(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
2 February 2012 (02.02.2012)

PCT

(10) International Publication Number
WO 2012/015949 A2

(51) International Patent Classification:
C12P 7/02 (2006.01)

(21) International Application Number:
PCT/US2011/045572

(22) International Filing Date:
27 July 2011 (27.07.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/369,004 29 July 2010 (29.07.2010) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

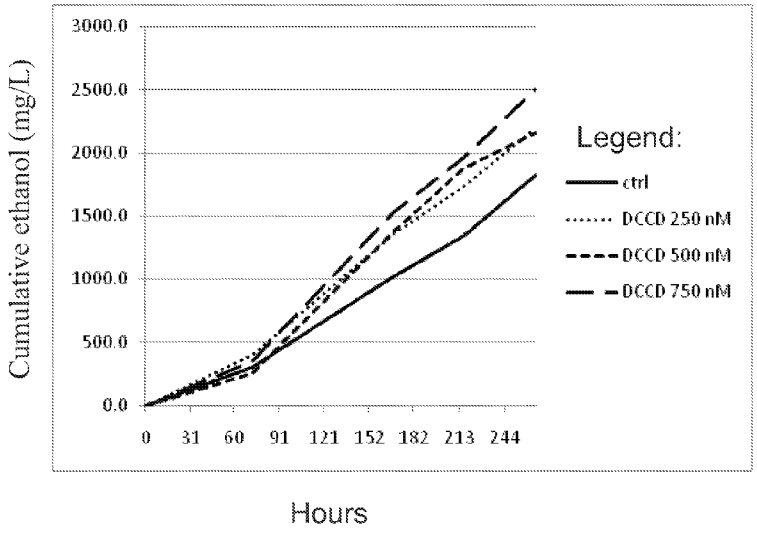
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

(54) **Title**: METHODS AND COMPOSITIONS FOR IMPROVING YIELDS OF REDUCED PRODUCTS OF PHOTOSYNTHETIC MICROORGANISMS

Figure 2



(57) **Abstract**: The invention provides methods and compositions for the improved biosynthesis of ethanol in a host cell. Genes encoding enzymes for futile cycle pathways to increase ethanol production, methods of optimizing expression of these enzymes in host cells, and methods for the production of ethanol by these host cells are disclosed. Exogenous compounds for increasing ethanol productivity by the host cells are also provided.

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METHODS AND COMPOSITIONS FOR IMPROVING YIELDS OF REDUCED PRODUCTS OF PHOTOSYNTHETIC MICROORGANISMS

BACKGROUND OF THE INVENTION

[0001] The desire for an efficient biosynthetic production of biofuel product has led to the development of photosynthetic microorganisms that produce carbon-based products of interest, *e.g.* reduced biofuel products, etc. While the yields of such products have increased over time, the yields reported to date have not reached the theoretical maximum. Thus, there is a need in the art for methods and compositions which increase the productivity of engineered photosynthetic cells, particularly with respect to the yields of desirable reduced products (e.g., biofuels) synthesized by such cells.

SUMMARY OF THE INVENTION

[0002] The present invention relates to methods and compositions for increasing a host cell's production of a carbon-based product of interest, including a reduced product of interest. The invention provides, in certain embodiments, an engineered host cell, comprising one or more recombinant protein activities selected from at least one futile cycle pathway, wherein the recombinant protein activities facilitate dissipation of excess energy stored in the engineered host cell. In another aspect of the invention, dissipation of excess energy stored in a host cell is implemented exogenously, e.g., through addition of an energy-dissipating factor to the host cell growth medium.

[0003] In one embodiment of the present invention, the host cell is a gram-negative or gram-positive bacterium. In another embodiment, the host cell is a photosynthetic microbe. In yet another embodiment, the host cell is a cyanobacterium.

[0004] In one aspect, the present invention relates to methods and compositions which dissipate excess energy in a host cell. In another aspect, the excess energy is stored in an ATP molecule. In still another aspect, the excess energy is stored as a proton gradient. The proton gradient stores energy as a proton concentration gradient across membrane-bound compartments. It can be used to generate proton motive force and drive ATP synthesis. In one embodiment, the host cell is engineered to dissipate excess ATP. In another embodiment, the host cell is engineered to dissipate excess proton gradient. In still another embodiment, excess energy dissipation increases the host cell's production of a reduced product of interest.

[0005] The present invention also provides for dissipation of excess energy stored in ATP, wherein the excess energy is defined by an amount of ATP in a host cell modified to produce a carbon-based product of interest that is greater than the amount of ATP at the metabolic equilibrium of a background host cell, wherein the background host cell lacks such modifications. In one aspect, the excess energy is defined by the energy charge in a host cell modified to produce a carbon based product of interest, wherein said cell is at metabolic equilibrium, and the energy charge is higher than a corresponding characteristic energy charge of a background host cell lacking such modifications. The characteristic energy charge in a background host cell (e.g., the wild-type energy charge) is proportional to the mole fraction of ATP plus half the mole fraction of ADP in relationship to the mole fraction of ATP, ADP, and AMP combined. In one aspect of the present invention, the energy charge is decreased by the compositions and methods provided herein.

[0006] In one aspect the engineered host cell has excess energy stored in ATP, wherein the amount of ATP in the host cell is greater than the amount of ATP at metabolic equilibrium of the background host cell, wherein the engineered host cell is modified to produce a carbon-based product of interest, and wherein the background host cell is unmodified. The amount of ATP at metabolic equilibrium can be determined by the characteristic energy charge of the background host cell.

[0007] In one embodiment, the present invention provides an engineered host cell, comprising one or more recombinant protein activities selected from at least one futile cycle pathway, wherein the recombinant protein activities facilitate dissipation of an excess energy stored in an engineered host cell, and wherein the host cell produces a carbon-based product of interest. In another embodiment, the excess energy is produced during the production of a carbon-based product of interest. In yet another embodiment, the carbon-based product o finterest is a reduced product. In a further embodiment, the carbon-based product of interest is a hydrocarbon. In still another embodiment, the carbon-based product of interest is an alcohol.

[0008] In one embodiment of the invention, a method is provided to increase production of a reduced product in a host cell, comprising the step of manipulating the host cell to dissipate excess energy stored in the host cell, and culturing the host cell in a host cell growth medium. In another embodiment, an exogenous ATP depletion agent is added to the host cell growth medium. In still another embodiment, the reduced product is ethanol. In still another embodiment, the host cell comprises a recombinant NADPH-dependent *adh* gene.

[0009] In one aspect, the exogenous ATP depletion agent is administered in an amount effective to mitigate ATP synthesis in said host cell, wherein the yield of the reduced product by the host cell is increased by at least 5% relative to the yield of the reduced product by the host cell cultured in an otherwise identical host cell growth medium lacking said exogenous ATP depletion agent. In still another aspect, exogenous ATP depletion agent is selected from the group consisting of: N,N′-dicyclohexylcarbodiimide, 3,3′,4′,5-tetrachlorosalicylanilide, 2,4-dinitrophenol, and carbonyl cyanide-*p*-trifluoromethoxyhydrazone. In one particular embodiment, the exogenous ATP depletion agent is N,N′-dicyclohexylcarbodiimide and is present in the host cell growth medium at a concentration of 0.25μM to 1μM. In a further embodiment, the presence of N,N′-dicyclohexylcarbodiimide in the culture medium results in a 5 to 30% increase in ethanol productivity of the host cell relative to ethanol productivity of the host cell cultured in an otherwise identical culture medium lacking said 3,3′,4′,5-tetrachlorosalicylanilide. In another particular embodiment, the exogenous ATP depletion agent is 3,3′,4′,5-tetrachlorosalicylanilide and is present in the host cell growth medium at a concentration of 0.1μM to 0.25μM. In a further embodiment, the presence of 3,3′,4′,5-tetrachlorosalicylanilide in the culture medium results in a 5 to 30% increase in ethanol productivity of the host cell relative to ethanol productivity of the host cell cultured in an otherwise identical culture medium lacking said 3,3′,4′,5-tetrachlorosalicylanilide.

[0010] In addition, the present invention provides for a method to increase production of a reduced product in a host cell, comprising the step of manipulating the host cell to dissipate excess energy stored in the host cell, and culturing the host cell in a host cell growth medium. In one embodiment, the host cell is genetically modified to dissipate the excess energy. In a further embodiment, the genetically modified host cell comprises one or more recombinant protein activities selected from a futile cycle pathway. In one aspect, an exogenous ATP synthase inhibitor is added to the host cell growth medium. In a further aspect, the exogenous ATP synthase inhibitor is selected from the group consisting of: N,N′-dicyclohexylcarbodiimide, 3,3′,4′,5-tetrachlorosalicylanilide, 2,4-dinitrophenol, and carbonyl cyanide-p-trifluoromethoxyhydrazone.

[0011] In one embodiment, the invention provides for a method for increasing the rate of production of a carbon-based product of interest by an engineered host cell, comprising the step of introducing one or more recombinant protein activities from a futile cycle pathway into a host cell, and culturing the engineered host cell under conditions that promote production of the carbon-based product of interest. In another embodiment, the one or more recombinant protein activities are under the control of an inducible promoter. In a further

embodiment, the inducible promoter is P(*nir*07) (SEQ ID NO:1). In one embodiment, the one or more recombinant protein activities are selected from Table 7. In one aspect, the one or more recombinant protein activities are comprised of a gene product of a gene selected from the group consisting of *pntAB* and *udhA*. In another aspect, the one or more recombinant protein activities are selected from the group consisting of a membrane-bound proton-translocating transhydrogenase and a soluble pyridine nucleotide transhydrogenase. In still another aspect, the one or more recombinant protein activities comprise a gene product of a gene selected from the group consisting of *glgC*, *glgA*, and *glgP*. In one embodiment, the one or more recombinant activities are selected from the group consisting of glucose-1-phosphate adenylyltransferase, glucose pyrophosphorylase, and glycogen phosphorylase. In another embodiment, the one or more recombinant protein activities comprise a gene product of a gene selected from the group consisting of *pfkA* and *fbp*. In yet another embodiment, the one or more recombinant protein activities are selected from the group consisting of phosphofructokinase and fructose-bisphosphatase. In another embodiment, the one or more recombinant protein activities comprise a gene product of a gene selected from the group consisting of *pta*, *ackA*, and *acs*. In still another embodiment, the one or more recombinant protein activities are selected from the group consisting of phosphotransacetylase, acetate kinase, and acetyl coenzyme synthetase. In yet another embodiment, the one or more recombinant protein activities comprise a gene product of a gene selected from the group consisting of *pykF* and *ppsA*. In one aspect, the one or more recombinant protein activities are selected from the group consisting of pyruvate kinase and phosphoenolpyruvate synthase. In another aspect, the one or more recombinant protein activities comprise a gene product of a gene selected from the group consisting of *pyc*, *pck*, and *pykF*. In yet another aspect, the one or more recombinant protein activities are selected from the group consisting of pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and pyruvate kinase. In still another aspect, the one or more recombinant protein activities comprise a gene product of a gene selected from the group consisting of *mdh*, *sfcA*, and *pyc*. In one embodiment, the one or more recombinant protein activities are selected from the group consisting of malate dehydrogenase and pyruvate carboxylase. In a further embodiment, the one or more recombinant protein activities comprise a gene product of a gene selected from the group consisting of *mdh*, *ppc*, *sucCD*, *maeB*, *sad*, and *sucD*. In another embodiment, the one or more recombinant protein activities are selected from the group consisting of malate dehydrogenase, phosphoenolpyruvate carboxylase, succinyl-CoA synthetase, and succinate semialdehyde dehydrogenase. In another further embodiment, the

one or more recombinant protein activities comprise a gene product of a gene selected from the group consisting of *mdh*, *mdhP*, *gap2*, *pgk*, *dld*, *mgsA*, *gloA*, and *gloB*. In yet another embodiment, the one or more recombinant protein activities are selected from the group consisting of NAD-specific malate dehydrogenase, NADP-specific malate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, D-lactate dehydrogenase, methylglyoxal synthase, glyoxalase I, and glyoxalase II. In still another embodiment, the one or more recombinant protein activities comprise a gene product of a gene selected from the group consisting of *zwf*, *pgl*, and *gnd*. In one aspect, the one or more recombinant protein activities are selected from the group consisting of glucose-6-phosphate dehydrogenase, 6-phosphogluconolactonase, and gluconate-P dehydrogenase.

[0012] In one embodiment, activity of the futile cycle is increased without overexpression of the genes of the futile cycle pathway. In another embodiment, the activity of at least part of a futile cycle pathway is increased by overexpression of another gene. In one aspect, the gene is outside the futile cycle pathway. In another aspect, the gene is endogenous. In yet another aspect, the gene is recombinant. In one particular embodiment, the overexpression of the group 2 sigma factor *sigE* increases the activity of a futile cycle pathway. In a further embodiment, the futile cycle pathway is an oxidative pentose phosphate pathway comprising an enzyme expressed by a gene selected from the group consisting of *zwf* (glucose-6-phosphate dehydrogenase), *pgl* (6-phosphogluconolactonase), and *gnd* (gluconate-P dehydrogenase).

[0013] The present invention also relates to methods for mitigating ATP accumulation in an engineered host cell. In one embodiment of the invention, the method provides for addition of salt to the host cell medium. In another embodiment of the invention, the method provides for acidification or alkalinization of the host cell medium. In still another embodiment of the invention, the temperature of the host cell medium is adjusted. In yet another embodiment, a toxic compound is introduced to the medium. In a further embodiment, the toxic compound induces an efflux reaction in the host cell.

[0014] In addition, one embodiment of the present invention provides a method for production of a host cell engineered to produce a carbon-based product of interest, wherein said host cell dissipates accumulated excess ATP, comprising the steps of: (i) performing random mutagenesis on the host cell, and (ii) screening the host cell for dissipation of excess ATP.

[0015] A composition for producing ethanol, comprising a host cell engineered to produce ethanol, and a medium comprising an ATP depletion agent, is provided in one aspect

of the invention. In a further aspect, the ATP depletion agent is selected from the group consisting of: N,N′-dicyclohexylcarbodiimide, 3,3′,4′,5-tetrachlorosalicylanilide, 2,4-dinitrophenol, and carbonyl cyanide-p-trifluoromethoxyhydrazone. In another aspect, the ATP depletion agent is N,N′-dicyclohexylcarbodiimide at a concentration of 0.25 to 0.75μM. In still another aspect, the ATP depletion agent is 3,3′,4′,5-tetrachlorosalicylanilide at a concentration of 0.1 to 0.25μM. IN one embodiment, the host cell is a photosynthetic microbe. In another embodiment, the host cell is cyanobacterium. In still another embodiment, the host cell comprises a recombinant NADPH-dependent *adh* gene.

[0016] In one particular embodiment, the invention provides a method to increase the production of ethanol in a photosynthetic microbe comprising a recombinant NADPH-dependent *adh* gene, comprising the step of culturing said photosynthetic microbe in a medium comprising N,N′-dicyclohexylcarbodiimide at a concentration of 0.75μM, wherein said N,N′-dicyclohexylcarbodiimide increases the ethanol productivity of the photosynthetic microbe. In another particular embodiment the invention provides a method to increase the production of ethanol in a photosynthetic microbe comprising a recombinant NADPH-dependent *adh* gene, comprising the step of culturing said photosynthetic microbe in a medium comprising 3,3′,4′,5-tetrachlorosalicylanilide at a concentration of 0.1μM, wherein said 3,3′,4′,5-tetrachlorosalicylanilide increases the ethanol productivity of the photosynthetic microbe. In a further aspect, the photosynthetic microbe is cyanobacterium

[0017] In one particular aspect, a composition is provided for producing ethanol, comprising a photosynthetic microbe having a recombinant NADPH-dependent *adh* gene, and a medium comprising N,N′-dicyclohexylcarbodiimide at a concentration of 0.75μM. In another particular aspect, a composition is provided for producing ethanol, comprising a photosynthetic microbe having a recombinant NADPH-dependent *adh* gene, and a medium comprising 3,3′,4′,5-tetrachlorosalicylanilide at a concentration of 0.1μM. In a further aspect, the photosynthetic microbe is cyanobacterium.

BRIEF DESCRIPTION OF THE FIGURES

[0018] **Figure 1**: Effect of N,N'-dicyclohexylcarbodiimide on growth curves of JCC1581 as measured by optical density at 730 nm over a 264 hr time course.

[0019] **Figure 2**: Effect of N,N'-dicyclohexylcarbodiimide on cumulative ethanol production by JCC1581 as measured over a 264-hr time course.

[0020] **Figure 3**: Effect of N,N'-dicyclohexylcarbodiimide on acetaldehyde levels produced by JCC1581 as measured over a 264-hr time course.

DETAILED DESCRIPTION OF THE INVENTION

[0021] Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include the plural and plural terms shall include the singular. Generally, nomenclatures used in connection with, and techniques of, biochemistry, enzymology, molecular and cellular biology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those well known and commonly used in the art. The methods and techniques of the present invention are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. *See, e.g.*, Sambrook et al. Molecular Cloning: A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates (1992, and Supplements to 2002); Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1990); Taylor and Drickamer, Introduction to Glycobiology, Oxford Univ. Press (2003); Worthington Enzyme Manual, Worthington Biochemical Corp., Freehold, N.J.; Handbook of Biochemistry: Section A Proteins, Vol I, CRC Press (1976); Handbook of Biochemistry: Section A Proteins, Vol II, CRC Press (1976); Essentials of Glycobiology, Cold Spring Harbor Laboratory Press (1999).

[0022] All publications, patents and other references mentioned herein are hereby incorporated by reference in their entireties for all purposes.

[0023] The following terms, unless otherwise indicated, shall be understood to have the following meanings:

[0024] Unless otherwise indicated, and as an example for all sequences described herein under the general format "**SEQ ID NO:**", "nucleic acid comprising SEQ ID NO:1" refers to

a nucleic acid, at least a portion of which has either (i) the sequence of SEQ ID NO:1, or (ii) a sequence complementary to SEQ ID NO:1. The choice between the two is dictated by the context. For instance, if the nucleic acid is used as a probe, the choice between the two is dictated by the requirement that the probe be complementary to the desired target.

[0025] The term "**recombinant**" refers to a biomolecule, *e.g.*, a gene or protein, that (1) has been removed from its naturally occurring environment, (2) is not associated with all or a portion of a polynucleotide in which the gene is found in nature, (3) is operatively linked to a polynucleotide which it is not linked to in nature, or (4) does not occur in nature. The term "recombinant" can be used in reference to cloned DNA isolates, chemically synthesized polynucleotide analogs, or polynucleotide analogs that are biologically synthesized by heterologous systems, as well as proteins and/or mRNAs encoded by such nucleic acids.

[0026] As used herein, an endogenous nucleic acid sequence in the genome of an organism (or the encoded protein product of that sequence) is deemed "recombinant" herein if a heterologous sequence is placed adjacent to the endogenous nucleic acid sequence, such that the expression of this endogenous nucleic acid sequence is altered. In this context, a heterologous sequence is a sequence that is not naturally adjacent to the endogenous nucleic acid sequence, whether or not the heterologous sequence is itself endogenous (originating from the same host cell or progeny thereof) or exogenous (originating from a different host cell or progeny thereof). By way of example, a promoter sequence can be substituted (*e.g.*, by homologous recombination) for the native promoter of a gene in the genome of a host cell, such that this gene has an altered expression pattern. This gene would now become "recombinant" because it is separated from at least some of the sequences that naturally flank it.

[0027] A nucleic acid is also considered "recombinant" if it contains any modifications that do not naturally occur to the corresponding nucleic acid in a genome. For instance, an endogenous coding sequence is considered "recombinant" if it contains an insertion, deletion or a point mutation introduced artificially, *e.g.*, by human intervention. A "recombinant nucleic acid" also includes a nucleic acid integrated into a host cell chromosome at a heterologous site and a nucleic acid construct present as an episome.

[0028] The term "**vector**" as used herein is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which generally refers to a circular, double-stranded DNA loop into which additional DNA segments may be ligated, but also includes linear double-stranded molecules such as those resulting from amplification by the polymerase chain reaction (PCR) or from

treatment of a circular plasmid with a restriction enzyme. Other vectors include cosmids, bacterial artificial chromosomes (BAC) and yeast artificial chromosomes (YAC). Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome (discussed in more detail below). Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, vectors having an origin of replication which functions in the host cell). Other vectors can be integrated into the genome of a host cell upon introduction into the host cell, and are thereby replicated along with the host genome. Moreover, certain preferred vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply "expression vectors").

[0029] The term "**expression control sequence**" as used herein refers to polynucleotide sequences which are necessary to affect the expression of coding sequences to which they are operatively linked. Expression control sequences are sequences which control the transcription, post-transcriptional events and/or translation of nucleic acid sequences. Expression control sequences include, *e.g.*, appropriate transcription initiation, termination, promoter and enhancer sequences; efficient RNA processing signals such as splicing and polyadenylation signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (*e.g.*, ribosome binding sites); sequences that enhance protein stability; and when desired, sequences that enhance protein secretion. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription termination sequence. The term "control sequences" is intended to include, at a minimum, all components whose presence is essential for expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

[0030] The term "**recombinant host cell**" (or simply "host cell"), as used herein, is intended to refer to a cell into which a recombinant nucleic acid sequence has been introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein. A recombinant host cell may be an isolated cell or cell line grown in culture or may be a cell which resides in a living tissue or organism.

[0031] The term "**background host cell**" as used herein, is intended to refer to a cell that lacks one or more recombinant nucleic acid sequences that are to be introduced to create a recombinant host cell or host cell.

[0032] The term "**carbon-based products of interest**" as used herein includes alcohols such as ethanol, propanol, isopropanol, butanol, fatty alcohols, fatty acid esters, wax esters; hydrocarbons and alkanes such as propane, octane, diesel, Jet Propellant 8 (JP8); polymers such as terephthalate, 1,3-propanediol, 1,4-butanediol, polyols, Polyhydroxyalkanoates (PHA), poly-beta-hydroxybutyrate (PHB), acrylate, adipic acid, ε-caprolactone, isoprene, caprolactam, rubber; commodity chemicals such as lactate, Docosahexaenoic acid (DHA), 3-hydroxypropionate, γ-valerolactone, lysine, serine, aspartate, aspartic acid, sorbitol, ascorbate, ascorbic acid, isopentenol, lanosterol, omega-3 DHA, lycopene, itaconate, 1,3-butadiene, ethylene, propylene, succinate, citrate, citric acid, glutamate, malate, 3-hydroxypropionic acid (HPA), lactic acid, THF, gamma butyrolactone, pyrrolidones, hydroxybutyrate, glutamic acid, levulinic acid, acrylic acid, malonic acid; specialty chemicals such as carotenoids, isoprenoids, itaconic acid; pharmaceuticals and pharmaceutical intermediates such as 7-aminodeacetoxycephalosporanic acid (7-ADCA)/cephalosporin, erythromycin, polyketides, statins, paclitaxel, docetaxel, terpenes, peptides, steroids, omega fatty acids and other such suitable products of interest. Such products are useful in the context of biofuels, industrial and specialty chemicals, as intermediates used to make additional products, such as nutritional supplements, nutraceuticals, polymers, paraffin replacements, personal care products and pharmaceuticals.

[0033] The term "**biofuel**" as used herein refers to any fuel that derives from a biological source. Biofuel can refer to one or more hydrocarbons, one or more alcohols, one or more fatty esters or a mixture thereof.

[0034] The term "**hydrocarbon**" as used herein generally refers to a chemical compound that consists of the elements carbon (C), hydrogen (H) and optionally oxygen (O). There are essentially three types of hydrocarbons, *e.g.*, aromatic hydrocarbons, saturated hydrocarbons and unsaturated hydrocarbons such as alkenes, alkynes, and dienes. The term also includes fuels, biofuels, plastics, waxes, solvents and oils. Hydrocarbons encompass biofuels, as well as plastics, waxes, solvents and oils.

[0035] The term "**futile cycle**" as used herein refers to two metabolic pathways running simultaneously in opposite directions whose primary purpose is to dissipate energy. This can include consumption of ATP or reduction of a proton motive force in a host cell by rebalancing a proton gradient. The simultaneous carrying out of glycolysis and

gluconeogenesis is an example of a futile cycle, where glucose would be converted to pyruvate by glycolysis and then converted back to glucose by gluconeogenesis, resulting in an overall consumption of ATP. One or more genes encoding enzymes in pathways related through a futile cycle can be engineered into a host cell, where their expression results in an overall dissipation of excess energy.

[0036] The term "**reduced product**" as used herein refers to a product that has been synthesized by reducing a reactant via an endogenous or exogenous pathway in a host cell. The reduced product, such as, *e.g.*, ethanol, is a carbon-based product of interest.

[0037] The term "**proton gradient**" as used herein refers to an imbalance in the concentration of protons across one or more intracellular compartments separated by a membrane. The "proton gradient" can also be described as a charge separation providing electrical potential energy. It can be generated by a variety of phenomena, including the operation of an electron transport chain, and the hydrolysis of ATP by ATP synthase. Excess energy created by metabolic process in the host cell can be stored as a proton gradient.

[0038] The term "**proton motive force**" as used herein refers to energy that is generated by the transfer of protons or electrons across an energy-transducing membrane and can be used for chemical, osmotic, or mechanical work. The proton motive force can be described as the work performed by the proton or voltage gradient across a membrane.

[0039] The term "**metabolic equilibrium**" as used herein refers to a state within a host cell that is actively regulated by several complex biochemical interactions and reactions. Metabolic equilibrium in the present application refers to the metabolic equilibrium state of an unmodified (*i.e.,* background) host cell at homeostasis. A genetically modified or engineered host cell may have an internal metabolic environment which is altered from the metabolic equilibrium of the unmodified host cell which affects metabolic reaction rates, for example, *e.g.* addition of a pathway to produce reduced products may affect the normal energetic flow of a host cell, affecting the energy charge of the modified host cell in a way which inhibits production of reduced products.

[0040] The term "**energy charge**" as used herein refers to an index of the energy status of a host cell, which is proportional to the mole fraction of ATP plus half the mole fraction of ADP, given that ATP contains two anhydride bonds whereas ADP contains one, and where the mole fraction is defined as the fraction of the species over the total amount of ATP, ADP, and AMP, combined. Metabolic pathway reactions are affected by the energy charge of the host cell. A 'characteristic energy charge' is an inherent property of an unmodified (*i.e.,* background) host cell at metabolic equilibrium or homeostasis.

[0041] The term "**excess energy**" as used herein refers to an energy level in a host cell that is outside of metabolic equilibrium or homeostasis, or to an energy level that inhibits production of a reduced product in the host cell. Excess energy can also refer to an energy charge which is outside of the normal value for the host cell, *e.g.*, energy charge that significantly exceeds the value in a corresponding unmodified (*i.e.*, background) host cell. Excess energy in the host cell can be stored or can physically manifest itself, for example, as a proton gradient, capable of generating a proton motive force, or as excess ATP. Excess ATP is defined as an amount of ATP that gives a higher than normal energy charge for a host cell, or an amount that inhibits synthesis of a reduced product. Dissipation of an excess energy refers to a mechanism to reduce the physical manifestation of an excess energy.

[0042] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this present invention pertains. Exemplary methods and materials are described below, although methods and materials similar or equivalent to those described herein can also be used in the practice of the present invention and will be apparent to those of skill in the art. All publications and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. The materials, methods, and examples are illustrative only and not intended to be limiting.

[0043] Throughout this specification and claims, the word "comprise" or variations such as "comprises" or "comprising," used in association with a group of integers will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

General Methods for Engineering Microorganisms to Produce Carbon-Based Products

[0044] The methods of the invention are based on principles of metabolic engineering, and uses, e.g., engineered pathways as described in, e.g., WO 2007/136762 and WO 2007/139925 (each of which is incorporated by reference in its entirety for all purposes) to make products from energy captured by photoautotrophic organisms. Generally, carbon-based products of interest are produced by expressing a gene or a set of genes in a photoautotrophic microorganism, e.g., cyanobacteria, as described herein. Plasmids are constructed to express various proteins that are useful in production of carbon-based products, as described in the Examples herein, e.g., **Example 1**. The constructs can be synthetically made or made using standard molecular biology methods and all the cloned genes are put under the control of constitutive promoters or inducible promoters. Plasmids

containing the genes of interest are transformed into the host and corresponding transformants are selected in LB plate supplemented with antibiotics such as spectinomycin, carbenicillin, etc. Using standard molecular biology techniques, cells in which a nucleic acid molecule has been introduced are transformed to express or over-express desired genes while other nucleic acid molecules are attenuated or functionally deleted. Transformation techniques by which a nucleic acid molecule can be introduced into such a cell, including, but not limited to, transfection with viral vectors, conjugation, transformation with plasmid vectors, and introduction of naked DNA by electroporation, lipofection, and particle gun acceleration. Transformants are inoculated into a suitable medium. The samples containing the transformants are grown at suitable temperatures in a shaker until they reach at certain OD. The cells are then spun down at and the cell pellets are suspended. Separation techniques allows for the sample to be subjected to GC/MS analysis. Total yield is determined.

Selected or Engineered Microorganisms for the Production of

Carbon-Based Products of Interest

[0045] Microorganism: Includes prokaryotic and eukaryotic microbial species from the Domains *Archaea, Bacteria* and *Eucarya*, the latter including yeast and filamentous fungi, protozoa, algae, or higher Protista. The terms "microbial cells" and "microbes" are used interchangeably with the term microorganism.

[0046] A variety of host organisms can be transformed to produce a product of interest. Photoautotrophic organisms include eukaryotic plants and algae, as well as prokaryotic cyanobacteria, green-sulfur bacteria, green non-sulfur bacteria, purple sulfur bacteria, and purple non-sulfur bacteria.

[0047] The host cell can be a Gram-negative bacterial cell or a Gram-positive bacterial cell. A Gram-negative host cell of the invention can be, e.g., *Gluconobacter, Rhizobium, Bradyrhizobium, Alcaligenes, Rhodobacter, Rhodococcus. Azospirillum, Rhodospirillum, Sphingomonas, Burkholderia, Desuifomonas, Geospirillum, Succinomonas, Aeromonas, Shewanella, Halochromatium, Citrobacter, Escherichia, Klebsiella, Zymomonas Zymobacter, or Acetobacter.* A Gram-positive host cell of the invention can be, e.g., *Fibrobacter, Acidobacter, Bacteroides, Sphingobacterium, Actinomyces, Corynebacterium, Nocardia, Rhodococcus, Propionibacterium, Bifidobacterium, Bacillus, Geobacillus, Paenibacillus, Sulfobacillus, Clostridium, Anaerobacter, Eubacterium, Streptococcus, Lactobacillus, Leuconostoc, Enterococcus, Lactococcus, Thermobifida, Cellulomonas, or Sarcina.*

[0048] Extremophiles are also contemplated as suitable organisms. Such organisms withstand various environmental parameters such as temperature, radiation, pressure, gravity, vacuum, desiccation, salinity, pH, oxygen tension, and chemicals. They include hyperthermophiles, which grow at or above 80°C such as *Pyrolobus fumarii*; thermophiles, which grow between 60-80°C such as *Synechococcus lividis*; mesophiles, which grow between 15-60°C and psychrophiles, which grow at or below 15°C such as *Psychrobacter* and some insects. Radiation-tolerant organisms include *Deinococcus radiodurans*. Pressure-tolerant organisms include piezophiles or barophiles, which tolerate pressure of 130 MPa. Hypergravity- (e.g., >1g) hypogravity- (e.g., <1g) tolerant organisms are also contemplated. Vacuum-tolerant organisms include tardigrades, insects, microbes and seeds. Dessicant-tolerant and anhydrobiotic organisms include xerophiles such as *Artemia salina*; nematodes, microbes, fungi and lichens. Salt-tolerant organisms include halophiles (e.g., 2-5 M NaCl) *Halobacteriacea* and *Dunaliella salina*. pH-tolerant organisms include alkaliphiles such as *Natronobacterium, Bacillus firmus* OF4, *Spirulina spp.* (e.g., pH > 9) and acidophiles such as *Cyanidium caldarium*, *Ferroplasma sp.* (e.g., low pH). Anaerobes, which cannot tolerate O_2 such as *Methanococcus jannaschii*; microaerophils, which tolerate some O_2 such as *Clostridium* and aerobes, which require O_2 are also contemplated. Gas-tolerant organisms, which tolerate pure CO_2 include *Cyanidium caldarium* and metal-tolerant organisms include metalotolerants such as *Ferroplasma acidarmanus* (e.g., Cu, As, Cd, Zn), *Ralstonia* sp. CH34 (e.g., Zn, Co, Cd, Hg, Pb). Gross, Michael. *Life on the Edge: Amazing Creatures Thriving in Extreme Environments*. New York: Plenum (1998) and Seckbach, J. "Search for Life in the Universe with Terrestrial Microbes Which Thrive Under Extreme Conditions." In Cristiano Batalli Cosmovici, Stuart Bowyer, and Dan Wertheimer, eds., *Astronomical and Biochemical Origins and the Search for Life in the Universe*, p. 511. Milan: Editrice Compositori (1997).

[0049] Plants include but are not limited to the following genera: *Arabidopsis, Beta, Glycine, Jatropha, Miscanthus, Panicum, Phalaris, Populus, Saccharum, Salix, Simmondsia* and *Zea.*

[0050] Algae and cyanobacteria include but are not limited to the following genera: *Acanthoceras, Acanthococcus, Acaryochloris, Achnanthes, Achnanthidium, Actinastrum, Actinochloris, Actinocyclus, Actinotaenium, Amphichrysis, Amphidinium, Amphikrikos, Amphipleura, Amphiprora, Amphithrix, Amphora, Anabaena, Anabaenopsis, Aneumastus, Ankistrodesmus, Ankyra, Anomoeoneis, Apatococcus, Aphanizomenon, Aphanocapsa, Aphanochaete, Aphanothece, Apiocystis, Apistonema, Arthrodesmus, Artherospira, Ascochloris, Asterionella, Asterococcus, Audouinella, Aulacoseira, Bacillaria, Balbiania,*

Bambusina, Bangia, Basichlamys, Batrachospermum, Binuclearia, Bitrichia, Blidingia, Botrdiopsis, Botrydium, Botryococcus, Botryosphaerella, Brachiomonas, Brachysira, Brachytrichia, Brebissonia, Bulbochaete, Bumilleria, Bumilleriopsis, Caloneis, Calothrix, Campylodiscus, Capsosiphon, Carteria, Catena, Cavinula, Centritractus, Centronella, Ceratium, Chaetoceros, Chaetochloris, Chaetomorpha, Chaetonella, Chaetonema, Chaetopeltis, Chaetophora, Chaetosphaeridium, Chamaesiphon, Chara, Characiochloris, Characiopsis, Characium, Charales, Chilomonas, Chlainomonas, Chlamydoblepharis, Chlamydocapsa, Chlamydomonas, Chlamydomonopsis, Chlamydomyxa, Chlamydonephris, Chlorangiella, Chlorangiopsis, Chlorella, Chlorobotrys, Chlorobrachis, Chlorochytrium, Chlorococcum, Chlorogloea, Chlorogloeopsis, Chlorogonium, Chlorolobion, Chloromonas, Chlorophysema, Chlorophyta, Chlorosaccus, Chlorosarcina, Choricystis, Chromophyton, Chromulina, Chroococcidiopsis, Chroococcus, Chroodactylon, Chroomonas, Chroothece, Chrysamoeba, Chrysapsis, Chrysidiastrum, Chrysocapsa, Chrysocapsella, Chrysochaete, Chrysochromulina, Chrysococcus, Chrysocrinus, Chrysolepidomonas, Chrysolykos, Chrysonebula, Chrysophyta, Chrysopyxis, Chrysosaccus, Chrysophaerella, Chrysostephanosphaera, Clodophora, Clastidium, Closteriopsis, Closterium, Coccomyxa, Cocconeis, Coelastrella, Coelastrum, Coelosphaerium, Coenochloris, Coenococcus, Coenocystis, Colacium, Coleochaete, Collodictyon, Compsogonopsis, Compsopogon, Conjugatophyta, Conochaete, Coronastrum, Cosmarium, Cosmioneis, Cosmocladium, Crateriportula, Craticula, Crinalium, Crucigenia, Crucigeniella, Cryptoaulax, Cryptomonas, Cryptophyta, Ctenophora, Cyanodictyon, Cyanonephron, Cyanophora, Cyanophyta, Cyanothece, Cyanothomonas, Cyclonexis, Cyclostephanos, Cyclotella, Cylindrocapsa, Cylindrocystis, Cylindrospermum, Cylindrotheca, Cymatopleura, Cymbella, Cymbellonitzschia, Cystodinium Dactylococcopsis, Debarya, Denticula, Dermatochrysis, Dermocarpa, Dermocarpella, Desmatractum, Desmidium, Desmococcus, Desmonema, Desmosiphon, Diacanthos, Diacronema, Diadesmis, Diatoma, Diatomella, Dicellula, Dichothrix, Dichotomococcus, Dicranochaete, Dictyochloris, Dictyococcus, Dictyosphaerium, Didymocystis, Didymogenes, Didymosphenia, Dilabifilum, Dimorphococcus, Dinobryon, Dinococcus, Diplochloris, Diploneis, Diplostauron, Distrionella, Docidium, Draparnaldia, Dunaliella, Dysmorphococcus, Ecballocystis, Elakatothrix, Ellerbeckia, Encyonema, Enteromorpha, Entocladia, Entomoneis, Entophysalis, Epichrysis, Epipyxis, Epithemia, Eremosphaera, Euastropsis, Euastrum, Eucapsis, Eucocconeis, Eudorina, Euglena, Euglenophyta, Eunotia, Eustigmatophyta, Eutreptia, Fallacia, Fischerella, Fragilaria, Fragilariforma, Franceia, Frustulia, Curcilla,

Geminella, Genicularia, Glaucocystis, Glaucophyta, Glenodiniopsis, Glenodinium, Gloeocapsa, Gloeochaete, Gloeochrysis, Gloeococcus, Gloeocystis, Gloeodendron, Gloeomonas, Gloeoplax, Gloeothece, Gloeotila, Gloeotrichia, Gloiodictyon, Golenkinia, Golenkiniopsis, Gomontia, Gomphocymbella, Gomphonema, Gomphosphaeria, Gonatozygon, Gongrosia, Gongrosira, Goniochloris, Gonium, Gonyostomum, Granulochloris, Granulocystopsis, Groenbladia, Gymnodinium, Gymnozyga, Gyrosigma, Haematococcus, Hafniomonas, Hallassia, Hammatoidea, Hannaea, Hantzschia, Hapalosiphon, Haplotaenium, Haptophyta, Haslea, Hemidinium, Hemitoma, Heribaudiella, Heteromastix, Heterothrix, Hibberdia, Hildenbrandia, Hillea, Holopedium, Homoeothrix, Hormanthonema, Hormotila, Hyalobrachion, Hyalocardium, Hyalodiscus, Hyalogonium, Hyalotheca, Hydrianum, Hydrococcus, Hydrocoleum, Hydrocoryne, Hydrodictyon, Hydrosera, Hydrurus, Hyella, Hymenomonas, Isthmochloron, Johannesbaptistia, Juranyiella, Karayevia, Kathablepharis, Katodinium, Kephyrion, Keratococcus, Kirchneriella, Klebsormidium, Kolbesia, Koliella, Komarekia, Korshikoviella, Kraskella, Lagerheimia, Lagynion, Lamprothamnium, Lemanea, Lepocinclis, Leptosira, Lobococcus, Lobocystis, Lobomonas, Luticola, Lyngbya, Malleochloris, Mallomonas, Mantoniella, Marssoniella, Martyana, Mastigocoleus, Gastogloia, Melosira, Merismopedia, Mesostigma, Mesotaenium, Micractinium, Micrasterias, Microchaete, Microcoleus, Microcystis, Microglena, Micromonas, Microspora, Microthamnion, Mischococcus, Monochrysis, Monodus, Monomastix, Monoraphidium, Monostroma, Mougeotia, Mougeotiopsis, Myochloris, Myromecia, Myxosarcina, Naegeliella, Nannochloris, Nautococcus, Navicula, Neglectella, Neidium, Nephroclamys, Nephrocytium, Nephrodiella, Nephroselmis, Netrium, Nitella, Nitellopsis, Nitzschia, Nodularia, Nostoc, Ochromonas, Oedogonium, Oligochaetophora, Onychonema, Oocardium, Oocystis, Opephora, Ophiocytium, Orthoseira, Oscillatoria, Oxyneis, Pachycladella, Palmella, Palmodictyon, Pnadorina, Pannus, Paralia, Pascherina, Paulschulzia, Pediastrum, Pedinella, Pedinomonas, Pedinopera, Pelagodictyon, Penium, Peranema, Peridiniopsis, Peridinium, Peronia, Petroneis, Phacotus, Phacus, Phaeaster, Phaeodermatium, Phaeophyta, Phaeosphaera, Phaeothamnion, Phormidium, Phycopeltis, Phyllariochloris, Phyllocardium, Phyllomitas, Pinnularia, Pitophora, Placoneis, Planctonema, Planktosphaeria, Planothidium, Plectonema, Pleodorina, Pleurastrum, Pleurocapsa, Pleurocladia, Pleurodiscus, Pleurosigma, Pleurosira, Pleurotaenium, Pocillomonas, Podohedra, Polyblepharides, Polychaetophora, Polyedriella, Polyedriopsis, Polygoniochloris, Polyepidomonas, Polytaenia, Polytoma, Polytomella, Porphyridium, Posteriochromonas, Prasinochloris, Prasinocladus, Prasinophyta, Prasiola, Prochlorphyta,

Prochlorothrix, Protoderma, Protosiphon, Provasoliella, Prymnesium, Psammodictyon, Psammothidium, Pseudanabaena, Pseudenoclonium, Psuedocarteria, Pseudochate, Pseudocharacium, Pseudococcomyxa, Pseudodictyosphaerium, Pseudokephyrion, Pseudoncobyrsa, Pseudoquadrigula, Pseudosphaerocystis, Pseudostaurastrum, Pseudostaurosira, Pseudotetrastrum, Pteromonas, Punctastruata, Pyramichlamys, Pyramimonas, Pyrrophyta, Quadrichloris, Quadricoccus, Quadrigula, Radiococcus, Radiofilum, Raphidiopsis, Raphidocelis, Raphidonema, Raphidophyta, Peimeria, Rhabdoderma, Rhabdomonas, Rhizoclonium, Rhodomonas, Rhodophyta, Rhoicosphenia, Rhopalodia, Rivularia, Rosenvingiella, Rossithidium, Roya, Scenedesmus, Scherffelia, Schizochlamydella, Schizochlamys, Schizomeris, Schizothrix, Schroederia, Scolioneis, Scotiella, Scotiellopsis, Scourfieldia, Scytonema, Selenastrum, Selenochloris, Sellaphora, Semiorbis, Siderocelis, Diderocystopsis, Dimonsenia, Siphononema, Sirocladium, Sirogonium, Skeletonema, Sorastrum, Spermatozopsis, Sphaerellocystis, Sphaerellopsis, Sphaerodinium, Sphaeroplea, Sphaerozosma, Spiniferomonas, Spirogyra, Spirotaenia, Spirulina, Spondylomorum, Spondylosium, Sporotetras, Spumella, Staurastrum, Stauerodesmus, Stauroneis, Staurosira, Staurosirella, Stenopterobia, Stephanocostis, Stephanodiscus, Stephanoporos, Stephanosphaera, Stichococcus, Stichogloea, Stigeoclonium, Stigonema, Stipitococcus, Stokesiella, Strombomonas, Stylochrysalis, Stylodinium, Styloyxis, Stylosphaeridium, Surirella, Sykidion, Symploca, Synechococcus, Synechocystis, Synedra, Synochromonas, Synura, Tabellaria, Tabularia, Teilingia, Temnogametum, Tetmemorus, Tetrachlorella, Tetracyclus, Tetradesmus, Tetraedriella, Tetraedron, Tetraselmis, Tetraspora, Tetrastrum, Thalassiosira, Thamniochaete, Thorakochloris, Thorea, Tolypella, Tolypothrix, Trachelomonas, Trachydiscus, Trebouxia, Trentepholia, Treubaria, Tribonema, Trichodesmium, Trichodiscus, Trochiscia, Tryblionella, Ulothrix, Uroglena, Uronema, Urosolenia, Urospora, Uva, Vacuolaria, Vaucheria, Volvox, Volvulina, Westella, Woloszynskia, Xanthidium, Xanthophyta, Xenococcus, Zygnema, Zygnemopsis, and *Zygonium*.

[0051] Green non-sulfur bacteria include but are not limited to the following genera: *Chloroflexus, Chloronema, Oscillochloris, Heliothrix, Herpetosiphon, Roseiflexus*, and *Thermomicrobium*.

[0052] Green sulfur bacteria include but are not limited to the following genera: *Chlorobium, Clathrochloris*, and *Prosthecochloris*.

[0053] Purple sulfur bacteria include but are not limited to the following genera: *Allochromatium, Chromatium, Halochromatium, Isochromatium, Marichromatium, Rhodovulum, Thermochromatium, Thiocapsa, Thiorhodococcus*, and *Thiocystis*.

[0054] Purple non-sulfur bacteria include but are not limited to the following genera: *Phaeospirillum, Rhodobaca, Rhodobacter, Rhodomicrobium, Rhodopila, Rhodopseudomonas, Rhodothalassium, Rhodospirillum, Rodovibrio,* and *Roseospira*.

[0055] Aerobic chemolithotrophic bacteria include but are not limited to nitrifying bacteria such as *Nitrobacteraceae* sp., *Nitrobacter* sp., *Nitrospina* sp., *Nitrococcus* sp., *Nitrospira* sp., *Nitrosomonas* sp., *Nitrosococcus* sp., *Nitrosospira* sp., *Nitrosolobus* sp., *Nitrosovibrio* sp.; colorless sulfur bacteria such as, *Thiovulum* sp., *Thiobacillus* sp., *Thiomicrospira* sp., *Thiosphaera* sp., *Thermothrix* sp.; obligately chemolithotrophic hydrogen bacteria such as *Hydrogenobacter* sp., iron and manganese-oxidizing and/or depositing bacteria such as *Siderococcus* sp., and magnetotactic bacteria such as *Aquaspirillum* sp.

[0056] Archaeobacteria include but are not limited to methanogenic archaeobacteria such as *Methanobacterium* sp., *Methanobrevibacter* sp., *Methanothermus* sp., *Methanococcus* sp., *Methanomicrobium* sp., *Methanospirillum* sp., *Methanogenium* sp., *Methanosarcina* sp., *Methanolobus* sp., *Methanothrix* sp., *Methanococcoides* sp., *Methanoplanus* sp.; extremely thermophilic sulfur-metabolizers such as *Thermoproteus* sp., *Pyrodictium* sp., *Sulfolobus* sp., *Acidianus* sp. and other microorganisms such as, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Streptomyces* sp., *Ralstonia* sp., *Rhodococcus* sp., *Corynebacteria* sp., *Brevibacteria* sp., *Mycobacteria* sp., and oleaginous yeast.

[0057] Hyperphotosynthetic conversion requires extensive genetic modification; thus, in some embodiments the parental photoautotrophic organism can be transformed with exogenous DNA.

[0058] Organisms for hyperphotosynthetic conversion include: *Arabidopsis thaliana, Panicum virgatum, Miscanthus giganteus*, and *Zea mays* (plants), *Botryococcus braunii, Chlamydomonas reinhardtii* and *Dunaliela salina* (algae), *Synechococcus* sp. PCC 7002, *Synechococcus* sp. PCC 7942, *Synechocystis* sp. PCC 6803, and *Thermosynechococcus elongatus* BP-1 (cyanobacteria), *Chlorobium tepidum* (green sulfur bacteria), *Chloroflexus auranticus* (green non-sulfur bacteria), *Chromatium tepidum,* and *Chromatium vinosum* (purple sulfur bacteria), *Rhodospirillum rubrum, Rhodobacter capsulatus*, and *Rhodopseudomonas palusris* (purple non-sulfur bacteria).

[0059] Yet other suitable organisms include synthetic cells or cells produced by synthetic genomes as described in Venter *et al.* US Pat. Pub. No. 2007/0264688, and cell-like systems or synthetic cells as described in Glass *et al.* US Pat. Pub. No. 2007/0269862.

[0060] Still, other suitable organisms include microorganisms that can be engineered to fix carbon dioxide bacteria such as *Escherichia coli*, *Acetobacter aceti*, *Bacillus subtilis*, yeast and fungi such as *Clostridium ljungdahlii*, *Clostridium thermocellum*, *Penicillium chrysogenum*, *Pichia pastoris*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Pseudomonas fluorescens*, or *Zymomonas mobilis*.

[0061] A common theme in selecting or engineering a suitable organism is autotrophic fixation of CO_2 to products. This covers photosynthesis and methanogenesis. Acetogenesis, encompassing the three types of CO_2 fixation; Calvin cycle, acetyl-CoA pathway and reductive TCA pathway is also covered. The capability to use carbon dioxide as the sole source of cell carbon (autotrophy) is found in almost all major groups of prokaryotes. The CO_2 fixation pathways differ between groups, and there is no clear distribution pattern of the four presently-known autotrophic pathways. Fuchs, G. 1989. Alternative pathways of autotrophic CO_2 fixation, p. 365-382. *In* H. G. Schlegel, and B. Bowien (ed.), *Autotrophic bacteria*. Springer-Verlag, Berlin, Germany. The reductive pentose phosphate cycle (Calvin-Bassham-Benson cycle) represents the CO_2 fixation pathway in many aerobic autotrophic bacteria, for example, cyanobacteria.

Propagation of Selected Microorganisms

[0062] Methods for cultivation of photosynthetic organisms in liquid media and on agarose-containing plates are well known to those skilled in the art (*see, e.g.*, websites associated with ATCC, and with the Institute Pasteur). For example, *Synechococcus* sp. PCC 7002 cells (available from the Pasteur Culture Collection of Cyanobacteria) are cultured in BG-11 medium (17.65 mM NaNO3, 0.18 mM K2HPO4, 0.3 mM MgSO4, 0.25 mM CaCl2, 0.03 mM citric acid, 0.03 mM ferric ammonium citrate, 0.003 mM EDTA, 0.19 mM Na2CO3, 2.86 mg/L H3BO3, 1.81 mg/L MnCl2, 0.222 mg/L ZnSO4, 0.390 mg/L Na2MoO4, 0.079 mg/L CuSO4, and 0.049 mg/L Co(NO3)2, pH 7.4) supplemented with 16 μg/L biotin, 20 mM MgSO4, 8 mM KCl, and 300 mM NaCl (see, e.g., website associated with the Institute Pasteur, and Price GD, Woodger FJ, Badger MR, Howitt SM, Tucker L. "Identification of a SulP-type bicarbonate transporter in marine cyanobacteria. *Proc Natl. Acad. Sci.* USA (2004) 101(52):18228-33). Typically, cultures are maintained at 28°C and bubbled continuously with 5% CO2 under a light intensity of 120 μmol photons/m2/s.

Alternatively, as described in **Example 1**, *Synechococcus* sp. PCC 7002 cells are cultured in A^+ medium as previously described [Frigaard NU *et al.* (2004) "Gene inactivation in the cyanobacterium *Synechococcus* sp. PCC 7002 and the green sulfur bacterium *Chlorobium* tepidum using in vitro-made DNA constructs and natural transformation," *Methods Mol. Biol.,* 274:325-340].

[0063] The above define typical propagation conditions. As appropriate, incubations are performed using alternate media or gas compositions, alternate temperatures (5 – 75°C), and/or light fluxes (0-5500 µmol photons/m2/s).

[0064] Light is delivered through a variety of mechanisms, including natural illumination (sunlight), standard incandescent, fluorescent, or halogen bulbs, or via propagation in specially-designed illuminated growth chambers (for example Model LI15 Illuminated Growth Chamber (Sheldon Manufacturing, Inc. Cornelius, OR). For experiments requiring specific wavelengths and/or intensities, light is distributed via light emitting diodes (LEDs), in which wavelength spectra and intensity can be carefully controlled (Philips).

[0065] Carbon dioxide is supplied via inclusion of solid media supplements (i.e., sodium bicarbonate) or as a gas via its distribution into the growth incubator or media. Most experiments are performed using concentrated carbon dioxide gas, at concentrations between 1 and 30%, which is directly bubbled into the growth media at velocities sufficient to provide mixing for the organisms. When concentrated carbon dioxide gas is utilized, the gas originates in pure form from commercially-available cylinders, or preferentially from concentrated sources including off-gas or flue gas from coal plants, refineries, cement production facilities, natural gas facilities, breweries, and the like.

Transformation of Selected Microorganisms

[0066] *Synechococcus sp.* PCC 7002 cells are transformed according to the optimized protocol previously described [Essich ES, Stevens Jr E, Porter RD "Chromosomal Transformation in the *Cyanobacterium Agmenellum quadruplicatum*". J Bacteriol (1990). 172(4):1916-1922]. Cells are grown in Medium A (18 g/L NaCl, 5 g/L MgSO4. 7 H20, 30 mg/L Na2EDTA, 600 mg/L KCl, 370 mg/L CaCl2. 2 H2O, 1 g/L NaNO3, 50 mg/L KH2PO4, 1 g/L Trizma base pH 8.2, 4 µg/L Vitamin B12, 3.89 mg/L FeCl3. 6 H20, 34.3 mg/L H3BO3, 4.3 mg/L MnCl2. 4 H20, 315 µg/L ZnCl2, 30 µg/L MoO3, 3 µg/L CuSO4. 5 H20, 12.2 µg/L CoCl2. 6 H20) [Stevens SE, Patterson COP, and Myers J. "The production of hydrogen peroxide by green algae: a survey." J. Phycology (1973). 9:427-430] plus 5g/L of NaNO3 to approximately 108 cells/ mL. Nine volumes of cells are mixed with 1 volume of

1-10 μg/mL DNA in 0.15 M NaCl/0.015 M Na3citrate and incubated at 27-30 °C for 3 hours before addition of 1 volume of DNaseI to a final concentration of 10 μg/mL. The cells are plated in 2.5mL of 0.6% medium A overlay agar that was tempered at 45°C and incubated. Cells are challenged with antibiotic by under-laying 2.0 mL of 0.6% medium A agar containing appropriate concentration of antibiotic with a sterile Pasteur pipette. Transformants are picked 3-4 days later. Selections are typically performed using 200 μg/ml kanamycin, 8 μg/ml chloramphenicol, 10 μg/ml spectinomycin on solid media, whereas 150 μg/ml kanamycin, 7 μg/ml chloramphenicol, and 5 μg/ml spectinomycin are employed in liquid media. As described in Example 1, selection is performed using A+ containing 3 mM urea, 50 μg/ml kanamycin, and a 25 μg/ml spectinomycin underlay.

[0067] Exemplary methods and materials are described below, although methods and materials similar or equivalent to those described herein can also be used in the practice of the invention and will be apparent to those of skill in the art. All publications and other references mentioned herein are incorporated by reference in their entirety for all purposes. In case of conflict, the present specification, including definitions, will control. The materials, methods, and examples are illustrative only and not intended to be limiting.

EXAMPLE 1

Energy Rebalancing Using ATP Synthase Inhibitors

[0068] Non-genetic ATP-mitigating factors (*e.g.* inhibitors of ATP synthesis) were applied to increase the yields of a reduced compound in a photosynthetic host which had been engineered to produce a reduced compound. ATP synthase inhibitors were evaluated in the ethanol-producing strains JCC1581 and JCC1510, described herein.

[0069] Examples of ATP synthesis inhibitors that could be used in an embodiment of the invention are shown in Table 1.

Table 1: Examples of chemical compounds used to deplete the cellular ATP pool. PMF: proton motive force.

ATP depletion agent	Mechanism
DCCD (N,N'-dicyclohexylcarbodiimide)	Inhibits flux through the proton channel of F0F1 ATP synthase. Directly reduces ATP synthesis.
TCS (3,3',4',5-tetrachlorosalicylanilide)	Decreases membrane resistance to protons and indirectly reduces ATP synthesis.
2,4-DNP (2,4-dinitrophenol) and CCCP (carbonyl cyanide-*p*-trifluoromethoxyhydrazone)	Affects proton ionophores. Collapses PMF and indirectly reduces ATP synthesis.

Construction of the JCC1581 strain

[0070] The JCC1581 strain was constructed by standard homologous recombination techniques. The starting material was wild-type *Synechococcus* sp. PCC7002 (JCC138), which was obtained from the Pasteur Collection or ATCC. Gene, promoter, terminator, and marker constructs made synthetically were obtained from DNA2.0 or by PCR, oligonucleotides for PCR and sequence confirmation from IDT. DCCD (N,N'-dicyclohexylcarbodiimide), TCS (3,3',4',5-tetrachlorosalicylanilide), 2,4-DNP (2,4-dinitrophenol), and CCCP (carbonyl cyanide-*p*-trifluoromethoxyhydrazone) were obtained from Sigma.

[0071] As previously described in published PCT application WO2010/044960, a pAQ7 *Δldh* targeting plasmid (*see* Genbank # CP000957) was constructed containing the *Moorella* alcohol dehydrogenase gene (*adhAM*) under the control of the lambda cI promoter. This plasmid (pJB594) was naturally transformed into JCC138 (see Table 2) using a standard cyanobacterial transformation protocol, yielding strain JCC1034. Briefly, JCC138 culture was grown to an OD_{730} of approximately 1.0, after which 5-10 μg of plasmid DNA was added to 1 ml of neat JCC138 culture. The cell-DNA mixture was incubated at 37 °C for 4 hours in the dark with gentle mixing, plated onto A+ plates, and incubated in a photoincubator (Percival) for 24 hours, at which point kanamycin was underlaid to a final concentration of 50 μg/ml. Kanamycin-resistant colonies appeared after 5-8 days of further incubation under 24 hr-light conditions (~100 μmol photons m^{-2} s^{-1}). One round of colony purification was performed on A+ plates supplemented with 50 μg/ml kanamycin. Single colonies of each of the six transformed strains was grown in test-tubes for 4-8 days at 37 °C at 150 rpm in 3 % CO_2-enriched air at ~100 μmol photons m^{-2} s^{-1} in a Multitron II (Infors) shaking photoincubator. The growth medium for liquid culture was A+ with 50 μg/ml

kanamycin. JCC1034 was then transformed with pJB1156 which introduced a two-gene operon, driven by P(*nir07*) (SEQ ID NO: 1), to an ectopic location on pAQ3. This operon contained *adhAM* as well as the pyruvate decarboxylase gene from *Zymomonas mobilis* (*pdcZm*). The resulting strain, JCC1581, therefore had two independent *adhAM* transgenes and one *pdcZm* transgene. The protocol used to transform pJB1156 into JCC1034 generating JCC1581 was the same with the exception that the selection media is A+ containing 3 mM urea, 50 μg/ml kanamycin, and a 25 μg/ml spectinomycin underlay.

Table 2: Transformation of host cell with integrative plasmid generated JCC1034 and JCC1518 strains from wild-type *Synechococcus* sp. PCC7002 (JCC138).

Strain	Host	Integrative Plasmid
JCC1034	JCC138	pJB594 (pAQ7::*Δldh_kan_*P(*cI*)*_adhAM*)
JCC1581	JCC1034	pJB1156 (pAQ3::P(*nir07*)*_pdcZm_adhAM_spec*)

SEQ ID NO: 1

P(*nir*07) promoter; this promoter is a synthetic construct based on the *nirA* promoter from *Synechococcus* sp. PCC 7942. (Shin-Ichi Maeda *et al.* (1998). *cis*-Acting Sequences Required for NtcB-Dependent, Nitrite-Responsive Positive Regulation of the Nitrate Assimilation Operon in the Cyanobacterium *Synechococcus* sp. Strain PCC 7942. *J. Bacteriol.* 180:4080–4088. The ATG in bold represents the start codon of an expressed gene.

GCTTGTAGCAATTGCTACTAAAAACTGCGATCGCTGCTGAAATGAGCTGGAATTTTGTCCCT
CTCAGCTCAAAAAGTATCAATGATTACTTAATGTTTGTTCTGCGCAAACTTCTTGCAGAACA
TGCATGATTTACAAAAAGTTGTAGTTTCTGTTACCAATTGCGAATCGAGAACTGCCTAATCT
GCCGAGTATGCGATCCTTTAGCAGGAGGAAAACCAT**ATG** **(SEQ ID NO: 1)**

Effect of ATP depletion agents on the production of ethanol in JCC1581

[0072] The effect of inhibition of ATP synthesis by the inhibitory compound DCCD on the yield of reduced products (*e.g.* ethanol) is described. Improved ethanol production in an engineered organism through modification of the expression levels and/or identity of enzymes involved in ethanol production is provided.

[0073] A single colony of one clone from the original streak plate from each transformation was inoculated into 5 ml A^+ broth with 5 mM urea, 50 μg/ml kanamycin, and 100 μg/ml spectinomycin in a 16mM x 150mM plastic-capped culture tube and incubated in the Infors incubator (37C, 150rpm, 2%CO_2) at a ~60^0 angle. After 3 days the cultures were diluted into fresh media (30 ml JB2.1 (as described in PCT application US2009/006516,

herein incorporated by reference in its entirety) with 3 mM urea, 50 μg/ml kanamycin, and 100 μg/ml spectinomycin) in foam-plugged 125-ml Erlenmeyer flasks to an OD_{730} of ~0.1. One flask served as the control flask while the others received DCCD to final concentrations of 250, 500, and 750 nM. The stock solution of DCCD is 500 mM in DMSO. At roughly 24-hour intervals, samples were taken for growth rate and ethanol/acetaldehyde concentration measurements. OD_{730} measurements and derived growth curves are shown in Figure 1. The levels of ethanol and acetaldehyde in the media were determined as described previously and the data is presented in Figure 2 and Figure 3, respectively.

[0074] As can be seen from the Figures, the levels of ethanol produced by JCC1581 were higher in the presence of DCCD, with 750 nM giving the most pronounced improvement. Addition of 750 nM DCCD did not strongly impact growth as measured by OD_{730}, and acetaldehyde accumulation was similar in all conditions. Therefore, enhanced levels of ethanol resulted from the direct inhibition of cellular ATP synthesis in an engineered cyanobacterial strain.

[0075] A similar experiment was conducted with ethanologen JCC1581, except in duplicate with 0.75 μM DCCD, 0.1 μM TCS, or 0.2 μM TCS. In this experiment, to control for the fact that DCCD and TCS stock solutions had been made in DMSO, a flask with a representative concentration of DMSO was included. Table 3 shows that again DCCD and TCS showed improved ethanol production over the controls. TCS at 0.1 μM was observed to be optimal, similar to the experiment using the JCC1510 strain as shown below. The three lowest producers were the two controls and the DMSO-only culture.

Table 3: Ethanol productivities in JCC1581 at various added DCCD and TCS concentrations.

Added factor (μM)	Productivity (mg/L-h)	Relative productivity
none	11.58 ± 0.36	1.00
DCCD (0.75)	12.49 ± 0.23	1.08
TCS (0.1)	15.13 ± 0.48	1.31
TCS (0.25)	12.77 ± 0.69	1.10
DMSO	12.28	1.06

Construction of the JCC1510 strain

[0076] The JCC1510 strain was constructed by standard homologous recombination techniques from a cyanobacterial strain. The engineered strain comprises a pyruvate decarboxylase gene under the control of a p(nir07) promoter, and an alcohol dehydrogenase gene under the control of a lambda cI promoter (Table 4).

Table 4: Transformation of a cyanobacterial host cell generated JCC1510

Strain	Genes
JCC1510	**Chromosome**::P(*nir07*)-*pdc*$_{Zm}$-kan-P(*cI*)-*adhA*$_{M}$

Effect of ATP depletion agents on the production of ethanol in JCC1510

[0077] The ethanologen JCC1510 was grown in 250-mL Erlenmeyer flasks (30 mL working volume) in JB2.1 medium containing 3 mM urea from an initial inoculum of OD_{730} = 0.1. Three control cultures were not supplemented, while an otherwise identical series of five cultures was supplemented with DCCD at 0.25, 0.5, 0.75, 1.0, and 1.25 μM, and another otherwise identical series of four cultures was supplemented with TCS at 0.1, 0.5, 1.0, and 2.0 μM. Tables 5 and 6 show the ethanol productivity in each culture (with the exception of TCS at 1.0 and 2.0 μM, which stopped producing ethanol at around 96 h). TCS at 0.1 μM and DCCD at 0.75 μM provided a beneficial effect.

Table 5: Ethanol productivities in JCC1510 at selected DCCD concentrations

DCCD (μM)	**Ethanol Productivity (mg/L-h)**	**Relative productivity**
0	9.46 ± 0.49	1.00
0.25	8.61	0.91
0.5	8.74	0.92
0.75	10.48	1.11
1	9.33	0.99
1.25	7.61	0.80

Table 6: Ethanol productivities in JCC1510 at selected TCS concentrations

TCS (μM)	Ethanol Productivity (mg/L-h)	Relative productivity
0	9.46 ± 0.49	1.00
0.1	10.02	1.06
0.5	8.79	0.93
1	N/A	N/A
2	N/A	N/A

[0078] Other methods are available that will mitigate ATP production and enhance host cell production of reduced products such as ethanol. These include, for example, addition of salt to the host cell medium, acidification or alkalinization of the medium, temperature shift, and addition of a toxic compound to the medium, *e.g.* a toxic compound that induces its own pmf-dependent efflux.

EXAMPLE 2

Energy Rebalancing Using Pathway Engineered Genes from Metabolic Futile Cycles

[0079] Improved yield of a reduced product, e.g. ethanol or diesel, results from futile cycle pathways engineered into a host cell producing a reduced product. Futile cycling to increase the glycolytic rate in *E. coli* has been shown in, *e.g.*, Patnaik, R., et al., Stimulation of glucose catabolism in *Escherichia coli* by a potential futile cycle., *J. Bacteriol.*, 174(23):7527-7532 (1992).

[0080] Genetic modification of a host cell producing a carbon-based product of interest to mitigate excess energy accumulation is performed to enhance production of the carbon-based product of interest. Genetic strategies for mitigating excess energy accumulation include but are not restricted to the sets of futile-cycle genes given in **Table 7**. Futile cycle pathways are inserted into modified host cells according to standard homologous recombination techniques.

[0081] When the parental organism encodes a native gene with the indicated enzymatic activity of a futile cycle pathway, it is useful to overexpress these components. It is also useful to increase the activity of the futile cycle pathways by overexpressing a homologous or heterologous gene that is not part of the futile cycle pathway, but whose overexpression increases the activity of the futile cycle pathway. Accordingly, in one aspect, *sigE* is overexpressed in a host cell, *e.g.*, *Synechocystis* sp., resulting an increased activity of at least

part of a futile cycle pathway, *e.g.*, the oxidative pentose phosphate pathway during ethanol production in light.

Table 7: Futile cycle sets of genes for dissipating excess ATP or proton motive force

Futile cycle #	Gene	EC #	Reaction
1	*pntAB*	1.6.1.2	NADH + Hxt = NADPH
	udhA	1.6.1.1	NADPH = NADH
2	*glgC*	2.7.7.27	Glucose-1-Phosphate + ATP = ADP-Glucose
	glgA	2.4.1.21	ADP-Glucose = Glycogen
	glgP	2.4.1.1	Glycogen = Glucose-1-Phosphate
3	*pfkA*	2.7.1.11	Fructose-6-Phosphate + ATP = Fructose Bisphosphate
	fbp	3.1.3.11	Fructose Bisphosphate = Fructose-6-Phosphate
4	*pta*	2.3.1.8	Acetyl-CoA = Acetyl Phosphate + CoA
	ackA	2.7.2.1	Acetyl Phosphate = Acetate + ATP
	acs	6.2.1.1	Acetate + 2 ATP + CoA = Acetyl-CoA
5	*pykF*	2.7.1.40	Phosphoenolpyruvate = Pyruvate + ATP
	ppsA	2.7.9.2	Pyruvate + 2 ATP = Phosphoenolpyruvate
6	*pyc*	6.4.1.1	Pyruvate + ATP + CO_2 = Oxaloacetate
	pck	4.1.1.49	Oxaloacetate + ATP = Phosphoenolpyruvate + CO_2
	pykF	2.7.1.40	Phosphoenolpyruvate = Pyruvate + ATP
7	*mdh*	1.1.1.37	Oxaloacetate + NADH = Malate
	sfcA	1.1.1.38	Malate = Pyruvate + NADH + CO_2
	pyc	6.4.1.1	Pyruvate + ATP + CO_2 = Oxaloacetate
8	*mdh*	1.1.1.37	Oxaloacetate + NADH = Malate
	ppc	4.1.1.31	Phosphoenolpyruvate + CO2 = Oxaloacetate
	sucCD	6.2.1.5	Succinate + ATP + CoA = Succinyl-CoA
	maeB	1.1.1.40	Malate = Pyruvate + NADPH + CO_2
	sad	1.2.1.16	Succinate Semialdehyde = Succinate + NADPH
	*sucD**	1.2.1.16	Succinyl-CoA + NADPH = Succinate Semialdehyde + CoA
	Net Reaction for Futile Cycle #8		Phosphoenolpyruvate + ATP = Pyruvate

*GenBank locus EDK35023; as described in Söhling, B. and Gottschalk, G., *J. Bacteriol.* **178:**871-80 (1996).

Table 7 - Continued

Futile cycle #	Gene	EC #	Reaction
9	*mdh*	1.1.1.37	Oxaloacetate + NADH = Malate
	mdhP	1.1.1.82	Malate = Oxaloacetate + NADPH
	gap2	1.2.1.13	1,3-Bisphosphoglycerate + NADPH = Glyceraldehyde Phosphate
	pgk	2.7.2.3	3-Phosphoglycerate + ATP = 1,3-Bisphosphoglycerate
	dld	1.1.2.4	D-Lactate = Pyruvate + NADH
	mgsA	4.2.3.3	Dihydroxyacetone phosphate = D-Lactate
	gloA	4.4.1.5	Methylglyoxal = S-Lactoyl-Glutathione
	gloB	3.1.2.6	S-Lactoyl-Glutathione = D-Lactate
	Net Reaction for Futile Cycle #9		Dihydroxyacetone phosphate + 3-Phosphoglycerate + ATP = Glyceraldehyde Phosphate + Pyruvate
10	*zwf*	1.1.1.49	Glucose-6-P = D-Gluconolactone-6-P + NADPH
	pgl	3.1.1.31	D-Gluconolactone-6-P = 6-Phosphogluconate
	gnd	1.1.1.44	6-Phosphogluconate = Ribulose-5-P + CO_2 + NADPH
	(Calvin cycle)		(endogenous)

[0082] Other futile cycles, such as those involving solute transport, are found in reviews, e.g., Russel, J.B., The energy spilling reactions of bacteria and other organisms., *J. Molec. Microbiol. Biotechnol.*, 12:1-11 (2007)

[0083] Genes selected from a futile cycle are cloned or synthesized and transformed into production hosts, where they are expressed. Expression of the genes in the production host is inducible under certain conditions. These conditions include, for example, absence of ammonia, absence of copper, presence of nickel, or presence of a gratuitous inducer, such as IPTG.

EXAMPLE 3

Use of a urea-repressible, nitrate-inducible *nirA*-type promoter, P(*nir*07)

[0084] Example 1 describes the engineering of an ethanologen comprising, in part, a urea-repressible, nitrate-inducible *nirA*-type promoter, P(*nir*07) (**SEQ ID NO: 1**) which controls expression of an operon. The P(*nir*07) promoter is a versatile inducible promoter

and can be used to control expression of other genes and operons, e.g., genes encoding AAR and ADM enzymes as described in U.S. Patent Application 12/833821, filed July 9, 2010.

CLAIMS

What is claimed is:

1. A method to increase production of a reduced product in a host cell, comprising the step of manipulating the host cell to dissipate excess energy stored in said host cell, and culturing said host cell in a host cell growth medium.

2. The method of claim 1, comprising addition of an exogenous ATP depletion agent to the host cell growth medium.

3. The method of claim 2, wherein said reduced product is ethanol.

4. The method of claim 2, wherein said host cell is a photosynthetic cell.

5. The method of claim 2, wherein said host cell is a cyanobacterium.

6. The method of claim 2, wherein said host cell comprises a recombinant NADPH-dependent *adh* gene.

7. The method of any of claims 2-6, wherein said exogenous ATP depletion agent is administered in an amount effective to mitigate ATP synthesis in said host cell, wherein the yield of the reduced product by said host cell is increased by at least 5% relative to the yield of the reduced product by said host cell cultured in an otherwise identical host cell growth medium lacking said exogenous ATP depletion agent.

8. The method of any of claims 2-6, wherein said exogenous ATP depletion agent is selected from the group consisting of: N,N′-dicyclohexylcarbodiimide, 3,3′,4′,5-tetrachlorosalicylanilide, 2,4-dinitrophenol, and carbonyl cyanide-*p*-trifluoromethoxyhydrazone.

9. The method of any of claims 2-6, wherein said exogenous ATP depletion agent is N,N′-dicyclohexylcarbodiimide.

10. The method of claim 9, wherein said N,N′-dicyclohexylcarbodiimide is present in the host cell growth medium at a concentration of 0.25μM to 1μM.

11. The method of claim 9 or 10, wherein said N,N′-dicyclohexylcarbodiimide results in a 5 to 30% increase in ethanol productivity of the host cell relative to ethanol productivity of

the host cell cultured in an otherwise identical culture medium lacking said 3,3′,4′,5-tetrachlorosalicylanilide.

12. The method of any of claims 2-6, wherein said exogenous ATP depletion agent is 3,3′,4′,5-tetrachlorosalicylanilide.

13. The method of claim 11, wherein said 3,3′,4′,5-tetrachlorosalicylanilide is present in the host cell growth medium at a concentration of 0.1μM to 0.25μM.

14. The method of claim 11, wherein said 3,3′,4′,5-tetrachlorosalicylanilide results in a 5 to 30% increase in ethanol productivity of the host cell relative to ethanol productivity of the host cell cultured in an otherwise identical culture medium lacking said 3,3′,4′,5-tetrachlorosalicylanilide.

15. The method of claim 1, wherein said host cell is genetically modified to dissipate said excess energy.

16. The method of claim 14, wherein said genetically modified host cell comprises one or more recombinant protein activities selected from a futile cycle pathway.

17. The method of claim 1, wherein said excess energy is stored in ATP.

18. The method of claim 1, wherein said excess energy is stored as a proton gradient.

19. The method of claim 1, wherein said dissipation of said excess energy increases production of the reduced product in said engineered host cell.

20. The method of claim 1, wherein said excess energy is stored in ATP, and wherein the amount of ATP in said host cell is greater than the amount of ATP at metabolic equilibrium of a background host cell, wherein said host cell is modified to produce a carbon-based product of interest, and wherein said background host cell is unmodified.

21. The method of claim 20, wherein said amount of ATP at metabolic equilibrium is determined by the characteristic energy charge of said background host cell.

22. The method of claim 1, wherein said host cell is used for the production of a carbon-based product of interest.

23. The method of claim 22, wherein said excess energy is produced during said production of a carbon-based product of interest.

24. The method of claim 22, wherein said carbon-based product of interest is a reduced product.

25. The method of claim 22, wherein said carbon-based product of interest is a hydrocarbon.

26. The method of claim 22, wherein said carbon-based product of interest is an alcohol.

27. The method of claim 1, wherein said host cell is capable of photosynthesis.

28. A method to increase production of a reduced product in an engineered photosynthetic cell, comprising the step of culturing said engineered photosynthetic cell in a host cell growth medium comprising an ATP-mitigating agent, wherein said agent is administered in an amount effective to mitigate ATP synthesis in said engineered photosynthetic cell, wherein said ATP-mitigation results in the increased production of a reduced product by said engineered photosynthetic cell.

29. An engineered host cell, comprising one or more recombinant protein activities selected from Table 7, wherein said recombinant protein activities facilitate dissipation of excess energy stored in said engineered host cell.

30. The host cell of claim 28, wherein said engineered host cell is a gram-negative or gram-positive bacteria.

31. The host cell of claim 28, wherein said engineered host cell is a photosynthetic microbe.

32. The host cell of claim 28, wherein said engineered host cell is a cyanobacterium.

33. The host cell of claim 28, wherein said excess energy is stored in ATP.

34. The host cell of claim 28, wherein said excess energy is stored as a proton gradient.

35. The host cell of claim 28, wherein said host cell is engineered to dissipate excess ATP.

36. The host cell of claim 28, wherein said host cell is engineered to dissipate excess proton gradient.

37. The host cell of claim 28, wherein said dissipation of excess energy increases production of a reduced product in said engineered host cell.

38. The host cell of claim 28, wherein said excess energy is stored in ATP, and wherein the amount of ATP in said host cell is greater than the amount of ATP at metabolic equilibrium of a background host cell, wherein said host cell is modified to produce a carbon-based product of interest, and wherein said background host cell is unmodified.

39. The host cell of claim 38, wherein said amount of ATP at metabolic equilibrium is determined by the characteristic energy charge of said background host cell.

40. The host cell of claim 28, wherein said engineered host cell produces a carbon-based product of interest.

41. The host cell of claim 40, wherein said excess energy is produced during the production of a carbon-based product of interest.

42. The host cell of claim 40, wherein said carbon-based product of interest is a reduced product.

43. The host cell of claim 40, wherein said carbon-based product of interest is a hydrocarbon.

44. The host cell of claim 40, wherein said carbon-based product of interest is an alcohol.

45. A method for increasing the rate of production of a carbon-based product of interest by an engineered host cell comprising the step of introducing one or more recombinant protein activities from a futile cycle pathway into a host cell, and culturing said engineered host cell under conditions that promote production of the carbon-based product of interest.

46. The method of claims 16 or 45, wherein said one or more recombinant protein activities are selected from Table 7.

47. The method of claims 16 or 45, wherein said one or more recombinant proteins are under the control of an inducible promoter.

48. The method of claim 47, wherein said inducible promoter is P(*nir*07).

49. The method of claims 16 or 45, wherein said one or more recombinant protein activities comprise a gene product of a gene selected from the group consisting of *pntAB* and *udhA*.

50. The method of claims 16 or 45, wherein said one or more recombinant protein activities are selected from the group consisting of a membrane-bound proton-translocating transhydrogenase and a soluble pyridine nucleotide transhydrogenase.

51. The method of claims 16 or 45, wherein said one or more recombinant protein activities comprise a gene product of a gene selected from the group consisting of *glgC*, *glgA* and *glgP*.

52. The method of claims 16 or 45, wherein said one or more recombinant protein activities are selected from the group consisting of glucose-1-phosphate adenylyltransferase, glucose pyrophosphorylase and glycogen phosphorylase.

53. The method of claims 16 or 45, wherein said one or more recombinant protein activities comprise a gene product of a gene selected from the group consisting of *pfkA* and *fbp*.

54. The method of claims 16 or 45, wherein said one or more recombinant protein activities are selected from the group consisting of phosphofructokinase and fructose-bisphosphatase.

55. The method of claims 16 or 45, wherein said one or more recombinant protein activities comprise a gene product of a gene selected from the group consisting of *pta*, *ackA*, and *acs*.

56. The method of claims 16 or 45, wherein said one or more recombinant protein activities are selected from the group consisting of phosphotransacetylase, acetate kinase, and acetyl coenzyme synthetase.

57. The method of claims 16 or 45, wherein said one or more recombinant protein activities comprise a gene product of a gene selected from the group consisting of *pykF* and *ppsA*.

58. The method of claims 16 or 45, wherein said one or more recombinant protein activities are selected from the group consisting of pyruvate kinase and phosphoenolpyruvate synthase.

59. The method of claims 16 or 45, wherein said one or more recombinant protein activities comprise a gene product of a gene selected from the group consisting of *pyc*, *pck*, and *pykF*.

60. The method of claims 16 or 45, wherein said one or more recombinant protein activities are selected from the group consisting of pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and pyruvate kinase.

61. The method of claims 16 or 45, wherein said one or more recombinant protein activities comprise a gene product of a gene selected from the group consisting of *mdh*, *sfcA*, and *pyc*.

62. The method of claims 16 or 45, wherein said one or more recombinant protein activities are selected from the group consisting of malate dehydrogenase and pyruvate carboxylase.

63. The method of claims 16 or 45, wherein said one or more recombinant protein activities comprise a gene product of a gene selected from the group consisting of *mdh*, *ppc*, *sucCD*, *maeB*, *sad*, and *sucD*.

64. The method of claims 16 or 45, wherein said one or more recombinant protein activities are selected from the group consisting of malate dehydrogenase, phosphoenolpyruvate carboxylase, succinyl-CoA synthetase, and succinate semialdehyde dehydrogenase.

65. The method of claims 16 or 45, wherein said one or more recombinant protein activities comprise a gene product of a gene selected from the group consisting of *mdh*, *mdhP*, *gap2*, *pgk*, *dld*, *mgsA*, *gloA*, and *gloB*.

66. The method of claims 16 or 45, wherein said one or more recombinant protein activities are selected from the group consisting of NAD-specific malate dehydrogenase, NADP-specific malate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, D-lactate dehydrogenase, methylglyoxal synthase, glyoxalase I, and glyoxalase II.

67. The method of claims 16 or 45, wherein said one or more recombinant protein activities comprise a gene product of a gene selected from the group consisting of *zwf*, *pgl*, and *gnd*.

68. The method of claims 16 or 45, wherein said one or more recombinant protein activities are selected from the group consisting of glucose-6-phosphate dehydrogenase, 6-phosphogluconolactonase, and gluconate-P dehydrogenase.

69. A method for increasing the rate of production of a carbon-based product of interest by an engineered host cell comprising the step of introducing one or more recombinant protein activities which increase the activity of at least part of a futile cycle pathway in a host cell, and culturing said engineered host cell under conditions that promote production of the carbon-based product of interest.

70. The method of claim 69, wherein said gene is endogenous.

71. The method of claim 69, wherein said gene is group 2 sigma factor *sigE*.

72. The method of claim 71, wherein futile cycle pathway is an oxidative pentose phosphate pathway.

73. The method of claim 72, wherein said oxidative pentose phosphate pathway comprises an enzyme expressed by a gene selected from the group consisting of *zwf*, *pgl*, and *gnd*.

74. A method for mitigating ATP accumulation in an engineered host cell, comprising at least one step selected from the group consisting of: addition of salt to the host cell medium, acidification or alkalinization of the host cell medium, adjusting the temperature of the host cell medium, and introduction of a toxic compound to the medium.

75. The method of claim 69, wherein said method step of introduction of a toxic compound to the medium induces an efflux reaction in said host cell.

76. A method for production of a host cell engineered to produce a carbon-based product of interest wherein said host cell dissipates accumulated excess ATP comprising the steps of:

(i) performing random mutagenesis on said host cell, and

(ii) screening said host cell for dissipation of excess ATP.

77. A composition for producing ethanol, comprising a host cell engineered to produce ethanol, and a medium comprising an ATP depletion agent.

78. The composition of claim 77, wherein said ATP depletion agent is selected from the group consisting of: N,N′-dicyclohexylcarbodiimide, 3,3′,4′,5-tetrachlorosalicylanilide, 2,4-dinitrophenol, and carbonyl cyanide-*p*-trifluoromethoxyhydrazone.

79. The composition of claim 77, wherein said ATP depletion agent is N,N′-dicyclohexylcarbodiimide at a concentration of 0.25 to 0.75μM.

80. The composition of claim 77, wherein said ATP depletion agent is 3,3′,4′,5-tetrachlorosalicylanilide at a concentration of 0.1 to 0.25μM.

81. The composition of claim 77, wherein said host cell is a photosynthetic microbe.

82. The composition of claim 77, wherein said host cell is a cyanobacterium.

83. The composition of claim 77, wherein said host cell comprises a recombinant NADPH-dependent *adh* gene.

84. A method to increase the production of ethanol in a photosynthetic microbe, wherein said photosynthetic microbe comprises a recombinant NADPH-dependent *adh* gene, comprising the step of culturing said photosynthetic microbe in a medium comprising N,N′-dicyclohexylcarbodiimide at a concentration of 0.75μM, wherein said N,N′-dicyclohexylcarbodiimide increases the ethanol productivity of the photosynthetic microbe.

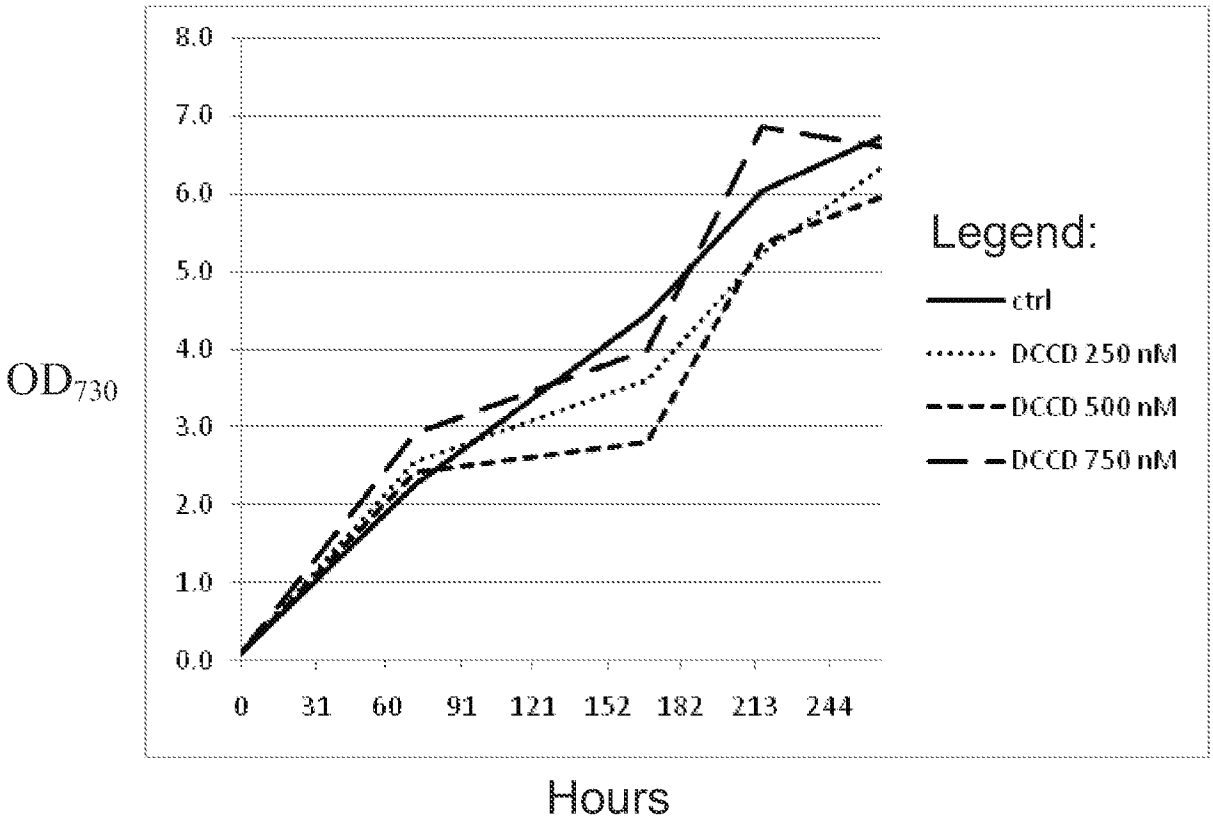
85. A method to increase the production of ethanol in a photosynthetic microbe, wherein said photosynthetic microbe comprises a recombinant NADPH-dependent *adh* gene, comprising the step of culturing said photosynthetic microbe in a medium comprising 3,3′,4′,5-tetrachlorosalicylanilide at a concentration of 0.1μM, wherein said 3,3′,4′,5-tetrachlorosalicylanilide increases the ethanol productivity of the photosynthetic microbe.

86. The method of claim 84 or 85, wherein said photosynthetic microbe is cyanobacterium.

87. A composition for producing ethanol, comprising a photosynthetic microbe comprising a recombinant NADPH-dependent *adh* gene, and a medium comprising N,N′-dicyclohexylcarbodiimide at a concentration of 0.75μM.

88. A composition for producing ethanol, comprising a photosynthetic microbe comprising a recombinant NADPH-dependent *adh* gene, and a medium comprising 3,3′,4′,5-tetrachlorosalicylanilide at a concentration of 0.1μM.

89. The composition of claim 86 or 88, wherein said photosynthetic microbe is cyanobacterium.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31
60
91
121
152
182
213
244
Legend:
ctrl
DCCD 250 nM
DCCD 500 nM
DCCD 750 nM
OD730
Hours

Figure 1

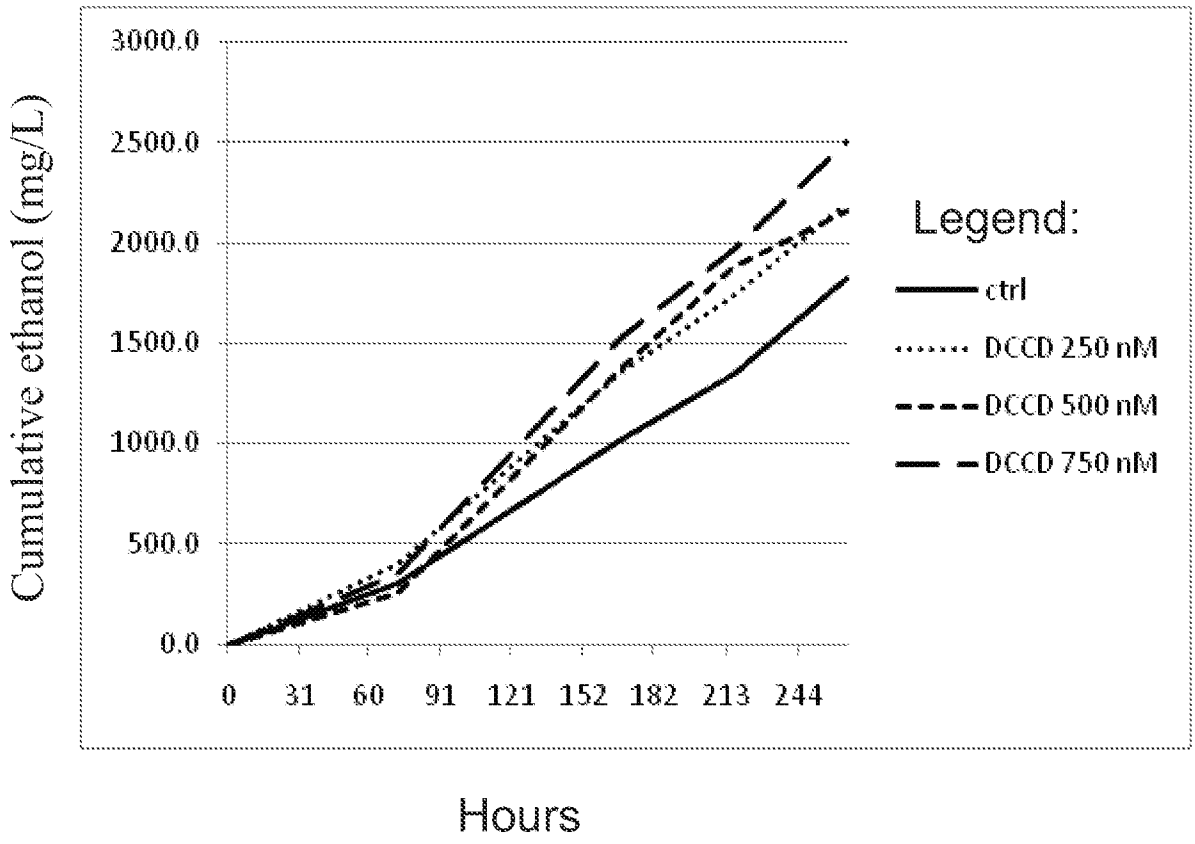
Cumulative ethanol (mg/L)
3000.0
2500.0
2000.0
1500.0
1000.0
500.0
0.0
0
31
60
91
121
152
182
213
244
Hours
Legend:
ctrl
DCCD 250 nM
DCCD 500 nM
DCCD 750 nM

Figure 2

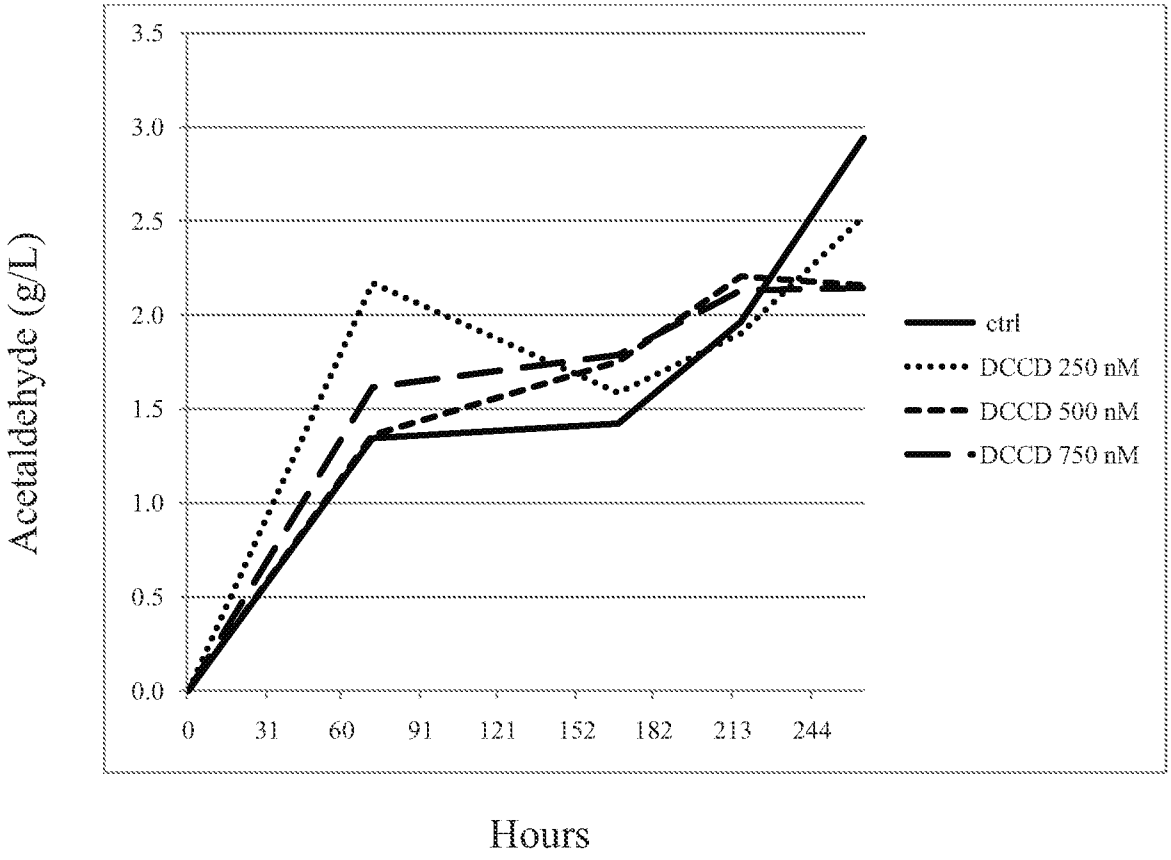
Acetaldehyde (g/L)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
31
60
91
121
152
182
213
244
Hours
ctrl
DCCD 250 nM
DCCD 500 nM
DCCD 750 nM

Figure 3