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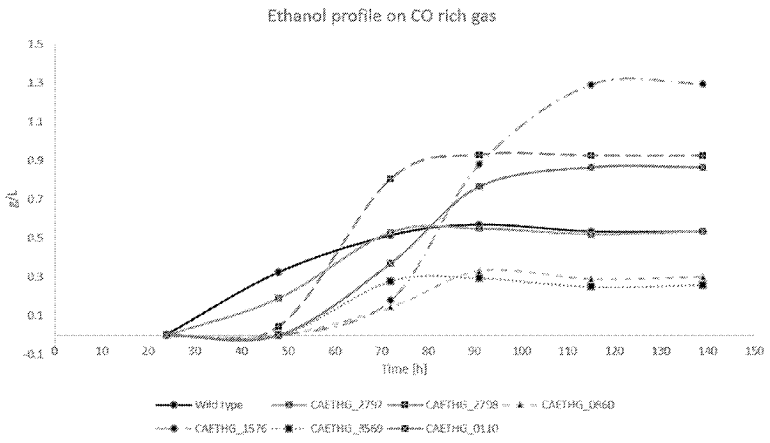


FIG. 1C

(57) Abstract: The invention provides genetically engineered microorganisms with modified hydrogenase activity and methods related thereto. Typically, the microorganisms are C1-fixing microorganisms with one or more disruptive mutations in a hydrogenase enzyme or a hydrogenase accessory enzyme. The microorganisms may have improved tolerance to toxins, such as acetylene, isocyanide, ammonium, or nitric oxide, improved production of products, such as ethanol, 2,3-butanediol, and isopropanol, and/or improved fixation of carbon, such as carbon derived from CO or CO2.

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MICROORGANISM WITH MODIFIED HYDROGENASE ACTIVITY

CROSS REFERENCE TO RELATED APPLICATIONS

0001 This application claims the benefit of U.S. Provisional Patent Application No. 62/271,466 filed December 28, 2015, the entirety of which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

0002 Carbon dioxide (CO_2) accounts for about 76% of global greenhouse gas emissions from human activities, with methane (16%), nitrous oxide (6%), and fluorinated gases (2%) accounting for the balance (United States Environmental Protection Agency). The majority of CO_2 comes from the burning fossil fuels to produce energy, although industrial and forestry practices also emit CO_2 into the atmosphere. Reduction of greenhouse gas emissions, particularly CO_2 emissions, is critical to halt the progression of global warming and the accompanying shifts in climate and weather.

0003 It has long been recognized that catalytic processes may be used to convert gases containing carbon dioxide (CO_2), carbon monoxide (CO), and/or hydrogen (H_2), such as industrial waste gas or syngas, into a variety of fuels and chemicals. Recently, however, gas fermentation has emerged as an alternative platform for the biological fixation of such gases. In particular, C1-fixing microorganisms have been demonstrated to convert gases containing CO_2, CO, and/or H_2 into products such as ethanol and 2,3-butanediol. Efficient production of such products may be limited, for example, by slow microbial growth, limited gas uptake, sensitivity to toxins, or diversion of carbon substrates into undesired byproducts. Accordingly, there remains a need for genetically engineered microorganisms having improved characteristics, especially improved carbon fixation, product production, and toxin resistance.

SUMMARY OF THE INVENTION

0004 The invention provides genetically engineered microorganisms with modified hydrogenase activity and methods related thereto. In particular, the invention provides a genetically engineered C1-fixing microorganism comprising a disruptive mutation in a hydrogenase enzyme or a hydrogenase accessory enzyme.

0005 For example, the hydrogenase enzyme may be an electron-bifurcating, NADP- and ferredoxin dependent [FeFe]-hydrogenase (e.g., CAETHG_2794-99 (HytABCDE1E2)); a

multi-subunit [FeFe]-hydrogenase (e.g., CAETHG_1576-78 or CAETHG_3569-71); a mono-subunit [FeFe]-hydrogenase (e.g., CAETHG_0110 or CAETHG_3841); and/or a [NiFe]-hydrogenase (e.g., CAETHG_0861-62). In a preferred embodiment, the hydrogenase enzyme is CAETHG_2794-99 (HytABCDE1E2) comprising a disruptive mutation in one or more of subunits HytA, HytB, HytC, HytD, HytE1, and HytE2. In another example, the hydrogenase accessory enzyme may be a [NiFe]-hydrogen maturation protease (e.g., CAETHG_0860).

0006 The microorganism of the invention may be derived from a parental microorganism such as *Acetobacterium woodii*, *Alkalibaculum bacchii*, *Blautia producta*, *Butyribacterium methylotrophicum*, *Clostridium aceticum*, *Clostridium autoethanogenum*, *Clostridium carboxidivorans*, *Clostridium coskatii*, *Clostridium drakei*, *Clostridium formicoaceticum*, *Clostridium ljungdahlii*, *Clostridium magnum*, *Clostridium ragsdalei*, *Clostridium scatologenes*, *Eubacterium limosum*, *Moorella thermautotrophica*, *Moorella thermoacetica*, *Oxobacter pfennigii*, *Sporomusa ovata*, *Sporomusa silvacetica*, *Sporomusa sphaeroides*, or *Thermoanaerobacter kiuvi*. In preferred embodiments, the microorganism of the invention may be derived from *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*.

0007 The microorganism of the invention may have a different product profile than the parental microorganism. For instance, the microorganism of the invention may produce more ethanol than a parental microorganism without the disruptive mutation.

0008 The microorganism of the invention may be capable of fixing carbon. For example, the microorganism of the invention may consume a gaseous substrate comprising a C1-carbon source comprising CO_2 and/or CO. In a preferred embodiment, the microorganism of the invention is capable of net carbon capture.

0009 The microorganism of the invention may also have different tolerances to toxins than the parental microorganism. For instance, the microorganism of the invention may be more tolerant of a toxin, e.g., acetylene, isocyanide, ammonium, or nitric oxide, than a parental microorganism without the disruptive mutation.

0010 The invention also provides a method of producing a product comprising culturing the microorganism of the invention in the presence of a gaseous substrate. Typically, the gaseous substrate comprises a C1-carbon source, e.g., CO_2 or CO. A wide variety of products may be produced, including native products, such as ethanol or 2,3-butanediol, or non-native

products, such as isopropanol. In certain embodiments, microbial biomass may also be considered a product.

0011 The invention further provides a method of fixing carbon comprising culturing the microorganism of the invention in the presence of a gaseous substrate comprising a C1-carbon source, whereby the microorganism consumes more C1-carbon than it produces. Typically, the gaseous substrate comprises a C1-carbon source, e.g., CO_2 or CO.

BRIEF DESCRIPTION OF THE DRAWINGS

0012 Figs. 1A-1C are graphs showing the results of growing *C. autoethanogenum* LZ1561 (control) and *C. autoethanogenum* mutants CAETHG_1576, CAETHG_2797, CAETHG_3569, CAETHG_2798, CAETHG_0110, and CAETHG_0860 on CO-rich gas, specifically the production of microbial biomass (Fig. 1A), acetate (Fig. 1B), and ethanol (Fig. 1C).

0013 Figs. 2A-2C are graphs showing the results of growing *C. autoethanogenum* LZ1561 (control) and *C. autoethanogenum* mutants CAETHG_1576, CAETHG_2797, CAETHG_3569, CAETHG_2798, CAETHG_0110, and CAETHG_0860 on H_2-rich gas, specifically the production of microbial biomass (Fig. 2A), acetate (Fig. 2B), and ethanol (Fig. 2C).

DETAILED DESCRIPTION OF THE INVENTION

0014 Hydrogenases play a central role in microbial energy metabolism. In particular, a "hydrogenase" is an enzyme that catalyzes the reversible oxidation of molecular hydrogen ($2H^+ + 2e^- \leftrightarrow H_2$). Hydrogenases can be distributed into three classes: the [Fe]-hydrogenases, the [NiFe]-hydrogenases, and the metal-free hydrogenases. The vast majority of known hydrogenases belong to the first two classes, and over 100 of these enzymes have been characterized genetically and/or biochemically. Compelling evidence from sequences and structures indicates that the [NiFe]- and [Fe]-hydrogenases are phylogenetically distinct classes of proteins. The catalytic core of the [NiFe]-hydrogenases is a heterodimeric protein, although additional subunits are present in many of these enzymes. Functional classes of [NiFe]-hydrogenases have been defined and they are consistent with categories defined by sequence similarity of the catalytic subunits. The catalytic core of the [Fe]-hydrogenases is a ca. 350-residue domain that accommodates the active site (H-cluster). A few monomeric [Fe]-hydrogenases are barely larger than the H-cluster domain. Many others are monomeric as well, but possess additional domains that contain redox centers, mostly iron-sulfur. Some

[Fe]-hydrogenases are oligomeric. The modular structure of hydrogenases is strikingly illustrated in recently unveiled sequences and structures. Microbial genome sequences are bringing forth a significant body of additional hydrogenase sequence data and contribute to the understanding of hydrogenase distribution and evolution (Vignais, *FEMS Microbiol Rev*, 25: 455-501, 2001).

0015 A "hydrogenase accessory enzyme" is an enzyme that affects the activity or expression of a hydrogenase enzyme, but is not itself a hydrogenase enzyme. For example, [NiFe]-hydrogen maturation protease (CAETHG_0860) catalyzes a proteolytic cleavage important to the maturation of [NiFe]-hydrogenase (CAETHG_0861-62) in *C. autoethanogenum*. Accordingly, [NiFe]-hydrogen maturation protease (CAETHG_0860) is a hydrogenase accessory enzyme that affects the activity of the hydrogenase enzyme [NiFe]-hydrogenase (CAETHG_0861-62). Herein, reference to hydrogenases enzymes (or disruption thereof) should be interpreted to also encompass hydrogenase accessory enzymes (or disruption thereof).

0016 Typically, *Clostridia* hydrogenases are capable of interacting with a wide variety of electron acceptors or donors, which gives *Clostridia* an evolutionary survival advantage across a variety of habitats (Calusinska, *Microbiol*, 156: 1575-1588, 2010). In particular, *C. autoethanogenum* has genes for six putative hydrogenases: (1) electron-bifurcating, NADP- and ferredoxin dependent [FeFe]-hydrogenase (CAETHG_2794-99) (Wang, *J Bacteriol*, 195: 4373-4386, 2013), (2) multi-subunit [FeFe]-hydrogenase (CAETHG_1576-78), (3) multi-subunit [FeFe]-hydrogenase (CAETHG_3569-71), (4) mono-subunit [FeFe]-hydrogenase (CAETHG_0110), (5) mono-subunit [FeFe]-hydrogenase (CAETHG_3841), and (6) [NiFe]-hydrogenase (CAETHG_0861-62).

0017 The electron-bifurcating, NADP- and ferredoxin dependent [FeFe]-hydrogenase is composed of the subunits HytABCDE1E2 and catalyzes the reversible reduction of $NADP^+$ and Fd_{ox} with 2 H_2.

0018 $2H_2 + NADP^+ + Fd_{ox} + \leftrightharpoons \quad NADPH + Fd_{red}^{2-} + 3H^+$

0019 HytA is the H-cluster harboring the [FeFe]-hydrogenase subunit, HytB is an iron-sulfur flavoprotein harboring the NADP binding site, and the other subunits are iron-sulfur proteins. The hydrogenase forms a tight complex with the selenium- and tungsten-dependent formate dehydrogenase FdhA. The hydrogenase (HytABCDE1E2) is encoded by the genes CAETHG_2794-99 (*hytABCDE1E2*), which form a transcription unit. Besides the structural

genes, genes required for [FeFe]-hydrogenase maturation, *hydE* (CAETHG_1691; 47 FPKM), *hydF* (CAETHG_2063; 21 FPKM) and *hydG* (CAETHG_0339; 148 FPKM) are present in *C. autoethanogenum* as well as in *C. ljungdahlii* (Mock, *J Bacteriol*, 197: 2965-2980, 2015). Fig. 2 of Mock, *J Bacteriol*, 197: 2965-2980, 2015 shows expression of the *C. autoethanogenum hyt-fdh* gene cluster and surrounding genes during growth in continuous culture.When grown in a CO ($E0'$ = -520 mV) rich atmosphere, the same HytABCDE1E2 NADP-specific hydrogenase is predicted to catalyze *in vivo* the formation of H_2 rather than the uptake of H_2 in order to re-oxidize reduced ferredoxin. It would be useful if these electrons could be used to fix carbon into more reduced products such as ethanol, isopropanol, and others.

0020 The genome of *C. autoethanogenum* harbors genes for two other multi-subunit [FeFe]-hydrogenases (CAETHG_1576-78; CAETHG_3569-71), which have a subunit structure and composition very similar to those of the electron-bifurcating and NAD- and ferredoxin-dependent [FeFe]-hydrogenases from *T. maritima*, *A. woodii*, *M. thermoacetica*, and *R. albus*. While CAETHG_3569-71 is hardly expressed (2 FPKM), CAETHG_1576-78 is the second highest expressed hydrogenase in *C. autoethanogenum*, but at significantly lower level (35 FPKM) than the characterized electron-bifurcating and NADP-dependent [FeFe]-hydrogenase. CAETHG_1576-78 is absent in *C. ljungdahlii* (Mock, *J Bacteriol*, 197: 2965-2980, 2015).

0021 The genome of *C. autoethanogenum* harbors genes for two mono-subunit [FeFe]-hydrogenases, CAETHG_0110 and CAETHG_3841. These generally use ferredoxin as electron acceptor/donor, are not electron bifurcating, and all show only very low expression (5-9 FPKM). There is also a third gene, CAETHG_0119, annotated as [FeFe]-hydrogenase but it lacks the sequence segments involved in H-cluster iron binding and can therefore not encode for a functional [FeFe]-hydrogenase (Mock, *J Bacteriol*, 197: 2965-2980, 2015).

0022 Genes for a [NiFe]-hydrogenase (CAETHG_0861-62) are also present and form a putative transcription unit with a [NiFe]-hydrogen maturation protease (CAETHG_0860). No expression for these three genes has been found (<0.1 FPKM). This has also been reported in transcriptomic datasets for *C. ljungdahlii* growing on either H_2/CO_2 or fructose. Consistently, not all the genes required for [NiFe]-hydrogenase maturation are present. Only the genes *hypECDF* (CAETHG_0372-0369) were found whereas the genes *hypAB* appear to be absent. Nevertheless, the *hypECDF* genes are expressed at a reasonable level (35 FPKM) (Mock, *J Bacteriol*, 197: 2965-2980, 2015).

0023 Additionally, (i) the reduction of NAD^+ with reduced ferredoxin via membrane-associated energy coupling reaction is catalyzed by Rnf A-B (CAETHG_3227-32), (ii) the reduction of NADP with reduced ferredoxin and NADH is catalyzed by Nfn (CAETHG_1580), (iii) the reduction of acetic acid to acetaldehyde by reduced ferredoxin is catalyzed by acetaldehyde:ferredoxin oxidoreductase (AOR) (CAETHG_0092, CAETHG_0102), and (iv) the reduction of acetyl-CoA plus CO_2 to pyruvate is catalyzed by pyruvate:ferredoxin oxidoreductase PFOR (CAETHG_0928, CAETHG_3029).

0024 *C. ljungdahlii* has homologues of five of the six hydrogenases found in *C. autoethanogenum*. The five hydrogenases of *C. ljungdahlii* are CLJU_c07030-07080 (homologue of CAETHG_2794-99), CLJU_c14700-20 (homologue of CAETHG_3569-71), CLJU_c20290 (homologue of CAETHG_0110), CLJU_c17280 (homologue of CAETHG_3841), and CLJU_c28650-60 (homologue of CAETHG_0861-62). The homologues of CAETHG_1576-78 are absent in *C. ljungdahlii* but a predicted iron-dependent hydrogenase (CLJU_c37220) is annotated in its place. One of the genes encoding the Rnf complex in *C. ljungdahlii* has been knocked out resulting in reduced growth under heterotrophic growth and no growth under autotrophic conditions on H_2/CO_2 or CO. The energy for minimal growth in the former case could be derived from substrate level phosphorylation by glycolysis.

0025 Similarly, *C. ragsdalei* has homologues of five of the six hydrogenases found in *C. autoethanogenum*. The homologue of CAETHG_1576-78 and CLJU_c37220 is not present in *C. ragsdalei*.

0026 Other C1-fixing microorganisms, including *Acetobacterium woodii*, *Alkalibaculum bacchii*, *Blautia producta*, *Butyribacterium methylotrophicum*, *Clostridium aceticum*, *Clostridium carboxidivorans*, *Clostridium coskatii*, *Clostridium drakei*, *Clostridium formicoaceticum*, *Clostridium magnum*, *Clostridium scatologenes*, *Eubacterium limosum*, *Moorella thermautotrophica*, *Moorella thermoacetica*, *Oxobacter pfennigii*, *Sporomusa ovata*, *Sporomusa silvacetica*, *Sporomusa sphaeroides*, and *Thermoanaerobacter kiuvi* have similar hydrogenases, the sequences of which are generally publically available.

0027 The inventors have surprisingly discovered that disruption of hydrogenases in C1-fixing microorganisms results in a variety of improved characteristics.

0028 Certain microorganisms are known to consume CO_2 or CO, rendering gas fermentation a potential route to both removing C1-carbon from the atmosphere and

sustainably producing useful fermentation products, such as fuels and chemicals. However, many of these microorganisms also produce some amount of CO_2, making it difficult to determine whether or not gas fermentation actually results in net carbon capture. The inventors have discovered that disruption of one or more hydrogenases in a microorganism causes the microorganism to consume more gaseous C1-carbon (e.g., in the form of CO_2 or CO) than it produces (e.g., in the form of CO_2), resulting in net carbon capture in organic fermentation products, such as ethanol. This can be illustrated by equations below. In particular, the more H_2 can be utilized, the less CO is required and the less CO_2 is produced per molecule of produced ethanol. At a certain point, CO_2 and H_2 can be utilized alone, in the absence of CO.

0029 When no H_2 is utilized, 6 molecules of CO are required per 1 molecule of ethanol and 4 molecules of CO_2 produced (Equation 1). When 3 molecules of H_2 are utilized, 3 molecules of CO are required per 1 molecule of ethanol and 1 molecule of CO_2 produced (Equation 2). When 4 molecules of H_2 are utilized, 2 molecules of CO are required per 1 molecule of ethanol produced (Equation 3); CO_2 may also be produced as a byproduct. When 6 molecules of H_2 are utilized, no CO is required per 1 molecule of ethanol produced (Equation 4). Additionally, 2 molecules of CO_2 are utilized and no CO_2 is produced.

0030 $6\ CO + 3\ H_2O \rightarrow CH_3CH_2OH + 4\ CO_2$ (Equation 1)

0031 $3\ CO + 3\ H_2 \rightarrow CH_3CH_2OH + CO_2$ (Equation 2)

0032 $2\ CO + 4\ H_2 \rightarrow CH_3CH_2OH + H_2O$ (Equation 3)

0033 $2\ CO_2 + 6\ H_2 \rightarrow CH_3CH_2OH + 3\ H_2O$ (Equation 4)

0034 Moreover, the inventors have found that some hydrogenases are redundant, such that a microorganism, e.g., *C. autoethanogenum*, can compensate for disruption of any one hydrogenase, e.g., HytABCDE1E2, by expressing other hydrogenase(s). In fact, the inventors have discovered that all six hydrogenases of *C. autoethanogenum* are redundant. Without wishing to be bound by any particular theory, the inventors believe disrupting certain hydrogenases leads to expression of other hydrogenase(s) with different uptake/production rates and/or cofactor dependencies, such that an overall shift in cofactor dependence is observed, e.g., from ferredoxin plus NADP to ferredoxin plus NAD or to ferredoxin only or direct reduction of CO_2 to formate with hydrogen. This results in a microorganism with a different gas uptake and/or carbon fixation profile. Additionally, this may result in a

microorganism that grows better on H_2-containing gases than the parental microorganism from which it was derived.

0035 Furthermore, the inventors have discovered that disruption of microbial hydrogenases causes the microorganism to produce greater amounts of other reduced fermentation products, such as ethanol, 2,3-butanediol, and/or isopropanol. Hydrogenase knockouts have been developed in non-C1-fixing microorganisms for the purpose of reducing hydrogen production. For example, hydrogen production in cellulolytic *C. thermocellum* was inhibited by knocking out the gene responsible for maturation of apoform hydrogenases (Biswas, *Biotechnol Biofuels*, 8: 20, 2015). However, results obtained in *C. thermocellum* are only applicable to the production of fuels and chemicals from carbohydrate feedstocks, such as lignocellulosic biomass. Such results cannot be extended to C1-fixing microorganisms, such as acetogenic bacteria, given the extensive differences in the metabolisms of carbohydrate-consuming microorganisms and C1-fixing microorganisms. Moreover, carbohydrate-consuming microorganisms (e.g., *C. thermocellum*) produce hydrogen, but typically do not utilize hydrogen, while C1-fixing microorganisms (e.g., *C. autoethanogenum*) are capable of utilizing hydrogen, such that hydrogenases play fundamentally different roles in these different types of microorganisms.

0036 Additionally, the inventors believe that disruption of microbial hydrogenases causes the microorganism to have improved resistance to toxins which would ordinarily inhibit hydrogenases. For example, acetylene inhibits the nickel-containing hydrogenases (NiFe and NiFeSe) of anaerobic sulfate reducing bacteria (He, *Biochem Biophys Res Commun*, 16: 127-133, 1989) and the Ni- and Fe- containing hydrogenase of *A. vinelandii* (Sun, *Biochem*, 31: 3158-3165, 1992). Isocyanides inhibit [Fe]-hydrogenases (Shima, *FEBS Lett*, 585: 353-356, 2011). Ammonium inhibits *C. ragsdalei* hydrogenases (Xu, *Biomass Bioenerg,* 45: 303-310, 2012). Nitric oxide inhibits *C. carboxidivorans* P7 uptake hydrogenase at 150 ppm (Ahmed, *Biomass Bioenerg*, 30: 665-672, 2006). Thus, the microorganism of the invention may have improved resistance to acetylene, isocyanide, ammonium, nitric oxide, or other toxins known to inhibit hydrogenases.

0037 *Definitions and background*

0038 The term "genetic modification" or "genetic engineering" broadly refers to manipulation of the genome or nucleic acids of a microorganism. Likewise, the term "genetically engineered" refers to a microorganism comprising a manipulated genome or

nucleic acids. Methods of genetic modification of include, for example, heterologous gene expression, gene or promoter insertion or deletion, nucleic acid mutation, altered gene expression or inactivation, enzyme engineering, directed evolution, knowledge-based design, random mutagenesis methods, gene shuffling, and codon optimization.

0039 "Recombinant" indicates that a nucleic acid, protein, or microorganism is the product of genetic modification, engineering, or recombination. Generally, the term "recombinant" refers to a nucleic acid, protein, or microorganism that contains or is encoded by genetic material derived from multiple sources, such as two or more different strains or species of microorganisms. As used herein, the term "recombinant" may also be used to describe a microorganism that comprises a mutated nucleic acid or protein, including a mutated form of an endogenous nucleic acid or protein.

0040 "Endogenous" refers to a nucleic acid or protein that is present or expressed in the wild-type or parental microorganism from which the microorganism of the invention is derived. For example, an endogenous gene is a gene that is natively present in the wild-type or parental microorganism from which the microorganism of the invention is derived. In one embodiment, the expression of an endogenous gene may be controlled by an exogenous regulatory element, such as an exogenous promoter.

0041 "Exogenous" refers to a nucleic acid or protein that is not present in the wild-type or parental microorganism from which the microorganism of the invention is derived. In one embodiment, an exogenous gene or enzyme may be derived from a heterologous (i.e., different) strain or species and introduced to or expressed in the microorganism of the invention. In another embodiment, an exogenous gene or enzyme may be artificially or recombinantly created and introduced to or expressed in the microorganism of the invention. Exogenous nucleic acids may be adapted to integrate into the genome of the microorganism of the invention or to remain in an extra-chromosomal state in the microorganism of the invention, for example, in a plasmid.

0042 "Enzyme activity," or simply "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 catalyze a reaction. Accordingly, "increasing" enzyme activity includes increasing the activity of an enzyme, increasing the amount of an enzyme, or increasing the availability of an enzyme to catalyze a reaction. Similarly, "decreasing"

enzyme activity includes decreasing the activity of an enzyme, decreasing the amount of an enzyme, or decreasing the availability of an enzyme to catalyze a reaction.

0043 "Mutated" refers to a nucleic acid or protein that has been modified in the microorganism of the invention compared to the wild-type or parental microorganism from which the microorganism of the invention is derived. In one embodiment, the mutation may be a deletion, insertion, or substitution in a gene encoding an enzyme. In another embodiment, the mutation may be a deletion, insertion, or substitution of one or more amino acids in an enzyme.

0044 In particular, a "disruptive mutation" is a mutation that reduces or eliminates (i.e., "disrupts") the expression or activity of a gene or enzyme. The disruptive mutation may partially inactivate, fully inactivate, or delete the gene or enzyme. The disruptive mutation may be a knockout (KO) mutation or a knock-down mutation. The disruptive mutation may be any mutation that reduces, prevents, or blocks the biosynthesis of a product produced by an enzyme. The disruptive mutation may include, for example, a mutation in a gene encoding an enzyme, a mutation in a genetic regulatory element involved in the expression of a gene encoding an enzyme, the introduction of a nucleic acid which produces a protein that reduces or inhibits the activity of an enzyme, or the introduction of a nucleic acid (e.g., antisense RNA, siRNA, CRISPR) or protein which inhibits the expression of an enzyme. The disruptive mutation may be introduced using any method known in the art. In the present invention, the disruptive mutation may be a disruptive mutation of a hydrogenase enzyme or a subunit thereof (or a gene encoding a hydrogenase enzyme or a subunit thereof) and/or a disruptive mutation of a hydrogenase accessory enzyme or a subunit thereof (or a gene encoding a hydrogenase accessory enzyme or a subunit thereof).

0045 "Codon optimization" refers to the mutation of a nucleic acid, such as a gene, for optimized or improved translation of the nucleic acid in a particular strain or species. Codon optimization may result in faster translation rates or higher translation accuracy. In a preferred embodiment, the genes of the invention are codon optimized for expression in *Clostridium*, particularly *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. In a further preferred embodiment, the genes of the invention are codon optimized for expression in *Clostridium autoethanogenum* LZ1561, which is deposited under DSMZ accession number DSM23693.

0046 "Overexpressed" refers to an increase in expression of a nucleic acid or protein in the microorganism of the invention compared to the wild-type or parental microorganism from which the microorganism 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.

0047 The term "variants" includes nucleic acids and proteins whose sequence varies from the sequence of a reference nucleic acid and protein, such as a sequence of a reference nucleic acid and protein disclosed in the prior art or exemplified herein. The invention may be practiced using variant nucleic acids or proteins that perform substantially the same function as the reference nucleic acid or protein. For example, a variant protein may perform substantially the same function or catalyze substantially the same reaction as a reference protein. A variant gene may encode the same or substantially the same protein as a reference gene. A variant promoter may have substantially the same ability to promote the expression of one or more genes as a reference promoter.

0048 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 may include allelic variants, fragments of a gene, mutated genes, polymorphisms, and the like. Homologous genes from other microorganisms are also examples of functionally equivalent variants. These include homologous genes in species such as *Clostridium acetobutylicum, Clostridium beijerinckii,* or *Clostridium ljungdahlii*, the details of which are publicly available on websites such as Genbank or NCBI. Functionally equivalent variants also include nucleic acids whose sequence varies as a result of codon optimization for a particular microorganism. 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 (percent homology) with the referenced 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 (percent homology) with the referenced protein. The functional equivalence of a variant nucleic acid or protein may be evaluated using any method known in the art.

0049 Nucleic acids may be delivered to a microorganism of the invention using any method known in the art. For example, nucleic acids may be delivered as naked nucleic acids or may be formulated with one or more agents, such as liposomes. The nucleic acids may be DNA,

RNA, cDNA, or combinations thereof, as is appropriate. Restriction inhibitors may be used in certain embodiments. Additional vectors may include plasmids, viruses, bacteriophages, cosmids, and artificial chromosomes. In a preferred embodiment, nucleic acids are delivered to the microorganism of the invention using a plasmid. By way of example, 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. In certain embodiments having active restriction enzyme systems, it may be necessary to methylate a nucleic acid before introduction of the nucleic acid into a microorganism.

0050 Furthermore, nucleic acids may be designed to comprise a regulatory element, such as a promoter, to increase or otherwise control expression of a particular nucleic acid. The promoter may be a constitutive promoter or an inducible promoter. Ideally, the promoter is a Wood-Ljungdahl pathway promoter, a ferredoxin promoter, a pyruvate:ferredoxin oxidoreductase promoter, an Rnf complex operon promoter, an ATP synthase operon promoter, or a phosphotransacetylase/acetate kinase operon promoter.

0051 A "microorganism" is a microscopic organism, especially a bacterium, archea, virus, or fungus. The microorganism of the invention is typically a bacterium. As used herein, recitation of "microorganism" should be taken to encompass "bacterium."

0052 A "parental microorganism" is a microorganism used to generate a microorganism of the invention. The parental microorganism may be a naturally-occurring microorganism (i.e., a wild-type microorganism) or a microorganism that has been previously modified (i.e., a mutant or recombinant microorganism). The microorganism of the invention may be modified to express or overexpress one or more enzymes that were not expressed or overexpressed in the parental microorganism. Similarly, the microorganism of the invention may be modified to contain one or more genes that were not contained by the parental microorganism. The microorganism of the invention may also be modified to not express or to express lower amounts of one or more enzymes that were expressed in the parental microorganism. In one embodiment, the parental microorganism is *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. In a preferred embodiment, the parental microorganism is *Clostridium autoethanogenum* LZ1561, which was deposited on June 7, 2010 with Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) located at Inhoffenstraß 7B, D-38124 Braunschweig, Germany

on June 7, 2010 under the terms of the Budapest Treaty and accorded accession number DSM23693.

0053 The term "derived from" indicates that a nucleic acid, protein, or microorganism is modified or adapted from a different (e.g., a parental or wild-type) nucleic acid, protein, or microorganism, so as to produce a new nucleic acid, protein, or microorganism. Such modifications or adaptations typically include insertion, deletion, mutation, or substitution of nucleic acids or genes. Generally, the microorganism of the invention is derived from a parental microorganism. In one embodiment, the microorganism of the invention is derived from *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. In a preferred embodiment, the microorganism of the invention is derived from *Clostridium autoethanogenum* LZ1561, which is deposited under DSMZ accession number DSM23693.

0054 The microorganism of the invention may be further classified based on functional characteristics. For example, the microorganism of the invention may be or may be derived from a C1-fixing microorganism, an anaerobe, an acetogen, an ethanologen, a carboxydotroph, and/or a methanotroph. Table 1 provides a representative list of microorganisms and identifies their functional characteristics.

Table 1

	C1-fixing	Anaerobe	Acetogen	Ethanologen	Autotroph	Carboxydotroph	Methanotroph
Acetobacterium woodii	+	+	+	+/- [1]	-	-	-
Alkalibaculum bacchii	+	+	+	+	+	+	-
Blautia producta	+	+	+	-	+	+	-
Butyribacterium methylotrophicum	+	+	+	+	+	+	-
Clostridium aceticum	+	+	+	-	+	+	-
Clostridium autoethanogenum	+	+	+	+	+	+	-
Clostridium carboxidivorans	+	+	+	+	+	+	-
Clostridium coskatii	+	+	+	+	+	+	-
Clostridium drakei	+	+	+	-	+	+	-
Clostridium formicoaceticum	+	+	+	-	+	+	-
Clostridium ljungdahlii	+	+	+	+	+	+	-
Clostridium magnum	+	+	+	-	+	+/- [2]	-

Clostridium ragsdalei	+	+	+	+	+	+	-
Clostridium scatologenes	+	+	+	-	+	+	-
Eubacterium limosum	+	+	+	-	+	+	-
Moorella thermautotrophica	+	+	+	+	+	+	-
Moorella thermoacetica (formerly *Clostridium thermoaceticum*)	+	+	+	- [3]	+	+	-
Oxobacter pfennigii	+	+	+	-	+	+	-
Sporomusa ovata	+	+	+	-	+	+/- [4]	-
Sporomusa silvacetica	+	+	+	-	+	+/- [5]	-
Sporomusa sphaeroides	+	+	+	-	+	+/- [6]	-
Thermoanaerobacter kiuvi	+	+	+	-	+	-	-

[1] *Acetobacterium woodi* can produce ethanol from fructose, but not from gas.

[2] It has not been investigated whether *Clostridium magnum* can grow on CO.

[3] One strain of *Moorella thermoacetica, Moorella* sp. HUC22-1, has been reported to produce ethanol from gas.

[4] It has not been investigated whether *Sporomusa ovata* can grow on CO.

[5] It has not been investigated whether *Sporomusa silvacetica* can grow on CO.

[6] It has not been investigated whether *Sporomusa sphaeroides* can grow on CO.

0055 "C1" refers to a one-carbon molecule, for example, CO, CO_2, CH_4, or CH_3OH. "C1-oxygenate" refers to a one-carbon molecule that also comprises at least one oxygen atom, for example, CO, CO_2, or CH_3OH. "C1-carbon source" refers a one carbon-molecule that serves as a partial or sole carbon source for the microorganism of the invention. For example, a C1-carbon source may comprise one or more of CO, CO_2, CH_4, CH_3OH, or CH_2O_2. Preferably, the C1-carbon source comprises one or both of CO and CO_2. A "C1-fixing microorganism" is a microorganism that has the ability to produce one or more products from a C1-carbon source. Typically, the microorganism of the invention is a C1-fixing bacterium. In a preferred embodiment, the microorganism of the invention is derived from a C1-fixing microorganism identified in Table 1.

0056 An "anaerobe" is a microorganism that does not require oxygen for growth. An anaerobe may react negatively or even die if oxygen is present above a certain threshold. Typically, the microorganism of the invention is an anaerobe. In a preferred embodiment, the microorganism of the invention is derived from an anaerobe identified in Table 1.

0057 An "acetogen" is a microorganism that produces or is capable of producing acetate (or acetic acid) as a product of anaerobic respiration. Typically, acetogens are obligately anaerobic bacteria that use the Wood-Ljungdahl pathway as their main mechanism for energy conservation and for synthesis of acetyl-CoA and acetyl-CoA-derived products, such as acetate (Ragsdale, *Biochim Biophys Acta*, 1784: 1873-1898, 2008). Acetogens use the acetyl-CoA pathway as a (1) mechanism for the reductive synthesis of acetyl-CoA from CO_2, (2) terminal electron-accepting, energy conserving process, (3) mechanism for the fixation (assimilation) of CO_2 in the synthesis of cell carbon (Drake, Acetogenic Prokaryotes, In: The Prokaryotes, 3rd edition, p. 354, New York, NY, 2006). All naturally occurring acetogens are C1-fixing, anaerobic, autotrophic, and non-methanotrophic. Typically, the microorganism of the invention is an acetogen. In a preferred embodiment, the microorganism of the invention is derived from an acetogen identified in Table 1.

0058 An "ethanologen" is a microorganism that produces or is capable of producing ethanol. Typically, the microorganism of the invention is an ethanologen. In a preferred embodiment, the microorganism of the invention is derived from an ethanologen identified in Table 1.

0059 An "autotroph" is a microorganism capable of growing in the absence of organic carbon. Instead, autotrophs use inorganic carbon sources, such as CO and/or CO_2. Typically, the microorganism of the invention is an autotroph. In a preferred embodiment, the microorganism of the invention is derived from an autotroph identified in Table 1.

0060 A "carboxydotroph" is a microorganism capable of utilizing CO as a sole source of carbon. Typically, the microorganism of the invention is a carboxydotroph. In a preferred embodiment, the microorganism of the invention is derived from a carboxydotroph identified in Table 1.

0061 A "methanotroph" is a microorganism capable of utilizing methane as a sole source of carbon and energy. In certain embodiments, the microorganism of the invention is derived from a methanotroph.

0062 More broadly, the microorganism of the invention may be derived from any genus or species identified in Table 1.

0063 In a preferred embodiment, the microorganism of the invention is derived from the cluster of *Clostridia* comprising the species *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei*. These species were first reported and characterized

by Abrini, *Arch Microbiol*, 161: 345-351, 1994 (*Clostridium autoethanogenum*), Tanner, *Int J System Bacteriol*, 43: 232-236, 1993 (*Clostridium ljungdahlii*), and Huhnke, WO 2008/028055 (*Clostridium ragsdalei*).

0064 These three species have many similarities. In particular, these species are all C1-fixing, anaerobic, acetogenic, ethanologenic, and carboxydotrophic members of the genus *Clostridium*. These species have similar genotypes and phenotypes and modes of energy conservation and fermentative metabolism. Moreover, these species are clustered in clostridial rRNA homology group I with 16S rRNA DNA that is more than 99% identical, have a DNA G + C content of about 22-30 mol%, are gram-positive, have similar morphology and size (logarithmic growing cells between 0.5-0.7 x 3-5 μm), are mesophilic (grow optimally at 30-37 °C), have similar pH ranges of about 4-7.5 (with an optimal pH of about 5.5-6), lack cytochromes, and conserve energy via an Rnf complex. Also, reduction of carboxylic acids into their corresponding alcohols has been shown in these species (Perez, *Biotechnol Bioeng*, 110:1066-1077, 2012). Importantly, these species also all show strong autotrophic growth on CO-containing gases, produce ethanol and acetate (or acetic acid) as main fermentation products, and produce small amounts of 2,3-butanediol and lactic acid under certain conditions.

0065 However, these three species also have a number of differences. These species were isolated from different sources: *Clostridium autoethanogenum* from rabbit gut, *Clostridium ljungdahlii* from chicken yard waste, and *Clostridium ragsdalei* from freshwater sediment. These species differ in utilization of various sugars (e.g., rhamnose, arabinose), acids (e.g., gluconate, citrate), amino acids (e.g., arginine, histidine), and other substrates (e.g., betaine, butanol). Moreover, these species differ in auxotrophy to certain vitamins (e.g., thiamine, biotin). These species have differences in nucleic and amino acid sequences of Wood-Ljungdahl pathway genes and proteins, although the general organization and number of these genes and proteins has been found to be the same in all species (Köpke, *Curr Opin Biotechnol*, 22: 320-325, 2011).

0066 Thus, in summary, many of the characteristics of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei* are not specific to that species, but are rather general characteristics for this cluster of C1-fixing, anaerobic, acetogenic, ethanologenic, and carboxydotrophic members of the genus *Clostridium*. However, since these species are, in fact, distinct, the genetic modification or manipulation of one of these

species may not have an identical effect in another of these species. For instance, differences in growth, performance, or product production may be observed.

0067 The microorganism of the invention may also be derived from an isolate or mutant of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. Isolates and mutants of *Clostridium autoethanogenum* include JA1-1 (DSM10061) (Abrini, *Arch Microbiol*, 161: 345-351, 1994), LBS1560 (DSM19630) (WO 2009/064200), and LZ1561 (DSM23693). Isolates and mutants of *Clostridium ljungdahlii* include ATCC 49587 (Tanner, *Int J Syst Bacteriol*, 43: 232-236, 1993), PETCT (DSM13528, ATCC 55383), ERI-2 (ATCC 55380) (US 5,593,886), C-01 (ATCC 55988) (US 6,368,819), O-52 (ATCC 55989) (US 6,368,819), and OTA-1 (Tirado-Acevedo, Production of bioethanol from synthesis gas using *Clostridium ljungdahlii*, PhD thesis, North Carolina State University, 2010). Isolates and mutants of *Clostridium ragsdalei* include PI 1 (ATCC BAA-622, ATCC PTA-7826) (WO 2008/028055).

0068 "Substrate" refers to a carbon and/or energy source for the microorganism of the invention. Typically, the substrate is gaseous and comprises a C1-carbon source, for example, CO, CO_2, and/or CH_4. Preferably, the substrate comprises a C1-carbon source of CO or CO + CO_2. The substrate may further comprise other non-carbon components, such as H_2, N_2, or electrons.

0069 The substrate may comprise at least some amount of CO, such as about 1, 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 mol% CO. The substrate may comprise a range of CO, such as about 20-80, 30-70, or 40-60 mol% CO. Preferably, the substrate comprises about 40-70 mol% CO (e.g., steel mill or blast furnace gas), about 20-30 mol% CO (e.g., basic oxygen furnace gas), or about 15-45 mol% CO (e.g., syngas). In some embodiments, the substrate may comprise a relatively low amount of CO, such as about 1-10 or 1-20 mol% CO. The microorganism of the invention typically converts at least a portion of the CO in the substrate to a product. In some embodiments, the substrate comprises no or substantially no (< 1 mol%) CO.

0070 The substrate may comprise some amount of H_2. For example, the substrate may comprise about 1, 2, 5, 10, 15, 20, or 30 mol% H_2. In some embodiments, the substrate may comprise a relatively high amount of H_2, such as about 60, 70, 80, or 90 mol% H_2. In further embodiments, the substrate comprises no or substantially no (< 1 mol%) H_2. The H_2 may be

derived from or produced by any suitable process, including the formation of H_2 using electrodes.

0071 The substrate may comprise some amount of CO_2. For example, the substrate may comprise about 1-80 or 1-30 mol% CO_2. In some embodiments, the substrate may comprise less than about 20, 15, 10, or 5 mol% CO_2. In another embodiment, the substrate comprises no or substantially no (< 1 mol%) CO_2.

0072 Although the substrate is typically gaseous, the substrate may also be provided in alternative forms. For example, the substrate may be dissolved in a liquid saturated with a CO-containing gas using a microbubble dispersion generator. By way of further example, the substrate may be adsorbed onto a solid support.

0073 The substrate and/or C1-carbon source may be a waste gas obtained as a byproduct of an industrial process or from some other source, such as from automobile exhaust fumes or biomass gasification. In certain embodiments, the industrial process is selected from the group consisting of ferrous metal products manufacturing, such as a steel mill manufacturing, non-ferrous products manufacturing, petroleum refining processes, coal gasification, electric power production, carbon black production, ammonia production, methanol production, and coke manufacturing. In these embodiments, the substrate and/or C1-carbon source may be captured from the industrial process before it is emitted into the atmosphere, using any convenient method.

0074 The substrate and/or C1-carbon source may be syngas, such as syngas obtained by gasification of coal or refinery residues, gasification of biomass or lignocellulosic material, or reforming of natural gas. In another embodiment, the syngas may be obtained from the gasification of municipal solid waste or industrial solid waste.

0075 The composition of the substrate may have a significant impact on the efficiency and/or cost of the reaction. For example, the presence of oxygen (O_2) may reduce the efficiency of an anaerobic fermentation process. Depending on the composition of the substrate, it may be desirable to treat, scrub, or filter the substrate to remove any undesired impurities, such as toxins, undesired components, or dust particles, and/or increase the concentration of desirable components.

0076 "Toxin," as used herein, refers to any chemical that inhibits or otherwise adversely affects a hydrogenase enzyme of a microorganism. The toxin may be, for example, acetylene, isocyanide, ammonium, or nitric oxide. The microorganism of the invention is

generally more resistant to (i.e., tolerant of) toxins than the parental microorganism from which the microorganism of the invention is derived, such that the microorganism of the invention is able to grow better and/or produce more products than the parental microorganism when exposed to the same amount of a toxin. Similarly, the microorganism of the invention may be able to grow and/or produce products in the presence of an amount of a toxin that would typically kill the parental microorganism.

0077 Herein, "carbon fixation" or "carbon capture" or "carbon assimilation" refers to the conversion process of inorganic carbon (e.g., CO and/or CO_2) to organic compounds (e.g., ethanol, 2,3-butanediol, acetic acid, and/or isopropanol) by living organisms. In preferred embodiments, the microorganism of the invention is capable of net carbon capture, consuming more gaseous C1-carbon than it produces. In particular, the microorganism of the invention may consume more gaseous C1-carbon in the form of CO and/or CO_2 than it produces in the form of CO_2. In certain embodiments, the microorganism of the invention is capable of net CO_2 capture, where the microorganism consumes more CO_2 than it produces.

0078 The microorganism of the invention may be cultured to produce one or more products. For instance, *Clostridium autoethanogenum* produces or can be engineered to produce ethanol (WO 2007/117157), acetate (WO 2007/117157), butanol (WO 2008/115080 and WO 2012/053905), butyrate (WO 2008/115080), 2,3-butanediol (WO 2009/151342), lactate (WO 2011/112103), butene (WO 2012/024522), butadiene (WO 2012/024522), methyl ethyl ketone (2-butanone) (WO 2012/024522 and WO 2013/185123), ethylene (WO 2012/026833), acetone (WO 2012/115527), isopropanol (WO 2012/115527), lipids (WO 2013/036147), 3-hydroxypropionate (3-HP) (WO 2013/180581), isoprene (WO 2013/180584), fatty acids (WO 2013/191567), 2-butanol (WO 2013/185123), 1,2-propanediol (WO 2014/0369152), and 1-propanol (WO 2014/0369152). In certain embodiments, microbial biomass itself may be considered a product.

0079 A "native product" is a product produced by a genetically unmodified microorganism. For example, ethanol, acetate, and 2,3-butanediol are native products of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei*. A "non-native product" is a product that is produced by a genetically modified microorganism, but is not produced by a genetically unmodified microorganism from which the genetically modified microorganism is derived. For example, isopropanol is a non-native product of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei*.

0080 "Selectivity" refers to the ratio of the production of a target product to the production of all fermentation products produced by a microorganism. The microorganism of the invention may be engineered to produce products at a certain selectivity or at a minimum selectivity. In one embodiment, a target product account for at least about 5%, 10%, 15%, 20%, 30%, 50%, or 75% of all fermentation products produced by the microorganism of the invention. In one embodiment, the target product accounts for at least 10% of all fermentation products produced by the microorganism of the invention, such that the microorganism of the invention has a selectivity for the target product of at least 10%. In another embodiment, the target product accounts for at least 30% of all fermentation products produced by the microorganism of the invention, such that the microorganism of the invention has a selectivity for the target product of at least 30%.

0081 "Increasing the efficiency," "increased efficiency," and the like include, but are not limited to, increasing growth rate, product production rate or volume, product volume per volume of substrate consumed, or product selectivity. Efficiency may be measured relative to the performance of parental microorganism from which the microorganism of the invention is derived.

0082 Typically, the culture is performed in a bioreactor. The term "bioreactor" includes a culture/fermentation device consisting of one or more vessels, towers, or piping arrangements, such as a continuous stirred tank reactor (CSTR), immobilized cell reactor (ICR), trickle bed reactor (TBR), bubble column, gas lift fermenter, static mixer, or other vessel or other device suitable for gas-liquid contact. In some embodiments, the bioreactor may comprise a first growth reactor and a second culture/fermentation reactor. The substrate may be provided to one or both of these reactors. As used herein, the terms "culture" and "fermentation" are used interchangeably. These terms encompass both the growth phase and product biosynthesis phase of the culture/fermentation process.

0083 The culture is generally 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 an anaerobic microbial growth medium, such as a minimal anaerobic microbial growth medium. Suitable media are well known in the art.

0084 The culture/fermentation should desirably be carried out under appropriate conditions for production of the target product. Typically, the culture/fermentation is performed under anaerobic conditions. Reaction conditions to consider include pressure (or partial 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 gas in the liquid phase does not become limiting, and maximum product concentrations to avoid product inhibition. In particular, the rate of introduction of the substrate may be controlled to ensure that the concentration of gas in the liquid phase does not become limiting, since products may be consumed by the culture under gas-limited conditions.

0085 Operating a bioreactor at elevated pressures allows for an increased rate of gas mass transfer from the gas phase to the liquid phase. Accordingly, it is generally preferable to perform the culture/fermentation at pressures higher than atmospheric pressure. Also, since a given gas conversion rate is, in part, a function of the substrate retention time and retention time 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 culture/fermentation equipment. 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 microorganism used. However, in general, it is preferable to operate the fermentation at a pressure higher than atmospheric pressure. Also, since a given gas conversion rate is in part a function of 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.

0086 Target products may be separated or purified from a fermentation broth using any method or combination of methods known in the art, including, for example, fractional distillation, evaporation, pervaporation, gas stripping, phase separation, and extractive fermentation, including for example, liquid-liquid extraction. In certain embodiments, target products are recovered from the fermentation broth by continuously removing a portion of the broth from the bioreactor, separating microbial cells from the broth (conveniently by filtration), and recovering one or more target products from the broth. Alcohols and/or acetone may be recovered, for example, by distillation. Acids may be recovered, for example, by adsorption on activated charcoal. Separated microbial cells are preferably returned to the bioreactor. The cell-free permeate remaining after target products have been removed is also preferably returned to the bioreactor. Additional nutrients (such as B

vitamins) may be added to the cell-free permeate to replenish the medium before it is returned to the bioreactor.

EXAMPLES

0087 The following examples further illustrate the invention but, of course, should not be construed to limit its scope in any way.

0088 *Example 1*

0089 This example describes the construction of *C. autoethanogenum* hydrogenase mutants.

0090 Microorganisms

0091 *C. autoethanogenum* LZ1561, i.e., DSM23693 (a derivate of DSM10061), was obtained from DSMZ. Growth was carried out at 37° C using strictly anaerobic conditions and techniques (Hungate, Meth Microbiol, 3B: 117-132, 1969; Wolfe, Adv Microb Physiol, 6: 107-146, 1971). Chemically defined PETC medium without yeast extract was used. A 30 psi CO-containing gas mix (44% CO, 32% N_2, 22% CO_2, 2% H_2) served as a sole source of carbon and energy.

PETC medium	**Per 1.0 L of medium**
NH_4Cl	1 g
KCl	0.1 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.8 g
KH_2PO_4	0.1 g
$CaCl_2$	0.02 g
Trace metal solution	10 ml
Wolfe's vitamin solution	10 ml
Resazurin (2 g/L stock)	0.5 ml
$NaHCO_3$	2 g
Reducing agent solution	0.006-0.008 % (v/v)
Distilled water	Up to 1.0 L pH 5.5 (adjusted with HCl)

Wolfe's vitamin solution	**Per 1.0 L of solution**
Biotin	2 mg
Folic acid	2 mg
Pyridoxine hydrochloride	10 mg
Riboflavin	5 mg
Nicotinic acid	5 mg
Calcium D-(+)-pantothenate	5 mg
Vitamin B12	0.1 mg
p-Aminobenzoic acid	5 mg

Lipoic acid	5 mg
Thiamine	5 mg
Distilled water	To 1.0 L

Trace metal solution	**Per 1.0 L of solution**
Nitrilotriacetic acid	2 g
$MnSO_4 \cdot H_2O$	1 g
$Fe(SO_4)_2(NH_4)_2 \cdot 6H_2O$	0.8 g
$CoCl_2 \cdot 6H_2O$	0.2 g
$ZnSO_4 \cdot 7H_2O$	0.2 mg
$CuCl_2 \cdot 2H_2O$	0.02 g
$NaMoO_4 \cdot 2H_2O$	0.02 g
Na_2SeO_3	0.02 g
$NiCl_2 \cdot 6H_2O$	0.02 g
$Na_2WO_4 \cdot 2H_2O$	0.02 g
Distilled water	To 1.0 L

Reducing agent solution	**Per 100 mL of solution**
NaOH	0.9 g
Cysteine • HCl	4 g
Na_2S	4 g
Distilled water	To 100 mL

0092 Analysis of metabolites

0093 To remove proteins and other cell residues, 400 µl samples were mixed with 100 µl of a 2 % (w/v) 5-sulfosalicylic acid and centrifuged at 14,000 x g for 3 min to separate precipitated residues. 10 µl of the supernatant was then injected into the HPLC for analyses. HPLC analysis of 2,3-butanediol, 2-butanol, and other metabolites was performed using an Agilent 1100 Series HPLC system equipped with a RID operated at 35 °C (Refractive Index Detector) and an Aminex HPX-87H column (300 x 7.8 mm, particle size 9 µm) kept at 35 °C. Slightly acidified water was used (0.005 M H_2SO_4) as mobile phase with a flow rate of 0.6 ml/min. For distinction of 2,3-butanediol sterioisomers, HPLC analysis was performed using an Agilent 1100 Series HPLC system equipped with a RID operated at 35 °C (Refractive Index Detector) and an Alltech IOA-2000 Organic acid column (150 x 6.5 mm, particle size 8 µm) kept at 60 °C. Slightly acidified water was used (0.005 M H_2SO_4) as mobile phase with a flow rate of 0.25 ml/min.

0094 Group II intron based insertional inactivation of hydrogenase genes in *C. autoethanogenum* LZ1561

0095 The hydrogenases in *C. autoethanogenum* LZ1561 were inactivated using ClosTron group II intron mediated gene disruption tool (Heap, *J Microbiol Meth*, 80: 49-55, 2010). The Perutka algorithm hosted on the ClosTron website was used to identify group II intron target sites and to design the intron targeting regions which were synthesized delivered in pMTL007C-E5 vector by DNA2.0. The resulting vectors contain a Retro-tranposition-Activated *ermB* Marker (RAM) which confers resistance to antibiotic clarithromycin upon insertion of group-II into the target site. The insertion of group II intron at the target site will most likely affect the expression of other genes in the same complex.

0096 Inactivation of hydrogenase genes CAETHG_0860, CAETHG_1576, CAETHG_3569, and CAETHG_0110 has already been reported earlier by Mock, *J Bacteriol*, 197: 2965-2980, 2015. However, the HytABCDE1E2 complex and another hydrogenase CAETHG_3841 could not be inactivated and were therefore concluded to be essential for growth on syngas. These hydrogenase genes, specifically CAETHG_2797 and CAETHG_2798 from the HytABCDE1E2 cluster and CAETHG_3841, were targeted again, but with group-II intron insertion sites of a lower score than that reported by Mock, *J Bacteriol*, 197: 2965-2980, 2015. Surprisingly, despite the lower predicted score by the Perutka algorithm these hydrogenase genes could be disrupted using the new group-II intron insertion sites, demonstrating that these are not essential and that any hydrogenase in *C. autoethanogenum* is redundant.

Target gene	Group II insertion site	Sequence of intron targeting region	Primers used for screening	
			Forward primer	Reverse primer
CAETHG_1576	1038|1039s	SEQ ID NO: 51	Og119f SEQ ID NO: 52	Og120r SEQ ID NO: 53
CAETHG_0110	123|124s	SEQ ID NO: 54	Og121f SEQ ID NO: 55	Og122r SEQ ID NO: 56
CAETHG_0860	807|808a	SEQ ID NO: 57	Og117f SEQ ID NO: 58	Og118r SEQ ID NO: 59
CAETHG_3569	931|932a	SEQ ID NO: 60	Og125f SEQ ID NO: 61	Og126r SEQ ID NO: 62
CAETHG_3841	73|74s	SEQ ID NO: 63	Og123f SEQ ID NO: 64	Og124r SEQ ID NO: 65
CAETHG_2797	190|191a	SEQ ID NO: 66	FS001for SEQ ID NO: 67	FS001rev SEQ ID NO: 68
CAETHG_2798	543|544s	SEQ ID NO: 69	FS003for SEQ ID NO: 70	FS003rev SEQ ID NO: 71

0097 To generate the mutants, the plasmids were introduced into *C. autoethanogenum* LZ1561 as described above. Streaks of single colonies were made sequentially first on

PETC-MES media containing 15 µg/ml thiamphenicol followed by on agar plates with PETC-MES media containing 5 µg/ml clarithromycin. Colonies were randomly screened for group II intron insertion by PCR using primers listed above. Amplification of PCR products of 300-500 bp with primers indicated the unmodified *C. autoethanogenum* LZ1561 locus. Amplification of PCR products of ~2.2 kb using the same set of primers indicated insertion of ClosTron group II intron in the target gene. These results confirm the disruption of hydrogenases in *C. autoethanogenum* LZ1561. The following positive clones were selected for further studies: ΔCAETHG_1576-Clone1, ΔCAETHG_0110-Clone5, ΔCAETHG_0860-Clone5, ΔCAETHG_3569-Clone8, ΔCAETHG_2797-Clone1, and ΔCAETHG_2798-Clone1.

Example 2

0098 This example profiles the growth of *C. autoethanogenum* hydrogenase mutants on a CO-rich gas mix containing 50% CO, 4% H_2, 25% CO_2, and 21% N_2.

0099 *C. autoethanogenum* LZ1561 was used as a control. ΔCAETHG_2797-Clone1 and ΔCAETHG_2798-Clone1 mutants essentially represent the knockout of one functional hydrogenase, HytABCDE1E2, which is most highly expressed under CO-rich gas conditions. All strains were grown in PETC-MES medium in 250 ml serum bottles and gassed to 30 psig with the CO-rich gas mix. The growth experiment was initiated by inoculating the media to a starting OD of 0.01. Growth and metabolite formation was followed for 139 hours.

0100 All hydrogenase mutants appear to grow in CO-rich gas, but at different growth rates (Fig. 1A) indicating that all five hydrogenase genes are redundant. The growth of ΔCAETHG_2797-Clone1 and ΔCAETHG_2798-Clone1 mutants is drastically impaired followed by ΔCAETHG_1576-Clone1 and ΔCAETHG_0110-Clone5. This is in accordance with the expression of these hydrogenases in *C. autoethanogenum* LZ1561 where the transcript abundance is CAETHG_2794-99 > CAETHG_1576-79 > CAETHG_0110 (Mock, *J Bacteriol*, 197: 2965-2980, 2015). A similar trend is observed with acetate production in *C. autoethanogenum* LZ1561 and hydrogenase mutant strains (Fig. 1B). Interestingly, ΔCAETHG_1576-Clone1 surprisingly produces double the ethanol, making up to 1.2 g/L whereas *C. autoethanogenum* LZ1561 only makes only 0.6 g/L (Fig. 1C) under the same conditions.

Example 3

0101 This example profiles growth of *C. autoethanogenum* hydrogenase mutants on a H_2-rich gas mix containing 65% H_2, 9.2% N_2, and 23% CO_2.

0102 Aside from the gas composition, the experimental setup was similar to that described in Example 2. The growth and metabolite profile of hydrogenase mutants on H_2-rich gas is interestingly different from that seen on the CO-rich gas of Example 2. ΔCAETHG_2797-Clone1 and ΔCAETHG_2798-Clone1 mutants grew to an OD600 of 1.2 while *C. autoethanogenum* LZ1561 and other mutants reached an OD600 of 0.8 (Fig. 2A). This implies that, in the absence of functional CAETHG_2794-99 cluster, other hydrogenases that rescue growth of the mutant help the mutant perform better in the H_2-rich gas. ΔCAETHG_1576-Clone1 and ΔCAETHG_0110-Clone5 grew poorly under the same conditions (Fig. 2A) indicating that these hydrogenases, CAETHG_1576-79 and CAETHG_0110, may be essential for survival on H_2 rich gas. Interestingly, the ΔCAETHG_2797-Clone1 and ΔCAETHG_2798-Clone1 mutants that had better growth, made 20-40% less acetate (3-4 g/L) compared to *C. autoethanogenum* LZ1561 (6.5 g/L) (Fig. 2B) and surprisingly over 20 times (2174%) more ethanol (up to 5 g/L) compared to *C. autoethanogenum* LZ1561 (0.23 g/L) (Fig. 2C) under same conditions.

Example 4

0103 This example describes transcriptomics of *C. autoethanogenum* LZ1561 grown on a H_2-rich gas mix.

0104 *C. autoethanogenum* LZ1561 was grown in a bioreactor on a gas mix containing 65% H_2, 25% CO_2, 6% N_2, and 4% according to the methods described in Mock, *J Bacteriol*, 197: 2965-2980, 2015. The cell pellets were harvested at steady state to investigate the expression of heptameric Hyt hydrogenase (HytABCDE1E2; CATHEG_2794-99) and trimeric Hyd hydrogenase over the course of 23 days using RNA transcriptomics (RNASeq) according to Marcellin, Low carbon fuels and commodity chemicals from waste gases – systematic approach to understand energy metabolism in a model acetogen, *Green Chem*, 2016.

0105 From the RNASeq data, it was evident that expression of the heptameric Hyt remained relatively stable from days 0-23. Hyt was the most highly expressed hydrogenase until day 10. However the expression of trimeric Hyd cluster, which was the second most highly expressed hydrogenase until about day 10, increased by approximately 5 fold between days 7 and 10 to reach similar levels of expression as Hyt. In contrast, the expression of Hyd did not change over time in cells growth on a CO-rich gas mix rather than a H_2-rich gas mix (Mock, *J Bacteriol*, 197: 2965-2980, 2015 and Marcellin, Low carbon fuels and commodity

chemicals from waste gases – systematic approach to understand energy metabolism in a model acetogen, *Green Chem*, 2016).

0106 The increase in expression of Hyd on a H_2-rich gas mix implies that Hyd plays a role in H_2 uptake.

0107 All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein. The reference to any prior art in this specification is not, and should not be taken as, an acknowledgement that that prior art forms part of the common general knowledge in the field of endeavour in any country.

0108 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.

0109 Preferred embodiments of this invention are described herein. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by

applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

CLAIMS

1. A genetically engineered C1-fixing microorganism comprising a disruptive mutation in a hydrogenase enzyme or a hydrogenase accessory enzyme.

2. The microorganism of claim 1, wherein the hydrogenase enzyme is selected from the group consisting of an electron-bifurcating, NADP- and ferredoxin dependent [FeFe]-hydrogenase; a multi-subunit [FeFe]-hydrogenase; a mono-subunit [FeFe]-hydrogenase; and a [NiFe]-hydrogenase.

3. The microorganism of claim 2, wherein the electron-bifurcating, NADP- and ferredoxin dependent [FeFe]-hydrogenase is CAETHG_2794-99 (HytABCDE1E2); the multi-subunit [FeFe]-hydrogenase is CAETHG_1576-78 or CAETHG_3569-71; the mono-subunit [FeFe]-hydrogenase is CAETHG_0110 or CAETHG_3841; or the [NiFe]-hydrogenase is CAETHG_0861-62.

4. The microorganism of claim 3, wherein the CAETHG_2794-99 (HytABCDE1E2) comprises a disruptive mutation in one or more of subunits HytA, HytB, HytC, HytD, HytE1, and HytE2.

5. The microorganism of claim 1, wherein the hydrogenase accessory enzyme is a [NiFe]-hydrogen maturation protease.

6. The microorganism of claim 5, wherein the [NiFe]-hydrogen maturation protease is CAETHG_0860.

7. The microorganism of claim 1, wherein the microorganism is derived from a parental microorganism selected from the group consisting of *Acetobacterium woodii*, *Alkalibaculum bacchii*, *Blautia producta*, *Butyribacterium methylotrophicum*, *Clostridium aceticum*, *Clostridium autoethanogenum*, *Clostridium carboxidivorans*, *Clostridium coskatii*, *Clostridium drakei*, *Clostridium formicoaceticum*, *Clostridium ljungdahlii*, *Clostridium magnum*, *Clostridium ragsdalei*, *Clostridium scatologenes*, *Eubacterium limosum*, *Moorella thermautotrophica*, *Moorella thermoacetica*, *Oxobacter pfennigii*, *Sporomusa ovata*, *Sporomusa silvacetica*, *Sporomusa sphaeroides*, and *Thermoanaerobacter kiuvi*.

8. The microorganism of claim 7, wherein the microorganism is derived from *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*.

9. The microorganism of claim 1, wherein the microorganism produces more ethanol than a parental microorganism without the disruptive mutation.

10. The microorganism of claim 1, wherein the microorganism consumes a gaseous substrate comprising a C1-carbon source comprising CO_2.

11. The microorganism of claim 1, wherein the microorganism consumes a gaseous substrate comprising a C1-carbon source comprising CO.

12. The microorganism of claim 1, wherein the microorganism is capable of net carbon capture.

13. The microorganism of claim 1, wherein the microorganism is more tolerant of a toxin than a parental microorganism without the disruptive mutation.

14. The microorganism of claim 13, wherein the toxin is acetylene, isocyanide, ammonium, or nitric oxide.

15. A method of producing a product comprising culturing the microorganism of claim 1 in the presence of a gaseous substrate, whereby the microorganism produces the product.

16. The method of claim 15, wherein the gaseous substrate comprises a C1-carbon source comprising CO_2.

17. The method of claim 15, wherein the gaseous substrate comprises a C1-carbon source comprising CO.

18. The method of claim 15, wherein the product is one or more of ethanol, 2,3-butanediol, and isopropanol.

19. The method of claim 15, wherein the product is biomass.

20. A method of fixing carbon comprising culturing the microorganism of claim 1 in the presence of a gaseous substrate comprising a C1-carbon source, whereby the microorganism consumes more C1-carbon than it produces.

21. The method of claim 20, wherein the C1-carbon source comprises CO_2.

22. The method of claim 20, wherein the C1-carbon source comprises CO.

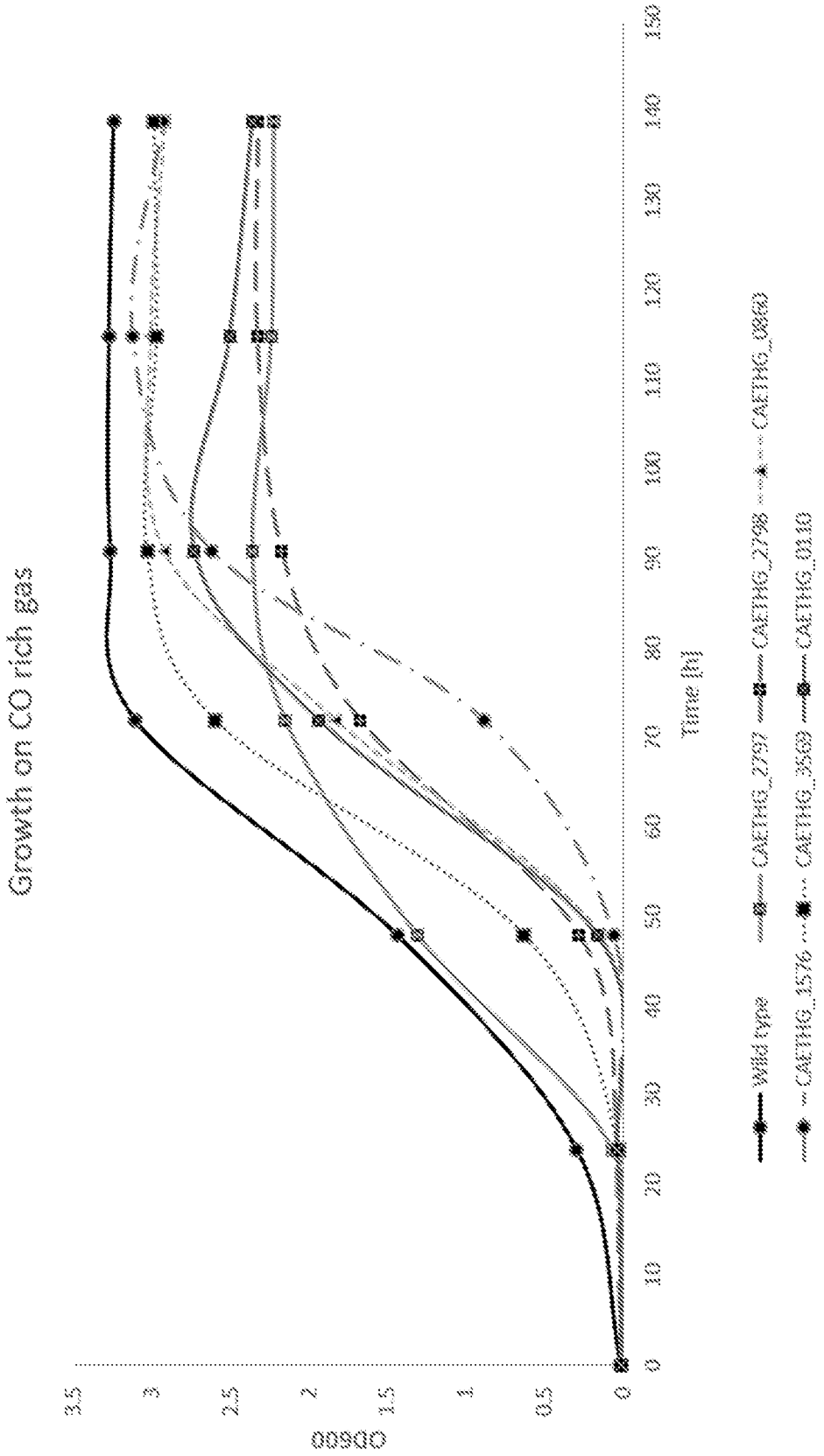

Growth on CO rich gas
OD600
Time [h]
Wild type
CAETHG_2797
CAETHG_2798
CAETHG_0860
CAETHG_1576
CAETHG_3589
CAETHG_0110

FIG. 1A

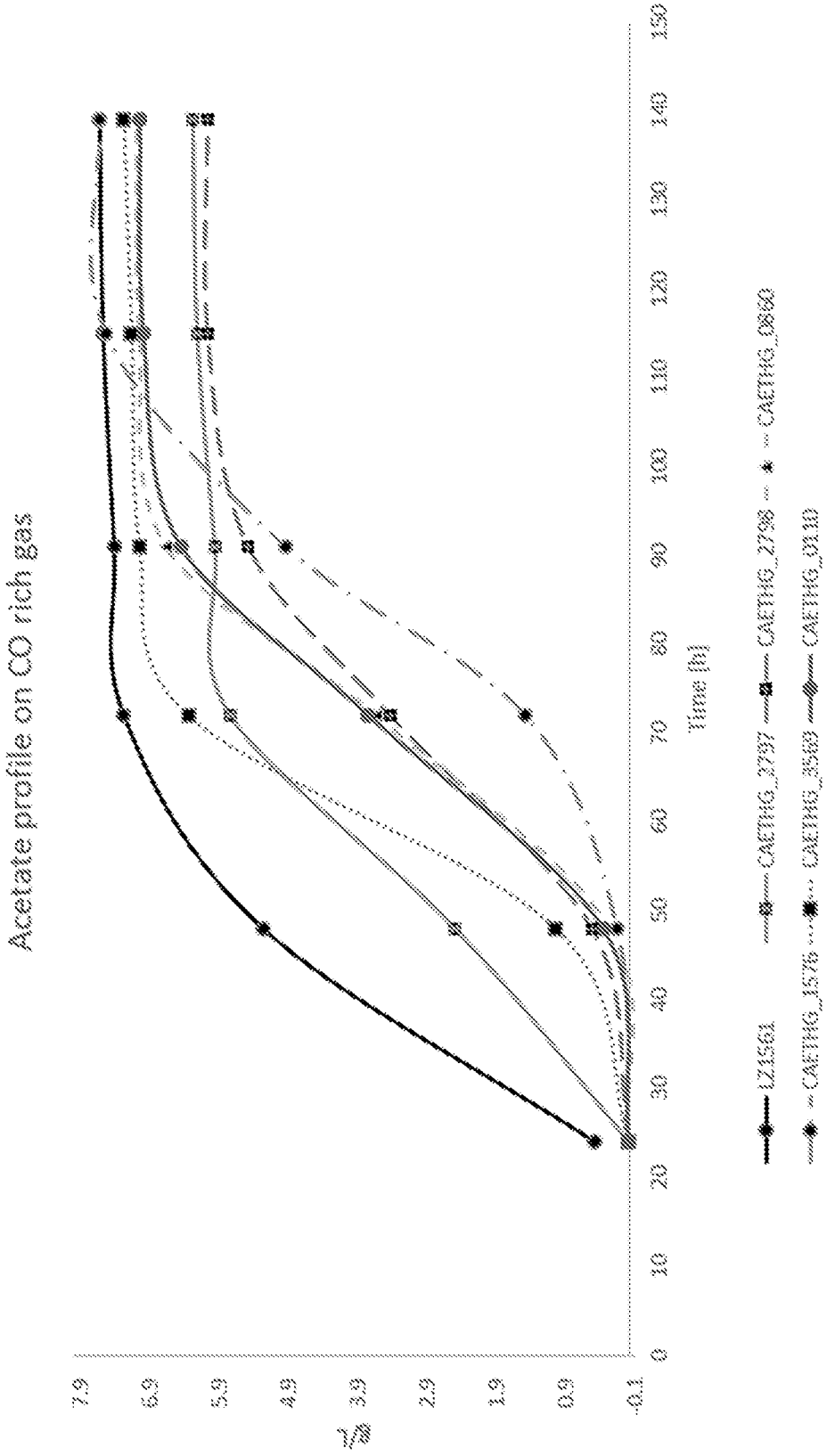

Acetate profile on CO rich gas
g/l
7.9
6.9
5.9
4.9
3.9
2.9
1.9
0.9
-0.1
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
Time [h]
LZ1561
CAETHG_2797
CAETHG_2798
CAETHG_0860
CAETHG_1576
CAETHG_3569
CAETHG_0110

FIG. 1B

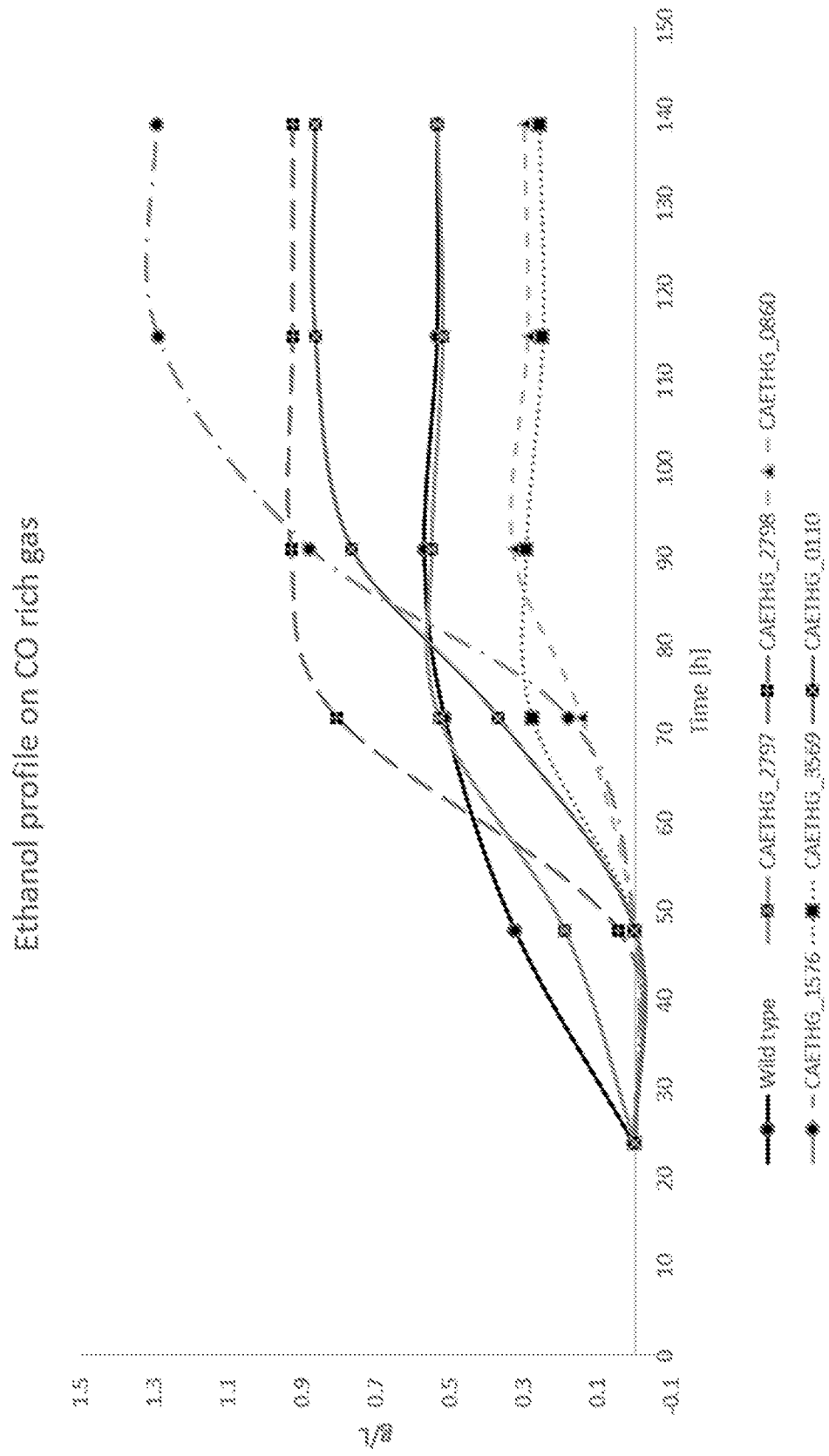

Ethanol profile on CO rich gas
g/l
Time [h]
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
Wild type
CAETHG_2797
CAETHG_2798
CAETHG_0860
CAETHG_1576
CAETHG_3569
CAETHG_0110

FIG. 1C

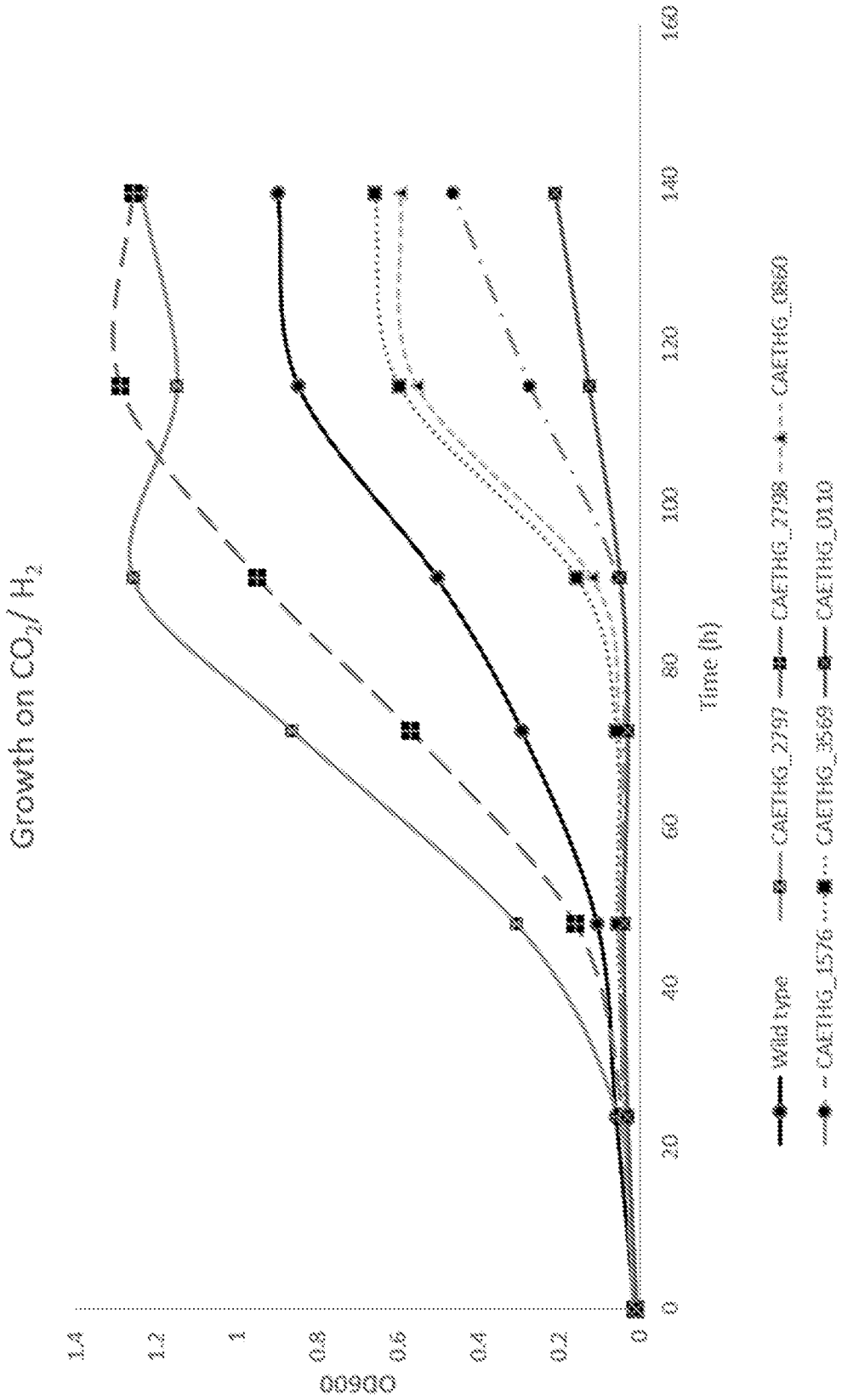

Growth on CO_2/ H_2
OD600
Time (h)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
0
20
40
60
80
100
120
140
160
Wild type
CAETHG_2797
CAETHG_2798
CAETHG_0860
CAETHG_1576
CAETHG_3569
CAETHG_0110

FIG. 2A

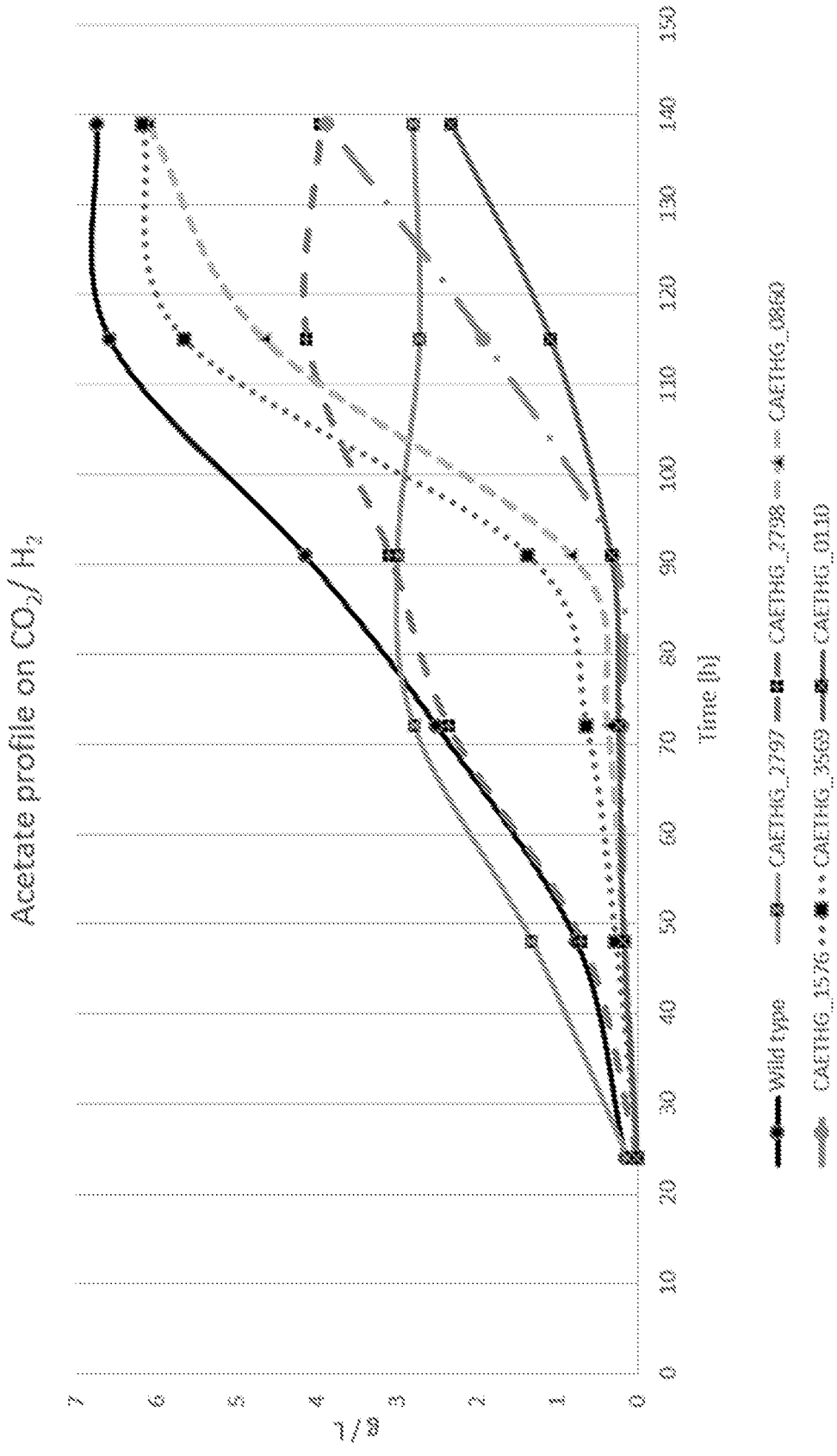

Acetate profile on CO_2/ H_2
1/8
Time (h)
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
1
2
3
4
5
6
7
Wild type
CAETHG_2797
CAETHG_2798
CAETHG_0860
CAETHG_1576
CAETHG_3569
CAETHG_0130

FIG. 2B

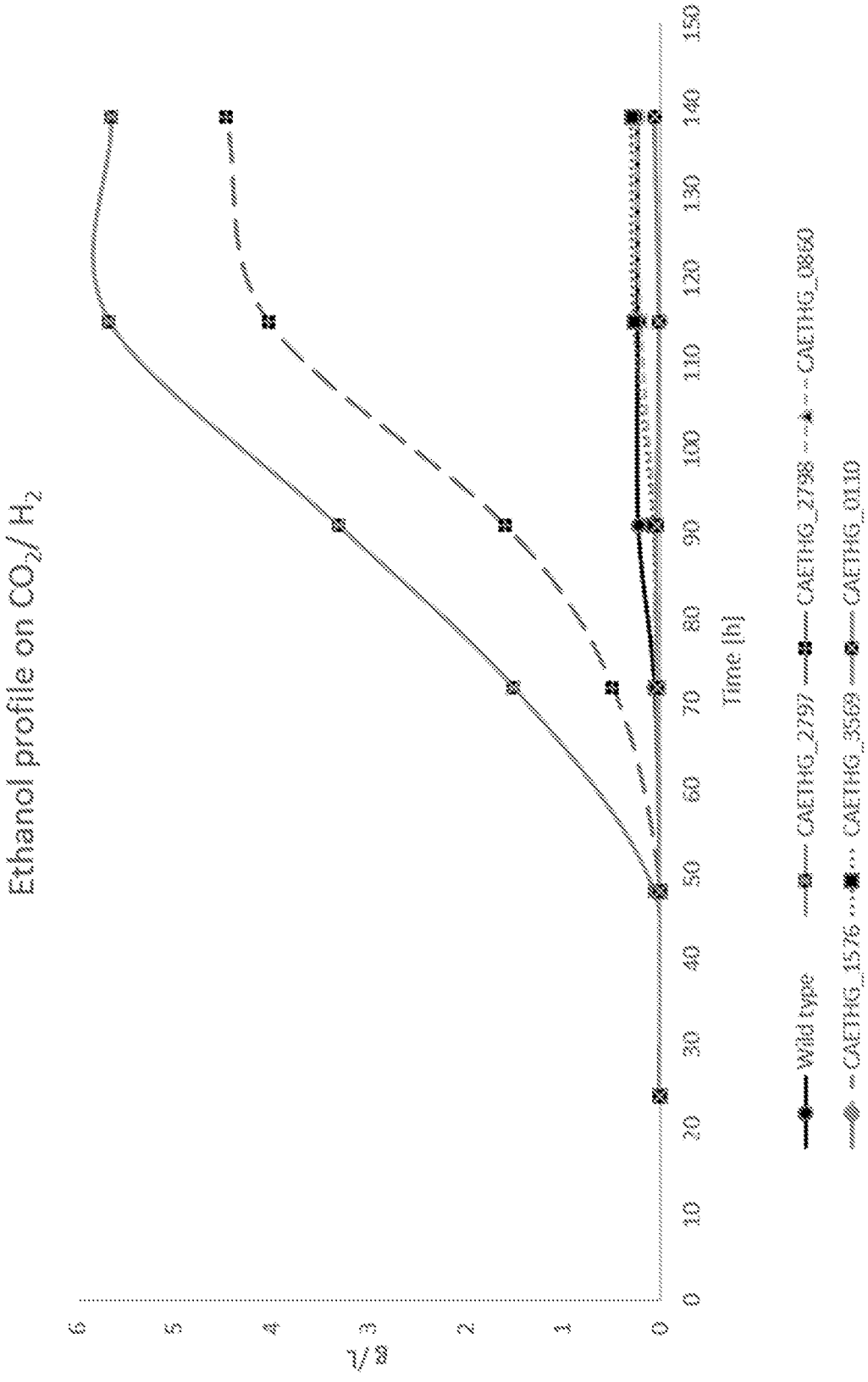

Ethanol profile on CO_2/ H_2
g/l
Time [h]
0
1
2
3
4
5
6
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
Wild type
CAETHG_2797
CAETHG_2798
CAETHG_0860
CAETHG_1576
CAETHG_3569
CAETHG_0110

FIG. 2C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/069044

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

      ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/069044

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/74(2006.01)i, C12N 9/02(2006.01)i, C12N 9/50(2006.01)i, C12P 7/04(2006.01)i, C12P 7/06(2006.01)i, C12P 7/18(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 15/74; A61K 39/108; A61K 39/112; C12N 9/02; C12N 9/50; C12P 7/04; C12P 7/06; C12P 7/18

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & keywords: hydrogenase, C1-fixing, microorganism, mutation, toxin resistance

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOCK et al., `Energy conservation associated with ethanol formation from H2 and CO2 in Clostridium autoethanogenum involving electron bifurcation`, Journal of Bacteriology, Vol.197, No.18, pp.2965-2980 (September 2015) See abstract; pages 2969-2971.	1-3,5-22
A		4
A	US 2009-0081257 A1 (MAIER et al.) 26 March 2009 See claims 1-6.	1-22
A	HUANG et al., `Electron bifurcation involved in the energy metabolism of the acetogenic bacterium Moorella thermoacetica growing on glucose or H2 plus CO2`, Journal of Bacteriology, Vol.194, No.14, pp.3689-3699 (July 2012) See the whole document.	1-22
A	WANG et al., `NADP-specific electron-bifurcating [FeFe]-hydrogenase in a functional complex with formate dehydrogenase in Clostridium autoethanogenum grown on CO`, Journal of Bacteriology, Vol.195, No.19, pp.4373-4386 (October 2013) See the whole document.	1-22

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
13 April 2017 (13.04.2017)	**13 April 2017 (13.04.2017)**

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/069044

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BISWAS et al., `Elimination of hydrogenase active site assembly blocks H2 production and increases ethanol yield in Clostridium thermocellum`, Biotechnology for Biofuels, Vol.8, Article No.20, pp.1-8 (Epub. 12 February 2015) See the whole document.	1-22

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2016/069044

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2009-0081257 A1	26/03/2009	US 7919081 B2	05/04/2011
		WO 2005-086669 A2	22/09/2005
		WO 2005-086669 A3	30/03/2006