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(54) Title: RECOMBINANT MICROORGANISMS EXHIBITING INCREASED FLUX THROUGH A FERMENTATION PATHWAY

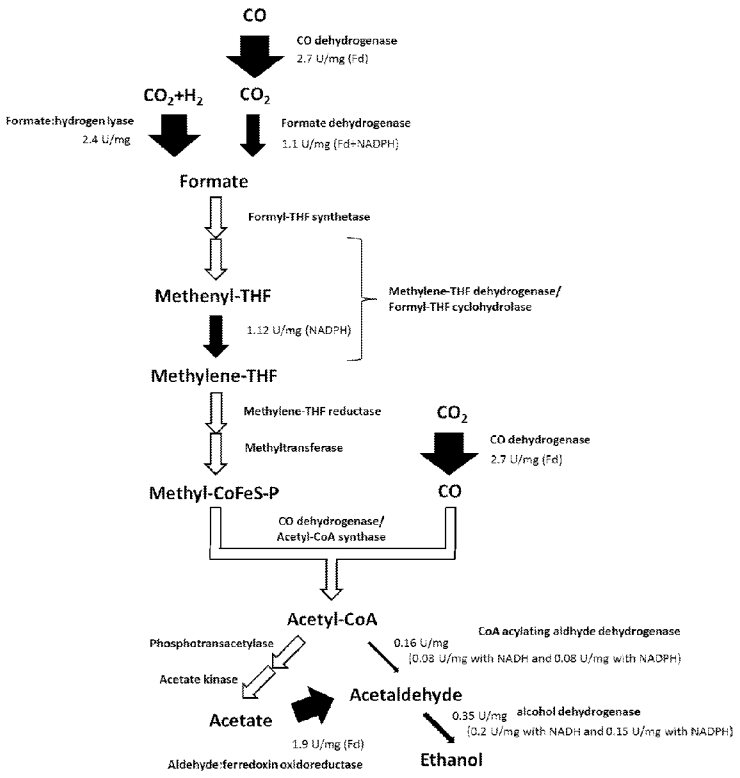


FIG. 1

(57) Abstract: The invention provides a recombinant, carboxydotrophic *Clostridium* bacterium that expresses one or more of pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1), acetolactate synthase (EC 2.2.1.6), and acetolactate decarboxylase (EC 4.1.1.5). The invention further provides a method of producing a fermentation product by fermenting the recombinant bacterium in the presence of a gaseous substrate comprising CO to produce one or more of ethanol, butanol, isopropanol, isobutanol, higher alcohols, butanediol, 2,3-butanediol, succinate, isoprenoids, fatty acids, biopolymers, and mixtures thereof.

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RECOMBINANT MICROORGANISMS EXHIBITING INCREASED FLUX THROUGH A FERMENTATION PATHWAY

CROSS REFERENCE TO RELATED APPLICATIONS

0001 This application claims the benefit of U.S. Patent Application 62/089,053 filed December 8, 2014 and U.S. Patent Application 62/168,969 filed June 1, 2015, the entirety of which are incorporated herein by reference.

BACKGROUND

0002 Carboxydotrophic microorganisms may be engineered to produce products, such as fuels and chemicals, through fermentation of a gaseous substrate. Efforts to improve product concentration and substrate utilization have historically focused on strain selection and optimization of fermentation conditions (Abubackar, *Bioresour Technol*, 114: 518-522, 2012). The metabolism of natural microorganisms, however, did not evolve to achieve commercial objectives of high yields, rates, and titers, such that certain commercial objectives cannot be achieved through mere strain selection and optimization of fermentation conditions. Accordingly, there remains a need for improved microorganisms and methods for the production of useful products, such as fuels and chemicals.

SUMMARY OF THE INVENTION

0003 The invention provides a recombinant, carboxydotrophic *Clostridium* bacterium comprising one or more enzymes selected from the group consisting of pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1), acetolactate synthase (EC 2.2.1.6), and acetolactate decarboxylase (EC 4.1.1.5), wherein each enzyme is an overexpressed endogenous enzyme, a mutated endogenous enzyme, or an exogenous enzyme. The recombinant bacterium may express one, two, or all three of these enzymes.

0004 The recombinant bacterium may be derived from any *Clostridium* microorganism. In one embodiment, the recombinant bacterium is derived from *C. autoethanogenum*, *C. ljungdahlii,* or *C. ragsdalei.* In a preferred embodiment, the recombinant bacterium is derived from *C. autoethanogenum* deposited under DSMZ Accession No. DSM23693 (*C. autoethanogenum* LZ1561).

0005 The invention further provides a method of producing a fermentation product, comprising fermenting the recombinant bacterium in the presence of a gaseous substrate comprising CO to produce one or more of ethanol, butanol, isopropanol, isobutanol, higher alcohols, butanediol, 2,3-butanediol, succinate, isoprenoids, fatty acids, biopolymers, and mixtures thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

0006 Fig. 1 is a flux map of the ethanol biosynthesis pathway detailing the measured enzyme activities and flux through a carboxydotrophic microorganism for ethanol formation via acetyl-CoA, which allows for the identification of rate-limiting pathway reactions. The thickness of the arrows is proportional to the activity of the particular pathway reaction.

0007 Fig. 2 is a flux map detailing the measured enzyme activities and flux through a carboxydotrophic microorganism for 2,3-butanediol formation via pyruvate, which allows for the identification of rate-limiting pathway reactions.

0008 Fig. 3 is a diagram showing the 2,3-butanediol pathway and the associated branched-chain amino acid biosynthesis pathway. Pyruvate is converted to α-acetolactate, the intermediate for both the 2,3-butanediol and the branched-chain amino acid biosynthesis pathways. Experiments were performed to overexpress *PFOR*, *alsS* and *alsD*.

0009 Fig. 4 is a schematic representation of the pMTL83159 plasmid. The plasmid contains a Gram negative origin of replication (*ColE1*) gene, a Gram positive origin of replication (*repH*) gene, the transfer gene *traJ*, the *catP* gene encoding for the chloramphenicol/thiamphenicol resistance, a multiple cloning site locating within lacZ alpha coding sequence and, a ferredoxin gene promoter (P_{fdx}).

0010 Fig. 5 is a set of graphs showing of the growth and metabolite profiles (biomass, 2,3-butanediol (BDO), acetic acid, and ethanol) versus time of five strains grown in Schott bottles.

0011 Fig. 6 is a set of graphs showing the metabolite profile (top graphs) and gas profile (bottom graphs) of the combined PFOR, alsS and alsD overexpression strain and the plasmid control strain at a 4 mol/L/d CO uptake over the course of 20 days.

0012 Fig. 7 is a set of graphs showing the metabolite and the gas profiles of the overexpression culture at an 8 mol/L/d CO uptake over the course of 11 days.

0013 Fig. 8 is a set of graphs showing biomass and butanediol production of cultures expressing *A. hydrophila alsD* (open squares), *L. lactis alsD* (closed squares), and empty plasmid control (triangles). Values are the average of three replicates and error bars represent one standard deviation.

DETAILED DESCRIPTION OF THE INVENTION

0014 A fermentation pathway is a cascade of biochemical reactions (pathway reactions) by which a substrate, preferably a gaseous substrate, is converted to a fermentation product. Pathway reactions typically involve enzymes that catalyse or increase the rate of the pathway reaction.

0015 "Flux" refers to the flow of metabolites through one or more reactions in a fermentation pathway. The flux through individual pathway reactions has an upper and lower limit. Therefore, the flux may be changed by adjusting conditions or factors that affect enzymatic activity. Adjustment of the flux through one pathway reaction may alter the overall flux of the fermentation pathway. Flux may be measured according to any method known in the art. By way of example, flux may be measured using flux-balance analysis (FBA) (Gianchandani, *Systems Biol Medicine*, 2: 372-382, 2010). Flux through the pathway may also be measured by the level of metabolites and products (metabolomics) (Patti, *Nat Rev Molec Cell Biol*, 13: 263-269, 2012) and/or labelling experiments as C13 (fluxomics) (Niittylae, *Methods Mol Biol*, 553: 355-372, 2009; Tang, *Mass Spectrom Rev*, 28:362-375, 2009).

0016 The efficiency of a fermentation pathway can be increased by increasing the reaction flux through the pathway. The increased flux results in one or more of: an increased rate of growth of microorganisms performing the fermentation, an increased rate of growth and/or product production rate at elevated product concentrations, an increased fermentation product concentration in the fermentation broth, an increased volume of fermentation product produced per volume of substrate consumed, an increased rate of production or level of

production of the fermentation product. Preferably, the increased efficiency results in an increased fermentation product production rate.

0017 One method to identify rate limiting reactions (bottlenecks) is to measure enzyme activities for all reactions involved in the fermentation pathway from substrate to product. This can be done by analysing the enzymatic activity of reactions in cells growing under process conditions to identify the reactions with the lowest rates. These can then be adjusted so as not to be rate limiting, thus increasing the flux throughout the system. Enzymatic activity may be measured by any method known in the art, such as the methods described in Huang, *J Bacteriol*, 194: 3689-3699, 2012.

0018 The inventors have analysed the activity of enzymes involved in fermentation pathways and found that some pathway reactions exhibit substantially lower enzymatic activity than other reactions in the same pathway. The recombinant microorganisms and methods described herein have particular utility for pathways where the product yield in a parental microorganism may lack the product yield to be a viable commercial target.

0019 Examples of fermentation pathways that are amenable to analysis of enzyme activity include the Wood-Ljungdahl pathway, fermentation pathways to produce ethanol, 2,3-butanediol or a precursor thereof such as acetyl-CoA and pyruvate, and biosynthesis pathways for cofactors tetrahydrofolate and cobalamine (B_{12}) which may be required in fermentation pathways. The Wood-Ljungdahl pathway is composed of a number of reactions catalysed by enzymes, as described in Fig. 1 and Fig. 2. The steps subsequent to the Wood-Ljungdahl pathway which lead to the production of desirable fermentation products are also considered to be part of the fermentation pathway. In a particular embodiment, the fermentation pathway results in the production of a fermentation product selected from the group consisting of ethanol, butanol, isopropanol, isobutanol, higher alcohols, butanediol, 2,3-butanediol, succinate, isoprenoids, fatty acids, biopolymers, and mixtures thereof.

0020 The invention provides a recombinant, carboxydotrophic *Clostridium* bacterium comprising one or more enzymes selected from the group consisting of pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1), acetolactate synthase (EC 2.2.1.6), and acetolactate decarboxylase (EC 4.1.1.5), wherein each enzyme is an overexpressed endogenous enzyme, a mutated endogenous enzyme, or an exogenous enzyme.

0021 A "parental microorganism" is a microorganism used to generate a recombinant microorganism of the invention. The parental microorganism may be one that occurs in nature (i.e., a wild type microorganism) or one that has been previously modified (i.e. a recombinant microorganism). The recombinant microorganisms of the invention may be modified to express or overexpress one or more enzymes that were or were not expressed or overexpressed in the parental microorganism, or may be modified to exhibit increased availability of one or more co-factors. In one embodiment, the parental organism may be *C. autoethanogenum*, *C. ljungdahlii*, or *C. ragsdalei*. In a particularly preferred embodiment, the parental organism is *C. autoethanogenum* LZ1561, which is deposited under DSMZ accession DSM23693.

0022 A "recombinant microorganism" is a microorganism that has undergone genetic modification when compared to a parental microorganism. Genetic modification includes insertion, deletion, or substitution of nucleic acids, for example.

0023 In general, the term "derived from" indicates that a nucleic acid, protein, or microorganism is modified or adapted from a different (i.e., a parental or wild-type) nucleic acid, protein, or microorganism, respectively, so as to produce a new recombinant microorganism.

0024 Methods of genetic modification of a parental microorganism include molecular methods such as heterologous gene expression, genome insertion or deletion, altered gene expression or inactivation of genes, or enzyme engineering methods. Such techniques are described, for example, in Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001; Pleiss, *Curr Opin Biotechnol*, 22: 611-617, 2011; Park, Protein Engineering and Design, CRC Press, 2010. Expression constructs/vectors may contain, for example, one or more promoters or ribosomal binding sites. Nucleic acids and construct/vector sequences described herein may also contain standard linker nucleotides such as those required for ribosome binding sites and/or restriction sites.

0025 Nucleic acids and nucleic acid constructs, including expression constructs/vectors of the invention may be constructed using any method known in the art. For example, chemical synthesis or recombinant techniques may be used. Such techniques are described, for

example, in Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989. Essentially, the individual genes and regulatory elements may be operably linked to one another such that the genes can be expressed to form the desired proteins. Suitable vectors will be appreciated by those of ordinary skill in the art. However, by way of example, the following vectors may be suitable: pMTL80000 vectors, pIMP1, pJIR750, and other plasmids.

0026 Nucleic acids may be delivered to a microorganism using any method known in the art. For example, nucleic acids may be delivered to a microorganism as naked nucleic acids or may be formulated with one or more agents (e.g., liposomes) to facilitate the transformation process to the microorganism. The nucleic acids may be DNA, RNA, cDNA, or combinations thereof, as is appropriate. Restriction inhibitors may be used in certain embodiments (Murray, *Microbiol Molec Biol Rev*, 64: 412-434, 2000). Additional vectors include include plasmids, viruses (including bacteriophage), cosmids, and artificial chromosomes. In a preferred embodiment, nucleic acids are delivered to a microorganism using a plasmid. By way of example only, transformation (including transduction or transfection) may be achieved by electroporation, ultrasonication, polyethylene glycol-mediated transformation, chemical or natural competence, protoplast transformation, prophage induction or conjugation. Suitable transformation techniques are described for example in, Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

0027 The use of electroporation has been reported for several carboxydotrophic acetogens, including *C. ljungdahlii* (Koepke, *PNAS*, 107:13087-13092, 2010; WO/2012/053905), *C. autoethanogenum* (WO/2012/053905), *C. aceticum* (Schiel-Bengelsdorf, *Synthetic Biol*, 15: 2191-2198, 2012), and *A. woodii* (Strätz, *Appl Environ Microbiol*, 60: 1033-1037, 1994). The use of electroporation has also been reported in *Clostridia*, including *C. acetobutylicum* (Mermelstein, *Biotechnol*, 10: 190-195, 1992), and *C. cellulolyticum* (Jennert, *Microbiol*, 146: 3071-3080, 2000). Additionally, prophage induction has been demonstrated for carboxydotrophic acetogens, including *C. scatologenes* (Parthasarathy, Development of a Genetic Modification System in *Clostridium scatologenes* ATCC 25775 for Generation of Mutants, Masters Project, Western Kentucky University, 2010), and conjugation has been described for many *Clostridia*, including *C. difficile* (Herbert, *FEMS Microbiol Lett*, 229:

103-110, 2003) and *C. acetobuylicum* (Williams, *J Gen Microbiol*, 136: 819-826, 1990). Similar methods could be used in carboxydotrophic acetogens.

0028 The invention provides a recombinant carboxydotrophic *Clostridium* bacterium adapted to exhibit increased flux through a fermentation pathway relative to a parental microorganism. In one particular embodiment of the invention, the parental microorganism is selected from the group of carboxydotrophic *Clostridia* comprising *C. autoethanogenum, C. ljungdahlii, C. ragsdalei, C. carboxidivorans, C. drakei, C. scatologenes, C. aceticum, C. formicoaceticum,* and *C. magnum.*

0029 The recombinant bacterium may be derived from the cluster of carboxydotrophic *Clostridia* comprising the species *C. autoethanogenum*, *C. ljungdahlii*, *C. ragsdalei,* and related isolates. These include but are not limited to strains *C. autoethanogenum* JAI-1T (DSM10061) (Abrini, *Arch Microbiol*, 161: 345-351, 1994), *C. autoethanogenum* LBS1560 (DSM19630) (WO 2009/064200), *C. autoethanogenum* LZ1561 (DSM23693), *C. ljungdahlii* PETCT (DSM13528 = ATCC 55383) (Tanner, *Int J Syst Bacteriol*, 43: 232-236, 1993), *C. ljungdahlii* ERI-2 (ATCC 55380) (U.S. Patent 5,593,886), *C. ljungdahlii* C-01 (ATCC 55988) (U.S. Patent 6,368,819), *C. ljungdahlii* O-52 (ATCC 55989) (U.S. Patent 6,368,819), *C. ragsdalei* P11T (ATCC BAA-622) (WO 2008/028055), related isolates such as "*C. coskatii*" (U.S. Publication 2011/0229947), or mutated strains such as *C. ljungdahlii* OTA-1 (Tirado-Acevedo, Production of Bioethanol from Synthesis Gas Using *Clostridium ljungdahlii*, PhD thesis, North Carolina State University, 2010). These strains form a subcluster within the *Clostridial* rRNA cluster I, and their 16S rRNA gene is more than 99% identical with a similar low GC content of around 30%. However, DNA-DNA reassociation and DNA fingerprinting experiments showed that these strains belong to distinct species (WO 2008/028055). The strains of this cluster are defined by common characteristics, having both a similar genotype and phenotype, and they all share the same mode of energy conservation and fermentative metabolism. The strains of this cluster lack cytochromes and conserve energy via an Rnf complex.

0030 All species of the above-referenced cluster have a similar morphology and size (logarithmic growing cells are between 0.5-0.7 x 3-5 μm), are mesophilic (optimal growth temperature between 30-37 °C), and are strictly anaerobic (Abrini, *Arch Microbiol*, 161: 345-

351, 1994; Tanner, *Int J Syst Bacteriol*, 43: 232-236, 1993; and WO 2008/028055). Moreover, they all share the same major phylogenetic traits, such as same pH range (pH 4-7.5, with an optimal initial pH of 5.5-6), strong autotrophic growth on CO-containing gases with similar growth rates, and a similar metabolic profile with ethanol and acetic acid as main fermentation end products, and small amounts of 2,3-butanediol and lactic acid formed under certain conditions (Abrini, *Arch Microbiol*, 161: 345-351, 1994; Köpke, *Curr Opin Biotechnol*, 22: 320-325, 2011; Tanner, *Int J Syst Bacteriol*, 43: 232-236, 1993; and WO 2008/028055). Indole production was observed with all three species as well. However, the species differentiate in substrate utilization of various sugars (e.g., rhamnose, arabinose), acids (e.g., gluconate, citrate), amino acids (e.g., arginine, histidine), or other substrates (e.g., betaine, butanol). Moreover some of the species were found to be auxotrophic to certain vitamins (e.g., thiamine, biotin) while others were not. The organization and number of Wood-Ljungdahl pathway genes, responsible for gas uptake, has been found to be the same in all species, despite differences in nucleic and amino acid sequences (Köpke, *Curr Opin Biotechnol*, 22: 320-325, 2011). Also, reduction of carboxylic acids into their corresponding alcohols has been shown in a range of these microorganisms (Perez, *Biotechnol Bioeng*, 110:1066-1077, 2012). These traits are therefore not specific to one organism like *C. autoethanogenum* or *C. ljungdahlii*, but rather general traits for carboxydotrophic, ethanol-synthesizing *Clostridia* and it can be anticipated that mechanisms work similarly across these strains, although there may be differences in performance.

0031 In one embodiment, the parental microorganism is *C. autoethanogenum, C. ljungdahlii,* or *C. ragsdalei.* Preferably, the parental microorganism is wild-type *C. autoethanogenum* or *C. autoethanogenum* deposited under DSMZ accession number DSM10061 or DSM23693 (*C. autoethanogenum* LZ1561). In one embodiment, the recombinant bacterium is derived from *C. autoethanogenum, C. ljungdahlii,* or *C. ragsdalei*. Preferably, the recombinant bacterium is derived from wild-type *C. autoethanogenum* or *C. autoethanogenum* deposited under DSMZ accession number DSM23693 (*C. autoethanogenum* LZ1561).

0032 The enzymes and genes of the invention may be overexpressed endogenous enzymes and genes, mutated endogenous enzymes and genes, or exogenous enzymes and genes.

0033 "Endogenous" refers to a nucleic acid or protein that is present in the wild-type or parental bacterium from which the recombinant bacterium of the invention is derived. In one embodiment, the expression of an endogenous gene may be controlled by an exogenous regulatory element, such as an exogenous promoter.

0034 "Exogenous" refers to a nucleic acid or protein that is not present in the wild-type or parental bacterium from which the recombinant bacterium of the invention is derived. In one embodiment, an exogenous gene or enzyme may be derived from a heterologous strain or species and introduced to or expressed in the recombinant bacterium. In another embodiment, an exogenous gene or enzyme may be artificially or recombinantly created. Exogenous nucleic acids may be adapted to integrate into the genome of the bacterium or to remain in an extra-chromosomal state in the bacterium, for example, in a plasmid.

0035 "Enzyme activity" refers broadly to enzymatic activity, including, but not limited, to the activity of an enzyme, the amount of an enzyme, or the availability of an enzyme to catalyse a reaction. Accordingly, "increasing" enzyme activity includes an increase in the activity of an enzyme, an increase in the amount of an enzyme, or an increase in the availability of an enzyme to catalyse a reaction.

0036 The genes and enzymes of the invention may be developed or engineered using any method known in the art, including, for example, directed evolution, knowledge-based design, random mutagenesis methods, gene shuffling, codon optimization, use of site-specific libraries, and use of site evaluation libraries.

0037 "Mutated" refers to a nucleic acid or protein that has been modified in the recombinant bacterium of the invention compared to the wild-type or parental bacterium from which the recombinant bacterium of the invention is derived. In one embodiment, the mutation may be a deletion, insertion, or substitution in a gene encoding an enzyme. In another embodiment, the mutation may be a deletion, insertion, or substitution of one or more amino acids in an enzyme.

0038 "Codon optimization" refers to the mutation of a nucleic acid, such as a gene, for optimized or improved translation of the nucleic acid in a particular strain or species. Codon optimization may result in faster translation rates or higher translation accuracy. In a

preferred embodiment, the genes encoding the enzymes of the invention are codon optimized for expression in *Clostridium*, particularly *C. autoethanogenum*, *C. ljungdahlii*, and/or *C. ragsdalei*. In a further preferred embodiment, the genes encoding the enzymes of the invention are codon optimized for expression in *C. autoethanogenum* LZ1561.

0039 "Overexpressed" refers to any increase in expression of a nucleic acid or protein in the recombinant bacterium of the invention compared to the wild-type or parental bacterium from which the recombinant bacterium of the invention is derived. Overexpression may be achieved by any means known in the art, including modifying gene copy number, gene transcription rate, gene translation rate, or enzyme degradation rate.

0040 "Overexpressed endogenous enzyme" refers to an endogenous enzyme that is present at higher levels in the recombinant bacterium of the invention compared to the wild-type or parental bacterium from which the recombinant bacterium of the invention is derived. The overexpressed endogenous enzyme may likewise be encoded by an endogenous gene, which may be modified, for example, to be controlled by a strong or constitutive promoter. Similarly, "overexpressed endogenous gene" refers to an endogenous gene that is present or transcripted at higher rates or levels in the recombinant bacterium of the invention compared to the wild-type or parental bacterium from which the recombinant bacterium of the invention is derived.

0041 "Mutated endogenous enzyme" refers to an endogenous enzyme that is mutated or modified in the recombinant bacterium of the invention compared to the wild-type or parental bacterium from which the recombinant bacterium of the invention is derived. Similarly, "mutated endogenous gene" refers to an endogenous gene that is mutated or modified in the recombinant bacterium of the invention compared to the wild-type or parental bacterium from which the recombinant bacterium of the invention is derived.

0042 "Exogenous enzyme" refers to an enzyme that is not present in the wild-type or parental bacterium from which the recombinant bacterium of the invention is derived. Similarly, "exogenous gene" refers to a gene that is not present in the wild-type or parental bacterium from which the recombinant bacterium of the invention is derived. Typically, the exogenous enzyme or gene is derived from a heterologous strain or species and introduced to or expressed in the recombinant bacterium.

0043 The invention may be practiced using variant nucleic acids or proteins whose sequence varies from the sequences specifically exemplified herein provided they perform substantially the same function. For nucleic acid sequences that encode a protein or peptide this means that the encoded protein or peptide has substantially the same function. For nucleic acid sequences that represent promoter sequences, the variant sequence will have a similar ability to promote expression of one or more genes. Such nucleic acids or proteins may be referred to herein as "functionally equivalent variants." By way of example, functionally equivalent variants of a nucleic acid include allelic variants, fragments of a gene, genes which include mutations (deletion, insertion, nucleotide substitutions and the like) and/or polymorphisms and the like. Homologous genes from other microorganisms may also be considered examples of functionally equivalent variants of the sequences specifically exemplified herein. These include homologous genes in species such as *C. acetobutylicum, C. beijerinckii,* or *C. ljungdahlii*, the details of which are publicly available on websites such as Genbank or NCBI. Functionally equivalent variants also includes nucleic acids whose sequence varies as a result of codon optimization for a particular organism. A functionally equivalent variant of a nucleic acid will preferably have at least approximately 70%, approximately 80%, approximately 85%, approximately 90%, approximately 95%, approximately 98%, or greater nucleic acid sequence identity with the specified nucleic acid. A functionally equivalent variant of a protein will preferably have at least approximately 70%, approximately 80%, approximately 85%, approximately 90%, approximately 95%, approximately 98%, or greater amino acid identity with the specified protein. Such variants include a fragment of a protein or peptide wherein the fragment comprises a truncated form of the protein or peptide wherein deletions may be from 1 to 5, to 10, to 15, to 20, to 25 amino acids, and may extend from residue 1 through 25 at either terminus of the polypeptide, and wherein deletions may be of any length within the region or may be at an internal location. The functional equivalence of a variant nucleic acid or protein may be evaluated using any method known in the art. However, by way of example, assays to test for the activity of certain enzymes are described in Huang, *J Bacteriol*, 194: 3689-3699, 2012.

0044 In certain embodiments having active restriction enzyme systems, it may be necessary to methylate a nucleic acid before introduction of the nucleic acid into a microorganism.

0045 Generally, methylation is performed using a shuttle microorganism, preferably a restriction negative shuttle microorganism, such as *E. coli*, *B. subtillis,* or *L. lactis*, that facilitates the methylation of the nucleic acid sequences that make up the expression construct/vector. The methylation construct/vector comprises a nucleic acid sequence encoding a methyltransferase. Once the expression construct/vector and the methylation construct/vector are introduced into the shuttle microorganism, the methyltransferase gene present on the methylation construct/vector is induced. Induction may be by any suitable promoter system although in one particular embodiment of the invention, the methylation construct/vector comprises an inducible lac promoter and is induced by addition of lactose or an analogue thereof, more preferably isopropyl-β-D-thio-galactoside (IPTG). Other suitable promoters include the ara, tet, or T7 system. In a further embodiment, the methylation construct/vector promoter is a constitutive promoter.

0046 In a particular embodiment, the methylation construct/vector has an origin of replication specific to the identity of the shuttle microorganism so that any genes present on the methylation construct/vector are expressed in the shuttle microorganism. Preferably, the expression construct/vector has an origin of replication specific to the identity of the destination microorganism so that any genes present on the expression construct/vector are expressed in the destination microorganism.

0047 Expression of the methyltransferase enzyme results in methylation of the genes present on the expression construct/vector. The expression construct/vector may then be isolated from the shuttle microorganism according to any method known in the art. In one embodiment, both construct/vector are concurrently isolated. The expression construct/vector may be introduced into the destination microorganism using any method known in the art. Since the expression construct/vector is methylated, the nucleic acid sequences present on the expression construct/vector are able to be incorporated into the destination microorganism and be successfully expressed.

0048 A methyltransferase gene may be introduced into a shuttle microorganism and overexpressed. Thus, in one embodiment, the resulting methyltransferase enzyme may be collected using known methods and used *in vitro* to methylate an expression plasmid. The expression construct/vector may then be introduced into the destination microorganism for

expression. In another embodiment, the methyltransferase gene is introduced into the genome of the shuttle microorganism followed by introduction of the expression construct/vector into the shuttle microorganism, isolation of one or more constructs/vectors from the shuttle microorganism and then introduction of the expression construct/vector into the destination microorganism.

0049 The expression construct/vector and the methylation construct/vector may be combined to provide a composition of matter. Such a composition has particular utility in circumventing restriction barrier mechanisms to produce the recombinant microorganisms of the invention. In one particular embodiment, the expression construct/vector and/or the methylation construct/vector are plasmids. A number of suitable methyltransferases may be used, including, for example, *B. subtilis* phage ΦT1 methyltransferase or the methyltransferase described in WO 2012/053905. Similarly, a number of constructs/vectors adapted to allow expression of a methyltransferase gene may be used to generate the methylation construct/vector.

0050 By way of example, in one embodiment, a recombinant microorganism of the invention may be produced by a method comprising (a) introduction into a shuttle microorganism of (i) of an expression construct/vector comprising a nucleic acid as described herein and (ii) a methylation construct/vector comprising a methyltransferase gene; and (b) expression of the methyltransferase gene; isolation of one or more constructs/vectors from the shuttle microorganism; and introduction of the one or more construct/vector into a destination microorganism. In one embodiment, the methyltransferase gene of step (b) is expressed constitutively. In another embodiment, expression of the methyltransferase gene of step (b) is induced.

0051 The recombinant bacterium of the invention comprises one or more of pyruvate:ferredoxin oxidoreductase, acetolactate synthase, and acetolactate decarboxylase.

0052 Pyruvate:ferredoxin oxidoreductase (PFOR or POR) (EC 1.2.7.1) is an enzyme belonging to a family of oxidoreductases that catalyses the transfer of electrons from one molecule (the reductant, or electron donor) to another (the oxidant, or electron acceptor). Specifically, pyruvate:ferredoxin oxidoreductase catalyses the interconversion of pyruvate and acetyl-CoA: pyruvate + CoA + 2 oxidized ferredoxin ↔ acetyl-CoA + CO_2 + 2 reduced

ferredoxin + 2 H^+. Conversion of acetyl-CoA to pyruvate links the Wood-Ljungdahl pathway of autotrophic CO(2) fixation to the reductive tricarboxylic acid cycle, which in autotrophic anaerobes is the stage for biosynthesis of all cellular macromolecules (Furdi, *J Biol Chem*, 15: 28494-28499, 2000). Pyruvate:ferredoxin oxidoreductase may also be known as pyruvate:ferredoxin 2-oxidoreductase (CoA-acetylating), pyruvate oxidoreductase, pyruvate synthase, pyruvate synthetase, or pyruvic-ferredoxin oxidoreductase.

0053 The pyruvate:ferredoxin oxidoreductase enzyme of the invention may be an overexpressed endogenous enzyme, a mutated endogenous enzyme, or an exogenous enzyme. Similarly, the pyruvate:ferredoxin oxidoreductase enzyme of the invention may be encoded by an endogenous pyruvate:ferredoxin oxidoreductase gene that has been engineered for overexpression, may be encoded by a mutated endogenous pyruvate:ferredoxin oxidoreductase gene, or may be encoded by an exogenous pyruvate:ferredoxin oxidoreductase gene. In a preferred embodiment, the pyruvate:ferredoxin oxidoreductase enzyme is overexpressed endogenous pyruvate:ferredoxin oxidoreductase, such as overexpressed endogenous *C. autoethanogenum, C. ljungdahlii,* or *C. ragsdalei* pyruvate:ferredoxin oxidoreductase. Pyruvate:ferredoxin oxidoreductase enzymes are often unstable in the presence of oxygen. In a preferred embodiment, the pyruvate:ferredoxin oxidoreductase enzyme is oxygen stable or demonstrates at least some degree of oxygen insensitivity. In a further preferred embodiment, the pyruvate:ferredoxin oxidoreductase enzyme is exogenous *Desulfovibrio africanus* pyruvate:ferredoxin oxidoreductase, or an enzyme derived therefrom. Expression of *D. africanus* pyruvate:ferredoxin oxidoreductase has been demonstrated in *E. coli* (Pieulle, *J Bacteriol*, 179: 5684-5692, 1997), but not in a *Clostridium* microorganism.

0054 Acetolactate synthase (Als) (EC 2.2.1.6) is an enzyme that catalyses the first step in the synthesis of branched-chain amino acids, such as valine, leucine, and isoleucine. In particular, acetolactate synthase is a transketolase that has both catabolic and anabolic forms, and catalyses the conversion of two pyruvate molecules to an acetolactate molecule and carbon dioxide: 2 $CH_3COCOO^- \leftrightarrow CH_3COCOHCH_3COO^- + CO_2$. Acetolactate synthase may also be known as acetohydroxy acid synthase.

0055 The acetolactate synthase enzyme of the invention may be an overexpressed endogenous enzyme, a mutated endogenous enzyme, or an exogenous enzyme. Similarly, the acetolactate synthase enzyme of the invention may be encoded by an endogenous acetolactate synthase gene that has been engineered for overexpression, may be encoded by a mutated endogenous acetolactate synthase gene, or may be encoded by an exogenous acetolactate synthase gene. The acetolactate synthase may be anabolic or catabolic. In a preferred embodiment, the acetolactate synthase enzyme is overexpressed endogenous acetolactate synthase, such as overexpressed endogenous *C. autoethanogenum, C. ljungdahlii,* or *C. ragsdalei* acetolactate synthase. In particular, the acetolactate synthase enzyme may be overexpressed endogenous IlvB, ILvB ORF2059, IlvB ORF2336, IlvC, IlvN, IlvBN, or AlsS acetolactate synthase. In a preferred embodiment, the acetolactate synthase enzyme is mutated endogenous acetolactate synthase, such as mutated acetolactate synthase derived from any endogenous *C. autoethanogenum, C. ljungdahlii,* or *C. ragsdalei* acetolactate synthase. In particular, the mutated endogenous acetolactate synthase may be feedback-insensitive IlvN acetolactate synthase. In a preferred embodiment, the acetolactate synthase enzyme is exogenous acetolactate synthase, such as *Bacillus subtilis* acetolactate synthesis, particularly feedback-insensitive *B. subtilis* AlsS acetolactate synthase. The expression of *B. subtilis* AlsS has been shown in *Synechococcus elongatus* sp. strain PCC 7942 (Oliver, *Metabol Eng*, 22: 76-82, 2014), but not in a *Clostridium* microorganism.

0056 Acetolactate decarboxylase (EC 4.1.1.5) is an enzyme belonging to a family of lyases, specifically the carboxy-lyases, which cleave carbon-carbon bonds. Acetolactate decarboxylase catalyses the reaction of (S)-2-hydroxy-2-methyl-3-oxobutanoate to (R)-2-acetoin and CO_2: (S)-2-hydroxy-2-methyl-3-oxobutanoate ↔ (R)-2-acetoin + CO_2. Acetolactate decarboxylase may also be known as alpha-acetolactate decarboxylase or (S)-2-hydroxy-2-methyl-3-oxobutanoate carboxy-lyase.

0057 The acetolactate decarboxylase enzyme of the invention may be an overexpressed endogenous enzyme, a mutated endogenous enzyme, or an exogenous enzyme. Similarly, the acetolactate decarboxylase enzyme of the invention may be encoded by an endogenous acetolactate decarboxylase gene that has been engineered for overexpression, may be encoded by a mutated endogenous acetolactate decarboxylase gene, or may be encoded by an exogenous acetolactate decarboxylase gene. In a preferred embodiment, the acetolactate

decarboxylase enzyme is overexpressed endogenous acetolactate decarboxylase, such as overexpressed endogenous *C. autoethanogenum, C. ljungdahlii,* or *C. ragsdalei* acetolactate decarboxylase. The overexpressed endogenous acetolactate decarboxylase may be BudA acetolactate decarboxylase or AlsD acetolactate decarboxylase. In a preferred embodiment, the acetolactate decarboxylase enzyme is exogenous acetolactate decarboxylase, such as *Aeromonas hydrophila* acetolactate decarboxylase or *Leuconostoc lactis* acetolactate decarboxylase. The expression of *B. subtilis* AlsD has been shown in *Synechococcus elongatus* sp. strain PCC 7942 (Oliver, *Metabol Eng*, 22: 76-82, 2014), but not in a *Clostridium* microorganism.

0058 The pyruvate:ferredoxin oxidoreductase, acetolactate synthase, and acetolactate dehydrogenase enzymes may comprise or may be derived from any of the amino acid sequences in the following table. Similarly, the genes encoding the pyruvate:ferredoxin oxidoreductase, acetolactate synthase, and acetolactate dehydrogenase enzymes may comprise or may be derived from any of the nucleic acid sequences in the following table. Moreover, any of the enzymes or genes may be variants of the sequences in the following table. For example, the enzymes or genes may have about 80%, about 90%, about 95%, or about 99% sequence identity to the sequences in the following table.

SEQ ID NO:	Description
1	native pyruvate:ferredoxin oxidoreductase, *C. autoethanogenum* LZ1561, nucleic acid sequence
2	native pyruvate:ferredoxin oxidoreductase, *C. autoethanogenum* LZ1561, amino acid sequence
3	codon-optimized pyruvate:ferredoxin oxidoreductase with XbaI and NheI, *C. autoethanogenum* LZ1561, nucleic acid sequence
4	codon-optimized pyruvate:ferredoxin oxidoreductase, *C. autoethanogenum* LZ1561, amino acid sequence
5	pyruvate:ferredoxin oxidoreductase with XbaI and NehI, *D. africanus*, nucleic acid sequence
6	pyruvate:ferredoxin oxidoreductase, *D. africanus*, amino acid sequence
7	native IlvB ORF2059 acetolactate synthase, *C. autoethanogenum* LZ1561, nucleic acid sequence
8	native IlvB ORF2059 acetolactate synthase, *C. autoethanogenum* LZ1561, amino acid sequence

9	native IlvB ORF2336 acetolactate synthase, *C. autoethanogenum* LZ1561, nucleic acid sequence
10	native IlvB ORF2336 acetolactate synthase, *C. autoethanogenum* LZ1561, amino acid sequence
11	native IlvN acetolactate synthase (regulatory subunit) with NdeI and SacI, *C. autoethanogenum* LZ1561, nucleic acid sequence
12	native IlvN acetolactate synthase (regulatory subunit), *C. autoethanogenum* LZ1561, amino acid sequence
13	mutant IlvN (G-10-D) acetolactate synthase (regulatory subunit) with NdeI and SacI, *C. autoethanogenum* LZ1561, nucleic acid sequence
14	mutant IlvN (G-10-D) acetolactate synthase (regulatory subunit), *C. autoethanogenum* LZ1561, amino acid sequence
15	native AlsS acetolactate synthase, *C. autoethanogenum* LZ1561, nucleic acid sequence
16	native AlsS acetolactate synthase, *C. autoethanogenum* LZ1561, amino acid sequence
17	codon-optimized AlsS acetolactate synthase with NdeI and SacI, *C. autoethanogenum* LZ1561, nucleic acid sequence
18	codon-optimized AlsS acetolactate synthase, *C. autoethanogenum* LZ1561, amino acid sequence
19	acetolactate synthase with NdeI and SacI, *B. subtillis*, nucleic acid sequence
20	acetolactate synthase, *B. subtillis*, amino acid sequence
21	native acetolactate decarboxylase, *C. autoethanogenum* LZ1561, nucleic acid sequence
22	native acetolactate decarboxylase, *C. autoethanogenum* LZ1561, amino acid sequence
23	codon-optimized acetolactate decarboxylase with SacI and KpnI, *C. autoethanogenum* LZ1561, nucleic acid sequence
24	codon-optimized acetolactate decarboxylase, *C. autoethanogenum* LZ1561, amino acid sequence
25	acetolactate decarboxylase, *A. hydrophila*, nucleic acid sequence
26	acetolactate decarboxylase, *A. hydrophila*, amino acid sequence
27	acetolactate decarboxylase with SacI and KpnI, *L. lactis*, nucleic acid sequence
28	acetolactate decarboxylase, *L. lactis*, amino acid sequence

0059 The recombinant bacterium of the invention may also comprise any combination of pyruvate:ferredoxin oxidoreductase, acetolactate synthase, and acetolactate decarboxylase. The bacterium may comprise pyruvate:ferredoxin oxidoreductase and acetolactate synthase, but not acetolactate decarboxylase. The bacterium may comprise pyruvate:ferredoxin oxidoreductase and acetolactate decarboxylase, but not acetolactate synthase. The bacterium may comprise acetolactate synthase and acetolactate decarboxylase, but not pyruvate:ferredoxin oxidoreductase. Finally, the bacterium may comprise each of pyruvate:ferredoxin oxidoreductase, acetolactate synthase, and acetolactate decarboxylase.

0060 The recombinant bacterium of the invention may further express or be engineered to express or overexpress one or more of alcohol dehydrogenase (EC 1.1.1.1), aldehyde dehydrogenase (acylating) (EC 1.2.1.10), formate dehydrogenase (EC 1.2.1.2), formyl-THF synthetase (EC 6.3.2.17), methylene-THF dehydrogenase/formyl-THF cyclohydrolase (EC:6.3.4.3), methylene-THF reductase (EC 1.1,1.58), CO dehydrogenase/acetyl-CoA synthase (EC 2.3.1.169), aldehyde ferredoxin oxidoreductase (EC 1.2.7.5), phosphotransacetylase (EC 2.3.1.8), acetate kinase (EC 2.7.2.1), CO dehydrogenase (EC 1.2.99.2), hydrogenase (EC 1.12.7.2), pyruvate:formate lyase (EC 2.3.1.54), 2,3-butanediol dehydrogenase (EC 1.1.1.4), primary:seconday alcohol dehydrogenase (EC 1.1.1.1), formate dehydrogenase (EC 1.2.1.2), formyl-THF synthetase (EC 6.3.2.17), methylene-THF dehydrogenase/formyl-THF cyclohydrolase (EC:6.3.4.3), methylene-THF reductase (EC 1.1,1.58), CO dehydrogenase/acetyl-CoA synthase (EC 2.3.1.169), CO dehydrogenase (EC 1.2.99.2), and hydrogenase (EC 1.12.7.2).

0061 An "enzyme co-factor" or simply a "co-factor" is a non-protein compound that binds to an enzyme to facilitate the biological function of the enzyme and thus the catalysis of a reaction. Non-limiting examples of co-factors include NAD+, NADP+, cobalamine, tetrahydrofolate and ferredoxin. "Nicotinamide adenine dinucleotide" (NADH) refers to either NAD+ (oxidized form), NADH + H+ (reduced form) or the the redox couple of both NAD+ and NADH + H+. "Nicotinamide adenine dinucleotide phosphate" (NADPH) refers to either NADP+ (oxidized form), NADPH + H+ (reduced form) or the redox couple of both NADP+ and NADPH + H+. Increase in the overall availability of the co-factor can increase the rate of a pathway reaction. Factors that may affect production of the co-factor include the expression of co-factor biosynthesis genes which may be altered to achieve increased availability of the co-factor. Other factors known to one of skill in the art may also be used to

achieve increased availability of the co-factor. Lack of availability of co-factors can have rate-limiting effects on pathway reactions. Methods for the determination of availability of co-factors are known in the art.

0062 The recombinant bacterium of the invention may further express or be engineered to express or overexpress an enzyme involved in the biosynthesis of a co-factor. In a particular embodiment, the co-factor comprises tetrahydrofolate. Enzymes that are involved in the biosynthesis of tetrahydrofolate are detailed below. Accordingly, in a particular embodiment, the recombinant microorganism exhibits increased expression of GTP cyclohydrolase I (EC 3.5.4.16), alkaline phosphatase (EC 3.1.3.1), dihydroneopterin aldolase (EC 4.1.2.25), 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase (EC 2.7.6.3), dihydropteroate synthase (2.5.1.15), dihydropteroate synthase (EC 2.5.1.15), dihydrofolate synthase (EC 6.3.2.12), folylpolyglutamate synthase (6.3.2.17), dihydrofolate reductase (EC 1.5.1.3), thymidylate synthase (EC 2.1.1.45), dihydromonapterin reductase (EC 1.5.1.-). In a particular embodiment, the co-factor comprises cobalamine (B_{12}). Enzymes that are involved in the biosynthesis of cobalamine are detailed below. Accordingly, in a particular embodiment, the recombinant microorganism exhibits increased expression of 5-aminolevulinate synthase (EC 2.3.1.37), 5-aminolevulinate:pyruvate aminotransferase (EC 2.6.1.43), adenosylcobinamide kinase / adenosylcobinamide-phosphate guanylyltransferase (EC 2.7.1.156 / 2.7.7.62), adenosylcobinamide-GDP ribazoletransferase (EC 2.7.8.26), adenosylcobinamide-phosphate synthase (EC 6.3.1.10), adenosylcobyric acid synthase (EC 6.3.5.10), alpha-ribazole phosphatase (EC 3.1.3.73), cob(I)alamin adenosyltransferase (EC 2.5.1.17), cob(II)yrinic acid a,c-diamide reductase (EC 1.16.8.1), cobalt-precorrin 5A hydrolase (EC 3.7.1.12), cobalt-precorrin-5B (C1)-methyltransferase (EC 2.1.1.195), cobalt-precorrin-7 (C15)-methyltransferase (EC 2.1.1.196), cobaltochelatase CobN (EC 6.6.1.2), cobyrinic acid a,c-diamide synthase (EC 6.3.5.9 / 6.3.5.11), ferritin (EC 1.16.3.1), glutamate-1-semialdehyde 2,1-aminomutase (EC 5.4.3.8), glutamyl-tRNA reductase (EC 1.2.1.70), glutamyl-tRNA synthetase (EC 6.1.1.17), hydroxymethylbilane synthase (EC 2.5.1.61), nicotinate-nucleotide-dimethylbenzimidazole phosphoribosyltransferase (EC 2.4.2.21), oxygen-independent coproporphyrinogen III oxidase (EC 1.3.99.22), porphobilinogen synthase (EC 4.2.1.24), precorrin-2 dehydrogenase / sirohydrochlorin ferrochelatase (EC 1.3.1.76 / 4.99.1.4), precorrin-2/cobalt-factor-2 C20-methyltransferase (EC 2.1.1.130 / 2.1.1.151), precorrin-3B synthase (EC 1.14.13.83),

precorrin-3B C17-methyltransferase (EC 2.1.1.131), precorrin-4 C11-methyltransferase (EC 2.1.1.133), precorrin-6X reductase (EC 1.3.1.54), precorrin-6Y C5,15-methyltransferase (EC 2.1.1.132), precorrin-8W decarboxylase (EC 1.-.-.-), precorrin-8X methylmutase (EC 5.4.1.2), sirohydrochlorin cobaltochelatase (EC 4.99.1.3), threonine-phosphate decarboxylase (EC 4.1.1.81), uroporphyrinogen decarboxylase (EC 4.1.1.37), uroporphyrinogen III methyltransferase / synthase (EC 2.1.1.107/ 4.2.1.75). Without wishing to be bound by theory, it is believed that an increase in the availability of a co-factor is achieved through over-expression of enzymes or genes involved in the biosynthesis pathway of said co-factor. As a result, reactions dependent on this co-factor are no longer limiting.

0063 The invention also provides methods for the production of one or more products by fermentation of a substrate comprising CO. Preferably, the product is one or more of ethanol, butanol, isopropanol, isobutanol, higher alcohols, butanediol, 2,3-butanediol, succinate, isoprenoids, fatty acids, biopolymers, and mixtures thereof.

0064 In one embodiment, the substrate comprising CO is a gaseous substrate comprising CO. In one embodiment, the substrate will typically contain a major proportion of CO, such as about 20% to about 100% CO by volume, from 20% to 70% CO by volume, from 30% to 60% CO by volume, or from 40% to 55% CO by volume. In particular embodiments, the substrate comprises about 25%, about 30%, about 35%, about 40%, about 45%, about 50% CO, about 55% CO, or about 60% CO by volume.

0065 While it is not necessary for the substrate to contain any hydrogen, the presence of hydrogen should not be detrimental to product formation in accordance with methods of the invention. In particular embodiments, the presence of hydrogen results in an improved overall efficiency of alcohol production. For example, in particular embodiments, the substrate may comprise an approx. 2:1, or 1:1, or 1:2 ratio of H_2:CO. In one embodiment the substrate comprises about 30% or less H_2 by volume, 20% or less H_2 by volume, about 15% or less H_2 by volume or about 10% or less H_2 by volume. In other embodiments, the substrate stream comprises low concentrations of H_2, for example, less than 5%, less than 4%, less than 3%, less than 2%, or less than 1% H_2. In further embodiments, the substrate stream is substantially hydrogen-free. The substrate may also contain some CO_2, for example, about 1% to about 80% CO_2 by volume, or 1% to about 30% CO_2 by volume. In

one embodiment the substrate comprises less than or equal to about 20% CO_2 by volume. In particular embodiments, the substrate comprises less than or equal to about 15% CO_2 by volume, less than or equal to about 10% CO_2 by volume, less than or equal to about 5% CO_2 by volume, or substantially no CO_2.

0066 Embodiments of the invention are described in terms of delivering and fermenting a "gaseous substrate containing CO." However, it should be appreciated that the gaseous substrate may be provided in alternative forms. For example, the gaseous substrate containing CO may be provided dissolved in a liquid. Essentially, a liquid is saturated with a carbon monoxide containing gas and then that liquid is added to the bioreactor. This may be achieved using standard methodology. By way of example, a microbubble dispersion generator (Hensirisak, *Appl Biochem Biotechnol*, 101: 211-227, 2002) could be used. By way of further example, the gaseous substrate containing CO may be adsorbed onto a solid support. Such alternative methods are encompassed by the term "substrate comprising CO" and the like.

0067 The gaseous substrate may be a CO-containing waste gas obtained as a by-product of an industrial process or from some other source, such as from automobile exhaust fumes or biomass gasification. In certain embodiments, the industrial process is selected from the group consisting of ferrous metal products manufacturing, such as a steel mill manufacturing, non-ferrous products manufacturing, petroleum refining processes, coal gasification, electric power production, carbon black production, ammonia production, methanol production, and coke manufacturing. In these embodiments, the CO-containing gas may be captured from the industrial process before it is emitted into the atmosphere, using any convenient method. The CO may be a component of syngas (gas comprising carbon monoxide and hydrogen). The CO produced from industrial processes is normally flared off to produce CO_2 and therefore the invention has particular utility in reducing CO_2 greenhouse gas emissions and producing a biofuel. Depending on the composition of the gaseous CO-containing substrate, it may also be desirable to treat it to remove any undesired impurities, such as dust particles before introducing it to the fermentation. For example, the gaseous substrate may be filtered or scrubbed using known methods.

0068 Typically, the fermentation is performed in a bioreactor. The term "bioreactor" includes a fermentation device consisting of one or more vessels and/or towers or piping arrangements, such as a continuous stirred tank reactor (CSTR), immobilized cell reactor (ICR), trickle bed reactor (TBR), bubble column, gas lift fermenter, static mixer, or other vessel or other device suitable for gas-liquid contact. In some embodiments the bioreactor may comprise a first growth reactor and a second fermentation reactor. As such, when referring to the addition of substrate to the bioreactor or fermentation reaction it should be understood to include addition to either or both of these reactors, where appropriate. As used herein, the terms "fermenting," "fermentation process," "fermentation reaction," and the like encompass both the growth phase and product biosynthesis phase of the fermentation process.

0069 In certain embodiments a culture of a bacterium of the invention is maintained in an aqueous culture medium that contains nutrients, vitamins, and/or minerals sufficient to permit growth of the microorganism. Preferably the aqueous culture medium is a minimal anaerobic microbial growth medium. Suitable media are known in the art and described for example in US Patent 5,173,429, US Patent 5,593,886, and WO 2002/008438.

0070 The fermentation should desirably be carried out under appropriate fermentation conditions for the production of the fermentation product to occur. Reaction conditions that should be considered include pressure, temperature, gas flow rate, liquid flow rate, media pH, media redox potential, agitation rate (if using a continuous stirred tank reactor), inoculum level, maximum gas substrate concentrations to ensure that CO in the liquid phase does not become limiting, and maximum product concentrations to avoid product inhibition.

0071 In addition, it is often desirable to increase the CO concentration of a substrate stream (or CO partial pressure in a gaseous substrate) and thus increase the efficiency of fermentation reactions where CO is a substrate. Operating at increased pressures allows a significant increase in the rate of CO transfer from the gas phase to the liquid phase where it can be taken up by the micro-organism as a carbon source for the production of fermentation. This in turn means 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. The optimum reaction conditions will

depend partly on the particular micro-organism of the invention used. However, in general, it is preferred that the fermentation be performed at pressure higher than ambient pressure. Also, since a given CO conversion rate is in part a function of the substrate retention time, and achieving a desired retention time in turn dictates the required volume of a bioreactor, the use of pressurized systems can greatly reduce the volume of the bioreactor required, and consequently the capital cost of the fermentation equipment. According to examples given in US Patent 5,593,886, 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.

0072 By way of example, the benefits of conducting a gas-to-ethanol fermentation at elevated pressures has been described. For example, WO 2002/008438 describes gas-to-ethanol fermentations performed under pressures of 30 psig and 75 psig, giving ethanol productivities of 150 g/l/day and 369 g/l/day respectively. However, example fermentations performed using similar media and input gas compositions at atmospheric pressure were found to produce between 10 and 20 times less ethanol per litre per day.

0073 It is also desirable that the rate of introduction of the CO-containing gaseous substrate is controlled to ensure that the concentration of CO in the liquid phase does not become limiting, since products may be consumed by the culture under CO-limited conditions.

0074 The composition of gas streams used to feed a fermentation reaction can have a significant impact on the efficiency and/or cost of the reaction. For example, O_2 may reduce the efficiency of an anaerobic fermentation process. Processing of unwanted or unnecessary gases in stages of a fermentation process before or after fermentation can increase the burden on such stages. For example, where the gas stream is compressed before entering a bioreactor, unnecessary energy may be used to compress gases that are not needed in the fermentation. Accordingly, it may be desirable to treat substrate streams, particularly substrate streams derived from industrial sources, to remove unwanted components and increase the concentration of desirable components.

EXAMPLES

0075 The following examples further illustrate the invention but, of course, should not be construed to limit its scope in any way.

Example 1

0076 This example describes the analysis of fermentation pathways of carboxydotrophic bacteria such as *C. autoethanogenum, C. ljungdahlii,* or *C. ragsdalei* for bottlenecks in the production of ethanol and 2,3-butanediol.

0077 Oxidoreductase enzyme steps of the Wood-Ljungdahl pathway and fermentation pathways to ethanol and 2,3-butanediol were assayed to determine their activity. Oxidoreductase reactions are particularly suitable since they are coupled with one or more co-factors whose reduction or oxidation can be measured. A synthetic redox dye such as methylviologen or benzylviologen can be used for this purpose as well. The enzymes in these pathways are involved in autotrophic growth including uptake and utilization of CO, CO_2, and H_2 gases, as well as product formation.

0078 The enzymes assayed and their activities are detailed in Fig. 1. All assays were performed using a synthetic redox dye as control, either methyl viologen (MV) or benzyl viologen (BV). Co-factors ferredoxin (Fd), NADH, and NADPH or a combination thereof were then tested. Enzyme assays were performed using crude extracts from a fermentation growing autotrophically on CO and hydrogen.

0079 Fermentations with *C. autoethanogenum* were carried out in 1.5L bioreactors at 37 °C using CO-containing steel mill gas as a sole energy and carbon source. The fermentation media contained, per litre, MgCl, $CaCl_2$ (0.5 mM), KCl (2 mM), H_3PO_4 (5 mM), Fe (100 μM), Ni, Zn (5 μM), Mn, B, W, Mo, and Se (2 μM). The media was transferred into a bioreactor and autoclaved at 121°C for 45 minutes. After autoclaving, the media was supplemented with thiamine, pantothenate (0.05 mg) and biotin (0.02 mg) and reduced with 3 mM cysteine-HCl. To achieve anaerobic conditions, the reactor vessel was sparged with nitrogen through a 0.2 μm filter. Prior to inoculation, the gas was switched to CO-containing

steel mill gas, feeding continuously to the reactor. The feed gas composition was 2% H_2, 42% CO, 20% CO_2, and 36% N_2. The pH of the culture was maintained between 5 and 5.2.

0080 At the time of harvesting the cells (biomass of 3.9 g cells/l fermentation broth), the gas consumption was 5 moles CO L^{-1} day^{-1} and 10 milimoles H_2 L^{-1} day^{-1}, with the following metabolites produced: 14 g L^{-1} day^{-1} acetate and 19.5 g L^{-1} day^{-1} ethanol. The pH of the culture was adjusted to pH 6 with K_2CO_3 and the reactor chilled in an ice-water bath. Approximately 1.2 L of culture was collected on ice. The culture was divided between two 1-L centrifuge bottles (this and all subsequent steps were carried out in an anaerobic chamber to ensure anoxic conditions to avoid inactivation of the enzymes) and cells were pelleted at 5000 rpm for 10 min. The supernatant was decanted and residual liquid removed. Each pellet was resuspended in approximately 30 mL of 50 mM K PO_4 pH 7.0 with 10 mM DTT. Resuspensions were transferred to pre weighed 50-mL-Falcon-tubes and cells repelleted at maximum speed (5000g) for 15 min. The tubes were removed from the anaerobic chamber and immediately frozen on liquid N_2 before assaying.

0081 Cells were harvested from a continuous reactor under anoxic conditions. They were disrupted by three passes through a French press. Unbroken cells and cell debris were removed by centrifugation at 20,000 × g and 4 °C for 30 min. The supernatant was used for enzyme assays. Except where indicated, all assays were performed at 37 °C in 1.5-ml anaerobic cuvettes closed with a rubber stopper filled with 0.8 ml reaction mixture and 0.7 ml N_2 or H_2 or CO at 1.2 x 10^5 Pa. Enzymes were assayed as described below or by Huang, *J Bacteriol*, 194: 3689-3699, 2012. After the start of the reaction with enzyme, the reduction of $NAD(P)^+$ or NAD^+ was monitored spectrophotometrically at 340 nm (ε = 6.2 mM^{-1} cm^{-1}) or at 380 nm (ε = 1.2 mM^{-1} cm^{-1}), *C. pasteurianum* ferredoxin reduction at 430 nm ($\varepsilon_{\Delta ox\text{-}red} \approx$ 13.1 mM^{-1} cm^{-1}), methyl viologen reduction at 578 nm (ε = 9.8 mM^{-1} cm^{-1}) and benzyl viologen reduction at 578 nm (ε = 8.6 mM^{-1} cm^{-1}).

0082 CO dehydrogenase was measured using an assay mixture that contained 100 mM Tris/HCl (pH 7.5), 2 mM DTT and about 30 μM ferredoxin and/or 1 mM NAD^+ or 1 mM $NADP^+$. The gas phase was 100% CO.

0083 Hydrogenase activity was measured using an assay mixture of 100 mM Tris/HCl (pH 7.5) or 100 mM potassium phosphate, 2 mM DTT and, 25 μM ferredoxin and/or 1 mM $NADP^+$ and/or 10 mM methyl viologen. The gas phase was 100% H_2.

0084 Formate-hydrogen lyase activity for reduction of CO_2 with H_2 to formate was measured with an assay mixture containing 100 mM potassium phosphate, 2 mM DTT, and 30 mM [^{14}C]K_2CO_3 (24,000 dpm/μmol). The gas phase was 100% H_2. The serum bottles were continuously shaken at 200 rpm to ensure equilibration of the gas phase with the liquid phase. After start of the reaction with enzyme, 100 μl liquid samples were withdrawn every 1.5 min and added into a 1.5-ml safe seal micro tube containing 100 μl of 150 mM acetic acid to stop the reaction by acidification. The 200 μl mixture was then incubated at 40°C for 10 min with shaking at 1,400 rpm in a Thermomixer to remove all $^{14}CO_2$ leaving behind the ^{14}C-formate formed. Subsequently, 100 μl of the mixture was added to 5 ml of Quicksave A scintillation fluid (Zinsser Analytic, Frankfurt, Germany) and analyzed for ^{14}C radioactivity in a Beckman LS6500 liquid scintillation counter (Fullerton, CA).

0085 Formate dehydrogenase measurement was carried out with an assay mixtures containing 100 mM Tris/HCl (pH 7.5) or 100 mM potassium phosphate, 2 mM DTT, 20 mM formate and, where indicated 25 μM ferredoxin, 1 mM $NADP^+$, 1 mM NAD^+ and/or 10 mM methyl viologen. The gas phase was 100% N_2.

0086 Methylene-H_4F dehydrogenase was measured using an assay mixture containing 100 mM MOPS/KOH (pH 6.5), 50 mM 2-mercaptoethanol, 0.4 mM tetrahydrofolate, 10 mM formaldehyde and 0.5 mM $NADP^+$ or 0.5 mM NAD^+. The gas phase was 100% N_2.

0087 Methylene-H_4F reductase was assayed under the following conditions. The assay mixtures contained 100 mM Tris/HCl (pH 7.5), 20 mM ascorbate, 10 μM FAD. 20 mM benzyl viologen and 1 mM methyl-H4F. Before start of the reaction with enzyme, benzyl viologen was reduced to an ΔA555 of 0.3 with sodium dithionite.

0088 Aldehyde:ferredoxin oxidoreductase was assayed using a mixture containing 100 mM Tris/HCl (pH 7.5), 2 mM DTT, 1.1 mM acetaldehyde, and about 25 μM ferredoxin. The gas phase was 100% N_2.

0089 CoA acetylating acetaldehyde dehydrogenase was measured using a mixture contained 100 mM Tris/HCl (pH 7.5), 2 mM DTT, 1.1 mM acetaldehyde, 1 mM coenzyme A, and 1 mM NADP+ or 1 mM NAD+. The gas phase was 100% N_2.

0090 Alcohol and butanediol dehydrogenases were measured in an assay with 100 mM potassium phosphate (pH 6), 2 mM DTT, 1.1 mM acetaldehyde or acetoin respectively and 1 mM NADPH or 1 mM NADH. The gas phase was 100% N_2.

0091 Ferredoxin was purified from *C. pasteurianum* as described by Schönheit, *FEBS Lett*, 89: 219-222, 1978.

0092 All oxidoreductase reactions in the pathways to ethanol and 2,3-butanediol of carboxydotrophic bacterium *C. autoethanogenum* were assayed and successfully detected, with the exception of the methylene-THF reductase which the inventors believe requires an as yet unknown coupling site (Köpke, *PNAS USA*, 107: 13087-13092, 2010; Poehlein, *PLoS One*, 7: e33439, 2012). Activity of this enzyme could not previously be detected in other organisms. Results are provided in Fig. 1 and Fig. 2. This data was used to analyze and determine bottlenecks in these pathways that would typically occur during a fermentation process.

Example 2

0093 This example demonstrates increasing the flux through a fermentation pathway.

0094 The general methods described in Example 3 of PCT/US2014/041188 may also be used to introduce pyruvate:ferredoxin oxidoreductase, acetolactate synthase, and/or acetolactate decarboxylase gene into the recombinant *Clostridium* microorganism of the invention.

Example 3

0095 This example identifies the conversion of acetyl CoA to pyruvate by pyruvate:ferredoxin oxidoreductase as a bottleneck in the production of 2,3-butanediol.

0096 As seen in Fig. 2, the bottleneck for 2,3-butanediol production is the reaction from acetyl CoA to pyruvate catalyzed by pyruvate:ferredoxin oxidoreductase. While all other measured reactions showed at least an activity of 1.1 U/mg, this rate limiting reaction exhibited an enzyme activity of only 0.11 U/mg (10 %) in the presence of ferredoxin. This is 90% less than all other reactions in the pathway. To go at least some way towards overcoming this bottleneck and increase the product yield from the fermentation, an endogenous pyruvate:ferredoxin oxidoreductase enzyme may be overexpressed or an exogenous pyruvate:ferredoxin oxidoreductase enzyme may be introduced and expressed.

Example 4

0097 This example demonstrates increasing the flux through the 2,3-butanediol production pathway by removing bottlenecks.

0098 The reaction catalysing the conversion of acetyl-CoA to pyruvate has been identified in Fig. 2 to be the rate limiting step in 2,3-butanediol formation in *C. autoethanogenum*, *C. ljungdahlii*, or *C. ragsdalei*. This can be overcome by overexpressing the gene that encodes pyruvate:ferredoxin oxidoreductase in *C. autoethanogenum*.

0099 The gene is codon-optimized to minimize issues with expression and designed to reduce homology to the native gene to prevent undesired integration events. The gene is flanked by restriction enzyme cut sites, *Xba*I (3'-end) and *Nhe*I (5'-end) for subcloning into pMTL83155. The synthesized construct and pMTL83155 are digested with *Xba*I and *Nhe*I (Fermentas), and the PFOR gene is ligated into pMTL83155 with T4 DNA ligase (Fermentas). The ligation mix is used to transform *E. coli* TOP10 (Invitrogen, LifeTechnologies) and colonies containing the desired plasmid are identified by plasmid miniprep (Zymo Research) and restriction digestion (Fermentas). The desired plasmid is methylated and transformed in *C. autoethanogenum*. Successful transformants are identified by thiamphenicol resistance and PCR analysis with primers repHF and CatR which will yield a 1584 base pair product when the plasmid is present.

0100 Transformants identified as containing the desired plasmid are grown in serum bottles containing PETC-MES media in the presence of mill gas, and their metabolite production, measured by HPLC analysis, is compared to that of a parental microorganism not harbouring

the plasmid. The pyruvate:ferredoxin oxidoreductase activity in the transformed strain is also measured in crude extracts to confirm that the observed bottleneck in the parental strain is alleviated. Overexpression of pyruvate:ferredoxin oxidoreductase increases the overall activity within the cell, alleviating the bottleneck in the pathway, and leading to an increase in the flux through pyruvate, and an increase in 2,3-butanediol production.

Example 5

0101 This example demonstrates an increase in flux from pyruvate to 2-hydroxy-2-methyl-3-ketobutyrate (acetolactate) via overexpression of native catabolic acetolactate synthase.

0102 The native catabolic acetolactate synthase gene (*alsS*) of *C. autoethanogenum* is cloned into the NdeI and NheI sites of pMTL83155 (WO 2013/185123) to generate an overexpression plasmid, expressing *alsS* under the control of the promoter region of the phosphotransacetylase-acetate kinase operon.

0103 The overexpression plasmid can be similarly produced using a catabolic acetolactate synthase from another microorganism, a native anabolic acetolactate synthase, or an anabolic acetolactate synthase from another microorganism.

0104 The use of either a catabolic acetolactate synthase from another microorganism or an anabolic acetolactate synthase from another microorganism may have a higher affinity toward pyruvate and faster reaction kinetics. An anabolic acetolactate synthase from another microorganism may be an enzyme which is identified to be insensitive to feedback inhibition. The small subunit of the anabolic acetolactate synthase mutant which is insensitive to feedback inhibitions may also be overexpressed.

0105 The overexpression plasmid is introduced into *C. autoethanogenum*. This results in a *C. autoethanogenum* strain adapted to increase flux from pyruvate to acetolactate.

Example 6

0106 This example describes a metabolic engineering approach to overexpression of pyruvate:ferredoxin oxidoreductase, acetolactate synthase, and/or acetolactate decarboxylase.

0107 In order to boost 2,3-butanediol production, the pool of pyruvate, a precursor molecule of 2,3-butanediol, was increased. The first target was the *PFOR* gene which encodes the PFOR enzyme, which catalyzes the conversion of acetyl-CoA to pyruvate. In the *C. autoethanogenum* genome, there are two copies of the *PFOR* gene. It is known that the PFOR gene (CAETHG 0928) is constitutively expressed at a high level while the other gene (CAETHG 3029) is only up-regulated at the end of the growth in a batch culture (Köpke, *Appl Environ Microbiol*, 77: 5467-5475, 2011). Thus, the highly expressed *PFOR* gene was chosen to be overexpressed.

0108 Acetolactate synthase, which links two pyruvate molecules to form α-acetolactate, is proposed to exist in three forms coded by three different genes in *C. autoethanogenum* and in closely related microorganisms (Köpke, *PNAS USA*, 107: 13087-13092, 2010; Köpke, *Appl Environ Microbiol*, 77: 5467-5475, 2011): one catabolic acetolactate synthase and two forms of anabolic acetolactate synthase. The *alsS* gene (CAETHG 1740) is predicted to code for the catabolic acetolactate synthase and to be involved in the formation of 2,3-butanediol. The two genes *ilvBN* (CAETHG 0406) and *ilvH* (CAETHG 0124) are predicted to code for anabolic acetolactate synthases which are likely to be involved in the formation of branched chain amino acids.

0109 α-acetolactate is decarboxylated to acetoin via the activity of acetolactate decarboxylase encoded by the *alsD* gene (CAETHG 2932). In other microorganisms, the *alsD* gene transcript levels and the enzyme activity have been found to be regulated by the concentration of branched-chain amino acids present in the cell. It is still unknown if the branched-chain amino acids in *C. autoethanogenum* produce any feedback inhibition of the *alsD* gene transcription or the activity of the corresponding enzyme.

0110 The reduction step from acetoin to 2,3-butanediol by the action of 2,3-butanediol dehydrogenase (EC 1.1.1.4) is not a rate limiting step. This has been demonstrated in batch and continuous cultures of *C. autoethanogenum* by the addition of acetoin to the fermentation media. In batch cultures, up to 40 g/L of acetoin was added and quantitatively converted to 2,3-butanediol after 24 hours of incubation. In fact, the putative 2,3-butanediol dehydrogenase gene was found to be expressed constitutively during both growth and stationary phase in a batch culture (Köpke, *Appl Environ Microbiol*, 77: 5467-5475, 2011).

Furthermore, it has been shown that *C. autoethanogenum* contains a strictly NADPH-dependent primary-secondary alcohol dehydrogenase which also reduces acetoin and other ketones to 2,3-butanediol and other secondary alcohols (Köpke, *Catalyst Rev*, 27: 7-12, 2014).

0111 The three native genes, *PFOR*, *alsS*, and *alsD*, were overexpressed individually and in combination of *alsS-alsD* and in combination of all three genes. To introduce the genes into *C. autoethanogenum*, the genes were cloned into a recombinant plasmid that carried an antibiotic resistant gene as a selection marker. For this reason, the control strain for this set of experiments carried the plasmid with the antibiotic resistance gene but without any active 2,3-butanediol gene insertion, so it could be exposed to antibiotic stress and compared to the performance of the overexpression strains. An important aspect of overexpression is the promoter choice to regulate the expression of inserted genes. In this research, the ferredoxin gene promoter (P_{fdx}) was chosen as it is known to be one of the strongest promoters in Clostridia. Furthermore, to avoid homologous recombination between the native gene in the genome and the gene present in the plasmid, the DNA sequence of the added genes was altered according to a proprietary optimizing process (GeneOptimizer) of the DNA synthesizing company (GeneArt).

0112 Four heterologous genes were also targeted. A PFOR gene isolated from *Desulfovibrio africanus* has been shown to produce a PFOR enzyme that is highly stable in the presence of oxygen which could be advantageous in commercial anaerobe fermentation (Pieulle, *J Bacteriol*, 179: 5684-5692, 1997). The *alsS* gene isolated from *Bacillus substilis* and two heterologous *alsD* genes isolated from *Leuconostoc lactis* and from *Aeromonas hydrophila* were also tested. The *alsS* gene isolated from *Bacillus substilis* was used to construct the 2,3-butanediol pathway in a number of heterologous hosts (Ng, *Microb Cell Factories*, 11: 68, 2012; Oliver, *PNAS*, 110: 1249-1254, 2013). The *alsD* isolated from *Aeromonas hydrophila* was shown to have highest enzyme activity among several other heterogolous *alsD* isolated from other microorganisms in a recent study (Oliver, *PNAS*, 110: 1249-1254, 2013). These properties make these genes the ideal candidates for genetic manipulation experiments.

Example 7

0113 This example describes cloning, conjugation, and characterization of strains overexpressing pyruvate:ferredoxin oxidoreductase, acetolactate synthase, and/or acetolactate decarboxylase.

0114 *C. autoethanogenum* strain LZ1561 (DSM23693) was used in this research. Two *E. coli* donor strains were used as tools for genetic manipulation; the TOP 10 (Invitrogen) strain was used for plasmid cloning and the CA434 strain was used for conjugation with *C. autoethanogenum*.

0115 A *Clostridium-E.coli* shuttle plasmid series pMTL83159 (4600 base pairs) was chosen to overexpress *PFOR*, *alsS*, and *alsD* genes (Heap, *J Microbiol Meth*, 78: 79-85, 2009). The plasmid was designed to contain a Gram-positive replicon, a Gram negative replicon, a *traJ* gene, an antibiotic resistant gene, the multiple cloning sites located within the lacZ alpha coding sequence, and the ferredoxin gene promoter (P_{fdx}). The Gram-positive replicon (*repH* gene) originated from the *C. butylicum* pCB102 plasmid. To clone the plasmid in *E. coli* the Gram-negative replicon *ColE1* was chosen due to the high copy number of plasmids it produces. A *traJ* or transfer gene allows the genetic material to be transferred between donor and recipient cell. The *catP* gene encoded for the chloramphenicol/thiamphenicol resistance was the selection marker.

0116 The three genes, *PFOR*, *alsS*, and *alsD*, were synthesized by a DNA synthesizing company (GeneArt). To clone them into the pMTL83159 plasmid, a pair of restriction enzyme recognition sequences and a ribosomal binding site sequence were added to each gene sequence and synthesized together with the gene. The original plasmids carrying the gene were first transformed into the *E. coli* TOP 10 strain. A ZYPPY™ plasmid miniprep kit (Zymo Research) was used to extract the plasmids. To transfer the targeted gene from its original plasmid to a pMTL83159 plasmid, both plasmids were cut with the same pair of restriction enzymes. The digested DNA was separated by gel electrophoresis on a 0.6% agarose gel which ran at 75 volts for one hour. After the plasmid pMTL83159 (vector) and the DNA sequence (insert) of each gene were recovered from the gel, they were ligated together by using T4 DNA ligase (Invitrogen). The ligated plasmids were then transformed into the *E. coli* Top10 culture. To ensure sure that the extracted plasmids contained the

insert, 1 μL of the plasmid was digested with appropriate restriction enzymes and the digested plasmid was then separated by gel electrophoresis on a 0.8% agarose gel.

0117 The plasmids were transformed into *E. coli* CA434 donor cells and then conjugated with *C. autoethanogenum*. To confirm the presence of the targeted plasmid in the *C. autoethanogenum* transformants, PCR was performed using samples taken from the cultures in serum bottles.

0118 The initial characterization of all the gene overexpression and gene disruption strains were first performed in 1L-Schott bottles to screen which strain had produced the highest concentration of 2,3-butanediol. These strains were then further tested in CSTR continuous cultures, which allows for accurate control of fermentation parameters such that metabolite and biomass selectivities can be calculated.

0119 The goals of the overexpression experiments were to overexpress three native genes in the 2,3-butanediol pathway individually and in combinations of two and three genes.

Overexpression strain	Strain availability	Schott bottle data availability
Plasmid control	+	+
Native PFOR	+	+
Native AlsS	+	+
Native AlsD	+	+
Native AlsS-AlsD	+	+
Native PFOR-AlsS-AlsD	+	-

0120 In Schott bottle experiments, the OD, metabolite concentrations in the media, pH of the media and headspace pressure were monitored over the course of eight days by analyzing daily samples. At that point no further growth or significant metabolic activity was observed, the pH of all the cultures had dropped to between 3.8 and 4, and no further gas consumption was measured. A graphic representation of the growth and metabolite profiles versus time of the five strains are shown in Fig. 5. All five strains with Schott bottle data availability quickly grew during the first two days of incubation. Thereafter, the growth rate reduced significantly and then ceased on day 4. The maximum optical density values were

approximately 0.75. Similarly to the biomass, the acetic acid concentrations increased steeply during the first two days in all the cultures. Between day 3 and day 4, the plasmid control and the *alsS* overexpression strains appeared to have stopped all metabolic activities, as no further changes in metabolite concentrations were observed. The other three strains showed activity until end of the experiments.

0121 Conversion of acetate to ethanol (AOR activity) could still be observed in three strains after the growth slowed down. The most notable drop in acetate was observed in the *alsD* overexpression strain, and as a result, the highest ethanol concentration of around 7 g/L was reached by this strain. Two strains, the *alsD* and the combined *alsS-alsD* overexpression strains, produced higher amounts of 2,3-butanediol than the other strains, producing most of it during the active growth phase. Thereafter, during the stationary phase from day 4 they continued producing 2,3-butanediol at slower rates until the end of the experiment on day 8. The PFOR overexpression strain was the other strain that produced 2,3-butanediol to a higher concentration than the plasmid control strain. In contrast to the first two strains, the PFOR overexpression strain started producing most of the 2,3-butanediol during the onset of the stationary phase. The production rate was similar to the rates of the other two strains during the stationary phase.

0122 Overexpression of the *alsS* gene alone did not appear to increase the amount of 2,3-butanediol. These results suggest that the 2,3-butanediol increase observed is primarily associated with the overexpression of the *alsD* gene. Overexpressing both *alsS* and *alsD* genes resulted in a slightly higher 2,3-butanediol concentration than just overexpressing the *alsD* gene alone. A positive additional effect of the *alsS* might be feasible. Overexpression of the PFOR gene appeared to have contributed to a higher 2,3-butanediol production during the stationary phase where no more growth was observed. Because of all of these positive results and the fact that no detrimental effect had been observed in these strains, the overexpression strain carrying all three gene was further tested in the continuous culture in CSTR.

0123 To explore the full potential of the microbe using a CO-containing gaseous substrate, the overexpression strain carrying all three native genes and the plasmid control strain were further characterized in CSTR-based continuous cultures. The media pH was controlled

during the entire fermentation by adding a base (5 M NH_4OH-solution) to compensate for the acid production and replenish media nitrogen levels. The substrate was continuously supplied by sparging a CO-containing gas mix through the stirred fermentation broth. The gas composition of the fresh incoming gas and the used outflowing gas was monitored hourly by gas chromatography. Based on the differences in gas composition between the inflowing and outflowing gases, the flow rate of the incoming gas, and the liquid volume of the fermentation; the gas utilization and rate of product synthesis at the time of sampling were calculated. The values are expressed in mol/L/d.

0124 The OD and metabolite concentrations were measured three times a day and the dilution rate and the specific growth rate were measured and calculated daily to determine the productivity of each metabolite. The product selectivity of each metabolite was calculated using the CO consumption, CO_2 production, and the metabolite productivity. CO uptake rates of 4 mol/L/d and 8 mol/L/d were established to determine whether the product selectivity is dependent on the volumetric productivity. At a CO uptake of 4 mol/L/d, the dilution rate of the system was maintained at 1 d^{-1} and the specific growth rate at 0.5 d^{-1}. At higher CO uptake of 8 mol/L/d and correspondingly higher metabolite production rates the dilution rate of the culture was increased to 1.7 d^{-1} to lower the metabolite concentrations to a similar range as in the 4 mol/L/d experiments. The specific growth rate was also increased to 0.75 d^{-1}.

0125 The metabolite and gas profile of the combined PFOR, *alsS*, and *alsD* overexpression strain and the plasmid control strain was monitored at a 4 mol/L/d CO uptake over the course of 20 days (Fig. 6). Although the preparation of the inoculum of each strain went well through several rounds of serum bottle sub-culturing in regular intervals, the CSTR cultures exhibited an unusual, almost identical long lag phase of five days. At around day 5.5, both CSTR cultures started normal exponential growth (within hours of each other).

0126 Despite the long lag phase in both cultures and the fluctuation in the growth pattern of the overexpression strain between days 8 and 10, both cultures were maintained at a stable gas uptake of 4 mol/L/d for 10 days. With a dilution rate of 1 d^{-1} and a specific growth rate of 0.5 d^{-1}, it takes about six days to replace 95% of the bacterial load in the fermenters. With the constant gas uptake measured during this period, the latest values were used for analysis.

The final results showed that the overexpression strain consistently produced higher 2,3-butanediol levels compared to the plasmid control strain.

0127 The metabolite and the gas profiles of the overexpression culture was monitored at an 8 mol/L/d CO uptake over the course of 11 days (Fig. 7). The culture was inoculated from the 4 mol/L/d CO uptake culture. No lag phase was observed in this culture, and an appropriate specific CO supply and dilution rate was applied during exponential growth to keep the culture under optimal growing conditions. Therefore a stable production of acetic acid was reached after day three of incubation while other metabolites continued to accumulate. The target of CO uptake was doubled from 4 mol/L/d to 8 mol /L/d.

0128 To avoid product inhibition, the overall dilution rate of the culture was increased from 1 d^{-1} to 1.7 d^{-1} and the specific growth rate was increased proportionally. Metabolite concentrations slowly increased and reached a stable production rate after seven days. Both the hydrogen uptake and the CO uptake were maintained in a stable manner for six days, indicating that the fermentation of the overexpression strain has the potential to be operated stably for extended time periods.

0129 Among the liquid products, ethanol was produced at the highest rate followed by acetate. The specific CO supply strategy was aimed to maintain a certain ratio of acetate to ethanol that allows the fermentation to be operated stably for extended periods of time. The LZ1561 strain and the plasmid control exhibited similar 2,3-butanediol and biomass profiles. The 2,3-butanediol to biomass production rate ratios were between 1.26 to 1.47 in these cultures. However, in the overexpression strain, this ratio was 2.45 at 4 mol/L/d CO uptake and 2.24 at the 8 mol/L/d CO uptake culture.

Strain	LZ1561	Control	Overexpression	LZ1561	Overexpression
			Gas uptake rate (mol/L/d)		
CO consumption	4	4	4	8	8
CO_2 production	2.45	2.53	2.43	4.53	4.34
Product			**Production rate (10^{-3} mol/L/d)**		
2,3-Butanediol	52	57	81	122	168
Biomass	37	44	33	83	75
Ethanol	447	450	367	798	823
Acetic acid	128	133	125	258	214

0130 The product selectivity for 2,3-butanediol, biomass, ethanol and acetate for the overexpression, control and LZ1561 strains was measured at a gas uptake of 4 mol/L/d and 8 mol/L/d. With the optimized fermentation parameters, more than 50% of the carbon was directed to ethanol formation. The data shows that 2,3-butanediol selectivity of the overexpression strain increased from an average of 15% in the LZ1561 and the plasmid control cultures to 22.5%. The elevated 2,3-butanediol selectivity of the overexpression strain appeared to be maintained at different CO uptake rates. The increase in 2,3-butanediol selectivity is contributed to the decrease of ethanol selectivity at 4 mol/L/d or the decrease in the acetate selectivity at 8 mol/L/d. The exact contribution of ethanol and acetate cannot be separated, because their selectivity can be influenced easily by the specific gas supplies, which in turn are easily influenced by small differences between run parameters. For instance, pH, impeller position, probe location among others, can all affect these results.

0131 *Example 8*

0132 This example demonstrates expression of exogenous acetolactate decarboxylase to increase flux toward 2,3-butanediol.

0133 Acetolactate decarboxylase genes from *A. hydrophila* and *L. lactis* were synthesized with codons selected for expression in *C. autoethanogenum* (GeneArt) and cloned into pMTL83159 as described above. The resulting plasmids, and pMTL83159 as a control, were transformed into *C. autoethanogenum* strain LZ1561 as described above.

0134 Strains were grown in 1-L Schott bottles containing 40 mL of PETC-MES medium with no yeast extract and the headspace was replaced with 1.5 bar(gauge) synthetic mill gas (50% CO, 29% N_2, 18% CO_2, and 3% H_2) as the carbon and energy source. To maintain the plasmid, 15mg L^{-1} thiamphenicol was added. Biomass and metabolite concentrations were recorded through the growth of the cultures.

0135 Expression of either exogenous acetolactate decarboxylase led to an increase of 2,3-butanediol production during growth on synthetic mill gas as compared to the strain harboring the empty plasmid as a control. Expression of *alsD* from *A. hydrophila* and *L. lactis* led to production of 2.3 ± 0.08 and 1.6 ± 0.16 g L^{-1} 2,3- butanediol, respectively, compared to a production of 0.3 ± 0.12 g L^{-1} by the empty-plasmid-control-strain (Fig. 8).

0136 All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein. The reference to any prior art in this specification is not, and should not be taken as, an acknowledgement that that prior art forms part of the common general knowledge in the field of endeavour in any country.

0137 The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to,") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

0138 Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible

variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

CLAIMS

1. A recombinant, carboxydotrophic *Clostridium* bacterium comprising one or more enzymes selected from the group consisting of pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1), acetolactate synthase (EC 2.2.1.6), and acetolactate decarboxylase (EC 4.1.1.5), wherein each enzyme is an overexpressed endogenous enzyme, a mutated endogenous enzyme, or an exogenous enzyme.

2. The bacterium of claim 1, comprising pyruvate:ferredoxin oxidoreductase and acetolactate synthase.

3. The bacterium of claim 1, comprising pyruvate:ferredoxin oxidoreductase and acetolactate decarboxylase.

4. The bacterium of claim 1, comprising acetolactate synthase and acetolactate decarboxylase.

5. The bacterium of claim 1, comprising pyruvate:ferredoxin oxidoreductase, acetolactate synthase, and acetolactate decarboxylase.

6. The bacterium of claim 1, wherein the enzyme is overexpressed endogenous pyruvate:ferredoxin oxidoreductase.

7. The bacterium of claim 1, wherein the enzyme is exogenous *Desulfovibrio africanus* pyruvate:ferredoxin oxidoreductase.

8. The bacterium of claim 1, wherein the enzyme is overexpressed endogenous IlvB ORF2059 acetolactate synthase, overexpressed endogenous IlvB ORF2336 acetolactate synthase, overexpressed endogenous IlvN acetolactate synthase, or overexpressed endogenous AlsS acetolactate synthase.

9. The bacterium of claim 1, wherein the enzyme is mutated endogenous IlvN acetolactate synthase.

10. The bacterium of claim 1, wherein the enzyme is exogenous *Bacillus subtilis* acetolactate synthase.

11. The bacterium of claim 1, wherein the enzyme is overexpressed endogenous AlsD acetolactate decarboxylase or overexpressed endogenous BudA acetolactate decarboxylase.

12. The bacterium of claim 1, wherein the enzyme is exogenous *Aeromonas hydrophila* acetolactate decarboxylase.

13. The bacterium of claim 1, wherein the enzyme is exogenous *Leuconostoc lactis* acetolactate decarboxylase.

14. The bacterium of claim 1, wherein the bacterium is derived from *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*.

15. The bacterium of claim 1, wherein the bacterium is derived from *Clostridium autoethanogenum*.

16. The bacterium of claim 1, wherein the bacterium is derived from *Clostridium autoethanogenum* deposited under DSMZ Accession No. DSM23693.

17. A method of producing a fermentation product, comprising fermenting the bacterium of claim 1 in the presence of a gaseous substrate comprising CO to produce a fermentation product.

18. The method of claim 17, wherein the fermentation product is selected from the group consisting of ethanol, butanol, isopropanol, isobutanol, higher alcohols, butanediol, 2,3-butanediol, succinate, isoprenoids, fatty acids, biopolymers, and mixtures thereof.

19. The method of claim 18, wherein the fermentation product is 2,3-butanediol.

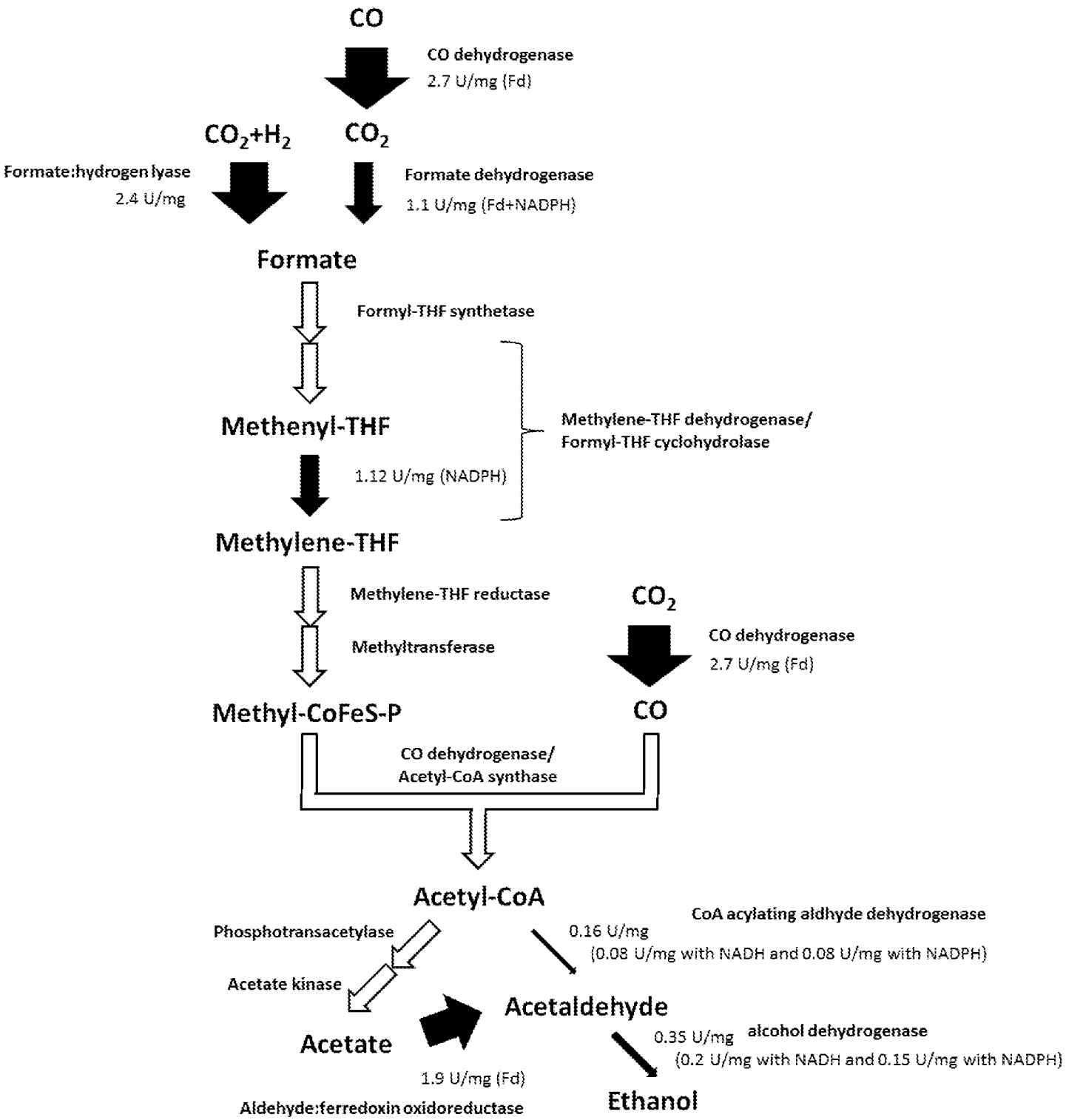

CO
CO dehydrogenase
2.7 U/mg (Fd)
CO2+H2
CO2
Formate:hydrogen lyase
2.4 U/mg
Formate dehydrogenase
1.1 U/mg (Fd+NADPH)
Formate
Formyl-THF synthetase
Methenyl-THF
Methylene-THF dehydrogenase/
Formyl-THF cyclohydrolase
1.12 U/mg (NADPH)
Methylene-THF
Methylene-THF reductase
Methyltransferase
CO2
CO dehydrogenase
2.7 U/mg (Fd)
Methyl-CoFeS-P
CO
CO dehydrogenase/
Acetyl-CoA synthase
Acetyl-CoA
CoA acylating aldhyde dehydrogenase
0.16 U/mg
(0.08 U/mg with NADH and 0.08 U/mg with NADPH)
Phosphotransacetylase
Acetate kinase
Acetaldehyde
Acetate
0.35 U/mg alcohol dehydrogenase
(0.2 U/mg with NADH and 0.15 U/mg with NADPH)
1.9 U/mg (Fd)
Aldehyde:ferredoxin oxidoreductase
Ethanol

FIG. 1

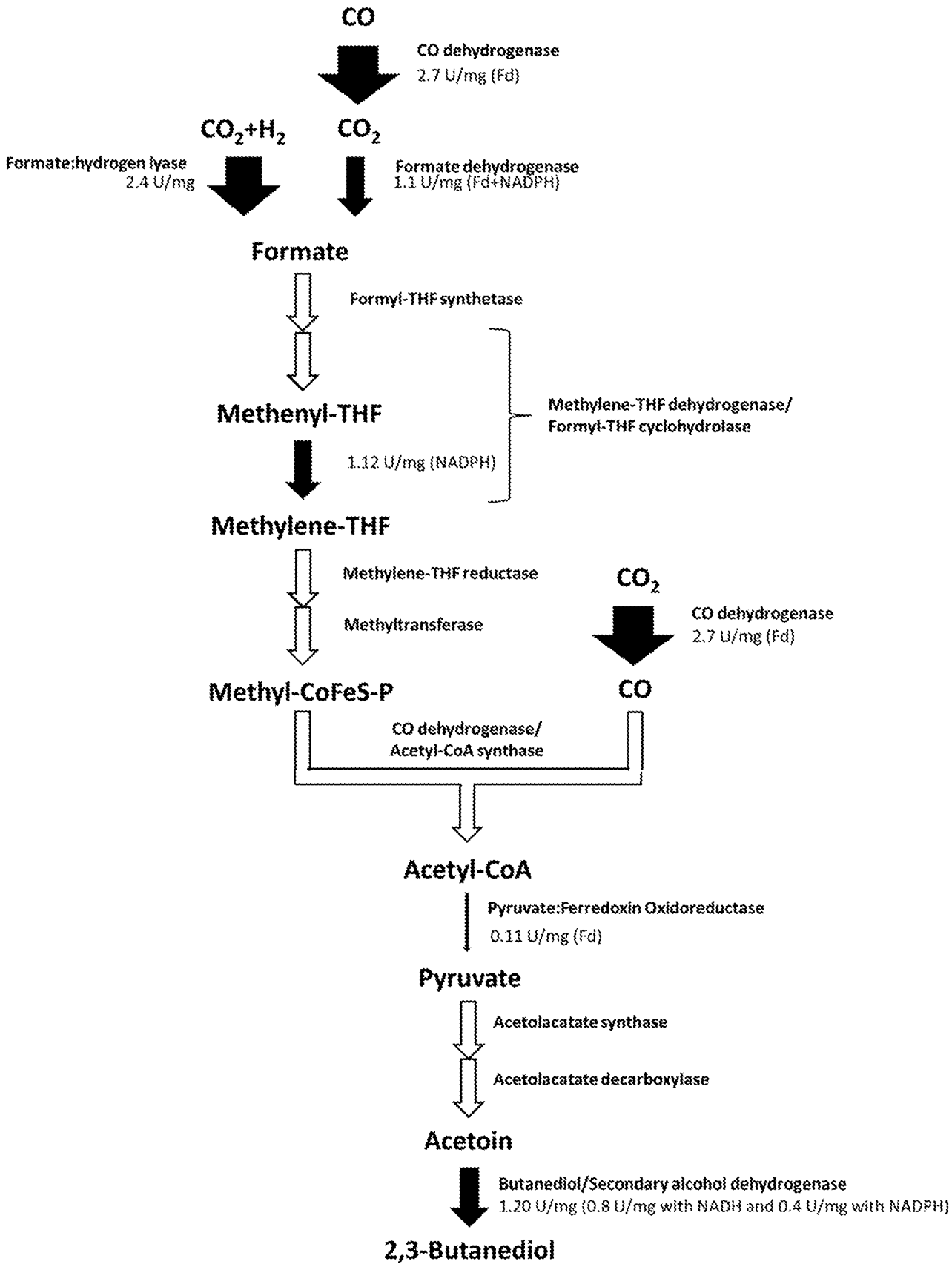

CO
CO dehydrogenase
2.7 U/mg (Fd)
CO_2+H_2
CO_2
Formate:hydrogen lyase
2.4 U/mg
Formate dehydrogenase
1.1 U/mg (Fd+NADPH)
Formate
Formyl-THF synthetase
Methenyl-THF
Methylene-THF dehydrogenase/
Formyl-THF cyclohydrolase
1.12 U/mg (NADPH)
Methylene-THF
Methylene-THF reductase
Methyltransferase
CO_2
CO dehydrogenase
2.7 U/mg (Fd)
Methyl-CoFeS-P
CO
CO dehydrogenase/
Acetyl-CoA synthase
Acetyl-CoA
Pyruvate:Ferredoxin Oxidoreductase
0.11 U/mg (Fd)
Pyruvate
Acetolacatate synthase
Acetolacatate decarboxylase
Acetoin
Butanediol/Secondary alcohol dehydrogenase
1.20 U/mg (0.8 U/mg with NADH and 0.4 U/mg with NADPH)
2,3-Butanediol

FIG. 2

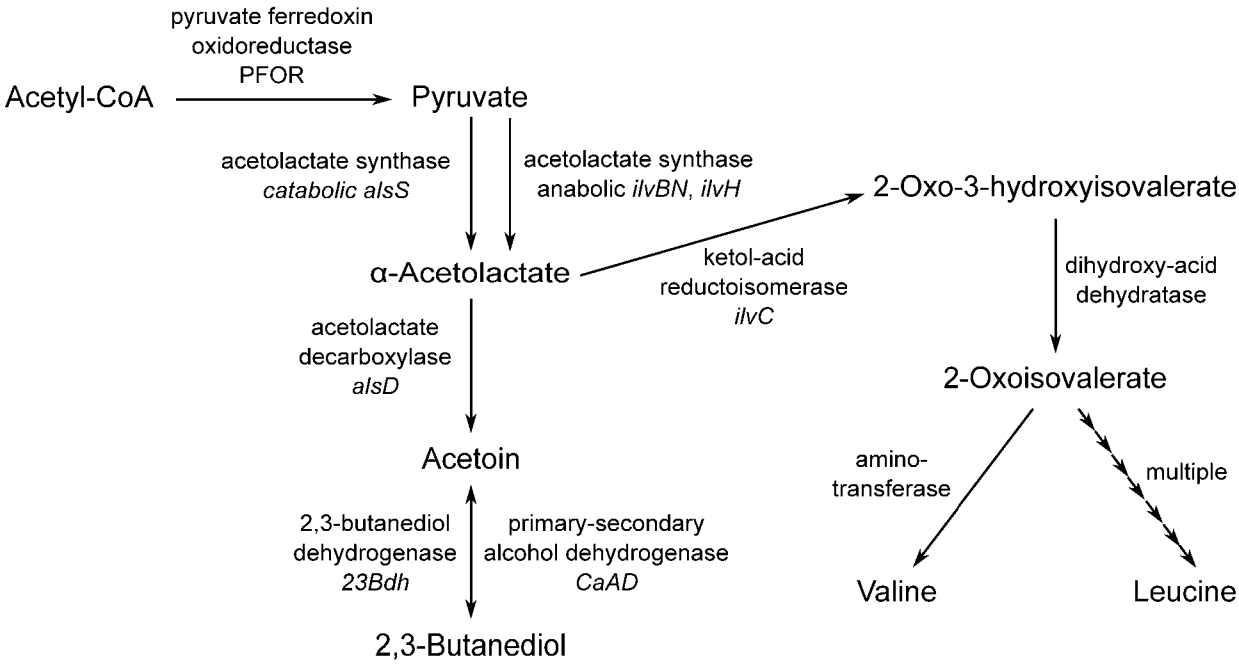

pyruvate ferredoxin oxidoreductase PFOR
Acetyl-CoA
Pyruvate
acetolactate synthase catabolic alsS
acetolactate synthase anabolic ilvBN, ilvH
α-Acetolactate
ketol-acid reductoisomerase ilvC
2-Oxo-3-hydroxyisovalerate
dihydroxy-acid dehydratase
2-Oxoisovalerate
acetolactate decarboxylase alsD
Acetoin
amino-transferase
multiple
Valine
Leucine
2,3-butanediol dehydrogenase 23Bdh
primary-secondary alcohol dehydrogenase CaAD
2,3-Butanediol

FIG. 3

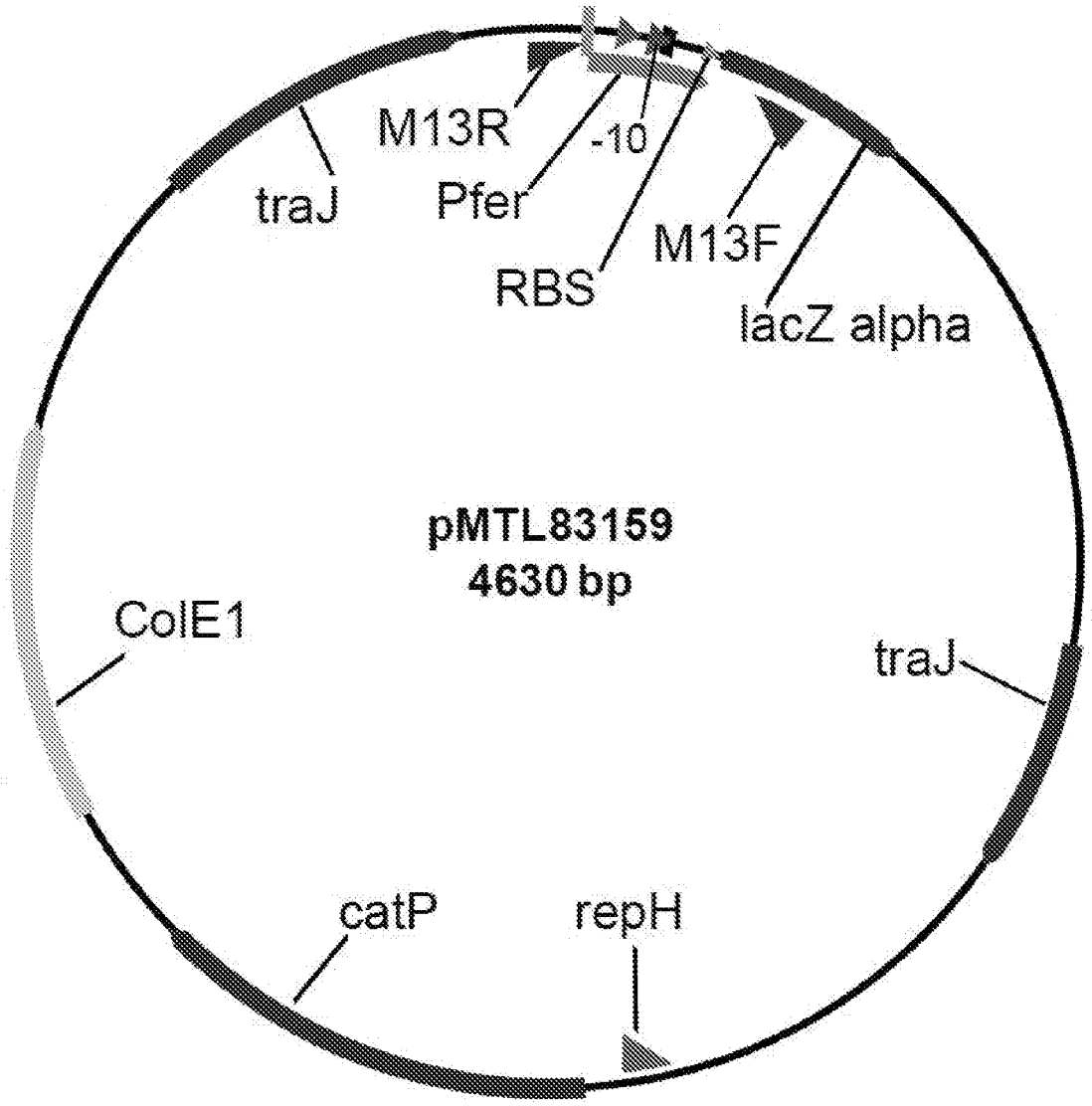

M13R
-10
traJ
Pfer
M13F
RBS
lacZ alpha
pMTL83159
4630 bp
ColE1
traJ
catP
repH

FIG. 4

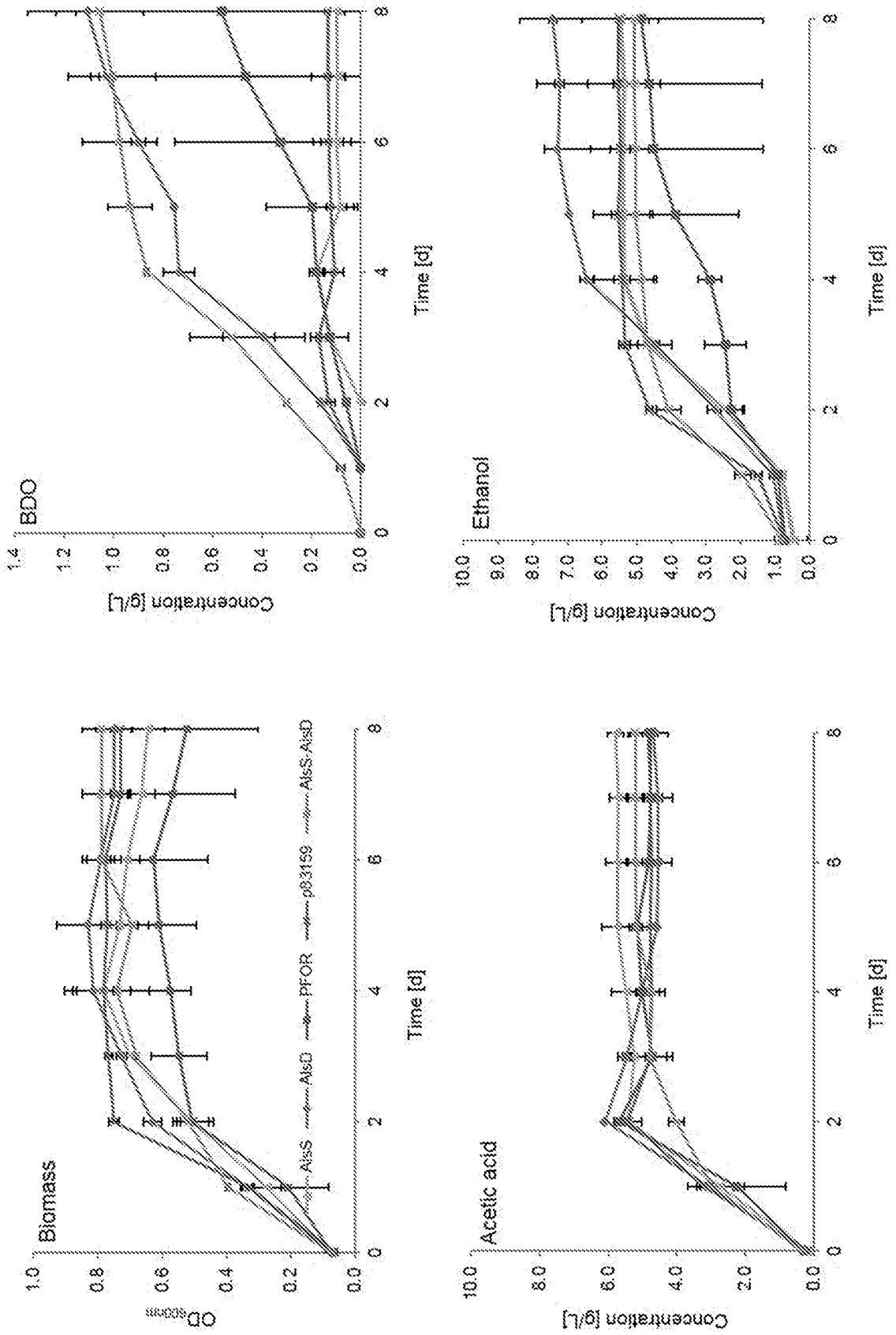

BDO
Concentration [g/L]
Time [d]
Ethanol
Concentration [g/L]
Time [d]
Biomass
OD600nm
Time [d]
AtoS
AtoD
PFOR
β3159
AtoS-AtoD
Acetic acid
Concentration [g/L]
Time [d]

FIG. 5

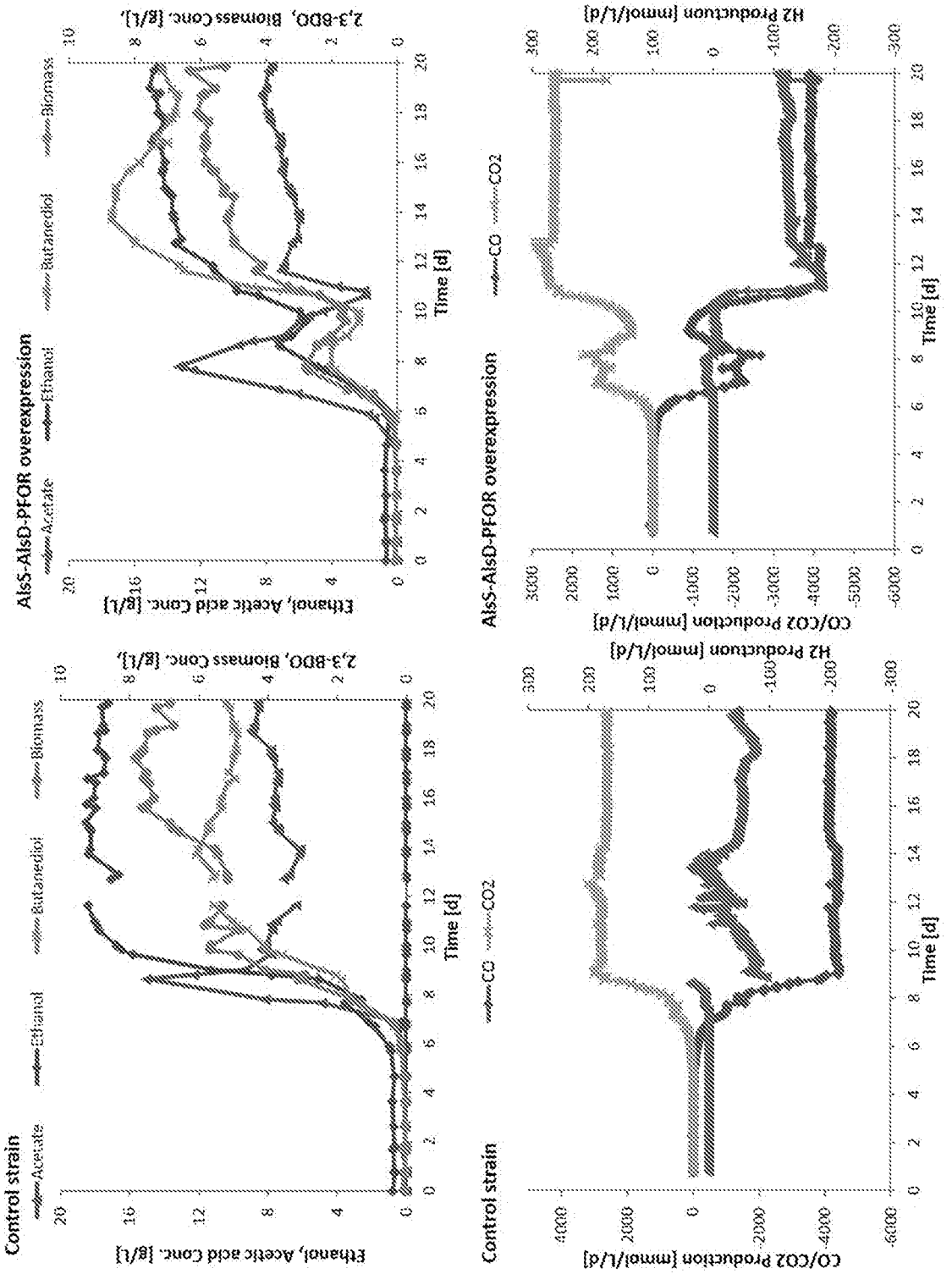

Control strain
AlsS-AlsD-PFOR overexpression
Acetate
Ethanol
Butanediol
Biomass
CO
CO2
Ethanol, Acetic acid Conc. [g/L]
2,3-BDO, Biomass Conc. [g/L],
Time [d]
CO/CO2 Production [mmol/L/d]
H2 Production [mmol/L/d]

FIG. 6

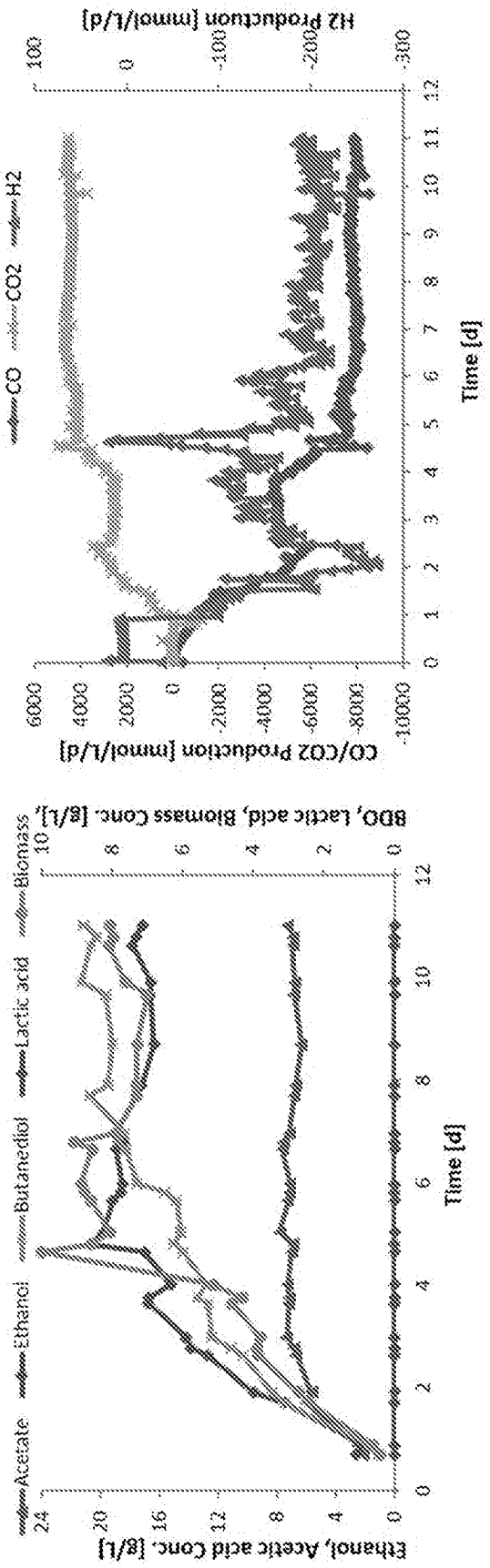

CO
CO2
H2
CO/CO2 Production [mmol/L/d]
H2 Production [mmol/L/d]
Time [d]
Acetate
Ethanol
Butanediol
Lactic acid
Biomass
Ethanol, Acetic acid Conc. [g/L]
BDO, Lactic acid, Biomass Conc. [g/L]
Time [d]

FIG. 7

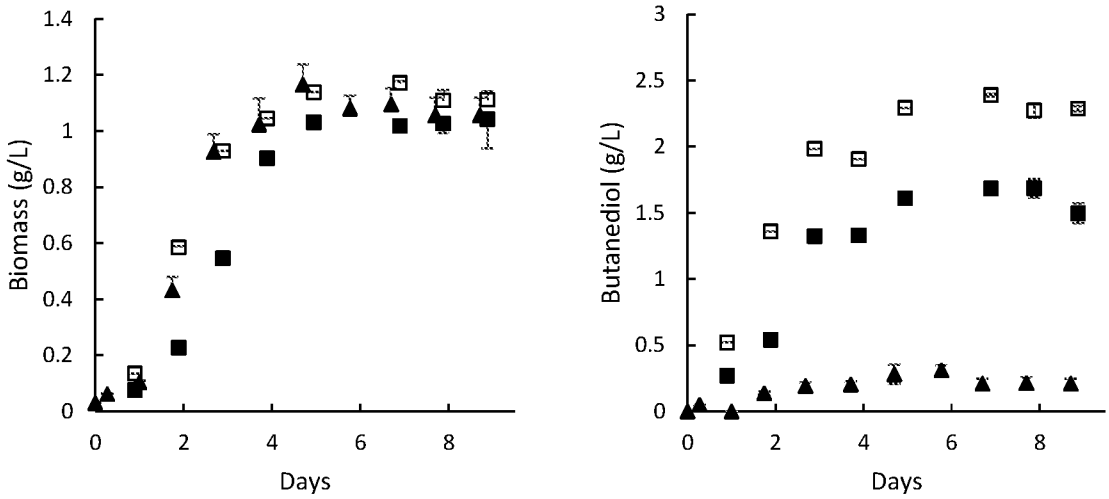

Biomass (g/L)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
Days
Butanediol (g/L)
3
2.5
2
1.5
1
0.5
0
0
2
4
6
8
Days

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/064351

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/74(2006.01)i, C12N 9/02(2006.01)i, C12N 9/10(2006.01)i, C12N 9/88(2006.01)i, C12P 1/04(2006.01)i, C12P 7/18(2006.01)i, C12R 1/145(2006.01)n

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 15/74; C12P 5/02; C12P 7/16; C12P 7/42; C12P 7/04; C12N 1/21; C12P 7/06; C12N 9/02; C12N 9/10; C12N 9/88; C12P 1/04; C12P 7/18; C12R 1/145

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: carboxydotrophic Clostridium, pyruvate:ferredoxin oxidoreductase, acetolactate synthase, acetolactate decarboxylase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2014-0256008 A1 (BOISART, SR., CEDRIC et al.) 11 September 2014 See claim 1; paragraphs [0053], [0099] and [0122].	1,8-10
Y		17-19
A		2-7,11-16
Y	KOPKE, MICHAEL et al., `2,3-butanediol production by acetogenic bacteria, an alternative route to chemical synthesis, using industrial waste gas`, Applied and Environmental Microbiology, 17 June 2011, Vol. 77, No. 15, pp. 5467-7475 See page 5470; abstract.	17-19
A	US 2013-0210090 A1 (PEARLMAN, PAUL S. et al.) 15 August 2013 See claims 1, 7 and 19.	1-19
A	US 2009-0203097 A1 (FLINT, DENNIS et al.) 13 August 2009 See claims 1, 4 and 16.	1-19
PX	WO 2014-197746 A1 (LANZATECH NEW ZEALAND LIMITED) 11 December 2014 See claims 1, 5 and 14-15; paragraph [0103].	1-6,10,14-15,17-19

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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

Date of the actual completion of the international search	Date of mailing of the international search report
30 March 2016 (30.03.2016)	**31 March 2016 (31.03.2016)**

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2015/064351

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 8852899 B2	07/10/2014
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		WO 2012-174430 A3	07/03/2013
US 2009-0203097 A1	13/08/2009	US 8518678 B2	27/08/2013
		WO 2009-082690 A1	02/07/2009
WO 2014-197746 A1	11/12/2014	AU 2014-274838 A1	11/12/2014
		US 2015-079650 A1	19/03/2015