Zang *et al.* (2007) *J. Microbiol.* 45: 241-245, and kanamycin resistant colonies were grown up and tested for the absence of the OCP gene and the presence of the kanamycin gene in separate PCR assays. One confirmed knockout isolate, designated  $\Delta$ OCP, was used for further study.

[00135] Chlorophyll fluorometry was used to verify that the OCP knockout strain was deficient in blue light induced NPQ, the established function of the OCP (Wilson *et al.* (2006) *The Plant Cell* 18: 992-1007; Boulay *et al.* (2008) *Biochimica et Biophysica Acta* 1777: 1344-1354).

[00136] Fluorescence after stimulation with blue light was determined using a Walz Dual-PAM-100 fluorometer according to established methods (see, for example, Wilson *et al.* (2006); Boulay *et al.* (2008)). To prepare the cells, cell cultures of  $\Delta$ OCP and wild type were grown in ~100 mL shake flasks under ~50 microEinsteins ( $\mu$ E) of light in the presence of ~1% CO<sub>2</sub> in BG-11 medium. A ~10 mL aliquot of each culture was removed, spun down at ~4,000 rpm for about 3 mins, and resuspended in ~2 mL of water, and the cells were dark-acclimated for about one hour prior to transferring to cuvettes for taking fluorescence measurements. The measuring light was turned on for

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~1 minute prior to exposing the cells to saturation flashes of ~10,000 E for measuring Fv/Fm. The samples were then exposed to saturating flashes (spikes) of blue light at ~400 E (start of exposure to blue light marked by an asterisks on the graphs of Figures 2A and 2B; blue light turned off marked by squares). The dark recovery of Fv/Fm was followed by the occasional application of saturating blue light flashes.

[00137] Figure 2A shows the fluorescence traces of wild type cells. The chlorophyll fluorescence was quenched when samples were shifted from dark to blue light (shown by a plus sign). The quenching was induced faster than it relaxed after a shift back to dark (shown by a minus sign). The wild-type (WT) had an OCP, while the strain without an OCP ( $\Delta$ OCP) shows no blue light induction of NPQ, as seen in Figure 2B.

[00138] Figure 2A also shows that NPQ was slow to relax in comparison to the time it took for induction.

#### Example 2: Production of Biomass

[00139] To investigate biomass accumulation, *Synechocystis* was grown in either controlled laboratory environments or in a greenhouse with natural light. Cell biomass was estimated using the standard ash-free dry weight method. Ash-free dry weights (a measurement of the weight of organic material in algal cultures) were determined by removing water from a sample of the growth culture, drying the sample, recording the weight, combusting the sample, and recording the weight of the remaining residue.

[00140] In the greenhouse growth experiments, cultures of *Synechocystis* sp. PCC 6803 wild type and *Synechocystis* sp. PCC 6803 Deleted Orange Carotenoid Protein strains were inoculated into ~150 mls of BG-11 media. After each ~24 hour period of greenhouse growth, the culture was brought up to a total volume of ~150 mls with distilled water to make up for any evaporation, and then half of the culture was removed and replaced with BG-11 media. About 25-35 mls of the harvested sample was assessed for ash-free dry weight (AFDW) by filtering the cells, drying the biomass in an oven, recording the weight of the dried biomass, combusting the dried sample in a furnace, and weighing the remaining residue after combusting. The dry weight (DW) and ash-free dry weight (AFDW) were calculated according to the formulas:

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$$DW(g/l) = \frac{(ovenweight(g) - filterweight(g)) * 1000(ml/l)}{samplevolume(ml)}$$

$$AFDW(g/l) = DW(g/l) - \left( \frac{(furnaceweight(g) - filterweight(g)) * 1000(ml/l)}{samplevolume(ml)} \right)$$

[00141] Where “ovenweight” is the weight of the sample after drying in the oven, and “furnaceweight” is the weight of the same sample after combusting in the muffle furnace.

[00142] Briefly, ~25 mls, ~30 mls, or ~35 mls of removed sample of each culture was transferred to a filtration assembly that included a side arm flask fitted with a stopper, funnel, and screen for supporting a filter held with a clamp. A pre-weighed Whatman 47mm GF/F glass microfiber filter was positioned over the screen. The sample was pipetted onto the surface of the filter, and a vacuum (about 5 – 10 psi) was applied via the side arm of the flask. Once all the liquid passed through the filter, the sides of the funnel were rinsed with ~9-12 mls of distilled water to bring down any cells that may have stuck to the side of the funnel. The rinsing step was repeated twice. The clamp was removed to disassemble the filtration unit, and the filter was removed from the base with forceps. The filter was placed in a pre-weighed aluminum weighing boat, and then the samples were placed in a ~105°C drying oven until the weight was constant, at least four hours. The dried samples were then place in a dessicator to cool, and then the weigh boat plus filter was weighed. Dry weight was calculated as:

$$DW(g/l) = \frac{(ovenweight(g) - vialweight(g)) * 1000(ml/l)}{samplevolume(ml)}$$

[00143] Samples were then place into a muffle furnace heated to ~550°C for ~1 hour. The samples were then removed using tongs and transferred to the desiccator to cool to room temperature. When the samples were cool, they were weighed using the same analytical balance used to weigh the dry samples.

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[00144] Ash Free Dry Weight (in g/l) was calculated as follows:

$$AFDW \left( \frac{g}{l} \right) = DW \left( \frac{g}{l} \right) - \left( \frac{\text{furnace weight (g)} - \text{vial weight (g)}}{\text{sample volume (ml)}} \right) * 1000 \left( \frac{ml}{l} \right)$$

The Ash Free Dry Weight of the culture was then calculated based on the culture volume.

[00145] In the laboratory the cells were grown in 250 ml tissue culture flasks and bubbled vigorously with air. Cells were grown at ~30°C, and light was supplied by white LEDs that were programmed according to: ~16 hours light; ~8 hours dark. Light was ~50 µE background with ~2000 µE flashes supplied every ~2 minutes. The length of the flash was randomized to vary between 10 seconds and 1 minute. Cells were diluted by about one half every day, and culture was harvested for ash-free dry weight. Over ~3 days in these conditions the wild-type culture produced ~0.09 grams per liter of the culture. The ΔOCP mutant produced ~0.12 grams of biomass per liter.

[00146] In the greenhouse, samples were: incubated to an equal optical density {OD at ~720 nm). The cultures were bubbled with air in ~250 ml tissue flasks that were submerged in a ~30°C water bath. To approximate the light environment of a dense production system, the bottom ~1/3 of the flask was made to be opaque, with only approximately 25% of the total culture volume exposed to full sunlight. Cell growth and ash-free dry weights were followed for several days in these conditions.

[00147] On day 0 cells were inoculated in flasks in the greenhouse. One day later, about one half of the culture was removed and replaced with an equal volume of fresh media. The removed cells were spun down and analyzed for ash-free dry weight. The same procedure was repeated on days 1, 2, 3, and 4. The accumulative biomass produced by the culture is shown in Table 1, and the daily biomass produced by the culture is shown in Figure 3.

Table 1: Accumulative biomass produced by WT and OCP knock-out ( $\Delta$ OCP)

	Biomass produced by WT (g/liter)	Biomass produced by $\Delta$ OCP (g/liter)
Day 1	0.05	0.08
Day 2	0.11	0.15
Day 3	0.18	0.24
Day 4	0.26	0.32

[00148] The results of the biomass accumulation experiment demonstrate that eliminating OCP and decreasing NPQ (as shown in Example 1) did not result have a deleterious effect on biomass accumulation as might be expected if excessive photodamage occurred. In fact, as shown in Figure 3, cells having a disrupted NPQ process due to knock out of the OCP gene accumulated approximately 28% more biomass than wild type (“non OCP-knock-out”) cells, suggesting that reduction of NPQ allowed a higher proportion of photons to provide energy for photochemistry and biochemical pathways that generate biomass.

## WHAT IS CLAIMED IS:

1. A method of producing biomass or at least one biomolecule comprising culturing a photosynthetic microorganism that comprises a disrupted Non-Photochemical Quenching (NPQ) process under intermittent light conditions in a suitable culture medium, and isolating biomass or at least one biomolecule from the culture.
2. The method of claim 1, wherein the amount of biomass or a biomolecule produced by the culture is at least 20% greater than the amount of biomass or a biomolecule produced by an identical culture of a microorganism identical in all respects except that it does not have a disrupted NPQ process.
3. The method of claim 1, wherein the photosynthetic microorganism is cultured phototrophically.
4. The method of claim 3, wherein the photosynthetic microorganism is cultured under a light/dark cycle.
5. The method of claim 3, wherein the photosynthetic microorganism is cultured under natural light.
6. The method of claim 1, wherein disrupting the NPQ process comprises disrupting the production of at least one carotenoid and/or reducing the expression of at least one carotenoid binding protein in the photosynthetic microorganism.
7. The method of claim 6, wherein the at least one carotenoid comprises echinenone and/or hydroxyechinenone.
8. The method of claim 6, wherein the at least one carotenoid binding protein comprises OCP.
9. The method of claim 8, wherein disrupting the NPQ process comprises inhibiting the expression of the OCP in the photosynthetic microorganism.
10. The method of claim 9, wherein all or a portion of an OCP gene in the photosynthetic microorganism is removed.

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11. The method of claim 1, wherein the photosynthetic microorganism is a cyanobacterium.
12. The method of claim 7, wherein the cyanobacterium is selected from a group of genera consisting of *Agmenellum*, *Anabaena*, *Anabaenopsis*, *Anacystis*, *Aphanizomenon*, *Arthrospira*, *Asterocapsa*, *Borzia*, *Calothrix*, *Chamaesiphon*, *Chlorogloeopsis*, *Chroococciopsis*, *Chroococcus*, *Crinalium*, *Crocospaera*, *Cyanobacterium*, *Cyanobium*, *Cyanocystis*, *Cyanospira*, *Cyanothece*, *Cylindrospermopsis*, *Cylindrospermum*, *Dactylococcopsis*, *Dermocarpella*, *Fischerella*, *Fremyella*, *Geitleria*, *Geitlerinema*, *Gloeobacter*, *Gloeocapsa*, *Gloeothece*, *Halospirulina*, *Iyengariella*, *Leptolyngbya*, *Limnothrix*, *Lyngbya*, *Microcoleus*, *Microcystis*, *Myxosarcina*, *Nodularia*, *Nostoc*, *Nostochopsis*, *Oscillatoria*, *Phormidium*, *Planktothrix*, *Pleurocapsa*, *Prochlorococcus*, *Prochloron*, *Prochlorothrix*, *Pseudanabaena*, *Rivularia*, *Schizothrix*, *Scytonema*, *Spirulina*, *Stanieria*, *Starria*, *Stigonema*, *Symploca*, *Synechococcus*, *Synechocystis*, *Thermocynechococcus*, *Tolypothrix*, *Trichodesmium*, *Tychonema*, and *Xenococcus*.
13. The method of claim 1, wherein the photosynthetic microorganism is cultured in a photobioreactor or pond.
14. The method of claim 13, wherein the culture undergoes active mixing.
15. The method of claim 1, wherein the photosynthetic microorganism produces at least 0.3 g/L of biomass.
16. The method of claim 1, wherein the microorganism comprises at least one non-native nucleic acid molecule encoding a thioesterase or polypeptide having lipolytic activity.
17. The method of claim 16, wherein the photosynthetic microorganism produces a free fatty acid or a fatty acid derivative.

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18. The method of claim 17, wherein the amount of free fatty acid or fatty acid derivative produced is greater than the amount of free fatty acid or fatty acid derivative produced by an identical culture of a photosynthetic microorganism identical in all respects except that it does not have a disrupted NPQ process that is grown under identical conditions.
19. The method of claim 17, wherein the fatty acid produced includes at least one C<sub>12</sub> to C<sub>24</sub> free fatty acid.
20. The method of claim 17, wherein the fatty acid produced includes at least one fatty acid derivative comprising an acyl chain length from 12 to 24 carbons.

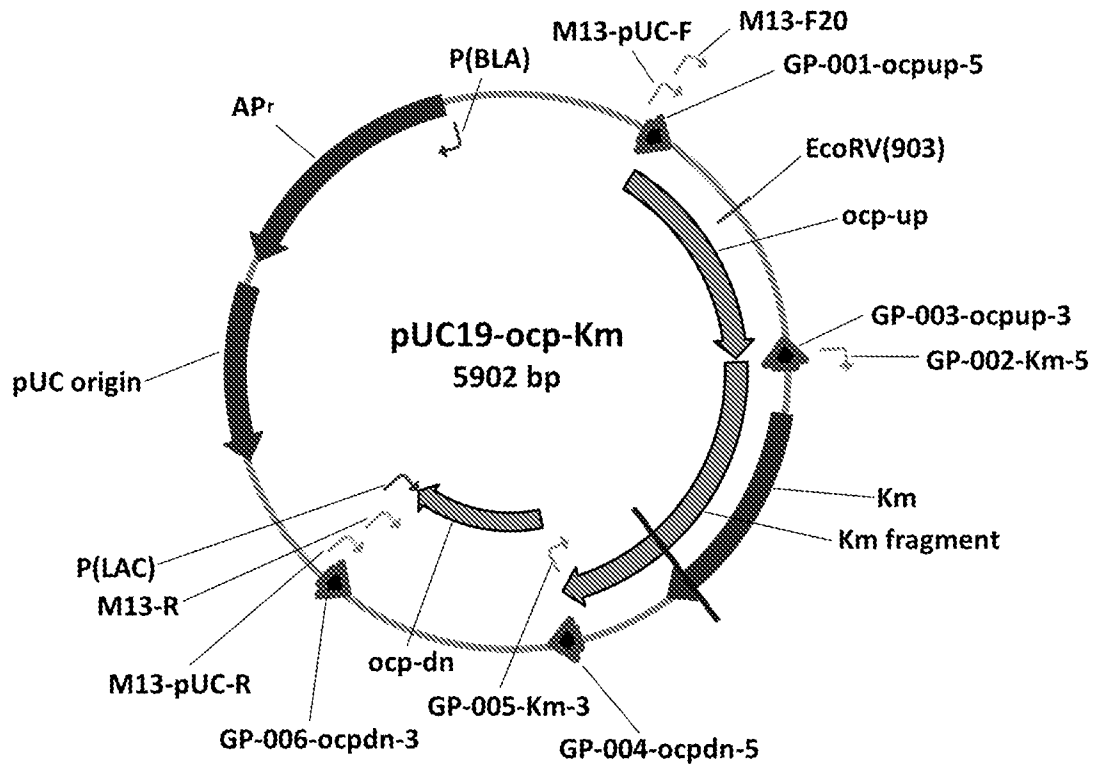


FIGURE 1

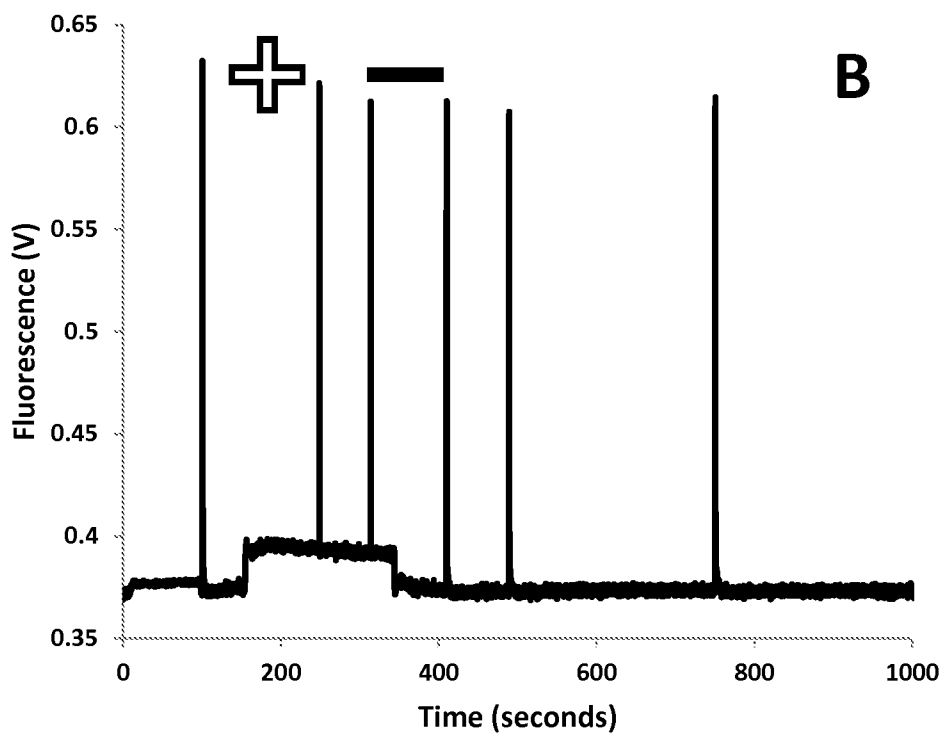
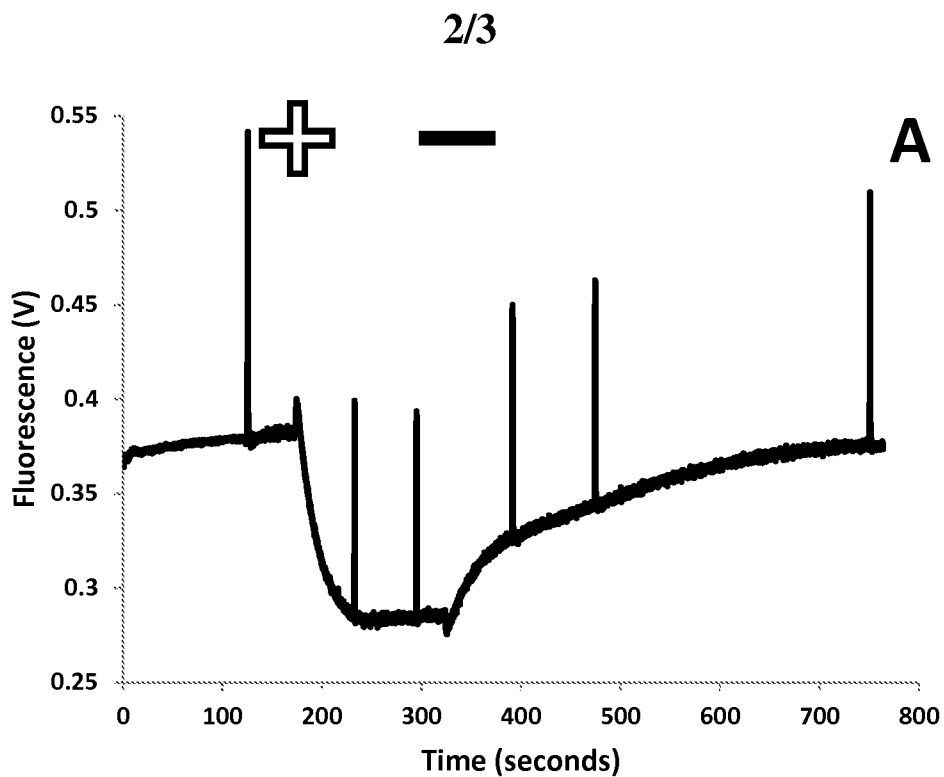


FIGURE 2

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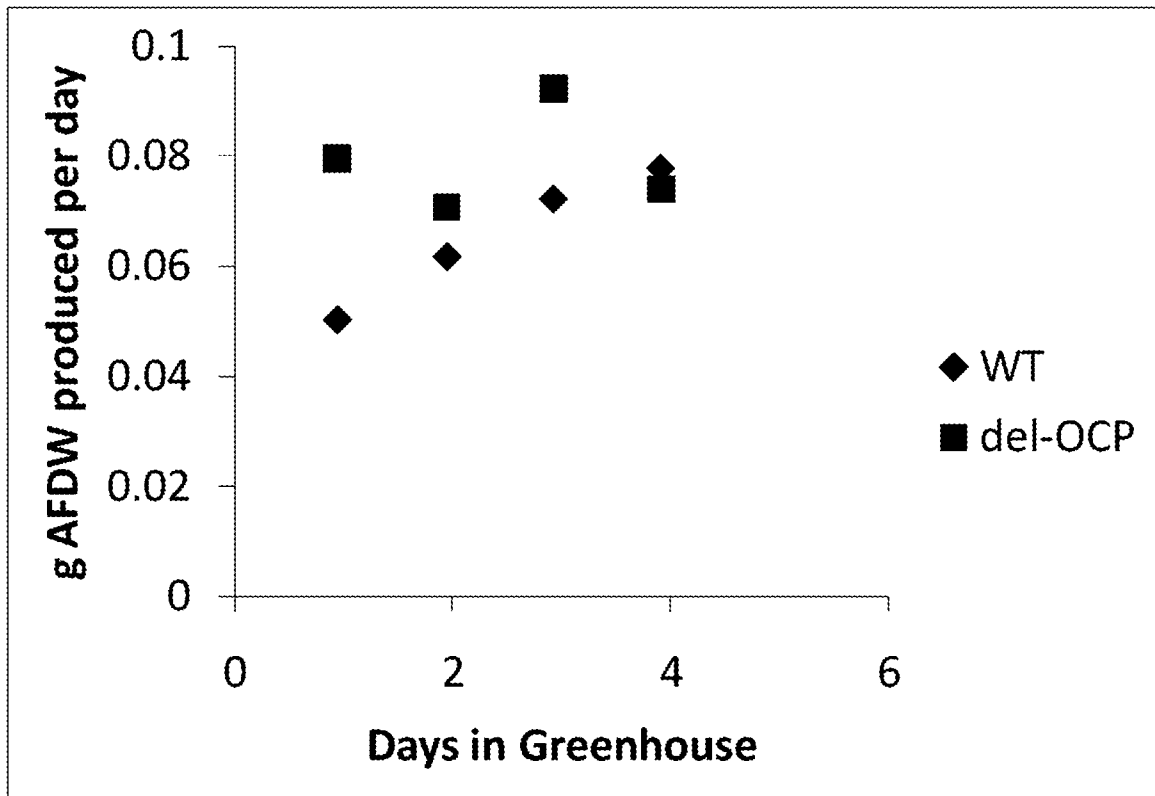


FIGURE 3

**INTERNATIONAL SEARCH REPORT**

International application No.  
PCT/US 11/66241

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC(8) - C12N 1/21, C12P 1/04, C12P 7/64 (2012.01)  
USPC - 435/170; 435/252.3, 435/134  
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
IPC(8): C12N 1/21, C12P 1/04, C12P 7/64 (2012.01)  
USPC: 435/170; 435/252.3, 435/134

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
USPC: 435/471, 435/41, 435/257.2

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
PubWEST (DB=PGPB,USPT,USOC,EPAB,JPAB; PLUR=NO; OP=ADJ), Google Scholar, Google Patents  
Search Terms Used:

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y --- A	WILSON et al. A Soluble Carotenoid Protein Involved in Phycobilisome-Related Energy Dissipation in Cyanobacteria. The Plant Cell, April 2006, Vol 18, No 4, pages 992-1007. esp: abstract; pg 993 section entitled "Construction of OCP Mutants"; pg 999 last paragraph; pg 1005 section entitled "Culture Conditions"; Fig. 1.	1, 3-6, 8-14 ----- 7, 15-20 ----- 2
Y	MOCHIMARU et al. The cyanobacterium Anabaena sp. PCC 7120 has two distinct beta-carotene ketolases: CrI0 for echinenone and CrIW for ketomyxol synthesis. FEBS Letters, 7 November 2005, Vol 579, No 27, pages 6111-6114. abstract; pg 6112 section entitled "3.2 Carotenoids in deleted mutans"; Fig. 3.	7
Y	US 2010/0297736 A1 (DUHRING et al.) 25 November 2010 (25.11.2010) esp: para [1289] Table 1.	15
Y	US 2010/0251601 A1 (HU et al.) 7 October 2010 (07.10.2010) esp: [0007]-[0010],[0018]-[0022],[0032], [0148], [0149], [0168].	16-20
A	BOULAY et al., Occurrence and function of the orange carotenoid protein in photoprotective mechanisms in various cyanobacteria. Biochim Biophys Acta, October 2008, Vol 1777, No 10, pages 1344-1354. entire document.	1-20
A	BOULAY et al., Identification of a protein required for recovery of full antenna capacity in OCP-related photoprotective mechanism in cyanobacteria. PNAS, 22 June 2010, Vol 107, No 25, pages 11620-11625. entire document	1-20

Further documents are listed in the continuation of Box C.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 30 March 2012 (30.03.2012)	Date of mailing of the international search report <b>03 MAY 2012</b>
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young  PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

**INTERNATIONAL SEARCH REPORT**

International application No.  
PCT/US 11/66241

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BRUNET et al., Can the xanthophyll cycle help extract the essence of the microalgal functional response to a variable light environment? Journal of Plankton Research, 17 August 2010, Vol 32, No 12, pages 1609-1617. entire document.	1-20
A	KIRILOVSKY. Photoprotection in cyanobacteria: the orange carotenoid protein (OCP)-related non-photochemical-quenching mechanism. Photosynth Res, July-September 2007, Vol 93, No 1-3, pages 7-16. entire document.	1-20
A	PUNGINELLI et al. Influence of zeaxanthin and echinenone binding on the activity of the orange carotenoid protein. Biochim Biophys Acta, April 2009, Vol 1787, No 4, pages 280-288. entire document.	1-20
A	US 2010/0285105 A1 (RADIANGTYAS) 11 November 2010 (11.11.2010) entire document.	16-20