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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 vol. 77, no. 15, 17 June 2011, pages 5467 - 7475, XP055104754

DESCRIPTION

BACKGROUND

[0001] 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

[0002] 0003 The invention provides a recombinant, carboxydotrophic *Clostridium* bacterium comprising:

1. (a) a pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1);
2. (b) an acetolactate decarboxylase (EC 4.1.1.5);
3. (c) a pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1) and an acetolactate synthase (EC 2.2.1.6);
4. (d) a pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1) and an acetolactate decarboxylase (EC 4.1.1.5);
5. (e) an acetolactate synthase (EC 2.2.1.6) and an acetolactate decarboxylase (EC 4.1.1.5); or
6. (f) a pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1), an acetolactate synthase (EC 2.2.1.6), and an acetolactate decarboxylase (EC 4.1.1.5);

wherein each enzyme is an overexpressed endogenous enzyme or an exogenous enzyme; and

wherein the bacterium is selected from the group consisting of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei*.

[0003] 0004 In a preferred embodiment, the recombinant bacterium is derived from *C. autoethanogenum* deposited under DSMZ Accession No. DSM23693 (*C. autoethanogenum* LZ1561).

[0004] 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, 2,3-butanediol, succinate, isoprenoids, fatty acids, biopolymers, and mixtures thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0005]

0006 Fig. 1 is a flux map of the ethanol biosynthesis pathway detailing the measured enzyme activities and flux through a carboxydutrophic 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 carboxydutrophic 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

[0006] 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.

[0007] 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).

[0008] 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.

[0009] 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.

[0010] 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.

[0011] 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 (B₁₂) 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, 2,3-butanediol, succinate, isoprenoids, fatty acids, biopolymers, and mixtures thereof.

[0012] 0020 The invention provides a recombinant, carboxydophilic *Clostridium* bacterium comprising:

1. (a) a pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1);
2. (b) an acetolactate decarboxylase (EC 4.1.1.5);
3. (c) a pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1) and an acetolactate synthase (EC 2.2.1.6);
4. (d) a pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1) and an acetolactate decarboxylase (EC 4.1.1.5);
5. (e) an acetolactate synthase (EC 2.2.1.6) and an acetolactate decarboxylase (EC 4.1.1.5); or
6. (f) a pyruvate:ferredoxin oxidoreductase (EC 1.2.7.1), an acetolactate synthase (EC 2.2.1.6), and an acetolactate decarboxylase (EC 4.1.1.5);

wherein each enzyme is an overexpressed endogenous enzyme or an exogenous enzyme; and

wherein the bacterium is selected from the group consisting of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei*. In a preferred embodiment, the recombinant bacterium is derived from *C. autoethanogenum* deposited under DSMZ Accession No. DSM23693 (*C. autoethanogenum* LZ1561).

[0013] 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.

[0014] 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.

[0015] 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.

[0016] 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.

[0017] 0025 Nucleic acids and nucleic acid constructs, including expression constructs/vectors 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.

[0018] 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 (Murray, *Microbiol Molec Biol Rev*, 64: 412-434, 2000). Additional vectors include include plasmids, viruses (including bacteriophage), cosmids, and artificial chromosomes. Nucleic acids may be 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.

[0019] 0027 The use of electroporation has been reported for several carboxydophilic 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.

[0020] 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*, and *C. ragsdalei*.

[0021] 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.

[0022] 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 carboxydophilic, ethanol-synthesizing *Clostridia* and it can be anticipated that mechanisms work similarly across these strains, although there may be differences in performance.

[0023] 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).

[0024] 0032 The enzymes and genes used in the invention may be overexpressed endogenous enzymes and genes, or exogenous enzymes and genes.

[0025] 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. The expression of an endogenous gene may be controlled by an exogenous regulatory element, such as an exogenous promoter.

[0026] 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. An exogenous gene or enzyme may be derived from a heterologous strain or species and introduced to or expressed in the recombinant bacterium. 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.

[0027] 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.

[0028] 0036 The genes and enzymes used in 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.

[0029] 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. The mutation may be a deletion, insertion, or substitution in a gene encoding an enzyme. The mutation may be a deletion, insertion, or substitution of one or more amino acids in an enzyme.

[0030] 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.

The genes encoding the enzymes may be codon optimized for expression in *Clostridium*, particularly *C. autoethanogenum*, *C. ljungdahlii*, and/or *C. ragsdalei*. The genes encoding the enzymes may be codon optimized for expression in *C. autoethanogenum* LZ1561.

[0031] 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.

[0032] 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 transcribed 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.

[0033] 0041 "Mutated endogenous enzyme" refers to an endogenous enzyme that is mutated or modified in the recombinant bacterium of the disclosure compared to the wild-type or parental bacterium from which the recombinant bacterium of the disclosure is derived. Similarly, "mutated endogenous gene" refers to an endogenous gene that is mutated or modified in the recombinant bacterium of the disclosure compared to the wild-type or parental bacterium from which the recombinant bacterium of the disclosure is derived.

[0034] 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.

[0035] 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.

[0036] 0044 It may be necessary to methylate a nucleic acid before introduction of the nucleic acid into a microorganism.

[0037] 0045 Generally, methylation is performed using a shuttle microorganism, preferably a restriction negative shuttle microorganism, such as *E. coli*, *B. subtilis*, 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, for example, the methylation construct/vector may comprise 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. The methylation construct/vector promoter may be a constitutive promoter.

[0038] 0046 The methylation construct/vector may have 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.

[0039] 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. Both construct/vector may be 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.

[0040] 0048 A methyltransferase gene may be introduced into a shuttle microorganism and overexpressed. Thus, 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. The methyltransferase gene may be 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.

[0041] 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. The expression construct/vector and/or the methylation construct/vector may be plasmids. A number of suitable methyltransferases may be used, including, for example, *B. subtilis* phage Φ T1 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.

[0042] 0050 By way of example, 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. The methyltransferase gene of step (b) may be expressed constitutively. Expression of the methyltransferase gene of step (b) may be induced.

[0043] 0051 The recombinant bacterium of the invention comprises one or more of pyruvate:ferredoxin oxidoreductase, and/or acetolactate decarboxylase, and in addition optionally acetolactate synthase.

[0044] 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: $\text{pyruvate} + \text{CoA} + 2 \text{ oxidized ferredoxin} \leftrightarrow \text{acetyl-CoA} + \text{CO}_2 + 2 \text{ reduced ferredoxin} + 2 \text{ H}^+$. 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.

[0045] 0053 The pyruvate:ferredoxin oxidoreductase enzyme used in the invention may be an overexpressed endogenous enzyme, a mutated endogenous enzyme, or an exogenous enzyme. Similarly, the pyruvate:ferredoxin oxidoreductase enzyme used in 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. The pyruvate:ferredoxin oxidoreductase enzyme may be 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. The pyruvate:ferredoxin oxidoreductase enzyme may be oxygen stable or demonstrates at least some degree of oxygen insensitivity. The pyruvate:ferredoxin oxidoreductase enzyme may be 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.

[0046] 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: $2 \text{ CH}_3\text{COCOO}^- \leftrightarrow \text{CH}_3\text{COCOHCH}_3\text{COO}^- + \text{CO}_2$. Acetolactate synthase may also be known as acetohydroxy acid synthase.

[0047] 0055 The acetolactate synthase enzyme used in the invention may be an overexpressed endogenous enzyme, a mutated endogenous enzyme, or an exogenous enzyme. Similarly, the acetolactate synthase enzyme used in 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. The acetolactate synthase enzyme may be 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. The acetolactate synthase enzyme may be 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. The acetolactate synthase enzyme may be 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.

[0048] 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 CO₂: (S)-2-hydroxy-2-methyl-3-oxobutanoate $\leftrightarrow$ (R)-2-acetoin + CO₂. Acetolactate decarboxylase may also be known as alpha-acetolactate decarboxylase or (S)-2-hydroxy-2-methyl-3-oxobutanoate carboxy-lyase.

[0049] 0057 The acetolactate decarboxylase enzyme used in the invention may be an overexpressed endogenous enzyme, a mutated endogenous enzyme, or an exogenous enzyme. Similarly, the acetolactate decarboxylase enzyme used in 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. The acetolactate decarboxylase enzyme may be 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. The acetolactate decarboxylase enzyme may be 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.

[0050] 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, <i>C. autoethanogenum</i> LZ1561, nucleic acid sequence
2	native pyruvate:ferredoxin oxidoreductase, <i>C. autoethanogenum</i> LZ1561, amino acid sequence
3	codon-optimized pyruvate:ferredoxin oxidoreductase with XbaI and NheI, <i>C. autoethanogenum</i> LZ1561, nucleic acid sequence
4	codon-optimized pyruvate:ferredoxin oxidoreductase, <i>C. autoethanogenum</i> LZ1561, amino acid sequence
5	pyruvate:ferredoxin oxidoreductase with XbaI and NheI, <i>D. africanus</i> , nucleic acid sequence
6	pyruvate:ferredoxin oxidoreductase, <i>D. africanus</i> , amino acid sequence
7	native IlvB ORF2059 acetolactate synthase, <i>C. autoethanogenum</i> LZ1561, nucleic acid sequence
8	native IlvB ORF2059 acetolactate synthase, <i>C. autoethanogenum</i> LZ1561, amino acid sequence
9	native IlvB ORF2336 acetolactate synthase, <i>C. autoethanogenum</i> LZ1561, nucleic acid sequence
10	native IlvB ORF2336 acetolactate synthase, <i>C. autoethanogenum</i> LZ1561, amino acid sequence
11	native IlvN acetolactate synthase (regulatory subunit) with NdeI and SacI, <i>C. autoethanogenum</i> LZ1561, nucleic acid sequence
12	native IlvN acetolactate synthase (regulatory subunit), <i>C. autoethanogenum</i> LZ1561, amino acid sequence
13	mutant IlvN (G-10-D) acetolactate synthase (regulatory subunit) with NdeI and SacI, <i>C. autoethanogenum</i> LZ1561, nucleic acid sequence
14	mutant IlvN (G-10-D) acetolactate synthase (regulatory subunit), <i>C. autoethanogenum</i> LZ1561, amino acid sequence
15	native AlsS acetolactate synthase, <i>C. autoethanogenum</i> LZ1561, nucleic acid sequence
16	native AlsS acetolactate synthase, <i>C. autoethanogenum</i> LZ1561, amino acid sequence
17	codon-optimized AlsS acetolactate synthase with NdeI and SacI, <i>C. autoethanogenum</i> LZ1561, nucleic acid sequence

SEQ ID NO:	Description
18	codon-optimized AlsS acetolactate synthase, <i>C. autoethanogenum</i> LZ1561, amino acid sequence
19	acetolactate synthase with NdeI and SacI, <i>B. subtilis</i> , nucleic acid sequence
20	acetolactate synthase, <i>B. subtilis</i> , amino acid sequence
21	native acetolactate decarboxylase, <i>C. autoethanogenum</i> LZ1561, nucleic acid sequence
22	native acetolactate decarboxylase, <i>C. autoethanogenum</i> LZ1561, amino acid sequence
23	codon-optimized acetolactate decarboxylase with SacI and KpnI, <i>C. autoethanogenum</i> LZ1561, nucleic acid sequence
24	codon-optimized acetolactate decarboxylase, <i>C. autoethanogenum</i> LZ1561, amino acid sequence
25	acetolactate decarboxylase, <i>A. hydrophila</i> , nucleic acid sequence
26	acetolactate decarboxylase, <i>A. hydrophila</i> , amino acid sequence
27	acetolactate decarboxylase with SacI and KpnI, <i>L. lactis</i> , nucleic acid sequence
28	acetolactate decarboxylase, <i>L. lactis</i> , amino acid sequence

[0051] 0059 The recombinant bacterium of the invention may also comprise any combination of pyruvate:ferredoxin oxidoreductase, and/or acetolactate decarboxylase, and in addition optionally acetolactate synthase. 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.

[0052] 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:secondary 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).

[0053] 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.

[0054] 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. The co-factor may comprise tetrahydrofolate. Enzymes that are involved in the biosynthesis of tetrahydrofolate are detailed below. The recombinant microorganism may exhibit 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.-). The co-factor may comprise cobalamine (B₁₂). Enzymes that are involved in the biosynthesis of cobalamine are detailed below. The recombinant microorganism may exhibit 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), cobaltochelataase CobN (EC 6.6.1.2), cobyric 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.

[0055] 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, 2,3-butanediol, succinate, isoprenoids, fatty acids, biopolymers, and mixtures thereof.

[0056] 0064 The substrate comprising CO may be a gaseous substrate comprising CO. 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. The substrate may comprise about 25%, about 30%, about 35%, about 40%, about 45%, about 50% CO, about 55% CO, or about 60% CO by volume.

[0057] 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. The presence of hydrogen may result in an improved overall efficiency of alcohol production. For example, the substrate may comprise an approx. 2:1, or 1:1, or 1:2 ratio of H₂:CO. The substrate may comprise about 30% or less H₂ by volume, 20% or less H₂ by volume, about 15% or less H₂ by volume or about 10% or less H₂ by volume. The substrate stream may comprise low concentrations of H₂, for example, less than 5%, less than 4%, less than 3%, less than 2%, or less than 1% H₂. The substrate stream may be substantially hydrogen-free. The substrate may also contain some CO₂, for example, about 1% to about 80% CO₂ by volume, or 1% to about 30% CO₂ by volume.

The substrate may comprise less than or equal to about 20% CO₂ by volume. The substrate may comprise less than or equal to about 15% CO₂ by volume, less than or equal to about 10% CO₂ by volume, less than or equal to about 5% CO₂ by volume, or substantially no CO₂.

[0058] 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.

[0059] 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. The industrial process may be 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. 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 CO₂ and therefore the invention has particular utility in reducing CO₂ 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.

[0060] 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. 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.

[0061] 0069 A culture of a bacterium of the invention may be 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.

[0062] 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.

[0063] 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.

[0064] 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.

[0065] 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.

[0066] 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, O₂ 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

[0067] 0075 The following examples further illustrate the invention but, of course, should not be construed to limit its scope in any way.

Example 1

[0068] 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.

[0069] 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, CO₂, and H₂ gases, as well as product formation.

[0070] 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.

[0071] 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, CaCl₂ (0.5 mM), KCl (2 mM), H₃PO₄ (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% H₂, 42% CO, 20% CO₂, and 36% N₂. The pH of the culture was maintained between 5 and 5.2.

[0072] 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 H₂ 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 K₂CO₃ 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 PO₄ 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 N₂ before assaying.

[0073] 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 N₂ or H₂ or CO at 1.2 × 10⁵ 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 ($\epsilon = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$) or at 380 nm ($\epsilon = 1.2 \text{ mM}^{-1} \text{ cm}^{-1}$), *C. pasteurianum* ferredoxin reduction at 430 nm ($\epsilon_{\Delta\text{ox-red}} \approx 13.1 \text{ mM}^{-1} \text{ cm}^{-1}$), methyl viologen reduction at 578 nm ($\epsilon = 9.8 \text{ mM}^{-1} \text{ cm}^{-1}$) and benzyl viologen reduction at 578 nm ($\epsilon = 8.6 \text{ mM}^{-1} \text{ cm}^{-1}$).

[0074] 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.

[0075] 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% H₂.

[0076] 0084 Formate-hydrogen lyase activity for reduction of CO₂ with H₂ to formate was measured with an assay mixture containing 100 mM potassium phosphate, 2 mM DTT, and 30 mM [¹⁴C]K₂CO₃ (24,000 dpm/µmol). The gas phase was 100% H₂. 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 ¹⁴CO₂ leaving behind the ¹⁴C-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 ¹⁴C radioactivity in a Beckman LS6500 liquid scintillation counter (Fullerton, CA).

[0077] 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% N₂.

[0078] 0086 Methylene-H₄F 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% N₂.

[0079] 0087 Methylene-H₄F 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.

[0080] 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% N₂.

[0081] 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% N₂.

[0082] 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% N₂.

[0083] 0091 Ferredoxin was purified from *C. pasteurianum* as described by Schönheit, FEBS Lett, 89: 219-222, 1978.

[0084] 0092 All oxidoreductase reactions in the pathways to ethanol and 2,3-butanediol of carboxydophilic 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

[0085] 0093 This example demonstrates increasing the flux through a fermentation pathway.

[0086] 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

[0087] 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.

[0088] 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

[0089] 0097 This example demonstrates increasing the flux through the 2,3-butanediol production pathway by removing bottlenecks.

[0090] 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*.

[0091] 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.

[0092] 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

[0093] 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.

[0094] 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.

[0095] 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.

[0096] 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.

[0097] 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

[0098] 0106 This example describes a metabolic engineering approach to overexpression of pyruvate:ferredoxin oxidoreductase, acetolactate synthase, and/or acetolactate decarboxylase.

[0099] 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.

[0100] 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.

[0101] 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.

[0102] 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).

[0103] 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).

[0104] 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).

[0105] 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 subtilis* and two heterologous *alsD* genes isolated from *Leuconostoc lactis* and from *Aeromonas hydrophila* were also tested. The *alsS* gene isolated from *Bacillus subtilis* 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 heterologous *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

[0106] 0113 This example describes cloning, conjugation, and characterization of strains overexpressing pyruvate:ferredoxin oxidoreductase, acetolactate synthase, and/or acetolactate decarboxylase.

[0107] 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*.

[0108] 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.

[0109] 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 ZYPHY™ 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 μ L 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.

[0110] 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.

[0111] 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.

[0112] 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	+	-

[0113] 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.

[0114] 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.

[0115] 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.

[0116] 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.

[0117] 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} .

[0118] 0125 The metabolite and gas profile of the combined PFOR, *a/sS*, and *a/sD* 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).

[0119] 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.

[0120] 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 .

[0121] 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.

[0122] 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
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

[0123] 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

[0124] 0132 This example demonstrates expression of exogenous acetolactate decarboxylase to increase flux toward 2,3-butanediol.

[0125] 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.

[0126] 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% N₂, 18% CO₂, and 3% H₂) 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.

[0127] 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).

[0128] 0136 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.

[0129] 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.

[0130] 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. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the disclosure unless otherwise indicated herein or otherwise clearly contradicted by context.

SEQUENCE LISTING

[0131]

<110> LanzaTech New Zealand Limited

<120> RECOMBINANT MICROORGANISMS EXHIBITING INCREASED FLUX THROUGH A FERMENTATION PATHWAY

<130> LT113WO1

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<213> Clostridium autoethanogenum LZ1561

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<211> 1170

<212> PRT

<213> Clostridium autoethanogenum LZ1561

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Asn	Ile	Phe	Gly	Gln	Gln	Val	Lys	Val	Met	Glu	Met	Gln	Ser	Glu	Ala
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Tyr	Lys	Ile	Ala	Gly	Glu	Leu	Leu	Pro	Gly	Val	Phe	His	Val	Ser	Ala
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Arg	Ala	Val	Ala	Ala	Asn	Ser	Leu	Asn	Ile	Phe	Gly	Asp	His	Gln	Asp
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Lys	Gly	Arg	Val	Pro	Phe	Val	Asn	Phe	Phe	Asp	Gly	Phe	Arg	Thr	Ser
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His	Glu	Ile	Gln	Lys	Ile	Glu	Leu	Leu	Glu	Tyr	Glu	Glu	Leu	Ala	Lys
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Leu	Val	Asp	Gln	Lys	Ala	Leu	Asn	Asp	Phe	Arg	Arg	Arg	Ala	Leu	Asn
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Pro	Asp	His	Pro	Val	Thr	Arg	Gly	Thr	Ala	Gln	Asn	Pro	Asp	Ile	Tyr
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Phe	Gln	Glu	Arg	Glu	Val	Ser	Asn	Thr	Tyr	Tyr	Glu	Ala	Leu	Pro	Glu
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Ile	Val	Glu	Gly	Tyr	Met	Gln	Glu	Met	Thr	Lys	Leu	Thr	Gly	Arg	Glu
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Tyr	His	Leu	Phe	Asn	Tyr	Tyr	Gly	Ala	Lys	Asp	Ala	Glu	Arg	Ile	Ile
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Lys Lys Ile Ala Val Leu Asp Arg Thr Lys Glu Pro Gly Ser Ile Gly
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Glu Pro Leu Tyr Leu Asp Val Lys Asn Ala Phe Tyr Gly Gln Glu Val
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Gln Pro Val Ile Val Gly Gly Arg Tyr Gly Leu Gly Ser Lys Asp Val
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Pro Lys Asp Arg Phe Thr Leu Ser Ile Val Asp Asp Val Thr Asn Thr
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His Leu Arg Phe Gly Lys Ser Pro Ile Lys Ser Pro Tyr Leu Ile Asp
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Lys Ala Asp Phe Val Ala Val His Asn Gln Ser Tyr Val His Lys Tyr
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Asp Val Leu Ala Gly Leu Lys Lys Gly Gly Asn Phe Leu Leu Asn Thr
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Val Trp Thr Gln Glu Glu Leu Glu Lys Glu Leu Pro Ala Ser Met Lys
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Lys Tyr Ile Ala Asn Asn Asp Ile Lys Phe Tyr Thr Leu Asn Ala Val
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Lys Ile Ala Gln Glu Ile Gly Leu Gly Gly Arg Ile Asn Met Ile Cys
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 Glu Ala Ala Ala Thr Lys Glu Leu Pro Lys Phe Ile Glu Lys Ile Val
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 Asn Pro Met Asn Arg Gln Glu Gly Asp Lys Leu Pro Val Ser Ala Phe
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 Val Gly Met Glu Asp Gly Thr Phe Pro Ala Gly Thr Ala Ala Tyr Glu
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 Lys Arg Gly Ile Ala Ile Asn Val Pro Glu Trp Gln Val Asp Lys Cys
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Ser Leu Phe Glu Asp Asn Ala Glu Tyr Gly Leu Gly Met Phe Leu Gly
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Gly Gly Asp Gly Trp Gly Tyr Asp Ile Gly Tyr Gly Gly Val Asp His
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Asp Leu Gly Met Met Ala Met Ser Tyr Gly Tyr Val Tyr Val Ala
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Gln Ile Ser Met Gly Ala Asp Lys Asn Gln Ala Leu Lys Ala Ile
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His Glu Ala Glu Ala Tyr His Gly Pro Ser Leu Ile Ile Ala Tyr
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Ala Pro Cys Ile Asn His Gly Leu Arg Val Gly Met Gly Lys Ser
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Gln Arg Glu Ala Lys Arg Ala Val Asp Cys Gly Tyr Trp Ala Leu
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Tyr Arg Tyr Asn Pro Glu Leu Lys Glu Glu Gly Lys Lys Ser Phe
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oxidoreductase

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<211> 1170

<212> PRT

<213> Artificial sequence

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<223> Codon-optimized Clostridium autoethanogenum LZ1561 pyruvate:ferredoxin

oxidoreductase

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Asn Ile Phe Gly Gln Gln Val Lys Val Met Glu Met Gln Ser Glu Ala
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Ser Thr Tyr Thr Ala Ser Gln Gly Leu Leu Leu Met Ile Pro Asn Met
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Tyr Lys Ile Ala Gly Glu Leu Leu Pro Gly Val Phe His Val Ser Ala
 100 105 110

Arg Ala Val Ala Ala Asn Ser Leu Asn Ile Phe Gly Asp His Gln Asp
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Val Met Ala Thr Arg Gln Thr Gly Phe Ala Leu Phe Ala Glu Ser Ser
 130 135 140

Val Gln Gln Val Met Asp Leu Ser Ala Val Ala His Leu Ser Ala Ile
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Lys Gly Arg Val Pro Phe Val Asn Phe Phe Asp Gly Phe Arg Thr Ser
 165 170 175

His Glu Ile Gln Lys Ile Glu Leu Leu Glu Tyr Glu Glu Leu Ala Lys
 180 185 190

Leu Val Asp Gln Lys Ala Leu Asn Asp Phe Arg Arg Arg Ala Leu Asn
 195 200 205

Pro Asp His Pro Val Thr Arg Gly Thr Ala Gln Asn Pro Asp Ile Tyr
 210 215 220

Phe Gln Glu Arg Glu Val Ser Asn Thr Tyr Tyr Glu Ala Leu Pro Glu
 225 230 235 240

Ile Val Glu Gly Tyr Met Gln Glu Met Thr Lys Leu Thr Gly Arg Glu
 245 250 255

Tyr His Leu Phe Asn Tyr Tyr Gly Ala Lys Asp Ala Glu Arg Ile Ile
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Ile Ala Met Gly Ser Val Cys Glu Thr Val Glu Glu Val Ile Asp Tyr
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Leu Thr Thr Lys Gly Glu Lys Val Gly Leu Leu Thr Val His Leu Tyr
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 Gln Pro Val Ile Val Gly Gly Arg Tyr Gly Leu Gly Ser Lys Asp Val
 355 360 365
 Leu Pro Ser His Ile Leu Pro Val Phe Glu Asn Leu Lys Ser Asp Lys
 370 375 380
 Pro Lys Asp Arg Phe Thr Leu Ser Ile Val Asp Asp Val Thr Asn Thr
 385 390 395 400
 Ser Leu Pro Val Gly Glu Asp Ile Asn Thr Thr Pro Glu Gly Thr Thr
 405 410 415
 Ala Cys Lys Phe Trp Gly Leu Gly Ser Asp Gly Thr Val Gly Ala Asn
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 Lys Ser Ala Ile Lys Ile Ile Gly Asp His Thr Asp Met Tyr Ala Gln
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 Gly Tyr Phe Ala Tyr Asp Ser Lys Lys Ser Gly Gly Ile Thr Val Ser
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 His Leu Arg Phe Gly Lys Ser Pro Ile Lys Ser Pro Tyr Leu Ile Asp
 465 470 475 480
 Lys Ala Asp Phe Val Ala Val His Asn Gln Ser Tyr Val His Lys Tyr
 485 490 495
 Asp Val Leu Ala Gly Leu Lys Lys Gly Gly Asn Phe Leu Leu Asn Thr
 500 505 510
 Val Trp Thr Gln Glu Glu Leu Glu Lys Glu Leu Pro Ala Ser Met Lys
 515 520 525
 Lys Tyr Ile Ala Asn Asn Asp Ile Lys Phe Tyr Thr Leu Asn Ala Val
 530 535 540
 Lys Ile Ala Gln Glu Ile Gly Leu Gly Gly Arg Ile Asn Met Ile Cys
 545 550 555 560
 Gln Ser Ala Phe Phe Lys Ile Ala Asn Ile Ile Pro Ile Asp Asp Ala
 565 570 575
 Val Lys Tyr Leu Lys Glu Ala Val Val Thr Ser Tyr Gly Lys Lys Gly
 580 585 590

Gln Lys Val Val Asp Met Asn Asn Ala Ala Ile Asp Lys Gly Val Asn
595 600 605

Ala Val Val Lys Ile Asp Val Pro Ala Ser Trp Lys Asp Ala Glu Asp
610 615 620

Glu Ala Ala Ala Thr Lys Glu Leu Pro Lys Phe Ile Glu Lys Ile Val
625 630 635 640

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Val Gly Met Glu Asp Gly Thr Phe Pro Ala Gly Thr Ala Ala Tyr Glu
660 665 670

Lys Arg Gly Ile Ala Ile Asn Val Pro Glu Trp Gln Val Asp Lys Cys
675 680 685

Ile Gln Cys Asn Gln Cys Ser Phe Val Cys Pro His Ala Ala Ile Arg
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Pro Val Leu Thr Thr Glu Glu Glu Leu Ala Lys Ala Pro Gln Gly Phe
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Glu Ala Lys Asp Ala Asn Gly Ala Lys Gly Leu Lys Phe Thr Met Ala
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Ile Ser Pro Leu Asp Cys Ser Gly Cys Gly Asn Cys Glu Asp Val Cys
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Pro Ala Lys Glu Lys Ala Leu Val Met Lys Pro Val Asp Thr Gln Leu
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Ser Lys Thr Glu Ala Trp Asp Tyr Ala Val Asn Ala Val Ala His Lys
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Asp Asn Pro Met Lys Asp Lys Tyr Ser Val Lys Ala Ser Gln Phe Glu
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Gln Pro Leu Leu Glu Phe Ser Gly Ala Cys Ala Gly Cys Gly Glu Thr
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Pro Tyr Val Lys Leu Val Thr Gln Leu Phe Gly Asp Arg Met Met Ile
820 825 830

Ala Asn Ala Thr Gly Cys Ser Ser Ile Trp Gly Ala Ser Ala Pro Ala
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Thr Pro Tyr Thr Thr Asn Tyr Arg Gly His Gly Pro Ser Trp Ala Asn
850 855 860

Ser Leu Phe Glu Asp Asn Ala Glu Tyr Gly Leu Gly Met Phe Leu Gly
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Val Lys Gln Thr Arg Glu Arg Leu Gln Asp Lys Ile Glu Glu Ala Leu
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Lys Gly Ser Leu Ser Ala Glu Leu Lys Ala Ala Phe Glu Asp Trp Ile
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Thr Ala Leu Leu Glu Lys Glu Lys Gly Ser Asn Asp Leu Leu Asn Asp
930 935 940

Ile Tyr Glu Asn Arg Asp Phe Leu Val Lys Arg Ser His Trp Ile Ile
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Gly Gly Asp Gly Trp Gly Tyr Asp Ile Gly Tyr Gly Gly Val Asp His
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Val Leu Ala Ser Asn Glu Asp Val Asn Ile Leu Val Leu Asp Thr Glu
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Val Tyr Ser Asn Thr Gly Gly Gln Ser Ser Lys Ser Thr Pro Thr Ala
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His Glu Ala Glu Ala Tyr His Gly Pro Ser Leu Ile Ile Ala Tyr
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Ala Pro Cys Ile Asn His Gly Leu Arg Val Gly Met Gly Lys Ser
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Tyr Arg Tyr Asn Pro Glu Leu Lys Glu Glu Gly Lys Lys Ser Phe
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Ser Leu Asp Ser Lys Glu Pro Thr Thr Asp Phe Lys Glu Phe Leu
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Met Gly Glu Val Arg Tyr Ser Ser Leu Ala Lys Gln Phe Pro Asp
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Gln Ala Asp Ala Leu Phe Glu Lys Thr Lys Lys Asp Ala Leu Gln
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<211> 3725

<212> DNA

<213> *Desulfovibrio africanus*

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<212> PRT
<213> Desulfovibrio africanus

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Ser Ser Thr Met Gly Glu Glu Ala Asp Asp Trp Ala Ala Gln Gly Arg
 35 40 45

Lys Asn Ile Phe Gly Gln Thr Leu Thr Ile Arg Glu Met Gln Ser Glu
 50 55 60

Ala Gly Ala Ala Gly Ala Val His Gly Ala Leu Ala Ala Gly Ala Leu
 65 70 75 80

Thr Thr Thr Phe Thr Ala Ser Gln Gly Leu Leu Leu Met Ile Pro Asn
 85 90 95

Met Tyr Lys Ile Ser Gly Glu Leu Leu Pro Gly Val Phe His Val Thr
 100 105 110

Ala Arg Ala Ile Ala Ala His Ala Leu Ser Ile Phe Gly Asp His Gln
 115 120 125

Asp Ile Tyr Ala Ala Arg Gln Thr Gly Phe Ala Met Leu Ala Ser Ser
 130 135 140

Ser Val Gln Glu Ala His Asp Met Ala Leu Val Ala His Leu Ala Ala
 145 150 155 160

Ile Glu Ser Asn Val Pro Phe Met His Phe Phe Asp Gly Phe Arg Thr
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Ser His Glu Ile Gln Lys Ile Glu Val Leu Asp Tyr Ala Asp Met Ala
 180 185 190

Ser Leu Val Asn Gln Lys Ala Leu Ala Glu Phe Arg Ala Lys Ser Met
 195 200 205

Asn Pro Glu His Pro His Val Arg Gly Thr Ala Gln Asn Pro Asp Ile
 210 215 220

Tyr Phe Gln Gly Arg Glu Ala Ala Asn Pro Tyr Tyr Leu Lys Val Pro
 225 230 235 240

Gly Ile Val Ala Glu Tyr Met Gln Lys Val Ala Ser Leu Thr Gly Arg
 245 250 255

Ser Tyr Lys Leu Phe Asp Tyr Val Gly Ala Pro Asp Ala Glu Arg Val
 260 265 270

Ile Val Ser Met Gly Ser Ser Cys Glu Thr Ile Glu Glu Val Ile Asn
 275 280 285

His Leu Ala Ala Lys Gly Glu Lys Ile Gly Leu Ile Lys Val Arg Leu
 290 295 300

Tyr Arg Pro Phe Val Ser Glu Ala Phe Phe Ala Ala Leu Pro Ala Ser
 305 310 315 320

Ala Lys Val Ile Thr Val Leu Asp Arg Thr Lys Glu Pro Gly Ala Pro
 325 330 335

Gly Asp Pro Leu Tyr Leu Asp Val Cys Ser Ala Phe Val Glu Arg Gly
 340 345 350

Glu Ala Met Pro Lys Ile Leu Ala Gly Arg Tyr Gly Leu Gly Ser Lys
 355 360 365

Glu Phe Ser Pro Ala Met Val Lys Ser Val Tyr Asp Asn Met Ser Gly
 370 375 380

Ala Lys Lys Asn His Phe Thr Val Gly Ile Glu Asp Asp Val Thr Gly
 385 390 395 400

Thr Ser Leu Pro Val Asp Asn Ala Phe Ala Asp Thr Thr Pro Lys Gly
 405 410 415

Thr Ile Gln Cys Gln Phe Trp Gly Leu Gly Ala Asp Gly Thr Val Gly
 420 425 430

Ala Asn Lys Gln Ala Ile Lys Ile Ile Gly Asp Asn Thr Asp Leu Phe
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Ala Gln Gly Tyr Phe Ser Tyr Asp Ser Lys Lys Ser Gly Gly Ile Thr
 450 455 460

Ile Ser His Leu Arg Phe Gly Glu Lys Pro Ile Gln Ser Thr Tyr Leu
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Val Asn Arg Ala Asp Tyr Val Ala Cys His Asn Pro Ala Tyr Val Gly
 485 490 495

Ile Tyr Asp Ile Leu Glu Gly Ile Lys Asp Gly Gly Thr Phe Val Leu
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Asn Ser Pro Trp Ser Ser Leu Glu Asp Met Asp Lys His Leu Pro Ser
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Gly Ile Lys Arg Thr Ile Ala Asn Lys Lys Leu Lys Phe Tyr Asn Ile
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Asp Ala Val Lys Ile Ala Thr Asp Val Gly Leu Gly Gly Arg Ile Asn
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Met Ile Met Gln Thr Ala Phe Phe Lys Leu Ala Gly Val Leu Pro Phe
 565 570 575

Glu Lys Ala Val Asp Leu Leu Lys Lys Ser Ile His Lys Ala Tyr Gly
 580 585 590

Lys Lys Gly Glu Lys Ile Val Lys Met Asn Thr Asp Ala Val Asp Gln
 595 600 605

Ala Val Thr Ser Leu Gln Glu Phe Lys Tyr Pro Asp Ser Trp Lys Asp
 610 615 620

Ala	Pro	Ala	Glu	Thr	Lys	Ala	Glu	Pro	Met	Thr	Asn	Glu	Phe	Phe	Lys	625	630	635	640
Asn	Val	Val	Lys	Pro	Ile	Leu	Thr	Gln	Gln	Gly	Asp	Lys	Leu	Pro	Val	645	650	655	
Ser	Ala	Phe	Glu	Ala	Asp	Gly	Arg	Phe	Pro	Leu	Gly	Thr	Ser	Gln	Phe	660	665	670	
Glu	Lys	Arg	Gly	Val	Ala	Ile	Asn	Val	Pro	Gln	Trp	Val	Pro	Glu	Asn	675	680	685	
Cys	Ile	Gln	Cys	Asn	Gln	Cys	Ala	Phe	Val	Cys	Pro	His	Ser	Ala	Ile	690	695	700	
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Phe	Thr	Ala	Leu	Glu	Ala	Lys	Gly	Lys	Glu	Leu	Lys	Gly	Tyr	Lys	Phe	725	730	735	
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Asp	Ile	Cys	Pro	Pro	Lys	Glu	Lys	Ala	Leu	Val	Met	Gln	Pro	Leu	Asp	755	760	765	
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Pro	Val	Lys	Ser	Glu	Val	Leu	Pro	Arg	Asp	Ser	Leu	Lys	Gly	Ser	Gln	785	790	795	800
Phe	Gln	Glu	Pro	Leu	Met	Glu	Phe	Ser	Gly	Ala	Cys	Ser	Gly	Cys	Gly	805	810	815	
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Phe	Ile	Ala	Asn	Ala	Thr	Gly	Cys	Ser	Ser	Ile	Trp	Gly	Ala	Ser	Ala	835	840	845	
Pro	Ser	Met	Pro	Tyr	Lys	Thr	Asn	Arg	Leu	Gly	Gln	Gly	Pro	Ala	Trp	850	855	860	
Gly	Asn	Ser	Leu	Phe	Glu	Asp	Ala	Ala	Glu	Tyr	Gly	Phe	Gly	Met	Asn	865	870	875	880
Met	Ser	Met	Phe	Ala	Arg	Arg	Thr	His	Leu	Ala	Asp	Leu	Ala	Ala	Lys	885	890	895	
Ala	Leu	Glu	Ser	Asp	Ala	Ser	Gly	Asp	Val	Lys	Glu	Ala	Leu	Gln	Gly	900	905	910	
Trp	Leu	Ala	Gly	Lys	Asn	Asp	Pro	Ile	Lys	Ser	Lys	Glu	Tyr	Gly	Asp	915	920	925	

Lys Leu Lys Lys Leu Leu Ala Gly Gln Lys Asp Gly Leu Leu Gly Gln
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 Gly Gly Asp Gly Trp Ala Tyr Asp Ile Gly Tyr Gly Gly Leu Asp His
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 Val Leu Ala Ser Gly Glu Asp Val Asn Val Phe Val Met Asp Thr Glu
 980 985 990
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 995 1000 1005
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<211> 1617

<212> DNA

<213> Clostridium autoethanogenum LZ1561

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<212> PRT

<213> Clostridium autoethanogenum LZ1561

<400> 8

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Tyr	Glu	Ala	Leu	Arg	Lys	Ser	Asp	Val	Lys	His	Ile	Leu	Val	Arg	Gln
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Glu	Gln	Ala	Ala	Gly	His	Ser	Ala	Ser	Gly	Tyr	Ala	Arg	Ser	Thr	Gly
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Glu	Val	Gly	Val	Cys	Ile	Val	Thr	Ser	Gly	Pro	Gly	Ala	Thr	Asn	Leu
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Ile	Thr	Ala	Ile	Ala	Ala	Ala	Tyr	Met	Asp	Ser	Ile	Pro	Leu	Val	Val
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Glu	Leu	Asp	Ile	Thr	Gly	Ala	Thr	Glu	Ser	Phe	Thr	Lys	Tyr	Asn	Phe
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Leu	Val	Arg	Asp	Ala	Lys	Ser	Ile	Pro	Lys	Thr	Ile	Lys	Glu	Ala	Phe
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Tyr	Ile	Ala	Glu	Thr	Gly	Arg	Lys	Gly	Pro	Val	Leu	Val	Asp	Ile	Pro
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Met	Asp	Ile	Met	Glu	Glu	Asp	Ile	Asp	Phe	Glu	Tyr	Pro	Glu	Ser	Val
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Asn	Ile	Arg	Gly	Tyr	Lys	Pro	Thr	Val	Lys	Gly	His	Ser	Gly	Gln	Ile
			180					185					190		

Lys	Lys	Ile	Ile	Asp	Arg	Ile	Lys	Val	Ser	Lys	Arg	Pro	Leu	Ile	Cys
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Ala	Gly	Gly	Gly	Val	Ile	Leu	Ala	Asn	Ala	Gln	Lys	Glu	Leu	Glu	Gln
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Phe	Val	Lys	Lys	Ser	His	Ile	Pro	Val	Val	His	Thr	Leu	Met	Gly	Lys
225					230					235					240

Gly	Cys	Ile	Asn	Glu	Asn	Ser	Asp	Tyr	Tyr	Val	Gly	Leu	Ile	Gly	Thr
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His	Gly	Phe	Ala	Tyr	Ala	Asn	Lys	Val	Val	Gln	Asn	Ala	Asp	Val	Leu
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Ile Leu Ile Gly Ala Arg Ala Ser Asp Arg Thr Val Ser Gly Val Lys
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 Ser Phe Ala Lys Asp Ala Asp Ile Ile His Ile Asp Ile Asp Pro Ala
 290 295 300
 Glu Ile Gly Lys Ile Leu Asn Thr Tyr Ile Pro Val Val Gly Asp Cys
 305 310 315 320
 Gly Ser Val Leu Ser Asp Leu Asn Lys Glu Ile Val Ala Pro Gln Thr
 325 330 335
 Glu Lys Trp Met Glu Glu Ile Lys Asn Trp Lys Lys Asp Leu Tyr Ile
 340 345 350
 Glu Arg Lys Pro Thr Asp Lys Val Asn Pro Lys Tyr Val Leu Lys Thr
 355 360 365
 Val Ser Asp Thr Leu Gly Glu Glu Val Ile Leu Thr Ala Asp Val Gly
 370 375 380
 Gln Asn Gln Leu Trp Cys Ala Arg Asn Phe Arg Met Thr Gly Asn Arg
 385 390 395 400
 Lys Phe Leu Thr Ser Gly Gly Leu Gly Thr Met Gly Tyr Ser Leu Pro
 405 410 415
 Ala Ala Ile Gly Ala Lys Ile Ala Cys Pro Asp Lys Gln Val Ile Ala
 420 425 430
 Phe Ala Gly Asp Gly Gly Phe Gln Met Ser Leu Phe Glu Leu Gly Thr
 435 440 445
 Ile Ala Glu Asn Asn Leu Asn Ile Ile Ile Val Leu Phe Asn Asn Ser
 450 455 460
 Gly Leu Gly Met Val Arg Glu Ile Gln Asp Asn Lys Tyr Ser Gly Glu
 465 470 475 480
 Phe Gly Val Asn Phe Arg Thr Asn Pro Asp Phe Val Lys Leu Ala Glu
 485 490 495
 Ala Tyr Gly Leu Lys Ala Lys Arg Val Glu Asn Asp Ser Glu Phe Asn
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 Cys Ile Val Asp Pro His Glu Arg Thr Phe
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<211> 1677

<212> DNA

<213> Clostridium autoethanogenum LZ1561

<400> 9

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ggaaagataa ctcacatatc gacttcacat gaacaagggg ctgctcatgc tgcagatgga	180
tatgcaagat ctacaggaaa ggtaggagtt gtaattgcaa catcagggcc gggagctact	240
aatacggtta cagcaattgc tacagcttat atggattccg tacctattgt agtatttaca	300
ggacaggttg cgagaagtct tcttggaag gattcttttc aagaagtaaa tattaaagat	360
attactgcat ccataactaa gaaaagttgc attgtagaaa aggtagagga tttagctgat	420
actgtaagag aggcatttca aattgcagtt agtggagac caggacctgt agtagtagat	480
atacctaaag acgtacaatc agctgaagta gaatatgagc cttttagaag taagctttct	540
gaaattaaag aaaagaaata ttttaattta aatgagtatg gagacagttt aaataaggca	600
atagatatga taaataggag tgagagacct gtaatttatt caggtggagg aactgtcaca	660
tcaggagctc aaaatgaatt gatggaactt gtagaaaaa tagattcacc aattacctgt	720
tcacttatgg gaataggagc tttcccggga aacaatgaat attatatggg tatggttgga	780
atgcatggaa gccgttgctc aaattatgca gtaagtaatt gtgacttatt aatagctata	840
ggagctaggt ttagtgatag ggttataagc aaggtaagtg cctttgctcc aaaagcaaga	900
ataatacaca tagacattga ccctaaggag tttggcaaaa acgtggatat agatgtagca	960
ataaaaggag atgtaaaaga ggtacttcaa aagattaatt gcaagttaga aaaggccgac	1020
cacagggatt ggatggagaa aataaaacag tggaaaagtg aacagtgtga accttttaaa	1080
gaatgtaaat taagtcctaa gtttataatg gataccttgt ataatcttac aggaggagaa	1140
tgcataatta ctacagaagt tggccaaaat caaatttgga ctgcacaata ttttaaattc	1200
ttaaagccaa gaacatttgt atcttcaggc ggacttgga ctatgggctt cggacttgga	1260
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tatggaaaga ggttttcaaa tacagaactt ggaccagatg ttgatttcat gaaacttgga	1500
gaagcgtatg gtataaagac ttttaagata gaagacaata gccaggtaga gaaatgctta	1560
aaggaagctc ttgacttaaa tgaacctgta attatagaat gtgatataga taggaaagaa	1620
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<210> 10

<211> 558

<212> PRT

<213> Clostridium autoethanogenum LZ1561

<400> 10

Met Lys Ile Lys Gly Ala Glu Val Leu Leu Lys Cys Met Met Glu Gln

1	5	10	15
Gly Val Asp Thr Val Phe Gly Tyr Pro Gly Gly Ala Val Leu Pro Ile	20	25	30
Tyr Asp Ala Leu Tyr Ala Ala Lys Gly Lys Ile Thr His Ile Ser Thr	35	40	45
Ser His Glu Gln Gly Ala Ala His Ala Ala Asp Gly Tyr Ala Arg Ser	50	55	60
Thr Gly Lys Val Gly Val Val Ile Ala Thr Ser Gly Pro Gly Ala Thr	65	70	75
Asn Thr Val Thr Ala Ile Ala Thr Ala Tyr Met Asp Ser Val Pro Ile	85	90	95
Val Val Phe Thr Gly Gln Val Ala Arg Ser Leu Leu Gly Lys Asp Ser	100	105	110
Phe Gln Glu Val Asn Ile Lys Asp Ile Thr Ala Ser Ile Thr Lys Lys	115	120	125
Ser Cys Ile Val Glu Lys Val Glu Asp Leu Ala Asp Thr Val Arg Glu	130	135	140
Ala Phe Gln Ile Ala Val Ser Gly Arg Pro Gly Pro Val Val Val Asp	145	150	155
Ile Pro Lys Asp Val Gln Ser Ala Glu Val Glu Tyr Glu Pro Phe Arg	165	170	175
Ser Lys Leu Ser Glu Ile Lys Glu Lys Lys Tyr Phe Asn Leu Asn Glu	180	185	190
Tyr Gly Asp Ser Leu Asn Lys Ala Ile Asp Met Ile Asn Arg Ser Glu	195	200	205
Arg Pro Val Ile Tyr Ser Gly Gly Gly Thr Val Thr Ser Gly Ala Gln	210	215	220
Asn Glu Leu Met Glu Leu Val Glu Lys Ile Asp Ser Pro Ile Thr Cys	225	230	235
Ser Leu Met Gly Ile Gly Ala Phe Pro Gly Asn Asn Glu Tyr Tyr Met	245	250	255
Gly Met Val Gly Met His Gly Ser Arg Cys Ser Asn Tyr Ala Val Ser	260	265	270
Asn Cys Asp Leu Leu Ile Ala Ile Gly Ala Arg Phe Ser Asp Arg Val	275	280	285
Ile Ser Lys Val Ser Ala Phe Ala Pro Lys Ala Arg Ile Ile His Ile	290	295	300

Asp Ile Asp Pro Lys Glu Phe Gly Lys Asn Val Asp Ile Asp Val Ala
305 310 315 320

Ile Lys Gly Asp Val Lys Glu Val Leu Gln Lys Ile Asn Cys Lys Leu
325 330 335

Glu Lys Ala Asp His Arg Asp Trp Met Glu Lys Ile Lys Gln Trp Lys
340 345 350

Ser Glu Gln Cys Glu Pro Phe Lys Glu Cys Lys Leu Ser Pro Lys Phe
355 360 365

Ile Met Asp Thr Leu Tyr Asn Leu Thr Gly Gly Glu Cys Ile Ile Thr
370 375 380

Thr Glu Val Gly Gln Asn Gln Ile Trp Thr Ala Gln Tyr Phe Lys Phe
385 390 395 400

Leu Lys Pro Arg Thr Phe Val Ser Ser Gly Gly Leu Gly Thr Met Gly
405 410 415

Phe Gly Leu Gly Ala Ser Ile Gly Ala Ser Met Gly Asn Pro Gly Lys
420 425 430

Lys Val Ile Asn Val Ala Gly Asp Gly Ser Phe Lys Met Asn Ser Thr
435 440 445

Glu Leu Ala Thr Val Ala Lys Tyr Lys Leu Pro Ile Val Gln Leu Leu
450 455 460

Leu Asn Asn Arg Ala Leu Gly Met Val Tyr Gln Trp Gln Asp Met Phe
465 470 475 480

Tyr Gly Lys Arg Phe Ser Asn Thr Glu Leu Gly Pro Asp Val Asp Phe
485 490 495

Met Lys Leu Gly Glu Ala Tyr Gly Ile Lys Thr Phe Lys Ile Glu Asp
500 505 510

Asn Ser Gln Val Glu Lys Cys Leu Lys Glu Ala Leu Asp Leu Asn Glu
515 520 525

Pro Val Ile Ile Glu Cys Asp Ile Asp Arg Lys Glu Lys Val Phe Pro
530 535 540

Ile Val Pro Pro Gly Ala Ala Ile Ser Asp Leu Val Glu Glu
545 550 555

<210> 11

<211> 486

<212> DNA

<213> Clostridium autoethanogenum LZ1561

<400> 11

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agtagaagag gatataacat tcatagttta actggttgag taactggtga tcctgaaata      120
agtagaatga ctatagtaag tattggagat gattatatgt ttgaacaaat atctaaacag      180
cttaataaat tgatagaagt aataaaagta atagaattaa atcctgatgc aagtgtatat      240
agagaattaa gtcttataaa agtaagtgc gaaagtaata acaaacttct tataatggaa      300
agtgtaaata ctttttagagg taaaatagta gatatgaatg aaaaagtat gataatagaa      360
ataactggaa atgaaaaaaaa aataagtgc tttatagaat taatgaaacc ttatggaata      420
aaagaaataa taagaactgg attaactgc ttacaaagag gatcaaaatt agaagattaa      480
gagctc                                     486

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<210> 12

<211> 158

<212> PRT

<213> Clostridium autoethanogenum LZ1561

<400> 12

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Met Ser Val Leu Val Glu Asn His Ser Gly Val Leu Ser Lys Val Ala
1           5           10           15

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Gly Leu Phe Ser Arg Arg Gly Tyr Asn Ile His Ser Leu Thr Val Gly
20           25           30

```

```

Val Thr Gly Asp Pro Glu Ile Ser Arg Met Thr Ile Val Ser Ile Gly
35           40           45

```

```

Asp Asp Tyr Met Phe Glu Gln Ile Ser Lys Gln Leu Asn Lys Leu Ile
50           55           60

```

```

Glu Val Ile Lys Val Ile Glu Leu Asn Pro Asp Ala Ser Val Tyr Arg
65           70           75           80

```

```

Glu Leu Ser Leu Ile Lys Val Ser Ala Glu Ser Asn Asn Lys Leu Leu
85           90           95

```

```

Ile Met Glu Ser Val Asn Thr Phe Arg Gly Lys Ile Val Asp Met Asn
100          105          110

```

```

Glu Lys Ser Met Ile Ile Glu Ile Thr Gly Asn Glu Lys Lys Ile Ser
115          120          125

```

```

Ala Phe Ile Glu Leu Met Lys Pro Tyr Gly Ile Lys Glu Ile Ile Arg
130          135          140

```

```

Thr Gly Leu Thr Ala Leu Gln Arg Gly Ser Lys Leu Glu Asp
145          150          155

```

<210> 13

<211> 486

<212> DNA

<213> Artificial sequence

<220>

<223> Mutant Clostridium autoethanogenum LZ1561 IlvN (G-10-D)
acetolactate synthase

<400> 13

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agtagaagag gatataacat tcatagttta actggttgag taactggtga tcctgaaata    120
agtagaatga ctatagtaag tattggagat gattatatgt ttgaacaaat atctaaacag    180
cttaataaat tgatagaagt aataaaagta atagaattaa atcctgatgc aagtgtatat    240
agagaattaa gtcttataaa agtaagtgcg gaaagtaata acaaacttct tataatggaa    300
agtgtaaata cttttagagg taaaatagta gatatgaatg aaaaagtat gataatagaa    360
ataactggaa atgaaaaaaaa aataagtgcg tttatagaat taatgaaacc ttatggaata    420
aaagaaataa taagaactgg attaactgcg ttacaaagag gatcaaaatt agaagattaa    480
gagctc                                         486

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<210> 14

<211> 158

<212> PRT

<213> Artificial sequence

<220>

<223> Mutant Clostridium autoethanogenum LZ1561 IlvN (G-10-D) acetolactate synthase

<400> 14

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Met Ser Val Leu Val Glu Asn His Ser Asp Val Leu Ser Lys Val Ala
1          5          10         15

Gly Leu Phe Ser Arg Arg Gly Tyr Asn Ile His Ser Leu Thr Val Gly
20         25         30

Val Thr Gly Asp Pro Glu Ile Ser Arg Met Thr Ile Val Ser Ile Gly
35         40         45

Asp Asp Tyr Met Phe Glu Gln Ile Ser Lys Gln Leu Asn Lys Leu Ile
50         55         60

Glu Val Ile Lys Val Ile Glu Leu Asn Pro Asp Ala Ser Val Tyr Arg
65         70         75         80

Glu Leu Ser Leu Ile Lys Val Ser Ala Glu Ser Asn Asn Lys Leu Leu
85         90         95

Ile Met Glu Ser Val Asn Thr Phe Arg Gly Lys Ile Val Asp Met Asn
100        105        110

Glu Lys Ser Met Ile Ile Glu Ile Thr Gly Asn Glu Lys Lys Ile Ser
115        120        125

```

Ala Phe Ile Glu Leu Met Lys Pro Tyr Gly Ile Lys Glu Ile Ile Arg
 130 135 140

Thr Gly Leu Thr Ala Leu Gln Arg Gly Ser Lys Leu Glu Asp
 145 150 155

<210> 15

<211> 1671

<212> DNA

<213> Clostridium autoethanogenum LZ1561

<400> 15

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ttagaagccg aaggagtaaa gtacatcttt ggtattcctg gtgaagaaaa cctagaaata      120
atgaatgcaa tttcagattc aactattgaa tttatcacia cccgtcatga gcaagggtgt      180
gcatttatgg ccgacgttta tggacgttta acaggaaaag caggtgtttg cctatcaaca      240
ctaggaccag gtgccactaa cttagtaact ggtgtagcag atgctgatag tgatgggtgt      300
ccggttggtg ctattacagg tcaagtaggt actgaaagaa tgcatataac atcgcaccaa      360
tttttagacc ttgcaaaat gttcgaacca atcaciaaga gaagtaaaca aatcgttcgt      420
cctgatactg taagtgagat tataagactt gtttttaagt atgctgaaag tgaaaagcct      480
ggagcatgcc acattgattt acctgtaaat attgcaaaaa tgcccgtagg tgcttttagaa      540
aagccttttg aaaagaagat tccaccaaag gaacatgcag atttatcaac aattgaggaa      600
gctgcaagtg aaatcttcaa agcaaaaaat cctattatct tagctggaag cgggtgctata      660
agaggaaatt cttcaaaagc tgttacggaa ttgcaacta aattgaaaat tccagtaatt      720
aatacgatga tggcaaaagg tattattcca atggataaca agtattcaat gtggacaata      780
ggtattccac aaaaagatta tgtaaataaa attattgaag aggctgattt agtaattaca      840
attggatatg atattgtaga atatgcccc acaaatgga atataaatgg ggacattaaa      900
attgtgcata tcgatgcaag accatcacac atcaataaac tttatcagcc catagtagaa      960
gtagttggtg atatttcaga tgctctatac aatatattga gaagaacttc tagcaaagat     1020
gaaccagtaa aagctttgga aattaaatca gaaatgctag ctgaacatga aagctatgca     1080
aatgacaatg cttttccaat gaaaccccaa agaattttta atgatgttag aaagggtcatg     1140
ggaccacatg acattgtcat atcagatgta ggtgcccata aaatgtggat tgccagacat     1200
tataactgct atgagcccaa tacatgtatt atttcaaacg gttttgctac aatgggtatt     1260
ggtgttccag gtgcaattgc agccaaatta attaatccag ataaaaaagt attggctatt     1320
gttgggtgat gcggtttcat gatgaataat caagaattag aaacagccct acgtattaaa     1380

actccaattg tagttttaat atttaatgac agtaactacg gtttaataaa gtggaaacaa     1440
gaagaacact atggtaaaag ctgttatgta gattttacta atccagactt tgtaaagctt     1500
gcagaaagta tgtatgcaaa aggatatcga gtagaaaaag cagaagattt aattccaact     1560
ttagaagaag ctttcaaaca aaatgtacct gcagttattg attgtcaagt tgactatggt     1620
gaaaatataa agcttacaaa gcatttaaaa gaagtttatg aaaatatgta a              1671

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<210> 16

<211> 556

<212> PRT

<213> Clostridium autoethanogenum LZ1561

<400> 16

Met	Asn	Arg	Asp	Ile	Lys	Lys	Glu	Val	Gln	Leu	Asn	Thr	Ala	Gln	Met
1				5					10					15	

Leu	Val	Lys	Cys	Leu	Glu	Ala	Glu	Gly	Val	Lys	Tyr	Ile	Phe	Gly	Ile
			20					25					30		

Pro	Gly	Glu	Glu	Asn	Leu	Glu	Ile	Met	Asn	Ala	Ile	Ser	Asp	Ser	Thr
		35					40					45			

Ile	Glu	Phe	Ile	Thr	Thr	Arg	His	Glu	Gln	Gly	Ala	Ala	Phe	Met	Ala
	50					55					60				

Asp	Val	Tyr	Gly	Arg	Leu	Thr	Gly	Lys	Ala	Gly	Val	Cys	Leu	Ser	Thr
65					70					75					80

Leu	Gly	Pro	Gly	Ala	Thr	Asn	Leu	Val	Thr	Gly	Val	Ala	Asp	Ala	Asp
				85					90					95	

Ser	Asp	Gly	Ala	Pro	Val	Val	Ala	Ile	Thr	Gly	Gln	Val	Gly	Thr	Glu
			100					105					110		

Arg	Met	His	Ile	Thr	Ser	His	Gln	Phe	Leu	Asp	Leu	Cys	Lys	Met	Phe
		115					120					125			

Glu	Pro	Ile	Thr	Lys	Arg	Ser	Lys	Gln	Ile	Val	Arg	Pro	Asp	Thr	Val
	130					135					140				

Ser	Glu	Ile	Ile	Arg	Leu	Val	Phe	Lys	Tyr	Ala	Glu	Ser	Glu	Lys	Pro
145					150					155					160

Gly	Ala	Cys	His	Ile	Asp	Leu	Pro	Val	Asn	Ile	Ala	Lys	Met	Pro	Val
			165						170					175	

Gly	Ala	Leu	Glu	Lys	Pro	Leu	Glu	Lys	Lys	Ile	Pro	Pro	Lys	Glu	His
-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

180

185

190

Ala	Asp	Leu	Ser	Thr	Ile	Glu	Glu	Ala	Ala	Ser	Glu	Ile	Phe	Lys	Ala
		195					200					205			

Lys	Asn	Pro	Ile	Ile	Leu	Ala	Gly	Ser	Gly	Ala	Ile	Arg	Gly	Asn	Ser
	210				215						220				

Ser	Lys	Ala	Val	Thr	Glu	Phe	Ala	Thr	Lys	Leu	Lys	Ile	Pro	Val	Ile
225					230					235					240

Asn	Thr	Met	Met	Ala	Lys	Gly	Ile	Ile	Pro	Met	Asp	Asn	Lys	Tyr	Ser
				245					250					255	

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Met Trp Thr Ile Gly Ile Pro Gln Lys Asp Tyr Val Asn Lys Ile Ile
      260                      265                      270

Glu Glu Ala Asp Leu Val Ile Thr Ile Gly Tyr Asp Ile Val Glu Tyr
      275                      280                      285

Ala Pro Ser Lys Trp Asn Ile Asn Gly Asp Ile Lys Ile Val His Ile
      290                      295                      300

Asp Ala Arg Pro Ser His Ile Asn Lys Leu Tyr Gln Pro Ile Val Glu
305                      310                      315                      320

Val Val Gly Asp Ile Ser Asp Ala Leu Tyr Asn Ile Leu Arg Arg Thr
      325                      330                      335

Ser Ser Lys Asp Glu Pro Val Lys Ala Leu Glu Ile Lys Ser Glu Met
      340                      345                      350

Leu Ala Glu His Glu Ser Tyr Ala Asn Asp Asn Ala Phe Pro Met Lys
      355                      360                      365

Pro Gln Arg Ile Leu Asn Asp Val Arg Lys Val Met Gly Pro His Asp
      370                      375                      380

Ile Val Ile Ser Asp Val Gly Ala His Lys Met Trp Ile Ala Arg His
385                      390                      395                      400

Tyr Asn Cys Tyr Glu Pro Asn Thr Cys Ile Ile Ser Asn Gly Phe Ala
      405                      410                      415

Thr Met Gly Ile Gly Val Pro Gly Ala Ile Ala Ala Lys Leu Ile Asn
      420                      425                      430

Pro Asp Lys Lys Val Leu Ala Ile Val Gly Asp Gly Gly Phe Met Met
      435                      440                      445

Asn Asn Gln Glu Leu Glu Thr Ala Leu Arg Ile Lys Thr Pro Ile Val
      450                      455                      460

Val Leu Ile Phe Asn Asp Ser Asn Tyr Gly Leu Ile Lys Trp Lys Gln
465                      470                      475                      480

Glu Glu His Tyr Gly Lys Ser Cys Tyr Val Asp Phe Thr Asn Pro Asp
      485                      490                      495

Phe Val Lys Leu Ala Glu Ser Met Tyr Ala Lys Gly Tyr Arg Val Glu
      500                      505                      510

Lys Ala Glu Asp Leu Ile Pro Thr Leu Glu Glu Ala Phe Lys Gln Asn
      515                      520                      525

Val Pro Ala Val Ile Asp Cys Gln Val Asp Tyr Gly Glu Asn Ile Lys
      530                      535                      540

Leu Thr Lys His Leu Lys Glu Val Tyr Glu Asn Met
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545

550

555

<210> 17

<211> 1680

<212> DNA

<213> Artificial sequence

<220>

<223> Codon-optimized *Clostridium autoethanogenum* LZ1561 AlsS acetolactate synthase

<400> 17

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ataatgaatg caatatctga tagtactata gaatttataa ctactagaca tgaacaagga	180
gcagcattta tggcagatgt atatggaaga ttaactggaa aagctggagt ttgtttaagt	240
actttaggac ctggagcaac taatttagta actggagtag cagatgcaga tagtgatgga	300
gcacctgtag tagcaataac tggacaagta ggaactgaaa gaatgcatat aactagtcac	360
caatTTTTtag atttatgtaa aatgtttgaa cctataacta aaagaagtaa acaaatagta	420
agacctgata ctgtaagtga aataataaga ttagtattta aatatgcaga aagtgaaaaa	480
cctggagcat gtcatataga ttacctgta aatatagcaa aaatgcctgt tggagcatta	540
gaaaaacctt tagaaaaaaa aatacctcct aaagaacatg cagattttaag tacaatagaa	600
gaagcagcat ctgaaatatt taaagcaaaa aatcctataa tattagctgg aagtggagca	660
ataagaggaa atagtagtaa agcagtaact gaatttgcaa ctaaattaaa aatacctgta	720
ataaatacta tgatggcaaa aggaataata cctatggata ataaatatag tatgtggact	780
ataggaatac ctcaaaaaga ttatgtaaat aaaataatag aagaagctga tttagtaata	840
actataggat atgatatagt agaatatgca cctagtaaata ggaatataaa tggagatata	900
aaaatagtac atatagatgc aagacctagt catataaata aattatatca acctatagta	960
gaagtagttg gagatataag tgatgcatta tataatatat taagaagaac tagttcaaaa	1020
gatgaacctg taaaagcatt agaaataaaa agtgaaatgt tagcagaaca tgaaagtatt	1080
gcaaatgata atgcatttcc tatgaaacct caaagaatat taaatgatgt aagaaaagta	1140
atgggacctc atgatatagt aataagtgat gttggagcac ataaaatgtg gatagcaaga	1200
cattataatt gttatgaacc taatacttgt ataataagta atggatttgc aacaatggga	1260
ataggagtac ctggagcaat agcagcaaaa ttaataaatc ctgataaaaa agtattagca	1320
atagttggag atggaggatt tatgatgaat aatcaagaat tagaaactgc attaagaata	1380
aaaactccta tagtagtatt aatatttaat gatagtaatt atggattaat aaaatggaaa	1440
caagaagaac attatggaaa aagttgttat gtagatttta ctaatcctga ttttgtaaaa	1500
ttagcagaaa gtatgtatgc aaaaggatat agagtagaaa aagcagaaga tttaatacct	1560
actttagaag aagcatttaa acaaaatgta cctgcagtaa tagattgtca agtagattat	1620
ggagaaaata taaaattaac taaacattta aaagaagtat atgaaaatat gtaagagctc	1680

<210> 18

<211> 556

<212> PRT

<213> Artificial sequence

<220>

<223> Codon-optimized Clostridium autoethanogenum LZ1561 AlsS acetolactate synthase

<400> 18

Met	Asn	Arg	Asp	Ile	Lys	Lys	Glu	Val	Gln	Leu	Asn	Thr	Ala	Gln	Met
1				5					10					15	

Leu	Val	Lys	Cys	Leu	Glu	Ala	Glu	Gly	Val	Lys	Tyr	Ile	Phe	Gly	Ile
			20					25					30		

Pro	Gly	Glu	Glu	Asn	Leu	Glu	Ile	Met	Asn	Ala	Ile	Ser	Asp	Ser	Thr
	35						40					45			

Ile	Glu	Phe	Ile	Thr	Thr	Arg	His	Glu	Gln	Gly	Ala	Ala	Phe	Met	Ala
	50					55					60				

Asp	Val	Tyr	Gly	Arg	Leu	Thr	Gly	Lys	Ala	Gly	Val	Cys	Leu	Ser	Thr
65					70					75				80	

Leu	Gly	Pro	Gly	Ala	Thr	Asn	Leu	Val	Thr	Gly	Val	Ala	Asp	Ala	Asp
			85						90					95	

Ser	Asp	Gly	Ala	Pro	Val	Val	Ala	Ile	Thr	Gly	Gln	Val	Gly	Thr	Glu
		100						105					110		

Arg	Met	His	Ile	Thr	Ser	His	Gln	Phe	Leu	Asp	Leu	Cys	Lys	Met	Phe
		115					120					125			

Glu	Pro	Ile	Thr	Lys	Arg	Ser	Lys	Gln	Ile	Val	Arg	Pro	Asp	Thr	Val
	130					135					140				

Ser	Glu	Ile	Ile	Arg	Leu	Val	Phe	Lys	Tyr	Ala	Glu	Ser	Glu	Lys	Pro
145					150					155					160

Gly	Ala	Cys	His	Ile	Asp	Leu	Pro	Val	Asn	Ile	Ala	Lys	Met	Pro	Val
			165						170					175	

Gly	Ala	Leu	Glu	Lys	Pro	Leu	Glu	Lys	Lys	Ile	Pro	Pro	Lys	Glu	His
		180						185					190		

Ala	Asp	Leu	Ser	Thr	Ile	Glu	Glu	Ala	Ala	Ser	Glu	Ile	Phe	Lys	Ala
		195					200					205			

Lys	Asn	Pro	Ile	Ile	Leu	Ala	Gly	Ser	Gly	Ala	Ile	Arg	Gly	Asn	Ser
	210					215					220				

Ser	Lys	Ala	Val	Thr	Glu	Phe	Ala	Thr	Lys	Leu	Lys	Ile	Pro	Val	Ile
225					230					235					240

Asn	Thr	Met	Met	Ala	Lys	Gly	Ile	Ile	Pro	Met	Asp	Asn	Lys	Tyr	Ser
				245					250					255	

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Met Trp Thr Ile Gly Ile Pro Gln Lys Asp Tyr Val Asn Lys Ile Ile
      260                      265                      270

Glu Glu Ala Asp Leu Val Ile Thr Ile Gly Tyr Asp Ile Val Glu Tyr
      275                      280                      285

Ala Pro Ser Lys Trp Asn Ile Asn Gly Asp Ile Lys Ile Val His Ile
      290                      295                      300

Asp Ala Arg Pro Ser His Ile Asn Lys Leu Tyr Gln Pro Ile Val Glu
305                      310                      315                      320

Val Val Gly Asp Ile Ser Asp Ala Leu Tyr Asn Ile Leu Arg Arg Thr
      325                      330                      335

Ser Ser Lys Asp Glu Pro Val Lys Ala Leu Glu Ile Lys Ser Glu Met
      340                      345                      350

Leu Ala Glu His Glu Ser Tyr Ala Asn Asp Asn Ala Phe Pro Met Lys
      355                      360                      365

Pro Gln Arg Ile Leu Asn Asp Val Arg Lys Val Met Gly Pro His Asp
      370                      375                      380

Ile Val Ile Ser Asp Val Gly Ala His Lys Met Trp Ile Ala Arg His
385                      390                      395                      400

Tyr Asn Cys Tyr Glu Pro Asn Thr Cys Ile Ile Ser Asn Gly Phe Ala
      405                      410                      415

Thr Met Gly Ile Gly Val Pro Gly Ala Ile Ala Ala Lys Leu Ile Asn
      420                      425                      430

Pro Asp Lys Lys Val Leu Ala Ile Val Gly Asp Gly Gly Phe Met Met
      435                      440                      445

Asn Asn Gln Glu Leu Glu Thr Ala Leu Arg Ile Lys Thr Pro Ile Val
      450                      455                      460

Val Leu Ile Phe Asn Asp Ser Asn Tyr Gly Leu Ile Lys Trp Lys Gln
465                      470                      475                      480

Glu Glu His Tyr Gly Lys Ser Cys Tyr Val Asp Phe Thr Asn Pro Asp
      485                      490                      495

Phe Val Lys Leu Ala Glu Ser Met Tyr Ala Lys Gly Tyr Arg Val Glu
      500                      505                      510

Lys Ala Glu Asp Leu Ile Pro Thr Leu Glu Glu Ala Phe Lys Gln Asn
      515                      520                      525

Val Pro Ala Val Ile Asp Cys Gln Val Asp Tyr Gly Glu Asn Ile Lys
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545                      550                      555

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<210> 19
 <211> 1722

<212> DNA

<213> *Bacillus subtilis*

<400> 19

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gaacaaaatg cagcatttat ggcacaagca gtaggaagat taactggaaa acctggtgta	240
gtacttgtaa ctagtggacc tgggtgcatca aatttagcaa ctggattatt aactgcaaat	300
actgaagggtg atcctgtagt agcattagct ggaaatgtaa taagagcaga tagattaaaa	360
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gaagtacaag atgtaaaaaa tatacctgaa gcagtaacta atgcatttag aatagcaagt	480
gcaggacaag ctggtgcagc ttttgtaagt tttcctcagg atgtagtaaa tgaagtaact	540
aataactaaa atgtaagagc agtagcagca cctaaattag gacctgcagc agatgatgca	600
ataagtgcag caatagcaaa aatacaaact gcaaaattac ctgtagtatt agtaggaatg	660
aaagggtggtg gacctgaagc aataaaaagca gtaagaaaat tacttaaaaa agtacaatta	720
ccttttgtag aaacttatca agcagctgga actttaagta gagatttaga agatcaatat	780
tttggaaaga taggattatt tagaaatcaa cctggtgatt tattacttga acaagcagat	840
gtagtattaa ctataggata tgatccaata gaatatgatc ctaaattttg gaatataaat	900
ggtgatagaa ctataataca tttagatgaa ataatagcag atatagatca tgcatatcaa	960
cctgatttag aattaattgg agatatacct agtactataa atcacataga acatgatgca	1020
gtaaaagtag aatttgcaga aagagaacaa aaaatactta gtgattttaa acaatatatg	1080
catgaagggtg aacaagtacc tgcagattgg aaaagtgata gagcacatcc tttagaaata	1140
gtaaaagaat taagaaatgc agtagatgat catgtaactg taacttgtga tataggatct	1200
catgcaatat ggatgagtag atatttttaga agttatgaac ctttaacttt aatgataagt	1260
aatggaatgc aaactcttgg agtagcatta ccttgggcaa ttggagcatc tttagtaaaa	1320
cctggtgaaa aagtagtaag tgtaagtggg gatggtggat ttcttttttag tgcaatggaa	1380
ttagaaactg cagtaagatt aaaagcacct atagtacata tagtatggaa tgatagtact	1440
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<211> 570

<212> PRT

<213> Bacillus subtilis

<400> 20

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Phe Gly Ile Pro Gly Ala Lys Ile Asp Ala Val Phe Asp Ala Leu Gln
35 40 45

Asp Lys Gly Pro Glu Ile Ile Val Ala Arg His Glu Gln Asn Ala Ala
50 55 60

Phe Met Ala Gln Ala Val Gly Arg Leu Thr Gly Lys Pro Gly Val Val
65 70 75 80

Leu Val Thr Ser Gly Pro Gly Ala Ser Asn Leu Ala Thr Gly Leu Leu
85 90 95

Thr Ala Asn Thr Glu Gly Asp Pro Val Val Ala Leu Ala Gly Asn Val
100 105 110

Ile Arg Ala Asp Arg Leu Lys Arg Thr His Gln Ser Leu Asp Asn Ala
115 120 125

Ala Leu Phe Gln Pro Ile Thr Lys Tyr Ser Val Glu Val Gln Asp Val
130 135 140

Lys Asn Ile Pro Glu Ala Val Thr Asn Ala Phe Arg Ile Ala Ser Ala
145 150 155 160

Gly Gln Ala Gly Ala Ala Phe Val Ser Phe Pro Gln Asp Val Val Asn
165 170 175

Glu Val Thr Asn Thr Lys Asn Val Arg Ala Val Ala Ala Pro Lys Leu
180 185 190

Gly Pro Ala Ala Asp Asp Ala Ile Ser Ala Ala Ile Ala Lys Ile Gln
195 200 205

Thr Ala Lys Leu Pro Val Val Leu Val Gly Met Lys Gly Gly Arg Pro
210 215 220

Glu Ala Ile Lys Ala Val Arg Lys Leu Leu Lys Lys Val Gln Leu Pro
225 230 235 240

Phe Val Glu Thr Tyr Gln Ala Ala Gly Thr Leu Ser Arg Asp Leu Glu
245 250 255

Asp Gln Tyr Phe Gly Arg Ile Gly Leu Phe Arg Asn Gln Pro Gly Asp
260 265 270

Leu Leu Leu Glu Gln Ala Asp Val Val Leu Thr Ile Glv Trp Asp Pro

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Ile Glu Tyr Asp Pro Lys Phe Trp Asn Ile Asn Gly Asp Arg Thr Ile
 290              295              300

Ile His Leu Asp Glu Ile Ile Ala Asp Ile Asp His Ala Tyr Gln Pro
 305              310              315              320

Asp Leu Glu Leu Ile Gly Asp Ile Pro Ser Thr Ile Asn His Ile Glu
      325              330              335

His Asp Ala Val Lys Val Glu Phe Ala Glu Arg Glu Gln Lys Ile Leu
      340              345              350

Ser Asp Leu Lys Gln Tyr Met His Glu Gly Glu Gln Val Pro Ala Asp
      355              360              365

Trp Lys Ser Asp Arg Ala His Pro Leu Glu Ile Val Lys Glu Leu Arg
      370              375              380

Asn Ala Val Asp Asp His Val Thr Val Thr Cys Asp Ile Gly Ser His
 385              390              395              400

Ala Ile Trp Met Ser Arg Tyr Phe Arg Ser Tyr Glu Pro Leu Thr Leu
      405              410              415

Met Ile Ser Asn Gly Met Gln Thr Leu Gly Val Ala Leu Pro Trp Ala
      420              425              430

Ile Gly Ala Ser Leu Val Lys Pro Gly Glu Lys Val Val Ser Val Ser
      435              440              445

Gly Asp Gly Gly Phe Leu Phe Ser Ala Met Glu Leu Glu Thr Ala Val
      450              455              460

Arg Leu Lys Ala Pro Ile Val His Ile Val Trp Asn Asp Ser Thr Tyr
 465              470              475              480

Asp Met Val Ala Phe Gln Gln Leu Lys Lys Tyr Asn Arg Thr Ser Ala
      485              490              495

Val Asp Phe Gly Asn Ile Asp Ile Val Lys Tyr Ala Glu Ser Phe Gly
      500              505              510

Ala Thr Gly Leu Arg Val Glu Ser Pro Asp Gln Leu Ala Asp Val Leu
      515              520              525

Arg Gln Gly Met Asn Ala Glu Gly Pro Val Ile Ile Asp Val Pro Val
      530              535              540

Asp Tyr Ser Asp Asn Ile Asn Leu Ala Ser Asp Lys Leu Pro Lys Glu
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Phe Gly Glu Leu Met Lys Thr Lys Ala Leu
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<210> 21

<211> 720

<212> DNA

<213> Clostridium autoethanogenum LZ1561

<400> 21

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gtagtcactg aactggaaaa ttataatact tataatatctc aaaatcgtac ttcttatgaa      300
gatataagaa aagaattgga cagctttata gaaagcaaaa atatatttta tgctttctat      360
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aagcctatgg ctgaagttgt taaagatcag cctatgtttg aatataacgg tgttgatgga      480
tatgtggttg gatttaggtg tcctgattat gttgaaggcc ttaatgtccc tggatatcat      540
tttcatttca taaataaaga taagaaattt ggtggacata taagtgaatt ttccattgaa      600
aatgcgaagg tttatgtaca gaactgttct tgcttttagga tggaacttcc taaaaatgaa      660
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<210> 22

<211> 239

<212> PRT

<213> Clostridium autoethanogenum LZ1561

<400> 22

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1          5          10          15

Ile Asn Ala Leu Val Ser Gly Leu Tyr Asp Gly Cys Val Ser Leu Ser
20        25        30

Lys Leu Leu Lys Lys Gly Asn Phe Gly Ile Gly Thr Phe Lys Gly Leu
35        40        45

Asp Gly Glu Leu Thr Leu Leu Asn Gly Thr Phe Tyr Arg Thr Lys Pro
50        55        60

Asp Gly Ser Val Tyr Val Cys Ser Lys Asn Val Ser Val Pro Phe Ala
65        70        75        80

Val Val Thr Glu Leu Glu Asn Tyr Asn Thr Tyr Asn Ile Gln Asn Arg
85        90        95

Thr Ser Tyr Glu Asp Ile Arg Lys Glu Leu Asp Ser Phe Ile Glu Ser
100       105       110

Lys Asn Ile Phe Tyr Ala Phe Tyr Met Glu Gly Lys Phe Asn Tyr Val
115       120       125

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Lys Thr Arg Thr Val Val Lys Gln Asn Met Pro Tyr Lys Pro Met Ala
130 135 140

Glu Val Val Lys Asp Gln Pro Met Phe Glu Tyr Asn Gly Val Asp Gly
145 150 155 160

Tyr Val Val Gly Phe Arg Cys Pro Asp Tyr Val Glu Gly Leu Asn Val
165 170 175

Pro Gly Tyr His Phe His Phe Ile Asn Lys Asp Lys Lys Phe Gly Gly
180 185 190

His Ile Ser Glu Phe Ser Ile Glu Asn Ala Lys Val Tyr Val Gln Asn
195 200 205

Cys Ser Cys Phe Arg Met Glu Leu Pro Lys Asn Glu Ser Phe Tyr Asn
210 215 220

Met Glu Val Gln Asp Arg Asn Asp Glu Ile Thr Ser Val Glu Lys
225 230 235

<210> 23
<211> 745
<212> DNA
<213> Artificial sequence

<220>
<223> Codon-optimized Clostridium autoethanogenum LZ1561 acetolactate decarboxylase

<400> 23

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attaaaaaaaa ggaaattttg gaataggaac ttttaaagga ttagatggag aattaacttt	180
attaaatgga actttttata gaactaaacc tgatggaagt gtatatgtat gtagtaaaaa	240
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aatagaact tcttatgaag atataagaaa agaattagat agttttatag aaagtaaaaa	360
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aagtgaattt agtatagaaa atgcaaaagt atatgtacaa aattgtagtt gttttagaat	660
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<210> 24
<211> 239
<212> PRT

<213> Artificial sequence

<220>

<223> Codon-optimized Clostridium autoethanogenum LZ1561 acetolactate decarboxylase

<400> 24

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Ile Asn Ala Leu Val Ser Gly Leu Tyr Asp Gly Cys Val Ser Leu Ser
20 25 30

Lys Leu Leu Lys Lys Gly Asn Phe Gly Ile Gly Thr Phe Lys Gly Leu
35 40 45

Asp Gly Glu Leu Thr Leu Leu Asn Gly Thr Phe Tyr Arg Thr Lys Pro
50 55 60

Asp Gly Ser Val Tyr Val Cys Ser Lys Asn Val Ser Val Pro Phe Ala
65 70 75 80

Val Val Thr Glu Leu Glu Asn Tyr Asn Thr Tyr Asn Ile Gln Asn Arg
85 90 95

Thr Ser Tyr Glu Asp Ile Arg Lys Glu Leu Asp Ser Phe Ile Glu Ser
100 105 110

Lys Asn Ile Phe Tyr Ala Phe Tyr Met Glu Gly Lys Phe Asn Tyr Val
115 120 125

Lys Thr Arg Thr Val Val Lys Gln Asn Met Pro Tyr Lys Pro Met Ala
130 135 140

Glu Val Val Lys Asp Gln Pro Met Phe Glu Tyr Asn Gly Val Asp Gly
145 150 155 160

Tyr Val Val Gly Phe Arg Cys Pro Asp Tyr Val Glu Gly Leu Asn Val
165 170 175

Pro Gly Tyr His Phe His Phe Ile Asn Lys Asp Lys Lys Phe Gly Gly
180 185 190

His Ile Ser Glu Phe Ser Ile Glu Asn Ala Lys Val Tyr Val Gln Asn
195 200 205

Cys Ser Cys Phe Arg Met Glu Leu Pro Lys Asn Glu Ser Phe Tyr Asn
210 215 220

Met Glu Val Gln Asp Arg Asn Asp Glu Ile Thr Ser Val Glu Lys
225 230 235

<210> 25

<211> 806

<212> DNA

<213> *Aeromonas hydrophila*

<400> 25

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aaactccttt tgcagtaatg actcatttta gacottgttt acaaagaaga tttgcacatc      360
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<210> 26

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<212> PRT

<213> *Aeromonas hydrophila*

<400> 26

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Ser Leu Met Ser Ala Leu Leu Ala Gly Val Tyr Glu Gly Glu Thr Thr
35          40          45

Met Ala Asp Leu Leu Arg His Gly Asp Phe Gly Leu Gly Thr Phe Asn
50          55          60

Arg Leu Asp Gly Glu Leu Ile Ala Phe Glu Arg Gln Ile His Gln Leu
65          70          75          80

Lys Ala Asp Gly Ser Ala Arg Pro Ala Arg Ala Glu Gln Lys Thr Pro
85          90          95

Phe Ala Val Met Thr His Phe Arg Pro Cys Leu Gln Arg Arg Phe Ala
100         105         110

His Pro Leu Ser Arg Glu Glu Ile His Gln Trp Val Asp Arg Leu Val
115         120         125

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Gly Thr Asp Asn Val Phe Val Ala Phe Arg Leu Asp Gly Leu Phe Glu
130 135 140

Gln Ala Gln Val Arg Thr Val Pro Cys Gln Ser Pro Pro Tyr Lys Pro
145 150 155 160

Met Leu Glu Ala Ile Glu Ala Gln Pro Leu Phe Ser Phe Ser Leu Arg
165 170 175

Arg Gly Thr Leu Val Gly Phe Arg Cys Pro Pro Phe Val Gln Gly Ile
180 185 190

Asn Val Ala Gly Tyr His Glu His Phe Ile Thr Glu Asp Arg Arg Gly
195 200 205

Gly Gly His Ile Leu Asp Tyr Ala Met Gly His Gly Gln Leu Gln Leu
210 215 220

Ser Val Val Gln His Leu Asn Ile Glu Leu Pro Arg Asn Pro Ala Phe
225 230 235 240

Gln Gln Ala Asp Leu Asn Pro Ala Asp Leu Asp Arg Ala Ile Arg Ala
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Ala Glu Gly

<210> 27
<211> 761
<212> DNA
<213> Leuconostoc lactis

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ttcttgatgg tgaagtttat caagcacaga gtgatggaca agtaaatacat ataactaatc 240
ctgatacttt aatgcctttt gcaagtgtac attttgatgc acctactcag cagttacctt 300
ttagtcaggt agattttagt actttaagtg caaaacttaa agcagaacaa ttacaaaatg 360
tatttgcagc agtgaaatth catggtgaat ttagtagagt acatgtaaga atagctccta 420
aacaagtacc tccttatcct tcattacttg cagtagcaga aaatcaacct gaatttgaaa 480
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ctgcagcagg atggcatgta cattttctta gtgatgatct tcaatttgca ggacatatat 600
tagattttag tgcaagtcaa ataagtggaa ctttacaaat atttgatgaa tttgtacaac 660
atttacctat acatgatcct gcatatagag aaatgactth agattttagg ggattattag 720
ctggaataga agcaagtga ggtggacaac aataaggtag c 761

<210> 28
<211> 244

<212> PRT

<213> *Leuconostoc lactis*

<400> 28

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20      25      30

Glu Leu Arg Gln His Gly Asp Thr Gly Ile Gly Thr Leu Thr Gly Leu
35      40      45

Asp Gly Glu Val Ile Leu Leu Asp Gly Glu Val Tyr Gln Ala Gln Ser
50      55      60

Asp Gly Gln Val Asn His Ile Thr Asn Pro Asp Thr Leu Met Pro Phe
65      70      75      80

Ala Ser Val His Phe Asp Ala Pro Thr Gln Gln Leu Pro Phe Ser Gln
85      90      95

Val Asp Phe Ser Thr Leu Ser Ala Lys Leu Lys Ala Glu Gln Leu Gln
100     105     110

Asn Val Phe Ala Ala Val Lys Phe His Gly Glu Phe Ser Arg Val His
115     120     125

Val Arg Ile Ala Pro Lys Gln Val Pro Pro Tyr Pro Ser Leu Leu Ala
130     135     140

Val Ala Glu Asn Gln Pro Glu Phe Glu Arg Glu His Val Thr Gly Thr
145     150     155     160

Val Val Gly Tyr Phe Ala Pro Gln Ile Phe Asn Gly Pro Thr Ala Ala
165     170     175

Gly Trp His Val His Phe Leu Ser Asp Asp Leu Gln Phe Ala Gly His
180     185     190

Ile Leu Asp Phe Ser Ala Ser Gln Ile Ser Gly Thr Leu Gln Ile Phe
195     200     205

Asp Glu Phe Val Gln His Leu Pro Ile His Asp Pro Ala Tyr Arg Glu
210     215     220

Met Thr Leu Asp Phe Asp Gly Leu Leu Ala Gly Ile Glu Ala Ser Glu
225     230     235     240

Gly Gly Gln Gln

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REFERENCES CITED IN THE DESCRIPTION

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PATENTKRAV

1. Rekombinant, carboxydotroft *Clostridium*-bakterie omfattende:

- (a) en pyruvat:ferredoxin-oxidoreduktase (EC 1.2.7.1);
 - 5 (b) en acetolactatdecarboxylase (EC 4.1.1.5);
 - (c) en pyruvat:ferredoxin-oxidoreduktase (EC 1.2.7.1) og en acetolactatsyntase (EC 2.2.1.6);
 - (d) en pyruvat:ferredoxin-oxidoreduktase (EC 1.2.7.1) og en acetolactatdecarboxylase (EC 4.1.1.5);
 - 10 (e) en acetolactatsyntase (EC 2.2.1.6) og en acetolactatdecarboxylase (EC 4.1.1.5); eller
 - (f) en pyruvat:ferredoxin-oxidoreduktase (EC 1.2.7.1), en acetolactatsyntase (EC 2.2.1.6) og en acetolactatdecarboxylase (EC 4.1.1.5);
- hvor hvert enzym er et overudtrykt endogent enzym eller et exogent enzym; og
- 15 hvor bakterien er udvalgt fra gruppen bestående af *Clostridium autoethanogenum*, *Clostridium ljungdahlii* og *Clostridium ragsdalei*.

2. Bakterie ifølge krav 1, hvor pyruvat:ferredoxin-oxidoreduktasen er en overudtrykt, endogen pyruvat:ferredoxin-oxidoreduktase.

20 3. Bakterie ifølge krav 1, hvor pyruvat:ferredoxin-oxidoreduktasen er en exogen *Desulfovibrio africanus*-pyruvat:ferredoxin-oxidoreduktase.

4. Bakterie ifølge krav 1, hvor acetolactatsyntasen er en overudtrykt endogen IlvB-ORF2059-acetolactatsyntase, en overudtrykt endogen IlvB-ORF2336-acetolactatsyntase, en overudtrykt endogen IlvN-acetolactatsyntase eller en overudtrykt endogen AlsS-acetolactatsyntase.

25 5. Bakterie ifølge krav 1, hvor acetolactatsyntasen er en exogen *Bacillus subtilis*-acetolactatsyntase.

6. Bakterie ifølge krav 1, hvor acetolactatsyntasen er en overudtrykt endogen AlsD-acetolactatdecarboxylase eller en overudtrykt endogen BudA-acetolactatdecarboxylase.

7. Bakterie ifølge krav 1, hvor acetolactatdecarboxylasen er en exogen
5 *Aeromonas hydrophila*-acetolactatdecarboxylase.

8. Bakterie ifølge krav 1, hvor acetolactatdecarboxylasen er en exogen *Leuconostoc lactis*-acetolactatdecarboxylase.

9. Bakterie ifølge krav 1, hvor bakterien er *Clostridium autoethanogenum* afledt af *Clostridium autoethanogenum* indleveret under DSMZ
10 accessionsnummer DSM23693.

10. Fremgangsmåde til frembringelse af 2,3-butanediol ved fermentering af CO ved hjælp af bakterien ifølge krav 1.

11. Fremgangsmåde ifølge krav 10, hvor bakterien endvidere frembringer et fermenteringsprodukt udvalgt fra gruppen bestående af ethanol, butanol,
15 isopropanol, isobutanol, højere alkoholer, succinat, isoprenoider, fedtsyrer, biopolymerer og blandinger deraf.

DRAWINGS

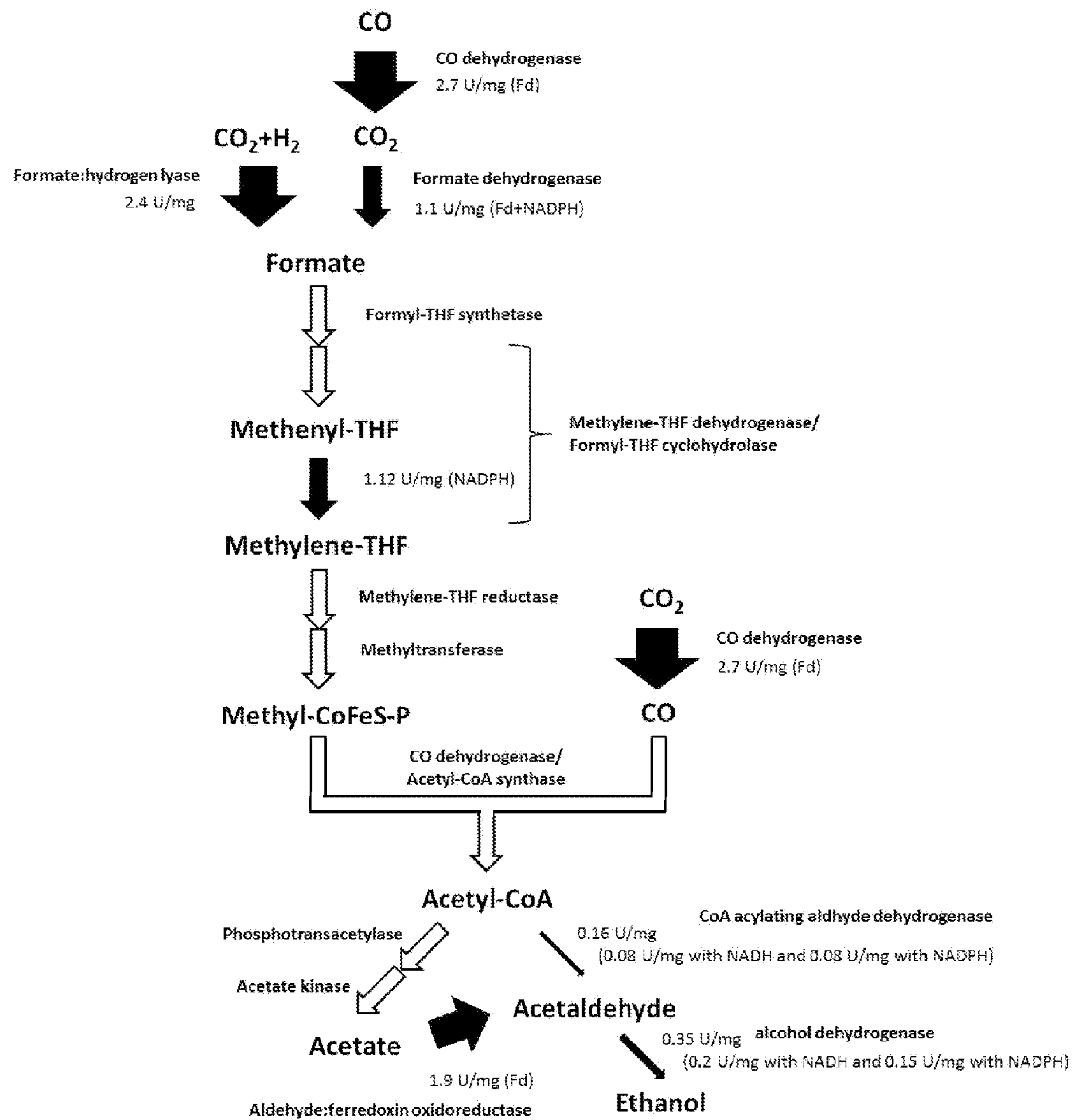


FIG. 1

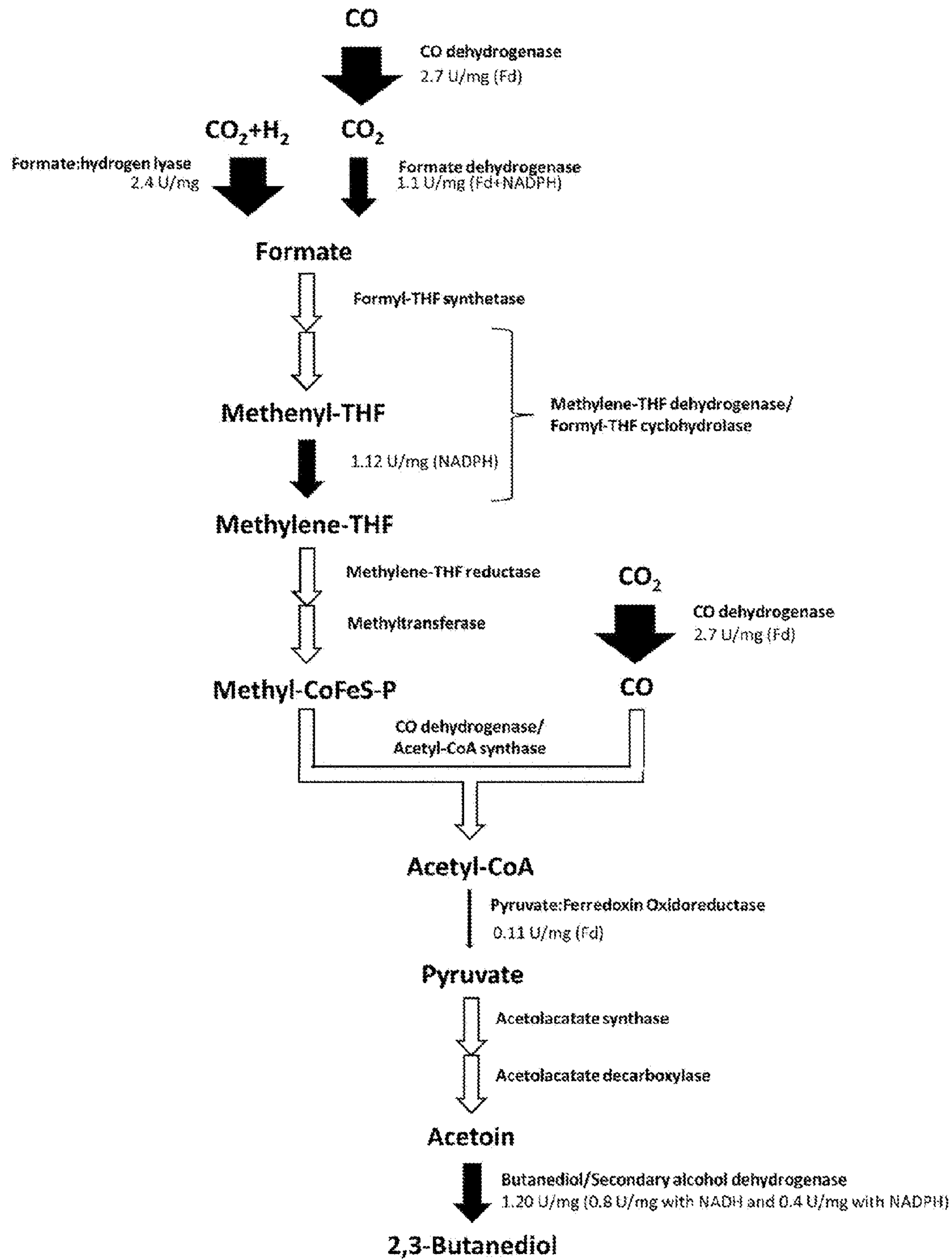


FIG. 2

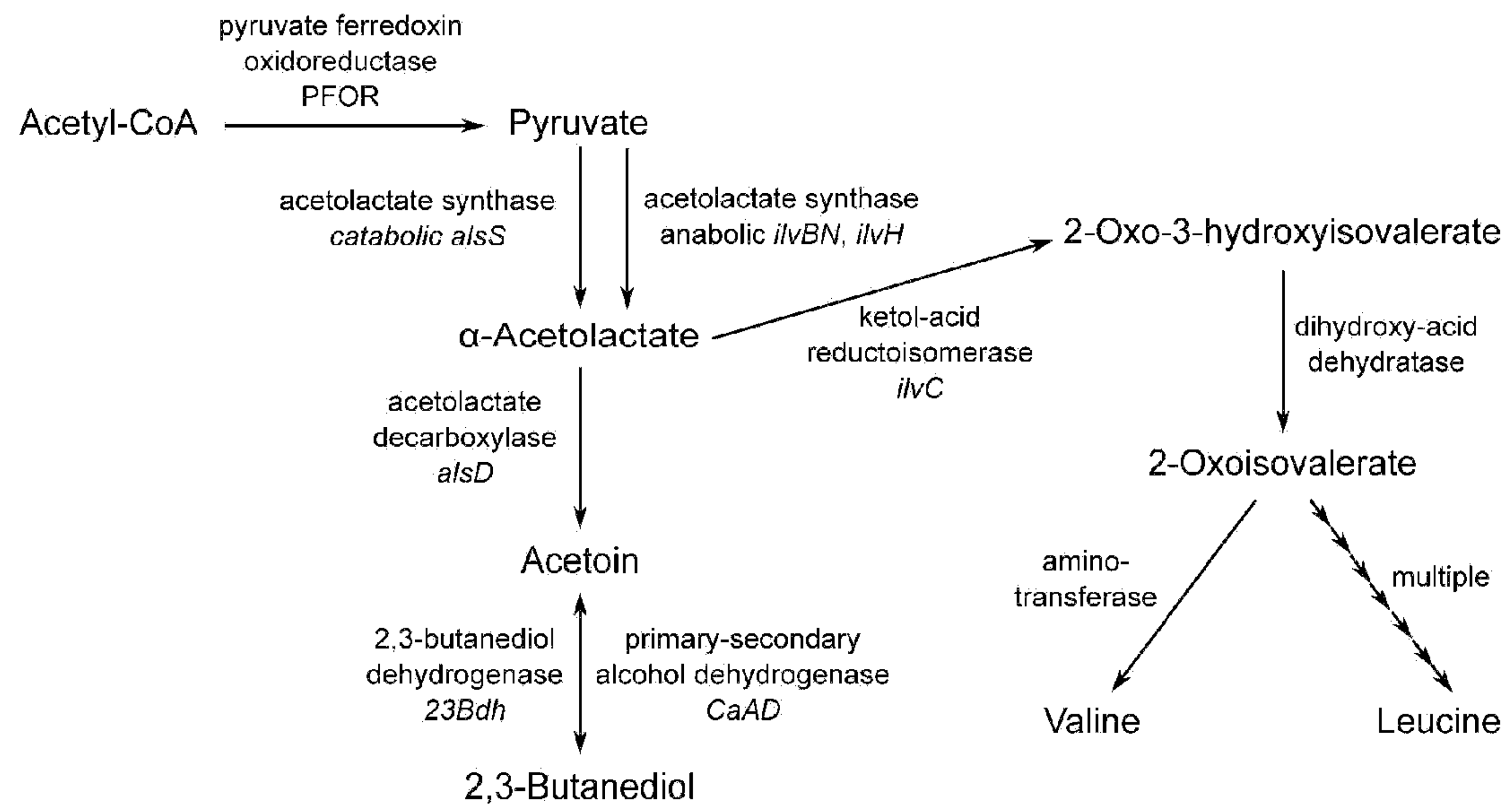


FIG. 3

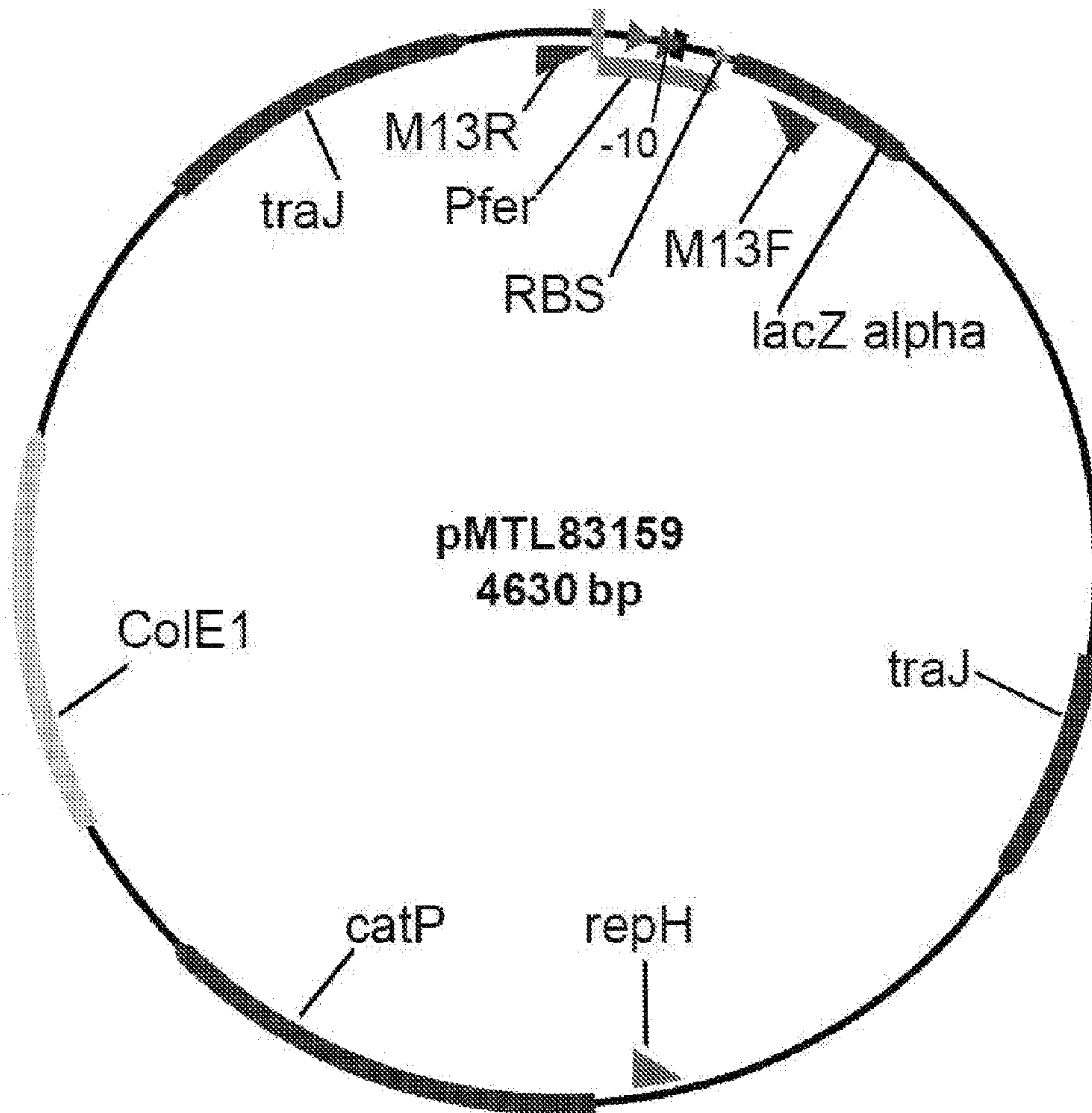


FIG. 4

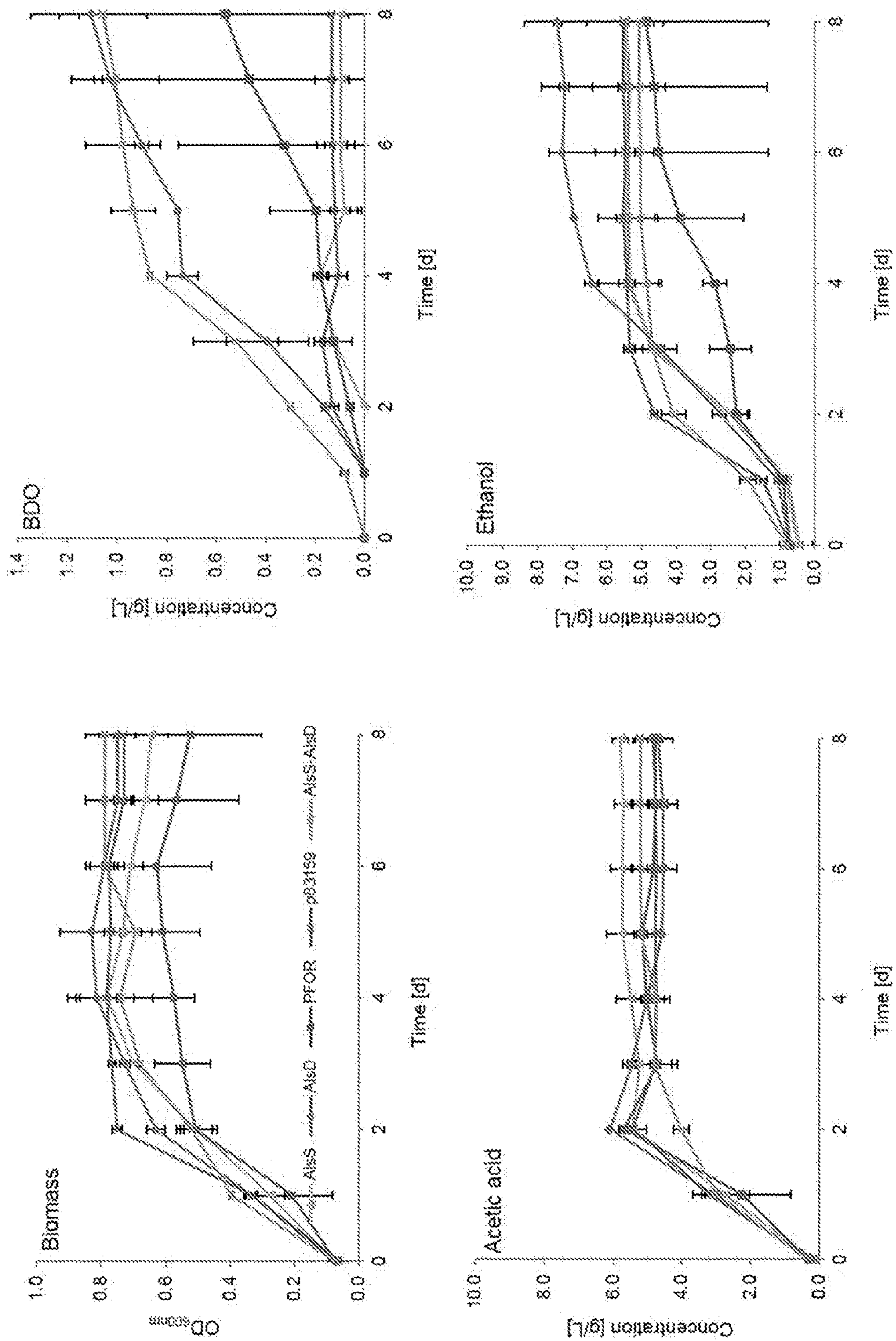


FIG. 5

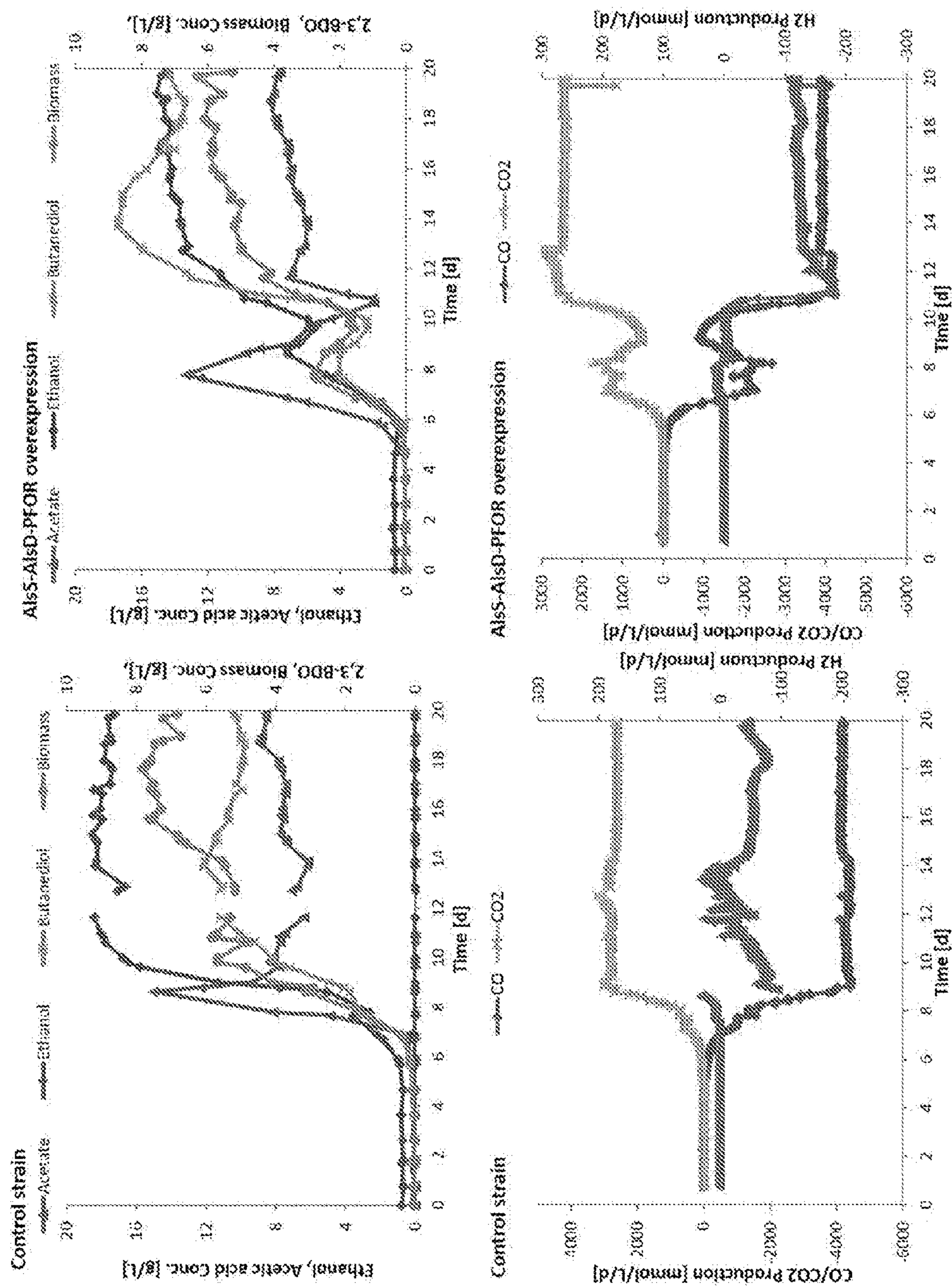


FIG. 6

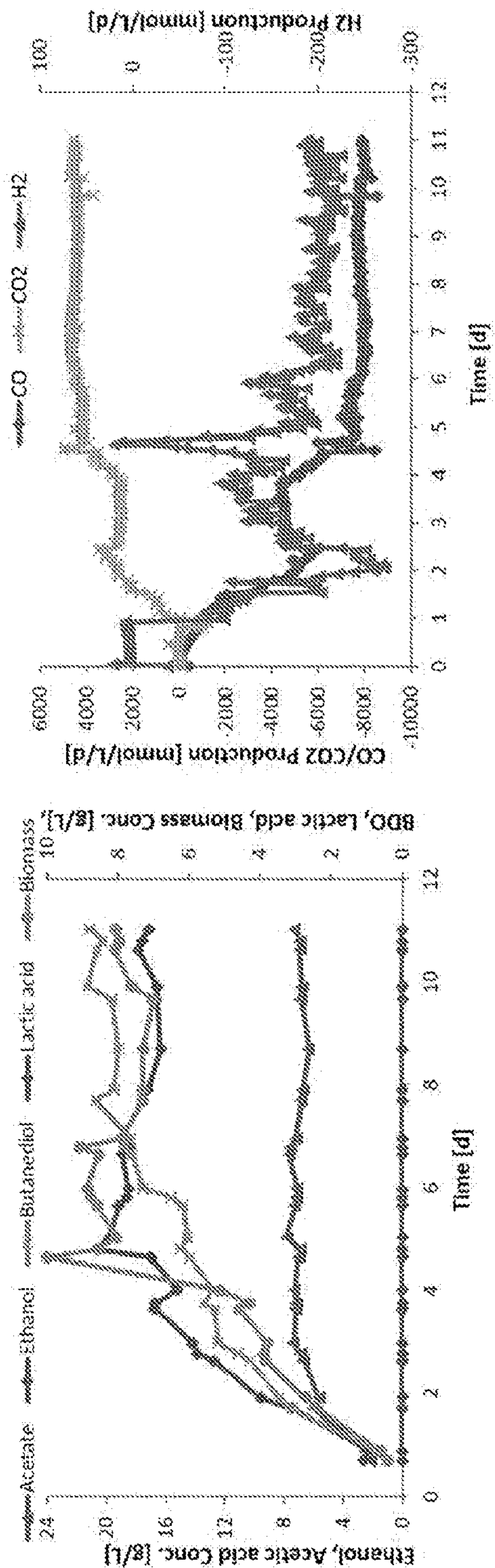


FIG. 7

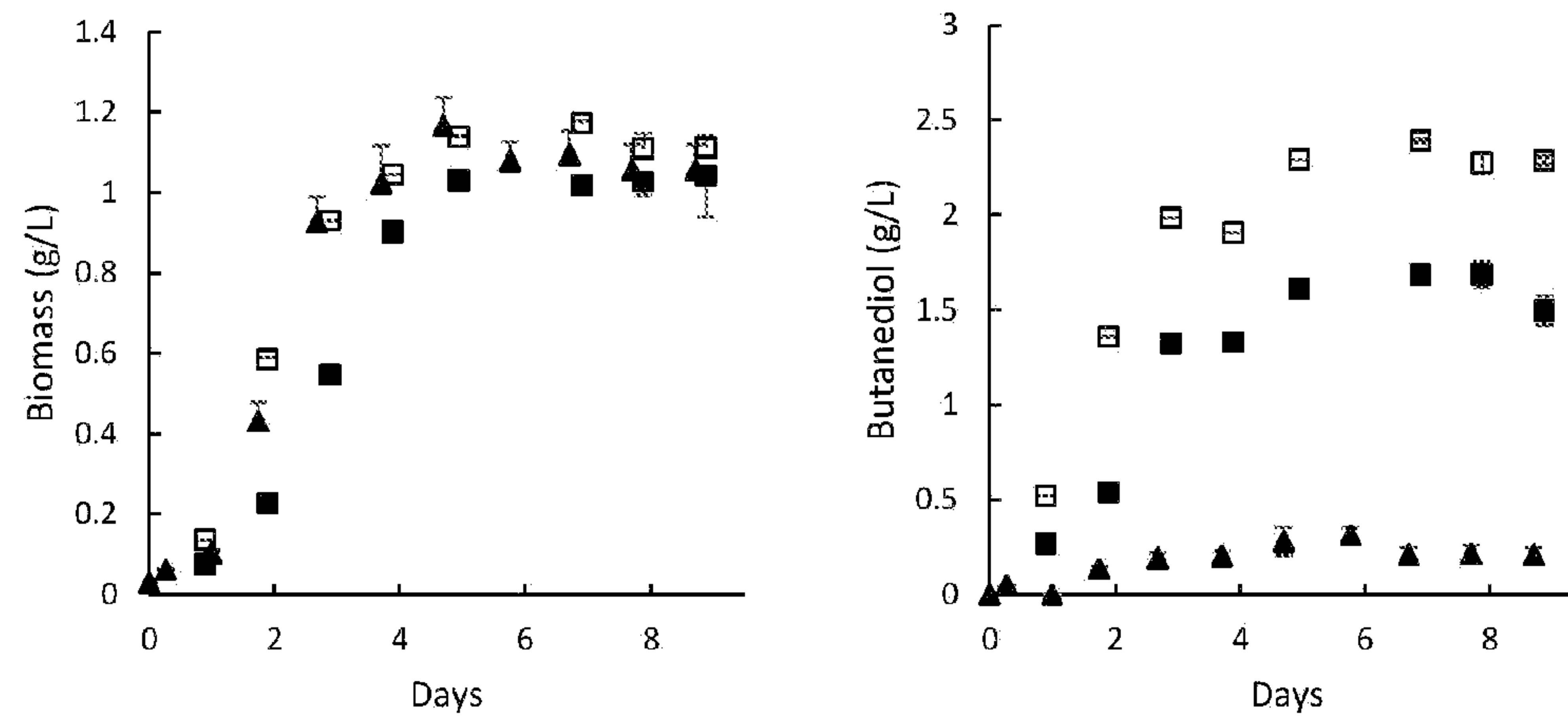


FIG. 8