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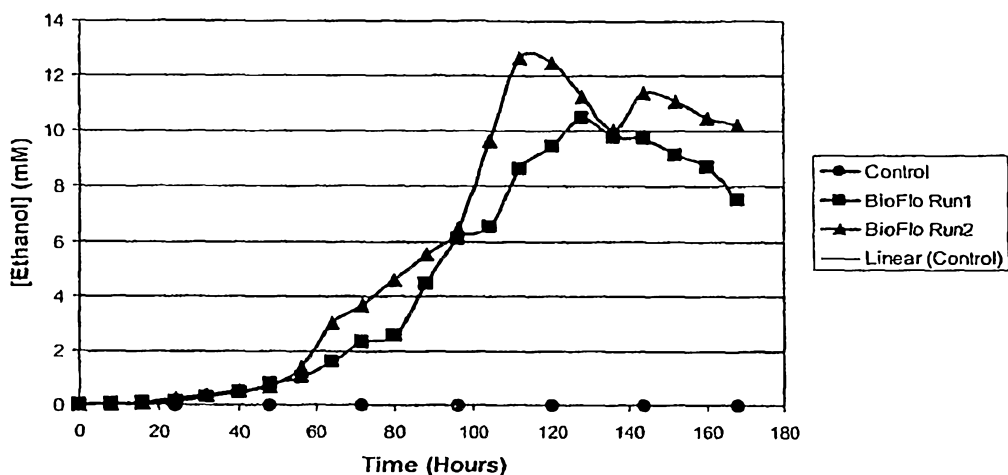
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(54) Title: METHODS AND COMPOSITIONS FOR ETHANOL PRODUCING CYANOBACTERIA

Ethanol Concentration vs. Time in Bioflo Reactor



- (57) Abstract: The present invention relates to methods and systems for the production of ethanol by cyanobacteria. More specifically, the methods can be used to produce ethanol using genetically engineered light responsive cyanobacteria.

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METHODS AND COMPOSITIONS FOR ETHANOL PRODUCING CYANOBACTERIA

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application Serial No. 60/758,683, filed January 13, 2006.

BACKGROUND OF THE INVENTION

Description of the Related Art

[0002] Development of renewable energy is rapidly embraced by society and industry to meet energy growth and emission reduction goals. Since the industrial revolution, the world's economy has relied heavily on fossil fuels as energy sources. Reliance on these energy sources has created several challenging problems, such as reduced supply of fossil fuel resources, environmental pollution and the consequent global warming effect. One alternative to fossil fuels is ethanol. The current world ethanol production is 60% from sugar crops, 33% from other crops and 7% from chemical synthesis. Traditional biomass ethanol production processes require vast quantities of arable land and energy input requirement for the growth of the feedstock. Furthermore, traditional fermentation methods release considerable quantities of CO₂ as a byproduct of the fermentation process. For example, a 40 MMGY (million gallons per year) biomass ethanol plant may release 121,000 tons of CO₂ each year into the environment (BBI, 2003). This greenhouse gas will worsen the global warming effect.

[0003] Bioethanol has recently surged to the forefront of renewable fuels technology. It provides a viable alternative to petroleum based fuels, offering control over both production and consumption processes. In addition, ethanol derived from biological systems is particularly attractive because it can be readily integrated into numerous existing infrastructures; considering both production and fuel industries. Various methods for ethanol production by living organisms have been investigated. The production of ethanol by microorganisms has, in large part, been investigated using the yeast *Saccharomyces cerevisiae* and the obligately ethanogenic bacteria *Zymomonas mobilis*. Both of these microorganisms contain the genetic information to produce the enzymes pyruvate decarboxylase (pdc) and alcohol dehydrogenase (adh), which are used to produce ethanol from pyruvate, a product of the glycolytic pathway. Woods et al. (U.S. Patent Nos. 6,306,639 and 6,699,696; *see also* Deng and Coleman, "Ethanol

Synthesis by Genetic Engineering in Cyanobacteria" *Applied and Environmental Microbiology* (1999) 65(2):523-428) disclose a genetically modified cyanobacterium useful for the production of ethanol. Woods et al. report an ethanol production level of 5 mM after 30 days of culture.

[0004] It is therefore desirable to find a simple, efficient and cost-effective biological system for producing substantial amounts of ethanol.

SUMMARY OF THE INVENTION

[0005] The technologies mentioned above suffer from various flaws. For example, the systems disclosed by Woods et al. suffer from low ethanol production levels, long fermentation times, and instability.

[0006] In some embodiments, what is needed is a simple, efficient and cost-effective system for producing substantial amounts of ethanol. The present invention relates to methods, compositions, host cells, and vectors for the optimization of ethanol production and tolerance of a host cell to economically relevant ethanol concentrations.

[0007] A nucleic acid construct is disclosed. The nucleic acid sequence comprises: a light responsive promoter, a sequence encoding a pyruvate decarboxylase (pdc) enzyme, and a sequence encoding an alcohol dehydrogenase (adh) enzyme.

[0008] An expression vector is disclosed. The expression vector comprises a nucleic acid comprising a light responsive promoter, a sequence encoding a pyruvate decarboxylase (pdc) enzyme, and a sequence encoding an alcohol dehydrogenase (adh) enzyme.

[0009] A *Synechocystis* host cell is disclosed in accordance with some embodiments of the present invention. The host cell comprises an expression vector comprising a nucleic acid comprising a light responsive promoter, which is operably linked to a sequence encoding a pyruvate decarboxylase (pdc) enzyme, and a sequence encoding an alcohol dehydrogenase (adh) enzyme.

[0010] A genetically engineered cyanobacterium is disclosed. The cyanobacterium comprises a construct comprising nucleic acid sequences encoding pyruvate decarboxylase (pdc) and alcohol dehydrogenase (adh) enzymes, wherein the cyanobacterium is capable of producing ethanol in recoverable quantities greater than about 10 mM ethanol after a 5 day fermentation.

[0011] A method of producing ethanol is disclosed in accordance with some embodiments of the present invention. The method comprises: culturing in a culture medium *Synechocystis* cyanobacteria, the cyanobacteria containing a construct comprising a light responsive promoter operably linked to DNA fragments encoding *pdh* and *adh* enzymes; and accumulating ethanol in the culture medium.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] FIG. 1 shows the map of the plasmid constructs (a) pPSBAIIKS and (b) pLOI295, used to create pMota.

[0013] FIG. 2 shows ethanol concentration vs. time in the BIOFLO® Reactor. Ethanol concentration reached 13 mM after five days of fermentation. The control is non-transformed (wild-type) *Synechocystis*.

[0014] While the subject matter of this application will now be described in detail with reference to the figures, it is done so in connection with the illustrative embodiments.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

I. Introduction

[0015] Nucleic acid sequences, vectors, host cells, and methods for the production of high levels of ethanol by cyanobacteria are disclosed in accordance with preferred embodiments of the present invention.

[0016] Ethanol production from cyanobacteria using sunlight, CO₂, and inorganic nutrients (possibly diverted from a wastewater stream) is an attractive pathway for obtaining a renewable fuel. By combining both the carbon fixation and ethanol generating pathways

into a single organism, the costs associated with plant growth/harvesting/processing are circumvented, reducing total input energy, and increasing net energy gain. In contrast to biomass ethanol production processes, the disclosed methods will directly utilize large quantities of CO₂ as a carbon source for fuel production and will thus help reduce this greenhouse gas from the atmosphere.

[0017] There are numerous benefits from producing ethanol using photosynthetic microorganisms such as *Synechocystis*, including: economic opportunities for biofuel production, positive environmental impacts, reduction in global warming, and improved food security. The present methods for producing ethanol from solar energy and CO₂ using cyanobacteria offer significant savings in both capital and operation costs, in comparison to the biomass-based ethanol production facilities. The decreased expenditure is achieved by factors such as: simplified production processes, absence of agricultural crops and residues, no solid wastes to be treated, no enzymes needed, etc. The cyanobacteria fermentation involves no hard cellulose or hemicellulose which is difficult to treat. As a result, there will be no emissions of hazardous air pollutants and volatile organic compounds from cyanobacterial ethanol production plants.

[0018] In comparison to traditional methods for biomass ethanol production, the disclosed methods and systems will help preserve agricultural space for food production. Furthermore, cyanobacterial ethanol production plants can be highly distributed without geographical limits because they do not require grain transportation to certain locations or pretreatment of the raw material. The infrastructure and equipment required for ethanol production using the presently disclosed systems are projected to be significantly less than those required for current yeast fermentation technology, allowing for smoother integration with fuel transportation and distribution platforms.

[0019] The initial product of photosynthetic fixation of carbon dioxide is 3-phosphoglycerate. 3-phosphoglycerate is used in the Calvin Cycle to regenerate ribulose-1,5-biphosphate, which is the acceptor of carbon dioxide. There are two major branching points where the intermediates of the Calvin Cycle are connected to other metabolic pathways. At one point, fructose-6-phosphate is converted into glucose-6-phosphate and glucose-phosphate, which are the substrates for the pentose phosphate pathway, the synthesis of cellulose (a major component of the cell wall) and the synthesis of glycogen (the major form

of carbohydrate reserve). At the other branching point, 3-phosphoglycerate is converted into 2-phosphoglycerate, phosphoenolpyruvate and pyruvate in a sequence of reactions catalysed by phosphoglycerate mutase, enolase and pyruvate kinase, respectively. Pyruvate is directed to the partial TCA cycle for the synthesis of amino acids, nucleotides, etc. in aerobic conditions. Pyruvate is also the substrate for ethanol synthesis.

[0020] To convert the carbohydrate reserves into ethanol, the carbohydrate reserves must be diverted to the glycolytic pathway. The presumed pathway for carbohydrate reserve metabolism in cyanobacteria is through both the glycolytic pathway and the phosphogluconate pathway. For the purposes of ethanol formation, the glycolytic pathway is of primary importance. Although not well characterized in cyanobacteria, glycogen is presumed to be metabolized into glucose 1-phosphate by a combination of glycogen phosphorylase and a 1,6-glycosidase. Phosphoglucomutase, phosphoglucoisomerase and phosphofructokinase convert glucose 1-phosphate into a molecule of fructose 1,6-bisphosphate. This compound is cleaved by the action of aldolase and triose phosphate isomerase into two molecules of glyceraldehyde 3-phosphate. This compound is converted into pyruvate through a sequential series of reactions catalysed by glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase and pyruvate kinase, respectively.

[0021] In some algae and cyanobacteria strains, a small amount of ethanol is synthesized as a fermentation product under dark and anaerobic conditions (Van der Oost et al., "Nucleotide sequence of the gene proposed to encode the small subunit of the soluble hydrogenase of the thermophilic unicellular cyanobacterium *Synechococcus* PCC 6716." *Nucleic Acids Res.* 1989 Dec 11;17(23):10098, incorporated herein by reference in its entirety). However, the dark-anaerobic fermentation process is generally operating at a very low level, only sufficient for the survival of the organisms under such stress conditions. The synthesis of ethanol under dark and anaerobic conditions is dependent on the degradation of glycogen reserve, as described above. Moreover, it has been found that ethanol synthesis under anaerobic conditions is totally inhibited by light. Thus, in photosynthetic microorganisms ethanol synthesis is not coupled with photosynthesis and can actually be inhibited by photosynthesis.

[0022] Therefore, it has been observed that cyanobacteria do not utilize carbon dioxide to produce ethanol. Furthermore, there are no known photosynthetic microorganisms, including genetically engineered photosynthetic microorganisms, which produce ethanol in relatively substantial amounts. A further complication is that some photosynthetic organisms have been shown to be inhibited by ethanol such that the addition of ethanol to the culture medium inhibits the expression of genes involved in photosynthesis.

[0023] In the present invention, it has been found that *Synechocystis* cyanobacteria can be successfully genetically engineered to produce a quantifiable amount of ethanol as opposed to utilizing a glycogen reserve as is done under anaerobic and dark conditions. Inorganic carbon is assimilated and is used for both cellular growth and for the production of ethanol via the insertion of the ethanol generating metabolic pathway consisting of the two aforementioned enzymes *pdc* and *adh*.

II. Definitions

[0024] Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0025] "Pyruvate decarboxylase" and "*pdc*" refer to an enzyme that catalyzes the decarboxylation of pyruvic acid to acetaldehyde and carbon dioxide. A "*pdc* gene" refers to the gene encoding an enzyme that catalyzes the decarboxylation of pyruvic acid to acetaldehyde and carbon dioxide. "Alcohol dehydrogenase" and "*adh*" refer to an enzyme that facilitates the interconversion between alcohols and aldehydes or ketones. An "*adh* gene" refers to the gene encoding an enzyme that facilitates the interconversion between alcohols and aldehydes or ketones. "*pdc/adh*" refers to the *pdc* and *adh* enzymes collectively. A "*pdc/adh* cassette" refers to a nucleic acid sequence encoding a *pdc* enzyme and an *adh* enzyme.

[0026] A "promoter" is an array of nucleic acid control sequences that direct transcription of an associated polynucleotide, which may be a heterologous or native polynucleotide. A promoter includes nucleic acid sequences near the start site of transcription, such as a polymerase binding site. The promoter also optionally includes distal

enhancer or repressor elements which can be located as much as several thousand base pairs from the start site of transcription.

[0027] A "light responsive promoter" refers to a promoter which is responsive to light.

[0028] "Polynucleotide" and "nucleic acid" refer to a polymer composed of nucleotide units (ribonucleotides, deoxyribonucleotides, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof) linked via phosphodiester bonds, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof. Thus, the term includes nucleotide polymers in which the nucleotides and the linkages between them include non-naturally occurring synthetic analogs. It will be understood that, where required by context, when a nucleotide sequence is represented by a DNA sequence (i.e., A, T, G, C), this also includes an RNA sequence (i.e., A, U, G, C) in which "U" replaces "T."

[0029] "Recombinant" refers to polynucleotides synthesized or otherwise manipulated in vitro ("recombinant polynucleotides") and to methods of using recombinant polynucleotides to produce gene products encoded by those polynucleotides in cells or other biological systems. For example, a cloned polynucleotide may be inserted into a suitable expression vector, such as a bacterial plasmid, and the plasmid can be used to transform a suitable host cell. A host cell that comprises the recombinant polynucleotide is referred to as a "recombinant host cell" or a "recombinant bacterium." The gene is then expressed in the recombinant host cell to produce, e.g., a "recombinant protein." A recombinant polynucleotide may serve a non-coding function (e.g., promoter, origin of replication, ribosome-binding site, etc.) as well.

[0030] The term "homologous recombination" refers to the process of recombination between two nucleic acid molecules based on nucleic acid sequence similarity. The term embraces both reciprocal and nonreciprocal recombination (also referred to as gene conversion). In addition, the recombination can be the result of equivalent or non-equivalent cross-over events. Equivalent crossing over occurs between two equivalent sequences or chromosome regions, whereas nonequivalent crossing over occurs between identical (or substantially identical) segments of nonequivalent sequences or chromosome regions. Unequal crossing over typically results in gene duplications and deletions. For a

description of the enzymes and mechanisms involved in homologous recombination see, Watson et al., *Molecular Biology of the Gene* pp 313-327, The Benjamin/Cummings Publishing Co. 4th ed. (1987).

[0031] The term “non-homologous or random integration” refers to any process by which DNA is integrated into the genome that does not involve homologous recombination. It appears to be a random process in which incorporation can occur at any of a large number of genomic locations.

[0032] A “heterologous polynucleotide sequence” or a “heterologous nucleic acid” is a relative term referring to a polynucleotide that is functionally related to another polynucleotide, such as a promoter sequence, in a manner so that the two polynucleotide sequences are not arranged in the same relationship to each other as in nature. Heterologous polynucleotide sequences include, e.g., a promoter operably linked to a heterologous nucleic acid, and a polynucleotide including its native promoter that is inserted into a heterologous vector for transformation into a recombinant host cell. Heterologous polynucleotide sequences are considered “exogenous” because they are introduced to the host cell via transformation techniques. However, the heterologous polynucleotide can originate from a foreign source or from the same source. Modification of the heterologous polynucleotide sequence may occur, e.g., by treating the polynucleotide with a restriction enzyme to generate a polynucleotide sequence that can be operably linked to a regulatory element. Modification can also occur by techniques such as site-directed mutagenesis.

[0033] The term “expressed endogenously” refers to polynucleotides that are native to the host cell and are naturally expressed in the host cell.

[0034] An “expression cassette” or “construct” refers to a series of polynucleotide elements that permit transcription of a gene in a host cell. Typically, the expression cassette includes a promoter and a heterologous or native polynucleotide sequence that is transcribed. Expression cassettes or constructs may also include, e.g., transcription termination signals, polyadenylation signals, and enhancer elements.

[0035] The term “operably linked” refers to a functional relationship between two parts in which the activity of one part (e.g., the ability to regulate transcription) results in an action on the other part (e.g., transcription of the sequence). Thus, a polynucleotide is “operably linked to a promoter” when there is a functional linkage between a polynucleotide

expression control sequence (such as a promoter or other transcription regulation sequences) and a second polynucleotide sequence (e.g., a native or a heterologous polynucleotide), where the expression control sequence directs transcription of the polynucleotide.

[0036] “Competent to express” refers to a host cell that provides a sufficient cellular environment for expression of endogenous and/or exogenous polynucleotides.

[0037] All numbers expressing quantities of ingredients, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about.” Accordingly, unless indicated to the contrary, the numerical parameters set forth in the specification and attached claims are approximations that can vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should be construed in light of the number of significant digits and ordinary rounding approaches.

III. Description of Embodiments

[0038] Nucleic acids and recombinant expression vectors for the optimization of ethanol production are disclosed in accordance with some embodiments of the present invention. Figure 1 shows one embodiment of a system, described more fully in Example 1 below, that can be used to perform a variety of methods or procedures. The sequence of the pMota recombinant expression vector for optimization of ethanol production is shown in SEQ ID NO: 1. The pMota vector contains the sequences for the *Synechocystis* psbAII promoter in a 500 base pair homologous upstream region nucleic acid sequence (SEQ ID NO: 2), the nucleic acid sequence encoding *Zymomonas mobilis* pdc from PLOI295 (SEQ ID NO: 3), and the nucleic acid sequence encoding *Zymomonas mobilis* adhII from PLOI295 (SEQ ID NO: 4). The pMota vector was created by subcloning the pdc/adh cassette from PLOI295 (Figure 1b) into the pPSBAIIKS plasmid (Figure 1a). The pMota vector was used to integrate these genes under the control of the psbAII light responsive promoter in the cyanobacterial genome.

[0039] A recombinant expression vector for transformation of a host cell and subsequent integration of the gene(s) of interest is prepared by first isolating the constituent polynucleotide sequences, as discussed herein. In some embodiments, the gene(s) of interest

are homologously integrated into the host cell genome. In other embodiments, the genes are non-homologously integrated into the host cell genome. Preferably, the gene(s) of interest are homologously integrated into the *Synechocystis* genome. In some embodiments, the pMota vector integrates into the psbAII locus via double homologous recombination. The polynucleotide sequences, e.g., a sequence encoding the pdc/adh enzymes driven by a promoter, are then ligated to create a recombinant expression vector, also referred to as a "pdc/adh construct," suitable for transformation of a host cell. Methods for isolating and preparing recombinant polynucleotides are well known to those skilled in the art. Sambrook et al., *Molecular Cloning. A Laboratory Manual* (2d ed. 1989); Ausubel et al., *Current Protocols in Molecular Biology* (1995)), provide information sufficient to direct persons of skill through many cloning exercises.

[0040] One preferred method for obtaining specific polynucleotides combines the use of synthetic oligonucleotide primers with polymerase extension or ligation on a mRNA or DNA template. Such a method, e.g., RT, PCR, or LCR, amplifies the desired nucleotide sequence (see U.S. Pat. Nos. 4,683,195 and 4,683,202). Restriction endonuclease sites can be incorporated into the primers. Amplified polynucleotides are purified and ligated to form an expression cassette. Alterations in the natural gene sequence can be introduced by techniques such as in vitro mutagenesis and PCR using primers that have been designed to incorporate appropriate mutations. Another preferred method of isolating polynucleotide sequences uses known restriction endonuclease sites to isolate nucleic acid fragments from plasmids. The genes of interest can also be isolated by one of skill in the art using primers based on the known gene sequence.

[0041] Promoters suitable for the present invention include any suitable light-responsive promoter such as, for example, the psbAII promoter and the nirA promoter. In some embodiments, the promoter is the in situ native psbAII promoter that, in wild type *Synechocystis* sp. PCC 6803 cells, mediates the transcription of the psbAII gene that encodes the D1 subunit of photosystem II. The psbAII light responsive promoter is located immediately upstream of the endogenous psbAII gene in *Synechocystis* sp PCC 6803. In some embodiments, the promoter is the nirA promoter, which is a 166 base pair *Synechococcus* sp. strain PCC 7942 promoter sequence described by Qi et al. (2005) in development of an inducible expression vector for *Synechocystis* sp. PCC 6803.

[0042] In some embodiments, the promoter comprises the nucleic acid sequence shown in SEQ ID NO: 2, which contains the *psbAII* promoter. In some embodiments, the promoter comprises the nucleic acid sequence shown in SEQ ID NO: 5. In other embodiments, the promoter comprises the *Synechococcus nirA* promoter sequence shown in SEQ ID NO: 6. In SEQ ID NO: 6, the TCC at the 3' terminus of the wild type *nirA* promoter was replaced with the sequence CAT in order to generate an *NdeI* restriction site at the start codon while maintaining the spatial integrity of the promoter/ORF construct. This allows for the creation of a system whereby the gene(s) of interest may be expressed via induction by addition of nitrate to the culture media. Ammonia may be used as the nitrogen source for growth prior to induction. (Qi *et al.*, 2005). In some embodiments, a 500 base pair homologous upstream region containing the *psbAII* promoter having the sequence shown in SEQ ID NO: 2 is used in constructing the recombinant expression vector. The 500 base pair homology targets the vector for integration into the *psbAII* locus via double homologous recombination.

[0043] Any *pdc* gene capable of being expressed may be used in the present invention. In some embodiments, the *pdc* gene is the *Zymomonas mobilis* *pdc* gene. In some embodiments, the *pdc* gene is obtained from the *Zymomonas mobilis* plasmid pLOI295. In some embodiments, the *pdc* gene comprises the nucleic acid sequence shown in SEQ ID NO: 3. In some embodiments, the *pdc* gene is a nucleic acid sequence encoding the protein shown in SEQ ID NO: 7. In other embodiments, the *pdc* gene is a nucleic acid encoding the *pdc* enzyme obtained from *Zymobacter palmae*. The NCBI accession number for the complete *pdc* protein sequence from *Zymobacter palmae* is AF474145 (SEQ ID NO: 8). In some embodiments, the *pdc* gene is a nucleic acid sequence encoding the amino acid sequence shown in SEQ ID NO: 8. There are other sources of *pdc* and *adh* enzymes, including *Saccharomyces cerevisiae*.

[0044] Any *adh* gene capable of being expressed may be used in the present invention. In some embodiments, the *adh* gene is the *Zymomonas mobilis* *adhII* gene. In some embodiments, the *adh* gene is obtained from the *Zymomonas mobilis* plasmid pLOI295. In some embodiments, the *adh* gene comprises the nucleic acid sequence shown in SEQ ID NO: 4. In some embodiments, the *pdc* gene is a nucleic acid sequence encoding the amino acid sequence shown in SEQ ID NO: 9.

[0045] The isolated polynucleotide sequence of choice, e.g., the *pdc/adh* genes driven by the promoter sequence discussed above, is inserted into an “expression vector,” “cloning vector,” or “vector,” terms which usually refer to plasmids or other nucleic acid molecules that are able to replicate in a chosen host cell. Expression vectors can replicate autonomously, or they can replicate by being inserted into the genome of the host cell.

[0046] Often, it is desirable for a vector to be usable in more than one host cell, e.g., in *E. coli* for cloning and construction, and in, e.g., *Synechocystis* for expression. Additional elements of the vector can include, for example, selectable markers, e.g., kanamycin resistance or ampicillin resistance, which permit detection and/or selection of those cells transformed with the desired polynucleotide sequences.

[0047] The particular vector used to transport the genetic information into the cell is also not particularly critical. Any suitable vector used for expression of recombinant proteins can be used. In preferred embodiments, a vector that is capable of being inserted into the genome of the host cell is used. In some embodiments, the vector is pSBAIKS, created and described by Lagarde et al. (2000). Expression vectors typically have an expression cassette that contains all the elements required for the expression of the polynucleotide of choice in a host cell. A typical expression cassette contains a promoter operably linked to the polynucleotide sequence of choice. The promoter used to direct expression of *pdc/adh* is as described above, and is operably linked to a sequence encoding the *pdc/adh* proteins. The promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

[0048] In addition to the *pdc/adh* genes, the expression vector for the optimization of ethanol production may include genes for the tolerance of a host cell to economically relevant ethanol concentrations. For example, genes such as *omrA*, *lmrA*, and *lmrCD* may be included in the expression vector. *OmrA* from wine lactic acid bacteria *Oenococcus oeni* and its homolog *LmrA* from *Lactococcus lactis* have been shown to increase the relative resistance of *tolC(-)* *E. Coli* by 100 to 10,000 times. (Bourdineaud et al., 2004) Therefore, it may be beneficial to incorporate *omrA*, *lmrA*, and other homologues in order to increase the ethanol tolerance of a host cell. In some embodiments, the expression vector comprising the

pdh/adh genes further comprises the omrA gene. In other embodiments, the expression vector comprising the pdh/adh genes further comprises the lmrA gene. In other embodiments, the expression vector comprising the pdh/adh genes further comprises the lmrCD gene. Any promoters suitable for driving the expression of a heterologous gene in a host cell can be used to drive the genes for the tolerance of a host cell, including those typically used in standard expression cassettes.

[0049] After construction and isolation of the recombinant expression vector, it is used to transform a host cell for ethanol production. The particular procedure used to introduce the genetic material into the host cell for expression of a protein is not particularly critical. Any of the well known procedures for introducing foreign polynucleotide sequences into host cells can be used. In some embodiments, the host cells can be transformed and screened sequentially via the protocol described by Williams (1988). This method exploits the natural transformability of the *Synechocystis* sp. PCC 6803 cyanobacteria, where transformation is possible via simple incubation of purified plasmid construct with exponentially growing cells.

[0050] Host cells for transformation with the recombinant expression vector described above include a suitable host cyanobacterium competent to produce ethanol, which is of the genus *Synechocystis*. Host cells suitable for use in the present invention include, for example, wild type *Synechocystis* sp. PCC 6803 and a mutant *Synechocystis* created by Howitt et al. (1999) that lacks a functional NDH type 2 dehydrogenase (NDH-2(-)). The type 2 dehydrogenase is specific for the regeneration of NAD⁺ from NADH. Flux through the ethanol pathway may be increased in the mutant. In particularly preferred embodiments, the host cells are *Synechocystis*. Host cells that are transformed with the pdh/adh construct are useful recombinant cyanobacteria for production of ethanol. Preferred subspecies of *Synechocystis* include, e.g., *Synechocystis* PCC 6803. A preferred strain is the *Synechocystis* sp. PCC 6803 NDH-2(-) mutant.

[0051] After the host cell is transformed with the pdh/adh construct, the host cell is incubated under conditions suitable for production of ethanol. Typically, the host cell will be grown in a photoautotrophic liquid culture in BG-11 media, with a 1L/min air sparge rate and a pH setpoint of 8.5, controlled via sparging with CO₂, and the temperature maintained at 30°C. Various media for growing cyanobacteria are known in the art. In some embodiments,

Synechocystis sp. PCC 6803 is cultured on standard BG-11 media plates, with or without the addition of (final concentration): 5 mM glucose, 5% sucrose, and/or either 5 $\mu\text{g ml}^{-1}$, 25 $\mu\text{g ml}^{-1}$, or 50 $\mu\text{g ml}^{-1}$ kanamycin. Plates containing *Synechocystis* sp. PCC 6803 were incubated at 30°C under ~ 100 microeinsteins $\text{m}^2 \text{s}^{-1}$. All *Synechocystis* liquid cultures were grown in standard BG-11, with the addition of 50 $\mu\text{g ml}^{-1}$ kanamycin when appropriate.

[0052] Enhanced secretion of ethanol is observed after host cells competent to produce ethanol are transformed with the *pdc/adh* construct and the cells are grown under suitable conditions as described above. Enhanced secretion of ethanol may be observed by standard methods, discussed more fully below in the Examples, known to those skilled in the art. In some embodiments, the host cells are grown using batch cultures. In some embodiments, the host cells are grown using photobioreactor fermentation. In some embodiments, the host cells are grown in a BIOFLO® Reactor. In some embodiments, the growth medium in which the host cells are grown is changed, thereby allowing increased levels of ethanol production. The number of medium changes may vary. Ethanol concentration levels may reach from about 5 mM to about 15 mM after about 2 to about 5 days of fermentation. In cases where the medium is changed, ethanol concentration levels may reach from about 25 to about 100 mM after 5 days of fermentation. In some embodiments, the ethanol production level is about 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9.0, 9.1, 9.2, 9.3, 9.4, 9.5, 9.6, 9.7, 9.8, 9.9, 10.0, 10.1, 10.2, 10.3, 10.4, 10.5, 10.6, 10.7, 10.8, 10.9, 11.0, 11.1, 11.2, 11.3, 11.4, 11.5, 11.6, 11.7, 11.8, 11.9, 12.0, 12.1, 12.2, 12.3, 12.4, 12.5, 12.6, 12.7, 12.8, 12.9, 13.0, 13.1, 13.2, 13.3, 13.4, 13.5, 13.6, 13.7, 13.8, 13.9, 14.0, 14.1, 14.2, 14.3, 14.4, 14.5, 14.6, 14.7, 14.8, 14.9 or 15.0 mM after about 5 days of fermentation. In cases where the medium is changed, in some embodiments, the ethanol production level is about 25.0, 25.1, 25.2, 25.3, 25.4, 25.5, 25.6, 25.7, 25.8, 25.9, 26.0, 26.1, 26.2, 26.3, 26.5, 26.6, 26.7, 26.8, 26.9, 27.0, 27.1, 27.2, 27.3, 27.5, 27.6, 27.7, 27.8, 27.9, 28.2, 28.2, 28.3, 28.5, 28.6, 28.7, 28.8, 28.9, 29.0, 29.1, 29.2, 29.3, 29.5, 29.6, 29.7, 29.8, 29.9, 30.0, 30.1, 30.2, 30.3, 30.4, 30.5, 30.6, 30.7, 30.8, 30.9, 31.0, 31.1, 31.2, 31.3, 31.4, 31.5, 31.6, 31.7, 31.8, 31.9, 32.0, 32.1, 32.2, 32.3, 32.4, 32.5, 32.6, 32.7, 32.8, 32.9, 33.0, 33.1, 33.2, 33.3, 33.4, 33.5, 33.6, 33.7, 33.8, 33.9, 34.0, 34.1, 34.2, 34.3, 34.4, 34.5, 34.6, 34.7, 34.8, 34.9, 35.0, 35.1, 35.2, 35.3, 35.4, 35.5, 35.6,

35.7, 35.8, 35.9, 36.0, 36.1, 36.2, 36.3, 36.5, 36.6, 36.7, 36.8, 36.9, 37.0, 37.1, 37.2, 37.3, 37.5, 37.6, 37.7, 37.8, 37.9, 38.2, 38.2, 38.3, 38.5, 38.6, 38.7, 38.8, 38.9, 39.0, 39.1, 39.2, 39.3, 39.5, 39.6, 39.7, 39.8, 39.9, 40.0, 40.1, 40.2, 40.3, 40.4, 40.5, 40.6, 40.7, 40.8, 40.9, 41.0, 41.1, 41.2, 41.3, 41.4, 41.5, 41.6, 41.7, 41.8, 41.9, 42.0, 42.1, 42.2, 42.3, 42.4, 42.5, 42.6, 42.7, 42.8, 42.9, 43.0, 43.1, 43.2, 43.3, 43.4, 43.5, 43.6, 43.7, 43.8, 43.9, 44.0, 44.1, 44.2, 44.3, 44.4, 44.5, 44.6, 44.7, 44.8, 44.9, 45.0, 45.1, 45.2, 45.3, 45.4, 45.5, 45.6, 45.7, 45.8, 45.9, 46.0, 46.1, 46.2, 46.3, 46.5, 46.6, 46.7, 46.8, 46.9, 47.0, 47.1, 47.2, 47.3, 47.5, 47.6, 47.7, 47.8, 47.9, 48.2, 48.2, 48.3, 48.5, 48.6, 48.7, 48.8, 48.9, 49.0, 49.1, 49.2, 49.3, 49.5, 49.6, 49.7, 49.8, 49.9 or 50.0 mM after about 5 days of fermentation. The fermentation times may vary from about 2 days to about 30 days of fermentation. In some embodiments, the fermentation time is about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 days.

[0053] The air sparge rate during host cell growth may be from 0.1L/min to 3.0L/min. In some embodiments, the air sparge rate during host cell growth is about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2.0, 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, 2.8, 2.9 or 3.0L/min. Preferably, the air sparge rate is 1L/min. The pH setpoint for host cell growth may be from 7.0 to 9.5. In some embodiments, the pH setpoint is about 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6, 7.7, 7.8, 7.9, 8.0, 8.1, 8.2, 8.3, 8.4, 8.5, 8.6, 8.7, 8.8, 8.9, 9.0, 9.1, 9.2, 9.3, 9.4, or 9.5. The temperature during host cell growth may be from about 25°C to 35°C. In some embodiments, the temperature is about 25.0, 25.1, 25.2, 25.3, 25.4, 25.5, 25.6, 25.7, 25.8, 25.9, 26.0, 26.1, 26.2, 26.3, 26.5, 26.6, 26.7, 26.8, 26.9, 27.0, 27.1, 27.2, 27.3, 27.5, 27.6, 27.7, 27.8, 27.9, 28.2, 28.2, 28.3, 28.5, 28.6, 28.7, 28.8, 28.9, 29.0, 29.1, 29.2, 29.3, 29.5, 29.6, 29.7, 29.8, 29.9, 30.0, 30.1, 30.2, 30.3, 30.4, 30.5, 30.6, 30.7, 30.8, 30.9, 31.0, 31.1, 31.2, 31.3, 31.4, 31.5, 31.6, 31.7, 31.8, 31.9, 32.0, 32.1, 32.2, 32.3, 32.4, 32.5, 32.6, 32.7, 32.8, 32.9, 33.0, 33.1, 33.2, 33.3, 33.4, 33.5, 33.6, 33.7, 33.8, 33.9, 34.0, 34.1, 34.2, 34.3, 34.4, 34.5, 34.6, 34.7, 34.8, 34.9 or 35.0°C.

[0054] The following examples are by way of illustration and not by way of limitation.

EXAMPLE 1

Creation of the transformation vector, pMota

[0055] This Example illustrates the preparation of the transformation vector, pMota.

[0056] PCR was used for amplification of the *pdh/adhII* cassette from pLOI295 and for the simultaneous introduction of NdeI and BamHI sites at the 5' and 3' ends, respectively. These sites then allowed for subcloning *pdh/adhII* into the backbone of the pPSBIIKS plasmid, resulting from removal of the *aphX/sacB* selection cassette via NdeI/BamHI dual digestion, yielding pMota. The following primers were used for the above PCR reaction (restriction sites are underlined, induced mutations are in bold: Upstream: 5'-ggAgTAAgCATATgAgTTATACTg - 3' (SEQ ID NO: 10) Downstream: 5' - ggATCTCgACTCTAgAggATCC - 3' (SEQ ID NO: 11). PCR was carried out as follows: Total reaction vol. of 50 µl, 0.36 µg of pLOI295 as template, 4 Units of Vent_R[®] polymerase, a final concentration of 0.5 µM for each primer, 300 µM of each dNTP. The reaction was run on the following program on an Eppendorf[®] Mastercycler[®]: Initial denaturation at 94°C for 2 min, followed by 35 cycles of 10 s denaturation at 94°C, 1 min annealing at 47°C, and 3.7 min extension at 68°C; finally, hold at 4°C.

[0057] All plasmid/PCR product cleanup kits and Taq DNA polymerase were acquired from Qiagen[®]. All restriction enzymes, Vent_R[®] Polymerase and T4 DNA ligase were obtained from New England Biolabs[®]. Plasmid PSBIIKS was obtained from Wim F. J. Vermaas at Arizona State University. Plasmid LOI295, containing the *Z. mobilis pdh* and *adhII* genes, was obtained from Lonnie O. Ingram at the University of Florida.

EXAMPLE 2

Transformation and Screening for Stable Ethanol Production

[0058] This Example illustrates the construction of a stable cyanobacteria line for production of ethanol.

[0059] Following creation of pMota (see Example 1 above), both wild type and NDH-2(-) mutant strains of *Synechocystis* sp. PCC 6803 were transformed and screened sequentially with pPSBIIKS and pMota via the protocol described by Williams (1988). After transformation with pPSBIIKS, serial replating on increasing kanamycin concentrations allowed for the isolation of strains WT_[r] and NDH-2(-)_[r], completely segregated with respect to the *aphX/sacB* selection cassette, as verified by a PCR based

assay. These in turn were grown in liquid seed culture, under the presence of 50 $\mu\text{g ml}^{-1}$ kanamycin, and subsequently transformed with pMota.

[0060] Transformants were screened on BG-11 plates containing 5% sucrose. Screening was performed via serial streaking of single colonies coupled with both an initial PCR based assay used for probing the *psbAII* loci and finally a seed reactor based assay for determination of stability of ethanol generation given the absence of selective pressure. The PCR assay consisted of three PCR reactions per sample, probing for the presence of the (1) WT *psbAII* gene, (2) *aphX/sacB* selection cassette, and (3) *pdh/adhII* ethanol pathway cassette. Each of the three reactions comprising the PCR assay shared a common upstream primer that lies outside of the *psbAII* gene loci, while each reaction is defined by the downstream primer that is specific for each of the three possible genetic constructs. The upstream primer used in all three reactions was: 5'- gTCAgTTCCAATCTgAACATCGA -3' (SEQ ID NO: 12), with the amplicon beginning 48 bp upstream of the *psbAII* start codon. The downstream primer for probing the WT *psbAII* gene: 5'- AATTTgTAACCGTAgtTCTgggAT - 3' (SEQ ID NO: 13), and the resultant amplicon is 749 bp. For probing the *aphX/sacB* selection cassette, downstream primer: 5' - TTggTgATTTTgAACTTTTgCTTgC - 3' (SEQ ID NO: 14), was used, resulting in a 3.1 kb amplicon. The downstream primer for probing the *pdh/adhII* cassette: 5' - TTgCAAgCgATTTCgAgATAAA - 3' (SEQ ID NO: 15), resulting in a 554 bp amplicon. All PCR reactions were formulated as described in the Qiagen® Taq Polymerase Handbook in the section for long PCR products, modified only by the exclusion of any high fidelity polymerase. The PCR assay utilized the following cycling program: Initial denaturation at 94°C for 3 min, followed by 35 cycles of 10 s denaturation at 94°C, 1 min annealing at 48°C, and 3.5 min extension at 68°C; a final 3 min extension at 68°C, hold at 4°C.

[0061] To perform the PCR assay on a given cyanobacterial sample, Whatman® brand FTA® cards were used for rapid preparation of genomic DNA for use as a template in the above PCR reaction. For testing a liquid culture, 5 μl was spotted onto the FTA® card. For testing cultures streaked on solid media, multiple colonies were lifted from the plate, streaked on the inside of a 1.5 ml tube and resuspended in 10 μl of BG-11 via vortexing; 5 μl were then spotted onto the FTA® card, as above. The FTA® protocol for preparation of the

archived DNA from a bacterial source was followed for preparation of the template for the PCR assay.

[0062] The primary seed reactor based assay was used to screen colonies that were shown to be completely segregated for the ethanol cassette for stable ethanol production. Seed reactors were inoculated with multiple colonies from a plate of a given isolate. The cells were grown to an OD_{730} of greater than 0.1, centrifuged at $3220 \times g$ for 6 min at room temperature, and resuspended in a fresh seed reactor at an initial $OD_{730} = 0.025$. This constituted the first experimental reactor in a series of five runs. The reactor was run for five days, at which point the cells were again collected by centrifugation and used to inoculate the second experimental reactor in the series to the above $OD_{730} = 0.025$. Of course, only a subset of the total cell biomass were used for this serial inoculation while the rest were discarded or glycerol stocked. Each day of a particular run, the OD_{730} were recorded, and a 550 μl aliquot were taken for ethanol concentration assay (the 'before' aliquot). The cells were then washed by collection via centrifugation (as above), discarding the supernatant, resuspension by vortexing of the entire pellet in 25 ml of fresh BG-11, and returned to the seed reactor. The OD_{730} were again recorded and another aliquot was taken for ethanol concentration assay (the so called 'after' aliquot). After isolation of a stable ethanol producing isolate, the PCR based assay was applied a final time for complete confirmation.

[0063] Wild Type (WT) *Synechocystis* sp. strain PCC 6803 and the NDH-2(-) mutant, lacking any functional NADH-oxidizing type II dehydrogenase, were obtained from Wim F. J. Vermaas at Arizona State University. C600 *E. coli* were obtained from the laboratory of Monto Kumagai, while at the University of Hawaii at Manoa.

[0064] *E. coli* was cultured on standard LB formulation in both liquid and solid media. Ampicillin and kanamycin were supplemented to the LB media plates at concentrations of $100 \mu g ml^{-1}$ and $50 \mu g ml^{-1}$, respectively.

[0065] All *Synechocystis* sp. PCC 6803 was cultured on standard BG-11 media plates, with or without the addition of (final concentration): 5 mM glucose, 5% sucrose, and/or either $5 \mu g ml^{-1}$, $25 \mu g ml^{-1}$, or $50 \mu g ml^{-1}$ kanamycin. Plates containing *Synechocystis* sp. PCC 6803 were incubated at $30^{\circ}C$ under ~ 100 microeinsteins $m^{-2} s^{-1}$. All *Synechocystis*

liquid cultures were grown in standard BG-11, with the addition of 50 $\mu\text{g ml}^{-1}$ kanamycin when appropriate.

EXAMPLE 3

Batch Growth Experiments

[0066] This Example illustrates batch growth experiments for productivity and stability studies.

[0067] A parallel batch culture system (six 100mL bioreactors) was established to grow the ethanol-producing *Synechocystis* strains developed. Standard BG-11 liquid media was used for the all the experiments. Agitation was set at 400 rpm. Lighting intensity was 200 microeinsteins on the formost face of the bioreactors. Compressed air was sparged to provide CO₂ and remove the oxygen produced by *Synechocystis*. Semi batch operation mode was used to test the ethanol production. The total cell growth period was 20 days. The seed cultures were started from a plate. Exponentially growing cells from a seed culture were inoculated into the reactors at OD₇₃₀ = 0.025. Batch cultures were conducted for about 4 days, and then terminated. The cells were spun down by centrifugation, resuspended in a reduced volume, and an aliquot was used to inoculate a bioreactor with fresh media.

EXAMPLE 4

Ethanol Concentration Assay

[0068] This Example illustrates determination of the ethanol concentration in a liquid culture.

[0069] For determination of ethanol concentration of a liquid culture, a 550 μl aliquot of the culture was taken, spun down at 12,100 x g for 5 min, and 500 μl (or other appropriate vol.) of the supernatant was placed in a fresh 1.5 ml tube and stored at -20°C until performing the assay. Given the linear range of the spectrophotometer and the sensitivity of the ethanol assay, dilution of the sample (up to 20 fold) was occasionally required. In this case, an appropriate volume of BG-11 was first added to the fresh 1.5 ml tube, to which the required vol. of clarified supernatant was added. This solution was used directly in the ethanol assay. Upon removal from -20°C and immediately before performing

the assay, the samples were spun down a second time at 12,100 x g for 5 min, also assisting in sample thawing.

[0070] The Boehringer Mannheim/r-biopharm[®] enzymatic ethanol detection kit was used for ethanol concentration determination. Briefly, this assay exploits the action of alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase in a phosphate-buffered solution of the NAD⁺ cofactor, which upon the addition of ethanol causes a conversion of NAD⁺ to NADH. Concentration of NADH is determined by light absorbance at 340 nm (A_{340}) and is then used to determine ethanol concentration. The assay was performed as given in the instructions, with the following modifications. As given under point 4 on the instruction sheet, the maximal sample volume ($v = 0.5$ ml), for maximum sensitivity, was used for the assay. Finally, all volumes in the assay (including the above $v = 0.5$ ml) were quartered. This allowed for reagent conservation, and the ability to retain a majority of the sample aliquot's volume, in case repetition was required. Thus, the sample volume used was actually $v = 0.125$ ml, in 0.75 ml of reaction mixture 2, and with the later addition of 12.5 μ l of (ADH) suspension 3. This conserved ratio volumetric reduction was determined to have no effect on the assay as performed. BG-11 was used as a blank.

EXAMPLE 5

Autotrophic Photobioreactor Fermentation

[0071] This Example illustrates production of ethanol using autotrophic photobioreactor fermentation.

[0072] Liquid seed cultures were grown at 24°C in an incubating shaker (Innova 4230 benchtop, New Brunswick) with a light which possesses a maximal surface flux of ~200 microeinsteins $m^{-2} s^{-1}$ on the seed reactor face, agitated via magnetic stir bar, and sparged with compressed air at a rate of approximately 0.5 L/min. Primary seed cultures consisted of a total volume of 25 ml in a standard 100 ml Pyrex[™] media bottle, with a two Pasteur pipettes serving as the sparge and the offgas tubes. Upstream of the sparge tube was a Whatman[®] PFTE 0.1 μ m filter and the offgas tube was loosely capped with aluminum foil. The secondary seed culture used for photobioreactor inoculation was identical to the primary seed culture, except the culture volume was 300 ml grown in a standard 1 L Pyrex[™] media bottle, the sparging pipette was replaced with a level C porosity diffuser, pore size 25-50

µm, from Ace Glass Inc. Lights used were 40W cool white fluorescent tubes. Cell growth in liquid culture was monitored by determination of optical density at 730 nm (OD_{730}) using a ThermoSpectronic® Genesys® 10-S Spectrophotometer. Plastibrand® UltraVette® 1.5 ml disposable cuvettes were used.

[0073] Exponentially growing seed culture from primary seed reactors were used to inoculate a secondary seed reactor, which was then grown to an $OD_{730} > 0.31$ and immediately used for inoculation of the autotrophic photobioractor. For inoculation, the OD_{730} was taken and used for determination of the seed culture volume required for initial bioreactor $OD_{730} = 0.02$ (total fermentation vol. was 3.1 L). Cells from this determined volume of seed culture were harvested via centrifugation and resuspended in 50 ml of fresh BG-11 by vortexing. After inoculation of the bioreactor with this 50 ml seed, an additional 50 ml of BG-11 was used for flushing of the inoculation tube.

[0074] A 7.5 L BIOFLO® 110 bioreactor system (New Brunswick Scientific, Edison, NJ) was used for the experiments. The 7.5 L BIOFLO® 110 bioreactor system includes controllers for temperature, pH and dissolved oxygen concentration (DO) adjustment. The *Synechocystis* cultivation process was monitored and controlled automatically by a Pentium® II (233 MHz, Windows 98) computer equipped with an interface board PCI-MIO-16E-10 (National Instruments Corp., Austin, TX). The data acquisition program was written in LabVIEW7.1 (National Instruments Corp., Austin, TX). The data from the BIOFLO® 110 bioreactor system, including pH, agitation, temperature and DO were acquired through the computer interface board. Six General Electric® 26W, F26DBX/SPX41/4P bulbs were arrayed around the reactor to give a maximal surface flux of ~ 1000 microeinsteins $m^{-2} s^{-1}$ on the reactor surface. The reactor was maintained at 27-29°C throughout the fermentation via an external fan circulating air around the lighting system. The reactor was sparged with air at a rate of 1 L/min, the pH was controlled via CO₂ injection with the setpoint at 8.5, and the agitation turbine was set to 300 rpm. The condenser on the offgas port was chilled to 8°C via a thermo circulating water bath (C10-K20, Haake, Berlin, Germany). Fermentation was maintained for eight days, with sampling every eight hours. For sampling, ~ 15 ml was drawn from the reactor via the sampling port (to clear the harvesting downtube), discarded, and a second ~ 15 ml aliquot, the sample, was drawn. From each sample OD_{730} was recorded and an aliquot was taken for the ethanol

concentration assay. The ethanol concentration was 12 mM after 112 hours of fermentation (Figure 2). After 5 days of fermentation, an ethanol concentration of 13 mM was reached (Figure 2).

[0075] The methods, procedures, and devices described herein are presently representative of preferred embodiments and are exemplary and are not intended as limitations on the scope of the invention. All numbers expressing quantities of ingredients, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters set forth in the specification and attached claims are approximations that can vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should be construed in light of the number of significant digits and ordinary rounding approaches.

[0076] It will be apparent to one skilled in the art that varying substitutions and modifications can be made to the invention disclosed herein.

[0077] Those skilled in the art recognize that the aspects and embodiments of the invention set forth herein can be practiced separate from each other or in conjunction with each other. Therefore, combinations of separate embodiments are within the scope of the invention as disclosed herein.

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[0092] The invention illustratively described herein suitably can be practiced in the absence of any element or elements, limitation or limitations that is not specifically disclosed herein. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions indicates the exclusion of equivalents of the features shown and described or portions thereof. It is recognized that various modifications are possible within the scope of the invention disclosed.

The claims defining the invention are as follows:

1. A Synechocystis host cell containing:
 - an expression vector comprising:
 - a nucleic acid construct including a light responsive promoter operably
5 linked to a sequence encoding a pyruvate decarboxylase (pdc) enzyme, and a
sequence encoding an alcohol dehydrogenase (adh) enzyme.
2. The Synechocystis host cell of claim 1, wherein the light responsive promoter
is the psbAII promoter.
3. The Synechocystis host cell of claim 1, wherein the light responsive promoter
10 is the nirA promoter.
4. The Synechocystis host cell of any of the claims 1 to 3, wherein the sequence
encoding a pdc enzyme comprises SEQ. ID NO:3 or a gene sequence that encodes pdc
and that is capable of expression in cyanobacteria.
5. A Synechocystis host cell containing:
 - an expression vector comprising:
 - a psbAII promoter or a nirA promoter operably linked to a sequence
15 encoding a pyruvate decarboxylase (pdc) enzyme, and a sequence encoding
an alcohol dehydrogenase (adh) enzyme.
6. The Synechocystis host cell of any of the claims 1 to 4, wherein the
20 expression vector is integrated into the host cell chromosome.
7. The Synechocystis host cell of any of the claims 1 to 6, wherein the host cell
is capable of producing ethanol in recoverable quantities greater than 10 mM ethanol after
a 5 day fermentation.
8. The Synechocystis host cell according to the previous claim, wherein the host
25 cell is capable of producing the 10 mM ethanol after the 5 day fermentation if subjected
to a light flux of around $1000 \mu\text{E m}^{-2} \text{s}^{-1}$.
9. A method of producing ethanol, comprising: culturing in a culture medium
Synechocystis host cells, the host cells containing a construct comprising a light
responsive promoter operably linked to DNA fragments encoding pdc and adh enzymes.
- 30 10. The method according to claim 9, further comprising accumulating ethanol in
the culture medium in an amount greater than 10 mM ethanol after a 5 day fermentation.
11. The method of any of the claims 9 or 10, wherein the construct is integrated
into the cyanobacteria chromosome.

12. The method of any of the claims 9 to 11, wherein the host cells are subjected to a light flux of around $1000 \mu\text{E m}^{-2} \text{s}^{-1}$.

Dated 24 March, 2011

University of Hawaii

Patent Attorneys for the Applicant/Nominated Person

SPRUSON & FERGUSON

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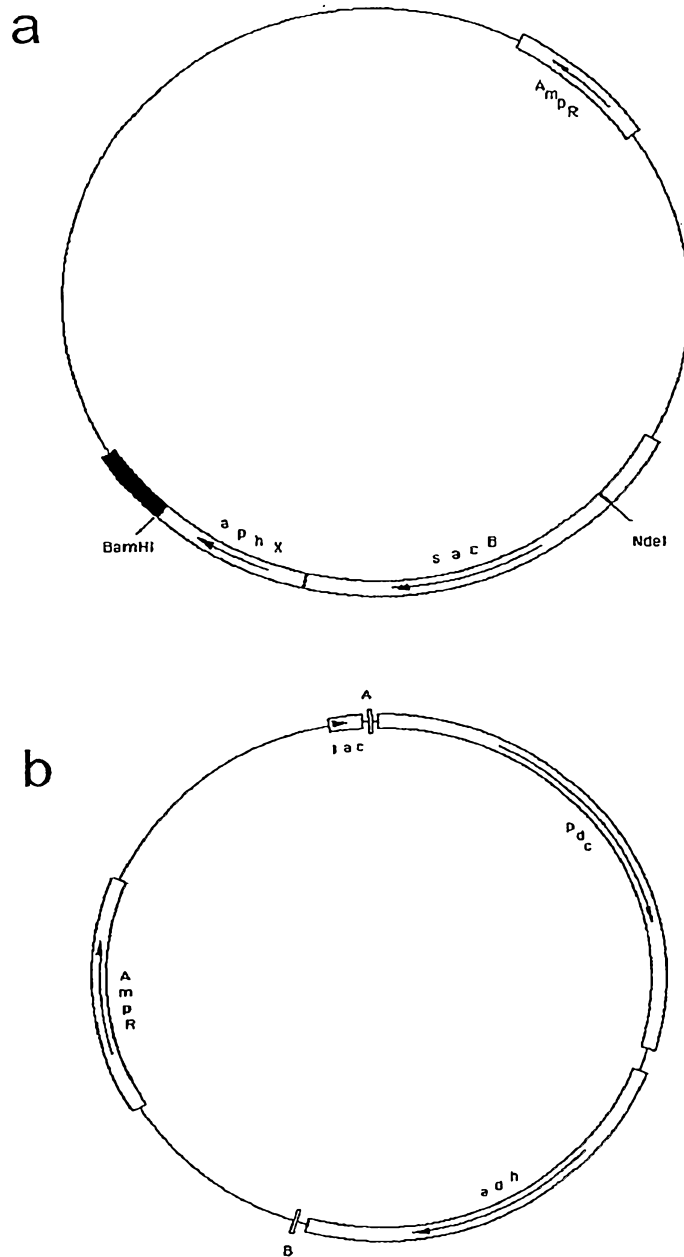


FIGURE 1

2/2

Ethanol Concentration vs. Time in Bioflo Reactor

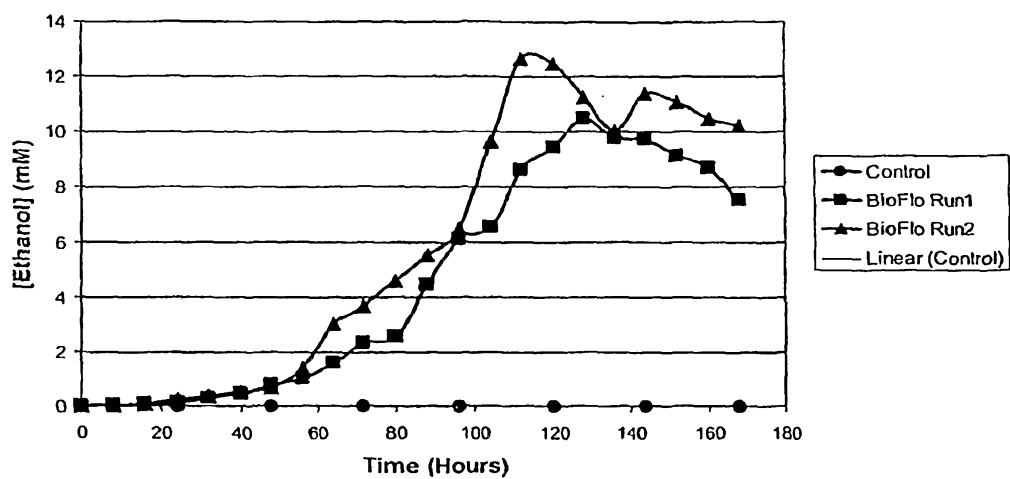


FIGURE 2

2007207635 12 Sep 2008

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 Pro Ala Lys Ile Asp His Val Ile Lys Thr Ala Leu Arg Glu Lys Lys

145	150	155	160
Pro Val Tyr Leu Glu Ile Ala Cys Asn Ile Ala Ser Met Pro Cys Ala			
	165	170	175
Ala Pro Gly Pro Ala Ser Ala Leu Phe Asn Asp Glu Ala Ser Asp Glu			
	180	185	190
Ala Ser Leu Asn Ala Ala Val Glu Glu Thr Leu Lys Phe Ile Ala Asn			
	195	200	205
Arg Asp Lys Val Ala Val Leu Val Gly Ser Lys Leu Arg Ala Ala Gly			
	210	215	220
Ala Glu Glu Ala Ala Val Lys Phe Ala Asp Ala Leu Gly Gly Ala Val			
	225	230	235
Ala Thr Met Ala Ala Ala Lys Ser Phe Phe Pro Glu Glu Asn Pro His			
	245	250	255
Tyr Ile Gly Thr Ser Trp Gly Glu Val Ser Tyr Pro Gly Val Glu Lys			
	260	265	270
Thr Met Lys Glu Ala Asp Ala Val Ile Ala Leu Ala Pro Val Phe Asn			
	275	280	285
Asp Tyr Ser Thr Thr Gly Trp Thr Asp Ile Pro Asp Pro Lys Lys Leu			
	290	295	300
Val Leu Ala Glu Pro Arg Ser Val Val Val Asn Gly Val Arg Phe Pro			
	305	310	315
Ser Val His Leu Lys Asp Tyr Leu Thr Arg Leu Ala Gln Lys Val Ser			
	325	330	335
Lys Lys Thr Gly Ala Leu Asp Phe Phe Lys Ser Leu Asn Ala Gly Glu			
	340	345	350
Leu Lys Lys Ala Ala Pro Ala Asp Pro Ser Ala Pro Leu Val Asn Ala			
	355	360	365
Glu Ile Ala Arg Gln Val Glu Ala Leu Leu Thr Pro Asn Thr Thr Val			
	370	375	380
Ile Ala Glu Thr Gly Asp Ser Trp Phe Asn Ala Gln Arg Met Lys Leu			
	385	390	395
Pro Asn Gly Ala Arg Val Glu Tyr Glu Met Gln Trp Gly His Ile Gly			
	405	410	415
Trp Ser Val Pro Ala Ala Phe Gly Tyr Ala Val Gly Ala Pro Glu Arg			
	420	425	430
Arg Asn Ile Leu Met Val Gly Asp Gly Ser Phe Gln Leu Thr Ala Gln			
	435	440	445
Glu Val Ala Gln Met Val Arg Leu Lys Leu Pro Val Ile Ile Phe Leu			
	450	455	460
Ile Asn Asn Tyr Gly Tyr Thr Ile Glu Val Met Ile His Asp Gly Pro			
	465	470	475
Tyr Asn Asn Ile Lys Asn Trp Asp Tyr Ala Gly Leu Met Glu Val Phe			
	485	490	495
Asn Gly Asn Gly Gly Tyr Asp Ser Gly Ala Gly Lys Gly Leu Lys Ala			
	500	505	510
Lys Thr Gly Gly Glu Leu Ala Glu Ala Ile Lys Val Ala Leu Ala Asn			
	515	520	525
Thr Asp Gly Pro Thr Leu Ile Glu Cys Phe Ile Gly Arg Glu Asp Cys			
	530	535	540
Thr Glu Glu Leu Val Lys Trp Gly Lys Arg Val Ala Ala Ala Asn Ser			
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Arg Lys Pro Val Asn Lys Leu Leu			
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<212> PRT

<213> Zymobacter palmae

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			20					25					30		
Asp	Gln	Leu	Leu	Leu	Asn	Lys	Asp	Met	Glu	Gln	Val	Tyr	Cys	Cys	Asn
		35					40					45			
Glu	Leu	Asn	Cys	Gly	Phe	Ser	Ala	Glu	Gly	Tyr	Ala	Arg	Ala	Arg	Gly
	50					55					60				
Ala	Ala	Ala	Ala	Ile	Val	Thr	Phe	Ser	Val	Gly	Ala	Ile	Ser	Ala	Met
65					70					75					80
Asn	Ala	Ile	Gly	Gly	Ala	Tyr	Ala	Glu	Asn	Leu	Pro	Val	Ile	Leu	Ile
				85					90					95	
Ser	Gly	Ser	Pro	Asn	Thr	Asn	Asp	Tyr	Gly	Thr	Gly	His	Ile	Leu	His
			100					105					110		
His	Thr	Ile	Gly	Thr	Thr	Asp	Tyr	Asn	Tyr	Gln	Leu	Glu	Met	Val	Lys
		115					120					125			
His	Val	Thr	Cys	Ala	Arg	Glu	Ser	Ile	Val	Ser	Ala	Glu	Glu	Ala	Pro
	130					135					140				
Ala	Lys	Ile	Asp	His	Val	Ile	Arg	Thr	Ala	Leu	Arg	Glu	Arg	Lys	Pro
145					150					155					160
Ala	Tyr	Leu	Glu	Ile	Ala	Cys	Asn	Val	Ala	Gly	Ala	Glu	Cys	Val	Arg
				165					170					175	
Pro	Gly	Pro	Ile	Asn	Ser	Leu	Leu	Arg	Glu	Leu	Glu	Val	Asp	Gln	Thr
			180					185					190		
Ser	Val	Thr	Ala	Ala	Val	Asp	Ala	Ala	Val	Glu	Trp	Leu	Gln	Asp	Arg
		195					200					205			
Gln	Asn	Val	Val	Met	Leu	Val	Gly	Ser	Lys	Leu	Arg	Ala	Ala	Ala	Ala
	210					215					220				
Glu	Lys	Gln	Ala	Val	Ala	Leu	Ala	Asp	Arg	Leu	Gly	Cys	Ala	Val	Thr
225					230					235					240
Ile	Met	Ala	Ala	Glu	Lys	Gly	Phe	Phe	Pro	Glu	Asp	His	Pro	Asn	Phe
				245					250					255	
Arg	Gly	Leu	Tyr	Trp	Gly	Glu	Val	Ser	Glu	Gly	Ala	Gln	Glu	Leu	
		260						265				270			
Val	Glu	Asn	Ala	Asp	Ala	Ile	Leu	Cys	Leu	Ala	Pro	Val	Phe	Asn	Asp
		275					280					285			
Tyr	Ala	Thr	Val	Gly	Trp	Asn	Ser	Trp	Pro	Lys	Gly	Asp	Asn	Val	Met
	290					295					300				
Val	Met	Asp	Thr	Asp	Arg	Val	Thr	Phe	Ala	Gly	Gln	Ser	Phe	Glu	Gly
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Leu	Ser	Leu	Ser	Thr	Phe	Ala	Ala	Ala	Leu	Ala	Glu	Lys	Ala	Pro	Ser
				325					330					335	
Arg	Pro	Ala	Thr	Thr	Gln	Gly	Thr	Gln	Ala	Pro	Val	Leu	Gly	Ile	Glu
			340					345					350		
Ala	Ala	Glu	Pro	Asn	Ala	Pro	Leu	Thr	Asn	Asp	Glu	Met	Thr	Arg	Gln
		355					360					365			
Ile	Gln	Ser	Leu	Ile	Thr	Ser	Asp	Thr	Thr	Leu	Thr	Ala	Glu	Thr	Gly
	370					375					380				
Asp	Ser	Trp	Phe	Asn	Ala	Ser	Arg	Met	Pro	Ile	Pro	Gly	Gly	Ala	Arg
385					390					395					400
Val	Glu	Leu	Glu	Met	Gln	Trp	Gly	His	Ile	Gly	Trp	Ser	Val	Pro	Ser
				405					410					415	
Ala	Phe	Gly	Asn	Ala	Val	Gly	Ser	Pro	Glu	Arg	Arg	His	Ile	Met	Met

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			420					425					430				
Val	Gly	Asp	Gly	Ser	Phe	Gln	Leu	Thr	Ala	Gln	Glu	Val	Ala	Gln	Met		
			435					440					445				
Ile	Arg	Tyr	Glu	Ile	Pro	Val	Ile	Ile	Phe	Leu	Ile	Asn	Asn	Arg	Gly		
			450					455					460				
Tyr	Val	Ile	Glu	Ile	Ala	Ile	His	Asp	Gly	Pro	Tyr	Asn	Tyr	Ile	Lys		
465					470						475					480	
Asn	Trp	Asn	Tyr	Ala	Gly	Leu	Ile	Asp	Val	Phe	Asn	Asp	Glu	Asp	Gly		
			485					490					495				
His	Gly	Leu	Gly	Leu	Lys	Ala	Ser	Thr	Gly	Ala	Glu	Leu	Glu	Gly	Ala		
			500					505					510				
Ile	Lys	Lys	Ala	Leu	Asp	Asn	Arg	Arg	Gly	Pro	Thr	Leu	Ile	Glu	Cys		
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Asn	Ile	Ala	Gln	Asp	Asp	Cys	Thr	Glu	Thr	Leu	Ile	Ala	Trp	Gly	Lys		
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			20					25					30			
Asn	Ala	Leu	Ile	Val	Ser	Asp	Ala	Phe	Met	Asn	Lys	Ser	Gly	Val	Val	
		35					40						45			
Lys	Gln	Val	Ala	Asp	Leu	Leu	Lys	Ala	Gln	Gly	Ile	Asn	Ser	Ala	Val	
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Tyr	Asp	Gly	Val	Met	Pro	Asn	Pro	Thr	Val	Thr	Ala	Val	Leu	Glu	Gly	
65					70					75					80	
Leu	Lys	Ile	Leu	Lys	Asp	Asn	Asn	Ser	Asp	Phe	Val	Ile	Ser	Leu	Gly	
				85					90					95		
Gly	Gly	Ser	Pro	His	Asp	Cys	Ala	Lys	Ala	Ile	Ala	Leu	Val	Ala	Thr	
			100					105					110			
Asn	Gly	Gly	Glu	Val	Lys	Asp	Tyr	Glu	Gly	Ile	Asp	Lys	Ser	Lys	Lys	
		115					120					125				
Pro	Ala	Leu	Pro	Leu	Met	Ser	Ile	Asn	Thr	Thr	Ala	Gly	Thr	Ala	Ser	
	130					135					140					
Glu	Met	Thr	Arg	Phe	Cys	Ile	Ile	Thr	Asp	Glu	Val	Arg	His	Val	Lys	
145					150					155					160	
Met	Ala	Ile	Val	Asp	Arg	His	Val	Thr	Pro	Met	Val	Ser	Val	Asn	Asp	
				165					170					175		
Pro	Leu	Leu	Met	Val	Gly	Met	Pro	Lys	Gly	Leu	Thr	Ala	Ala	Thr	Gly	
			180					185					190			
Met	Asp	Ala	Leu	Thr	His	Ala	Phe	Glu	Ala	Tyr	Ser	Ser	Thr	Ala	Ala	
	195						200					205				
Thr	Pro	Ile	Thr	Asp	Ala	Cys	Ala	Leu	Lys	Ala	Ala	Ser	Met	Ile	Ala	
	210					215					220					
Lys	Asn	Leu	Lys	Thr	Ala	Cys	Asp	Asn	Gly	Lys	Asp	Met	Pro	Ala	Arg	
225					230					235					240	
Glu	Ala	Met	Ala	Tyr	Ala	Gln	Phe	Leu	Ala	Gly	Met	Ala	Phe	Asn	Asn	
				245					250					255		

Ala	Ser	Leu	Gly	Tyr	Val	His	Ala	Met	Ala	His	Gln	Leu	Gly	Gly	Tyr
			260					265					270		
Tyr	Asn	Leu	Pro	His	Gly	Val	Cys	Asn	Ala	Val	Leu	Leu	Pro	His	Val
		275					280					285			
Leu	Ala	Tyr	Asn	Ala	Ser	Val	Val	Ala	Gly	Arg	Leu	Lys	Asp	Val	Gly
		290				295						300			
Val	Ala	Met	Gly	Leu	Asp	Ile	Ala	Asn	Leu	Gly	Asp	Lys	Glu	Gly	Ala
		305			310					315					320
Glu	Ala	Thr	Ile	Gln	Ala	Val	Arg	Asp	Leu	Ala	Ala	Ser	Ile	Gly	Ile
				325					330					335	
Pro	Ala	Asn	Leu	Thr	Glu	Leu	Gly	Ala	Lys	Lys	Glu	Asp	Val	Pro	Leu
			340					345					350		
Leu	Ala	Asp	His	Ala	Leu	Lys	Asp	Ala	Cys	Ala	Leu	Thr	Asn	Pro	Arg
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Gln	Gly	Asp	Gln	Lys	Glu	Val	Glu	Glu	Leu	Phe	Leu	Ser	Ala	Phe	
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