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(54) **Title**: PRODUCTION OF 1,2-PROPANEDIOL IN CYANOBACTERIA

(57) **Abstract**: Cyanobacterial host cells are modified to produce 1,2-propanediol.

TITLE

PRODUCTION OF 1,2-PROPANEDIOL IN CYANOBACTERIA

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 61/715,435, filed October 18, 2012, the disclosure of which is incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] Not Applicable.

REFERENCE TO SEQUENCE LISTING

[0003] This application contains a sequence listing submitted by EFS-Web, thereby satisfying the requirements of 37 C.F.R. §§ 1.821-1.825. The Sequence Listing, created on October 10, 2013, is named "1_2-Propanediol_SEQ_LIST_US_ST25", and is 160 KB in size.

FIELD OF THE INVENTION

[0004] The present invention relates to cyanobacterial host cells which are modified to produce 1,2-propanediol.

BACKGROUND OF THE INVENTION

[0005] Cyanobacteria (also known as "blue-green algae") are small, mainly aquatic, prokaryotic cells that have the ability to perform oxygenic photosynthesis and make biomass and organic compounds from the input of light, nutrients, and CO_2. Cyanobacteria can be genetically enhanced to produce valuable products, such as biofuels, pharmaceuticals, nutrients, carotenoids, etc. For example, the transformation of the cyanobacterial genus *Synechococcus* with genes that encode specific enzymes that can produce ethanol for biofuel production has been described (U.S. Patent Nos. 6,699,696 and 6,306,639, both to Woods et al.). The transformation of the cyanobacterial genus *Synechocystis* is described, for example, in PCT/EP2009/000892 and in PCT/EP2009/060526.

[0006] 1,2-propanediol (also termed propylene glycol, propane-1,2-diol, 1,2-dihydroxypropane, and methylethylene glycol) is a three-carbon diol that is chiral with an asymmetric carbon at the 2-position. 1,2-propanediol is a colorless, viscous, water-miscible liquid. 1,2-propanediol, as a racemic mixture, is used in many industrial applications, including as a solvent in pharmaceuticals, as a de-icer for aircraft wings, and in deodorant sticks. 1,2-propanediol also has a relatively low human toxicity. The current commonly used pathway of production of 1,2-propanediol is through propylene from crude oil.

[0007] A metabolically engineered biosynthetic pathway for production of 1,2-propanediol in *E. coli* has been elucidated (FIG. 2; Altaras, N.E. et al.,1999, Applied and Environ. Biol. 65:1180-1185; and Altaras, N.E. et al., (2000) Biotech. Progress 16:940-946). The pathway can produce either S-1,2-propanediol or R-1,2-propanediol, depending on the chosen enzymes.

[0008] Current methods of producing 1,2-propanediol require the input of an organic carbon source, such as fossil fuel or sugar. What is needed is a method of producing these compounds from CO_2 as the input carbon source, rather than from fossil fuels or from other organic starting materials.

SUMMARY OF THE INVENTION

[0009] In an aspect of the invention, a genetically enhanced nucleic acid sequence for the production of 1,2-propanediol in cyanobacteria is provided, having at least one promoter capable of regulating gene expression in cyanobacteria; and the following genes: *gld*A, *fuc*O, *mgs*A, and optionally *yqh*D. The nucleic acid sequence can be capable of replicating in a cyanobacterial cell. At least one of the genes can be located on an exogenously or endogenously derived plasmid, or on the cyanobacterial chromosome. The promoter can be, for example, Psrp (such as SEQ ID NO: 1), $PnblA_{7120}$ (such as SEQ ID NO: 2), $PrbcL_{6803}$ or derivatives (such as SEQ ID NO: 3, 4, 5, or 6), $PsmtA_{7002}$ (such as SEQ ID NO: 7), ziaR-$PziaA_{6803}$ (such as SEQ ID NO: 8), or PpetJ (such as SEQ ID NO: 9). The *gld*A gene can have at least 98% identity to SEQ ID NO: 11. The GldA polypeptide can have at least 98% identity to SEQ ID NO: 12. The *fuc*O gene can have at least 98% identity to SEQ ID NO: 13. The FucO polypeptide can have at least 98% identity to SEQ ID NO: 14. The *mgs*A gene can have at least 98% identity to SEQ ID NO: 15. The MgsA polypeptide can have at least 98% identity to SEQ ID NO: 16. The *yqh*D gene can have at least 98% identity to SEQ ID NO: 17. The YqhD polypeptide can have at least 98%

identity to SEQ ID NO: 18. In another aspect of the invention, a genetically modified cyanobacterial cell having a heterologous nucleic acid sequence of any one of the above sequences is provided, where the cell can produce 1,2-propanediol.

[0010] In another aspect of the invention, a genetically enhanced cyanobacterial cell is provided, having a *gld*A gene, a *fuc*O gene, an *mgs*A gene, and optionally a *yqh*D gene, wherein the cyanobacterial cell produces 1,2-propanediol. The genes can be located together under the control of one promoter, or at least one of the genes can be present in another location in the cell. The cyanobacterium can be, for example, *Synechocystis* sp. PCC 6803, or *Synechococcus* sp. PCC 7002.

[0011] In yet another aspect of the invention, a method of producing 1,2-propanediol in a cyanobacterial cell is provided, by introducing a nucleic acid sequence having a gene encoding a GldA enzyme, a gene encoding a FucO enzyme, a gene encoding an MgsA enzyme, and optionally a gene encoding a YqhD enzyme into a cyanobacterial cell; and then culturing the cyanobacterial cell under conditions to produce 1,2-propanediol.

In another aspect of the invention, a method of producing 1,2-propanediol in a cyanobacterial cell is provided, by transforming the cell with an *mgsA* gene, a gene encoding an enzyme capable of converting methylglyoxal to lactaldehyde, and a gene encoding an enzyme capable of converting lactaldehyde to 1,2-propanediol. The gene encoding the enzyme capable of converting methylglyoxal to lactaldehyde can be selected, for example, from GldA, SynADH, and SynAKR. The gene encoding the enzyme capable of converting lactaldehyde to 1,2-propanediol can be selected, for example, from FucO and GldA. Each of the inserted genes can be under the control of separate promoters, or they can be under the control of one promoter. In a further embodiment, the SynAKR gene has a sequence of SEQ ID NO: 19. In another embodiment, the SynAKR protein is SEQ ID NO: 20. In an embodiment, the SynADH gene has a sequence of SEQ ID NO: 21. In another embodiment, the SynADH protein is SEQ ID NO: 22.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] FIG. 1 is a diagram of one biosynthetic pathway used to produce 1,2-propanediol from the central carbon metabolites pyruvate and glycerone phosphate (DHAP). These metabolites can be produced through photosynthetic and gluconeogenic pathways using CO_2 as the input carbon source in cyanobacteria. As shown in the figure, the pathway involves the intermediate

compounds glycerone phosphate, methylglyoxal, acetol, and 2-hydroxypropionaldehyde ("lactaldehyde").

[0013] FIG. 2 is another diagram of a biosynthetic pathway that can be used to produce 1,2-propanediol. In this pathway, either the S- or the R- form of 1,2-propanediol can be formed. The pathway diagram is taken from Alteras et al., 1999.

[0014] FIG. 3 is another diagram of a biosynthetic pathway that can be used to produce 1,2-propanediol. Three possible alternative enzymes for the conversion of methylglyoxal to lactaldehyde are shown. Two possible alternative enzymes for the conversion of lactaldehyde to 1,2-propanediol are also shown. Further, the role of NADP and NADPH are also indicated.

[0015] FIG. 4 is a map of two gene cassettes (#728, #729) for transformation to cyanobacteria. Both of the gene cassettes encode the enzymes MgsA and synADH. The promoters, terminators, and relevant restriction sites are indicated.

[0016] FIG. 5 is a map of two gene cassettes (#747, #748) for transformation to cyanobacteria. Both of the gene cassettes encode the enzymes GldA, MgsA, and synADH deg, but different promoters are used to control the expression of each of the genes. The promoters, terminators, and relevant restriction sites are indicated.

[0017] FIG. 6 is a map of two gene cassettes (#749, #750) for transformation to cyanobacteria. Both of the gene cassettes encode the enzymes FucO, MgsA, and synADH deg., but different promoters are used to control the expression of each of the genes, as indicated. The terminator sequences and relevant restriction sites are also indicated.

[0018] FIG. 7 is a map of two gene cassettes (#767, #768) for transformation to cyanobacteria. Both of the gene cassettes encode the enzymes FucO, MgsA, and Syn7002AKR, but different promoters are used to control the expression of each of the genes, as indicated. The terminator sequences and relevant restriction sites are also indicated.

[0019] FIG. 8 is a map of two gene cassettes (#769, #770) for transformation to cyanobacteria. Both of the gene cassettes encode the enzymes GldA, MgsA, and Syn7002AKR, but different promoters are used to control the expression of each of the genes, as indicated. The terminator sequences and relevant restriction sites are also indicated.

[0020] FIG. 9 is a bar graph showing the *in vitro* MgsA and GldA/SynADH activity in *Synechocystis* PCC 6803 transformed with plasmids containing the #747 or #748 cassette, as indicated. The enzyme activity is measured in nmol per mg protein per minute.

[0021] FIG. 10 is a bar graph showing the GldA/SynADH *in vitro* activity in *Synechocystis* PCC 6803 transformed with plasmids containing the #747 or #748 cassette, as indicated.

[0022] FIG. 11 is a bar graph showing the production of hydroxyacetone and 1,2-propanediol in *Synechocystis* PCC 6803 transformed with plasmids containing the #749 or #750 cassette, as indicated.

[0023] FIG. 12 is a bar graph showing the *in vitro* activity of the enzyme MgsA for *Synechocystis* PCC 6803 cells transformed with plasmids containing either the #767-#768 constructs (FucO, MgsA, AKR) at day 2, 5, and 7, or the #769 construct (GldA, MgsA, AKR) at days 5 and 7. The enzyme activity is measured in nmol per mg protein per minute.

[0024] FIG. 13 is a bar graph showing the *in vitro* activity of 1) a combination of MgsA + SynAKR + FucO; 2) a combination of SynAKR + FucO; or 3) FucO activity alone. *Synechocystis* PCC 6803 cells were transformed with plasmids containing the #769 construct (GldA, MgsA, AKR). The cultures were measured at day 5 and day 7. The enzyme activity is measured in nmol per mg protein per minute.

[0025] FIG. 14 is a bar graph showing the *in vitro* activity of 1) a combination of MgsA + SynAKR + FucO; 2) a combination of SynAKR + FucO; or 3) FucO activity alone. *Synechocystis* PCC 6803 cells were transformed with plasmids containing the #767 or #768 construct (FucO, MgsA, and AKR) The cultures were measured at day 2, day 5, and day 7. The enzyme activity is measured in nmol per mg protein per minute.

[0026] FIG. 15 is a linear diagram of the genes and relevant features in the broad host range RSF1010-derivative plasmid pSL1211, which was used as the basis for the expression vectors described herein. Relevant restriction sites and terminator regions (TT) are indicated.

[0027] FIG. 16 is a linearized map of the pSL1211-derived plasmid (“pABb”) that was used as the framework plasmid for the insertion of the polycistronic propanediol genes described in Examples 10-12. The promoter, terminator (TT), and ribosomal binding site (RBS) are indicated.

[0028] FIG. 17 is a linearized map of the “GYFM” fragment that was inserted into plasmid pABb (FIG. 16) to create pAB1025 in order to produce 1,2-propanediol as described in Examples 10-12. The relevant restriction sites used for cloning are indicated.

[0029] FIG. 18 is a graph confirming the production of 1,2-propanediol in *Synechococcus* sp. PCC 7002. The graph represents a chromatographic trace of a 20 X concentrated

methanol/phosphate extract from a culture of PCC 7002 harboring the plasmid pAB1025. The trace was produced from a separation of 1,2 propanediol using gas chromatography and peaks were identified using mass spectroscopy. The peak at retention time 4.9 minutes was identified as 1,2 propanediol. This peak was not present in wild type *Synechococcus* sp. PCC 7002.

[0030] FIG. 19 is a graph confirming the production of 1,2-propanediol in *Synechocystis* sp. PCC 6803. The graph represents a chromatographic trace of a 15X concentrated methanol/phosphate extract from *Synechocystis* PCC 6803 harboring the plasmid pAB1025. The trace was produced from a separation of 1,2-propanediol using gas chromatography. The peaks were identified using mass spectroscopy. The peak having a retention time of 4.9 minutes was identified as 1,2-propanediol. This peak was not present in wild type *Synechocystis* sp. PCC 6803.

DETAILED DESCRIPTION

[0031] Cyanobacterial host cells can be genetically enhanced in order to produce various valuable chemical products, such as 1,2-propanediol. In an embodiment, genes involved in the biosynthetic pathways for 1,2-propanediol production can be transferred to a cyanobacterial host cell. The inserted heterologous genes can be present on extrachromosomal plasmids, or they can be present on the cyanobacterial chromosome. The cyanobacterial cells are then cultured following general cyanobacterial methods, and the propanediol is removed at the appropriate time. The production of 1,2-propanediol in cyanobacteria rather than by use of chemical means allows the compounds to be produced from carbon dioxide as the initial carbon source, rather than from crude oil or other organic carbon sources.

[0032] Aspects of the invention utilize techniques and methods common to the fields of molecular biology, microbiology and cell culture. Useful laboratory references for these types of methodologies are readily available to those skilled in the art. See, for example, Molecular Cloning: A Laboratory Manual (Third Edition), Sambrook, J., et al. (2001) Cold Spring Harbor Laboratory Press; Current Protocols in Microbiology (2007) Edited by Coico, R, et al., John Wiley and Sons, Inc.; The Molecular Biology of Cyanobacteria (1994) Donald Bryant (Ed.), Springer Netherlands; Handbook Of Microalgal Culture Biotechnology And Applied Phycology (2003) Richmond, A.; (ed.), Blackwell Publishing; and "The cyanobacteria, molecular Biology,

Genomics and Evolution", Edited by Antonia Herrero and Enrique Flores, Caister Academic Press, Norfolk, UK, 2008.

[0033] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Definitions

[0034] 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.

[0035] The term "about" is used herein to mean approximately, in the region of, roughly, or around. When the term "about" is used in conjunction with a numerical value/range, it modifies that value/range by extending the boundaries above and below the numerical value(s) set forth. In general, the term "about" is used herein to modify a numerical value(s) above and below the stated value(s) by a variance of 20%.

[0036] The term "Cyanobacterium" refers to a member from the group of photoautotrophic prokaryotic microorganisms which can utilize solar energy and fix carbon dioxide. Cyanobacteria are also referred to as blue-green algae.

[0037] The terms "host cell" and "recombinant host cell" are intended to include a cell suitable for metabolic manipulation, e.g., which can incorporate heterologous polynucleotide sequences, e.g., which can be transformed. The term is intended to include progeny of the cell originally transformed. In particular embodiments, the cell is a prokaryotic cell, e.g., a cyanobacterial cell. The term recombinant host cell is intended to include a cell that has already been selected or engineered to have certain desirable properties and to be suitable for further enhancement using the compositions and methods of the invention.

[0038] "Competent to express" refers to a host cell that provides a sufficient cellular environment for expression of endogenous and/or exogenous polynucleotides.

[0039] As used herein, the term "genetically enhanced" refers to any change in the endogenous genome of a wild type cell or to the addition of non-endogenous genetic code to a wild type cell, e.g., the introduction of a heterologous gene. More specifically, such changes are made by the hand of man through the use of recombinant DNA technology or mutagenesis. The changes can

involve protein coding sequences or non-protein coding sequences such as regulatory sequences as promoters or enhancers.

[0040] The terms "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."

[0041] The nucleic acids may be modified chemically or biochemically or may contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those of skill in the art. Such modifications include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as uncharged linkages, charged linkages, alkylators, intercalators, pendent moieties, modified linkages, and chelators. Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence via hydrogen bonding and other chemical interactions.

[0042] A "promoter" is a nucleic acid control sequence that directs transcription of an associated polynucleotide, which may be a heterologous polynucleotide or a 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. In one embodiment, the transcriptional control of a promoter results in an increase in expression of the gene of interest. In another embodiment, a promoter is placed 5' to the gene of interest.

[0043] A promoter can be used to replace the natural promoter, or can be used in addition to the natural promoter. A promoter can be endogenous with regard to the host cell in which it is used or it can be a heterologous polynucleotide sequence introduced into the host cell, e.g., exogenous with regard to the host cell in which it is used. A promoter can also be endogenous with regard to the host cell, but derived from a different original gene. In an embodiment, the promoter is a constitutive promoter. In another embodiment, the promoter is inducible, meaning that certain

exogenous stimuli (e.g., nutrient starvation, heat shock, mechanical stress, light exposure, etc.) will induce the promoter leading to the transcription of the gene.

[0044] In one aspect the invention also provides nucleic acids which are at least 60%, 70%, 80% 90%, 95%, 97%, 98%, 99%, or 99.5% identical to the nucleic acids disclosed herein.

[0045] The term "nucleic acid" (also referred to as polynucleotide) is also intended to include nucleic acid molecules having an open reading frame encoding a polypeptide, and can further include non-coding regulatory sequences and introns. In addition, the terms are intended to include one or more genes that map to a functional locus. In addition, the terms are intended to include a specific gene for a selected purpose. The gene can be endogenous to the host cell or can be recombinantly introduced into the host cell.

[0046] The percentage of identity of two nucleic acid sequences or two amino acid sequences can be determined using the algorithm of Thompson et al. (CLUSTALW, 1994, Nucleic Acids Research 22: 4673-4680). A nucleotide sequence or an amino acid sequence can also be used as a so-called "query sequence" to perform a search against public nucleic acid or protein sequence databases in order, for example, to identify further unknown homologous promoters, which can also be used in embodiments of this invention. In addition, any nucleic acid sequences or protein sequences disclosed in this patent application can also be used as a "query sequence" in order to identify yet unknown sequences in public databases, which can encode for example new enzymes, which could be useful in this invention. Such searches can be performed using the algorithm of Karlin and Altschul (1990, Proceedings of the National Academy of Sciences U.S.A. 87: 2,264 to 2,268), modified as in Karlin and Altschul (1993, Proceedings of the National Academy of Sciences U.S.A. 90: 5,873 to 5,877). Such an algorithm is incorporated in the NBLAST and XBLAST programs of Altschul et al. (1990, Journal of Molecular Biology 215: 403 to 410). Suitable parameters for these database searches with these programs are, for example, a score of 100 and a word length of 12 for BLAST nucleotide searches as performed with the NBLAST program. BLAST protein searches are performed with the XBLAST program with a score of 50 and a word length of 3. Where gaps exist between two sequences, gapped BLAST is utilized as described in Altschul et al. (1997, Nucleic Acids Research, 25: 3,389 to 3,402).

[0047] "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. In an embodiment, the recombinant polynucleotide can be located on an extrachromosomal plasmid. In another embodiment, the recombinant nucleic acid can be located on the cyanobacterial chromosome. A host cell that comprises the recombinant polynucleotide is referred to as a "recombinant host cell" or a "recombinant bacterium" or a "recombinant cyanobacterium." 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.

[0048] 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," pages 313-327, The Benjamin/Cummings Publishing Co. 4th ed. (1987).

[0049] 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.

[0050] The term "expressed endogenously" refers to polynucleotides that are native to the host cell and are naturally expressed in the host cell.

[0051] 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. The nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for regulation of expression (e.g., enhanced, increased, constitutive, basal, attenuated, decreased or repressed expression) of the nucleotide sequence and expression of a gene product encoded by the nucleotide sequence (e.g., when the recombinant nucleic acid molecule is included in a recombinant vector, as defined herein, and is introduced into a microorganism).

[0052] 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 molecule 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.

[0053] 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 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").

[0054] In an embodiment, the RSF1010 vector, originally derived from *E. coli*, is used as a base plasmid for expression of the propanediol genes in Cyanobacteria. This vector appears to be relatively stable and can exist in the cell at a copy number of about 15-20 per cell.

[0055] Other plasmids, such as plasmids derived from an endogenous vector of the host cell strain or another cyanobacterial cell, may also be used. An "endogenous vector" or "endogenous plasmid" refers to an extrachromosomal, circular nucleic acid molecule that is derived from the host cell organism.

[0056] The term "recombinant nucleic acid molecule" includes a nucleic acid molecule (e.g., a DNA molecule) that has been altered, modified or engineered such that it differs in nucleotide sequence from the native or natural nucleic acid molecule from which the recombinant nucleic acid molecule was derived (e.g., by addition, deletion or substitution of one or more nucleotides). The recombinant nucleic acid molecule (e.g., a recombinant DNA molecule) can also refer to a nucleic acid that originated in a different location on the DNA, or from a different organism.

[0057] The term “gene” refers to an assembly of nucleotides that encode a polypeptide, and includes cDNA and genomic DNA nucleic acids. “Gene” also refers to a nucleic acid fragment that expresses a specific protein or polypeptide, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence.

[0058] The term “endogenous gene” refers to a native gene in its natural location in the genome of an organism. A “foreign” gene or “heterologous” gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A “transgene” is a gene that has been introduced into the genome by a transformation procedure.

[0059] The term “nucleic acid fragment” will be understood to mean a nucleotide sequence of reduced length relative to the reference nucleic acid and comprising, over the common portion, a nucleotide sequence substantially identical to the reference nucleic acid. Such a nucleic acid fragment according to the invention may be, where appropriate, included in a larger polynucleotide of which it is a constituent. Such fragments comprise, or alternatively consist of, oligonucleotides ranging in length from at least about 6 to about 2200 or more consecutive nucleotides of a polynucleotide according to the invention.

[0060] The term “open reading frame,” abbreviated as “ORF,” refers to a length of nucleic acid sequence, either DNA, cDNA or RNA, that comprises a translation start signal or initiation codon, such as an ATG or AUG, and a termination codon and can be potentially translated into a polypeptide sequence.

[0061] The term “upstream” refers to a nucleotide sequence that is located 5' to reference nucleotide sequence. In particular, upstream nucleotide sequences generally relate to sequences that are located on the 5' side of a coding sequence or starting point of transcription. For example, most promoters are located upstream of the start site of transcription.

[0062] The term “downstream” refers to a nucleotide sequence that is located 3' to reference nucleotide sequence. In particular, downstream nucleotide sequences generally relate to sequences that follow the starting point of transcription. For example, the translation initiation codon of a gene is located downstream of the start site of transcription.

[0063] The term “homology” refers to the percent of identity between two polynucleotide or two polypeptide moieties. The correspondence between the sequence from one moiety to another can be determined by techniques known to the art. For example, homology can be determined by a

direct comparison of the sequence information between two polypeptide molecules by aligning the sequence information and using readily available computer programs. Alternatively, homology can be determined by hybridization of polynucleotides under conditions that form stable duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s) and size determination of the digested fragments.

[0064] As used herein, “substantially similar” refers to nucleic acid fragments wherein changes in one or more nucleotide bases results in substitution of one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence.

[0065] The term “substantially similar” also refers to modifications of the nucleic acid fragments, such as the deletion or insertion of one or more nucleotide bases that do not substantially affect the functional properties of the resulting transcript.

[0066] The terms “restriction endonuclease” and “restriction enzyme” refer to an enzyme that binds and cuts within a specific nucleotide sequence within double stranded DNA.

[0067] The term “expression” as used herein refers to the transcription and stable accumulation mRNA derived from a nucleic acid or polynucleotide. Expression may also refer to translation of mRNA into a protein or polypeptide.

[0068] The term “primer” is an oligonucleotide that hybridizes to a target nucleic acid sequence to create a double stranded nucleic acid region that can serve as an initiation point for DNA synthesis under suitable conditions. Such primers may be used in a polymerase chain reaction.

[0069] The term “polymerase chain reaction,” also termed “PCR,” refers to an in vitro method for enzymatically amplifying specific nucleic acid sequences. PCR involves a repetitive series of temperature cycles with each cycle comprising three stages: denaturation of the template nucleic acid to separate the strands of the target molecule, annealing a single stranded PCR oligonucleotide primer to the template nucleic acid, and extension of the annealed primer(s) by DNA polymerase. PCR provides a means to detect the presence of the target molecule and, under quantitative or semi-quantitative conditions, to determine the relative amount of that target molecule within the starting pool of nucleic acids.

[0070] An “expression cassette” or “expression 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 one or more heterologous or native polynucleotide sequences that are

transcribed. Expression cassettes or constructs may also include, e.g., transcription termination signals, polyadenylation signals, and enhancer elements.

[0071] The term “codon” refers to a triplet of nucleotides coding for a single amino acid.

[0072] The term “codon–anticodon recognition” refers to the interaction between a codon on an mRNA molecule and the corresponding anticodon on a tRNA molecule.

[0073] The term “codon bias” refers to the fact that not all codons are used equally frequently in the genes of a particular organism.

[0074] The term “codon optimization” refers to the modification of at least some of the codons present in a heterologous gene sequence from a triplet code that is not generally used in the host organism to a triplet code that is more common in the particular host organism. This can result in a higher expression level of the gene of interest.

[0075] The expression constructs can be designed taking into account such properties as codon usage frequencies of the organism in which the recombinant genes are to be expressed. Codon usage frequencies can be determined using known methods (see, e.g., Nakamura et al. Nucl. Acids Res. 28:292, 2000). Codon usage frequency tables, including those for cyanobacteria, are also available in the art (e.g., in codon usage databases of the Department of Plant Genome Research, Kazusa DNA Research Institute (www.kazusa.or.jp/codon).

[0076] The term “transformation” is used herein to mean the insertion of heterologous genetic material into the host cell. Typically, the genetic material is DNA on a plasmid vector, but other means can also be employed. General transformation methods and selectable markers for bacteria and cyanobacteria are known in the art (Wirth, Mol Gen Genet. 216:175-177 (1989); Koksharova, Appl Microbiol Biotechnol 58:123-137 (2002); Sambrook et al, supra).

[0077] The term “selectable marker” means an identifying factor, usually an antibiotic or chemical resistance gene, that is able to be selected for based upon the marker gene's effect, i.e., resistance to an antibiotic, resistance to a herbicide, colorimetric markers, enzymes, fluorescent markers, and the like, wherein the effect is used to track the inheritance of a nucleic acid of interest and/or to identify a cell or organism that has inherited the nucleic acid of interest. Examples of selectable marker genes known and used in the art include: genes providing resistance to ampicillin, streptomycin, gentamycin, spectinomycin, kanamycin, hygromycin, and the like.

[0078] A "polypeptide" is a polymeric compound comprised of covalently linked amino acid residues. A "protein" is a polypeptide that performs a structural or functional role in a living cell.

[0079] The invention also provides amino acid sequences of the enzymes involved in 1,2-propanediol formation, which are at least 60%, 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 99.5% identical to the amino acid sequences disclosed herein.

[0080] The EC numbers cited throughout this patent application are enzyme commission numbers. This is a numerical classification scheme for enzymes based on the chemical reactions which are catalyzed by the enzymes.

[0081] A "heterologous gene" refers to a gene that is not naturally present in the cell. Similarly, the term "heterologous nucleic acid" refers to a nucleic acid sequence that is not normally present in the cell.

[0082] A "heterologous protein" refers to a protein not naturally produced in the cell.

[0083] An "isolated polypeptide" or "isolated protein" is a polypeptide or protein that is substantially free of those compounds that are normally associated therewith in its natural state (e.g., other proteins or polypeptides, nucleic acids, carbohydrates, lipids).

[0084] The term "polypeptide fragment" of a polypeptide refers to a polypeptide whose amino acid sequence is shorter than that of the reference polypeptide. Such fragments of a polypeptide according to the invention may have a length of at least about 2 to about 750 or more amino acids.

[0085] A "variant" of a polypeptide or protein is any analogue, fragment, derivative, or mutant which is derived from a polypeptide or protein and which retains at least one biological property of the polypeptide or protein. Different variants of the polypeptide or protein may exist in nature. These variants may be allelic variations characterized by differences in the nucleotide sequences of the structural gene coding for the protein, or may involve differential splicing or post-translational modification. The skilled artisan can produce variants having single or multiple amino acid substitutions, deletions, additions, or replacements.

<u>Preparation of Recombinant Vectors for Genetic Modification of Cyanobacteria</u>

[0086] Cyanobacteria can be modified to add enzymatic pathways of interest as shown herein in order to produce 1,2-propanediol. The DNA sequences encoding the genes described herein can be amplified by polymerase chain reaction (PCR) using specific primers. The amplified PCR

fragments can be digested with the appropriate restriction enzymes and can then be cloned into either a self-replicating plasmid or an integrative plasmid.

[0087] In an embodiment, the nucleic acids of interest can be amplified from nucleic acid samples using amplification techniques. PCR can be used to amplify the sequences of the genes directly from mRNA, from cDNA, from genomic libraries or cDNA libraries. PCR and other in vitro amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of the desired mRNA in samples, and for nucleic acid sequencing.

[0088] In order to use isolated sequences in the above techniques, recombinant DNA vectors suitable for transformation of cyanobacteria can be prepared. Techniques for transformation are well known and described in the technical and scientific literature. For example, a DNA sequence encoding one or more of the genes described herein can be combined with transcriptional and other regulatory sequences which will direct the transcription of the sequence from the gene in the transformed cyanobacteria.

[0089] In an embodiment, an antibiotic resistance cassette for selection of positive clones can be present on the plasmid to aid in selection of transformed cells. For example, genes conferring resistance to ampicillin, gentamycin, kanamycin, or other antibiotics can be inserted into the vector, under the control of a suitable promoter. Other antibiotic resistance genes can be used if desired. In some embodiments, the vector contains more than one antibiotic resistance gene. The presence of a foreign gene encoding antibiotic resistance can be selected, for example, by placing the putative transformed cells into a suitable amount of the corresponding antibiotic, and picking the cells that survive.

[0090] In an embodiment, the genes of interest are inserted into the cyanobacterial chromosome. When the cell is polyploid, the gene insertions can be present in all of the copies of the chromosome, or in some of the copies of the chromosome.

[0091] In another embodiment, the inserted genes are present on an extrachromosomal plasmid. The extrachromosomal plasmids can be present in a high number or a low number within the genetically enhanced cyanobacterium.

[0092] The extrachromosomal plasmid can be derived from an outside source, such as, for example, RSF1010-based plasmid vectors, or it can be derived from an endogenous plasmid from the cyanobacterial cell or from another species of cyanobacteria.

[0093] Many cyanobacterial species harbor endogenous vectors that can be used to carry production genes. The cyanobacterium *Synechococcus* PCC 7002, for example, contains six endogenous plasmids having different numbers of copies in the cyanobacterial cell (Xu et al., 2011, "Expression of genes in cyanobacteria: Adaption of Endogenous Plasmids as platforms for High-Level gene Expression in *Synechococcus* PCC 7002", Photosynthesis Research Protocols, Methods in Molecular Biology, 684:273-293). The endogenous plasmid pAQ1 is present in a number of 50 copies per cell (high-copy), the plasmid pAQ3 with 27 copies, the plasmid pAQ4 with 15 copies and the plasmid pAQ5 with 10 copies per cell (low-copy). In an embodiment, these endogenous plasmids can be used as an integration platform for the 1,2-propanediol genes described herein. The propanediol pathway genes can be integrated into the endogenous cyanobacterial plasmids via homologous recombination, or by other suitable means. It is also possible to create a "shuttle vector" based on the backbone of an endogenous vector, in combination with portions of self-replicating *E.coli* vectors, for ease of genetic manipulation. Such vectors can be easily manipulated in *E. coli*, for example, then the vectors can be transferred to the cyanobacterial host strain for the production of 1,2-propanediol.

[0094] In an embodiment, the inserted genes are present on an extrachromosomal plasmid, wherein the plasmid has multiple copies per cell. The plasmid can be present, for example, at about 1, 3, 5, 8, 10, 15, 20, 30, 40, 50, 60, 70, 80, 90, or more copies per host cyanobacterial cell. In an embodiment, the plasmids are fully segregated.

[0095] In another embodiment, the inserted genes are present on one cassette driven by one promoter. In another embodiment, the inserted genes are present on separate plasmids, or on different cassettes.

[0096] In another embodiment, the inserted genes are modified for optimal expression by modifying the nucleic acid sequence to accommodate the cyanobacterial cell's protein translation system. Modifying the nucleic acid sequences in this manner can result in an increased expression of the genes.

[0097] The inserted genes can be regulated by one promoter, or they can be regulated by individual promoters. The promoters can be constitutive or inducible. The promoter sequences can be derived, for example, from the host cell, from another organism, or can be synthetically derived.

[0098] Any desired promoter can be used to regulate the expression of the genes for 1,2-propanediol production. Exemplary promoter types include but are not limited to, for example, constitutive promoters, inducible promoters (e.g., by nutrient starvation, heat shock, mechanical stress, environmental stress, metal concentration, light exposure, etc.), endogenous promoters, heterologous promoters, and the like.

[0099] In an embodiment, the inserted genes for 1,2-propanediol production are placed under the transcriptional control of promoters selected from a group consisting of: rbcL, ntcA, nblA, isiA, petJ, petE, sigB, lrtA, htpG, hspA, clpBl, hliB, ggpS, psbA2, psaA, nirA, crhC, and srp. The promoters hspA, clpBl, and hliB can be induced by heat shock (raising the growth temperature of the host cell culture from 30°C to 40°C), cold shock (reducing the growth temperature of the cell culture from 30°C to 20°C), oxidative stress (for example by adding oxidants such as hydrogen peroxide to the culture), or osmotic stress (for example by increasing the salinity). The promoter sigB can be induced by stationary growth, heat shock, and osmotic stress. The promoters ntcA and nblA can be induced by decreasing the concentration of nitrogen in the growth medium and the promoters psaA and psbA2 can be induced by low light or high light conditions. The promoter htpG can be induced by osmotic stress and heat shock. The promoter crhC can be induced by cold shock. An increase in copper concentration can be used in order to induce the promoter petE, whereas the promoter petJ is induced by decreasing the copper concentration. The promoter srp can be induced by the addition of IPTG (isopropyl β-D-1-thiogalactopyranoside). Additional details of these promoters can be found, for example, in PCT/EP2009/060526, which is incorporated by reference herein in its entirety.

[00100] In an embodiment, the inducible promoters are selected from the group consisting of: PntcA, PnblA, PisiA, PpetJ, PpetE, PggpS, PpsbA2, PpsaA, PsigB, PlrtA, PhtpG, PnirA, PhspA, PclpB1, PhliB, PcrhC, PziaA, PsmtA, PcorT, PnrsB, PaztA, PbmtA, Pbxa1, PzntA, PczrB, PnmtA and Psrp.

[00101] In certain other embodiments, truncated or partially truncated versions of these promoters including only a small portion of the native promoters upstream of the transcription start point, such as the region ranging from -35 to the transcription start can often be used. Furthermore, the introduction of nucleotide changes into the promoter sequence, e.g. into the TATA box, the operator sequence and/or the ribosomal binding site (RBS) can be used to tailor

or optimize the promoter strength and/or its induction conditions, such as the concentration of inducer compound.

[00102] In an embodiment, the promoter used to regulate expression of 1,2-propanediol pathway genes is the Psrp promoter (SEQ ID NO: 1). In another embodiment, the promoter is $PnblA_{7120}$ (the phycobilisome degradation protein promoter from *Nostoc* sp. PCC 7120 (SEQ ID NO: 2).

[00103] In an embodiment, the promoter is $PrbcL_{6803}$ (the constitutive ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit promoter from *Synechocystis* sp. PCC 6803 (SEQ ID NO: 3). The promoter can also be a derivative or a variant of an rbcL promoter, such as the PrbcL promoter from *Synechocystis* sp. PCC 6803, or from another organism. Thus, in an embodiment, the promoter is $Prbc\text{-}_{PCC\ 6803}$ (SEQ ID NO: 4). In another embodiment, the promoter is $PrbcL^{*}\text{-}_{PCC\ 6803}$ (SEQ ID NO: 5). In another embodiment, the promoter is $Prbc^{*}\text{-}_{PCC\ 6803}$ (SEQ ID NO: 6).

[00104] Examples of other promoters that can be used are $PsmtA_{7002}$ (the promoter for prokaryotic metallothionein-related protein from *Synechococcus* sp. PCC 7002; (SEQ ID NO: 7); and the repressor/promoter system ziaR-$PziaA_{6803}$ (the zinc-inducible promoter from *Synechocystis* sp. PCC 6803; (SEQ ID NO: 8). Additionally, the promoter PpetJ from *Synechocystis* sp. PCC 6803 (SEQ ID NO: 9) can also be used, as shown in FIG. 4 through FIG. 8.

[00105] A terminator region can be inserted at the 3' end of the genes of interest. An exemplary terminator sequence is the Oop lamda phage terminator (SEQ ID NO: 10), as shown in the gene cassette maps shown in FIG. 4 through FIG. 7.

1,2-Propanediol

[00106] 1,2-propanediol (also termed propylene glycol, propane-1,2-diol, 1,2-dihydroxypropane, and methylethylene glycol) is a three-carbon diol with a stereogenic center at the central carbon atom. The enantiomerically pure 1,2-propanediol generated from biological processes is a high value commodity chemical with a broad application as solvent, food additive, de-icing compounds etc.

[00107] In an embodiment, the biochemical pathway from CO_2 to 1,2-propanediol involves several steps, as shown in FIG. 1. Briefly, these steps are:

CO_2→ → → Dihydroxyacetone phosphate (DHAP) →Methylglyoxal→Lactaldehyde→1,2-Propanediol

or

CO_2→ → →Dihydroxyacetone phosphate→Methylglyoxal→Acetol→1,2-Propanediol

[00108] FIG. 2 shows another diagram of 1,2-propanediol production in *E. coli* (Alteras et al.,1999, Applied and Envir. Biol. 65:1180-1185). The diagram shows biosynthetic pathway variations that can be used to produce either S-1,2-propanediol or R-1,2-propanediol in *E. coli* cells.

[00109] Yet another biosynthetic pathway diagram is shown in FIG. 3, where possible alternate enzymes for some of the steps are indicated. Examples 3 through 6 demonstrate how the gene cassettes encoding the various enzymes were transferred to cyanobacterial cells, then tested for activity in cyanobacteria.

Production of 1,2-Propanediol in Cyanobacteria

[00110] Cyanobacteria can be modified to produce 1,2-propanediol. In an embodiment of the invention, in order to create the 1,2-propanediol biosynthetic pathway from CO_2 as the carbon source, the following genes can be inserted into the cyanobacterial cell:

*gld*A - *fuc*O - *mgs*A ("GFM")

[00111] In another embodiment of the invention, the gene *yqhD* can also be inserted into the cyanobacterial cell. As shown in FIG. 1, the biosynthetic pathway for 1,2-propanediol production can include a branch from the intermediate methylglyoxal – both the enzymes GldA and YqhD can utilize methylglyoxal to move the pathway toward 1,2-propanediol production. Methylglyoxal is partially toxic to many cell types, so the inclusion of another enzyme and another biosynthetic pathway branch can help deplete any methylglyoxal that accumulates from the prior steps. Further, since YqhD acts on methylglyoxal to create acetol, which, in the presence of GldA, becomes 1,2-propanediol, the added branching step can also produce more 1,2-propanediol.

gldA – *yqhD* - *fucO* - *mgsA* ("GYFM")

[00112] In another embodiment, the biosynthetic pathway from:

CO_2→ → → DHAP→Methylglyoxal→Lactaldehyde→1,2-Propanediol

can also be achieved with a choice of several enzymes, as shown in FIG. 3. The conversion of methylglyoxal to lactaldehyde can be achieved, for example, by either GldA, ADH, or AKR. The conversion of lactaldehyde to 1,2-propanediol can be achieved, for example, by either FucO or GldA. To determine the effect of each of these enzyme choices on the production of the intermediates and the end product 1,2-propanediol, different combinations of the above-described enzymes were constructed as shown in Example 3. Plasmids containing the various gene cassettes as shown in Table 3 (such as those shown in FIGS. 4 through 8) were transferred to cyanobacterial host cells using a shuttle vector system. The enzyme activity of the resulting transformed cultures was confirmed as shown in Table 3. In certain cases, such as for the #748 and #749 constructs, the intermediates and the end product 1,2-propanediol were also tested and confirmed. When transferred to a cyanobacterial cell, the enzyme activities and levels of intermediates could be determined, as demonstrated in FIGs. 9 through 14.

[00113] A demonstration of the construction of plasmids for the production of 1,2-propanediol is shown in Examples 3 and 10. An example of a successful transformation to cyanobacteria is shown in Example 12. Verification of the successful transformation is shown in Example 13. A suitable method for determining the level of 1,2-propanediol that is produced is shown in Example 14.

[00114] The terms “gldA” and “glycerol dehydrogenase” refer to an enzyme that facilitates the formation of 1,2-propanediol from acetol, or, alternatively, the formation of 2-hydroxypropionaldehyde (lactaldehyde) from methylglyoxal. A “gldA gene” is a nucleic acid that encodes the enzyme. In an embodiment, the gene is originally derived from *E. coli*. In an embodiment, the gene is nucleic acid sequence Accession No. NC_010473.1; encoding a protein having Accession No. YP_001732735.1. In another embodiment, the invention provides a recombinant photosynthetic microorganism that includes at least one heterologous DNA sequence encoding at least one polypeptide that catalyzes a substrate to product conversion that leads to the synthesis of 1,2-propanediol from acetol, or, alternatively, the formation of 2-hydroxypropionaldehyde from methylglyoxal. In an embodiment, the GldA enzyme is in the enzyme class EC#1.1.1.6. In an embodiment, the GldA nucleotide sequence is SEQ ID NO: 11 and the amino acid sequence is SEQ ID NO: 12.

[00115] The terms "FucO" and "L-1,2-propanediol oxidoreductase" refer to an enzyme that facilitates the formation of 1,2-propanediol from 2-hydroxypropionaldehyde (lactaldehyde), as shown in FIG. 1. A "fucO gene" refers to the gene encoding the enzyme. In an embodiment, the gene is originally derived from *E. coli*. In another embodiment, the gene is nucleic acid sequence Accession No. NC_010473.1; encoding a protein having accession # YP_001731690.1. In another embodiment, the invention provides a recombinant photosynthetic microorganism that includes at least one heterologous DNA sequence encoding at least one polypeptide that catalyzes a substrate to product conversion that leads to the synthesis of 1,2-propanediol from 2-hydroxypropionaldehyde (lactaldehyde). In an embodiment, the FucO enzyme is in the enzyme class EC#1.1.1.77. In another embodiment, the FucO enzyme is present in the broader enzyme class EC#1.1.1.21. In an embodiment, the FucO nucleotide sequence is SEQ ID NO: 13, and the FucO amino acid sequence is SEQ ID NO: 14.

[00116] The terms "MgsA" and "methylglyoxal synthase" refer to an enzyme that facilitates the formation of methylglyoxal from glycerone phosphate. An "mgsA gene" refers to the gene encoding the enzyme. Details of the regulation of this enzyme in *E. coli*, as well as an assay for its activity, can be found in Hopper et al., (1971), FEBS Letters 13:213-216. In an embodiment, the gene is originally derived from *E. coli*. In another embodiment, the gene sequence is nucleic acid sequence Accession No. NC_010473.1; encoding a protein having accession # YP_001729941.1. In another embodiment, the invention provides a recombinant photosynthetic microorganism that includes at least one heterologous DNA sequence encoding at least one polypeptide that catalyzes a substrate to product conversion that leads to the synthesis of methylglyoxal from glycerone phosphate. In an embodiment, the MgsA enzyme is a member of the enzyme class EC# 4.2.3.3. In an embodiment, the mgsA nucleotide sequence is SEQ ID NO: 15, and the mgsA amino acid sequence is SEQ ID NO: 16.

[00117] The term "yqhD" refers to a gene encoding an alcohol dehydrogenase. The enzyme can be utilized to form acetol from methylglyoxal (FIG. 1). In an embodiment, the gene is derived from *E. coli*. In an additional embodiment, the gene is nucleic acid accession # NC_010473.1:3251122..3252285 and the protein accession is # YP_001731875.1. In an embodiment, the YqhD nucleotide sequence is SEQ ID NO: 17, and the YqhD amino acid sequence is SEQ ID NO: 18.

[00118] The term "AKR" or "sacR1" refers to a gene encoding the enzyme aldo/keto reductase. In an embodiment, the gene is from a cyanobacterial species, such as *Synechococcus*. In an embodiment, the gene is Cyanobase ID # SYNPCC7002_A1474. In an embodiment, the AKR sequence is synAKR, and its nucleotide sequence is SEQ ID NO: 19, while the synAKR amino acid sequence is SEQ ID NO: 20.

[00119] The term "synADH" or "ADH" refers to a gene encoding the enzyme alcohol dehydrogenase. As shown in FIG. 3, it is possible that this enzyme can catalyze the conversion of methylglyoxal to lactaldehyde, which is a portion of the biosynthetic pathway leading to the production of 1,2-propanediol. In an embodiment, the synADH gene is a codon optimized version of the gene originally derived from *Synechocystis* PCC 6803 (nucleic acid SEQ ID NO: 21, amino acid SEQ ID NO: 22). In another embodiment, ADH from another source can be used.

[00120] In certain cyanobacterial strains that are capable of metabolizing glycerol, the input carbon source can be glycerol in addition to, or instead of CO_2. The cyanobacterial strain *Synechococcus* sp. PCC 7002, for example, naturally contains the genes capable of glycerol metabolism. Thus, this strain is a good candidate for using a glycerol feed to produce 1,2-propanediol.

[00121] The invention also comprises recombinant nucleic acids having 80%, 85%, 90%, 95%, 97%, 98%, 99% identity to SEQ ID NOs: 11, 13, 15, 17, 19 or 21.

Transformation of Cyanobacterial Cells

[00122] Cyanobacteria can be transformed by several suitable methods. Exemplary cyanobacteria that can be transformed with the nucleic acids described herein include, but are not limited to, *Synechocystis*, *Synechococcus*, *Acaryochloris*, *Anabaena*, *Thermosynechococcus*, *Chamaesiphon*, *Chroococcus*, *Cyanobacterium*, *Cyanobium*, *Dactylococcopsis*, *Gloeobacter*, *Gloeocapsa*, *Gloeothece*, *Microcystis*, *Prochlorococcus*, *Prochloron*, *Chroococcidiopsis*, *Cyanocystis*, *Dermocarpella*, *Myxosarcina*, *Pleurocapsa*, *Stanieria, Xenococcus*, *Arthrospira*, *Borzia*, *Crinalium*, *Geitlerinema*, *Halospirulina*, *Leptolyngbya*, *Limnothrix, Lyngbya, Microcoleus, Cyanodictyon, Aphanocapsa, Oscillatoria, Planktothrix, Prochlorothrix, Pseudanabaena, Spirulina, Starria, Symploca, Trichodesmium, Tychonema, Anabaenopsis, Aphanizomenon, Calothrix, Cyanospira, Cylindrospermopsis, Cylindrospermum, Nodularia,*

*Nostoc, Chlorogloeopsis, Fischerella, Geitleria, Nostochopsis, Iyengariella, Stigonema, Rivularia, Scytonema, Tolypothrix, Cyanothece, Phormidium, Adrianema, a*nd the like.

[00123] Exemplary methods suitable for transformation of Cyanobacteria, include, as nonlimiting examples, natural DNA uptake (Chung, et al. (1998) FEMS Microbiol. Lett. 164: 353-361; Frigaard, et al. (2004) Methods Mol. Biol. 274: 325-40; Zang, et al. (2007) J. Microbiol. 45: 241-245), conjugation, transduction, glass bead transformation (Kindle, et al. (1989) J. Cell Biol. 109: 2589-601; Feng, et al. (2009) Mol. Biol. Rep. 36: 1433-9; U.S. Pat. No. 5,661,017), silicon carbide whisker transformation (Dunahay, et al. (1997) Methods Mol. Biol. (1997) 62: 503-9), biolistics (Dawson, et al. (1997) Curr. Microbiol. 35: 356-62; Hallmann, et al. (1997) Proc. Natl. Acad. USA 94: 7469-7474; Jakobiak, et al. (2004) Protist 155:381-93; Tan, et al. (2005) J. Microbiol. 43: 361-365; Steinbrenner, et al. (2006) Appl Environ. Microbiol. 72: 7477-7484; Kroth (2007) Methods Mol. Biol. 390: 257-267; U.S. Pat. No. 5,661,017) electroporation (Kjaerulff, et al. (1994) Photosynth. Res. 41: 277-283; Iwai, et al. (2004) Plant Cell Physiol. 45: 171-5; Ravindran, et al. (2006) J. Microbiol. Methods 66: 174-6; Sun, et al. (2006) Gene 377: 140-149; Wang, et al. (2007) Appl. Microbiol. Biotechnol. 76: 651-657; Chaurasia, et al. (2008) J. Microbiol. Methods 73: 133-141; Ludwig, et al. (2008) Appl. Microbiol. Biotechnol. 78: 729-35), laser-mediated transformation, or incubation with DNA in the presence of or after pre-treatment with any of poly(amidoamine) dendrimers (Pasupathy, et al. (2008) Biotechnol. J. 3: 1078-82), polyethylene glycol (Ohnuma, et al. (2008) Plant Cell Physiol. 49: 117-120), cationic lipids (Muradawa, et al. (2008) J. Biosci. Bioeng. 105: 77-80), dextran, calcium phosphate, or calcium chloride (Mendez-Alvarez, et al. (1994) J. Bacteriol. 176: 7395-7397), optionally after treatment of the cells with cell wall-degrading enzymes (Perrone, et al. (1998) Mol. Biol. Cell 9: 3351-3365); and biolistic methods (see, for example, Ramesh, et al. (2004) Methods Mol. Biol. 274: 355-307; Doestch, et al. (2001) Curr. Genet. 39: 49-60; all of which are incorporated herein by reference in their entireties).

Culturing the Cyanobacterial Cells

[00124] In an embodiment, 1,2-propanediol is synthesized in cyanobacterial cultures by preparing host cyanobacterial cells having the gene constructs discussed herein, and then growing cultures of the cells.

[00125] The choice of culture medium can depend on the cyanobacterial species. In an embodiment of the invention, the following BG-11 medium for growing cyanobacteria can be

used (Table 1 and Table 2, below). When salt water species are grown, Instant Ocean (35 g/L) and vitamin B_{12} (1 μg/ml) can be added to the culture medium.

Table 1: Exemplary Culture Medium Composition

Compound	**Amount (per liter)**	**Final Concentration**
$NaNO_3$	1.5 g	17.6 mM
K_2HPO_4	0.04 g	0.23 mM
$MgSO_4 \cdot 7H_2O$	0.75 g	3.04 mM
$CaCl_2 \cdot 2H_2O$	0.036 g	0.24 mM
Citric acid	0.006 g	0.031 mM
Ferric ammonium citrate	0.006 g	- -
EDTA (disodium salt)	0.001 g	0.0030 mM
$NaCO_3$	0.02 g	0.19 mM
Trace metal mix A5	1.0 ml	- -

Table 2: Trace Metal Mix

Trace Metal mix A5		**Concentration in Final Medium**
H_3BO_3	2.86 g	46.26 μM
$MnCl_2 \cdot 4H_2O$	1.81 g	9.15 μM
$ZnSO_4 \cdot 7H_2O$	0.222 g	0.772 μM
$NaMoO_4 \cdot 2H_2O$	0.39 g	1.61 μM
$CuSO_4 \cdot 5H_2O$	0.079 g	0.32 μM
$Co(NO_3)_2 \cdot 6H_2O$	49.4 mg	0.170 μM
Distilled water	1.0 L	- -

[00126] In an embodiment, the cells are grown autotrophically, and the only carbon source is CO_2. In another embodiment, the cells are grown mixotrophically, for example with the addition of a carbon source such as glycerol.

[00127] The cultures can be grown indoors or outdoors. The cultures can be axenic or non-axenic. In another embodiment, the cultures are grown indoors, with continuous light, in a sterile environment. In another embodiment, the cultures are grown outdoors in an open pond type of photobioreactor.

[00128] In an embodiment, the cyanobacteria are grown in enclosed bioreactors in quantities of at least about 100 liters, 500 liters, 1000 liters, 2000 liters, 5,000 liters, or more. In an embodiment, the cyanobacterial cell cultures are grown in disposable, flexible, tubular photobioreactors made of a clear plastic material.

[00129] The light cycle can be set as desired, for example: continuous light, or 16 hours on and 8 hours off, or 14 hours on and 10 hours off, or 12 hours on and 12 hours off.

Confirmation of Transformation and Enzyme Activity in the Transformed Cyanobacterial Cells

[00130] The presence of the 1,2-propanediol pathway genes in the shuttle vector or in the transformed host cell can be determined by several means. In an embodiment, the gene pathway cassette is confirmed using PCR-based methods. The presence of the expressed mRNA can be determined, for example, using RT-PCR or a northern blot, or by any other suitable means. The presence of the expressed enzymes themselves can be determined, for example, by an SDS-PAGE followed by transfer to a western blot. In order to confirm that the genes encoding the 1,2-propanediol pathway enzymes are actually functional when expressed in cyanobacteria, chemical analysis, such as gas chromatography, can be performed. Specific enzymatic assays can also be performed. Examples of several enzyme activity assays for mgsA, synAKR, and FucO are described in Examples 6 through 9.

Isolation and Purification of 1,2-Propanediol from the Cyanobacterial Cultures

[00131] Various methods can be used to remove 1,2-propanediol from the cyanobacterial culture medium. In an embodiment, the propanediol is separated from the culture medium periodically as the culture is growing. For example, the culture medium can be separated from the cells, followed by a filtration step. The propanediol can then be removed from the filtrate. The culture medium can be recycled back into the culture, if desired, or new culture medium can be added. In another embodiment, the propanediol is removed from the culture at the end of the batch run.

[00132] Another method of separating polyol products from the culture producing it is described in International Patent Application No. WO/2000/024918 to Fisher et al. This application describes a pre-treatment step that can be used to separate the cells from the polyol-containing solution without killing the cell culture. Additional steps can include flotation or flocculation to remove proteinaceous materials, followed by ion exchange chromatography, activated carbon treatment, evaporative concentration, precipitation and crystallization.

[00133] A process for reclaiming 1,2-propylene glycol from operative fluids such as antifreeze solutions, heat transfer fluids, deicers, lubricants, hydraulic fluids, quenchants, solvents and absorbents, is disclosed in U.S. Patent No. 5,194,159 to George et al. The method involves contacting the fluid with semi-permeable membranes under reverse osmosis.

[00134] U.S. Patent No. 5,510,036 to Woyciesjes et al. discloses a process for the purification and removal of contaminants (such as heavy metals oils and organic contaminants) in a polyol-containing solution, wherein the process involves lowering the pH and adding precipitating, flocculating, or coagulating agents, which can be followed by filtration and an ion exchange chromatography step.

[00135] The present invention is further described by the following non-limiting examples. However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

EXAMPLES

Example 1

General Methods

[00136] Restriction endonucleases were purchased from New England Biolabs (New England Biolabs (NEB), Ipswich, MA), unless otherwise noted. PCR was performed using an Eppendorf Mastercycler thermocycler (Eppendorf, Hauppauge, NY), using Phire II Hot Start polymerase or Taq DNA polymerase (NEB) for diagnostic amplifications, and Phusion polymerase or Crimson LongAmp Taq Polymerase (NEB) for high fidelity amplifications. PCR

temperature profiles were set up as recommended by the polymerase manufacturer. Cloning was performed in *E. coli* using XL10-Gold Ultracompetent cells (Agilent Technologies, Santa Clara, CA) following the manufacturer's protocol. TOPO cloning kits (Zero Blunt TOPO PCR Cloning kit) were purchased from Invitrogen (Invitrogen, Carlsbad, CA), and were used according to the manufacturer's protocol.

[00137] BG-11 stock solution was purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, MO). Marine BG-11 (MBG-11) was prepared by dissolving 35 g Instant Ocean (United Pet Group, Inc, Cincinnati, OH) in 1 L water and supplementing with BG-11 stock solution. Vitamin B_{12} (Sigma Aldrich) was supplemented to MBG-11 to achieve a final concentration of 1 μg/L, as needed. Solid media (agar plates) were prepared similarly to liquid media, with the addition of 1% (w/v) phyto agar (Research Products International Corp, Mt. Prospect, IL). Stock solutions of the antibiotics spectinomycin (100 mg/ml) and kanamycin (50 mg/ml) were purchased from Teknova (Teknova, Hollister, CA). Stock solution of the antibiotic gentamycin (10 mg/ml) was purchased from MP Biomedicals (MP Biomedicals, Solon, OH).

Example 2

SLIC Method (sequence- and ligation-independent cloning)

[00138] Primers were designed with 5' sequences that overlapped the target vector at the desired restriction site, or which overlapped the next PCR product if inserting more than one product at a time. The overlapping sequence was typically 30 base pairs (bp) long. PCR products were amplified from genomic DNA (*Klebsiella* or *Saccharomyces*) or from whole cells (*E. coli*) and gel-purified. Target vectors were digested with appropriate restriction enzymes and gel-purified. To generate the 30-bp sticky ends, digested target vector (200 ng - 1 μg) and each PCR product (20 ng - 1 μg) were treated with 0.5 U of T4 DNA polymerase from NEB in NEB buffer 2 plus BSA (with no dNTP's) and incubated at room temperature for 15 minutes per 10 bp overlap (45 minutes for a 30 bp overlap). Reactions were stopped by adding 1/10 volume of 10 mM dCTP (or other single dNTP). Equimolar amounts (1:1 or 1:1:1, etc.) of T4-treated vector and insert(s) were combined in 8 μl volume in a PCR tube. 10X T4 ligase buffer, 1 μl, was added to the tube. Using a thermal cycler, reactions were heated to 65°C for 10 minutes, then slowly ramped down to 37°C (10% ramp speed). RecA protein from NEB, 20 ng in 1 ml 10X

RecA buffer, was added to the tube, which was incubated at 37°C for 30 minutes. 5 μl of the reaction was used for *E. coli* transformation.

Example 3

Construction of Various Gene Cassettes for Confirmation of Activity of Certain Pathway Enzymes for 1,2-Propanediol Production

[00139] FIG. 3 shows a biosynthetic pathway scheme for production of 1,2-propanediol in Cyanobacteria. To confirm whether each of the putative pathway enzymes would actually be correctly expressed and have normal activity in a cyanobacterial species, various constructs were prepared as shown below in Table 3. Maps of the gene constructs are shown in FIG. 4 through FIG. 8. The constructs varied in the choice of promoter, the choice of antibiotic resistance gene used, and the choice of enzyme.

Table 3: Gene Cassettes for Portions of 1,2-Propanediol Pathway

Plasmid #	Plasmid Name	Promoter	Antibiotic Resistance Gene	Primer used	Restriction Sites	Restriction Sites	Comments	Enzyme Assays
550	pVZ325-PpetJ-PDC-PrbcL*-synADH deg.	petJ, rbcL*	Gm/Spec	/	SpeI/PstI	SpeI/PstI	Recipients; Plasmid SEQ ID NO: 23	
707	pJet-PpetJ-fbpI	petJ	Amp	/	XbaI/EcoRI	XbaI/EcoRI	recipient for EcMgsA 712 Plasmid SEQ ID NO: 24	
713	pJet-Prbc-fbpI	rbc	Amp	/	EcoRI/XbaI	EcoRI/XbaI	recipient for EcMgsA 712 Plasmid SEQ ID NO: 26	
712	pJet-EcMgsA		Amp	313 and 314	blunt end cloning		E.coli MgsA aimed for cloning into #550, #707 and #713 Plasmid SEQ ID NO: 25	
717	pJet-PpetJ:EcMgsA	petJ	Amp	/	EcoRI/XhoI	EcoRI/XhoI	PpetJ:EcMgsA aimed for cloning into #550 Plasmid SEQ ID NO: 27	
718	pJet-Prbc:EcMgsA	rbc	Amp	/	EcoRI/XhoI	EcoRI/XhoI	Prbc:EcMgsA aimed for cloning into #550 Plasmid SEQ ID NO: 28	
728	pVZ326-PpetJ:EcMgsA PrbcL*:SynADH deg	petJ,rbcL	Gm	/	XbaI/SalI	XbaI/XhoI	PpetJ:EcMgsA out of #717 and cloned into #550 Cassette SEQ ID NO: 46	MgsA+ SynADH+
729	pVZ326-Prbc:EcMgsA PrbcL*:SynADH deg	rbc,rbcL	Gm	/	XbaI/SalI	XbaI/XhoI	PpetJ:EcMgsA out of #718 and cloned into #550 Cassette SEQ ID NO:	MgsA+ SynADH+

							47	
733	pJet Syncoccus7002 akr:oop NdeI PstI		Amp	328 and 329	blunt end cloning		Aldo-Ketoreductase from Synechococcus7002 aimed for cloning into 728/729 Plasmid SEQ ID NO: 29	
734	pJet Prbc*:EcGldA XmaI XbaI	Prbc*	Amp	326 and 327	blunt end cloning		E.coli K12 Glycerol Dehydrogenase aimed for cloning into 728/729 Plasmid SEQ ID NO: 30	
735	pJet Prbc*:EcFuc0:oop Xma-Xba	Prbc*	Amp	330 and 331	blunt end cloning		E.coli K12 Lactaldehyde Oxidoreductase aimed for cloning into 728/729 Plasmid SEQ ID NO: 31	
747	pVZ326-Prbc*:EcGldA-PpetJ:EcMgsA-PrbcL*-synADH deg.	Prbc*,PpetJ, PrbcL*	Gm	-	SmaI/XbaI	SmaI/XbaI	E. coli gldA cut from #734, cloned into #728 Cassette SEQ ID NO: 48	MgsA+ SynADH+ GldA+
748	pVZ326-Prbc*:EcGldA-Prbc:EcMgsA-PrbcL*-synADH deg.	Prbc*,Prbc, PrbcL*	Gm	-	SmaI/XbaI	SmaI/XbaI	E. coli gldA cut from #734, cloned into #729 Cassette SEQ ID NO: 49	MgsA+ SynADH+ GldA+
749	pVZ326-Prbc*:EcFuc0-PpetJ:EcMgsA-PrbcL*-synADH deg.	Prbc*,PpetJ, PrbcL*	Gm		SmaI/XbaI	SmaI/XbaI	E.coli fuc0 cut from #735, cloned into #728 Cassette SEQ ID NO: 50	MgsA+ SynADH+ FucO+
750	pVZ326-Prbc*:Fuc0-Prbc:EcMgsA-PrbcL*-synADH deg.	Prbc*,Prbc, PrbcL*	Gm		SmaI/XbaI	SmaI/XbaI	E.coli fuc0 cut from #735, cloned into #729 Cassette SEQ ID NO: 51	MgsA+ SynADH+ FucO+
767	pVZ326 Prbc*:EcFuc0-PpetJ:EcMgsA-PrbcL*:Syn7002Akr	rbc*, petJ, rbcL*	Gm	-	NdeI/SbfI	NdeI/PstI	Syn7002 Akr cut from #733, cloned into #749 Cassette SEQ ID NO: 52	MgsA+ Akr+ FucO+
768	pVZ326 Prbc*:Fuc0-Prbc:EcMgsA-PrbcL*:Syn7002Akr	rbc*, rbc, rbcL*	Gm	-	NdeI/SbfI	NdeI/PstI	Syn7002 Akr cut from #733, cloned into #750 Cassette SEQ ID NO: 53	MgsA+ Akr+ FucO+
769	pVZ326 Prbc*:EcGldA-PpetJ:EcMgsA-PrbcL*:Syn7002Akr	rbc*, petJ, rbcL*	Gm	-	NdeI/PstI	NdeI/PstI	Syn7002 Akr cut from #733, cloned into #747 Cassette SEQ ID NO: 54	MgsA+ Akr+ GldA+

Example 4

Alternate Enzymes for Conversion of Methylglyoxal to Lactaldehyde

[00140] As shown in FIG. 3, it is possible that multiple enzymes (GldA, SynADH, and SynAKR) can catalyze the conversion of methylglyoxal to lactaldehyde. To confirm this, and to quantitate the products of the reaction using the different enzymes, the genes encoding these

enzymes were transformed to *Synechocystis* sp. PCC 6803. The host cells were then measured for the presence of the gene construct and for the ability to produce the intermediate. The results show that all three enzymes GldA, SynADH and SynAKR were able to convert methylglyoxal to lactaldehyde (FIG. 10, 13 and 14).

[00141] It was found that the enzymes GldA and SynAKR appeared to be better than SynADH for converting methylglyoxal to lactaldehyde. This may be because SynADH has a fairly low affinity to methylglyoxal (as shown in in vitro studies), causing a buildup of toxic methylglyoxal. Indeed, cultures of host cells containing the 728 and 729 plasmids, which carry the MgsA and SynADH genes, grew poorly or were lethal, which was likely to be due to the effects of accumulation of the toxic intermediate methylglyoxal in those cells.

Example 5

Alternate Enzymes for Conversion of Lactaldehyde to 1,2-Propanediol

[00142] As shown in FIG. 3, it is possible that multiple enzymes (FucO, GldA) can catalyze the conversion of lactaldehyde to 1,2-propanediol. To confirm this, and further to quantitate the products of the reaction using the different enzymes, the genes encoding these enzymes were transformed to *Synechocystis* sp. PCC 6803. The host cells were then measured for the presence of the gene construct and for the ability to produce 1,2-propanediol. The results show that both enzymes GldA and FucO were successfully expressed and their activity could be determined in vitro (FIG. 10, 13 and 14). The activity for GldA in converting methylglyoxal to lactadehyde was higher than the rate for the conversion to 1,2-propanediol. Similar results were observed for the FucO enzymatic assay. It was concluded that the final step in this metabolic pathway is the rate limiting step for the production of 1,2-propanediol.

Example 6

MgsA and GldA/SynADH Activity in Host Cells transformed with genes encoding GldA, MgsA, and SynADH

[00143] Host cyanobacterial cells were transformed with the construct #747 and #748 (FIG. 5; Table 3). Each of these constructs contained the genes encoding GldA, MgsA, and SynADH. Each of the inserted genes was controlled by its own promoter, as shown in Table 3.

[00144] To determine MgsA activity in converting DHAP to methylglyoxal, the following method was used. Frozen pelleted cells were suspended in imidazole buffer (40 mM, pH 7) and total protein was extracted by grinding with glass beads at 30Hz for 10 minutes. DHAP (750 μM) was added to the protein cell extract to start the reaction. Enzyme activity of MgsA was indicated by the rate of the production of methylglyoxal. Methylglyoxal reacts spontaneously with reduced glutathione, forming hemithioacetal, which is then converted to S-lactoglutathione. Thus, MgsA activity can be indirectly measured by the increase of absorption of S-lactoglutathione at OD_{240} over time. By use of this method, the activity of MgsA was confirmed in the transformed host cells. (FIG. 9, FIG. 12).

[00145] Both GldA and SynADH are NADPH-dependent enzymes (FIG. 3). Therefore, the NADPH level was measured to determine the combined activity of the GldA and SynADH enzymes. The following method was used: Frozen pelleted cells were suspended in imidazole buffer (40 mM, pH 7) and total protein was extracted by grinding with glass beads at 30Hz for 10 minutes. NADPH (200 μM) was added to the protein cell extract. Addition of 10 mM methylglyoxal started the reaction for combined GldA/synAKR activity or activity of SynADH alone, respectively. The change in OD_{340} (maximum absorbance of NADPH) was measured to indicate enzyme activity.

[00146] The activity of MgsA or GldA/SynADH was then determined, as shown in FIG. 9. It was found that when the in vitro reaction was started with the substrate DHAP for the first step in the reaction, strain # 748 had a high level of MgsA activity (about 3,000 nmol/mg protein*minute). When the same reaction was started with the intermediate substrate methylglyoxal, instead, the strains showed GldA/SynADH activity: #747: about 275 nmol/mg protein*minute; #748: about 610 nmol/mg protein*minute (FIG. 10).

Example 7

Determination of enzymatic activity of the SynAKR enzyme

[00147] The NADPH-dependent enzyme synAKR is capable of catalyzing the conversion of methylglyoxal to lactaldehyde. The following method was used to measure the activity of synAKR. Frozen pelleted cells were suspended in imidazole buffer (40 mM, pH 7) and total protein was extracted by grinding with glass beads at 30Hz for 10 minutes. NADPH (200μM)

was added to the protein cell extract. Methylglyoxal (10 mM) was added to start the reaction. The change in the absorption OD_{340} (maximum absorbance of NADPH) was measured to indicate enzyme activity.

Example 8

Determination of Enzymatic Activity of the FucO enzyme

[00148] The enzyme FucO is capable of catalyzing the NADPH-dependent conversion of hydroxyacetone to 1,2-propanediol. To determine the enzymatic activity of FucO, the following method was used. Frozen pelleted cells were suspended in imidazole buffer (40 mM, pH 7) and total protein was extracted by grinding with glass beads at 30Hz for 10 minutes. NADPH (200μM) was added to the protein cell extract. Hydroxyacetone (10 mM) was added to start the reaction. The change in the absorption OD_{340} (maximum absorbance of NADPH) was measured to indicate enzyme activity.

[00149] By use of this method, the activity of the enzyme FucO was confirmed in the host cells transformed with the *FucO* gene. Additionally, the increased product from the combination of synAKR and FucO or MgsA, SynAKR, and FucO was shown (FIG. 13, FIG. 14).

Example 9

Calculations of enzymatic activity

[00150] The activity of each of the enzymes described above (Examples 6-8) was determined as follows. The maximum slope in the linear area (ΔE) was determined based on the detected change in extinction over time. The protein concentration was determined by the Lowry method in order to calculate the specific activity, as shown below.

Beer-Lambert-Law:

$$E\lambda = \varepsilon\lambda * c * d$$

where:

$\varepsilon\lambda$: molar Extinction coefficient of sample at specific wavelength λ

c : concentration of sample

d : layer thickness (here d = 1cm)

Thus the change of extinction over time (ΔE) is:

$$\Delta E = E\lambda * min^{-1} = \varepsilon\lambda * c * d * min^{-1}$$

This leads to the change of concentration over time $c * min^{-1}$

$$c * min^{-1} = \Delta E * \varepsilon\lambda$$

$$-1 * d^{-1} * min^{-1}$$

Considering the dilution factor b (Volume of sample/ Volume of used cell extract) and the overall protein concentration c_{ov} , leads to the specific activity A_{spec}

$$A_{spec} = c * min^{-1} * b * c_{ov}$$

-1

The basal specific activities of the wild type cells on the corresponding day were subtracted from each value. The resulting enzyme activity measurements are shown in FIGs. 9-10 and 12-14.

Example 10

Construction of Polycistronic Plasmids for 1,2-Propanediol Production in Cyanobacteria

[00151] The gene cassettes for 1,2-propanediol production shown in Example 3, above, contain genes that were each regulated by their own upstream promoter. To determine whether the genes could be regulated by just one upstream promoter controlling expression of several or all of the pathway genes, several polycistronic gene cassette arrangements were also prepared and tested, as detailed below.

[00152] Many of the broad-host range plasmids described herein are derived from the RSF1010-derivative plasmid pSL1211, as shown in FIG. 15. An IPTG-inducible *srp* promoter and a kanamycin resistance gene were ligated into pSL1211, generating the plasmid pABb, to be used as a backbone plasmid for the heterologous expression of propanediol genes (FIG. 16).

[00153] To determine whether all of the 1,2-propanediol pathway genes could be regulated by just one upstream promoter controlling expression of all of the pathway genes, a gene cassette having the following four enzymes (*gldA - yqhD - fucO - mgsA*), designed to have polycistronic expression driven by the Psrp promoter in a single operon, was prepared as shown in FIG. 17.

[00154] Each of the genes had its own RBS (ribosome-binding site). The genes were inserted into an RSF1010-derived plasmid backbone, as shown in Table 5. One construct was termed “pAB1025”. The genes were amplified from wild type *E. coli* using the primers listed

below in Table 4, following the manufacturer's protocol for Phusion polymerase. Overlap PCR was used to combine *gldA* and *yqhD* into a single PCR product and to combine *fucO* and *mgsA* into a single PCR product. These were ligated into TOPO blunt cloning vectors according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). The TOPO vector containing *gldA-yqhD* was digested with *Nhe*I/*Spe*I, while the genes *fucO-mgsA* were digested out of the TOPO vector with *Acc*65I/*Bpu*10I. These were combined in a standard ligation reaction to make a TOPO-based plasmid containing the full operon, named pAB1012. The operon was amplified from pAB1012 by PCR with primers gldA F5 and mgsA R5 and inserted into the pABb plasmid (FIG. 3) digested with *Eco*RI /*Sbf*I in a standard SLIC reaction to create pAB1025.

[00155] Additional plasmid constructs pAB1030 (SEQ ID NO: 44) and pAB1068 (SEQ ID NO: 45), differing only by the choice of promoter, were also prepared. These were constructed by amplifying the operon with primers which had the appropriate 5' ends for inserting the PCR product into the appropriate vector by SLIC or by recombination cloning using GENEART Seamless Cloning and Assembly Kit from Invitrogen (Carlsbad, CA, USA). Plasmid pAB1030, which was the same as pAB1025 except for the presence of a $PnblA_{7120}$ promoter rather than Psrp to drive the 1,2-propanediol gene expression, was prepared as described above, except the vector used was pAB412, an RSF1010-derived plasmid containing a spectinomycin selection marker and a gene cassette (ZmPDC-SynADH) under the control of an $nblA_{7120}$ promoter. The pAB412 vector was digested with *Eco*RI and *Pst*I to remove the existing ZmPDC-SynADH gene cassette which created ends compatible for the previously described SLIC reaction. Plasmid pAB1068, which was the same as the pAB1025 plasmid except that it contained an $smtA_{7002}$ promoter to drive the expression of the 1,2-propanediol genes rather than an srp promoter, was prepared as described above, except the vector used was pAB421, an RSF1010-derived plasmid containing a gentamycin and spectinomycin resistance selection markers and a gene cassette (ZmPDC-SynADH) under the control of the $smtA_{7002}$ promoter. The pAB421 vector was digested with *Eco*RI and *Pst*I to remove the existing ZmPDC-SynADH gene cassette which created ends compatible for the previously described SLIC reaction.

Table 4: Primers for Construction of 1,2-Propanediol-Producing Plasmids

Primer name	PCR Primer sequences
gldA F5	aatgtgtggatcagcaggacgcactgaccgGAATTCGGCGCGCCagaggagaaCTTAAG atggaccgcattattcaatcac (SEQ ID NO: 32)
gldA R5	ttgttcatatgtagatctcctGTTAATTAAttattcccactcttgcaggaaac (SEQ ID NO: 33)
yqhD F5	agtgggaataaTTAATTAACaggagatctacatatgaacaactttaatctgcacacc (SEQ ID NO: 34)
yqhD R5	agccatcatGCTAGCtctcctcGGCCGGCCgcttagcgggcggcttcg (SEQ ID NO: 35)
fucO F5	gcccgctaaGGCCGGCCgaggagaGCTAGCatgatggctaacagaatgattctg (SEQ ID NO: 36)
fucO R5	cagttccatGCTAGCtctcctcGGCCGGCCttaccaggcggtatggtaaagc (SEQ ID NO: 37)
mgsA F5	gcctggtaaGGCCGGCCgaggagaGCTAGCatggaactgacgactcgcac (SEQ ID NO: 38)
mgsA R5	cgctactgccgccaggcaaattctgtttccTGCAGGCGCGCCttacttcagacggtccgcga (SEQ ID NO: 39)
gldA R	GGCACTGGCTGAACTGTGCTACAA (SEQ ID NO: 40)
fucO L	ACTTGCGCCGTTTCTCTTCGTC (SEQ ID NO: 41)
SpcF3	CTCGGGCATCCAAGCAGC (SEQ ID NO: 42)
SpcF	GTAGAGCTATTCACTTTAGGTTTAG (SEQ ID NO: 43)

Example 11

Confirmation of Plasmid Sequences and 1,2-Propanediol Production in *E. coli*

[00156] Several plasmid constructs (pAB1025, pAB1030 (SEQ ID NO: 44), pAB1061, pAB1062, pAB1068 (SEQ ID NO: 45), pAB1012, pAB1007, and pAB1008), each having the 1,2-propanediol pathway genes described in Example 10 were prepared and confirmed. The constructs differed in the choice of promoter, *E. coli* origin of replication, and cyanobacterial origin of replication, as shown below in Table 5. The sequence of the above-described plasmid pAB1025 was confirmed by digestion with the restriction enzyme *Ava*II and by sequencing. Plasmid pAB1030 was confirmed by digestion with the restriction enzyme *Bam*HI and by sequencing. Plasmid pAB1068 was confirmed by digestion with the restriction enzyme *Xmn*I and by sequencing. The ability of the plasmid to produce 1,2-propanediol was first confirmed in *E. coli*. Once propanediol production in *E. coli* was confirmed, the plasmids were ready for transformation to cyanobacteria.

Table 5: 1,2-Propanediol Polycistronic Plasmids

Plasmid Name	Promoter	Gene Cassette	E coli Origin of Replication	Cyanobacterial Origin of Replication
pAB1025	Psrp	*gldA-yqhD-fucO-mgsA*	RSF1010	RSF1010
pAB1030	$PnblA_{7120}$	*gldA-yqhD-fucO-mgsA*	RSF1010	RSF1010
pAB1061	ziaR-$PziaA_{6803}$	*gldA-yqhD-fucO-mgsA*	RSF1010	RSF1010
pAB1062	$PsmtA_{7002}$	*gldA-yqhD-fucO-mgsA*	pUC	pAQ1
pAB1068	$PsmtA_{7002}$	*gldA-yqhD-fucO-mgsA*	RSF1010	RSF1010
pAB1012	Plac	*gldA-yqhD-fucO-mgsA*	pBR	N/A
pAB1007	Plac	*gldA-yqhD*	pBR	N/A
pAB1008	Plac	*fucO-mgsA*	pBR	N/A

Example 12

Transformation of Plasmids Harboring the Polycistronic 1,2-Propanediol-Producing Genes to Cyanobacteria

[00157] Cyanobacterial strains *Synechococcus* sp. PCC 7002 and *Synechocystis* sp. PCC 6803 were transformed with the plasmids harboring the 1,2-propanediol-producing genes. *Synechocystis* sp. PCC 6803 was transformed with plasmids pAB1025, pAB1030, and pAB1068. *Synechococcus* sp. PCC 7002 was transformed with plasmids pAB1025, pAB1030, and pAB1068. Transformation procedures were performed via conjugation as follows. One week before the day of conjugation, cyanobacterial cells (e.g. PCC 7002 and PCC 6803) were inoculated with a fresh culture using a ~ 1:10 dilution of an older (1 week) culture. *E. coli* cultures containing the plasmid(s) of interest and the helper plasmid pRL443 (described in Elhai et al. (1997), Journal of Bacteriology, 179:1998-2005) were started the night before the planned conjugation in ~3 ml LB supplemented with the appropriate antibiotic(s). Four hours prior to conjugation, 30 ml of fresh LB medium (with appropriate antibiotic(s)) was inoculated with ~0.5 ml of the overnight culture. The *E. coli* and cyanobacterial cultures were transferred to a 50 ml conical tube and centrifuged at 2,500 x g for 10 minutes at room temperature to pellet the cells. The supernatant was decanted, and the cell pellets were resuspended in 1 ml LB (for the *E. coli* cultures) or (M)BG-11 (for cyanobacteria). The cells were then transferred to a microcentrifuge tube and centrifuged at 2,500 x g for 10 minutes at room temperature. The decanting, resuspension, and centrifuge steps were repeated, resuspending each pellet in 300 µl LB or (M)BG-11, as appropriate. The cell resuspensions were diluted and the cells were counted.

[00158] The next step involved preparing a mixture of cyanobacterial cells, the helper plasmid, and the plasmids harboring the 1,2-propanediol-producing genes in about a 1:1:1 cell count ratio. Approximately 3.6×10^8 cells each of cyanobacteria, *E. coli* with plasmid pRL443, and *E. coli* with the plasmid of interest was placed in a microcentrifuge tube. The cell mixture was then centrifuged at 2,500 x g for 5 minutes at room temperature. The supernatant was decanted and the pellet was resuspended in 950 µl (M)BG-11 and 50 µl LB. Sterilized cellulose nitrate membrane filters (Whatman) were transferred to (M)BG-11 (vB_{12}) + 5% LB agar plates. A 200 µl aliquot of the mixture was spread evenly on the filter. The agar plate was then placed in low light for two days. The filter was then transferred onto a fresh (M)BG-11 (vB_{12}) agar

plate containing the appropriate selective antibiotic. MBG-11 vB_{12} plates had the following final antibiotic concentrations: spectinomycin, 100 μg/ml; kanamycin, 40 μg/ml. BG-11 plates had the following final antibiotic concentrations: spectinomycin, 15 μg/ml; kanamycin, 10 μg/ml. After 8-12 days, the presence of single colonies on the filters was monitored. Once single colonies were observed, the colonies were streaked onto a fresh selective plate (1st pass plate). The process was repeated (2nd pass plate). Once colonies were observed on the 2nd pass plate, the patch was taken and streaked onto an LB plate to check for potential *E. coli* contamination. Clean patches were used to perform colony PCR to test for the plasmid of interest.

Example 13

Colony PCR for Verification of Transformation

[00159] To confirm the presence of the 1,2-propanediol genes in the cyanobacterial host cells, streaks from colonies were resuspended in TE buffer (MediaTech, Inc, Manassas, VA, USA, and the cells were disrupted with glass beads. The supernatants were used as a DNA template for PCR amplifications of fragments of the 1,2-propanediol genes using the gldA R and fucO L primers. The results of the PCR analysis confirmed the presence of the 1,2-propanediol genes in the host cells.

[00160] Cells from verified streaks were then used to inoculate 3 ml liquid BG-11 or MBG-11 vB_{12} cultures supplemented with the appropriate antibiotics (MBG-11 vB_{12} medium had the following final antibiotic concentrations: spectinomycin, 100 μg/ml; kanamycin, 40 μg/ml; BG-11 medium had the following final antibiotic concentrations: spectinomycin, 15 μg/ml; kanamycin, 10 μg/ml) and incubated under a light intensity of 10-20 μmol m^{-2} s^{-1} at 37°C.

Example 14

Extraction and Detection of 1,2-Propanediol

[00161] A methanol/phosphate extraction was used to separate 1,2-propanediol produced from the culture. Five ml of cyanobacterial culture was saturated with dipotassium phosphate (~6 g). This mixture was amended with methanol to a final methanol concentration of 30%, and was then vigorously shaken three times with five minute rest intervals. This extraction was left overnight at room temperature to allow phase separation. The upper methanol layer was

collected avoiding the interface and evaporated to ~100 μl (15X concentration) in a benchtop centrifugal evaporator. This extract was passed through a 0.2 μm filter prior to analysis. The methanol extract was loaded onto a GC/MS using a liquid injection. 1,2-propanediol was measured using gas chromatography with flame ionization detection. A Stabilwax column (30 m length, 0.53 mm diameter, 1 μm film) was used on an Agilent 7890A GC system equipped with a 7683B liquid injector. A cyclo-uniliner was installed on the split/splitless injector and heated to 225°C. Two microliters were injected using a pulsed splitless program at 10 psi for 0.1 min. Using helium as the carrier gas at 50 cm/sec, separation was performed by running a linear thermal program from 80°C to 200°C at 24°C/min with a 5 minute hold at 200°C. Using this method, the retention time of 1,2-propanediol was 4.9 minutes. The cyanobacterial *Synechococcus* sp. PCC 7002 transformed with the plasmid pAB1025 produced ~1 μM or 72 μg/L 1,2-propanediol, as shown in FIG. 18. The cyanobacterial strain *Synechocystis* sp. PCC 6803 transformed with plasmid pAB1025 produced ~2.5 μM or 0.2 mg/L 1,2-propanediol, as shown in FIG. 19.

Example 15

Production of S-1,2-Propanediol

[00162] In a prophetic example, S-1,2-propanediol can be produced by following certain portions of the propanediol pathways shown herein. As shown in FIG. 2, the 1,2-propanediol intermediate acetol can be converted to the S form of 1,2-propanediol. Further, as demonstrated in Example 8, the enzyme FucO is capable of catalyzing the NADPH-dependent conversion of hydroxyacetone to 1,2-propanediol. In this prophetic example, a host cyanobacterial cell is transformed with a shuttle vector containing a plasmid carrying genes encoding the enzymes MgsA, methylglyoxyl reductase, and FucO. The production of S-1,2-propanediol is confirmed by chemical analysis. By use of this method, S-1,2-propanediol is produced in cyanobacteria.

Example 16

Tolerance Testing for Determination of Suitable Cyanobacterial Strains for 1,2-Propanediol Production

[00163] The tolerance of cyanobacterial strains PCC 6803 and PCC 7002 to the presence of accumulated 1,2-propanediol in the culture medium was examined by adding a one time bolus of varying amounts of 1,2-propanediol (1%, 2%, 3% and 5%) to exponential phase cultures and comparing the growth of these cultures to a wild type culture with no addition. Growth was monitored by optical density (OD_{750}) for one week. There was no difference in the growth of cultures containing up to 3% 1,2-propanediol compared to wild type for either strain. The addition of 5% 1,2-propanediol was found to inhibit *Synechocystis* sp. PCC 6803 causing less growth, discoloration and clumping, but the culture did not bleach out and die. The same effect was observed with the addition of 5% 1,2-propanediol to *Synechococcus* sp. PCC 7002. No lethal effect (complete bleaching) was observed within these parameters.

Example 17

Production of 1,2-Propanediol from a *Synechococcus* Culture in a 500 Liter Outdoor Photobioreactor

[00164] A strain of *Synechococcus* PCC 7002 cells modified to contain a 1,2-propanediol gene cassette is inoculated into a 500 L enclosed outdoor photobioreactor in seawater containing BG-11 nutrients and vitamin B_{12} (1 μg/ml) and grown for three months. Every two weeks, 50% of the culture medium is separated from the remaining cells and removed from the culture, and fresh replacement medium is added to the photobioreactor. The spent culture medium is filtered, pH treated, flocculated, filtered once again, then the resulting liquid is treated with a distillation procedure to result in substantially purified 1,2-propanediol. Following this method, a healthy, continuously growing cyanobacterial culture is able to produce 1,2-propanediol continuously for a range of time from about several months, to a year or more.

[00165] Although the present invention has been described in considerable detail with reference to certain embodiments thereof, other embodiments are possible. Therefore, the spirit and scope of the appended claims should not be limited to the description of the embodiments contained therein.

CLAIMS

What is Claimed is:

1. A genetically enhanced cyanobacterial cell, comprising:

a) at least one promoter capable of regulating gene expression in cyanobacteria; and

b) a *gldA* gene, a *fucO* gene, and an *mgsA* gene,

wherein said at least one promoter is operably linked to said *gldA*, *fucO*, and *mgsA* genes, further wherein said cell produces 1,2-propanediol.

2. The genetically enhanced cyanobacterial cell of claim 1, further comprising a *yqhD* gene.

3. The genetically enhanced cyanobacterial cell of claim 1 or 2, wherein at least one of said genes is present in a location selected from the group consisting of an exogenously derived extrachromosomal plasmid, an endogenous plasmid-derived extrachromosomal plasmid, and on the cyanobacterial chromosome.

4. The genetically enhanced cyanobacterial cell of claim 1 or 2, wherein said at least one promoter is selected from the group consisting of: Psrp, $PnblA_{7120}$, $PrbcL_{6803}$, $PsmtA_{7002}$, and ziaR-$PziaA_{6803}$.

5. The genetically enhanced cyanobacterial cell of claim 1 or 2, wherein the *gldA* gene has at least 98% identity to SEQ ID NO: 11.

6. The genetically enhanced cyanobacterial cell of claim 1 or 2, wherein the *gldA* gene encodes a polypeptide having at least 98% identity to SEQ ID NO: 12.

7. The genetically enhanced cyanobacterial cell of claim 1 or 2, wherein the *fucO* gene has at least 98% identity to SEQ ID NO: 13.

8. The genetically enhanced cyanobacterial cell of claim 1 or 2, wherein the *fucO* gene encodes a polypeptide having at least 98% identity to SEQ ID NO: 14.

9. The genetically enhanced cyanobacterial cell of claim 1 or 2, wherein the *mgsA* gene has at least 98% identity to SEQ ID NO: 15.

10. The genetically enhanced cyanobacterial cell of claim 1 or 2, wherein the *mgsA* gene encodes a polypeptide having at least 98% identity to SEQ ID NO: 16.

11. The genetically enhanced cyanobacterial cell of claim 2, wherein the *yqhD* gene has at least 98% identity to SEQ ID NO: 17.

12. The genetically enhanced cyanobacterial cell of claim 2, wherein the *yqhD* gene encodes a polypeptide having at least 98% identity to SEQ ID NO: 18.

13. The genetically enhanced cyanobacterial cell of claim 1 or 2, wherein said genes are located together under the regulation of one promoter.

14. The genetically enhanced cyanobacterial cell of claim 1 or 2, wherein at least one of the genes is present in a separate genetic region in the cell.

15. The genetically enhanced cyanobacterial cell of claim 14, wherein said separate genetic region in the cell is a different plasmid vector or a different chromosome.

16. The cyanobacterial cell of any one of claims 1-15, wherein said cyanobacterial cell is selected from the group consisting of *Synechocystis* sp. PCC6803 and *Synechococcus* sp. PCC7002.

17. A method of producing 1,2-propanediol in a cyanobacterial cell, comprising culturing a genetically enhanced cyanobacterial cell of claim 1 or 2 under conditions wherein the cyanobacterial cell produces 1,2-propanediol.

18. A method of producing 1,2-propanediol in a cyanobacterial cell, comprising:

a) transforming said cell with an mgsA gene, a gene encoding an enzyme capable of converting methylglyoxal to lactaldehyde, and a gene encoding an enzyme capable of converting lactaldehyde to 1,2-propanediol, and

b) producing 1,2-propanediol from the cyanobacterial cell.

19. The method of claim 18, wherein said gene encoding an enzyme capable of converting methylglyoxal to lactaldehyde is selected from the group consisting of GldA, SynADH, and SynAKR.

20. The method of claim 18, wherein said gene encoding an enzyme capable of converting lactaldehyde to 1,2-propanediol is selected from FucO and GldA.

21. The method of claim 18, wherein each of said genes is under the control of a separate promoter.

22. The method of claim 18, wherein all of the said genes are under the control of one promoter.

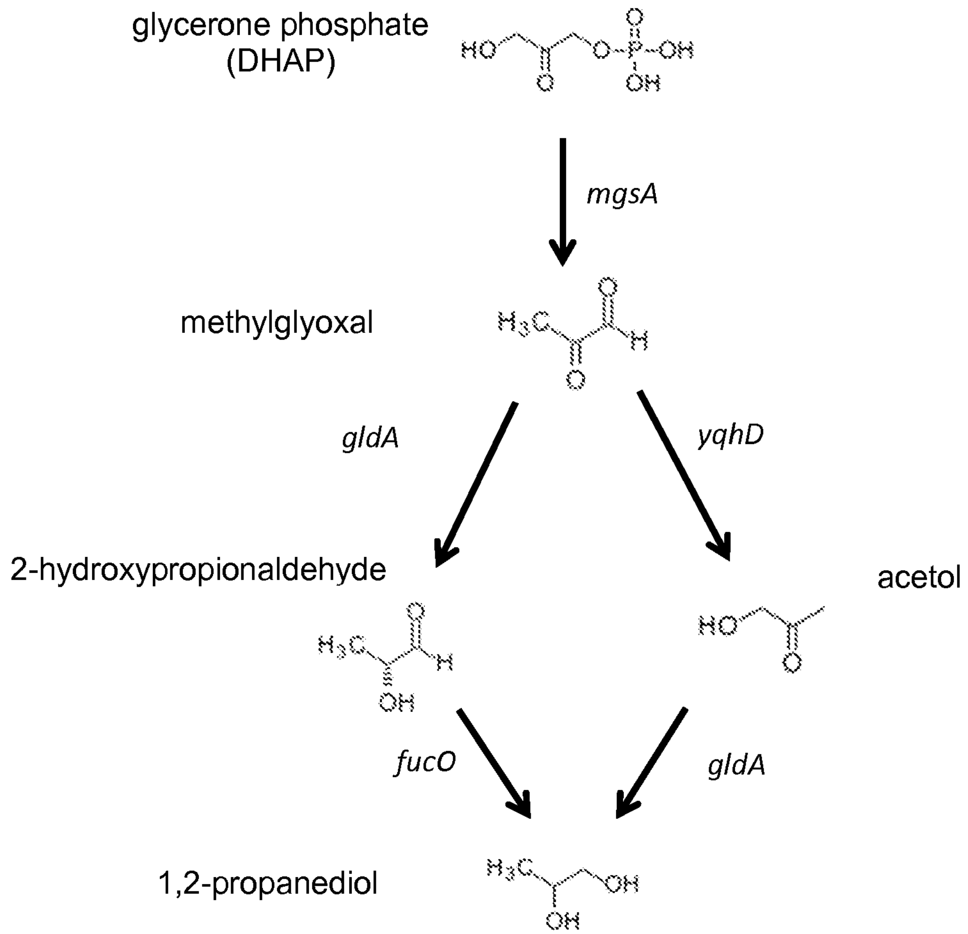


FIG. 1

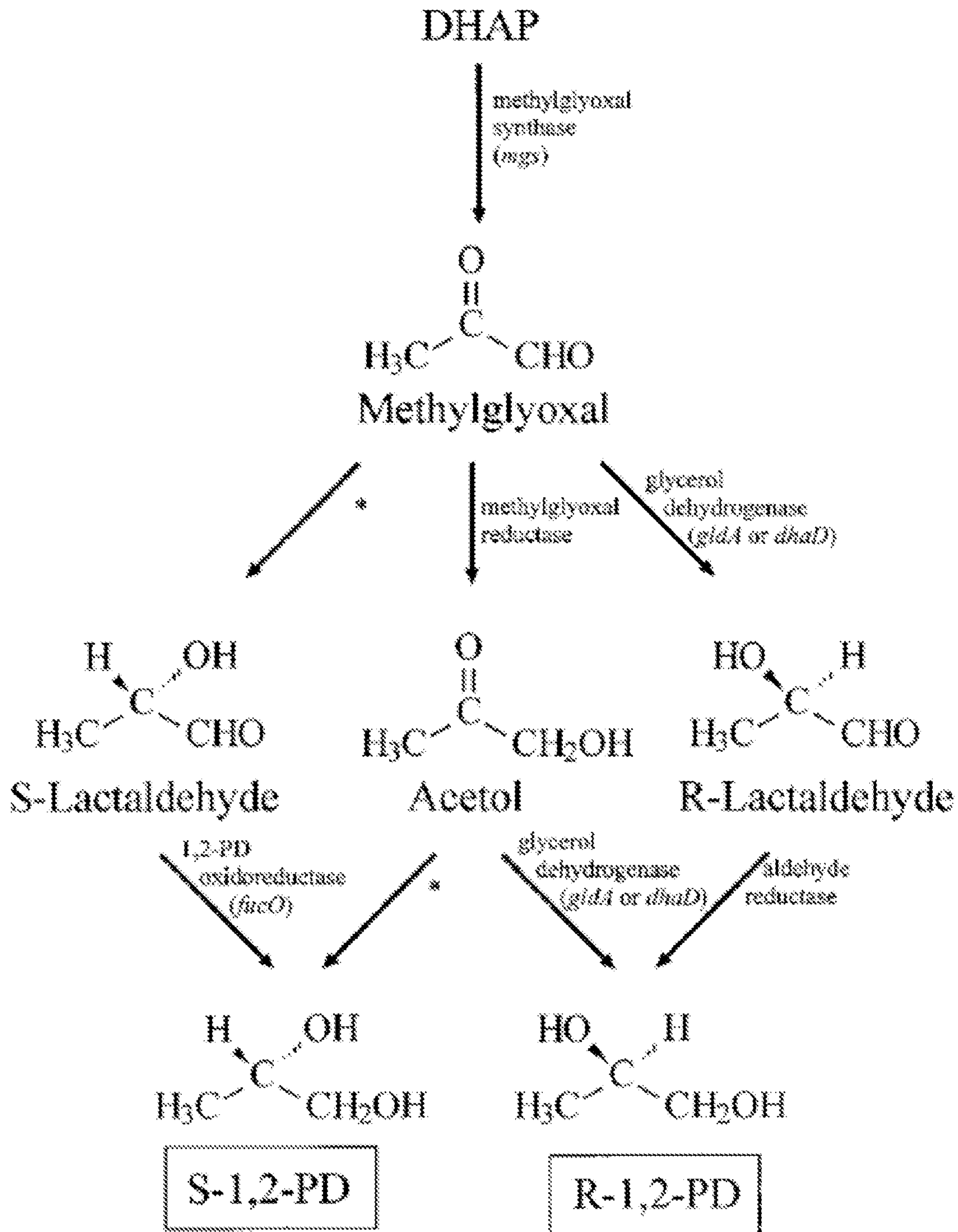


FIG. 2

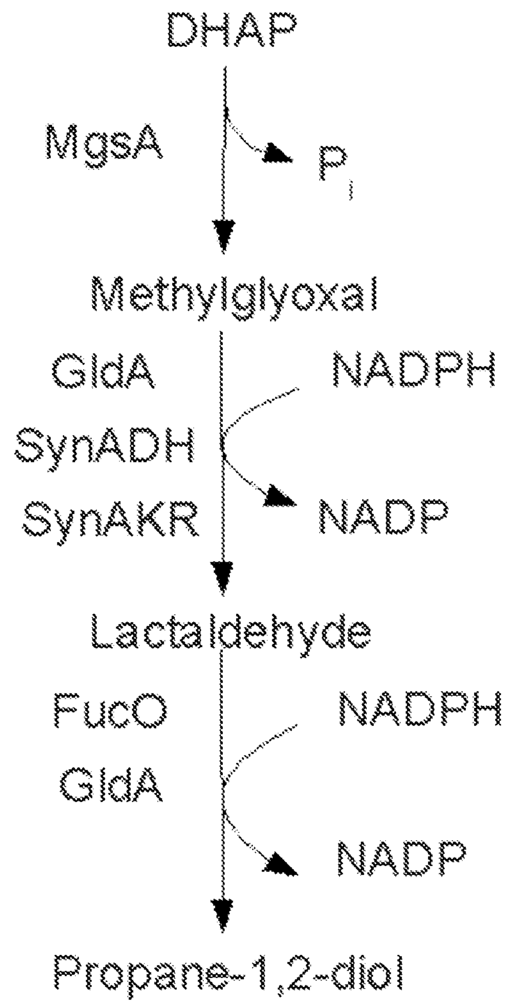
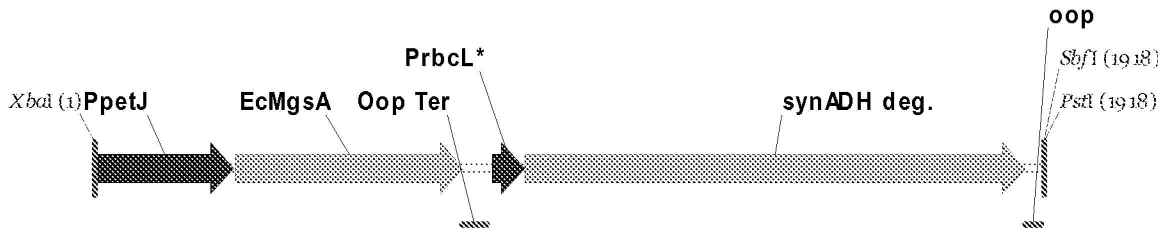
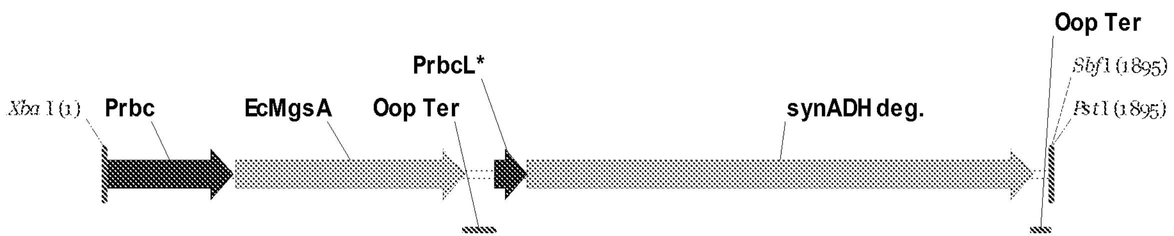


FIG. 3

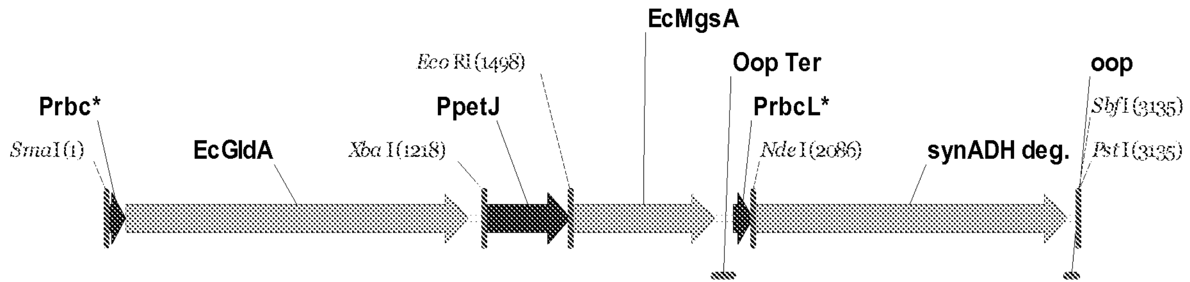


Fragment of #728 pVZ326-PpetJ:EcMgsA-PrbcL*-synADH deg.
1921 bp (molecule 10192 bp)

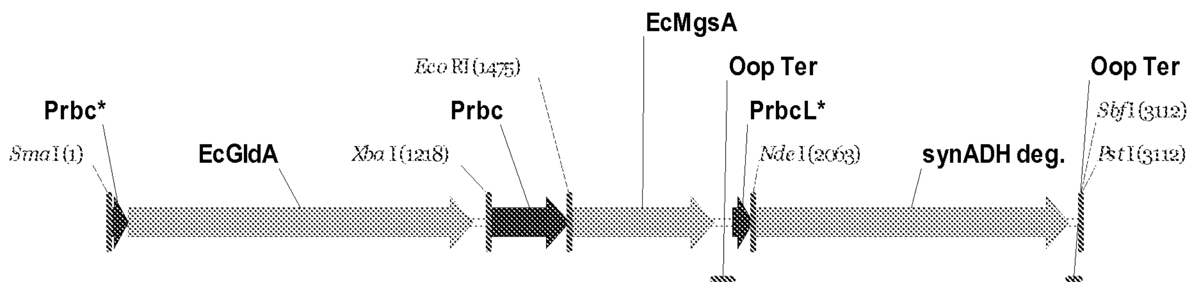


Fragment of #729 pVZ326-Prbc:EcMgsA-PrbcL*-synADH deg.
1897 bp (molecule 10169 bp)

FIG. 4

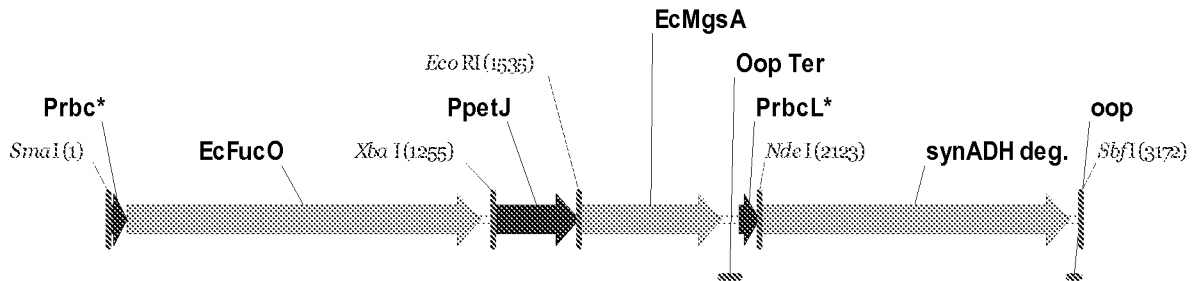


Fragment of #747 pVZ326-Prbc*:EcGldA-PpetJ:EcMgsA-PrbcL*-synADH deg.
3140 bp (molecule 10476 bp)

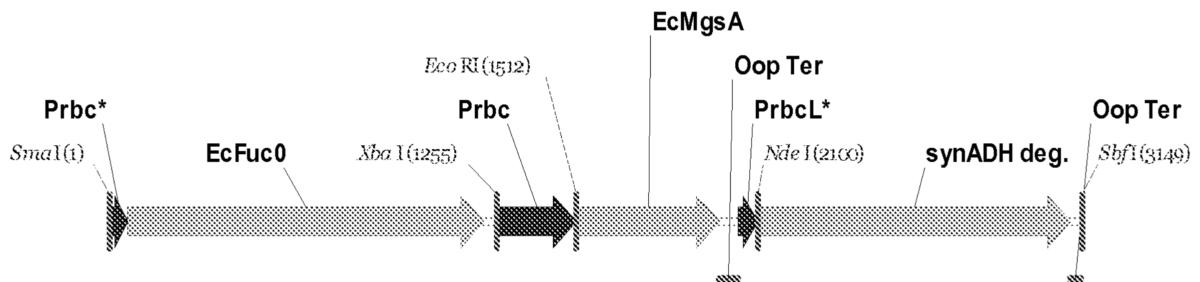


Fragment of #748 pVZ326-Prbc*:EcGldA-Prbc:EcMgsA-PrbcL*-synADH deg.
3117 bp (molecule 10453 bp)

FIG. 5

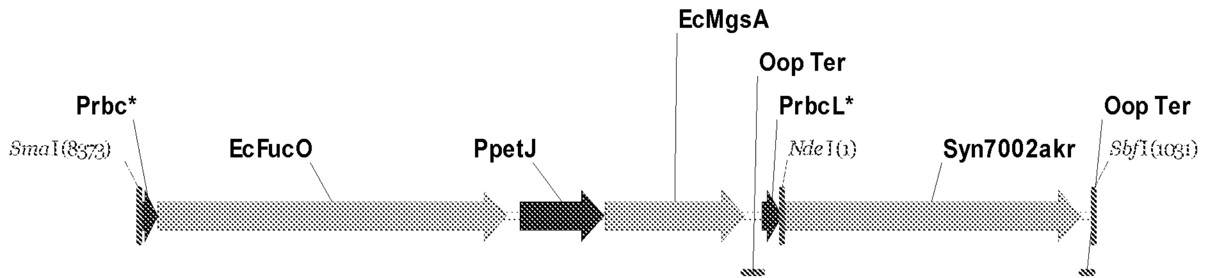


Fragment of #749 pVZ326-Prbc*:EcFuc0-PpetJ:EcMgsA-PrbcL*-synADH deg.
3176 bp (molecule 10513 bp)



Fragment of #750 pVZ326-Prbc*:Fuc0-Prbc:EcMgsA-PrbcL*-synADH deg.
3154 bp (molecule 10490 bp)

FIG. 6



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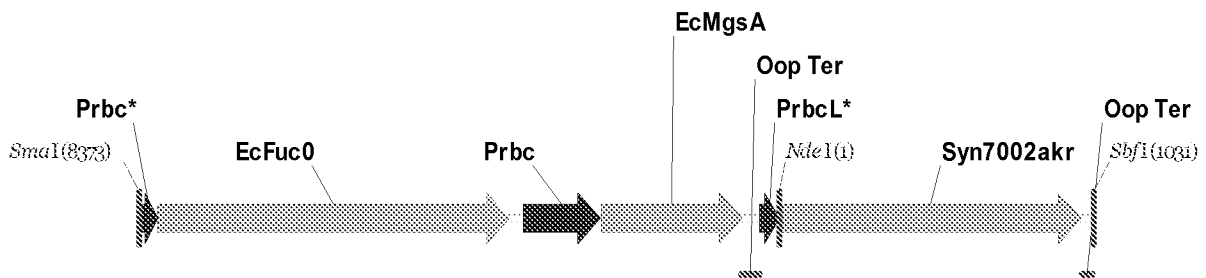
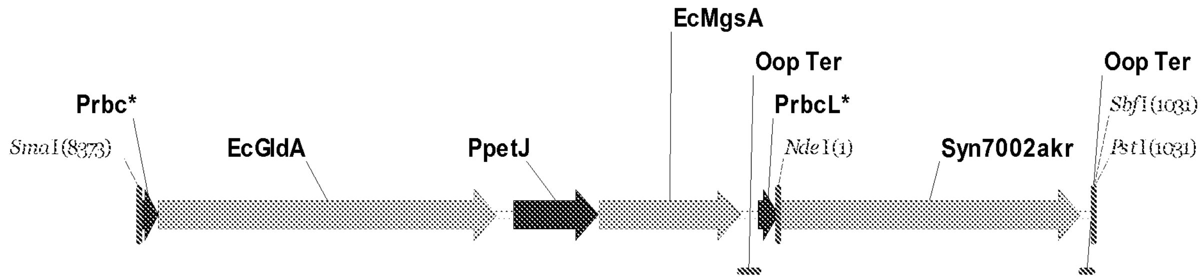
3158 bp (molecule 10494 bp)

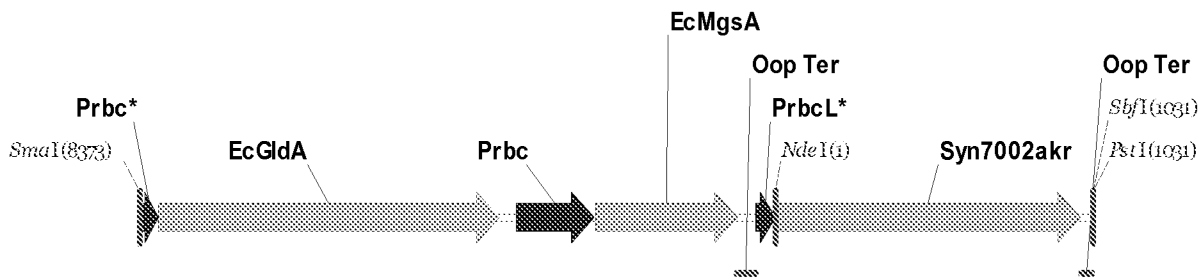


Fragment of #768 pVZ326 Prbc*:EcFuc0-Prbc:EcMgsA-PrbcL*:Syn7002Akr
3135 bp (molecule 10471 bp)

FIG. 7



Fragment of #769 pVZ326 Prbc*:EcGldA-PpetJ:EcMgsA-PrbcL*:Syn7002Akr
3122 bp (molecule 10457 bp)



Fragment of #770 pVZ326 Prbc*:EcGldA-Prbc:EcMgsA-PrbcL*:SynAkr
3097 bp (molecule 10434 bp)

FIG. 8

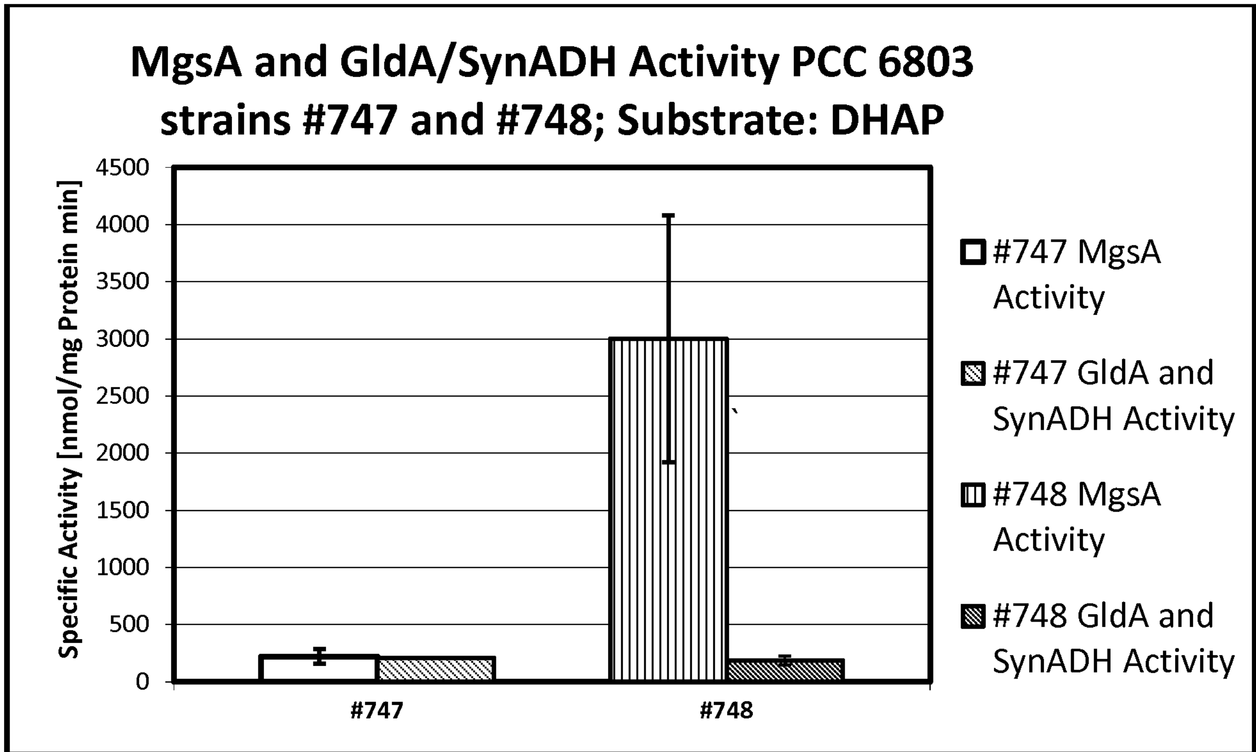


FIG. 9

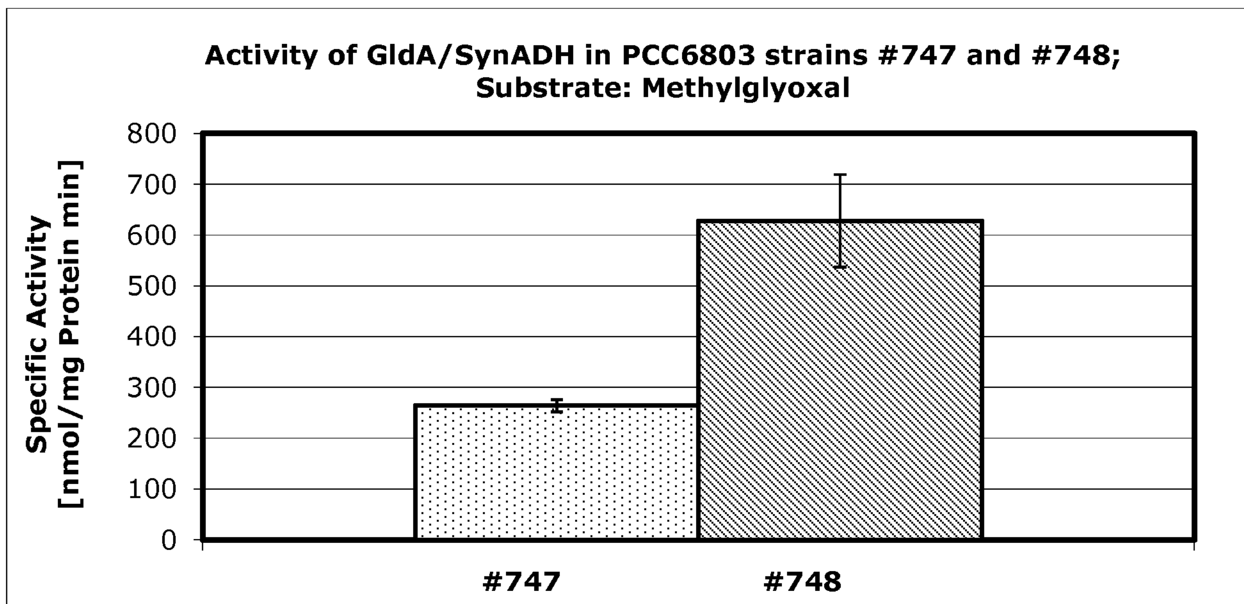


FIG. 10

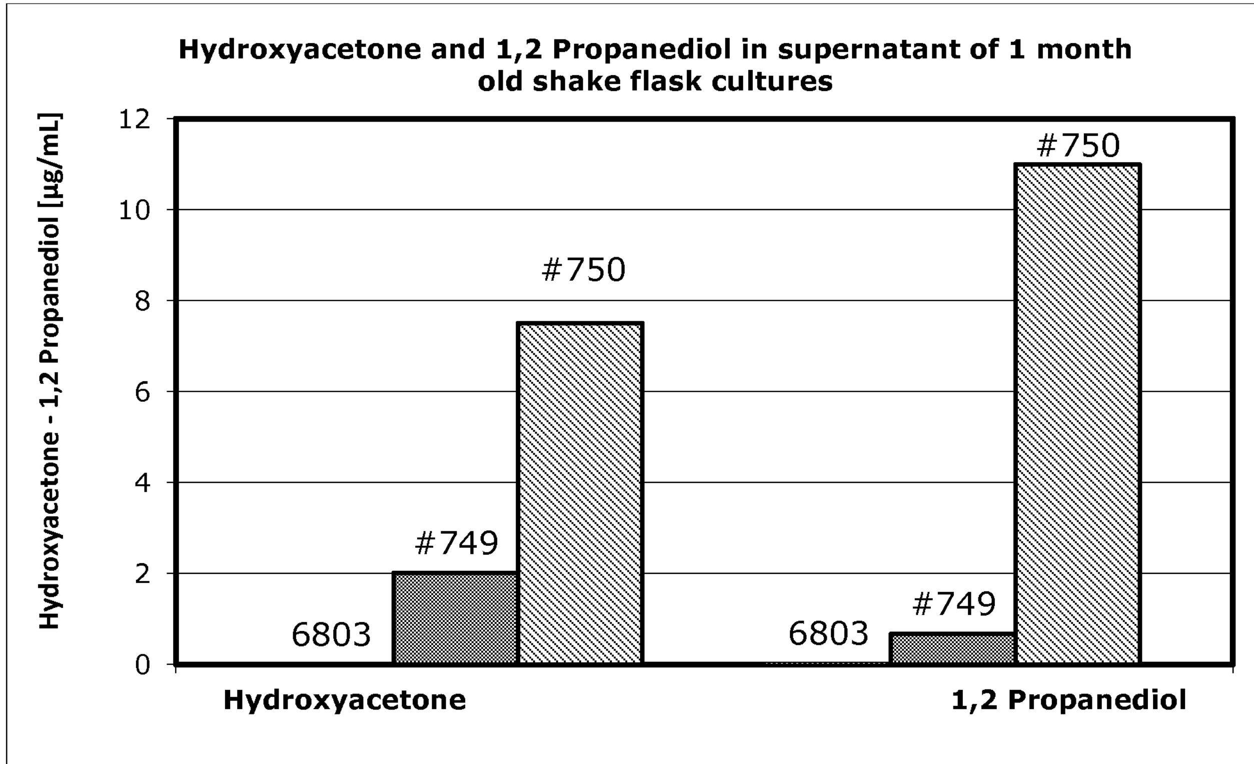


FIG. 11

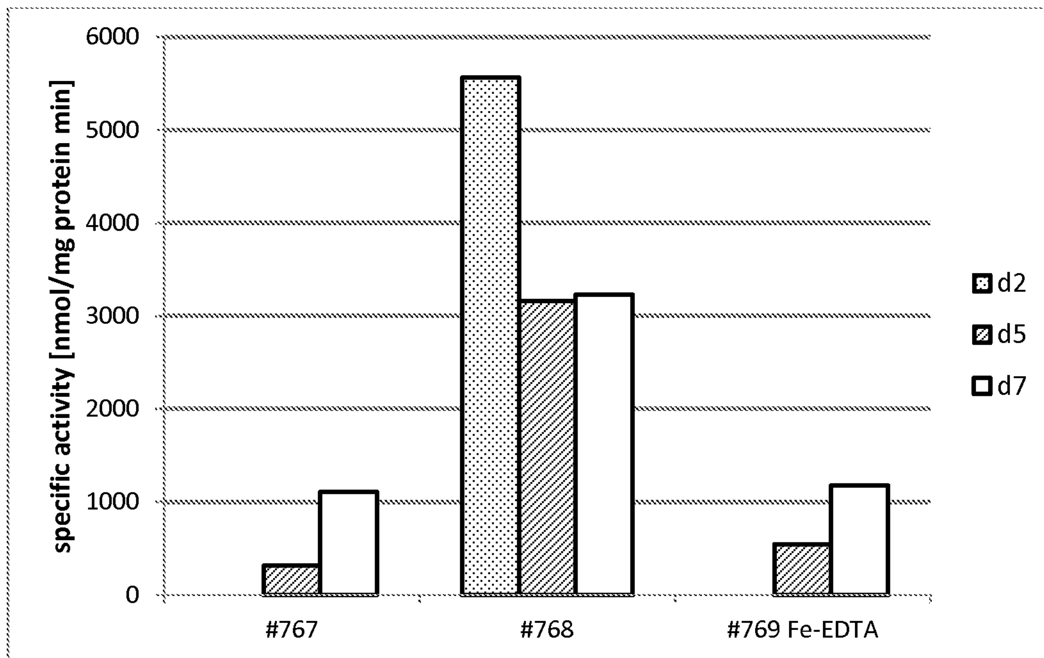


FIG. 12

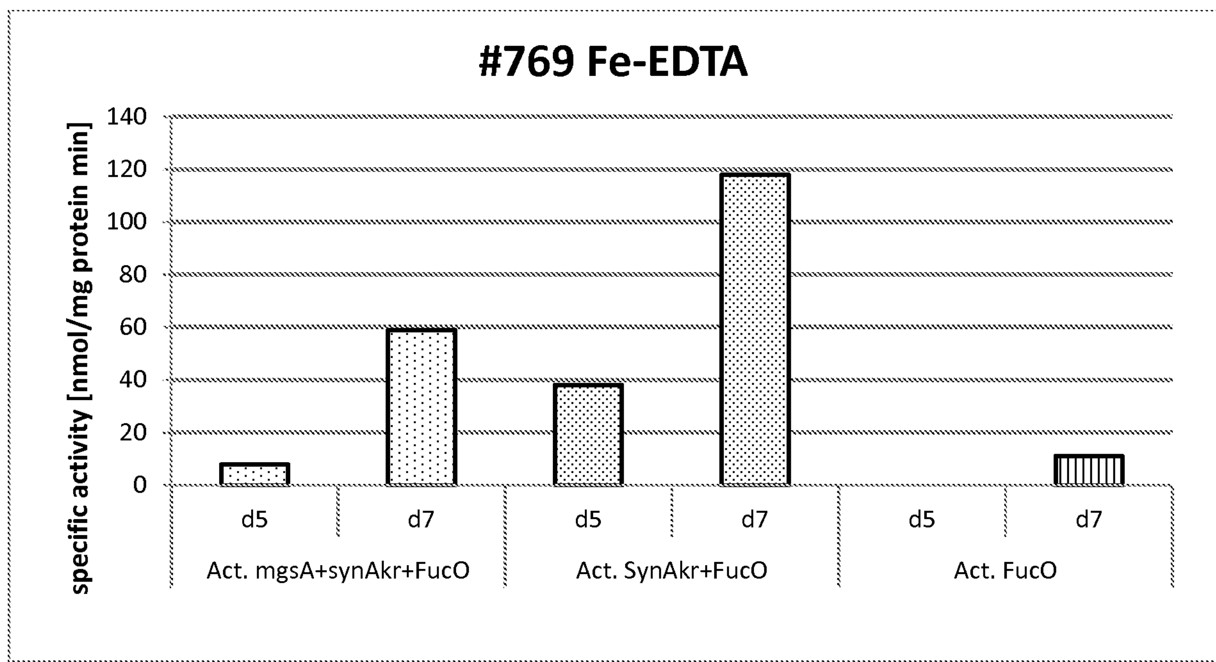


FIG. 13

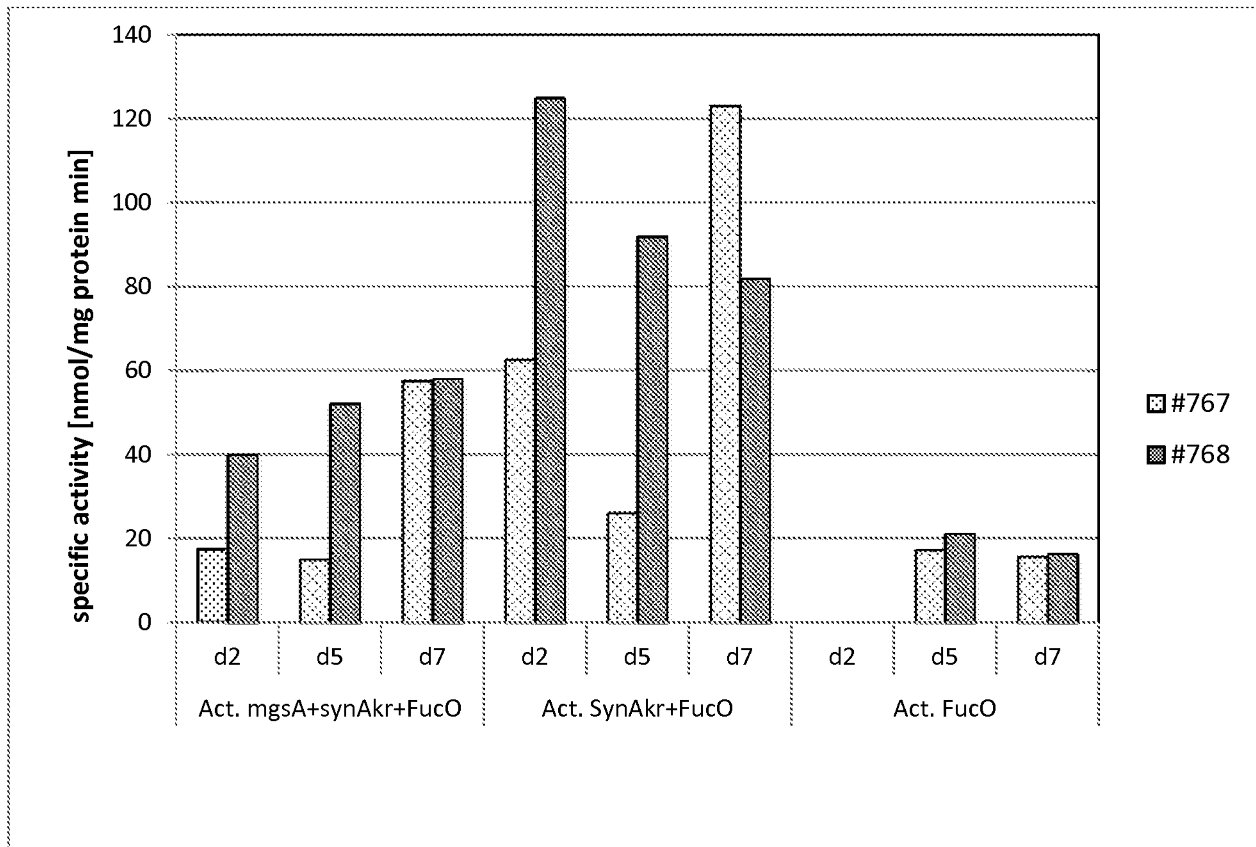


FIG. 14

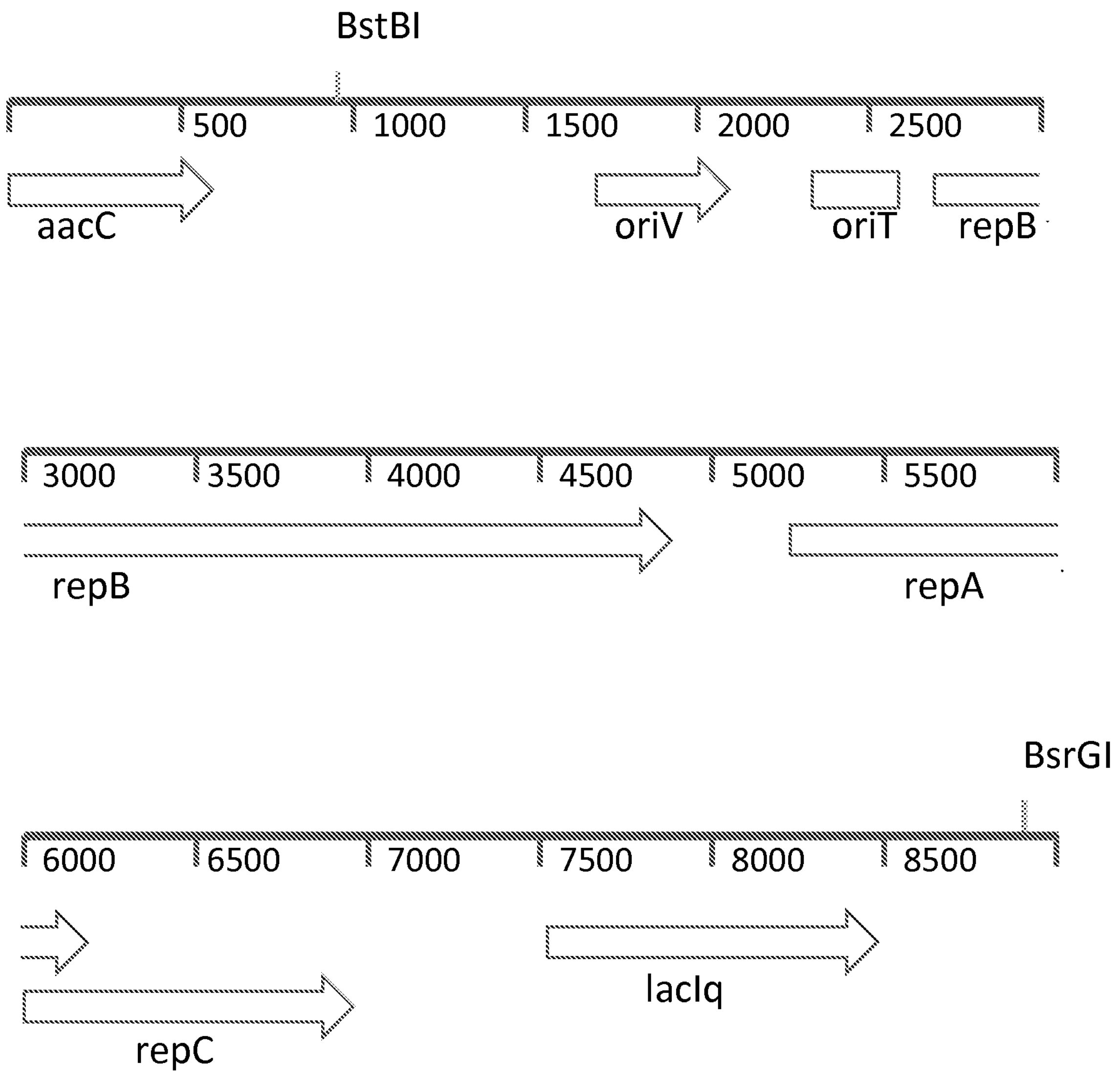


FIG. 15

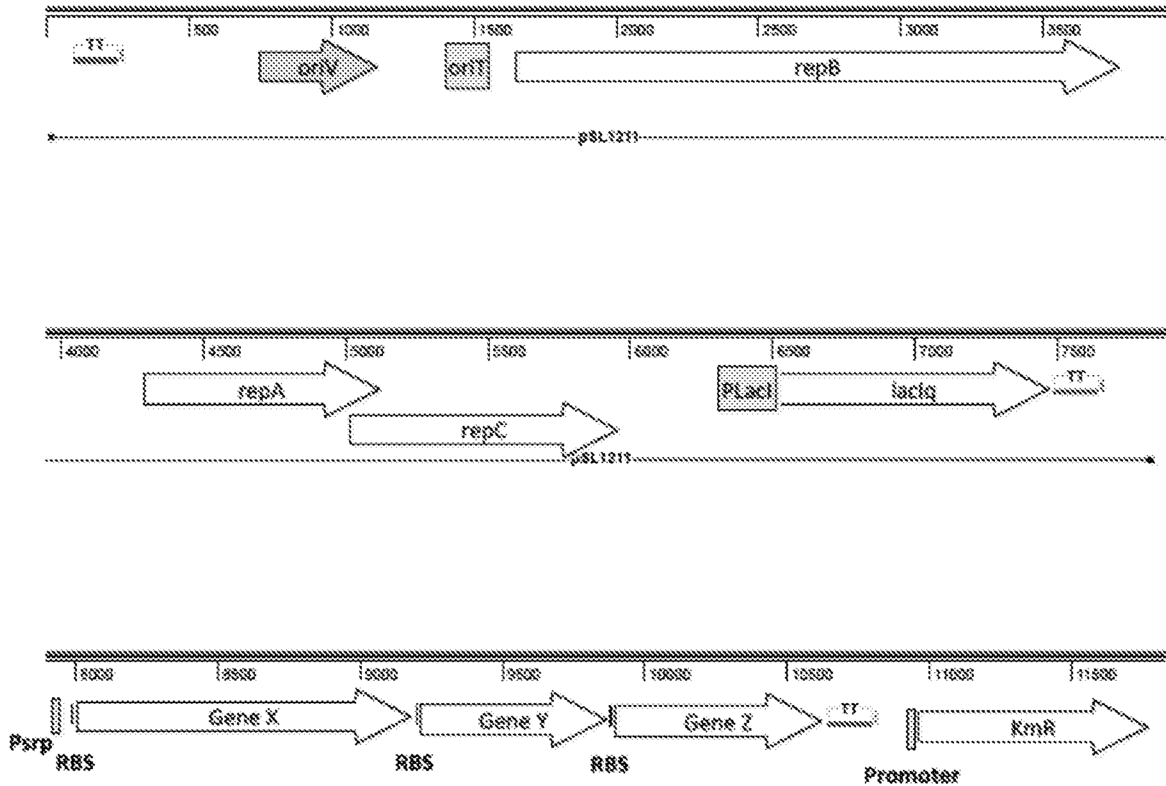


FIG. 16

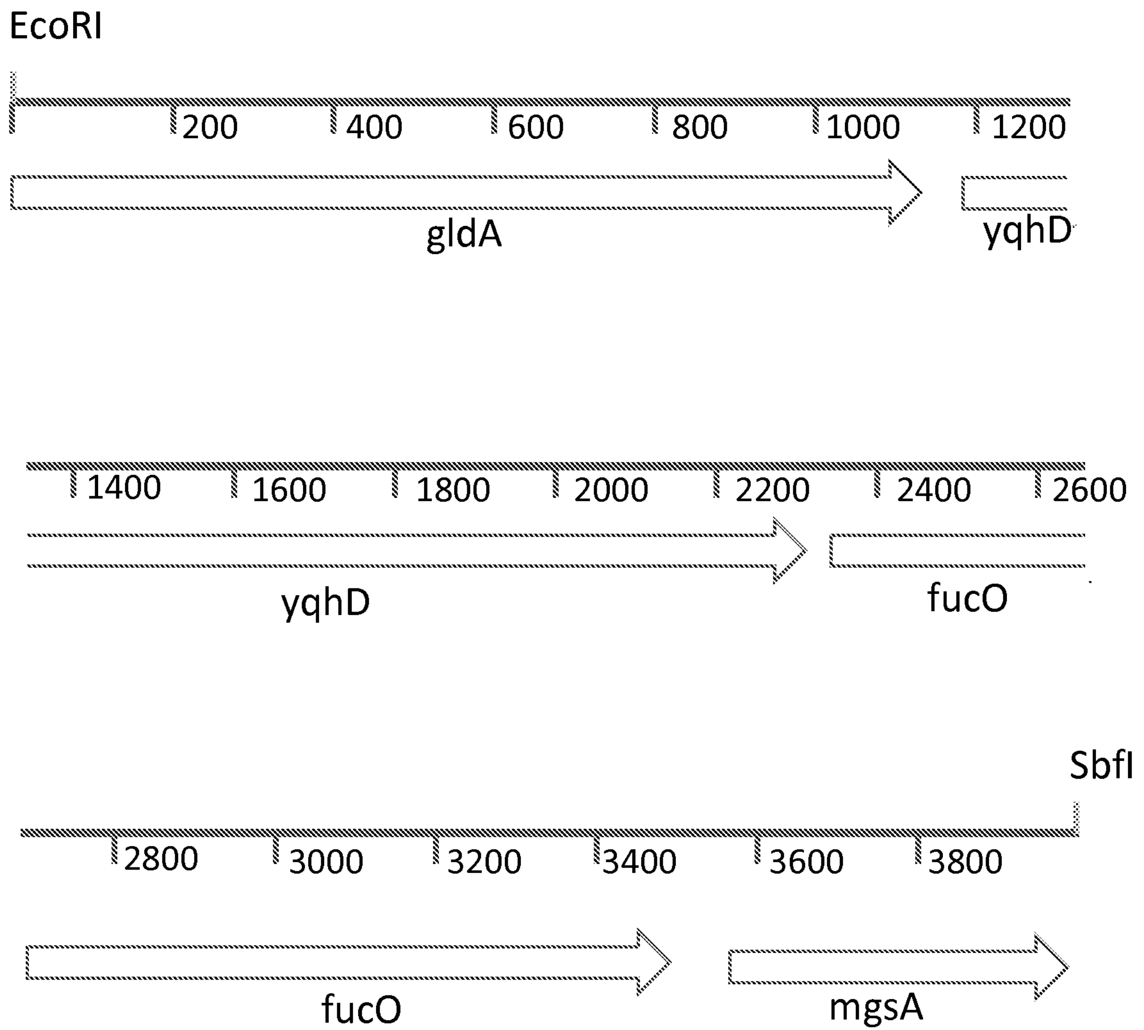


FIG. 17

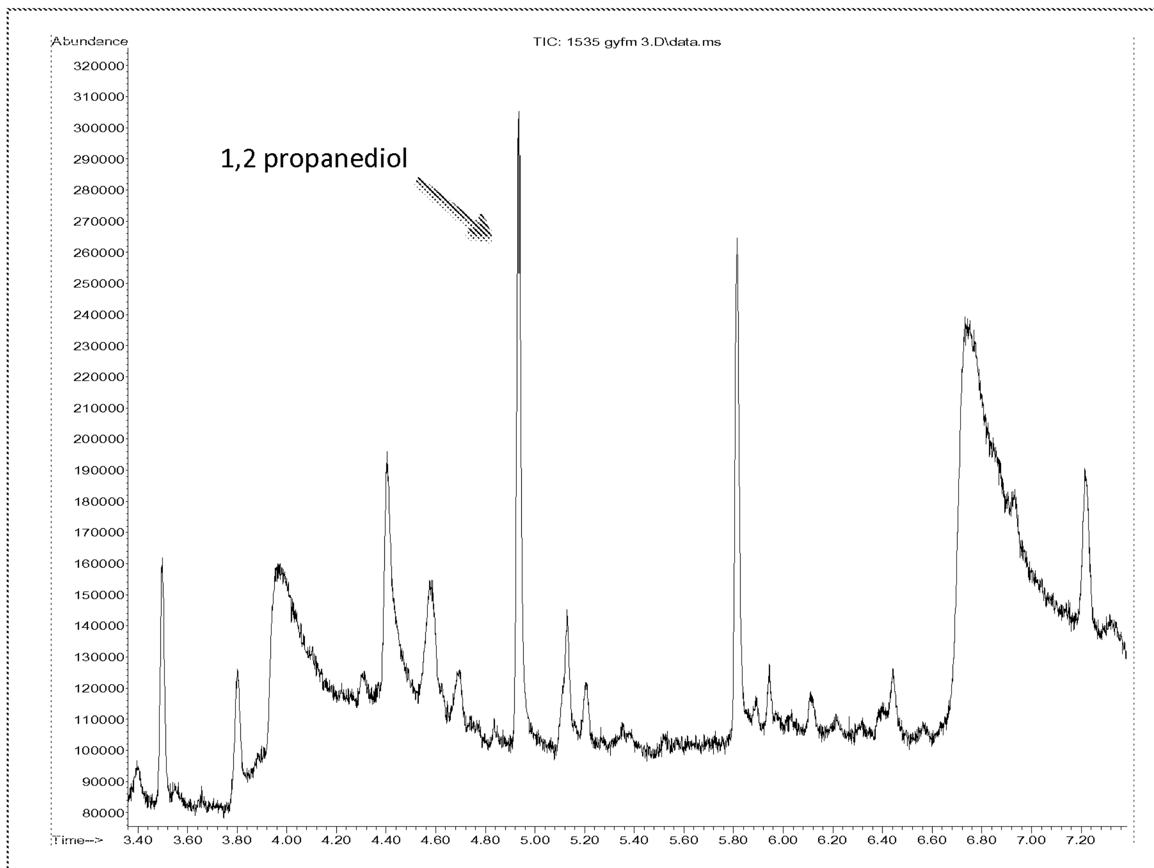


FIG. 18

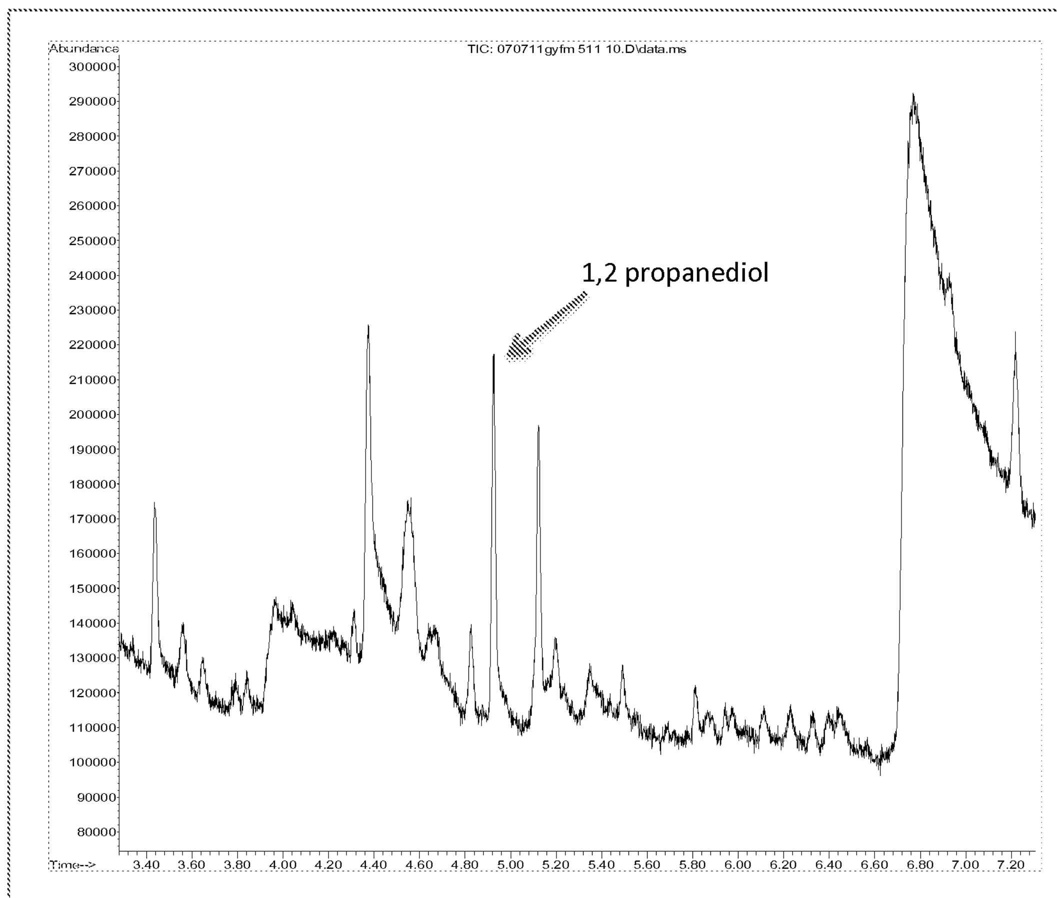


FIG. 19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/065568

A. CLASSIFICATION OF SUBJECT MATTER

C12N 1/21(2006.01)i, C12N 15/31(2006.01)i, C12P 7/04(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 1/21; C12N 1/20; C12N 15/31; C12P 7/04

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: 1,2-propanediol, cyanobacteria, gldA, fucO, mgsA, yqhD, biofuel

C. DOCUMENTS CONSIDERED TO BE RELEVANT

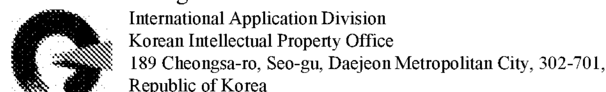
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CLOMBURG, JAMES M. et al., 'Metabolic engineering of Escherichia coli for the production of 1,2-propanediol from glycerol', Biotechnology and Bioengineering, 2011, Vol.108, No.4, pp.867-879 See abstract; pages 868 and 874; table I; and figure 1.	1-3,5-15,17-22
A		4
Y	LAN, ETHAN I. et al., 'Metabolic engineering of cyanobacteria for 1-butanol production from carbon dioxide', Metabolic Engineering, 2011, Vol.13, No.4, pp.353-363 See abstract and page 355.	1-3,5-15,17-22
A		4
Y	NCBI, GenBank accession no. CP002291.1 (09 April 2012) See the whole document.	5,7,9,11
Y	NCBI, GenBank accession no. YP_006151638.1 (27 September 2012) See the whole document.	6
Y	NCBI, GenBank accession no. NP_289354.1 (27 September 2012) See the whole document.	8

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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

Date of the actual completion of the international search	Date of mailing of the international search report
27 February 2014 (27.02.2014)	**27 February 2014 (27.02.2014)**

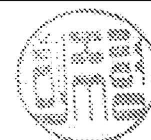
Name and mailing address of the ISA/KR


International Application Division
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea

Facsimile No. +82-42-472-7140

Authorized officer

HEO, Joo Hyung



Telephone No. +82-42-481-8150

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NCBI, GenBank accession no. NP_415483.2 (11 January 2012) See the whole document.	10
Y	NCBI, GenBank accession no. YP_006150597.1 (27 September 2012) See the whole document.	12
A	WO 2010-012604 A1 (CLARIANT INTERNATIONAL LTD) 04 February 2010 See the whole document.	1-15,17-22
A	JUNG, JOON-YOUNG et al., 'Production of 1,2-propanediol from glycerol in Saccharomyces cerevisiae', Journal of Microbiology and Biotechnology, 2011, Vol.21, No.8, pp.846-853 See the whole document.	1-15,17-22
A	JARBOE, LAURA R., 'YqhD: a broad-substrate range aldehyde reductase with various applications in production of biorenewable fuels and chemicals', Applied Microbiology and Biotechnology, 2011, Vol.89, No.2, pp.249-257 See the whole document.	1-15,17-22
PX	LI, HAN et al., 'Engineering a cyanobacterium as the catalyst for the photosynthetic conversion of CO2 to 1,2-propanediol', Microbial Cell Factories, 22 January 2013, Vol.12, No.1, pp.1-9 See the whole document.	1-15,17-22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/065568

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 16
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		CA 2732204 A1	04/02/2010
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