

METHODS AND MICROORGANISMS FOR MAKING 2,3-BUTANEDIOL AND DERIVATIVES THEREOF FROM C1 CARBONS

CROSS-REFERENCE

[0001] This application claims priority benefit of U.S. Provisional Application Nos. 62/451,819, filed January 30, 2017; 62/504,626, filed May 11, 2017; 62/512,312, filed May 30, 2017; and 62/588,985, filed November 21, 2017, which are hereby incorporated by reference in their entireties.

SEQUENCE LISTING

[0002] This application contains a Sequence Listing, which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. The ASCII copy, created on January 29, 2018, is named INX00382_SL.txt and is 65,784 bytes in size.

BACKGROUND OF THE INVENTION

[0003] The compound 2,3-butanediol ("2,3-BDO"), also known as 2,3-butylene glycol, dimethylene glycol, dimethylethylene glycol and butane-2,3-diol ($C_4H_{10}O_2$; CAS No. 513-85-9) is a high value chemical that is currently produced mainly from petroleum sources. 2,3-BDO has a large number of industrial applications. For example, 2,3-BDO can be used as a precursor for various plastics, pesticides, synthetic rubber, printing inks, perfumes, fumigants, moistening and softening agents, explosives, plasticizers, food, and pharmaceuticals. (Garg, S.K., and Jain, A., "Fermentative production of 2,3-butanediol," *Bioresource Technology*, p. 103-109 (1995)).

[0004] 2,3-BDO is currently produced through the use of crude oil. However, 2,3-BDO is also produced by a variety of microorganisms and can be found in cocoa butter, the roots of *Ruta graveolens*, sweet corn, and rotten mussels. 2,3-BDO is also a by-product of alcoholic fermentation by yeast and usually one of the most abundant minor constituent of wine. It originates from the reduction of acetoin. (Romano, P. and Suzzi, G., "Origin and Production of Acetoin during Wine Yeast Fermentation," *Applied and Environmental Microbiology*, p. 309-315 (1996)).

[0005] There has been some interest in recent years to produce 2,3-BDO by fermentation. Fermentation typically involves taking a carbon source (usually sugar) and fermenting it using a microorganism that is capable of converting the carbon source into a desired product.

[0006] Numerous attempts have been made to engineer *Saccharomyces cerevisiae* strains with reduced acetoin yields, by re-orienting carbons toward glycerol and 2,3-BDO to obtain low-alcohol yeasts with desirable organoleptic features, permitting the decrease of the ethanol contents in wines

by up to 3°C. (Ehsani, M., *et al.*, “Engineering of 2,3-butanediol dehydrogenase to reduce acetoin formation by glycerol-overproducing, low-alcohol *Saccharomyces cerevisiae*,” *Applied and Environmental Microbiology*, p. 3196-3205 (2009)).

[0007] Costs to produce chemicals such as 2,3-BDO by fermentation typically depend on the carbon source used. Sugars are generally higher cost carbon sources that also result in a decrease of food supply. One carbon source is currently extremely cost-effective and abundant is natural gas. The primary source of carbon within natural gas is methane (CH₄), a C1 carbon. By using cheap carbon sources such as methane, 2,3-BDO can be produced economically.

[0008] 2,3-BDO is also currently produced by some non-genetically modified microorganisms at very small titers. At these titers, the cost of fermentation would be too great to be economically feasible. Thus, genetic engineering is required to produce 2,3-BDO at an economically viable level. The challenge lies in engineering fermentation methods and microorganisms to efficiently convert cheap carbon sources, such as methane, into 2,3-BDO, using fermentative processes.

[0009] The subject matter of the present invention relates to microorganisms, such as methanotrophs or yeast, genetically modified so as to substantially improve 2,3-BDO biosynthesis.

INCORPORATION BY REFERENCE

[0010] All publications, patents, and patent applications herein are incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference. In the event of a conflict between a term herein and a term in an incorporated reference, the term herein controls.

[0011] The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

SUMMARY

[0012] Disclosed herein are genetically modified microorganisms that are capable of producing a desired organic compound, starting from a single carbon containing hydrocarbon molecule such as methane. Various methods of producing the desired organic compounds, including by using a genetically modified microorganism are disclosed.

[0013] For example, a genetically modified microorganism capable of converting a C1 carbon to 2,3-butanediol (2,3-BDO) is disclosed. Examples of C1 carbons that can be converted by the

microorganisms can be carbon monoxide (CO), carbon dioxide (CO₂), methane (CH₄), or any combination thereof. The genetically modified microorganism can comprise one or more genes encoding heterologous enzymes such as, for example, an acetoin reductase, alpha-acetolactate decarboxylase (budA), and/or acetolactate synthase (AlsS). The AlsS gene in these microorganisms can be temporarily expressed. One or more of the genes can be under the control of a switch, such as an inducible or repressible promoter that is responsive to the presence or absence of a component in the media, *e.g.*, a sugar such as arabinose or a rare earth element such as lanthanum.

[0014] The AlsS encoded can, for example, comprise an amino acid sequence that is at least 90% identical to any one of SEQ ID NOs: 1, 3, or 19. The alpha-acetolactate decarboxylase (budA) encoded can, for example, comprise an amino acid sequence that is at least 90% identical to SEQ ID NO: 7. The acetoin reductase encoded can, for example, comprise an amino acid sequence that is at least 90% identical to SEQ ID NO: 9. The acetoin reductase encoded can, for example, be a gram positive bacterium NADPH-dependent acetoin reductase, such as from the genus *Clostridium*, for example, *Clostridium autoethanogenum*. In some cases the acetoin reductase can be NADPH-dependent. In some cases, the acetoin reductase can be NADH-dependent.

[0015] The genetically modified microorganism can, for example, be a methanotroph, such as from the genera *Methylobacter*, *Methylomicrobium*, *Methylomonas*, *Methylocaldum*, *Methylococcus*, *Methylosoma*, *Methylosarcina*, *Methylothermus*, *Methylohalobius*, *Methylogaea*, *Methylovulum*, *Crenothrix*, *Clonothrix*, *Methylosphaera*, *Methylocapsa*, *Methylocella*, *Methylosinus*, *Methylocystis*, or *Methyloacidophilum*. The methanotroph can be from the genus *Methylococcus*, for example, from the species *Methylococcus capsulatus*.

[0016] The genetically modified microorganism can also be a prokaryote. In some cases, the genetically modified microorganism can be a bacterium, yeast, or algae.

[0017] In some cases, the genetically modified microorganisms can also produce a greater amount of 2,3-BDO at 42 °C compared to the same organism at 37°C. In some cases, the genetically modified microorganisms can also produce a greater amount of 2,3-BDO at 41 °C compared to the same organism at 37°C. In some cases, the genetically modified microorganism can produce a greater amount of 2,3-BDO at 42 °C compared to the same organism at 45 °C. In some cases, the genetically modified microorganism can produce a greater amount of 2,3-BDO at 41 °C compared to the same organism at 45 °C. In some cases, the genetically modified microorganism can produce a greater amount of 2,3-BDO at 37 °C compared to the same organism at 45 °C.

[0018] In some cases, the heterologous gene encoding for the acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase is integrated by an integration vector into the genome of the microorganism. In some cases, the heterologous gene encoding for the acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase is expressed on an episomal vector.

[0019] In some cases, the heterologous gene encoding the acetolactate synthase is 5' in relation to any other heterologous gene. In some cases, the heterologous gene encoding the acetoin reductase is 3' in relation to any other heterologous gene. In some cases, the heterologous gene encoding the alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other heterologous gene.

[0020] The order of the heterologous genes can be present on the vector prior, during, or after contact with the microorganism. For example, a gene can be 5' in relation to any other heterologous gene on the vector prior to contact with the microorganism. However, after contact with the microorganism, the gene can be inserted into a position or another gene can be inserted into the vector where the gene is then neither 5' or 3' in relation to any other heterologous gene on the vector. For instance, the vectors can be modified within a microorganism in such a way that the order of the genes are altered. In some cases, the specific order of the genes can be achieved after one or more heterologous gene(s) have been inserted into the genome of the microorganism. For example, different integration vectors can be used in order to achieve a specific gene order within the genome of the microorganism.

[0021] Disclosed herein are also vectors that comprise two or more of: an acetoin reductase (*e.g.*, NADPH-dependent) gene, alpha-acetolactate decarboxylase (*budA*) gene, and an *AlsS* gene. In some cases, the gene encoding the acetolactate synthase is 5' in relation to any other heterologous gene. In some cases, the gene encoding the acetoin reductase is 3' in relation to any other heterologous gene. In some cases, the gene encoding the alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other heterologous gene. In some cases, the different genes can be under the control of a switch, such as an inducible or repressible promoter that is responsive to the presence or absence of a component in the media, *e.g.*, a sugar such as arabinose or a rare earth element such as lanthanum. The different genes can be under the control of different promoters, such as a constitutively expressed promoter or a non-constitutively expressed promoter. The promoters used can also be active within a methanotroph. Examples of such vectors include those that comprise a nucleotide sequence that is at least 90% identical to any one of SEQ ID NOs: 15 to 18. In some cases, the vector is an integration vector, whereas in other cases, the vector is an episomally expressed vector.

[0022] Also disclosed herein is a method of making a genetically modified microorganism capable of converting a C1 carbon to 2,3-BDO comprising transforming a microorganism with a nucleic acid that expresses at least one heterologous gene encoding i) an acetoin reductase (*e.g.*, NADPH-dependent); ii) an alpha-acetolactate decarboxylase (*budA*); iii) an acetolactate synthase (*AlsS*), or iv) any combination thereof. The at least one heterologous gene can, for example, be under the control of a switch, such as an inducible or repressible promoter that is responsive to the presence or absence of a component in the media, *e.g.*, a sugar such as arabinose or a rare earth element such as lanthanum. In some cases, the method can make genetically modified microorganisms that can produce a greater amount of 2,3-BDO at 42 °C compared to the same organism at 37°C. In some cases, the method can make genetically modified microorganisms that can produce a greater amount of 2,3-BDO at 41 °C compared to the same organism at 37°C. In some cases, the method can make genetically modified microorganisms that can produce a greater amount of 2,3-BDO at 42 °C compared to the same organism at 45 °C. In some cases, the method can make genetically modified microorganisms that can produce a greater amount of 2,3-BDO at 41 °C compared to the same organism at 45 °C. In some cases, the method can make genetically modified microorganisms that can produce a greater amount of 2,3-BDO at 37 °C compared to the same organism at 45 °C.

[0023] In some cases, the methods can comprise microorganisms in which the gene(s) encoding for the heterologous acetoin reductase, heterologous alpha-acetolactate decarboxylase, and/or heterologous acetolactate synthase is integrated by an integration vector into the genome of the microorganism. In some cases, the methods can comprise microorganisms in which the gene(s) encoding for the heterologous acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase is expressed on an episomal vector. In some cases, the method can comprises microorganism in which the gene(s) encoding for the heterologous acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase are expressed on both an episomal vector and integrated into the genome of the microorganism (*e.g.*, by an integration vector).

[0024] In some cases, the heterologous gene encoding the acetolactate synthase is 5' in relation to any other heterologous gene. In some cases, the heterologous gene encoding the acetoin reductase is 3' in relation to any other heterologous gene. In some cases, the heterologous gene encoding the alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other heterologous gene.

[0025] Further disclosed is a method of making 2,3-BDO comprising (a) contacting a genetically modified microorganism with a C1 carbon, where the microorganism comprises at least one

heterologous gene encoding for (i) an acetoin reductase (*e.g.*, NADPH-dependent); (ii) an alpha-acetolactate decarboxylase (*budA*); (iii) an *AlsS*; or (iv) any combination thereof; and (b) growing the microorganism to produce 2,3-BDO. In some cases, the heterologous gene encoding for the acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase is integrated by an integration vector into the genome of the microorganism. In some cases, the heterologous gene encoding for the acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase is expressed on an episomal vector. In some cases, the method can comprise microorganism in which the gene(s) encoding for the heterologous acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase are expressed on both an episomal vector and integrated into the genome of the microorganism (*e.g.*, by an integration vector). In some cases, the heterologous gene encoding the acetolactate synthase is 5' in relation to any other heterologous gene. In some cases, the heterologous gene encoding the acetoin reductase is 3' in relation to any other heterologous gene. In some cases, the heterologous gene encoding the alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other heterologous gene. The method can comprise growing the microorganism at a temperature of between 32 °C to 49 °C. In some cases, the microorganism can be grown at a temperature of between 37 °C to 42 °C. In some cases, the microorganism can be grown at a temperature of about 42 °C. In some cases, the microorganism can be grown at a temperature of about 41 °C. In some cases, the at least one heterologous gene can, for example, be under the control of a switch, such as an inducible or repressible promoter that is responsive to the presence or absence of a component in the media, *e.g.*, a sugar such as arabinose or a rare earth element such as lanthanum. Also the microorganism can be first grown in media that contains a rare earth metal such as lanthanum (*e.g.*, at least 1µM lanthanum) and then subsequently the rare earth metal such as lanthanum can be diluted. This can occur before growing the microorganism to produce 2,3-BDO. The 2,3-BDO produced from this method can be recovered and, in some cases, be substantially pure.

[0026] Additionally disclosed is a method of making acetoin comprising (a) contacting a genetically modified microorganism with a C1 carbon, where the microorganism comprises a heterologous gene encoding for alpha-acetolactate decarboxylase (*budA*); and (b) growing the microorganism to produce acetoin. In some cases, the heterologous gene encoding for the alpha-acetolactate decarboxylase is integrated by an integration vector into the genome of the microorganism. In some cases, the heterologous gene encoding for the alpha-acetolactate decarboxylase is expressed on an episomal vector. In some cases, the heterologous gene encoding

for the alpha-acetolactate decarboxylase is expressed on both an episomal vector and integrated into the genome of the microorganism (*e.g.*, by an integration vector). The method can comprise growing the microorganism at a temperature of between 32 °C to 49 °C. In some cases, the microorganism can be grown at a temperature of between 37 °C to 42 °C. In some cases, the microorganism can be grown at a temperature of about 42 °C. In some cases, the microorganism can be grown at a temperature of about 41 °C. In some cases, the *budA* gene can, for example, be under the control of a switch, such as an inducible or repressible switch, *e.g.*, an arabinose or lanthanum switch. In some cases, the microorganism can be first grown in media containing lanthanum (*e.g.*, at least 1µM lanthanum). The lanthanum can be subsequently consumed, removed, and/or diluted. This can occur before growing the microorganism to produce acetoin. The acetoin produced from this method can be recovered and, in some cases, be substantially pure. Should the acetoin produced by this method be not substantially pure, the non-acetoin by-products, such as 2,3-BDO, can be recovered as well.

[0027] Once 2,3-BDO is made, it can be converted into other desired products, for example, such as butadiene or methyl ethyl ketone (MEK). Thus, also disclosed herein is a method of making butadiene comprising (a) contacting a genetically modified microorganism with a C1 carbon substrate, where the microorganism comprises at least one heterologous gene encoding (i) an NADPH-dependent acetoin reductase; (ii) an alpha-acetolactate decarboxylase (*budA*); (iii) an *AlsS*; or (iv) any combination thereof; and (b) growing the microorganism to produce 2,3-BDO; and (c) contacting the 2,3-BDO from (b) with a catalyst to produce butadiene. In some cases, the 2,3-BDO from (b) is removed prior to (c). In some cases, the heterologous gene encoding for the acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase is integrated by an integration vector into the genome of the microorganism. In some cases, the heterologous gene encoding for the acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase is expressed on an episomal vector. In some cases, the heterologous gene encoding for the acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase is expressed on both an episomal vector and integrated into the genome of the microorganism (*e.g.*, by an integration vector). In some cases, the heterologous gene encoding the acetolactate synthase is 5' in relation to any other heterologous gene. In some cases, the heterologous gene encoding the acetoin reductase is 3' in relation to any other heterologous gene. In some cases, the heterologous gene encoding the alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other heterologous gene. The method can comprise growing the microorganism at a temperature of

between 32 °C to 49 °C. In some cases, the microorganism can be grown at a temperature of between 37 °C to 42 °C. In some cases, the microorganism can be grown at a temperature of about 42 °C. In some cases, the microorganism can be grown at a temperature of about 41 °C. Additionally, the catalyst can be any catalyst that is capable of dehydrating 2,3-BDO, such as a SiO₂-supported cesium dihydrogen phosphate (CsH₂PO₄) catalyst. The butadiene produced from this method can be recovered and in some cases can be substantially pure. The butadiene can also be further processed into a synthetic rubber.

[0028] Disclosed also is a method of making MEK comprising (a) contacting a genetically modified microorganism with a C1 carbon substrate, where the microorganism comprises at least one heterologous gene encoding (i) an NADPH-dependent acetoin reductase; (ii) an alpha-acetolactate decarboxylase (*budA*); (iii) an acetolactate synthase (*AlsS*); or (iv) any combination thereof; and (b) growing the microorganism to produce 2,3-BDO; and (c) contacting the 2,3-BDO from (b) with a catalyst to produce MEK. In some cases, the heterologous gene encoding for the acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase is integrated by an integration vector into the genome of the microorganism. In some cases, the heterologous gene encoding for the acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase is expressed on an episomal vector. In some cases, the heterologous gene encoding for the acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase are expressed on both an episomal vector and integrated into the genome of the microorganism (*e.g.*, by an integration vector). In some cases, the heterologous gene encoding the acetolactate synthase is 5' in relation to any other heterologous gene. In some cases, the heterologous gene encoding the acetoin reductase is 3' in relation to any other heterologous gene. In some cases, the heterologous gene encoding the alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other heterologous gene. The method can comprise growing the microorganism at a temperature of between 32 °C to 49 °C. In some cases, the microorganism can be grown at a temperature of between 37 °C to 42 °C. In some cases, the microorganism can be grown at a temperature of about 42 °C. In some cases, the microorganism can be grown at a temperature of about 41 °C. In some cases, the catalyst is a solid acid catalyst. The MEK produced from this method can be recovered and in some cases be substantially pure. The MEK can also be further processed into plastics, textiles, paraffin wax, lacquer, varnishes, paint remover, glues, and/or cleaning agents. In some cases, the catalyst from (c) can be a diol dehydratase (B12). In some cases, a diol dehydratase gene can be expressed by the same or different genetically modified microorganism.

Thus, disclosed herein is a method of making MEK comprising (a) contacting a genetically modified microorganism with a C1 carbon substrate, where the microorganism comprises at least one heterologous gene encoding (i) an NADPH-dependent acetoin reductase; (ii) an alpha-acetolactate decarboxylase (budA); (iii) an AlsS; (iv) a diol dehydratase or (v) any combination thereof; and (b) growing the microorganism to produce MEK.

[0029] Also disclosed are isolated polynucleic acids comprising nucleotide sequences that are at least 84% identical to SEQ ID NO: 2, at least 88% identical to SEQ ID NO: 4, or at least 60% identical to SEQ ID NO: 20. These nucleotide sequences can encode for a protein that has acetolactate synthase activity. Further disclosed are, isolated polynucleic acids comprising a nucleotide sequence that is at least 85% identical to SEQ ID NOs: 6 or 8. These nucleotide sequences can encode a protein that has alpha-acetolactate decarboxylase activity. Additionally disclosed are isolated polynucleic acids that are at least 85% identical to any one of SEQ ID NOs: 10, 12, or 14. These nucleotide sequences can encode a protein that has butanediol dehydrogenase activity.

BRIEF DESCRIPTION OF THE DRAWINGS

[0030] FIG. 1 shows a metabolic pathway from methane (CH₄) to 2,3-BDO.

[0031] FIG. 2 shows the performance of engineered strains in a high-cell density fermentation experiment over 72 hours. From left to right are the following strains that were tested: XZ58 (MF1904), XZ06 (MF1905), XZ59(MF1906), and XZ08 (MF1907). 2,3-BDO and acetoin production was tested at various time points.

[0032] FIG. 3 shows the nucleotide sequence of the 2,3-BDO pathway gene expression cassette in strain XZ58 (SEQ ID NO: 15). Proceeding in the 5' to 3' direction, the underlined uppercase sequences represent the pBAD promoter. The initiator ATG and terminator TAA for the g.Bsu AlsS (acetolactate synthase) gene are indicated by bold uppercase while the coding region is indicated in lowercase. The ribosomal binding site rbsGTW001 is indicated by boxed uppercase text. The initiator ATG and terminator TGA for the g.Kpn BudA gene are indicated by bold italics uppercase while the coding region is indicated in italics lowercase. The terminator rrnB is indicated in uppercase, followed by a pmxaF promoter that indicated by underlined italics uppercase. The initiator ATG and terminator TGA for the g.Cau ButA gene are indicated by bold italics uppercase while the coding region is indicated in bold italics lowercase. The terminator lambda T0 is indicated in italics uppercase.

[0033] FIG. 4 shows the nucleotide sequence of the 2,3-BDO pathway genes expression cassette in strain XZ59 (SEQ ID NO: 16). Proceeding in the 5' to 3' direction, the underlined uppercase sequences represent the pBAD promoter. The initiator ATG and terminator TAA for the g.Bsu AlsS gene are indicated by bold uppercase while the coding region is indicated in lowercase. The ribosomal binding site rbsGTW001 is indicated by boxed uppercase text. The initiator ATG and terminator TGA for the g.Kpn BudA gene are indicated by bold italics uppercase while the coding region is indicated in italics lowercase. The terminator rrnB is indicated in uppercase, followed by a pmxaF promoter that indicated by underlined italics uppercase. The initiator ATG and terminator TGA for the g.Bsu ButA gene are indicated by bold italics uppercase while the coding region is indicated in bold italics lowercase. The terminator lambda T0 is indicated in italics uppercase.

[0034] FIG. 5 shows the nucleotide sequence of the 2,3-BDO pathway genes expression cassette in strain XZ06 (SEQ ID NO: 17). Proceeding in the 5' to 3' direction, the underlined uppercase sequences represent the pBAD promoter. The initiator ATG and terminator TAA for the g.Bsu AlsS gene are indicated by bold uppercase while the coding region is indicated in lowercase. The ribosomal binding site rbsGTW001 is indicated by boxed uppercase text. The initiator ATG and terminator TGA for the g.Kpn BudA gene are indicated by bold italics uppercase while the coding region is indicated in italics lowercase, followed by an additional ribosomal binding site rbsGTW001 that is indicated by boxed uppercase text. The initiator ATG and terminator TGA for the g.Cau ButA gene are indicated by bold italics uppercase while the coding region is indicated in bold italics lowercase. The terminator rrnB is indicated in uppercase.

[0035] FIG. 6 demonstrates the nucleotide sequence of the 2,3-BDO pathway genes expression cassette in strain XZ08 (SEQ ID NO: 18). Proceeding in the 5' to 3' direction, the underlined uppercase sequences represent the pBAD promoter. The initiator ATG and terminator TAA for the g.Bsu AlsS gene are indicated by bold uppercase while the coding region is indicated in lowercase. The ribosomal binding site rbsGTW001 is indicated by boxed uppercase text. The initiator ATG and terminator TGA for the g.Kpn BudA gene are indicated by bold italics uppercase while the coding region is indicated in italics lowercase, followed by an additional ribosomal binding site rbsGTW001 that is indicated by boxed uppercase text. The initiator ATG and terminator TGA for the g.Bsu ButA gene are indicated by bold italics uppercase while the coding region is indicated in bold italics lowercase. The terminator rrnB is indicated in uppercase.

[0036] FIGs 7A and 7B. FIG. 7A demonstrates acetoin and 2,3-BDO production of 21 different strains after 96 hours following dilution of lanthanum containing media. The strains and genotypes

of the strains are listed in Table 3 and 4. For strains 1 to 21, production titers were measured 96 hours after a 1:10 (10X) dilution of the culture into fresh medium whereas for strains 22 to 42 production titers were measured after a 1:50 (50X) dilution. Strains 22 to 27 produced high levels of 2,3-BDO, compared to strains subjected to less dilution prior to the 2,3-BDO production phase. **FIG. 7B** demonstrates acetoin and 2,3-BDO production of 21 different strains after 120 hours following dilution of lanthanum containing media. The strains and genotypes of the strains are listed in Table 3 and 4. For strains 1 to 21, production titers were measured 120 hours after a 1:10 (10X) dilution of the culture into fresh medium, whereas for strains 22 to 42 production titers were measured after a 1:50 (50X) dilution. Strains 22 to 27 produced high levels of 2,3-BDO, compared to strains subjected to less dilution prior to the 2,3-BDO production phase.

[0037] FIG. 8 shows that strains expressing a *Bacillus licheniformis* AlsS exhibited significantly improved 2,3-BDO titers. In one strain expressing *Bacillus licheniformis* AlsS (XZ562) the 2,3-BDO titers increased an average of 44.6% over the XZ58 strain (described in **FIG. 3**) over the fermentation run. Another biological replicate (XZ561), produced also significantly higher average 2,3-BDO titers compared to the XZ58 strain.

[0038] FIG. 9 shows 2,3-BDO titers of 7 strains of methanotrophs genetically engineered to produce 2,3-BDO fermented at either 42 °C or 37 °C. As seen, all strains produced higher 2,3-BDO titers when incubated at 42 °C. Strain MBC2122 produced approximately 50% more 2,3-BDO at 42 °C compared to at 37 °C.

[0039] FIG. 10 shows 2,3-BDO titers of 3 strains of methanotrophs genetically engineered to produce 2,3-BDO fermented at either 45 °C or 37 °C. As seen, all strains produced lower 2,3-BDO titers when incubated at 45 °C. Most of the strains produced approximately 50% less 2,3-BDO at 45 °C compared to at 37 °C. The episomal strain MBC1322 performed better with antibiotic selective pressure, at both 37 °C and 45 °C, indicating a stability issue. On the contrary, the integrated strains were able to maintain its productivity without selective pressure.

[0040] FIG. 11 shows the productivity (2,3-BDO/OD ratio) of a methanotroph strain genetically engineered to produce 2,3-BDO fermented at 37°C, 41°C, 43°C, 45°C, and 47°C. The best productivity was observed at a temperature of 41°C.

[0041] FIG. 12 shows that methanotroph strains that contained additional copies of 2,3-BDO pathway enzymes (**Strains B and C**) within an episomal vector produced over 3 times as much 2,3-BDO compared to the singly integrated strain (**Strain A**) in shake bottle experiments. **Strain B** and **Strain C** were precultured in 10 µM lanthanum in the presence of Kanamycin at 37°C for 48 hours.

Strain A was precultured in the same conditions except that no Kanamycin was added. After 48 hours, the lanthanum was diluted by 50 fold (50X) and 2,3-BDO titers were measured after 96 hours.

[0042] **FIG. 13** shows the effect (in a shake bottle experiment) of preculturing a methanotroph strain having genes encoding for 2,3-BDO pathway enzymes when those genes are episomally expressed (**Strain D**). This methanotroph strain was precultured in 10uM, 3uM, and 1uM lanthanum. The production media was subsequently diluted by 50-fold. Higher lanthanum concentrations resulted in higher 2,3BDO titers for episomally expressed methanotroph strains.

[0043] **FIG. 14** shows the effect (in a shake bottle experiment) of preculturing a methanotroph strain having genes encoding for 2,3-BDO pathway enzymes when those genes are integrated into the methanotroph's genome (**Strain A**). This strain was precultured in 35 μ M, 10 μ M, 3 μ M, 1 μ M, and 0 μ M lanthanum. The production media was subsequently diluted by 50 fold (50X). Levels of 2,3-BDO production increased when the strain was precultured with lower concentration of lanthanum.

[0044] **FIG. 15** shows the effect of preculturing at different lanthanum concentrations (10 μ M, 3 μ M, and 1 μ M) of the two strains from **FIG. 12** (**Strains B** and **C**) in a shake bottle experiment. Both strains contained both an integrated and an episomally expressed copy of the 2,3-BDO pathway enzymes. Generally, the strains having episomally expressed 2,3-BDO pathway enzymes, yield higher 2,3-BDO titers when precultured in 10 μ M lanthanum.

[0045] **FIG. 16** shows a summary of the genotypes of the **Strains A, B, C, and D**.

DETAILED DESCRIPTION OF THE INVENTION

[0046] As summarized above, aspects of the invention include genetically modified microorganisms that can convert carbon substrates into chemical products such as 2,3-BDO. The genetically modified microorganisms include methanotrophs, which are capable of generating 2,3-BDO at high titers from a methane source. Also disclosed are methods of making and using such genetically modified microorganisms.

[0047] Before the present invention is described in greater detail, it is to be understood that this invention is not limited to particular cases described, as such can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular cases only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0048] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is

encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

I. DEFINITIONS

[0049] The term “2,3-butanediol” or “2,3-BDO” and their grammatical equivalents as used herein can refer to all enantiomeric and diastereomeric forms of the compound, including (R,R), (S,S) and mesa forms, in racemic, partially stereoisomerically pure and/or substantially stereoisomerically pure forms.

[0050] The term “butene” or “butylene” and their grammatical equivalents as used herein can refer to all structural isomers of the alkene including 2-butene, but-1-ene, 2-methylpropene, and all stereoisomeric and geometric isomeric forms of the compound, including Z-but-2-ene, E-but-2-ene, in mixtures of isomers and pure and/or substantially pure forms.

[0051] The term “butadiene” and their grammatical equivalents as used herein can refer to all geometric isomers of the diene including cis and trans 1,3-butadiene, in mixtures of isomers and pure and/or substantially pure forms.

[0052] The term “methyl ethyl ketone” or “MEK” or “butanone” and their grammatical equivalents as used herein can refer to all isomers of the ketone in partially pure and/or substantially pure forms.

[0053] The term “about” in relation to a reference numerical value and its grammatical equivalents as used herein can include the numerical value itself and a range of values plus or minus 10% from that numerical value. For example, the amount “about 10” includes 10 and any amounts from 9 to 11. For example, the term “about” in relation to a reference numerical value can also include a range of values plus or minus 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% from that value. In some cases, the numerical value disclosed throughout can be “about” that numerical value even without specifically mentioning the term “about.”

[0054] It is noted that, as used herein and in the appended claims, the singular forms “a”, “an”, and “the” include plural referents unless the context clearly dictates otherwise. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as “solely,” “only” and the like in connection with the recitation of claim elements, or use of a “negative” limitation.

[0055] The term “genetic modification” or “genetically modified” and their grammatical equivalents as used herein can refer to one or more alterations of a nucleic acid, *e.g.*, the nucleic acid within a microorganism’s genome. For example, genetic modification can refer to alterations, additions, and/or deletion of nucleic acid (*e.g.*, whole genes or fragments of genes).

[0056] The term “disrupting” and its grammatical equivalents as used herein can refer to a process of altering a gene, *e.g.*, by deletion, insertion, mutation, rearrangement, or any combination thereof. For example, a gene can be disrupted by knockout. Disrupting a gene can be partially reducing or completely suppressing expression (*e.g.*, mRNA and/or protein expression) of the gene. Disrupting can also include inhibitory technology, such as shRNA, siRNA, microRNA, dominant negative, or any other means to inhibit functionality or expression of a gene or protein.

[0057] The term “gene editing” and its grammatical equivalents as used herein can refer to genetic engineering in which one or more nucleotides are inserted, replaced, or removed from a genome. For example, gene editing can be performed using a nuclease (*e.g.*, a natural-existing nuclease or an artificially engineered nuclease).

[0058] The terms “and/or” and “any combination thereof” and their grammatical equivalents as used herein, can be used interchangeably. These terms can convey that any combination is specifically contemplated. Solely for illustrative purposes, the following phrases “A, B, and/or C” or “A, B, C, or any combination thereof” can mean “A individually; B individually; C individually; A and B; B and C; A and C; and A, B, and C.”

[0059] As will be apparent to those of skill in the art upon reading this disclosure, each of the individual cases described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several cases without departing from the scope or spirit of the present invention. Any recited method can be carried out in the order of events recited or in any other order which is logically possible.

[0060] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, representative illustrative methods and materials are now described.

[0061] The term “polynucleic acid” and its grammatical equivalents as used herein can refer to an organic polymer composed of two or more monomers including nucleotides, nucleosides or analogs thereof, including but not limited to single stranded or double stranded, sense or antisense

deoxyribonucleic acid (DNA) of any length and, where appropriate, single stranded or double stranded, sense or antisense ribonucleic acid (RNA) of any length, including siRNA. The term “nucleotide” refers to any of several compounds that consist of a ribose or deoxyribose sugar joined to a purine or a pyrimidine base and to a phosphate group, and that are the basic structural units of nucleic acids. The nucleotides can be naturally occurring, artificial and/or modified nucleotides. The term “nucleoside” refers to a compound (as guanosine or adenosine) that consists of a purine or pyrimidine base combined with deoxyribose or ribose and is found especially in polynucleic acids. The term “nucleotide analog” or “nucleoside analog” refers, respectively, to a nucleotide or nucleoside in which one or more individual atoms have been replaced with a different atom or with a different functional group. The term “polynucleic acid,” as used herein, includes nucleic acids of any length, including DNA, RNA, open reading frames, analogs and fragments thereof.

[0062] Examples of polynucleic acids include oligonucleotides that typically range in length from 2 nucleotides to about 100 nucleotides, and polynucleotides, which typically have a length greater than about 100 nucleotides. It is understood that the polynucleic acids described herein include polynucleotides such as “genes,” “promoters,” “operons,” and/or “vectors.” As used herein, the term “gene” and its grammatical equivalents refers to a polynucleotide that codes for a particular sequence of amino acids, which comprise all or part of one or more proteins or enzymes, and may include regulatory (non-transcribed) DNA sequences, such as promoter sequences, which determine for example the conditions under which the gene is expressed. The transcribed region of the gene may include untranslated regions, including introns, 5'-untranslated region (UTR), and 3'-UTR, as well as the coding sequence.

[0063] The term “promoter” and its grammatical equivalents as used herein can refer to a nucleic acid sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic nucleic acid segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental or physiological conditions. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as “constitutive promoters”. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

[0064] The term “operably linked” and its grammatical equivalents as used herein can refer to the association of nucleic acid sequences on a single polynucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of effecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

[0065] The term “codon optimized” and its grammatical equivalents as used herein insofar as it refers to genes or coding regions of nucleic acid molecules (or open reading frames) for transformation of various hosts, can refer to the alteration of codons in the gene or coding regions of the nucleic acid molecules to reflect the typical codon usage of the host organism without altering the polypeptide encoded by the DNA.

[0066] The term “open reading frame” (“ORF”) and its grammatical equivalents as used herein can refer to a polynucleic acid or nucleic acid sequence (whether naturally occurring, non-naturally occurring, or synthetic) comprising an uninterrupted reading frame consisting of (i) an initiation codon, (ii) a series of two (2) or more codons representing amino acids, and (iii) a termination codon, the ORF being read (or translated) in the 5’ to 3’ direction.

[0067] The term “operon” and its grammatical equivalents as used herein can refer to two or more genes which are transcribed as a single transcriptional unit from a common promoter. In certain cases, the genes, polynucleotides or ORFs comprising the operon are contiguous. It is understood that transcription of an entire operon can be modified (i.e., increased, decreased, or eliminated) by modifying the common promoter. Alternatively, any gene, polynucleotide or ORF, or any combination thereof in an operon can be modified to alter the function or activity of the encoded polypeptide. The modification can result in an increase or a decrease in the activity or function of the encoded polypeptide. Further, the modification can impart new activities on the encoded polypeptide.

[0068] The term “vector” and its grammatical equivalents as used herein can refer to any means by which a nucleic acid can be propagated and/or transferred between organisms, cells, or cellular components. Vectors include viruses, bacteriophage, pro-viruses, plasmids, phagemids, transposons, and artificial chromosomes such as YACs (yeast artificial chromosomes), BACs (bacterial artificial chromosomes), and PLACs (plant artificial chromosomes), and the like, that are “episomes”, that is, that replicate autonomously or can integrate into a chromosome of a host microorganism. A vector can also be a naked RNA polynucleotide, a naked DNA polynucleotide, a polynucleotide composed

of both DNA and RNA within the same strand, a poly-lysine-conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that are not episomal in nature, or it can be an organism which comprises one or more of the above polynucleotide constructs such as an agrobacterium or a bacterium.

[0069] The term “polypeptide” and its grammatical equivalents as used herein can refer to any organic polymer comprising two or more amino acids, regardless of its size. Although “protein” is often used in reference to relatively large polypeptides, and “peptide” is often used in reference to small polypeptides, usage of these terms in the art overlaps and varies. The term “polypeptide” as used herein refers to peptides, polypeptides and proteins, unless otherwise noted. As used herein, the terms “protein,” “polypeptide,” and “peptide” are used interchangeably herein when referring to a gene product. Unless otherwise indicated, a particular polypeptide also implicitly encompasses conservatively-substituted variants thereof.

[0070] The term “enzyme” and its grammatical equivalents as used herein can refer to any of numerous proteins that act as a biological catalyst. Similar to traditional chemical catalysts, enzymes speed the rate of biological reactions by producing a transition state with a lower energy of activation than the uncatalyzed reaction. In other words, enzymes are proteins specialized for the reactions they catalyze. Examples of enzymes described herein include acetolactate synthase (encoded by the *AlsS* gene), alpha-acetolactate decarboxylase (encoded by the gene *BudA*), and acetoin reductase (encoded by the gene *ButA*).

[0071] The phrases “recombinant host cell,” “genetically engineered host cell,” “engineered host cell,” “genetically modified host cell,” and their grammatical equivalents as used herein may be used interchangeably and can refer to host cells that have been genetically modified to: (a) express one or more exogenous polynucleic acids; (b) over-express one or more endogenous and/or one or more exogenous polynucleic acids, such as those included in a vector, or which have an alteration in expression of an endogenous gene; or (c) knock-out or down-regulate an endogenous gene. In addition, certain genes may be physically removed from the genome (e.g., knock-outs) or they may be engineered to have reduced, altered or enhanced activity. The phrases “recombinant host cell,” “genetically engineered host cell,” “engineered host cell,” and “genetically modified host cell” refer not only to the particular subject host cell, but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term(s) as used herein.

[0072] The term “*in vitro*” and its grammatical equivalents as used herein can refer to outside of a living cell, regardless of the location of the cell. The term “*in vivo*” and its grammatical equivalents as used herein can refer to inside a living cell, regardless of the location of the cell.

[0073] The terms “engineer,” “genetically engineer,” “modify,” “genetically modify,” and their grammatical equivalents as used herein can refer to any manipulation of a microorganism that results in a detectable change in the microorganism, where the manipulation includes, but is not limited to, introducing non-native metabolic functionality via heterologous (exogenous) polynucleic acids or removing native-functionality via polynucleic acid deletions, mutations or knock-outs. The term “metabolically engineered” generally involves rational pathway design and assembly of biosynthetic genes (or open reading frames), genes associated with operons, and control elements of such polynucleic acids, for the production of a desired metabolite. “Metabolically engineered” may further include optimization of metabolic flux by regulation and optimization of transcription, translation, protein stability and protein functionality using genetic engineering and appropriate culture condition including the reduction of, disruption, or knocking out of, a competing metabolic pathway that competes with an intermediate leading to a desired pathway.

[0074] The term “switch” and its grammatical equivalents as used herein can mean a regulatory unit of a gene or genes that is capable of responding to a particular stimulus to either induce or repress expression. For example, switches can include regulatory units that respond to sugar (*e.g.*, arabinose) or rare earth metals (*e.g.*, lanthanum).

[0075] As used herein, the terms “genetic modification,” “genetically modified” and their grammatical equivalents can refer to any modification of a polynucleic acid and/or polypeptide that results in an altered nucleic acid or polypeptide (*i.e.*, relative to the wild-type nucleic acid or polypeptide sequence). Genetic modification includes, for example, point mutations, substitutions, deletions, or insertions of single or multiple residues in a polynucleic acid (or the encoded polypeptide), which includes alterations arising within a protein-encoding region of a gene as well as alterations in regions outside of a protein-encoding sequence, such as, but not limited to, regulatory or promoter sequences. A genetic modification may be an alteration of any type. For instance, the modification may be a deletion, insertion, mutation, rearrangement, or any combination thereof. In certain cases, a portion of a genetically modified microorganism’s genome may be replaced with one or more heterologous (exogenous) polynucleic acids. In some cases, the modification is naturally-occurring. In other cases, the modification is the results of artificial selection pressure. In still other cases, the modification is the result of genetic engineering. One form of genetic modification is

disruption, such as by knockout. As used herein, the term “introducing,” and its grammatical equivalents as used in phrases such as “introducing into the host cell” at least one polynucleic acid includes methods known in the art for introducing polynucleic acids into a cell, including, but not limited to transformation (e.g., calcium chloride, electroporation), transduction, transfection, conjugation and the like.

[0076] As used herein, the terms “expression” or “expressed,” and their grammatical equivalents with respect to a gene sequence, ORF sequence, or polynucleic acid sequence, can refer to transcription of the gene, open reading frame or polynucleic acid and, as appropriate, translation of the resulting mRNA transcript to a protein. Thus, as will be clear from the context, expression of a protein results from transcription and translation of the open reading frame sequence. The level of expression of a desired end product in a host cell may be determined on the basis of either the amount of corresponding mRNA that is present in the host cell, or the amount of the desired end product encoded by the selected sequence. For example, mRNA transcribed from a selected sequence can be quantitated by PCR or by northern hybridization (see, e.g., Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, 1989). Protein encoded by a selected sequence can be quantitated by various methods (e.g., by ELISA, by assaying for the biological activity of the protein, or by employing assays that are independent of such activity, such as western blotting or radioimmunoassay, using antibodies that recognize and bind reacting the protein).

[0077] As used herein, the term “endogenous,” and its grammatical equivalents when used in reference to polynucleic acids (and the polypeptides encoded therein), refer to polynucleic acids and polypeptides that are expressed in the organism in which they originated (*i.e.*, they are innate to the organism). In contrast, the terms “heterologous” and “exogenous” are used interchangeably, and as defined herein with reference to polynucleic acids (and the polypeptides encoded therein), indicates polynucleic acids and polypeptides that are expressed in an organism other than the organism from which they (*i.e.*, the polynucleic acid or polypeptide sequences) originated or where derived. In some cases, the term “heterologous” and its grammatical equivalents can mean derived from a different species. For example, a “heterologous gene” can mean a gene that is from a species different than the reference species. For example, a methanotroph comprising a “heterologous gene” comprises a gene that is not from the same methanotroph. The gene can be from a different microorganism such as yeast or from a different species such as a different methanotroph species.

[0078] As used herein, the term “substrate” and its grammatical equivalents can refer to any substance or compound that is converted, or meant to be converted, into another compound by the action of an enzyme. The term includes not only a single compound, but also combinations of compounds, such as solutions, mixtures and other materials which contain at least one substrate, or derivatives thereof.

[0079] As used herein, the terms “C1 carbon,” “C1-carbon substrates” and their grammatical equivalents can refer to any organic compound that contains a single carbon atom. Examples include, but are not limited to, carbon monoxide (CO), methane (CH₄), and carbon dioxide (CO₂).

[0080] As used herein, the term “fermentation” or “fermentation process,” and its grammatical equivalents, can be a process in which a host cell is cultivated in a culture medium containing raw materials, such as feedstock and nutrients, where the cell converts raw materials, such as a feedstock, into desirable end products.

[0081] As used herein, the term “homolog” and its grammatical equivalents, as used with respect to an original protein, polypeptide, gene, or polynucleic acid (or ORF encoding the same) of a first family or species, can refer to distinct proteins, genes, or polynucleic acids of a second family or species that correspond (structurally, functionally, and/or genomically) to the original protein, gene, or polynucleic acid of the first family or species. Most often, “homologs” will have functional, structural or genomic similarities. Techniques are known by which homologs of an protein, gene or polynucleic acid can readily be cloned using genetic probes and PCR. Identity of cloned sequences as “homologs” can be confirmed using functional assays and/or by genomic mapping of the genes.

[0082] A polypeptide (or protein or enzyme) has “homology” or is “homologous” to a second polypeptide if the nucleic acid sequence that encodes the polypeptide has a similar sequence to the nucleic acid sequence that encodes the second polypeptide. Alternatively, a polypeptide has homology to a second polypeptide if the two proteins have “similar” amino acid sequences. Thus, the terms “homologous proteins” or “homologous polypeptides” and their grammatical equivalents can refer to two polypeptides have similar amino acid sequences. In certain cases of the invention, polynucleotides and polypeptides homologous to one or more polynucleotides and/or polypeptides set forth in Table 1 may be readily identified using methods known in the art for sequence analysis and comparison.

[0083] A homologous polynucleotide or polypeptide sequence of the invention may also be determined or identified by BLAST analysis (Basic Local Alignment Search Tool) or similar bioinformatic tools, which compare a query nucleotide or polypeptide sequence to a database of

known sequences. For example, a search analysis may be done using BLAST to determine sequence identity or similarity to previously published sequences, and if the sequence has not yet been published, can give relevant insight into the function of the DNA or protein sequence.

[0084] As used herein, the term “substantially pure” and its grammatical equivalents can refer to a particular substance that does not contain a majority of another substance. For example, “substantially pure 2,3-BDO” can mean at least 90% 2,3-BDO. In some instances, “substantially pure 2,3-BDO” can mean at least 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, 99.9%, 99.99%, 99.999%, or 99.9999% 2,3-BDO. For example, substantially pure 2,3-BDO can mean at least 70% 2,3-BDO. In some cases, substantially pure 2,3-BDO can mean at least 75% 2,3-BDO. In some cases, substantially pure 2,3-BDO can mean at least 80% 2,3-BDO. In some cases, substantially pure 2,3-BDO can mean at least 85% 2,3-BDO. In some cases, substantially pure 2,3-BDO can mean at least 90% 2,3-BDO. In some cases, substantially pure 2,3-BDO can mean at least 91% 2,3-BDO. In some cases, substantially pure 2,3-BDO can mean at least 92% 2,3-BDO. In some cases, substantially pure 2,3-BDO can mean at least 93% 2,3-BDO. In some cases, substantially pure 2,3-BDO can mean at least 94% 2,3-BDO. In some cases, substantially pure 2,3-BDO can mean at least 95% 2,3-BDO. In some cases, substantially pure 2,3-BDO can mean at least 96% 2,3-BDO. In some cases, substantially pure 2,3-BDO can mean at least 97% 2,3-BDO. In some cases, substantially pure 2,3-BDO can mean at least 98% 2,3-BDO. In some cases, substantially pure 2,3-BDO can mean at least 99% 2,3-BDO.

[0085] As used herein, the term “substantially similar” and its grammatical equivalents, when used in reference to the similarity between a sequence and a reference sequence, means that the sequences are at least 50% (but not 100%) identical. In some cases, the sequences are 55%, 60%, 65%, 70%, 75%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical. In some cases, the term substantially similar refers to a sequence that is at least 50% identical. In some instances, the term substantially similar refers to a sequence that is 55% identical. In some instances, the term substantially similar refers to a sequence that is 60% identical. In some instances, the term substantially similar refers to a sequence that is 65% identical. In some instances, the term substantially similar refers to a sequence that is 70% identical. In some instances, the term substantially similar refers to a sequence that is 75% identical. In some instances, the term substantially similar refers to a sequence that is 80% identical. In other instances, the term substantially similar refers to a sequence that is 81% identical. In other instances, the term

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Acid Res 22(22): 4673-4680 together with the BLOSUM 62 scoring matrix (Henikoff S & Henikoff, J G, 1992, Proc. Natl. Acad. Sci. USA 89: 10915-10919) using a gap opening penalty of 10 and a gap extension penalty of 0.1, so that the highest order match obtained between two sequences where at least 50% of the total length of one of the two sequences is involved in the alignment.

II. GENETICALLY MODIFIED MICROORGANISMS AND METHODS OF MAKING THE SAME

[0086] The present disclosure is directed, in part, to genetically modified microorganisms that have dramatically improved 2,3-BDO biosynthesis rates as compared to those seen in wild-type microorganisms. In some cases, the biosynthesis rates are orders of magnitude higher than what could be normally produced. In some instances, the microorganisms that do not naturally produce 2,3-BDO have been genetically modified to synthesize 2,3-BDO, including at significantly high levels.

Microorganisms

[0087] In some cases, the microorganisms can use C1 carbon substrates, such as, CO, CO₂, and CH₄, to synthesize a desired end product. This, however, does not mean that these microorganisms use solely C1 carbons. Some of the microorganisms can be made to utilize additional carbon substrates, including carbon substrates that the microorganism naturally uses. For example, if the microorganism naturally uses sugar for carbon substrates, this microorganism can be made to utilize a different carbon source such as a C1 carbon.

[0088] The microorganisms can be a prokaryote or a eukaryote. In some cases, for example, the microorganisms can be bacteria, yeast, or algae.

[0089] Microorganisms that can convert C1 carbon substrates into desired products include those capable of using natural gas as a carbon substrate. For example, the microorganism can use methane contained within the natural gas as a carbon source to make such desired products. Such microorganisms can include methanotrophs. Methanotrophs that can be particularly useful include those from the genera *Methylobacter*, *Methylomicrobium*, *Methylomonas*, *Methylocaldum*, *Methylococcus*, *Methylosoma*, *Methylosarcina*, *Methylothermus*, *Methylohalobius*, *Methylogaea*, *Methylovulum*, *Crenothrix*, *Clonothrix*, *Methylosphaera*, *Methylocapsa*, *Methylocella*, *Methylosinus*, *Methylocystis*, *Methyloacidophilum*, or any combinations thereof. In some cases, the methanotroph is from the genus *Methylococcus*. In one instance, the methanotroph can be a methanotroph from the species *Methylococcus capsulatus*.

[0090] Some microorganisms are capable of using CO₂ as a substrate. Such microorganisms include methanogens. Microorganisms that are capable of using CO₂ as a substrate can contain chlorophyll. Examples thereof include algae and cyanobacteria.

[0091] Some microorganisms are capable of using CO as a substrate. Examples include anaerobic microorganisms such as *Clostridium*. These microorganism can be genetically modified so as to make substantial amounts of 2,3-BDO.

[0092] In some cases, the genetically modified microorganisms described throughout can produce a desired product at higher titers when fermented at a higher temperature. For example, the genetically modified microorganisms can be made to produce higher titers of products, such as 2,3-BDO, butadiene, and/or MEK, when incubated at a temperature of greater than 37 °C (but no greater than a temperature of 100 °C). In some cases, the genetically modified microorganisms can be made to produce higher product titers when incubated at 42 °C compared to at 37 °C. In some cases, the genetically modified microorganisms can be made to produce higher product titers when incubated at 41 °C compared to at 37 °C. In some cases, the genetically modified microorganism can be made to produce higher product titers when incubated at 42 °C compared to at 45 °C. In some cases, the genetically modified microorganism can be made to produce higher product titers when incubated at 41 °C compared to at 45 °C. In some cases, the genetically modified microorganisms can be made to produce higher product titers when incubated at 37 °C compared to at 45 °C. In some cases, the genetic modifications produce the increased tolerance/preference for higher temperatures.

Enzymes

[0093] In order to genetically engineer certain microorganisms to produce certain useful products, such as 2,3-BDO, butadiene, and/or MEK, microorganisms can be transformed with one or more genes that encode specific enzymes. These genes can be heterologous to the microorganism.

[0094] For example, in order to engineer a microorganism that can produce 2,3-BDO, one or more genes (*e.g.*, heterologous genes) can be transformed or transfected into the microorganism, either transiently or stably. In some cases, one or more of these genes can be episomally expressed. In some cases, one or more of these genes can be integrated into the genome of the microorganism. In some cases, one or more of these genes can be episomally expressed whereas one or more of these genes can also be integrated into the genome of the microorganism. In some cases, the engineered microorganism can utilize one or more of the following enzymes: (i) acetolactate synthase, (ii) alpha-acetolactate decarboxylase, and/or (iii) acetoin reductase. Acetolactate synthase (encoded by the gene *AlsS*) converts two molecules of pyruvate into 2-acetolactate. Alpha-acetolactate

decarboxylase (encoded by the gene BudA) converts 2-acetolactate into acetoin. Acetoin reductase (encoded by the gene ButA) converts acetoin into 2,3-BDO using NADPH or NADH as a reduced cofactor. Acetoin reductases that use NADPH as a cofactor are referred to as “NADPH-dependent acetoin reductase(s).” Acetoin reductases that use NADH as a cofactor are referred to as “NADH-dependent acetoin reductase(s).” In some cases, when using a vector to express or integrate a gene into a microorganism, the gene encoding the acetolactate synthase can be 5' in relation to any other gene on the vector. In some cases, the gene encoding the acetoin reductase can be 3' in relation to any other gene on a vector. In some cases, the gene encoding the alpha-acetolactate decarboxylase can be neither 5' or 3' in relation to any other gene on a vector (*e.g.*, there is at least one gene that is 5' as well as at least one gene that is 3' of the alpha-acetolactate decarboxylase gene). The order of the genes can be present on the vector prior, during, or after contact with the microorganism. For example, a gene can be 5' in relation to any other gene on the vector prior to contact with the microorganism. After contact with the microorganism, the gene can be inserted into a genomic position of the microorganism where the gene is then 3' in relation to any other gene on a vector or neither 3' or 5' in relation to any other gene on a vector. In some cases, the gene can remain 5' in relation to any other gene. For instance, the vectors can be modified within a microorganism in such a way that the order of the genes can be altered. In some cases, after contact with the microorganism, the gene can be inserted into the vector where the gene is then 3' in relation to any other gene on the vector or neither 3' or 5' in relation to any other gene on the vector. In some cases, the specific order of the genes can be achieved after one or more heterologous gene(s) have been inserted into the genome of the microorganism. For example, different integration vectors can be used in order to achieve a specific gene order within the genome of the microorganism. In some cases, the order of the genes is determined after the heterologous gene has been inserted in the genome of the microorganism.

[0095] Described herein are microorganisms used to make 2,3-BDO from a C1 carbon (*e.g.*, methane). In some cases, the microorganism herein can be transformed with a gene encoding one or more of the following enzymes: (i) acetoin reductase (NADPH-dependent and/or NADH-dependent); (ii) alpha-acetolactate decarboxylase; and/or (iii) acetolactate synthase (AlsS). For example, the microorganism can be transformed with a gene encoding a NADPH-or NADH-dependent acetoin reductase. As another example, the microorganism can be transformed with a gene encoding an alpha-acetolactate decarboxylase. As yet another example, the microorganism can be transformed with a gene encoding an acetolactate synthase. These genes can be heterologous to

the microorganism. In some cases, these genes can be episomally expressed, integrated into the genome of the microorganism (*e.g.*, through the use of an integration vector), or any combination of thereof. In some cases, the gene encoding the heterologous acetolactate synthase is 5' in relation to any other heterologous gene. In some cases, the heterologous gene encoding the acetoin reductase is 3' in relation to any other heterologous gene. In some cases, the heterologous gene encoding the alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other heterologous gene.

[0096] In some cases, the microorganism can be transformed with two or more genes such as those encoding NADPH- and/or NADH-dependent acetoin reductase and alpha-acetolactate decarboxylase. As another example, the microorganism can also be transformed with genes encoding NADPH- or NADH-dependent acetoin reductase and acetolactate synthase. As yet another example, the microorganism can be transformed with genes encoding an alpha-acetolactate decarboxylase and acetolactate synthase. One or more of the genes can be heterologous to the microorganism. In some cases, these genes can be episomally expressed, integrated into the genome of the microorganism, or any combination of thereof.

[0097] In some cases, the microorganism can be transformed with at least three or more genes such as those encoding NADPH- and/or NADH-dependent acetoin reductase, alpha-acetolactate decarboxylase, and acetolactate synthase. One or more of the genes can be heterologous to the microorganism. In some cases, these genes can be episomally expressed, integrated into the genome of the microorganism, or any combination of thereof.

[0098] The gene encoding the NADPH-dependent acetoin reductase can be from a bacteria (*e.g.*, a gram positive or gram negative bacterium). The bacterium can, for example, be from the genus *Clostridium*, for example, *Clostridium autoethanogenum*.

[0099] The NADPH-dependent acetoin reductase can comprise an amino acid sequence that is substantially similar to SEQ ID NO: 9. For example, the NADPH-dependent acetoin reductase can comprise an amino acid sequence that is at least 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 9. In some cases, the NADPH-dependent acetoin reductase can comprise an amino acid sequence that is at least 60% identical to SEQ ID NO: 9. In some cases, the NADPH-dependent acetoin reductase can comprise an amino acid sequence that is at least 65% identical to SEQ ID NO: 9. In some cases, the NADPH-dependent acetoin reductase can comprise an amino acid sequence that is at least 70% identical to SEQ ID NO: 9. In some cases, the NADPH-dependent acetoin reductase can comprise an amino acid sequence that is at least 75% identical to SEQ ID NO: 9. In some cases, the NADPH-dependent acetoin

reductase can comprise an amino acid sequence that is at least 80% identical to SEQ ID NO: 9. In some cases, the NADPH-dependent acetoin reductase can comprise an amino acid sequence that is at least 85% identical to SEQ ID NO: 9. In some cases, the NADPH-dependent acetoin reductase can comprise an amino acid sequence that is at least 90% identical to SEQ ID NO: 9. In some cases, the NADPH-dependent acetoin reductase can comprise an amino acid sequence that is at least 91% identical to SEQ ID NO: 9. In some cases, the NADPH-dependent acetoin reductase can comprise an amino acid sequence that is at least 92% identical to SEQ ID NO: 9. In some cases, the NADPH-dependent acetoin reductase can comprise an amino acid sequence that is at least 93% identical to SEQ ID NO: 9. In some cases, the NADPH-dependent acetoin reductase can comprise an amino acid sequence that is at least 94% identical to SEQ ID NO: 9. In some cases, the NADPH-dependent acetoin reductase can comprise an amino acid sequence that is at least 95% identical to SEQ ID NO: 9. In some cases, the NADPH-dependent acetoin reductase can comprise an amino acid sequence that is at least 96% identical to SEQ ID NO: 9. In some cases, the NADPH-dependent acetoin reductase can comprise an amino acid sequence that is at least 97% identical to SEQ ID NO: 9. In some cases, the NADPH-dependent acetoin reductase can comprise an amino acid sequence that is at least 98% identical to SEQ ID NO: 9. In some cases, the NADPH-dependent acetoin reductase can comprise an amino acid sequence that is at least 99% identical to SEQ ID NO: 9. In some cases, the NADPH-dependent acetoin reductase can comprise an amino acid sequence that is SEQ ID NO: 9. In some cases, the NADPH-dependent acetoin reductase comprise the amino acid sequence SEQ ID NO: 9.

[00100] The gene encoding the NADH-dependent acetoin reductase can be from a bacteria (*e.g.*, a gram positive or gram negative bacteria). Examples include those from the genus *Bacillus*, for example *Bacillus subtilis*. The bacteria can be from the genus *Paenibacillus*, for example *Paenibacillus polymyxa*.

[00101] In some cases, the NADH-dependent acetoin reductase can comprise an amino acid sequence that is substantially similar to SEQ ID NOs: 11 or 13. For example, the NADH-dependent acetoin reductase can comprises an amino acid sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NOs: 11 or 13. In some cases, the NADH-dependent acetoin reductase can comprise an amino acid sequence that is at least 60% identical to SEQ ID NOs: 11 or 13. In some cases, the NADH-dependent acetoin reductase can comprise an amino acid sequence that is at least 65% identical to SEQ ID NOs: 11 or 13. In some cases, the NADH-dependent acetoin reductase can comprise an amino acid sequence

that is at least 70% identical to SEQ ID NOs: 11 or 13. In some cases, the NADH-dependent acetoin reductase can comprise an amino acid sequence that is at least 75% identical to SEQ ID NOs: 11 or 13. In some cases, the NADH-dependent acetoin reductase can comprise an amino acid sequence that is at least 80% identical to SEQ ID NOs: 11 or 13. In some cases, the NADH-dependent acetoin reductase can comprise an amino acid sequence that is at least 85% identical to SEQ ID NOs: 11 or 13. In some cases, the NADH-dependent acetoin reductase can comprise an amino acid sequence that is at least 90% identical to SEQ ID NOs: 11 or 13. In some cases, the NADH-dependent acetoin reductase can comprise an amino acid sequence that is at least 91% identical to SEQ ID NOs: 11 or 13. In some cases, the NADH-dependent acetoin reductase can comprise an amino acid sequence that is at least 92% identical to SEQ ID NOs: 11 or 13. In some cases, the NADH-dependent acetoin reductase can comprise an amino acid sequence that is at least 93% identical to SEQ ID NOs: 11 or 13. In some cases, the NADH-dependent acetoin reductase can comprise an amino acid sequence that is at least 94% identical to SEQ ID NOs: 11 or 13. In some cases, the NADH-dependent acetoin reductase can comprise an amino acid sequence that is at least 95% identical to SEQ ID NOs: 11 or 13. In some cases, the NADH-dependent acetoin reductase can comprise an amino acid sequence that is at least 96% identical to SEQ ID NOs: 11 or 13. In some cases, the NADH-dependent acetoin reductase can comprise an amino acid sequence that is at least 97% identical to SEQ ID NOs: 11 or 13. In some cases, the NADH-dependent acetoin reductase can comprise an amino acid sequence that is at least 98% identical to SEQ ID NOs: 11 or 13. In some cases, the NADH-dependent acetoin reductase can comprise an amino acid sequence that is at least 99% identical to SEQ ID NOs: 11 or 13. In some cases, the NADH-dependent acetoin reductase comprises the amino acid sequence SEQ ID NOs: 11 or 13.

[00102] The gene encoding the alpha-acetolactate decarboxylase (budA) can be from a bacterium (e.g., a gram positive bacteria or gram negative bacterium). Examples include those from the genus *Clostridium*, for example *Clostridium autoethanogenum*. Other examples include those from the genus *Klebsiella*, for example *Klebsiella pneumoniae*.

[00103] In some cases, the alpha-acetolactate decarboxylase can comprise an amino acid sequence that is substantially similar to SEQ ID NOs: 5 or 7. For example, the alpha-acetolactate decarboxylase can comprise an amino acid sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NOs: 5 or 7. In some cases, the alpha-acetolactate decarboxylase can comprise an amino acid sequence that is at least 60% identical to SEQ ID NOs: 5 or 7. For example, the alpha-acetolactate decarboxylase can

comprise an amino acid sequence that is at least 65% identical to SEQ ID NOs: 5 or 7. In some cases, the alpha-acetolactate decarboxylase can comprise an amino acid sequence that is at least 70% identical to SEQ ID NOs: 5 or 7. For example, the alpha-acetolactate decarboxylase can comprise an amino acid sequence that is at least 75% identical to SEQ ID NOs: 5 or 7. In some cases, the alpha-acetolactate decarboxylase can comprise an amino acid sequence that is at least 80% identical to SEQ ID NOs: 5 or 7. For example, the alpha-acetolactate decarboxylase can comprise an amino acid sequence that is at least 85% identical to SEQ ID NOs: 5 or 7. In some cases, the alpha-acetolactate decarboxylase can comprise an amino acid sequence that is at least 90% identical to SEQ ID NOs: 5 or 7. In some cases, the alpha-acetolactate decarboxylase can comprise an amino acid sequence that is at least 91% identical to SEQ ID NOs: 5 or 7. In some cases, the alpha-acetolactate decarboxylase can comprise an amino acid sequence that is at least 92% identical to SEQ ID NOs: 5 or 7. In some cases, the alpha-acetolactate decarboxylase can comprise an amino acid sequence that is at least 93% identical to SEQ ID NOs: 5 or 7. In some cases, the alpha-acetolactate decarboxylase can comprise an amino acid sequence that is at least 94% identical to SEQ ID NOs: 5 or 7. In some cases, the alpha-acetolactate decarboxylase can comprise an amino acid sequence that is at least 95% identical to SEQ ID NOs: 5 or 7. In some cases, the alpha-acetolactate decarboxylase can comprise an amino acid sequence that is at least 96% identical to SEQ ID NOs: 5 or 7. In some cases, the alpha-acetolactate decarboxylase can comprise an amino acid sequence that is at least 97% identical to SEQ ID NOs: 5 or 7. In some cases, the alpha-acetolactate decarboxylase can comprise an amino acid sequence that is at least 98% identical to SEQ ID NOs: 5 or 7. In some cases, the alpha-acetolactate decarboxylase can comprise an amino acid sequence that is at least 99% identical to SEQ ID NOs: 5 or 7. In some cases, the alpha-acetolactate decarboxylase can comprise an amino acid sequence that is identical to SEQ ID NOs: 5 or 7. In some cases, the alpha-acetolactate decarboxylase comprises the amino acid sequence SEQ ID NOs: 5 or 7.

[00104] The gene encoding the acetolactate synthase (AlsS) can be from a bacterium (*e.g.*, a gram positive bacterium). Examples include those from the genus *Clostridium*, for example *Clostridium autoethanogenum*. Other examples include those from the genus *Bacillus*, for example *Bacillus subtilis*. Additional species examples include *Bacillus licheniformis*.

[00105] In some cases, the acetolactate synthase can comprise an amino acid sequence that is substantially similar to anyone of SEQ ID NOs: 1, 3, or 19. In some cases, the acetolactate synthase can comprise an amino acid sequence that is at least 60% 65%, 70%, 75%, 80%, 85%, 90%, 91%,

92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to anyone of SEQ ID NOs: 1, 3, or 19. In some cases, the acetolactate synthase can comprise an amino acid sequence that is at least 60% identical to anyone of SEQ ID NOs: 1, 3, or 19. In some cases, the acetolactate synthase can comprise an amino acid sequence that is at least 65% identical to anyone of SEQ ID NOs: 1, 3, or 19. In some cases, the acetolactate synthase can comprise an amino acid sequence that is at least 70% identical to anyone of SEQ ID NOs: 1, 3, or 19. In some cases, the acetolactate synthase can comprise an amino acid sequence that is at least 75% identical to anyone of SEQ ID NOs: 1, 3, or 19. In some cases, the acetolactate synthase can comprise an amino acid sequence that is at least 80% identical to anyone of SEQ ID NOs: 1, 3, or 19. In some cases, the acetolactate synthase can comprise an amino acid sequence that is at least 85% identical to anyone of SEQ ID NOs: 1, 3, or 19. In some cases, the acetolactate synthase can comprise an amino acid sequence that is at least 90% identical to anyone of SEQ ID NOs: 1, 3, or 19. In some cases, the acetolactate synthase can comprise an amino acid sequence that is at least 91% identical to anyone of SEQ ID NOs: 1, 3, or 19. In some cases, the acetolactate synthase can comprise an amino acid sequence that is at least 92% identical to anyone of SEQ ID NOs: 1, 3, or 19. In some cases, the acetolactate synthase can comprise an amino acid sequence that is at least 93% identical to anyone of SEQ ID NOs: 1, 3, or 19. In some cases, the acetolactate synthase can comprise an amino acid sequence that is at least 94% identical to anyone of SEQ ID NOs: 1, 3, or 19. In some cases, the acetolactate synthase can comprise an amino acid sequence that is at least 95% identical to anyone of SEQ ID NOs: 1, 3, or 19. In some cases, the acetolactate synthase can comprise an amino acid sequence that is at least 96% identical to anyone of SEQ ID NOs: 1, 3, or 19. In some cases, the acetolactate synthase can comprise an amino acid sequence that is at least 97% identical to anyone of SEQ ID NOs: 1, 3, or 19. In some cases, the acetolactate synthase can comprise an amino acid sequence that is at least 98% identical to anyone of SEQ ID NOs: 1, 3, or 19. In some cases, the acetolactate synthase can comprise an amino acid sequence that is at least 99% identical to anyone of SEQ ID NOs: 1, 3, or 19. In some cases, the acetolactate synthase can comprise an amino acid sequence that is identical to anyone of SEQ ID NOs: 1, 3, or 19.

[00106] In some cases, additional enzymes can be provided to the microorganism to yield other desired end products by fermentation.

[00107] In some cases, the amino acid sequence can be optimized based on the microorganism in which the genes will be provided or the enzymes will be expressed. In such cases, conservative

amino acids substitutions can be made based on whether the microorganism typically uses a specific amino acid or how much of that particular amino acid is available for use within the microorganism.

Vectors

[00108] Polynucleotide constructs prepared for introduction into a prokaryotic or eukaryotic host may typically, but not always, comprise a replication system (*i.e.* vector) recognized by the host. The vector includes the intended polynucleotide fragment encoding the desired polypeptide and, optionally, transcription and translational initiation regulatory sequences operably linked to the polypeptide-encoding segment. Expression systems (such as expression vectors) can include, for example, an origin of replication or autonomously replicating sequence (ARS), expression control sequences, a promoter, an enhancer and necessary processing information sites, such as ribosome - binding sites, RNA splice sites, polyadenylation sites, transcriptional terminator sequences, mRNA stabilizing sequences, nucleotide sequences homologous to host chromosomal DNA, and/or a multiple cloning site. Signal peptides can also be included where appropriate, preferably from secreted polypeptides of the same or related species, which allow the protein to cross and/or lodge in cell membranes or be secreted from the cell.

[00109] The vectors can be constructed using standard methods (see, *e.g.*, Sambrook *et al.*, Molecular Biology: A Laboratory Manual, Cold Spring Harbor, N.Y. 1989; and Ausubel, *et al.*, Current Protocols in Molecular Biology, Greene Publishing, Co. N.Y, 1995).

[00110] Manipulation of polynucleotides that encode the enzymes disclosed herein is typically carried out in recombinant vectors. Vectors which may be employed include bacterial plasmids, bacteriophage, artificial chromosomes, episomal vectors and gene expression vectors. Vectors may be selected to accommodate a polynucleotide encoding a protein of a desired size. Following production of a selected vector, a suitable host cell (*e.g.*, the microorganisms described herein) is transfected or transformed with the vector. Each vector contains various functional components, which generally include a cloning site, an origin of replication and at least one selectable marker gene. A vector may additionally possess one or more of the following elements: an enhancer, promoter, a transcription termination sequence and/or other signal sequences. Such sequence elements may be optimized for the selected host species. Such sequence elements may be positioned in the vicinity of the cloning site, such that they are operatively linked to the gene encoding a preselected enzyme.

[00111] Vectors, including cloning and expression vectors, can contain nucleic acid sequences that enable the vector to replicate in one or more selected microorganisms. For example, the sequence

can be one that enables the vector to replicate independently of the host chromosomal DNA and can include origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast and viruses. For example, the origin of replication from the plasmid pBR322 is suitable for most gram-negative bacteria, the origin of replication for 2 micron plasmid is suitable for yeast, and various viral origins of replication (*e.g.* SV40, adenovirus) are useful for cloning vectors.

[00112] A cloning or expression vector may contain a selection gene, also referred to as a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed microorganisms in a selective culture medium. Microorganisms not transformed with the vector containing the selection gene will therefore not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics and other toxins, *e.g.* ampicillin, neomycin, methotrexate, hygromycin, thiostrepton, apramycin or tetracycline, complement auxotrophic deficiencies, or supply critical nutrients not available in the growth media.

[00113] The replication of vectors may be performed in *E. coli*. An example of a *E. coli*-selectable marker is the β -lactamase gene, which confers resistance to the antibiotic ampicillin. These selectable markers can be obtained from *E. coli* plasmids, such as pBR322 or a pUC plasmid such as pUC18 or pUC19, or pUC119.

[00114] The vectors of the present invention can comprise one or more switches, such as an inducible or repressible switch, *e.g.*, an arabinose or lanthanum sensitive switch. The vectors can also comprise one or more different/same promoters.

Promoters

[00115] Vectors can contain a promoter that is recognized by the host microorganism. The promoter can be operably linked to a coding sequence of interest. Such a promoter can be inducible or constitutive. Polynucleotides are operably linked when the polynucleotides are in a relationship permitting them to function in their intended manner.

[00116] Different promoters can be used to drive the expression of the genes. For example, if temporary gene expression (*i.e.*, non-constitutively expressed) is desired, expression can be driven by inducible promoters.

[00117] In some cases, the AlsS gene is expressed temporarily. In other words, the AlsS gene is not constitutively expressed. The expression of the AlsS gene can be driven by inducible or repressible promoters. Examples of inducible or repressible promoters include, but are not limited to, those promoters inducible or repressible by: (a) sugars such as arabinose and lactose (or non metabolizable

analogs, e.g., isopropyl β -D-1-thiogalactopyranoside (IPTG)); (b) metals such as lanthanum, copper, calcium; (c) temperature; (d) Nitrogen-source; (e) oxygen; (f) cell state (growth or stationary); (g) metabolites such as phosphate; (h) CRISPRi; (i) jun; (j) fos, (k) metallothionein and/or (l) heat shock. These promoters can be used in a methanotroph system. An example of an inducible promoter that can be used within methanotrophs is a pBAD promoter or a pMxaF promoter.

[00118] Inducible or repressible promoters that can be particularly useful are sugar and rare earth metal switches. For example, promoters that are sensitive to the sugar arabinose can be used as an inducible switch. In some cases, arabinose switches can be used to drive expression of one or more genes. For example, in the presence arabinose, the 2,3-BDO producing machinery can be “turned-on.” The arabinose switch can turn on the expression of an acetoin reductase. The arabinose switch can also turn on the expression of an alpha-acetolactate decarboxylase (budA). The arabinose switch can also turn on the expression of acetolactate synthase (AlsS).

[00119] Other particularly useful switches can be rare earth metal switches, such as lanthanum switches (or cerium, praseodymium, or neodymium switches). In some cases, the rare earth metal (e.g., lanthanum, cerium, praseodymium, or neodymium) switch can be a repressible switch that can be used to repress expression of one or more genes, until the repressor is removed, after which the genes are “turned-on”. For example, in the presence the metal lanthanum, the 2,3-BDO producing machinery can be “turned-off.” The lanthanum switch can turned off (and expression of the genes induced) by either removing the lanthanum from the media or diluting the lanthanum in the media to levels where its repressible effects are reduced, minimized, or eliminated. In some cases, the rare earth metal (e.g., lanthanum, cerium, praseodymium, or neodymium) switch can control the expression of an NADPH-dependent acetoin reductase. The rare earth metal (e.g., lanthanum, cerium, praseodymium, or neodymium) switch can be used to control the expression of an alpha-acetolactate decarboxylase (budA). Further, the rare earth metal (e.g., lanthanum, cerium, praseodymium, or neodymium) switch can be used to control the expression of an acetolactate synthase (AlsS).

[00120] Constitutively expressed promoters can also be used in the vector systems herein. For example, the expression of NADPH- or NADH-dependent acetoin reductase and/or alpha-acetolactate decarboxylase can be controlled by constitutively active promoters. Examples of such promoters include but are not limited to pMxaF and p.Bba.J23111.

[00121] Promoters suitable for use with prokaryotic hosts can include, for example, the α -lactamase and lactose promoter systems, alkaline phosphatase, the tryptophan (trp) promoter system, the

erythromycin promoter, apramycin promoter, hygromycin promoter, methylenomycin promoter and hybrid promoters such as the tac promoter. Promoters for use in bacterial systems will also generally contain a Shine-Dalgarno sequence operably linked to the coding sequence.

[00122] Generally, a strong promoter may be employed to provide for high level transcription and expression of the desired product.

[00123] One or more promoters of a transcription unit can be an inducible promoter. For example, a GFP can be expressed from a constitutive promoter while an inducible promoter is used to drive transcription of a gene coding for one or more enzymes as disclosed herein and/or the amplifiable selectable marker.

[00124] Some vectors may contain prokaryotic sequences that facilitate the propagation of the vector in bacteria. Thus, the vectors may have other components such as an origin of replication (*e.g.*, a nucleic acid sequence that enables the vector to replicate in one or more selected microorganisms), antibiotic resistance genes for selection in bacteria, and/or an amber stop codon which can permit translation to read through the codon. Additional selectable gene(s) may also be incorporated. Generally, in cloning vectors, the origin of replication is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences can include the ColEI origin of replication in bacteria or other known sequences.

Genes

[00125] The vectors described throughout can comprise a nucleic acid sequence of one or more genes encoding: i) acetoin reductase (NADPH-dependent and/or NADH-dependent); ii) alpha-acetolactate decarboxylase; and/or iii) acetolactate synthase (AlsS). For example, the vector can comprise an NADPH- or NADH-dependent acetoin reductase gene. The vector can comprise an alpha-acetolactate decarboxylase gene. The vector can comprise an acetolactate synthase gene. These vectors can also contain one or more regulatory elements (inducible and/or repressible promoters) that control the expression of the genes within the vectors. In some cases, the switches that can be used include, but are not limited to, inducible or repressible switches, *e.g.*, an arabinose or lanthanum switches. These genes can be heterologous to the microorganism in which the vector is contacted with (and eventually transformed with). In some cases, these genes can be in an episomally vector. In some cases, the vector can be one that can be used to integrate one or more into the genome of the microorganism. In some cases, both an episomal vector and an integration vector can be used. In some cases, the gene encoding the heterologous acetolactate synthase is 5' in

relation to any other heterologous gene. In some cases, the heterologous gene encoding the acetoin reductase is 3' in relation to any other heterologous gene. In some cases, the heterologous gene encoding the alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other heterologous gene. The order of the genes can be present on the vector prior, during, or after contact with the microorganism. For example, a gene can be 5' in relation to any other gene on the vector prior to contact with the microorganism. After contact with the microorganism, the gene can be inserted into a genomic position of the microorganism where the gene is then 3' in relation to any other gene on a vector or is neither 3' or 5' in relation to any other gene on a vector. In some cases, the gene can remain 5' in relation to any other gene on the vector. For instance, the vectors can be modified within a microorganism in such a way that the order of the genes can be altered. In some cases, after contact with the microorganism, the gene can be inserted into the vector where the gene is then positioned 3' in relation to any other gene on the vector or is positioned as neither 3' or 5' in relation to any other gene on the vector. In some cases, the specific order of the genes can be achieved after one or more heterologous gene(s) have been inserted into the genome of the microorganism. For example, different integration vectors can be used in order to achieve a specific gene order within the genome of the microorganism. In some cases, the order of the genes can be determined after the heterologous gene has been inserted in the genome of the microorganism.

[00126] In some cases, the vector can comprise two or more genes encoding: i) acetoin reductase (NADPH-dependent and/or NADH-dependent); ii) alpha-acetolactate decarboxylase; and/or iii) acetolactate synthase (AlsS). For example, the vector can include an NADPH- or NADH-dependent acetoin reductase. In this case, one or more of the genes can be heterologous to the microorganism in which the vector is contacted with (and eventually transformed with). In some cases, these genes can be in an episomally vector. In some cases, the vector can be one that can be used to integrate one or more into the genome of the microorganism. In some cases, these genes can be in an episomally vector as well as in an integration vector. In some cases, the gene encoding the heterologous acetolactate synthase is 5' in relation to any other heterologous gene. In some cases, the heterologous gene encoding the acetoin reductase is 3' in relation to any other heterologous gene. In some cases, the heterologous gene encoding the alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other heterologous gene.

[00127] In one case, the vector can comprise at least three or more genes encoding NADPH- and/or NADH-dependent acetoin reductase, alpha-acetolactate decarboxylase, and acetolactate synthase. One or more of the genes can be heterologous to the microorganism in which the vector is contacted

with (and eventually transformed with). In some cases, these genes can be in an episomally vector. In some cases, the vector can be one that can be used to integrate one or more into the genome of the microorganism. In some cases, these genes can be in an episomally vector as well as in an integration vector.

[00128] In one instance, the acetoin reductase gene is from a bacteria (e.g., a gram positive bacterium). The bacterium can be from the genus *Clostridium*, for example the species *Clostridium autoethanogenum*.

[00129] The NADPH-dependent acetoin reductase gene can comprise a nucleotide sequence that is substantially similar to SEQ ID NO: 10. For example, the NADPH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 60%, , 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 10. For example, the NADPH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 60% identical to SEQ ID NO: 10. In some cases, the NADPH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 65% identical to SEQ ID NO: 10. In some cases, the NADPH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 70% identical to SEQ ID NO: 10. In some cases, the NADPH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 75% identical to SEQ ID NO: 10. In some cases, the NADPH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 80% identical to SEQ ID NO: 10. In some cases, the NADPH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 85% identical to SEQ ID NO: 10. In some cases, the NADPH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 90% identical to SEQ ID NO: 10. In some cases, the NADPH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 91% identical to SEQ ID NO: 10. In some cases, the NADPH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 92% identical to SEQ ID NO: 10. In some cases, the NADPH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 93% identical to SEQ ID NO: 10. In some cases, the NADPH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 94% identical to SEQ ID NO: 10. In some cases, the NADPH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 95% identical to SEQ ID NO: 10. In some cases, the NADPH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 96% identical to SEQ ID NO: 10. In some cases, the NADPH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 97% identical to SEQ ID NO: 10. In some cases, the

NADPH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 98% identical to SEQ ID NO: 10. In some cases, the NADPH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 99% identical to SEQ ID NO: 10. In some cases, the NADPH-dependent acetoin reductase gene can comprise a nucleotide sequence that is SEQ ID NO: 10.

[00130] When a NADH-dependent acetoin reductase is desired, the NADH-dependent acetoin reductase gene can be from a bacteria (*e.g.*, a gram positive bacterium). Examples of such bacteria include those from the genus *Bacillus*, for example the species *Bacillus subtilis*, and the genus *Paenibacillus*, for example the species *Paenibacillus polymyxa*.

[00131] The NADH-dependent acetoin reductase gene can comprise a nucleotide sequence that is substantially similar to SEQ ID NOs: 12 or 14. For example NADH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NOs: 12 or 14. In some cases, the NADH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 65% identical to SEQ ID NOs: 12 or 14. In some cases, the NADH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 70% identical to SEQ ID NOs: 12 or 14. In some cases, the NADH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 75% identical to SEQ ID NOs: 12 or 14. In some cases, the NADH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 80% identical to SEQ ID NOs: 12 or 14. In some cases, the NADH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 85% identical to SEQ ID NOs: 12 or 14. In some cases, the NADH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 90% identical to SEQ ID NOs: 12 or 14. In some cases, the NADH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 91% identical to SEQ ID NOs: 12 or 14. In some cases, the NADH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 92% identical to SEQ ID NOs: 12 or 14. In some cases, the NADH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 93% identical to SEQ ID NOs: 12 or 14. In some cases, the NADH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 94% identical to SEQ ID NOs: 12 or 14. In some cases, the NADH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 95% identical to SEQ ID NOs: 12 or 14. In some cases, the NADH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 96% identical to SEQ ID NOs: 12

or 14. In some cases, the NADH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 97% identical to SEQ ID NOs: 12 or 14. In some cases, the NADH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 98% identical to SEQ ID NOs: 12 or 14. In some cases, the NADH-dependent acetoin reductase gene can comprise a nucleotide sequence that is at least 99% identical to SEQ ID NOs: 12 or 14. In some cases, the NADH-dependent acetoin reductase gene can comprise a nucleotide sequence that is identical to SEQ ID NOs: 12 or 14.

[00132] When an alpha-acetolactate decarboxylase (budA) is desired, the alpha-acetolactate decarboxylase gene can be from a bacterium. Examples of such bacterium include those from the genus *Clostridium*, for example the species *Clostridium autoethanogenum*, and those from the genus *Klebsiella*, for example the species *Klebsiella pneumoniae*. The alpha-acetolactate decarboxylase gene can be from a gram positive or gram negative bacterium.

[00133] The alpha-acetolactate decarboxylase gene can comprise a nucleotide sequence that is substantially similar to SEQ ID NOs: 6 or 8. For example, the alpha-acetolactate decarboxylase gene can comprise a nucleotide sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NOs: 6 or 8. In some cases, the alpha-acetolactate decarboxylase gene can comprise a nucleotide sequence that is at least 60% identical to SEQ ID NOs: 6 or 8. In some cases, the alpha-acetolactate decarboxylase gene can comprise a nucleotide sequence that is at least 65% identical to SEQ ID NOs: 6 or 8. In some cases, the alpha-acetolactate decarboxylase gene can comprise a nucleotide sequence that is at least 70% identical to SEQ ID NOs: 6 or 8. In some cases, the alpha-acetolactate decarboxylase gene can comprise a nucleotide sequence that is at least 75% identical to SEQ ID NOs: 6 or 8. In some cases, the alpha-acetolactate decarboxylase gene can comprise a nucleotide sequence that is at least 80% identical to SEQ ID NOs: 6 or 8. In some cases, the alpha-acetolactate decarboxylase gene can comprise a nucleotide sequence that is at least 85% identical to SEQ ID NOs: 6 or 8. In some cases, the alpha-acetolactate decarboxylase gene can comprise a nucleotide sequence that is at least 90% identical to SEQ ID NOs: 6 or 8. In some cases, the alpha-acetolactate decarboxylase gene can comprise a nucleotide sequence that is at least 91% identical to SEQ ID NOs: 6 or 8. In some cases, the alpha-acetolactate decarboxylase gene can comprise a nucleotide sequence that is at least 92% identical to SEQ ID NOs: 6 or 8. In some cases, the alpha-acetolactate decarboxylase gene can comprise a nucleotide sequence that is at least 93% identical to SEQ ID NOs: 6 or 8. In some cases, the alpha-acetolactate decarboxylase gene can comprise a nucleotide sequence that is at least 94%

identical to SEQ ID NOs: 6 or 8. In some cases, the alpha-acetolactate decarboxylase gene can comprise a nucleotide sequence that is at least 95% identical to SEQ ID NOs: 6 or 8. In some cases, the alpha-acetolactate decarboxylase gene can comprise a nucleotide sequence that is at least 96% identical to SEQ ID NOs: 6 or 8. In some cases, the alpha-acetolactate decarboxylase gene can comprise a nucleotide sequence that is at least 97% identical to SEQ ID NOs: 6 or 8. In some cases, the alpha-acetolactate decarboxylase gene can comprise a nucleotide sequence that is at least 98% identical to SEQ ID NOs: 6 or 8. In some cases, the alpha-acetolactate decarboxylase gene can comprise a nucleotide sequence that is at least 99% identical to SEQ ID NOs: 6 or 8. In some cases, the alpha-acetolactate decarboxylase gene can comprise a nucleotide sequence that is identical to SEQ ID NOs: 6 or 8.

[00134] When an acetolactate synthase (AlsS) is desired, the acetolactate synthase can be from a bacterium (*e.g.*, a gram positive bacterium). Examples of such bacterium include those from the genus *Clostridium*, for example the species *Clostridium autoethanogenum*, and those from the genus *Bacillus*, for example the species *Bacillus subtilis*. Additional species examples include *Bacillus licheniformis*.

[00135] The acetolactate synthase gene can comprise a nucleotide sequence that is substantially similar to any one of SEQ ID NOs: 2, 4, or 20. For example, the acetolactate synthase gene can comprise a nucleotide sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to any one of SEQ ID NOs: 2, 4, or 20. The acetolactate synthase gene can comprise a nucleotide sequence that is substantially similar to any one of SEQ ID NOs: 2, 4, or 20. For example, the acetolactate synthase gene can comprise a nucleotide sequence that is at least 60% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the acetolactate synthase gene can comprise a nucleotide sequence that is at least 65% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the acetolactate synthase gene can comprise a nucleotide sequence that is at least 70% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the acetolactate synthase gene can comprise a nucleotide sequence that is at least 75% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the acetolactate synthase gene can comprise a nucleotide sequence that is at least 80% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the acetolactate synthase gene can comprise a nucleotide sequence that is at least 85% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the acetolactate synthase gene can comprise a nucleotide sequence that is at least 90% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the acetolactate synthase gene can comprise a nucleotide sequence that is at least 91%

identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the acetolactate synthase gene can comprise a nucleotide sequence that is at least 92% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the acetolactate synthase gene can comprise a nucleotide sequence that is at least 93% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the acetolactate synthase gene can comprise a nucleotide sequence that is at least 94% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the acetolactate synthase gene can comprise a nucleotide sequence that is at least 95% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the acetolactate synthase gene can comprise a nucleotide sequence that is at least 96% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the acetolactate synthase gene can comprise a nucleotide sequence that is at least 97% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the acetolactate synthase gene can comprise a nucleotide sequence that is at least 98% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the acetolactate synthase gene can comprise a nucleotide sequence that is at least 99% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the acetolactate synthase gene can comprise a nucleotide sequence that is identical to any one of SEQ ID NOs: 2, 4, or 20.

[00136] Additional genes can be placed inside the microorganism in order to make other desired end products by fermentation.

[00137] The nucleotide sequence (or more specifically the codons that are encoded by the nucleotide sequences) can be optimized based on the microorganism in which the nucleotide sequences will be expressed. The nucleotide sequences can be codon optimized based on the amount of tRNA available within each individual microorganism. In other words, conservative codon substitutions can be made based on whether the respective microorganism typically uses a specific codon or how much of a particular tRNA is available within the microorganism.

Gene Copy Number

[00138] Any of the genes disclosed throughout can have one or more copies of the genes (whether integrated, episomally expressed, or both). For example, each of the acetoin reductase (NADPH-dependent and/or NADH-dependent); ii) alpha-acetolactate decarboxylase; and/or iii) acetolactate synthase (AlsS) can have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more copies of the genes. In some cases, the gene copy number is 1. In some cases, the gene copy number is 2. In some cases, the gene copy number is 3. In some cases, the gene copy number is 4. In some cases, the gene copy number is 5. In some cases, the gene copy number is 6. In some cases, the gene copy number is 7. In some cases, the gene copy number is 8. In some cases, the gene copy number is 9. In some cases, the gene copy number is 10. In some cases, the gene

copy number is 11. In some cases, the gene copy number is 12. In some cases, the gene copy number is 13. In some cases, the gene copy number is 14. In some cases, the gene copy number is 15. In some cases, the gene copy number is 16. In some cases, the gene copy number is 17. In some cases, the gene copy number is 18. In some cases, the gene copy number is 19. In some cases, the gene copy number is 20. In some cases, the gene copy number is 21. In some cases, the gene copy number is 22. In some cases, the gene copy number is 23. In some cases, the gene copy number is 24. In some cases, the gene copy number is 25. Typically, the gene copy number is under 25 copies, however, in some cases, it can be more. Therefore, in some cases, the gene copy number is more than 25.

[00139] Further, any of genes described throughout (*e.g.*, acetoin reductase; alpha-acetolactate decarboxylase; and/or acetolactate synthase) can have the same number of copies of genes. For example, the gene encoding acetolactate synthase can have 1 to 25 copies, the gene encoding alpha-acetolactate decarboxylase can have 1 to 25 copies, and the gene encoding acetoin reductase can have 1 to 25 copies, where all the genes are present in the same number. In some cases, any of the genes described throughout can have different number of copies of genes. For example, the gene encoding acetolactate synthase can have 1 to 25 copies, the gene encoding alpha-acetolactate decarboxylase can have 1 to 25 copies, and the gene encoding acetoin reductase can have 1 to 25 copies, where all the genes are present in different numbers. In one particular example, the gene encoding an acetolactate synthase can have 5 to 10 copies, while the gene encoding acetoin reductase can have 1 to 6 copies, while the gene encoding an alpha-acetolactate decarboxylase can have 3 to 8 copies. Again, typically the gene copy number is under 25 copies of any given gene. However, in some cases it can be more.

[00140] Any number of the genes can be expressed in an integration vector, episomal vector, or both. In regards to the example above, the gene encoding an acetolactate synthase can have 5 to 10 copies, where 2 to 5 can be integrated and 3 to 5 copies can be expressed in the episomal vector. In regards to the example above, the gene encoding an acetoin reductase decarboxylase can have 1 to 6 copies, where 1 to 4 copies can be integrated and 0 to 1 copies can be expressed in the episomal vector. In regards to the example above, the gene encoding an alpha-acetolactate decarboxylase can have 3 to 8 copies, where 2 to 6 copies can be integrated and 1 to 2 copies can be expressed in the episomal vector.

[00141] In some cases, a gene copy can include a partial sequence of the full gene. In some cases, the partial sequence of the full gene can include the active site of the enzyme encoded by the gene.

Any combination of the full gene and partial sequence of the gene is contemplated. For example, there can be 4 copies of the full gene and 2 copies of the partial sequence of the gene. This can be considered 6 copies of the gene.

Isolated Nucleic Acids

[00142] The genes described herein can be in the form of an isolated polynucleic acid. In other words, the genes can be in forms that do not exist in nature, isolated from a chromosome. The isolated polynucleic acids can comprise a nucleic acid sequence of one or more genes encoding: i) acetoin reductase (NADPH-dependent and/or NADH-dependent); ii) alpha-acetolactate decarboxylase; and/or iii) acetolactate synthase (AlsS). For example, the isolated polynucleic acid can comprise an NADPH-dependent and/or NADH-dependent acetoin reductase gene. The isolated polynucleic acid can comprise an alpha-acetolactate decarboxylase gene. The isolated polynucleic acid can comprise an acetolactate synthase gene.

[00143] In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that can encode a NADPH-dependent acetoin reductase gene. For example, the isolated polynucleic acid can comprise a nucleotide sequence that is substantially similar to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprises a nucleotide sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 60% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 65% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 70% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 75% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 80% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 81% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 82% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 83% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 84% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 85% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 86% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a

nucleotide sequence that is at least 87% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 88% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 89% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 90% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 91% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 92% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 93% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 94% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 95% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 96% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 97% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 98% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 99% identical to SEQ ID NO: 10. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is SEQ ID NO: 10.

[00144] In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that can encode a NADH-dependent acetoin reductase. For example, the isolated polynucleic acid can comprise a nucleotide sequence that is substantially similar to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can encode for an NADH-dependent acetoin reductase. For example, the isolated polynucleic acid can comprise a nucleotide sequence that is substantially similar to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 60% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 65% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 70% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 75% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at

least 80% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 81% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 82% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 83% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 84% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 85% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 86% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 87% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 88% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 89% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 90% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 91% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 92% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 93% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 94% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 95% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 96% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 97% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 98% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 99% identical to SEQ ID NOs: 12 or 14. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is identical to SEQ ID NOs: 12 or 14.

[00145] In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that encodes for an alpha-acetolactate decarboxylase. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is substantially similar to SEQ ID NOs: 6 or 8. For example,

[illegible]

polynucleic acid can comprise a nucleotide sequence that is at least 98% identical to SEQ ID NOs: 6 or 8. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 99% identical to SEQ ID NOs: 6 or 8. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is identical to SEQ ID NOs: 6 or 8

[00146] In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that encodes an acetolactate synthase. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is substantially similar to any one of SEQ ID NOs: 2, 4, or 20. For example, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to any one of SEQ ID NOs: 2, 4, or 20. The isolated polynucleic acid can comprise a nucleotide sequence that is at least 60% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 65% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 70% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 75% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 80% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 81% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 82% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 83% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 84% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 85% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 86% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 87% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 88% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 89% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 90% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the

isolated polynucleic acid can comprise a nucleotide sequence that is at least 91% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 92% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 93% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 94% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 95% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 96% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 97% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 98% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is at least 99% identical to any one of SEQ ID NOs: 2, 4, or 20. In some cases, the isolated polynucleic acid can comprise a nucleotide sequence that is identical to any one of SEQ ID NOs: 2, 4, or 20.

Exemplary Vector Sequences

[00147] Disclosed herein are also vectors. These vectors can be integrated into various microorganisms, such as methanotrophs are disclosed herein (*e.g.*, any one of SEQ ID NOs: 15 to 18). In some cases, these vectors can be episomally expressed. In some cases, these vectors can be both integrated and episomally expressed. In some cases, minor changes can be made to the vectors without significant changes in the effectiveness of the vectors or the amount of enzymes the vectors are able to produce. Thus, the vectors can be substantially similar to any one of SEQ ID NOs: 15 to 18.

[00148] In some cases, the expression cassette of SEQ ID NO: 15 or a sequence that is substantially similar thereto, can be contacted with (and inserted into) a microorganism. This expression cassette comprises a pBAD promoter; a g.Bsu AlsS gene (which includes an initiator ATG and a terminator TAA); a ribosomal binding site rbsGTW001; a g.Kpn BudA gene (which includes an initiator ATG and a terminator TGA); a terminator rrnB; a pmxαF promoter; g.Cau ButA gene (which includes an initiator ATG and a terminator TGA) and a terminator lambda T0. Once a microorganism (such as a methanotroph) is transformed with this expression cassette, the microorganism is referred to as strain XZ58. In some cases, the g.Bsu AlsS gene can be substituted for g.Blic_AlsS gene (*e.g.*, SEQ ID NO: 20).

[00149] In some cases, the expression cassette of SEQ ID NO: 16 or a sequence that is substantially similar, can be contacted with (and inserted into) a microorganism. This expression cassette comprises a pBAD promoter; a g.Bsu AlsS gene (which includes an initiator ATG and a terminator TAA); a ribosomal binding site rbsGTW001; a g.Kpn BudA gene (which includes an initiator ATG and a terminator TGA); a terminator rrnB; a pmxαF promoter; g.Bsu ButA gene (which includes an initiator ATG and a terminator TGA) and a terminator lambda T0. Once a microorganism (such as a methanotroph) is transformed with this expression cassette, the microorganism is referred to as strain XZ59. In some cases, the g.Bsu AlsS gene can be substituted for g.Blic_AlsS gene (*e.g.*, SEQ ID NO: 20).

[00150] In some cases, the expression cassette of SEQ ID NO: 17 or a sequence that is substantially similar, can be contacted with (and inserted into) a microorganism. This expression cassette comprises a pBAD promoter; a g.Bsu AlsS gene (which includes an initiator ATG and a terminator TAA); a ribosomal binding site rbsGTW001; a g.Kpn BudA gene (which includes an initiator ATG and a terminator TGA); an additional ribosomal binding site rbsGTW001; a terminator rrnB; a pmxαF promoter; g.Cau ButA gene (which includes an initiator ATG and a terminator TGA) and a terminator rrnB. Once a microorganism (such as a methanotroph) is transformed with this expression cassette, the microorganism is referred to as strain XZ06. In some cases, the g.Bsu AlsS gene can be substituted for g.Blic_AlsS gene (*e.g.*, SEQ ID NO: 20).

[00151] In some cases, the expression cassette of SEQ ID NO: 18 or a sequence that is substantially similar, can be contacted with (and inserted into) a microorganism. This expression cassette comprises a pBAD promoter; a g.Bsu AlsS gene (which includes an initiator ATG and a terminator TAA); a ribosomal binding site rbsGTW001; a g.Kpn BudA gene (which includes an initiator ATG and a terminator TGA); an additional ribosomal binding site rbsGTW001; a terminator rrnB; a pmxαF promoter; g.Bsu ButA gene (which includes an initiator ATG and a terminator TGA) and a terminator rrnB. Once a microorganism (such as a methanotroph) is transformed with this expression cassette, the microorganism is referred to as strain XZ08. In some cases, the g.Bsu AlsS gene can be substituted for g.Blic_AlsS gene (*e.g.*, SEQ ID NO: 20).

III. Method of Making Genetically Modified Microorganisms

[00152] The genetically modified microorganisms disclosed throughout can be made by a variety of ways. A microorganism may be modified (*e.g.*, genetically engineered) by any method to comprise and/or express one or more polynucleotides encoding for enzymes in a pathway that catalyze a

[00153] The microorganism used in this method can be any described above, including but not limited to a prokaryote. Other microorganisms such as bacteria, yeast, or algae can be used. Microorganisms of particular interest include methanotrophs, such as those from the genera *Methylobacter*, *Methylomicrobium*, *Methylomonas*, *Methylocaldum*, *Methylococcus*, *Methylosoma*, *Methylosarcina*, *Methylothermus*, *Methylohalobius*, *Methylogaea*, *Methylovulum*, *Crenothrix*, *Clonothrix*, *Methylosphaera*, *Methylocapsa*, *Methylocella*, *Methylosinus*, *Methylocystis*, or *Methyloacidophilum*. One desired species can include a *Methylococcus capsulatus*.

[00154] An exemplary method of making a genetically modified microorganism disclosed herein is contacting (or transforming) a microorganism with a nucleic acid that expresses at least one heterologous gene encoding i) an acetoin reductase (e.g., NADPH- or NADH- dependent); ii) an alpha-acetolactate decarboxylase (budA); iii) a acetolactate synthase, or iv) any combination thereof. The microorganism can be any microorganism that is capable of converting a C1 carbon to a product. In some cases, the product is 2,3-BDO.

[00155] The NADPH- or NADH-dependent acetoin reductase, alpha-acetolactate decarboxylase (budA); and/or acetolactate synthase can be any of the variations described above. For example, the acetoin reductase can be from a gram positive bacterium, such as from the genus *Clostridium*, like the species *Clostridium autoethanogenum*.

[00156] The genes that are inserted into a microorganism can be heterologous to the microorganism itself. For example, if the microorganism is a methanotroph, the inserted genes can, for example, be from yeast, a bacterium, or a different species of methanotroph. Further, the genes can be endogenously part of the genome of the microorganism.

[00157] The genes can be inserted into a microorganism through the use of vectors. In some cases, the genes can be inserted into the genome of the microorganism. In some cases, both techniques can be used when two or more genes are inserted into a microorganism. For example, a gene can be inserted into the genome of a microorganism by, for example, use of an integration vector. Subsequently, an additional gene can be transformed into the microorganism through an episomal vector. In some cases, the vector can present a specific order of genes. For example, in some cases the vector can comprise a gene encoding for an acetolactate synthase, where the gene is 5' in relation to any other gene on the vector. In some cases, the vector can comprise a gene encoding for an acetoin reductase, where the gene is 3' in relation to any other gene on the vector. In some cases, the vector can comprise a gene encoding for an alpha-acetolactate decarboxylase, which is neither 5' or 3' in relation to any other gene on the vector.

[00158] In some cases, the genetically modified microorganisms made by the methods described throughout, can produce a desired product at higher titers when fermented at a higher temperature. For example, the genetically modified microorganisms can be made to produce higher titers of products, such as 2,3-BDO, butadiene, and/or MEK, when incubated at a temperature of greater than 37 °C (but no greater than a temperature of 100 °C). In some cases, the genetically modified microorganisms can be made to produce higher product titers when incubated at 42 °C compared to at 37 °C. In some cases, the genetically modified microorganisms can be made to produce higher

product titers when incubated at 41 °C compared to at 37 °C. In some cases, the genetically modified microorganism can be made to produce higher product titers when incubated at 42 °C compared to at 45 °C. In some cases, the genetically modified microorganism can be made to produce higher product titers when incubated at 41 °C compared to at 45 °C. In some cases, the genetically modified microorganisms can be made to produce higher product titers when incubated at 37 °C compared to at 45 °C. In some cases, the genetic modifications produce the increased tolerance/preference for higher temperatures.

Techniques for Genetic Modification

[00159] The microorganisms disclosed herein may be genetically engineered by using classic microbiological techniques. Some of such techniques are generally disclosed, for example, in Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Labs Press.

[00160] The genetically modified microorganisms disclosed herein may include a polynucleotide that has been inserted, deleted or modified (*i.e.*, mutated; *e.g.*, by insertion, deletion, substitution, and/or inversion of nucleotides), in such a manner that such modifications provide the desired effect of expression (*e.g.*, over-expression) of one or more enzymes as provided herein within the microorganism. Genetic modifications which result in an increase in gene expression or function can be referred to as amplification, overproduction, overexpression, activation, enhancement, addition, or up-regulation of a gene. Addition of a gene to increase gene expression can include maintaining the gene(s) on replicating plasmids or integrating the cloned gene(s) into the genome of the production microorganism. Furthermore, increasing the expression of desired genes can include operatively linking the cloned gene(s) to native or heterologous transcriptional control elements.

[00161] Where desired, the expression of one or more of the enzymes provided herein is under the control of a regulatory sequence that controls directly or indirectly the enzyme expression in a time-dependent fashion during the fermentation. In some cases, inducible promoters can be used to achieve this.

[00162] In some cases, a microorganism is transformed or transfected with a genetic vehicle, such as an expression vector comprising a heterologous polynucleotide sequence coding for the enzymes are provided herein. In some cases, the vector(s) can be an episomal vector, the gene sequence can be integrated into the genome of the microorganism, or any combination thereof. In some cases, the vectors comprising the heterologous polynucleotide sequence encoding for the enzymes provided herein are integrated into the genome of the microorganism.

[00163] To facilitate insertion and expression of different genes coding for the enzymes as disclosed herein from the constructs and expression vectors, the constructs may be designed with at least one cloning site for insertion of any gene coding for any enzyme disclosed herein. The cloning site may be a multiple cloning site, *e.g.*, containing multiple restriction sites.

Transfection

[00164] Standard transfection techniques can be used to insert genes into a microorganism. As used herein, the term “transfection” or “transformation” and their grammatical equivalents can refer to the insertion of an exogenous nucleic acid or polynucleotide into a host cell. The exogenous nucleic acid or polynucleotide may be maintained as a non-integrated vector, for example, a plasmid or episomal vector, or alternatively, can be integrated into the host cell genome. The term transfecting or transfection is intended to encompass all conventional techniques for introducing nucleic acid or polynucleotide into microorganisms. Examples of transfection techniques include, but are not limited to, calcium phosphate precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation, microinjection, rubidium chloride or polycation mediated transfection, protoplast fusion, and sonication. The transfection method that provides optimal transfection frequency and expression of the construct in the particular host cell line and type is favored. For stable transfectants, the constructs are integrated so as to be stably maintained within the host chromosome. In some cases, the preferred transfection is a stable transfection. In some cases, the integration of the gene occurs at a specific locus within the genome of the microorganism.

Transformation

[00165] Expression vectors or other nucleic acids may be introduced to selected microorganisms by any of a number of suitable methods. For example, vector constructs may be introduced to appropriate cells by any of a number of transformation methods for plasmid vectors. Standard calcium-chloride-mediated bacterial transformation is still commonly used to introduce naked DNA to bacteria (see, *e.g.*, Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), but electroporation and conjugation may also be used (see, *e.g.*, Ausubel *et al.*, 1988, Current Protocols in Molecular Biology, John Wiley & Sons, Inc., NY, N.Y.).

[00166] For the introduction of vector constructs to yeast or other fungal cells, chemical transformation methods can be used (*e.g.*, Rose *et al.*, 1990, Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). Transformed cells may be isolated on

selective media appropriate to the selectable marker used. Alternatively, or in addition, plates or filters lifted from plates may be scanned for GFP fluorescence to identify transformed clones.

[00167] For the introduction of vectors comprising differentially expressed sequences to certain types of cells, the method used may depend on the form of the vector. Plasmid vectors may be introduced by any of a number of transfection methods, including, for example, lipid-mediated transfection ("lipofection"), DEAE-dextran-mediated transfection, electroporation or calcium phosphate precipitation (see, *e.g.*, Ausubel *et al.*, 1988, Current Protocols in Molecular Biology, John Wiley & Sons, Inc., NY, N.Y.).

[00168] Lipofection reagents and methods suitable for transient transfection of a wide variety of transformed and non-transformed or primary cells are widely available, making lipofection an attractive method of introducing constructs to eukaryotic, and particularly mammalian cells in culture. Many companies offer kits and ways for this type of transfection.

[00169] The host cell may be capable of expressing the construct encoding the desired protein, processing the protein and transporting a secreted protein to the cell surface for secretion. Processing includes co- and post-translational modification such as leader peptide cleavage, GPI attachment, glycosylation, ubiquitination, and disulfide bond formation.

[00170] Microorganisms can be transformed or transfected with the above-described expression vectors or polynucleotides coding for one or more enzymes as disclosed herein and cultured in nutrient media modified as appropriate for the specific microorganism, inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. In some cases, electroporation methods can be used to deliver an expression vector.

[00171] Expression of a vector (and the gene contained in the vector) can be verified by an expression assay, for example, qPCR or by measuring levels of RNA. Expression level can be indicative also of copy number. For example, if expression levels are extremely high, this can indicate that more than one copy of a gene was integrated in a genome. Alternatively, high expression can indicate that a gene was integrated in a highly transcribed area, for example, near a highly expressed promoter. Expression can also be verified by measuring protein levels, such as through Western blotting.

CRISPR/cas system

[00172] The methods disclosed throughout can involve pinpoint insertion of genes or the deletion of genes (or parts of genes). Methods described herein can use a CRISPR/cas system. For example, double-strand breaks (DSBs) can be generated using a CRISPR/cas system, *e.g.*, a type II

CRISPR/cas system. A Cas enzyme used in the methods disclosed herein can be Cas9, which catalyzes DNA cleavage. Enzymatic action by Cas9 from *Streptococcus pyogenes* or any closely related Cas9 can generate double stranded breaks at target site sequences which hybridize to 20 nucleotides of a guide sequence and have a protospacer-adjacent motif (PAM) following the 20 nucleotides of the target sequence.

[00173] A vector can be operably linked to an enzyme-coding sequence encoding a CRISPR enzyme, such as a Cas protein. Cas proteins that can be used include class 1 and class 2. Non-limiting examples of Cas proteins include Cas1, Cas1B, Cas2, Cas3, Cas4, Cas5, Cas5d, Cas5t, Cas5h, Cas5a, Cas6, Cas7, Cas8, Cas9 (also known as Csn1 or Csx12), Cas10, Csy1, Csy2, Csy3, Csy4, Cse1, Cse2, Cse3, Cse4, Cse5e, Csc1, Csc2, Csa5, Csn1, Csn2, Csm1, Csm2, Csm3, Csm4, Csm5, Csm6, Cmr1, Cmr3, Cmr4, Cmr5, Cmr6, Csb1, Csb2, Csb3, Csx17, Csx14, Csx10, Csx16, CsaX, Csx3, Csx1, Csx1S, Csf1, Csf2, CsO, Csf4, Csd1, Csd2, Cst1, Cst2, Csh1, Csh2, Csa1, Csa2, Csa3, Csa4, Csa5, C2c1, C2c2, C2c3, Cpf1, CARE, DinG, homologues thereof, or modified versions thereof. An unmodified CRISPR enzyme can have DNA cleavage activity, such as Cas9. A CRISPR enzyme can direct cleavage of one or both strands at a target sequence, such as within a target sequence and/or within a complement of a target sequence. For example, a CRISPR enzyme can direct cleavage of one or both strands within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, 300, 400, 500, or more base pairs from the first or last nucleotide of a target sequence. A vector that encodes a CRISPR enzyme that is mutated to with respect, to a corresponding wild-type enzyme such that the mutated CRISPR enzyme lacks the ability to cleave one or both strands of a target polynucleotide containing a target sequence can be used.

[00174] A vector that encodes a CRISPR enzyme comprising one or more nuclear localization sequences (NLSs) can be used. For example, there can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 NLSs used. A CRISPR enzyme can comprise the NLSs at or near the ammo-terminus (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 NLSs), or at or near the carboxy-terminus (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 NLSs), or any combination of these (*e.g.*, one or more NLS at the ammo-terminus and one or more NLS at the carboxy terminus). When more than one NLS is present, each can be selected independently of others, such that a single NLS can be present in more than one copy and/or in combination with one or more other NLSs present in one or more copies.

[00175] CRISPR enzymes used in the methods can comprise at most 6 NLSs. An NLS is considered near the N- or C-terminus when the nearest amino acid to the NLS is within 50 amino

acids along a polypeptide chain from the N- or C-terminus, *e.g.*, within 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, or 50 amino acids.

Guide RNA

[00176] As used herein, the term “guide RNA” and its grammatical equivalents can refer to an RNA which can be specific for a target DNA and can form a complex with Cas protein. An RNA/Cas complex can assist in “guiding” Cas protein to a target DNA.

[00177] A method disclosed herein also can comprise introducing into a cell or embryo at least one guide RNA or nucleic acid, *e.g.*, DNA encoding at least one guide RNA. A guide RNA can interact with a RNA-guided endonuclease to direct the endonuclease to a specific target site, at which site the 5' end of the guide RNA base pairs with a specific protospacer sequence in a chromosomal sequence.

[00178] A guide RNA can comprise two RNAs, *e.g.*, CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA). A guide RNA can sometimes comprise a single-chain RNA, or single guide RNA (sgRNA) formed by fusion of a portion (*e.g.*, a functional portion) of crRNA and tracrRNA. A guide RNA can also be a dualRNA comprising a crRNA and a tracrRNA. Furthermore, a crRNA can hybridize with a target DNA.

[00179] As discussed above, a guide RNA can be an expression product. For example, a DNA that encodes a guide RNA can be a vector comprising a sequence coding for the guide RNA. A guide RNA can be transferred into a cell or microorganism by transfecting the cell or microorganism with an isolated guide RNA or plasmid DNA comprising a sequence coding for the guide RNA and a promoter. A guide RNA can also be transferred into a cell or microorganism in other way, such as using virus-mediated gene delivery.

[00180] A guide RNA can be isolated. For example, a guide RNA can be transfected in the form of an isolated RNA into a cell or microorganism. A guide RNA can be prepared by *in vitro* transcription using any *in vitro* transcription system. A guide RNA can be transferred to a cell in the form of isolated RNA rather than in the form of plasmid comprising encoding sequence for a guide RNA.

[00181] A guide RNA can comprise three regions: a first region at the 5' end that can be complementary to a target site in a chromosomal sequence, a second internal region that can form a stem loop structure, and a third 3' region that can be single-stranded. A first region of each guide RNA can also be different such that each guide RNA guides a fusion protein to a specific target site. Further, second and third regions of each guide RNA can be identical in all guide RNAs.

[00182] A first region of a guide RNA can be complementary to sequence at a target site in a chromosomal sequence such that the first region of the guide RNA can base pair with the target site. In some cases, a first region of a guide RNA can comprise from 10 nucleotides to 25 nucleotides (*i.e.*, from 10 nts to 25nts; or 10nts to 25 nts; or from 10 nts to 25nts; or from 10 nts to 25 nts) or more. For example, a region of base pairing between a first region of a guide RNA and a target site in a chromosomal sequence can be 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 23, 24, 25, or more nucleotides in length. Sometimes, a first region of a guide RNA can be 19, 20, or 21 nucleotides in length.

[00183] A guide RNA can also comprises a second region that forms a secondary structure. For example, a secondary structure formed by a guide RNA can comprise a stem (or hairpin) and a loop. A length of a loop and a stem can vary. For example, a loop can range from 3 to 10 nucleotides in length, and a stem can range from 6 to 20 base pairs in length. A stem can comprise one or more bulges of 1 to 10 nucleotides. The overall length of a second region can range from 16 to 60 nucleotides in length. For example, a loop can be 4 nucleotides in length and a stem can be 12 base pairs.

[00184] A guide RNA can also comprise a third region at the 3' end that can be essentially single-stranded. For example, a third region is sometimes not complementarity to any chromosomal sequence in a cell of interest and is sometimes not complementarity to the rest of a guide RNA. Further, the length of a third region can vary. A third region can be more than 4 nucleotides in length. For example, the length of a third region can range from 5 to 60 nucleotides in length.

[00185] A guide RNA can be introduced into a cell or embryo as an RNA molecule. For example, a RNA molecule can be transcribed *in vitro* and/or can be chemically synthesized. An RNA can be transcribed from a synthetic DNA molecule, *e.g.*, a gBlocks[®] gene fragment. A guide RNA can then be introduced into a cell or embryo as an RNA molecule. A guide RNA can also be introduced into a cell or embryo in the form of a non-RNA nucleic acid molecule, *e.g.*, DNA molecule. For example, a DNA encoding a guide RNA can be operably linked to promoter control sequence for expression of the guide RNA in a cell or embryo of interest. A RNA coding sequence can be operably linked to a promoter sequence that is recognized by RNA polymerase III (Pol III). Plasmid vectors that can be used to express guide RNA include, but are not limited to, px330 vectors and px333 vectors. In some cases, a plasmid vector (*e.g.*, px333 vector) can comprise two guide RNA-encoding DNA sequences.

[00186] A DNA sequence encoding a guide RNA can also be part of a vector. Further, a vector can comprise additional expression control sequences (*e.g.*, enhancer sequences, Kozak sequences, polyadenylation sequences, transcriptional termination sequences, *etc.*), selectable marker sequences (*e.g.*, antibiotic resistance genes), origins of replication, and the like. A DNA molecule encoding a guide RNA can also be linear. A DNA molecule encoding a guide RNA can also be circular.

[00187] When DNA sequences encoding an RNA-guided endonuclease and a guide RNA are introduced into a cell, each DNA sequence can be part of a separate molecule (*e.g.*, one vector containing an RNA-guided endonuclease coding sequence and a second vector containing a guide RNA coding sequence) or both can be part of a same molecule (*e.g.*, one vector containing coding (and regulatory) sequence for both an RNA-guided endonuclease and a guide RNA).

Site-specific insertion

[00188] Insertion of the genes can be site-specific. For example, one or more genes can be inserted adjacent to a promoter.

[00189] Modification of a targeted locus of a microorganism can be produced by introducing DNA into microorganisms, where the DNA has homology to the target locus. DNA can include a marker gene, allowing for selection of cells comprising the integrated construct. Homologous DNA in a target vector can recombine with DNA at a target locus. A marker gene can be flanked on both sides by homologous DNA sequences, a 3' recombination arm, and a 5' recombination arm.

[00190] A variety of enzymes can catalyze insertion of foreign DNA into a microorganism genome. For example, site-specific recombinases can be clustered into two protein families with distinct biochemical properties, namely tyrosine recombinases (in which DNA is covalently attached to a tyrosine residue) and serine recombinases (where covalent attachment occurs at a serine residue). In some cases, recombinases can comprise Cre, $\Phi 31$ integrase (a serine recombinase derived from *Streptomyces* phage $\Phi 31$), or bacteriophage derived site-specific recombinases (including Flp, lambda integrase, bacteriophage HK022 recombinase, bacteriophage R4 integrase and phage TP901-1 integrase).

[00191] The CRISPR/Cas system can be used to perform site specific insertion. For example, a nick on an insertion site in the genome can be made by CRISPR/cas to facilitate the insertion of a transgene at the insertion site.

[00192] The methods described herein, can utilize techniques which can be used to allow a DNA or RNA construct entry into a host cell include, but are not limited to, calcium phosphate/DNA coprecipitation, microinjection of DNA into a nucleus, electroporation, bacterial protoplast fusion

with intact cells, transfection, lipofection, infection, particle bombardment, sperm mediated gene transfer, or any other technique.

[00193] Certain aspects disclosed herein can utilize vectors (including the ones described above). Any plasmids and vectors can be used as long as they are replicable and viable in a selected host microorganism. Vectors known in the art and those commercially available (and variants or derivatives thereof) can be engineered to include one or more recombination sites for use in the methods. Vectors that can be used include, but not limited to eukaryotic expression vectors such as pFastBac, pFastBacHT, pFastBacDUAL, pSFV, and pTet-Splice (Invitrogen), pEUK-C1, pPUR, pMAM, pMAMneo, pBI101, pBI121, pDR2, pCMVEBNA, and pYACneo (Clontech), pSVK3, pSVL, pMSG, pCH110, and pKK232-8 (Pharmacia, Inc.), pXT1, pSG5, pPbac, pMbac, pMCIneo, and pOG44 (Stratagene, Inc.), and pYES2, pAC360, pBlueBa-cHis A, B, and C, pVL1392, pBlueBac111, pCDM8, pcDNA1, pZeoSV, pcDNA3, pREP4, pCEP4, and pEBVHis (Invitrogen, Corp.), and variants or derivatives thereof.

[00194] These vectors can be used to express a gene or portion of a gene of interest. A gene of portion or a gene can be inserted by using known methods, such as restriction enzyme-based techniques.

IV. Other Methods

Methods of Making Useful Chemicals

[00195] The genetically modified microorganisms described herein can be used to make useful chemicals, including but not limited to acetoin, 2,3-BDO, MEK, butadiene, and/or butene.

[00196] The microorganism can be any of the microorganisms discussed throughout including but not limited to a prokaryote, such as a methanotroph.

[00197] The carbon substrate can be any carbon substrate discussed throughout including but not limited to a C1 carbon substrate, such as methane.

[00198] The fermentation conditions used during the making of the useful chemicals can be any condition described throughout, such as temperature. For example, temperature of fermentation can be performed at between 37 °C to 45 °C. In some cases, the temperature fermentation can be performed at 42 °C. In some cases, the temperature fermentation can be performed at 41 °C.

2-acetolactate

[00199] Disclosed herein is a method of making 2-acetolactate comprising contacting a genetically modified microorganism with a carbon substrate, where the microorganism comprises a heterologous gene encoding acetolactate synthase (AlsS). In some cases, the heterologous gene is

integrated into the genome of the microorganism. In some cases, the heterologous gene is episomally expressed. In some cases, the heterologous gene is both episomally expressed and integrated into the genome of the microorganism. In some cases, the heterologous acetolactate synthase gene is 5' in relation to any other heterologous gene (whether expressed on an episomal vector or integrated into the genome of the microorganism). In some cases, where the microorganism used has more than one chromosome, the terms 5' or 3' can be in reference to the genes contained on a single chromosome. The method can further include growing the microorganism to produce 2-acetolactate. The AlsS can be substantially similar to any one of SEQ ID NO: 1, 3, or 19. In some cases, the AlsS can be encoded by a nucleic acid that is substantially similar to any one of SEQ ID NO: 2, 4, or 20. The AlsS gene can be under the control of a switch, such as an inducible or repressible promoter that is responsive to the presence or absence of a component in the media, *e.g.*, a sugar such as arabinose or a rare earth element such as lanthanum. Also the microorganism can be first grown in media that contains lanthanum (*e.g.*, at least 1 μ M lanthanum) and then subsequently the lanthanum can be diluted. This can occur before growing the microorganism to produce 2-acetolactate. The 2-acetolactate produced can be substantially pure. The 2-acetolactate that is produced can be recovered. Additionally, 2-acetolactate products (*i.e.*, by-products) can also be recovered. For example, diacetyl can be recovered as a by-product.

[00200] The 2-acetolactate can be further processed by the same microorganism, a different microorganism, or outside the microorganism (*i.e.*, *in vitro*). Various enzymatic reactions or even spontaneous reactions can occur. For example, 2-acetolactate can be spontaneously converted into diacetyl. 2-acetolactate can also be converted to acetoin through the use of an alpha-acetolactate decarboxylase. Further 2-acetolactate can also be converted to 2,3-dihydroxy-2-methylbutanoic acid through a reduction reaction by 3-ketoacid reductase.

[00201] Therefore, the same microorganism can comprise an alpha-acetolactate decarboxylase and/or a 3-ketoacid reductase. In other instances, a different microorganism can comprise an alpha-acetolactate decarboxylase and/or a 3-ketoacid reductase or the alpha-acetolactate decarboxylase and/or a 3-ketoacid reductase is isolated from a cell. If the alpha-acetolactate decarboxylase and/or a 3-ketoacid reductase is in a different microorganism or is isolated from a cell, the microorganism/isolated enzyme can convert 2-acetolactate that is in the culture media (either by supplemental addition or by secretion by an acetoin producing microorganism). The conversion of 2-acetolactate by alpha-acetolactate decarboxylase and/or a 3-ketoacid reductase can produce acetoin and/or 2,3-dihydroxy-2-methylbutanoic acid, respectively.

Acetoin

[00202] Disclosed herein is a method of making acetoin comprising contacting a genetically modified microorganism with a carbon substrate, where the microorganism comprises a heterologous gene encoding for alpha-acetolactate decarboxylase (budA). In some cases, the heterologous gene is integrated into the genome of the microorganism. In some cases, the heterologous gene is episomally expressed. In some cases, the heterologous gene is both episomally expressed and integrated into the genome of the microorganism. In some cases, the alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other heterologous gene (whether expressed on an episomal vector or integrated into the genome of the microorganism). For example, with regarding to the acetolactate decarboxylase gene, there is at least one heterologous gene that is upstream (further 5') of the acetolactate decarboxylase gene as well as at least one additional heterologous gene that is downstream (further 3') of the acetolactate decarboxylase gene. In some cases, where the microorganism used has more than one chromosome, the terms 5' or 3' can be in reference to the genes contained on a single chromosome. The method can further comprise growing the microorganism to produce acetoin. The budA gene can be under the control of a switch, such as an inducible or repressible promoter that is responsive to the presence or absence of a component in the media, *e.g.*, a sugar such as arabinose or a rare earth element such as lanthanum. Also the microorganism can be first grown in media that contains lanthanum (*e.g.*, at least 1 μ M lanthanum) and then subsequently the lanthanum can be diluted. This can occur before growing the microorganism to produce acetoin. The acetoin produced can be substantially pure. The acetoin that is produced can be recovered. Additionally, non-acetoin products (*i.e.*, by-products) can also be recovered.

[00203] The acetoin can be further processed by the same microorganism, a different microorganism, or outside a microorganism (*i.e.*, *in vitro*) through an acetoin reductase (*e.g.*, an NADH-dependent or NADPH-dependent acetoin reductase). The same microorganism can comprise an acetoin reductase (*e.g.*, an NADH-dependent or NADPH-dependent acetoin reductase). In other instances, a different microorganism can comprise an acetoin reductase or the acetoin reductase is isolated from a cell. If the acetoin reductase is in a different microorganism or is isolated from a cell, the microorganism/isolated enzyme can convert acetoin reductase that is in the culture media (either by supplemental addition or by secretion by an acetoin producing microorganism). The reduction of acetoin by acetoin reductase can produce 2,3-BDO.

[00204] Further conversion of 2,3-BDO into various products can occur through different fermentation processes or by different catalytic conversions.

2,3-BDO

[00205] Disclosed herein is a method of making 2,3-BDO comprising contacting a genetically modified microorganism with a carbon substrate, where the microorganism comprises at least one heterologous gene encoding: (i) an acetoin reductase; (ii) an alpha-acetolactate decarboxylase (budA); (iii) an AlsS; or (iv) any combination thereof. In some cases, the at least one heterologous gene is integrated into the genome of the microorganism. In some cases, the at least one heterologous gene is episomally expressed. In some cases, the at least one heterologous gene is both episomally expressed and integrated into the genome of the microorganism. In some cases, the heterologous gene encoding the acetolactate synthase is 5' in relation to any other heterologous gene. In some cases, where the microorganism used has more than one chromosome, the terms 5' or 3' can be in reference to the genes contained on a single chromosome. In some cases, the heterologous gene encoding the acetoin reductase is the 3' heterologous gene in relation to any other heterologous gene. In some cases, the heterologous gene encoding the alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other heterologous gene. The method can further comprise growing the microorganism to produce 2,3-BDO. At least one heterologous gene can be under the control of a switch, such as an inducible or repressible promoter that is responsive to the presence or absence of a component in the media, *e.g.*, a sugar such as arabinose or a rare earth element such as lanthanum. Also the microorganism can be first grown in media that contains lanthanum (*e.g.*, at least 1 μ M lanthanum) and then subsequently the lanthanum can be diluted. This can occur before growing the microorganism to produce 2,3-BDO.

[00206] The 2,3-BDO that is produced from these methods can be substantially pure. The 2,3-BDO produced can be recovered. Additionally, non-2,3-BDO products (*i.e.*, by-products) can also be recovered, such as 2-acetolactate and acetoin.

MEK

[00207] Disclosed herein is a method for making MEK comprising contacting a genetically modified microorganism with a carbon substrate, where the microorganism comprises at least one heterologous gene encoding: (i) an acetoin reductase; (ii) an alpha-acetolactate decarboxylase (budA); (iii) an AlsS; or (iv) any combination thereof. In some cases, the at least one heterologous gene is integrated into the genome of the microorganism. In some cases, the at least one heterologous gene is episomally expressed. In some cases, the at least one heterologous gene is both

episomally expressed and integrated into the genome of the microorganism. In some cases, the heterologous gene encoding the acetolactate synthase is 5' in relation to any other heterologous gene. In some cases, where the microorganism used has more than one chromosome, the terms 5' or 3' can be in reference to the genes contained on a single chromosome. In some cases, the heterologous gene encoding the acetoin reductase is the 3' in relation to any other heterologous gene. In some cases, the heterologous gene encoding the alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other heterologous gene. The method can further comprise growing the microorganism to produce 2,3-BDO. In addition, the method can also further comprise contacting the 2,3-BDO produced with a catalyst to produce MEK. At least one heterologous gene can be under the control of a switch, such as an inducible or repressible promoter that is responsive to the presence or absence of a component in the media, *e.g.*, a sugar such as arabinose or a rare earth element such as lanthanum. Also the microorganism can be first grown in media that contains lanthanum (*e.g.*, at least 1 μ M lanthanum) and then subsequently the lanthanum can be diluted. This can occur before growing the microorganism to produce 2,3-BDO. In some cases, the 2,3-BDO can be isolated or purified before proceeding to contacting the 2,3-BDO produced with a catalyst to produce MEK.

[00208] The catalyst can be an enzymatic catalyst or a non-enzymatic catalyst. The catalyst can include any catalyst that is capable of producing MEK. For example, MEK can be obtained by the direct dehydration of 2,3-BDO over a variety of catalysts such as alumina, direct reaction with sulfuric acid, Cu, AlO₃, and/or zeolite (or other solid acid catalysts) (see *e.g.*, Emerson, R.R., *et al.*, "Kinetics of dehydration of aqueous 2,3-butanediol to methyl ethyl ketone," Ind. Eng. Chem. Prod. Res. Dev., p. 473-477 (1982)). General acid catalysts such as metal oxides and zeolites mainly produce MEK, IBA, butenes, and C₁-C₃ gaseous compounds. For example, 10% H₃PO₄/Silica gel 60 produces 43% 1,3-butadiene 43%, 41% MEK and 8% IBA. Use of the zeolite ZSM-5 produces greater than 80% MEK. In some cases ZSM-5 produces over 90% MEK. In some cases, the catalyst and/or the reaction mixture does not contain alkali metals, such as potassium (K), Rubidium (Rb), and/or Cesium (Cs).

[00209] The acid-catalyzed dehydration of one water molecule from 2,3-BDO produces a carbocation intermediate. A pinacol rearrangement produces methyl ethyl ketone (MEK) and isobutyraldehyde (IBA).

[00210] Additionally, the 2,3-BDO made by the microorganisms and the methods described throughout can be further processed by a diol dehydratase (B12). This enzymatic reaction can

produce MEK (also known as butan-2-one). Thus, disclosed is a method of making MEK comprising: (a) contacting a genetically modified microorganism with a carbon substrate, where the microorganism comprises a heterologous gene encoding for diol dehydratase; and (b) growing the microorganism to produce MEK. The microorganism can also comprise an acetolactate synthase (AlsS), alpha-acetolactate (budA), and/or acetoin reductase (butA).

[00211] In some cases, the MEK can be further processed by an alcohol dehydrogenase. This enzymatic reaction can produce butan-2-ol (also known as 2-butanol). Thus, disclosed is a method of making butan-2-ol comprising: (a) contacting a genetically modified microorganism with a carbon substrate, where the microorganism comprises a heterologous gene encoding for an alcohol dehydrogenase; and (b) growing the microorganism to produce butan-2-ol. The microorganism can also comprise an acetolactate synthase (AlsS), alpha-acetolactate (budA), acetoin reductase (butA), and/or diol dehydratase (B12).

1,3-butadiene (butadiene)

[00212] Disclosed herein is a method for making butadiene, where the method comprises contacting a genetically modified microorganism with a carbon substrate, where the microorganism comprises at least one heterologous gene encoding: (i) an acetoin reductase; (ii) an alpha-acetolactate decarboxylase (budA); (iii) an AlsS; or (iv) any combination thereof. In some cases, the at least one heterologous gene is integrated into the genome of the microorganism. In some cases, the at least one heterologous gene is episomally expressed. In some cases, the at least one heterologous gene is both episomally expressed and integrated into the genome of the microorganism. In some cases, the heterologous gene encoding the acetolactate synthase is 5' in relation to any other heterologous gene. In some cases, where the microorganism used has more than one chromosome, the terms 5' or 3' can be in reference to the genes contained on a single chromosome. In some cases, the heterologous gene encoding the acetoin reductase is 3' in relation to any other heterologous gene. In some cases, the heterologous gene encoding the alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other heterologous gene. The method can further comprise growing the microorganism to produce 2,3-BDO. The method can also further comprise contacting the 2,3-BDO produced with a catalyst to produce butadiene. At least one heterologous gene can be under the control of a switch, such as an inducible or repressible promoter that is responsive to the presence or absence of a component in the media, *e.g.*, a sugar such as arabinose or a rare earth element such as lanthanum. Also the microorganism can be first grown in media that contains lanthanum (*e.g.*, at

least 1 μ M lanthanum) and then subsequently the lanthanum can be diluted. This can occur before growing the microorganism to produce 2,3-BDO.

[00213] The catalyst can be an enzymatic catalyst or a non-enzymatic catalyst. The catalyst can include any catalyst that is capable of dehydrating 2,3-BDO. The catalyst can include any catalyst that is capable of producing a hydride shift. For example, alumina or a direct reaction with sulfuric acid (Emerson, R.R., *et al.*, "Kinetics of dehydration of aqueous 2,3-butanediol to methyl ethyl ketone," *Ind. Eng. Chem. Prod. Res. Dev.*, 21(3), pp. 473-477 (1982)). Additionally, greater than 80% conversion of 2,3-BDO to butadiene can be made by using a SiO₂-supported cesium dihydrogen phosphate (CsH₂PO₄) catalyst. In generally, 10% of a CsH₂PO₄ catalyst can be used. In some cases, the conversion can be greater than 90% conversion. Examples of catalysts that can be used include, but are not limited to 10% CsH₂PO₄/CARiACT Q6 and/or 10% CsH₂PO₄/CARiACT Q10. (Further, the use of a catalyst in which the alkali metal, such as, is K, Rb, or Cs, can lead to a higher butadiene production, and less of a MEK production. (*See e.g.*, U.S. Pat. Appl. Pub. No. 2016/0229765). Thus, the method disclosed herein can use additional alkali metals.

[00214] By way of further example, butadiene can also be obtained by the direct dehydration of 2,3-BDO over thoria catalyst, although most other dehydration catalysts give methyl ethyl ketone as the main product (Winfield, M.E., "The Catalytic Dehydration of 2,3-Butanediol to Butadiene. II. Adsorption Equilibri," *Australian Journal of Scientific Research, Series A: Physical Sciences*, vol. 3, p. 290-305 (1945)).

2-Butene (butene)

[00215] Disclosed herein is a method for making butene, where the method comprises contacting a genetically modified microorganism with a carbon substrate, where the microorganism comprises at least one heterologous gene encoding for: (i) an acetoin reductase; (ii) an alpha-acetolactate decarboxylase (budA); (iii) an AlsS; or (iv) any combination thereof. In some cases, the at least one heterologous gene is integrated into the genome of the microorganism. In some cases, the at least one heterologous gene is episomally expressed. In some cases, the at least one heterologous gene is both episomally expressed and integrated into the genome of the microorganism. In some cases, the heterologous gene encoding the acetolactate synthase is 5' in relation to any other heterologous gene. In some cases, where the microorganism used has more than one chromosome, the terms 5' or 3' can be in reference to the genes contained on a single chromosome. In some cases, the heterologous gene encoding the acetoin reductase is 3' in relation to any other heterologous gene. In some cases, the heterologous gene encoding the alpha-acetolactate decarboxylase is neither 5' or 3'

in relation to any other heterologous gene. The method can further comprise growing the microorganism to produce 2,3-BDO. The method can also further comprise contacting the 2,3-BDO produced with a catalyst to produce butene. At least one heterologous gene can be under the control of a switch, such as an inducible or repressible promoter that is responsive to the presence or absence of a component in the media, *e.g.*, a sugar such as arabinose or a rare earth element such as lanthanum. Also the microorganism can be first grown in media that contains lanthanum (*e.g.*, at least 1 μ M lanthanum) and then subsequently the lanthanum can be diluted. This can occur before growing the microorganism to produce 2,3-BDO.

[00216] Butene can be produced from 2,3-BDO. For example, treatment of the dial with HBr, followed by Zn powder can result in butene. The debrominations proceed with a high degree of anti-stereospecificity (House, H.O, and Ro, R.S, "The Stereochemistry of Elimination Reactions Involving Halohydrin Derivatives and Metals," *J. Am. Chem. Soc.* 80(1), p. 182-187(1958); Gordon, M., and Hay, J.V., "Stereochemistry of vapor phase dehalogenation of meso- and DL-2,3-dibromobutane with zinc," *J. Org. Chem.* 33(1), p. 427-427 (1968)), the mesa isomer giving the trans butene, and the(+) isomer the cis butene.

[00217] Butene can be converted into butadiene. For example, butenes can be catalytically dehydrogenated to 1,3-butadiene in the presence of superheated steam as a diluent and a heating medium (Voloch, M., *et al.*, "2,3-Butanediol," *Industrial Chemicals, Biochemicals and Fuels, Chapter 45*, p. 933-947 (1985).

Methods of Making Commercially Useful Products

[00218] Although 2,3-BDO in itself can be used in some commercial useful aspects, many of the downstream products of 2,3-BDO can be used to produce additional commercially useful products. For example, butadiene, butene, and MEK can subsequently be used in a variety of processes for producing commercially useful products.

[00219] For example, butene may be used in the production of gasoline and butadiene. Butene can also be used as a component or precursor in the manufacture of C₁₂ paraffins, such as iso paraffins used as aviation fuels (see *e.g.*, U.S. Pat. No. 7,338,541).

[00220] MEK can be used to dissolve many substances and may be used, for example, as a solvent in processes involving gums, resins, cellulose acetate, and nitrocellulose coatings and in vinyl films. Therefore, MEK can be useful in the manufacture of plastics, textiles, paraffin wax, and in household products such as lacquer, varnishes and paint remover, glues, and as a cleaning agent. MEK can also be used as a denaturing agent for denatured alcohol. MEK can also be used in dry

erase markers as the solvent of the erasable dye. In addition, MEK is the precursor to methyl ethyl ketone peroxide, a catalyst used in some polymerization reactions. Further, MEK can be converted to 2-butanol by contacting the MEK with a catalyst such as ruthenium on carbon. MEK can also be converted to 2-butanol by contact with an alcohol dehydrogenase.

[00221] One of the most useful chemicals that can be produced by the processes herein is butadiene. Butadiene may be used to produce a variety of very useful products such as synthetic rubbers and polymer resins. While polybutadiene itself is a very soft, almost liquid material, polymers prepared from mixtures of butadiene with styrene or acrylonitrile, such as ABS, are both tough and elastic. Styrene-butadiene rubber is the material most commonly used for the production of automobile tires. Butadiene may also be used to make nylon via the intermediate adiponitrile, other synthetic rubber materials such as chloroprene, and the solvent sulfolane. In addition, butadiene may be used in the industrial production of 4-vinylcyclohexene via a dimerization reaction and cyclododecatriene via a trimerization reaction. Butadiene is also useful in the synthesis of cycloalkanes and cycloalkenes, as it reacts with double and triple carbon-carbon bonds through the Diels-Alder reaction. By way of further example, butadiene may be used in the manufacture of cycloalkanes, cycloalkenes, dodecandioic acid (DDDA), adiponitrile, caprolactam, styrene, ethylidene norbornene, lauryl lactam and 1,5-cyclooctadiene (COD).

[00222] It should be appreciated that the methods of the invention may be integrated or linked with one or more methods for the production of downstream products from butene, butadiene and/or MEK. For example, the methods of the invention may feed butene, butadiene and/or MEK directly or indirectly to chemical processes or reactions sufficient for the conversion or production of other useful chemical products. In some cases, as noted herein before, 2,3-BDO can be converted to one or more chemical products directly via the intermediate compounds butene, butadiene and/or MEK without the need for recovery of butene, butadiene and/or MEK from the method before subsequent use in production of the one or more chemical products.

[00223] In particular cases, methane is converted to 2,3-BDO, which is then subsequently converted to butene, butadiene and/or MEK by one or more chemical processes, which in turn is converted to one or more chemical products by one or more chemical processes. In particular cases, the one or more chemical products are produced without recovering the butane, butadiene and/or MEK. In another embodiment, 2,3-BDO is converted to one or more chemical products in a single chemical process via one or more of the butane, butadiene and/or MEK intermediate compounds.

V. Fermentation

[00224] In general, the microorganisms disclosed herein should be used in fermentation conditions that are appropriate to convert a C1 carbon (such as methane) to 2,3-BDO (or other desired product). Reaction conditions that should be considered include temperature, media flow rate, pH, media redox potential, agitation rate (if using a continuous stirred tank reactor), inoculum level, maximum substrate concentrations and rates of introduction of the substrate to the bioreactor to ensure that substrate level does not become limiting, and maximum product concentrations to avoid product inhibition.

[00225] The optimum reaction conditions will depend partly on the particular microorganism of used. However, in general, the fermentation can be performed at a pressure higher than ambient pressure. Operating at increased pressures can allow for a significant increase in the rate of C1-carbon transfer from the gas phase to the liquid phase where it can be taken up by the microorganism as a carbon source for the production of 2,3-BDO. This in turn can mean that the retention time (defined as the liquid volume in the bioreactor divided by the input gas flow rate) can be reduced when bioreactors are maintained at elevated pressure rather than atmospheric pressure.

[00226] The use of pressurized systems can greatly reduce the volume of the bioreactor required, and consequently the capital cost of the fermentation equipment. In some cases, reactor volume can be reduced in linear proportion to increases in reactor operating pressure, *i.e.* bioreactors operated at 10 atmospheres of pressure need only be one tenth the volume of those operated at 1 atmosphere of pressure.

[00227] It is also desirable that the rate of introduction of the C1-carbon substrate (such as methane) containing gaseous substrate is such as to ensure that the concentration of the C1-carbon substrate (such as methane) in the liquid phase does not become limiting. This is because a consequence of C1-carbon substrate (*e.g.*, methane) limited conditions may be that the 2,3-BDO product (or other desired product) is consumed by the culture.

Preculturing

[00228] Preculturing the various strains of genetically microorganisms described throughout can lead to increase titers of the multicarbon products described throughout such as 2,3-BDO, butadiene, and/or MEK.

[00229] Preculturing can include adding to the media an antibiotic, such as kanamycin, a carbon source, such as methane or other C1 carbon, and/or substances to activate or repress a molecular switch.

[00230] Typically, preculturing can be performed for a time of less than 240 hours, depending on the species of the microorganism used. Preculturing allows for sufficient biomass to be created in order to efficiently increase product titers. In some cases, the preculturing is less than 240 hours. In some cases, the preculturing is less than 220 hours. In some cases, the preculturing is less than 200 hours. In some cases, the preculturing is less than 180 hours. In some cases, the preculturing is less than 160 hours. In some cases, the preculturing is less than 140 hours. In some cases, the preculturing is less than 120 hours. In some cases, the preculturing is less than 100 hours. In some cases, the preculturing is less than 96 hours. In some cases, the preculturing is less than 90 hours. In some cases, the preculturing is less than 84 hours. In some cases, the preculturing is less than 78 hours. In some cases, the preculturing is less than 72 hours. In some cases, the preculturing is less than 68 hours. In some cases, the preculturing is less than 62 hours. In some cases, the preculturing is less than 56 hours. In some cases, the preculturing is less than 50 hours. In some cases, the preculturing is less than 48 hours. In some cases, the preculturing is less than 46 hours. In some cases, the preculturing is less than 44 hours. In some cases, the preculturing is less than 42 hours. In some cases, the preculturing is less than 40 hours. In some cases, the preculturing is less than 38 hours. In some cases, the preculturing is less than 36 hours. In some cases, the preculturing is less than 34 hours. In some cases, the preculturing is less than 32 hours. In some cases, the preculturing is less than 30 hours. In some cases, the preculturing is less than 28 hours. In some cases, the preculturing is less than 26 hours. In some cases, the preculturing is less than 24 hours. In some cases, the preculturing is less than 22 hours. In some cases, the preculturing is less than 20 hours. In some cases, the preculturing is less than 18 hours. In some cases, the preculturing is less than 16 hours. In some cases, the preculturing is less than 14 hours. In some cases, the preculturing is less than 12 hours. In some cases, the preculturing is less than 10 hours. In some cases, the preculturing is less than 8 hours. In some cases, the preculturing is less than 6 hours. In some cases, the preculturing is less than 4 hours. In some cases, the preculturing is less than 2 hours. In some cases, the preculturing is less than 1 hour. In some cases, there is no preculturing.

[00231] In some cases, the preculturing is between 240 hours and 1 hour. In some cases, the preculturing is between 220 hours. In some cases, the preculturing is between 200 hours and 2 hours. In some cases, the preculturing is between 180 hours and 4 hours. In some cases, the preculturing is between 160 hours and 6 hours. In some cases, the preculturing is between 140 hours and 8 hours. In some cases, the preculturing is between 120 hours and 10 hours. In some cases, the preculturing is between 100 hours and 12 hours. In some cases, the preculturing is between 96 hours

and 18 hours. In some cases, the preculturing is between 90 hours and 24 hours. In some cases, the preculturing is between 84 hours and 36 hours. In some cases, the preculturing is between 78 hours and 40 hours. In some cases, the preculturing is between 72 hours and 42 hours. In some cases, the preculturing is between 56 hours and 44 hours. In some cases, the preculturing is between 50 hours and 46 hours.

[00232] When a substance to activate or repress a molecular switch is added to the preculture, the amount can vary. For example, in some cases, if the substance to activate or repress a molecular switch is a rare earth metal (such as lanthanum), the genetically modified microorganism that comprise only integrated genes, do not require any preculture containing the rare earth metal (such as lanthanum). However, in some cases, these integrated strains do require the rare earth metal (such as lanthanum), however, in small amounts. For example, the amount of the rare earth metal (such as lanthanum) required for preculture of the integrated strains can require less than 25 μM of the rare earth metal (such as lanthanum). In some cases, the amount of the rare earth metal (such as lanthanum) required for preculture of the integrated strains can require less than 20 μM . The amount of the rare earth metal (such as lanthanum) required for preculture of the integrated strains can require less than 15 μM . The amount of the rare earth metal (such as lanthanum) required for preculture of the integrated strains can require less than 10 μM . The amount of the rare earth metal (such as lanthanum) required for preculture of the integrated strains can require less than 7.5 μM . The amount of the rare earth metal (such as lanthanum) required for preculture of the integrated strains can require less than 5 μM . The amount of the rare earth metal (such as lanthanum) required for preculture of the integrated strains can require less than 2.5 μM . The amount of the rare earth metal (such as lanthanum) required for preculture of the integrated strains can require less than 1 μM .

[00233] However, when the microorganism comprises an episomally expressed genes (whether or not an additional integrated set of genes is already present in the microorganism), more of the rare earth metal (such as lanthanum) is required during preculture to optimize production. In some cases, these episomal strains require in preculture more than 1 μM of the rare earth metal (such as lanthanum). In some cases, these episomal strains require in preculture more than 2.5 μM of the rare earth metal (such as lanthanum). In some cases, these episomal strains require in preculture more than 5.0 μM of the rare earth metal (such as lanthanum). In some cases, these episomal strains require in preculture more than 7.5 μM of the rare earth metal (such as lanthanum). In some cases, these episomal strains require in preculture more than 10 μM of the rare earth metal (such as

lanthanum). In some cases, these episomal strains require in preculture more than 12.5 μM of the rare earth metal (such as lanthanum). In some cases, these episomal strains require in preculture more than 15 μM of the rare earth metal (such as lanthanum). In some cases, these episomal strains require in preculture more than 17.5 μM of the rare earth metal (such as lanthanum). In some cases, these episomal strains require in preculture more than 20 μM of the rare earth metal (such as lanthanum). In some cases, these episomal strains require in preculture more than 25 μM of the rare earth metal (such as lanthanum). In some cases, these episomal strains require in preculture more than 30 μM of the rare earth metal (such as lanthanum). In some cases, these episomal strains require in preculture more than 35 μM of the rare earth metal (such as lanthanum). In some cases, these episomal strains require in preculture more than 40 μM of the rare earth metal (such as lanthanum). In some cases, these episomal strains require in preculture more than 45 μM of the rare earth metal (such as lanthanum). In some cases, these episomal strains require in preculture more than 50 μM of the rare earth metal (such as lanthanum). In some cases, these episomal strains require in preculture more than 75 μM of the rare earth metal (such as lanthanum). In some cases, these episomal strains require in preculture more than 100 μM of the rare earth metal (such as lanthanum).

[00234] In some cases, the amount of the rare earth metal (such as lanthanum) that is required in preculture to optimize production can be in a range of 0.5 μM to 100 μM of the rare earth metal (such as lanthanum). In some cases, a range of 0.5 μM to 50 μM of the rare earth metal (such as lanthanum) is required in preculture to optimize production. In other cases, a range of 1 μM to 20 μM of the rare earth metal (such as lanthanum) is required in preculture to optimize production. In some cases, a range of 2 μM to 15 μM of the rare earth metal (such as lanthanum) is required in preculture to optimize production. In some cases, a range of 3 μM to 12.5 μM of the rare earth metal (such as lanthanum) is required in preculture to optimize production. In some cases, a range of 4 μM to 12 μM of the rare earth metal (such as lanthanum) is required in preculture to optimize production. In some cases, a range of 5 μM to 11.5 μM of the rare earth metal (such as lanthanum) is required in preculture to optimize production. In some cases, a range of 6 μM to 11 μM of the rare earth metal (such as lanthanum) is required in preculture to optimize production. In some cases, a range of 7 μM to 10.5 μM of the rare earth metal (such as lanthanum) is required in preculture to optimize production. In some cases, a range of 8 μM to 10 μM of the rare earth metal (such as lanthanum) is required in preculture to optimize production.

[00235] In some cases, other rare earth metals, such as cerium (Ce), dysprosium (Dy), erbium (Er), europium (Eu), gadolinium (Gd), holmium (Ho), lutetium (Lu), neodymium (Nd), praseodymium (Pr), promethium (Pm), samarium (Sm), scandium (Sc), terbium (Tb), thulium (Tm), ytterbium (Yb), yttrium (Y), or any combination thereof, can be used. In other cases, sugars, such as IPTG and arabinose can be used.

Fermentation Conditions

[00236] pH can be optimized based on the microorganism used. For example, the pH used during the methanotroph fermentation of methane to a desired product, can be from 4 to 10. In other instances, the pH can be from 5 to 9; 6 to 8; 6.1 to 7.9; 6.2 to 7.8; 6.3 to 7.7; 6.4 to 7.6; or 6.5 to 7.5. For example, the pH can be from 6.6 to 7.4. In some cases, the pH can be from 5 to 9. In some cases, the pH can be from 6 to 8. In some cases, the pH can be from 6.1 to 7.9. In some cases, the pH can be from 6.2 to 7.8. In some cases, the pH can be from 6.3 to 7.7. In some cases, the pH can be from 6.4 to 7.6. In some cases, the pH can be from 6.5 to 7.5. In some cases, the pH used for the fermentation of methanotrophs can be greater than 6.

[00237] Temperature can also be adjusted based on the microorganism used. For example, the temperature used during the methanotroph fermentation of methane to a desired product, can be from 30 C° to 45 C°. In other instances, the temperature of the fermentation can be from 30 C° to 45 C°; 31 C° to 44 C°; 32 C° to 43 C°; 33 C° to 42 C°; 34 C° to 41 C°; 35 C° to 40 C°. For example, the temperature can be from 36 C° to 39 C° (*e.g.*, 36 C°, 37 C°, 38 C°, or 39 C°). In some cases, the temperature can be from 30 C° to 45 C° (*e.g.*, 30 C°, 31 C°, 32 C°, 33 C°, 34 C°, 35 C°, 36 C°, 37 C°, 38 C°, 39 C°, 40 C°, 41 C°, 42 C°, 43 C°, 44 C°, or 45 C°). In some cases, the temperature can be from 31 C° to 44 C° (*e.g.*, 31 C°, 32 C°, 33 C°, 34 C°, 35 C°, 36 C°, 37 C°, 38 C°, 39 C°, 40 C°, 41 C°, 42 C°, 43 C°, or 44 C°). In some cases, the temperature can be from 32 C° to 43 C°. In some cases, the temperature can be from 33 C° to 42 C° (*e.g.*, 33 C°, 34 C°, 35 C°, 36 C°, 37 C°, 38 C°, 39 C°, 40 C°, 41 C°, or 42 C°). In some cases, the temperature can be from 34 C° to 41 C° (*e.g.*, 34 C°, 35 C°, 36 C°, 37 C°, 38 C°, 39 C°, 40 C°, or 41 C°). In some cases, the temperature can be from 35 C° to 40 C° (*e.g.*, 35 C°, 36 C°, 37 C°, 38 C°, 39 C°, or 40 C°).

[00238] In some cases, the temperatures can be within one tenth of a degree. For example, in some cases, the temperature of fermentation can be 37.0 C°, 37.1 C°, 37.2 C°, 37.3 C°, 37.4 C°, 37.5 C°, 37.6 C°, 37.7 C°, 37.8 C°, 37.9 C°, 38.0 C°, 38.1 C°, 38.2 C°, 38.3 C°, 38.4 C°, 38.5 C°, 38.6 C°, 38.7 C°, 38.8 C°, 38.9 C°, 39.0 C°, 39.1 C°, 39.2 C°, 39.3 C°, 39.4 C°, 39.5 C°, 39.6 C°, 39.7 C°, 39.8 C°, 39.9 C°, 40.0 C°, 40.1 C°, 40.2 C°, 40.3 C°, 40.4 C°, 40.5 C°, 40.6 C°, 40.7 C°, 40.8 C°,

40.9 C°, 41.0 C°, 41.1 C°, 41.2 C°, 41.3 C°, 41.4 C°, 41.5 C°, 41.6 C°, 41.7 C°, 41.8 C°, 41.9 C°, 42.0 C°, 42.1 C°, 42.2 C°, 42.3 C°, 42.4 C°, 42.5 C°, 42.6 C°, 42.7 C°, 42.8 C°, 42.9 C°, 43.0 C°, 43.1 C°, 43.2 C°, 43.3 C°, 43.4 C°, 43.5 C°, 43.6 C°, 43.7 C°, 43.8 C°, 43.9 C°, 44.0 C°, 44.1 C°, 44.2 C°, 44.3 C°, 44.4 C°, 44.5 C°, 44.6 C°, 44.7 C°, 44.8 C°, 44.9 C°, 45.0 C°, 45.1 C°, 45.2 C°, 45.3 C°, 45.4 C°, 45.5 C°, 45.6 C°, 45.7 C°, 45.8 C°, 45.9 C°, 46.0 C°, 46.1 C°, 46.2 C°, 46.3 C°, 46.4 C°, 46.5 C°, 46.6 C°, 46.7 C°, 46.8 C°, 46.9 C°, 47.0 C°, 47.1 C°, 47.2 C°, 47.3 C°, 47.4 C°, 47.5 C°, 47.6 C°, 47.7 C°, 47.8 C°, or 47.9 C°.

[00239] In some cases, the temperature of fermentation can be from 37.0 C° to 47.9 C°. In some cases, the temperature of fermentation can be from 37.1 C° to 47.8 C°. In some cases, the temperature of fermentation can be from 37.2 C° to 47.7 C°. In some cases, the temperature of fermentation can be from 37.3 C° to 47.6 C°. In some cases, the temperature of fermentation can be from 37.4 C° to 47.5 C°. In some cases, the temperature of fermentation can be from 37.5 C° to 47.4 C°. In some cases, the temperature of fermentation can be from 37.6 C° to 47.3 C°. In some cases, the temperature of fermentation can be from 37.7 C° to 47.2 C°. In some cases, the temperature of fermentation can be from 37.8 C° to 47.1 C°. In some cases, the temperature of fermentation can be from 37.9 C° to 47.0 C°. In some cases, the temperature of fermentation can be from 38.0 C° to 46.9 C°. In some cases, the temperature of fermentation can be from 38.1 C° to 46.8 C°. In some cases, the temperature of fermentation can be from 38.2 C° to 46.7 C°. In some cases, the temperature of fermentation can be from 38.3 C° to 46.6 C°. In some cases, the temperature of fermentation can be from 38.4 C° to 46.5 C°. In some cases, the temperature of fermentation can be from 38.5 C° to 46.4 C°. In some cases, the temperature of fermentation can be from 38.6 C° to 46.3 C°. In some cases, the temperature of fermentation can be from 38.7 C° to 46.2 C°. In some cases, the temperature of fermentation can be from 38.8 C° to 46.1 C°. In some cases, the temperature of fermentation can be from 38.9 C° to 46.0 C°. In some cases, the temperature of fermentation can be from 39.0 C° to 45.9 C°. In some cases, the temperature of fermentation can be from 39.1 C° to 45.8 C°. In some cases, the temperature of fermentation can be from 39.2 C° to 45.7 C°. In some cases, the temperature of fermentation can be from 39.3 C° to 45.6 C°. In some cases, the temperature of fermentation can be from 39.4 C° to 45.5 C°. In some cases, the temperature of fermentation can be from 39.5 C° to 45.4 C°. In some cases, the temperature of fermentation can be from 39.6 C° to 45.3 C°. In some cases, the temperature of fermentation can be from 39.7 C° to 45.2 C°. In some cases, the temperature of fermentation can be from 39.8 C° to 45.1 C°. In some cases, the temperature of fermentation can be from 39.9 C° to 45.0 C°. In some cases, the temperature of fermentation can be from 40.0 C° to 44.9

C°. In some cases, the temperature of fermentation can be from 40.1 C° to 44.8 C°. In some cases, the temperature of fermentation can be from 40.2 C° to 44.7 C°. In some cases, the temperature of fermentation can be from 40.3 C° to 44.6 C°. In some cases, the temperature of fermentation can be from 40.4 C° to 44.5 C°. In some cases, the temperature of fermentation can be from 40.5 C° to 44.4 C°. In some cases, the temperature of fermentation can be from 40.6 C° to 44.3 C°. In some cases, the temperature of fermentation can be from 40.7 C° to 44.2 C°. In some cases, the temperature of fermentation can be from 40.8 C° to 44.1 C°. In some cases, the temperature of fermentation can be from 40.9 C° to 44.0 C°. In some cases, the temperature of fermentation can be from 41.0 C° to 43.9 C°. In some cases, the temperature of fermentation can be from 41.1 C° to 43.8 C°. In some cases, the temperature of fermentation can be from 41.2 C° to 43.7 C°. In some cases, the temperature of fermentation can be from 41.3 C° to 43.6 C°. In some cases, the temperature of fermentation can be from 41.4 C° to 43.5 C°. In some cases, the temperature of fermentation can be from 41.5 C° to 43.4 C°. In some cases, the temperature of fermentation can be from 41.6 C° to 43.3 C°. In some cases, the temperature of fermentation can be from 41.7 C° to 43.2 C°. In some cases, the temperature of fermentation can be from 41.8 C° to 43.1 C°. In some cases, the temperature of fermentation can be from 41.9 C° to 43.0 C°. In some cases, the temperature of fermentation can be from 42.0 C° to 42.9 C°. In some cases, the temperature of fermentation can be from 42.1 C° to 42.8 C°. In some cases, the temperature of fermentation can be from 42.2 C° to 42.7 C°. In some cases, the temperature of fermentation can be from 42.3 C° to 42.6 C°. In some cases, the temperature of fermentation can be from 42.4 C° to 42.5 C°.

[00240] Availability of oxygen and other gases such as gaseous C1-carbon substrates (such as methane) can affect yield and fermentation rate. For example, when considering oxygen availability, the percent of dissolved oxygen (DO) within the fermentation media can be from 1% to 40%. In certain instances, the DO concentration can be from 1.5% to 35%; 2% to 30%; 2.5% to 25%; 3% to 20%; 4% to 19%; 5% to 18%; 6% to 17%; 7% to 16%; 8% to 15%; 9% to 14%; 10% to 13%; or 11% to 12%. For example, in some cases the DO concentration can be from 2% to 30%. In other cases, the DO can be from 3% to 20%. In some cases, the DO can be from 4% to 10%. In some cases, the DO can be from 1.5% to 35%. In some cases, the DO can be from 2.5% to 25%. In some cases, the DO can be from 4% to 19%. In some cases, the DO can be from 5% to 18%. In some cases, the DO can be from 6% to 17%. In some cases, the DO can be from 7% to 16%. In some cases, the DO can be from 8% to 15%. In some cases, the DO can be from 9% to 14%. In some cases, the DO can be from 10% to 13%. In some cases, the DO can be from 11% to 12%.

[00241] When using a methanotroph, the type of methane substances can have an effect on yield and fermentation rates. For example, natural gas can be used, which typically has a methane content of above 85% (*e.g.*, above 90%) methane. Other components within natural gas can include but is not limited to ethane, propane, iso-butane, normal-butane, iso-pentane, normal pentane, hexanes plus, nitrogen, carbon dioxide, oxygen, hydrogen, and hydrogen sulfides.

[00242] “Pure” methane can be used as well. In these cases, the methane typically comes from a tank. The methane contained within these tanks can range from 90% or greater methane content and the remaining gas are other gases (such as carbon dioxide). For example, gas having a methane content of greater than 90% can be used during the fermentation process. In certain instances, the methane concentration can be greater than 90%; 91%; 92%; 93%; 94%; 95%; 96%; 97%; 98%; 99%; or 99.9%. In some cases, the methane concentration can be 90% methane and 10% are other gases (such as carbon dioxide). In other instances, the methane concentration can be 91% methane and 9% are other gases (such as carbon dioxide). In some cases, the methane concentration can be 92% methane and 8% are other gases (such as carbon dioxide). In some cases, the methane concentration can be 93% methane and 7% are other gases (such as carbon dioxide). In some cases, the methane concentration can be 94% methane and 6% are other gases (such as carbon dioxide). In some cases, the methane concentration can be 95% methane and 5% are other gases (such as carbon dioxide). In other instances, the methane concentration can be 96% methane and 4% are other gases (such as carbon dioxide). In some cases, the methane concentration can be 97% methane and 3% are other gases (such as carbon dioxide). In some cases, the methane concentration can be 98% methane and 2% are other gases (such as carbon dioxide). In some cases, the methane concentration can be 99% methane and 1% are other gases (such as carbon dioxide). In some cases, the methane concentration can be 99.9% methane and 0.1% are other gases (such as carbon dioxide).

[00243] In cases where a switch is used, the media can comprise the molecule that induces or represses the switch. For example, when a lanthanum switch is used to repress the expression of one or more of the genes described herein, the media can comprise lanthanum, which will repress expression of the one or more genes under the control of the switch. In the case of lanthanum any one of the following concentrations can effectively repress expression of the one or more genes: 0.1 μM ; 0.5 μM ; 1 μM ; 2 μM ; 3 μM ; 4 μM ; 5 μM ; 6 μM ; 7 μM ; 8 μM ; 9 μM ; 10 μM ; 12.5 μM ; 15 μM ; 17.5 μM ; 20 μM ; 25 μM ; 50 μM ; 100 μM or more. In one case, 0.1 μM lanthanum can be used to repress expression of the one or more genes under the control of a lanthanum switch. In other cases, at least 0.5 μM lanthanum can be used. In other cases, at least 1 μM lanthanum can be used.

In other cases, at least 2 μM lanthanum can be used. In other cases, at least 3 μM lanthanum can be used. In other cases, at least 4 μM lanthanum can be used. In other cases, at least 5 μM lanthanum can be used. In other cases, at least 6 μM lanthanum can be used. In other cases, at least 7 μM lanthanum can be used. In other cases, at least 8 μM lanthanum can be used. In other cases, at least 9 μM lanthanum can be used. In other cases, at least 10 μM lanthanum can be used. In other cases, at least 12.5 μM lanthanum can be used. In other cases, at least 15 μM lanthanum can be used. In other cases, at least 17.5 μM lanthanum can be used. In other cases, at least 20 μM lanthanum can be used. In other cases, at least 25 μM lanthanum can be used. In other cases, at least 50 μM lanthanum can be used. In other cases, at least 100 μM lanthanum can be used. In some cases, a range of 0.5 μM lanthanum to 100 μM lanthanum will effectively repress gene expression. In some cases, a range of 0.5 μM lanthanum to 50 μM lanthanum will repress gene expression. In other cases, a range of 1 μM lanthanum to 20 μM lanthanum will repress gene expression. In some cases, a range of 2 μM lanthanum to 15 μM lanthanum will repress gene expression. In some cases, a range of 3 μM lanthanum to 12.5 μM lanthanum will repress gene expression. In some cases, a range of 4 μM lanthanum to 12 μM lanthanum will repress gene expression. In some cases, a range of 5 μM lanthanum to 11.5 μM lanthanum will repress gene expression. In some cases, a range of 6 μM lanthanum to 11 μM lanthanum will repress gene expression. In some cases, a range of 7 μM lanthanum to 10.5 μM lanthanum will repress gene expression. In some cases, a range of 8 μM lanthanum to 10 μM lanthanum will repress gene expression.

[00244] In some cases, the lanthanum in the media can be diluted to turn on expression of the one or more lanthanum repressed genes. For example, in some cases, the dilution of lanthanum containing media can be 1:1 (1 part lanthanum containing media to 1 part non-lanthanum containing media). In some cases, the dilution can be at least 1:2; 1:3; 1:4; 1:5; 1:7.5; 1:10; 1:15; 1:20; 1:25; 1:30; 1:35; 1:40; 1:45; 1:50; 1:75; 1:100; 1:200; 1:300; 1:400; 1:500; 1:1,000; or 1:10,000. For example, in some cases, a 1:2 dilution can be used. In some cases, at least a 1:3 dilution can be used. In some cases, at least a 1:4 dilution can be used. In some cases, at least a 1:5 dilution can be used. In some cases, at least a 1:7.5 dilution can be used. In some cases, at least a 1:10 dilution can be used. In some cases, at least a 1:15 dilution can be used. In some cases, at least a 1:20 dilution can be used. In some cases, at least a 1:25 dilution can be used. In some cases, at least a 1:30 dilution can be used. In some cases, at least a 1:35 dilution can be used. In some cases, at least a 1:40 dilution can be used. In some cases, at least a 1:45 dilution can be used. In some cases, at least a 1:50 dilution can be used. In some cases, at least a 1:75 dilution can be used. In some cases, at least

a 1:100 dilution can be used. In some cases, at least a 1:200 dilution can be used. In some cases, at least a 1:300 dilution can be used. In some cases, at least a 1:400 dilution can be used. In some cases, at least a 1:500 dilution can be used. In some cases, at least a 1:1,000 dilution can be used. In some cases, at least a 1:10,000 dilution can be used.

[00245] In some cases, the microorganism can be grown in media comprising lanthanum. The media can then be diluted to effectively turn on the expression of the lanthanum repressed genes. The microorganism can be then grown in conditions to promote the production of desired products, such as 2,3-BDO and acetoin (or others disclosed throughout).

[00246] In some cases, other rare earth metals can be used. For example, other rare earth metals such as cerium (Ce), dysprosium (Dy), erbium (Er), europium (Eu), gadolinium (Gd), holmium (Ho), lutetium (Lu), neodymium (Nd), praseodymium (Pr), promethium (Pm), samarium (Sm), scandium (Sc), terbium (Tb), thulium (Tm), ytterbium (Yb), yttrium (Y), or any combination thereof, can be used to repress or activate a molecular switch.

Bioreactor

[00247] Fermentation reactions may be carried out in any suitable bioreactor. In some cases of the invention, the bioreactor can comprise a first, growth reactor in which the micro-organisms are cultured, and a second, fermentation reactor, to which broth from the growth reactor is fed and in which most of the fermentation product (2,3-BDO, for example) is produced.

Product Recovery

[00248] The fermentation of the microorganisms disclosed herein can produce a fermentation broth comprising a desired product (*e.g.*, 2,3-BDO, MEK, and/or butadiene) and/or one or more by-products as well as the microorganisms (*e.g.*, a genetically modified methanotroph), in a nutrient medium.

[00249] The microorganisms and the methods herein can produce 2,3-BDO at surprisingly high efficiency, more so than other known 2,3-BDO fermentation processes. For example, the microorganisms and the methods disclosed herein can convert a C1-carbon substrate (such as methane) at a rate of greater than 50%. This means that at least 50% of the C1-carbons within the systems are converted into product, such as 2,3-BDO. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 60%, 70%, 80%, 81%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 60%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 70%. In some cases, the conversion of a C1-carbon substrate

into 2,3-BDO can be at least 80%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 81%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 82%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 83%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 84%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 85%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 86%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 87%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 88%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 89%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 90%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 91%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 92%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 93%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 94%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 95%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 96%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 97%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 98%. In some cases, the conversion of a C1-carbon substrate into 2,3-BDO can be at least 99%.

[00250] In certain methods when producing 2,3-BDO, the concentration of 2,3-BDO in the fermentation broth is at least 1g/L. For example, the concentration of 2,3-BDO produced in the fermentation broth can be from 1 g/L to 5 g/L, 2 g/L to 6 g/L, 3 g/L to 7 g/L, 4 g/L to 8 g/L, 5 g/L to 9 g/L, or 6 g/L to 10 g/L. In some cases, the concentration of 2,3-BDO can be at least 9g/L. In some cases, the concentration of 2,3-BDO can be from 1 g/L to 5 g/L. In some cases, the concentration of 2,3-BDO can be from 2 g/L to 6 g/L. In some cases, the concentration of 2,3-BDO can be from 3 g/L to 7 g/L. In some cases, the concentration of 2,3-BDO can be from 4 g/L to 8 g/L. In some cases, the concentration of 2,3-BDO can be from 5 g/L to 9 g/L. In some cases, the concentration of 2,3-BDO can be from 6 g/L to 10 g/L.

[00251] In other cases, when microorganisms are used that normally produce at least some 2,3-BDO, after genetic modification and fermentation, the genetically modified microorganism can produce 2,3-BDO in concentrations that are at least 1.1X the amount that is normally produced. In some cases, the genetically modified microorganism can produce at least 2X, 3X, 4X, 5X, 6X, 7X,

8X, 9X, 10X, 15X, 20X, 25X, 30X, 35X, 40X, 45X, 50X, 60X, 70X, 80X, 90X or 100X the amount that is normally produced (*e.g.*, produced by a microorganism that is unmodified and of the same species as the genetically modified microorganism). In some cases, the genetically modified microorganism can produce at least 2X, 3X, 4X, 5X, 10X, 25X, 50X, and or 100X the amount that is normally produced. In some cases, the genetically modified microorganism can produce at least 2X the amount that is normally produced. In some cases, the genetically modified microorganism can produce at least 3X the amount that is normally produced. In some cases, the genetically modified microorganism can produce at least 4X the amount that is normally produced. In some cases, the genetically modified microorganism can produce at least 5X the amount that is normally produced. In some cases, the genetically modified microorganism can produce at least 10X the amount that is normally produced. In some cases, the genetically modified microorganism can produce at least 25X the amount that is normally produced. In some cases, the genetically modified microorganism can produce at least 50X the amount that is normally produced. In some cases, the genetically modified microorganism can produce at least 100X the amount that is normally produced.

[00252] As discussed above, in certain cases the 2,3-BDO produced in the fermentation reaction is converted to MEK, butene, and/or butadiene (or other products) directly from the fermentation broth. In other cases, the 2,3-BDO is first recovered from the fermentation broth before conversion to MEK, butene, and/or butadiene.

[00253] In some cases, 2,3-BDO can be continuously removed from a portion of broth and recovered as purified 2,3-BDO. In particular cases, the recovery of 2,3-BDO includes passing the removed portion of the broth containing 2,3-BDO through a separation unit to separate the microorganisms (*e.g.*, genetically modified methanotroph) from the broth, to produce a cell-free 2,3-BDO containing permeate, and returning the microorganisms to the bioreactor. The cell-free 2,3-BDO-containing permeate may then can be stored or be used for subsequent conversion to butene, MEK and/or butadiene (or other desired product).

[00254] The recovering of 2,3-BDO and/or one or more other products or by-products produced in the fermentation reaction can comprise continuously removing a portion of the broth and recovering separately 2,3-BDO and one or more other products from the removed portion of the broth. In some cases the recovery of 2,3-BDO and/or one or more other products includes passing the removed portion of the broth containing 2,3-BDO and/or one or more other products through a separation unit to separate microorganisms from the 2,3-BDO and/or one or more other products, to produce a cell-

free 2,3-BDO and one or more other product-containing permeate, and returning the microorganisms to the bioreactor.

[00255] In the above cases, the recovery of 2,3-BDO and one or more other products can include first removing 2,3-BDO from the cell-free permeate followed by removing the one or more other products from the cell-free permeate. The cell-free permeate can also then be returned to the bioreactor.

[00256] 2,3-BDO, or a mixed product stream containing 2,3-BDO, can be recovered from the fermentation broth. For example, methods that can be used can include but are not limited to, fractional distillation or evaporation, pervaporation, and extractive fermentation. Further examples include: recovery using steam from whole fermentation broths; reverse osmosis combined with distillation; Liquid-liquid extraction techniques involving solvent extraction of 2,3-BDO; aqueous two-phase extraction of 2,3-BDO in PEG/dextran system; solvent extraction using alcohols or esters, *e.g.*, ethyl acetate, tributylphosphate, diethyl ether, *n*-butanol, dodecanol, oleyl alcohol, and an ethanol/phosphate system; aqueous two-phase systems composed of hydrophilic solvents and inorganic salts. *See generally*, Voloch, M., *et al.*, (1985) and U.S. Pat. Pub. Appl. No. 2012/0045807.

[00257] In some cases prior to exposure to solvent, the fermentation broth is dewatered by evaporation or both microfiltration and reverse osmosis because of the low partition coefficient and the low selectivity of 2,3-BDO. Repulsive extraction or salting out using potassium chloride (KCl) or dehydrated K_2CO_3 has also been investigated on the recovery of 2,3-BD (Syu 2001) like the salting-out effect of K_2CO_3 on extraction of butanol in acetone-O butanol-ethanol fermentation. The removal of water from the fermentation broth was also tested before salting out because the concentration of 2,3-BDO in the broth was too low to be salted out even if at a saturated KCl or K_2CO_3 solution. *See generally*, U.S. Pat. Pub. Appl. No. 2012/0045807.

[00258] A yet further example of a method to recover 2,3-BDO is to react it with formaldehyde to form a formal under catalysis of acid. The 2,3-BDO formal is collected in the top oil phase and allowed to react with acid methanol to form 2,3-BDO and methylal. Methylal can be hydrolyzed to methanol and formaldehyde. *See generally*, U.S. Pat. Pub. Appl. No. 2012/0045807.

[00259] A further example, may be the use of ionic liquids to extract the ethanol/2,3-BD from clarified broth. Ionic liquids can be tailored in many ways to change physical properties. An advantage of this approach is that ionic liquids are not volatile. Some are water sensitive but others are not.

[00260] Pervaporation or vacuum membrane distillation, used previously in ethanol and butanol fermentations, can be used to concentrate 2,3-BDO in water as an extract from the fermentation broth. A microporous polytetrafluoroethylene (PTFE) membrane is used in the integrated process, while a silicone membrane is usually used in pervaporative ethanol or butanol fermentations. *See generally*, U.S. Pat. Pub. Appl. No. 2012/0045807.

[00261] By-products such as acids including acetate and butyrate may also be recovered from the fermentation broth using methods known in the art. For example, an adsorption system involving an activated charcoal filter or electrodialysis can be used.

[00262] In certain cases of the invention, 2,3-BDO and by-products are recovered from the fermentation broth by continuously removing a portion of the broth from the bioreactor, separating microbial cells from the broth (conveniently by filtration, for example), and recovering 2,3-BDO and optionally other alcohols and acids from the broth. Alcohols may conveniently be recovered for example by distillation, and acids may be recovered for example by adsorption on activated charcoal. The separated microbial cells can be returned to the fermentation bioreactor. The cell free permeate remaining after the alcohol(s) and acid(s) have been removed can also be returned to the fermentation bioreactor. Additional nutrients may be added to the cell free permeate to replenish the nutrient medium before it is returned to the bioreactor.

[00263] Also, if the pH of the broth was adjusted during recovery of 2,3-BDO and/or by-products, the pH should be re-adjusted to a similar pH to that of the broth in the fermentation bioreactor, before being returned to the bioreactor.

[00264] In certain cases, the 2,3-BDO is continuously recovered from the fermentation broth or bioreactor and fed directly for chemical conversion to one or more of butene, butadiene and methyl ethyl ketone. For example, the 2,3-BDO may be fed directly through a conduit to one or more vessel suitable for chemical synthesis of one or more of butene, butadiene and methyl ethyl ketone or other down-stream chemical products.

[00265] While some cases have been shown and described herein, such cases are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the cases of the invention described herein will be employed in practicing the invention.

EXAMPLES

Example 1: Genetic engineering of methanotrophs

[00266] To engineer a methanotroph to produce 2,3-BDO, we started with *M. capsulatus* and tested several 2,3-BDO biosynthetic genes from *Bacillus subtilis*, *Clostridium autoethanogenum*, *Klebsiella pneumoniae*, and *Paenibacillus polymyxa*. **Table 1** below shows the genes tested and the origin of the genes.

Table 1:

Gene category		Gene name	Microbial species
2,3-BDO pathway genes	AlsS	BsuAlsS	<i>Bacillus subtilis</i>
		CauAlsS	<i>Clostridium autoethanogenum</i>
	BudA	CauBudA	<i>Clostridium autoethanogenum</i>
		KpnBudA	<i>Klebsiella pneumoniae</i>
	ButA	CauButA	<i>Clostridium autoethanogenum</i>
		BsuButA	<i>Bacillus subtilis</i>
		PpoButA	<i>Paenibacillus polymyxa</i>

[00267] For the first gene of the pathway, AlsS, which encodes for an enzyme that converts two molecules of pyruvate into 2-acetolactate, we tested AlsS homologs from *Bacillus subtilis* and *Clostridium autoethanogenum*. For the second gene in the pathway, BudA, which encodes for an enzyme that converts 2-acetolactate into acetoin, we tested BudA homologs from *Clostridium autoethanogenum* and *Klebsiella pneumoniae*. For the third gene in the pathway, ButA, which encodes an enzyme that converts acetoin into 2,3-BDO using NAD(P)H as a reduced cofactor, we tested ButA homologs from *Clostridium autoethanogenum* (NADPH-dependent), *Bacillus subtilis* (NADH-dependent), and *Paenibacillus polymyxa* (NADH-dependent).

[00268] We generated 48 different broad host plasmids containing variations in the 3-gene 2,3-BDO pathway, as shown in Table 2 (below in Example 2). **Table 2** shows the genotypes of the resulting engineered strains.

Example 2: 2,3-BDO productivity

[00269] We transformed the aforementioned plasmids (in Example 1) into a transformation competent methanotroph strain, RL83A, and evaluated 74 resulting strains (including biological replicate strains) for 2,3-BDO production in small scale microtiter plate fermentation using methane as the carbon source.

[00270] As shown in **Table 2**, the constructs that constitutively expressed AlsS genes resulted in either no transformants or no production of 2,3-BDO, indicating that there was a negative impact

when AlsS genes were strongly and constitutively expressed. The results showed that the strains that produced the most 2,3-BDO contained the gene combinations BsuAlsA-KpnBudA-CauButA or BsuAlsA-KpnBudA-BsuButA (**Table 2**).

Table 2

DNA construct		Genotype*			Resulted <i>M. capsulatus</i> Strain **		2,3BDO production in Tier1 assay (mg/L)	
asm178.1	pBAD	BsuAlsS	CauBudA	CauButA	XZ01	NT	55.4	/
asm178.2	pBAD	BsuAlsS	CauBudA	BsuButA	XZ02	XZ03	49.6	49.9
asm178.3	pBAD	BsuAlsS	CauBudA	PpoButA	XZ04	XZ05	47.2	48
asm178.4	pBAD	BsuAlsS	KpnBudA	CauButA	XZ06	XZ07	179.6	175.6
asm178.5	pBAD	BsuAlsS	KpnBudA	BsuButA	XZ08	XZ09	135.3	132.4
asm178.6	pBAD	BsuAlsS	KpnBudA	PpoButA	XZ10	XZ11	28.3	27.5
asm178.7	pBAD	CauAlsS	CauBudA	CauButA	XZ12	XZ13	2.1	1.8
asm178.8	pBAD	CauAlsS	CauBudA	BsuButA	XZ14	XZ15	1.9	1.6
asm178.9	pBAD	CauAlsS	CauBudA	PpoButA	XZ16	XZ17	0	0
asm178.10	pBAD	CauAlsS	KpnBudA	CauButA	XZ18	XZ19	79.2	75.2
asm178.11	pBAD	CauAlsS	KpnBudA	BsuButA	XZ20	XZ21	50.1	49.7
asm178.12	pBAD	CauAlsS	KpnBudA	PpoButA	XZ22	XZ23	2.4	1.8
asm178.13	pMxgF	BsuAlsS	CauBudA	CauButA	NT	NT	/	/
asm178.14	pMxgF	BsuAlsS	CauBudA	BsuButA	NT	NT	/	/
asm178.15	pMxgF	BsuAlsS	CauBudA	PpoButA	NT	NT	/	/
asm178.16	pMxgF	BsuAlsS	KpnBudA	CauButA	NT	NT	/	/
asm178.17	pMxgF	BsuAlsS	KpnBudA	BsuButA	NT	NT	/	/
asm178.18	pMxgF	BsuAlsS	KpnBudA	PpoButA	NT	NT	/	/
asm178.19	pMxgF	CauAlsS	CauBudA	CauButA	XZ29	XZ30	0	0
asm178.20	pMxgF	CauAlsS	CauBudA	BsuButA	XZ31	XZ32	0	0
asm178.21	pMxgF	CauAlsS	CauBudA	PpoButA	XZ33	XZ34	0	0
asm178.22	pMxgF	CauAlsS	KpnBudA	CauButA	XZ35	NT	0	/
asm178.23	pMxgF	CauAlsS	KpnBudA	BsuButA	XZ36	NT	0	/
asm178.24	pMxgF	CauAlsS	KpnBudA	PpoButA	XZ37	XZ38	0	0
asm178.25	pMxgF	BsuAlsS	CauBudA	<u>p.Bga./23111</u> CauButA	XZ39	XZ40	8.4	0
asm178.26	pMxgF	BsuAlsS	CauBudA	<u>p.Bga./23111</u> BsuButA	XZ41	XZ42	0	0
asm178.27	pMxgF	BsuAlsS	CauBudA	<u>p.Bga./23111</u> PpoButA	XZ43	XZ44	0	0
asm178.28	pMxgF	BsuAlsS	KpnBudA	<u>p.Bga./23111</u> CauButA	NT	NT	/	/
asm178.29	pMxgF	BsuAlsS	KpnBudA	<u>p.Bga./23111</u> BsuButA	XZ45	NT	11.6	/
asm178.30	pMxgF	BsuAlsS	KpnBudA	<u>p.Bga./23111</u> PpoButA	XZ46	NT	0	/
asm178.31	pMxgF	CauAlsS	CauBudA	<u>p.Bga./23111</u> CauButA	XZ47	XZ48	0	0
asm178.32	pMxgF	CauAlsS	CauBudA	<u>p.Bga./23111</u> BsuButA	XZ49	XZ50	0	0
asm178.33	pMxgF	CauAlsS	CauBudA	<u>p.Bga./23111</u> PpoButA	XZ51	XZ52	0	0
asm178.34	pMxgF	CauAlsS	KpnBudA	<u>p.Bga./23111</u> CauButA	NT	NT	/	/
asm178.35	pMxgF	CauAlsS	KpnBudA	<u>p.Bga./23111</u> BsuButA	NT	NT	/	/
asm178.36	pMxgF	CauAlsS	KpnBudA	<u>p.Bga./23111</u> PpoButA	NT	NT	/	/
asm178.37	pBAD	BsuAlsS	CauBudA	pMxgF CauButA	NT	NT	/	/
asm178.38	pBAD	BsuAlsS	CauBudA	pMxgF BsuButA	XZ53	XZ54	54.6	63.7
asm178.39	pBAD	BsuAlsS	CauBudA	pMxgF PpoButA	XZ55	XZ56	47.4	32.6
asm178.40	pBAD	BsuAlsS	KpnBudA	pMxgF CauButA	XZ57	XZ58	180.8	194.3
asm178.41	pBAD	BsuAlsS	KpnBudA	pMxgF BsuButA	XZ59	XZ60	144.4	125.9
asm178.42	pBAD	BsuAlsS	KpnBudA	pMxgF PpoButA	XZ61	XZ62	1.8	2.7
asm178.43	pBAD	CauAlsS	CauBudA	pMxgF CauButA	XZ63	XZ64	1.8	1.7
asm178.44	pBAD	CauAlsS	CauBudA	pMxgF BsuButA	XZ65	XZ66	1.5	1.8
asm178.45	pBAD	CauAlsS	CauBudA	pMxgF PpoButA	XZ67	XZ68	0	0
asm178.46	pBAD	CauAlsS	KpnBudA	pMxgF CauButA	XZ69	XZ70	83.3	78.5
asm178.47	pBAD	CauAlsS	KpnBudA	pMxgF BsuButA	XZ71	XZ72	39.2	38.3
asm178.48	pBAD	CauAlsS	KpnBudA	pMxgF PpoButA	XZ73	XZ74	2	1.6

[00271] To confirm the results from the primary microplate assay, we evaluated four top strains, XZ58 and XZ06 (BsuAlsA-KpnBudA-CauButA), XZ59 and XZ08 (BsuAlsA-KpnBudA-BsuButA),

in a high cell-density fermentation experiment in a 1 L bioreactor. As shown in **FIG. 2**, strains XZ58 and XZ06 produced 9.3g/L and 8.5g/L 2,3-BDO, respectively, with no penultimate product, acetoin, produced (Fig 2, left panel). The data demonstrates that strains expressing NADPH-dependent acetoin reductase (CauButA) produce exclusively 2,3-BDO with no acetoin co-product. In contrast, strains expressing NADH-dependent acetoin reductase produced a significant amount of the 2,3-BDO precursor, acetoin (**FIG. 2**, right panel). Inefficient conversion of acetoin to 2,3-BDO with NADH-dependent pathways could be due to either poor enzyme expression or low NADH precursor pools in *M. capsulatus*.

Example 3: Genetic Switches used for 2,3-BDO production

[00272] In order to effectively control 2,3-BDO production at specific times, a genetic switch system was implemented. Methanotrophs were transformed with genes placed under the control of a lanthanum “switch”. Lanthanum switches repress expression of genes in the presence of the metal lanthanum. Upon removal or dilution of lanthanum in the media, the repressed genes are “switched” on.

[00273] Different strains of methanotrophs (as shown in **Table 3** below shows the strains and genotype of the strains) were pre-culturing in the presence of 10 μ M lanthanum.

Table 3	
Strain	Genotype
XZ685	pmxaF>Bsu.alsA>rbs.GTW0001_Kpn.BudA>rbs.GTW0001_Cau.ButA
XZ686	
XZ687	
XZ688	pmxaF>Bsu.alsA>rbs.Mca.mxaF_Kpn.BudA>rbs.Mca.mxaF_Cau.ButA
XZ689	
XZ690	
XZ691	pmxaF>Blic.alsA>rbs.GTW0001_Kpn.BudA>rbs.GTW0001_Cau.ButA
XZ692	
XZ693	
XZ694	pmxaF>Blic.alsA>rbs.Mca.mxaF_Kpn.BudA>rbs.Mca.mxaF_Cau.ButA
XZ695	
XZ696	
XZ697	pmxaF>Bsu.alsA>rbs.GTW0001_Kpn.BudA>rbs.GTW0001_Cau.ButA
XZ698	
XZ699	
XZ700	pmxaF>Bsu.alsA>rbs.Mca.mxaF_Kpn.BudA>rbs.Mca.mxaF_Cau.ButA
XZ701	
XZ702	
XZ703	pmxaF>Blic.alsA>rbs.Mca.mxaF_Kpn.BudA>rbs.Mca.mxaF_Cau.ButA
XZ704	
XZ705	

[00274] After growing precultures to ~ 3 OD₆₀₀, the medium containing lanthanum was diluted at a ratio of 1:10 (lanthanum containing media: non-lanthanum containing media) or 1:50. After 96 or 120 hours, the cultures assessed for production of 2,3-BDO and acetoin (**FIG. 7A, 96 hours**) or (**FIG. 7B, 120 hours**). Shown below in **Table 4** are strains, dilution levels, acetoin production titers after 96 hours, 2,3-BDO production titers after 96 hours, acetoin production titers after 120 hours, and 2,3-BDO production titers after 120 hours.

TABLE 4						
	Strain	Dilution	Acetoin (mg/L)_96h	2,3BDO (mg/L)_96h	Acetoin (mg/L)_120h	2,3BDO (mg/L)_120h
1	XZ685	10X	5.847	71.342	8.617	150.864
2	XZ686	10X	4.86	51.439	10.539	135.871
3	XZ687	10X	6.306	53.591	10.475	137.104
4	XZ688	10X	5.311	73.08	8.79	158.947
5	XZ689	10X	5.282	71.059	6.944	149.219
6	XZ690	10X	5.679	51.291	13.412	165.688
7	XZ691	10X	4.014	0	0	19.231
8	XZ692	10X	3.719	4.679	7.984	23.991
9	XZ693	10X	3.778	4.082	6.2	18.78
10	XZ694	10X	43.925	0	54.582	4.871
11	XZ695	10X	44.734	0	55.632	3.363
12	XZ696	10X	46.473	0	57.444	5.39
13	XZ697	10X	5.982	48.549	12.221	131.618
14	XZ698	10X	3.441	52.775	12.594	137.996
15	XZ699	10X	5.747	44.14	12.937	131.699
16	XZ700	10X	5.101	50.998	12.26	117.653
17	XZ701	10X	4.82	52.523	12.167	120.369
18	XZ702	10X	5.802	54.763	12.119	133.294
19	XZ703	10X	38.501	0	51.296	6.482
20	XZ704	10X	33.64	0	44.195	5.021
21	XZ705	10X	40.326	0	52.443	5.978
22	XZ685	50X	4.245	95.288	8.993	184.974
23	XZ686	50X	3.028	87.876	0	173.315
24	XZ687	50X	4.341	85.562	10.06	193.196
25	XZ688	50X	4.088	73.804	8.181	156.027
26	XZ689	50X	4.273	78.782	0	156.435
27	XZ690	50X	4.383	79.226	7.879	155.869
28	XZ691	50X	3.178	10.35	8.197	32.164
29	XZ692	50X	2.838	14.16	7.839	38.733
30	XZ693	50X	2.865	12.942	9.163	41.967
31	XZ694	50X	53.138	0	64.47	4.259

32	XZ695	50X	66.256	0	79.665	4.736
33	XZ696	50X	66.139	0	78.46	4.746
34	XZ697	50X	4.664	57.939	9.411	122.655
35	XZ698	50X	5.749	67.055	10.424	151.002
36	XZ699	50X	4.182	63.379	10.386	137.577
37	XZ700	50X	3.558	59.187	9.519	121.163
38	XZ701	50X	3.085	63.574	10.265	140.806
39	XZ702	50X	5.039	66.163	11.252	131.931
40	XZ703	50X	58.687	0	72.718	6.653
41	XZ704	50X	55.523	0	70.855	6.577
42	XZ705	50X	59.577	0	76.618	4.793

[00275] As shown **FIGs. 7A, 7B**, and **Table 4**, strains XZ685, XZ686, XZ687, XZ688, XZ689, and XZ690 (referring to 22 to 27, respectively in **FIGs. 7A** and **7B**) produced the highest titers of 2,3-BDO at both 96 and 120 hours when diluting the lanthanum containing medium 1:50 (50X). Using a 1:10 dilution protocol also resulted in significant production of 2,3-BDO but the titers were lower than those using the 1:50 dilution protocol at both 96 and 120 hours. Strains XZ697, XZ698, XZ699, XZ700, XZ701, and XZ702 (referring to 34 to 39, respectively in **FIGs. 7A** and **7B**) produced lower titers at both 1:10 and 1:50 dilutions at both 96 and 120 hours.

Example 4: Acetolactate Synthase

[00276] The plasmids described in Table 5 (below), were transformed into a transformation competent methanotroph strain. The resulting strains were tested for 2,3-BDO production titers in small scale microtiter plate fermentation using methane as the carbon source.

TABLE 5			
Strain	Strain Genotype	Average Titer (mg/L)	% diff. vs. XZ58
XZ58	p.BAD>g.Bsu.alsS>(rbs.GTW0001)g.Kpn.BudA>p.mxaF>g.Cau.ButA	372	0.0%
XZ557	p.BAD>g.Blic.alsS>(rbs.GTW0001)g.Kpn.BudA-p.mxaF>g.Cau.ButA	421	16.1%
XZ546	p.BAD>g.Bsu.alsS-(rbs.Mca.MxaF)g.Kpn.BudA-p.mxaF>g.Cau.ButA	373	0.3%
XZ562	p.BAD>g.Blic.alsS-(rbs.Mca.MxaF)g.Kpn.BudA-p.mxaF>g.Cau.ButA	538	44.6%

[00277] As shown in **Table 5** and in **FIG. 8**, the strains that expressed *Bacillus licheniformis* AlsS gene, showed better 2,3-BDO production titers than the strains that expressed *Bacillus subtilis* AlsS (e.g., the strains described in Example 2). In one example, a strain that has a substitution of only the AlsS gene (e.g., strains XZ557) exhibited an increase of 2,3-BDO production titer of up to 16.1% compared to strain XZ58. In another sample, strain XZ546, a strain having a substitution of only the ribosome binding site for the Kpn.BudA gene, showed virtually no increase of 2,3-BDO titers

compared with strain XZ58. However, remarkably, a strain that contained rbs.Mca.MxaF for the Kpn.BudA, instead of a rbs.GTW0001 and expressed a *Bacillus licheniformis* AlsS gene (e.g., strain XZ562), exhibited a significant increase in 2,3-BDO titers, up to 44.6% compared to strain XZ58.

Example 5: Temperature during Fermentation

[00278] In order to prevent instability sometimes present in plasmid expressing strains, we generated strains with 2,3-BDO pathway genes integrated in chromosome at the Glgc locus. A total of 21 strains were generated.

[00279] Seven of the strains were tested in shake bottles for their ability to produce 2,3-BDO at 37°C and at 42°C. Fermentation occurred over a 96 hour period. The genotypes of the strains are presented in **TABLE 6** below:

TABLE6

	Strain	Background	Genotypes					
Integrated	MBC2115	RL83	p.Mcap.mxaF	cds.Bsub.AlsS	rbs synt.GTW0001	cds.Kpne.BudA	rbs synt.GTW0001	cds.Caut.ButA
Integrated	MBC2116	RL83	p.Mcap.mxaF	cds.Bsub.AlsS	rbs synt.GTW0001	cds.Kpne.BudA	rbs synt.GTW0001	cds.Caut.ButA
Integrated	MBC2117	RL83	p.Mcap.mxaF	cds.Bsub.AlsS	rbs synt.GTW0001	cds.Kpne.BudA	rbs synt.GTW0001	cds.Caut.ButA
Integrated	MBC2122	RL83	p.Mcap.mxaF	cds.Caut.ButA	rbs.Mcap.MxaF	cds.Kpne.BudA	rbs.Mcap.MxaF	cds.Blic.Als
Integrated	MBC2131	XZ715	p.Mcap.mxaF	cds.Caut.ButA	rbs.Mcap.MxaF	cds.Kpne.BudA	rbs.Mcap.MxaF	cds.Bsub.AlsS
Episomal	XZ585	RL83	p.Mcap.mxaF	cds.Bsub.AlsS	rbs synt.GTW0001	cds.Kpne.BudA	rbs synt.GTW0001	cds.Caut.ButA
Episomal	MBC1322	MH436	p.Mcap.mxaF	cds.Bsub.AlsS	rbs synt.GTW0001	cds.Kpne.BudA	rbs synt.GTW0001	cds.Caut.ButA

[00280] As seen in **FIG. 9**, all strains tested produced significantly more 2,3-BDO when fermentation occurred at 42°C. Some strains, such as MBC2122, produced approximately double the amount of 2,3-BDO at 42°C rather than 37°C.

[00281] Three of the strains were further tested when fermented at 45°C, with and without antibiotic selective pressure. Compared to the same strain incubated at 37°C, the strains that were fermented at 45°C produced significantly less 2,3-BDO. See **FIG. 10**. The episomal strain MBC1322 performed better with antibiotic selective pressure, at both 37 °C and 45 °C, indicating a stability issue. On the contrary, the integrated strains were able to maintain its productivity without selective pressure.

[00282] Additionally testing was performed at 37°C, 41°C, 43°C, 45°C, and 47°C. 2,3-BDO titers and OD ratios were calculated. As seen in **FIG. 11** the best productivity was observed at a temperature of 41°C.

Example 6: Additional Gene Copies

[00283] A methanotroph strain that contained additional copies of 2,3-BDO pathway enzymes (**Strains B and C**) within an episomal vector produced increased 2,3-BDO compared to the singly

integrated strain (**Strain A**) in shake bottle experiments. **Strain B** and **Strain C** were precultured in 10 μ M lanthanum in the presence of Kanamycin at 37°C for 48 hours. **Strain A** was precultured in the same conditions except that no Kanamycin was added. After 48 hours, the lanthanum was diluted by 50 fold (50X) and 2,3-BDO titer were measured after 96 hours. As seen in **FIG. 12**, the strains that contained additional copies of 2,3-BDO pathway enzymes (**Strains B** and **C**) within an episomal vector produced over 3 times as much 2,3-BDO compared to the singly integrated strain (**Strain A**).

Example 7: Preculturing

[00284] The effect of preculturing was tested in both episomal and integration strains as well as strains having both episomally expressed 2,3-BDO pathway genes and integrated copies of the genes.

[00285] As seen in **FIG. 13**, the effect of preculturing a methanotroph strain (in a shake bottle) in which the genes encoding for 2,3-BDO pathway enzymes were episomally expressed (**Strain D**). The methanotroph strain was precultured in 10 μ M, 3 μ M, and 1 μ M lanthanum during pre-culture. The production media was later diluted by 50-fold. Higher lanthanum concentrations resulted in higher 2,3BDO titers for episomally expressed strains.

[00286] As seen in **FIG. 14**, the effect of preculturing a methanotroph strain (in a shake bottle) in which the genes encoding for 2,3-BDO pathway enzymes are integrated into the methanotroph's genome. The methanotroph strain (**Strain A**) was precultured in 35 μ M, 10 μ M, 3 μ M, 1 μ M, and 0 μ M lanthanum. The production media was later diluted by 50 fold (50X). Levels of 2,3-BDO production increased when the strain are precultured with lower concentration of lanthanum.

[00287] As seen in **FIG. 15**, the shows the effect of preculturing at different lanthanum concentrations (10 μ M, 3 μ M, and 1 μ M) of the two strains from **FIG. 12** (**Strains B** and **C**) in a shake bottle experiment. Both strains contain one integrated copy and episomally expressed copies of the 2,3-BDO pathway enzymes. Generally, the strains having episomally expressed 2,3-BDO pathway enzymes, yield higher 2,3-BDO titers when precultured in 10 μ M lanthanum.

WHAT IS CLAIMED IS:

1. A genetically modified microorganism capable of converting a C1 carbon to 2,3-BDO comprising a heterologous acetoin reductase.
2. A genetically modified microorganism capable of converting a C1 carbon to 2,3-BDO comprising: (i) a heterologous acetoin reductase, (ii) a heterologous alpha-acetolactate decarboxylase, and (iii) a heterologous acetolactate synthase.
3. The genetically modified microorganism of claim 1, wherein said genetically modified microorganism further comprises a heterologous alpha-acetolactate decarboxylase or a heterologous acetolactate synthase.
4. The genetically modified microorganism of any one of claims 1 to 3, wherein said genetically modified microorganism comprises a heterologous acetoin reductase and a heterologous acetolactate synthase.
5. The genetically modified microorganism of any one of claims 1 to 4, wherein said genetically modified microorganism comprises a heterologous acetoin reductase and a heterologous alpha-acetolactate decarboxylase.
6. The genetically modified microorganism of any one of claims 2 to 5, wherein said heterologous acetolactate synthase is non-constitutively expressed.
7. The genetically modified microorganism of any one of claims 2 to 6, wherein said heterologous acetolactate synthase comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 1 or 19.
8. The genetically modified microorganism of any one of claims 2 to 7, wherein said heterologous alpha-acetolactate decarboxylase comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 7.
9. The genetically modified microorganism of any one of claims 1 to 8, wherein said heterologous acetoin reductase is NADPH-dependent.
10. The genetically modified microorganism of any one of claims 1 to 9, wherein said heterologous acetoin reductase comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 9.
11. The genetically modified microorganism of any one of claims 1 to 9, wherein said heterologous acetoin reductase is a gram positive bacterium acetoin reductase.
12. The genetically modified microorganism of claim 11, wherein said gram positive bacterium is from the genus *Clostridium* or *Bacillus*.

13. The genetically modified microorganism of claim 12, wherein said gram positive bacterium is a *Clostridium autoethanogenum* or *Bacillus subtilis*.
14. The genetically modified microorganism of any one of claims 1 to 13, wherein said genetically modified microorganism is a methanotroph.
15. The genetically modified microorganism of claim 14, wherein said methanotroph is from the genera *Methylobacter*, *Methylomicrobium*, *Methylomonas*, *Methylocaldum*, *Methylococcus*, *Methylosoma*, *Methylosarcina*, *Methylothermus*, *Methylohalobius*, *Methylogaea*, *Methylovulum*, *Crenothrix*, *Clonothrix*, *Methylosphaera*, *Methylocapsa*, *Methylocella*, *Methylosinus*, *Methylocystis*, or *Methyloacidophilum*.
16. The genetically modified microorganism of claim 15, wherein said methanotroph is a *Methylococcus*.
17. The genetically modified microorganism of claim 16, wherein said *Methylococcus* is *Methylococcus capsulatus*.
18. The genetically modified microorganism of any one of claims 1 to 17, wherein said C1 carbon is carbon monoxide (CO), carbon dioxide (CO₂), methane (CH₄), or any combination thereof.
19. The genetically modified microorganism of any one of claims 1 to 18, wherein said C1 carbon is CH₄.
20. The genetically modified microorganism of any one of claims 1 to 19, wherein said microorganism produces a greater amount of 2,3-BDO at 42 °C compared to the same organism at 37°C.
21. The genetically modified microorganism of any one of claims 1 to 20, wherein said microorganism produces a greater amount of 2,3-BDO at 42 °C compared to the same organism at 45 °C.
22. The genetically modified microorganism of any one of claims 1 to 21, wherein said microorganism produces a greater amount of 2,3-BDO at 37 °C compared to the same organism at 45 °C.
23. The genetically modified microorganism of any one of claims 1 to 22, wherein a heterologous gene encoding for said heterologous acetoin reductase, heterologous alpha-acetolactate decarboxylase, and/or heterologous acetolactate synthase is integrated into the genome of said microorganism.

24. The genetically modified microorganism of any one of claims 1 to 23, wherein a heterologous gene encoding for said heterologous acetoin reductase, heterologous alpha-acetolactate decarboxylase, and/or heterologous acetolactate synthase is expressed on an episomal vector.
25. The genetically modified microorganism of claim 23 or 24, wherein said heterologous gene encoding for said heterologous acetolactate synthase is 5' in relation to any other heterologous gene.
26. The genetically modified microorganism of any one of claims 23 to 25, wherein said heterologous gene encoding for said heterologous acetoin reductase is 3' in relation to any other heterologous gene.
27. The genetically modified microorganism of any one of claims 23 to 26, wherein said heterologous gene encoding for said heterologous alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other heterologous gene.
28. The genetically modified microorganism of any one of claims 23 to 27, wherein said heterologous gene encoding for said heterologous acetoin reductase, heterologous alpha-acetolactate decarboxylase, and/or heterologous acetolactate synthase is under the control of a switch.
29. The genetically modified microorganism of claim 28, wherein said switch is a rare earth metal switch.
30. The genetically modified microorganism of claim 29, wherein said rare earth metal is lanthanum, cerium, praseodymium, and/or neodymium.
31. The genetically modified microorganism of claim 30, wherein said rare earth metal is lanthanum.
32. A vector comprising two or more genes encoding: an acetoin reductase, an alpha-acetolactate decarboxylase, or an acetolactate synthase.
33. The vector of claim 32, wherein said gene encoding said acetolactate synthase is 5' in relation to any other gene on said vector.
34. The vector of claim 32 or 33, wherein said gene encoding said acetoin reductase is 3' in relation to any other gene on said vector.
35. The vector of any one of claims 32 to 34, wherein said gene encoding said alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other gene on said vector.
36. The vector of any one of claims 32 to 35, comprising a gene encoding an acetoin reductase, wherein said gene encoding an acetoin reductase comprises a nucleotide sequence that is at least 85% identical to SEQ ID NO: 10.

37. The vector of any one of claims 32 to 36, comprising a gene encoding an alpha-acetolactate decarboxylase, wherein the gene encoding an alpha-acetolactate decarboxylase comprises a nucleotide sequence that is at least 86% identical to SEQ ID NO: 8.
38. The vector of any one of claims 32 to 37, comprising a gene encoding an acetolactate synthase, wherein the gene encoding an acetolactate synthase comprises a nucleotide sequence that is at least 85% identical to SEQ ID NO: 2 or at least 60% identical to SEQ ID NO: 20.
39. The vector of any one of claims 32 to 38, further comprising a promoter that is active within a methanotroph.
40. The vector of any one of claims 32 to 39, wherein said vector is an integration vector.
41. The vector of any one of claims 32 to 40, wherein said vector is an episomal vector.
42. A method of making a genetically modified microorganism capable of converting a C1 carbon to 2,3-BDO comprising transforming a microorganism with a heterologous nucleic acid comprising a gene encoding an acetoin reductase.
43. The method of claim 42, further comprising transforming said genetically modified microorganism with a heterologous nucleic acid comprising a gene encoding alpha-acetolactate decarboxylase and/or an acetolactate synthase.
44. The method of claim 42 to 43, wherein said acetoin reductase is NADPH-dependent.
45. The method of any one of claims 42 to 44, wherein said acetoin reductase is a gram positive bacterium acetoin reductase.
46. The method of any one of claims 42 to 45, wherein said NADPH-dependent acetoin reductase comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 5.
47. The method of any one of claims 43 to 46, wherein said alpha-acetolactate decarboxylase comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 7.
48. The method of any one of claims 43 to 47, wherein said acetolactate synthase comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 1 or 19.
49. The method of any one of claims 42 to 48, wherein said C1 carbon is methane.
50. The method of any one of claims 42 to 49 wherein said microorganism is a methanotroph.
51. The method of any one of claims 42 to 50, wherein said microorganism produces a greater amount of 2,3-BDO at 42 °C compared to the same organism at 37°C.
52. The method of any one of claims 42 to 51, wherein said microorganism produces a greater amount of 2,3-BDO at 42 °C compared to the same organism at 45 °C.

53. The method of any one of claims 42 to 52, wherein said microorganism produces a greater amount of 2,3-BDO at 37 °C compared to the same organism at 45 °C.
54. The method of any one of claims 42 to 53, wherein said gene encoding for said acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase is integrated by an integration vector into the genome of said microorganism.
55. The method of any one of claims 42 to 54, wherein said gene encoding for said acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase is expressed on an episomal vector.
56. The method of claim 54 or 55, wherein said gene encoding said acetolactate synthase is 5' in relation to any other heterologous gene.
57. The method of any one of claims 54 to 56, wherein said gene encoding said acetoin reductase is 3' in relation to any other heterologous gene.
58. The method of any one of claims 54 to 57, wherein said gene encoding said alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other heterologous gene.
59. The method of any one of claims 42 to 58, wherein one or more of said gene encoding an acetoin reductase, an alpha-acetolactate decarboxylase, and/or an acetolactate synthase are under the control of a switch.
60. The method of claim 59, wherein said switch is an arabinose switch.
61. The method of claim 59, wherein said switch is under the control of a rare earth metal switch.
62. The method of any one of claims 42 to 61, wherein said acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase are heterologous to said genetically modified microorganism.
63. A method of making 2,3-BDO comprising:
- a) contacting a genetically modified microorganism with a C1 carbon, wherein said microorganism comprises at least one heterologous gene encoding:
 - i. an acetoin reductase;
 - ii. an alpha-acetolactate decarboxylase ;
 - iii. an AlsS; or
 - iv. any combination thereof; and
 - b) growing said microorganism to produce 2,3-BDO.

64. The method of claim 63, wherein said heterologous gene encoding for said acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase is integrated into the genome of said microorganism.
65. The method of claim 63 or 64, wherein said heterologous gene encoding for said acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase is expressed on an episomal vector.
66. The method of claim 64 or 65, wherein said heterologous gene encoding said acetolactate synthase is 5' in relation to any other heterologous gene.
67. The method of any one of claims 64 to 66, wherein said heterologous gene encoding said acetoin reductase is 3' in relation to any other heterologous gene.
68. The method of any one of claims 64 to 67, wherein said heterologous gene encoding said alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other heterologous gene.
69. The method of any one of claims 63 to 68, wherein said microorganism is a methanotroph.
70. The method of any one of claims 63 to 69, wherein said C1 carbon is methane.
71. The method of any one of claims 63 to 70, wherein said microorganism is grown at a temperature of between 32 °C to 49 °C.
72. The method of any one of claims 63 to 71, wherein said at least one heterologous gene is under the control of a switch.
73. The method of claim 72, wherein said switch is a rare earth metal switch.
74. The method of claim 73, wherein said rare earth metal switch is a lanthanum switch.
75. The method of any one of claims 63 to 74, further comprising contacting said microorganism with media containing at least 1 μM lanthanum before growing said microorganism to produce 2,3-BDO.
76. The method of claim 75, further comprising diluting said lanthanum in media before growing said microorganism to produce 2,3-BDO.
77. A method of making acetoin comprising:
- a) contacting a genetically modified microorganism with a C1 carbon, wherein said microorganism comprises a heterologous gene encoding alpha-acetolactate decarboxylase; and
 - b) growing said microorganism to produce acetoin.
78. The method of claim 77, wherein said heterologous gene encoding for said alpha-acetolactate decarboxylase is integrated into the genome of said microorganism.

79. The method of claim 77 or 78, wherein said heterologous gene encoding for said alpha-acetolactate decarboxylase is expressed on an episomal vector.
80. The method of any one of claims 77 to 79, wherein said microorganism is a methanotroph.
81. The method of claim 77 or 80, wherein said C1 carbon is methane.
82. The method of any one of claims 77 to 81, wherein said microorganism is grown at a temperature of between 32 °C to 49 °C.
83. The method of any one of claims 77 to 82, wherein said heterologous gene encoding alpha-acetolactate decarboxylase is under the control of a switch.
84. The method of claim 83, wherein said switch is a rare earth metal switch.
85. The method of claim 84, wherein said rare earth metal is lanthanum, cerium, praseodymium, and/or neodymium.
86. The method of claim 85, wherein said rare earth metal switch is a lanthanum switch.
87. The method of claim 86, further comprising contacting said microorganism with media containing at least 1µM lanthanum before growing said microorganism to produce acetoin.
88. The method of claim 87, further comprising diluting said lanthanum in media before growing said microorganism to produce acetoin.
89. A method of making butadiene comprising:
- a) contacting a genetically modified microorganism with a C1 carbon substrate, wherein said microorganism comprises at least one heterologous gene encoding for:
 - i. an NADPH-dependent acetoin reductase;
 - ii. an alpha-acetolactate decarboxylase (budA);
 - iii. an AlsS; or
 - iv. any combination thereof; and
 - b) growing said microorganism to produce 2,3-BDO; and
 - c) contacting said 2,3-BDO from (b) with a catalyst to produce butadiene.
90. The method of claim 89, wherein said heterologous gene encoding for said acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase is integrated by an integration vector into the genome of said microorganism.
91. The method of claim 89 or 90, wherein said heterologous gene encoding for said acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase is expressed on an episomal vector.

92. The method of claim 90 or 91, wherein said heterologous gene encoding said acetolactate synthase is 5' in relation to any other heterologous gene.
93. The method of any one of claims 90 to 92, wherein said heterologous gene encoding said acetoin reductase is 3' in relation to any other heterologous gene.
94. The method of any one of claims 90 to 93, wherein said heterologous gene encoding said alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other heterologous gene.
95. The method of any one of claims 89 to 94, wherein said microorganism is a methanotroph.
96. The method of claim 89 or 95, wherein said C1 carbon is methane.
97. The method of any one of claims 89 to 96, wherein said microorganism is grown at a temperature of between 32 °C to 49 °C.
98. The method of any one of claims 89 to 97, wherein said one or more heterologous genes encoding for acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase are under the control of a switch.
99. The method of claim 98, wherein said switch is a rare earth metal switch.
100. The method of claim 99, wherein said rare earth metal switch is a lanthanum switch.
101. The method of any one of claims 89 to 100, further comprising contacting said microorganism with media containing at least 1 μM lanthanum before growing said microorganism to produce 2,3-BDO.
102. The method of claim 101, further comprising diluting said lanthanum in media before growing said microorganism to produce 2,3-BDO.
103. The method of any one of claims 89 to 102, wherein said 2,3-BDO from (b) is isolated prior to (c).
104. The method of any one of claims 89 to 103, wherein said catalyst is capable of dehydrating 2,3-BDO.
105. The method of any one of claims 89 to 104, wherein said catalyst is a SiO₂-supported cesium dihydrogen phosphate (CsH₂PO₄) catalyst.
106. A method of making methyl ethyl ketone (MEK) comprising:
- a) contacting a genetically modified microorganism with a C1 carbon substrate, wherein said microorganism comprises at least one heterologous gene encoding for:
 - i. an NADPH-dependent acetoin reductase;
 - ii. an alpha-acetolactate decarboxylase (budA);
 - iii. an AlsS; or

iv. any combination thereof;

b) growing said microorganism to produce 2,3-BDO; and

c) contacting said 2,3-BDO from (b) with a catalyst to produce methyl ethyl ketone.

107. The method of claim 106, wherein said heterologous gene encoding for said acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase is integrated by an integration vector into the genome of said microorganism.

108. The method of claim 106 or 107, wherein said heterologous gene encoding for said acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase is expressed on an episomal vector.

109. The method of claim 107 or 108, wherein said heterologous gene encoding said acetolactate synthase is 5' in relation to any other heterologous gene.

110. The method of any one of claims 107 to 109, wherein said heterologous gene encoding said acetoin reductase is 3' in relation to any other heterologous gene.

111. The method of any one of claims 107 to 110, wherein said heterologous gene encoding said alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other heterologous gene.

112. The method of any one of claims 106 to 111, wherein said microorganism is grown at a temperature of between 32 °C to 49 °C.

113. The method of any one of claims 106 to 112, wherein said heterologous gene(s) encoding acetoin reductase, alpha-acetolactate decarboxylase, and/or acetolactate synthase are under the control of a switch.

114. The method of claim 113, wherein said switch is a rare earth metal switch.

115. The method of claim 113 or 114, wherein said rare earth metal is lanthanum.

116. The method of any one of claims 106 to 115, further comprising contacting said microorganism with media containing at least 1µM lanthanum before growing said microorganism to produce 2,3-BDO.

117. The method of any one of claims 106 to 116, further comprising diluting said lanthanum in media before growing said microorganism to produce 2,3-BDO.

118. The method of any one of claims 106 to 117, wherein said 2,3-BDO from (b) is isolated prior to (c).

119. The method of any one of claims 106 to 118, wherein said catalyst is a solid acid catalyst.

120. An isolated polynucleic acid comprising a nucleotide sequence that is at least 84% identical to SEQ ID NO: 2.

121. An isolated polynucleic acid comprising a nucleotide sequence that is at least 88% identical to SEQ ID NO: 4.
122. An isolated polynucleic acid comprising a nucleotide sequence that is at least 85% identical to SEQ ID NO: 6.
123. An isolated polynucleic acid comprising a nucleotide sequence that is at least 85% identical to SEQ ID NO: 8.
124. An isolated polynucleic acid comprising a nucleotide sequence that is at least 85% identical to SEQ ID NO: 10.
125. An isolated polynucleic acid comprising a nucleotide sequence that is at least 85% identical to SEQ ID NO: 12.
126. An isolated polynucleic acid comprising a nucleotide sequence that is at least 85% identical to SEQ ID NO: 14.
127. An isolated polynucleic acid comprising a nucleotide sequence that is at least 60% identical to SEQ ID NO: 20.
128. A genetically modified microorganism capable of converting a C1 carbon to 2,3-BDO, comprising a heterologous gene encoding an acetoin reductase.
129. The genetically modified microorganism of claim 128, further comprising a heterologous gene encoding an alpha-acetolactate decarboxylase and/or a heterologous gene encoding an acetolactate synthase.
130. The genetically modified microorganism of claim 128 or 129, wherein said heterologous gene encoding for said acetoin reductase, heterologous gene encoding for said alpha-acetolactate decarboxylase, and/or heterologous gene encoding for said acetolactate synthase is integrated by an integration vector into the genome of said microorganism.
131. The genetically modified microorganism of any one of claims 128 to 130, wherein said heterologous gene encoding for said acetoin reductase, heterologous gene encoding for said alpha-acetolactate decarboxylase, and/or heterologous gene encoding for said acetolactate synthase is expressed on an episomal vector.
132. The genetically modified microorganism of claim 130 or 131, wherein said heterologous gene encoding said acetolactate synthase is 5' in relation to any other heterologous gene.
133. The genetically modified microorganism of any one of claims 130 to 132, wherein said heterologous gene encoding said acetoin reductase is 3' in relation to any other heterologous gene.

134. The genetically modified microorganism of any one of claims 130 to 133, wherein said heterologous gene encoding said alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other heterologous gene.
135. The genetically modified microorganism of any one of claims 128 to 134, wherein at least one of said heterologous gene(s) is under the control of a switch.
136. The genetically modified microorganism of claim 135, wherein said switch is an inducible switch.
137. The genetically modified microorganism of claim 136, wherein said inducible switch is an arabinose switch.
138. The genetically modified microorganism of claim 135, wherein said switch is a repressible switch.
139. The genetically modified microorganism of claim 138, wherein said repressible switch is a rare earth metal switch.
140. A vector comprising one or more genes encoding an acetoin reductase, an alpha-acetolactate decarboxylase, and/or an acetolactate synthase, wherein said one or more genes is under the control of a switch.
141. The vector of claim 140, wherein said gene encoding said acetolactate synthase is 5' in relation to any other gene on said vector.
142. The vector of claim 140 or 141, wherein said gene encoding said acetoin reductase is 3' in relation to any other gene on said vector.
143. The vector of any one of claims 140 to 142, wherein said gene encoding said alpha-acetolactate decarboxylase is neither 5' or 3' in relation to any other gene on said vector.
144. The vector of any one of claims 140 to 143, wherein said switch is an inducible switch or repressible switch.
145. The vector of claim 144, comprising an inducible switch, wherein said inducible switch is an arabinose switch.
146. The vector of claim 144, comprising a repressible switch, wherein said repressible switch is a rare-earth metal switch.