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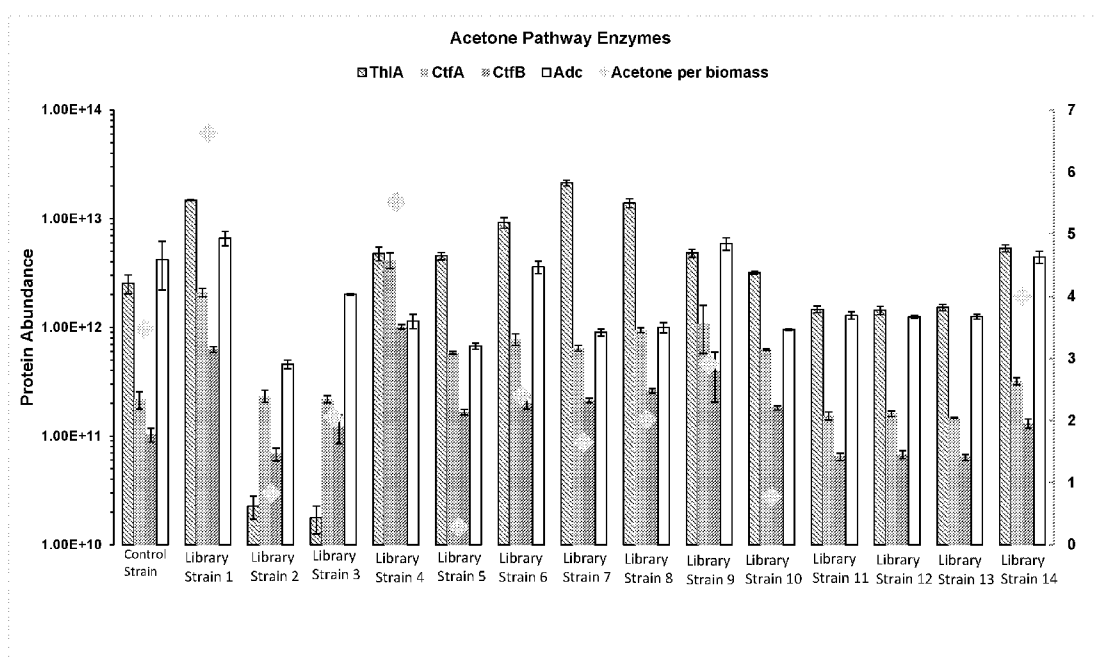


Fig. 5

(57) Abstract: Provided is a genetically engineered microorganism comprising expression of multiple CoA transferases conferring certain advantages, including increased product production and fermentation stability. Also provided is a method for increasing production of a product comprising culturing the genetically engineered microorganism in the presence of a gaseous substrate wherein the gaseous substrate may comprise a C1-carbon source comprising one or more of CO, CO₂, and H₂.

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RECOMBINANT MICROORGANISMS AND USES THEREFOR

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 63/083,257, filed September 25, 2020, the entirety of which is incorporated herein by reference.

FIELD

[0002] This application relates to genetically engineered microorganisms and use of those microorganisms for the fermentative production of products from substrates comprising carbon dioxide (CO₂), carbon monoxide (CO), and/or hydrogen (H₂).

BACKGROUND

[0003] Mitigation of impending climate change requires drastic reductions in emissions of greenhouse gases (GHGs), such as those generated through the burning of fossil fuels like coal and oil. Although sustainable sources of fuels and chemicals are currently insufficient to significantly displace our dependence on fossil carbon, gas fermentation has recently emerged as an alternative platform for the biological fixation of such gases such as CO, CO₂, and/or H₂ into sustainable fuels and chemicals. In particular, gas fermentation technology can utilize a wide range of feedstocks including gasified carbonaceous matter (e.g., municipal solid waste or agricultural waste) or industrial waste gases (e.g., from steel mills or oil refineries) to produce ethanol, jet fuel, and a variety of other products. Gas fermentation alone could displace 30% of crude oil use and reduce global CO₂ emissions by 10%, but, as with any disruptive technology, many technical challenges must be overcome before this potential is fully achieved.

[0004] In particular, there remains a need for additional microorganisms with improved stability and substrate selectivity for the increased production of native and nonnative products from gaseous substrates.

SUMMARY

[0005] The disclosure is directed to a genetically engineered Wood-Ljungdahl microorganism comprising a first exogenous CoA transferase, and at least one additional exogenous CoA transferase. The first exogenous CoA transferase may replace a coding region of an acetolactate decarboxylase gene. The at least one additional exogenous CoA transferase may replace a coding region of an aldehyde-alcohol dehydrogenase gene. The

microorganism may further comprise an exogenous thiolase and an exogenous decarboxylase selected from acetoacetate decarboxylase or alpha-ketoisovalerate decarboxylase, with the first exogenous CoA transferase. The exogenous CoA transferases may be *Clostridium acetobutylicum* CtfA and CtfB, or *Clostridium beijerinckii* CtfA and CtfB. The exogenous

5 CoA transferases may be nonnative to the microorganism, or the exogenous CoA transferases may be native to the microorganism. The exogenous CoA transferases may be the same or the exogenous CoA transferases may be different. The exogenous CoA transferases may be under the control of both an acetolactate decarboxylase promoter and at least one other promoter. The microorganism may not produce 2,3-butanediol. The microorganism may

10 produce one or more of acetone and isopropanol. The microorganism may further comprise a disruptive mutation in a primary-secondary alcohol dehydrogenase gene, a thioesterase gene, a 3-hydroxybutyryl coA dehydrogenase gene, or any combination thereof. The exogenous CoA transferases may enable production of 1-butanol, butyrate, butene, butadiene, methyl ethyl ketone, ethylene, acetone, isopropanol, lipids, 3-hydroxypropionate, terpenes, isoprene,

15 fatty acids, 2-butanol, 1,2-propanediol, 1-propanol, 1-hexanol, 1-octanol, chorismate-derived products, 3-hydroxybutyrate, 1,3-butanediol, 2-hydroxyisobutyrate or 2-hydroxyisobutyric acid, isobutylene, adipic acid, keto-adipic acid, 1,3-hexanediol, 3-methyl-2-butanol, 2-buten-1-ol, isovalerate, isoamyl alcohol, or monoethylene glycol. The microorganism may be a C1-fixing microorganism. The microorganism may further comprise an exogenous thiolase, an

20 exogenous decarboxylase selected from acetoacetate decarboxylase or alpha-ketoisovalerate decarboxylase, or any combination thereof, with the at least one additional exogenous CoA transferase. The microorganism may be a bacterium. The microorganism may be a member of a genus selected from *Acetobacterium*, *Alkalibaculum*, *Blautia*, *Butyribacterium*, *Clostridium*, *Eubacterium*, *Moorella*, *Oxobacter*, *Sporomusa*, and *Thermoanaerobacter*.

25 **[0006]** The disclosure is further directed to a method for increasing production of a product comprising culturing a genetically engineered Wood-Ljungdahl microorganism comprising a first exogenous CoA transferase, and at least one additional exogenous CoA transferase in the presence of a gaseous substrate. The gaseous substrate may comprise a C1-carbon source comprising one or more of CO, CO₂, CH₄ and H₂. The gaseous substrate may comprise

30 syngas or industrial waste gas. The product may be one or more of acetone and isopropanol.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007] Fig. 1 is a set of graphs depicting fermentation of a microorganism with a disrupted acetolactate decarboxylase gene ($\Delta budA$) and a disrupted primary-secondary alcohol

dehydrogenase gene ($\Delta secAdh$). The top panel shows metabolite production (ethanol and acetate). The bottom panel shows gas consumption and production (CO, CO₂, and H₂).

[0008] Fig. 2 is a set of graphs depicting fermentation of a $\Delta budA \Delta secAdh$ microorganism with expression of an acetone pathway (*thlA*, *ctfAB*, *adc*) on a plasmid. The acetone pathway is under the control of a P_{fer} promoter. The top panel shows metabolite production (ethanol, acetate, and acetone). The bottom panel shows gas consumption and production (CO, CO₂, and H₂).

[0009] Fig. 3 is a set of graphs depicting fermentation of a $\Delta secAdh$ microorganism with knock-in of an acetone pathway (*thlA*, *ctfAB*, *adc*) at an acetolactate decarboxylase gene (*budA*) locus. The acetone pathway is under the control of a P_{budA} promoter and a P_{fer} promoter. The top panel shows metabolite production (ethanol, acetate, and acetone). The bottom panel shows gas consumption and production (CO, CO₂, and H₂).

[0010] Fig. 4 is a set of graphs depicting fermentation of a microorganism with knock-in of an acetone pathway (*thlA*, *ctfAB*, *adc*) at bifunctional aldehyde-alcohol dehydrogenase (*adhE1+adhE2*) gene locus and a functional primary-secondary alcohol dehydrogenase (*secAdh*) gene. Primary-secondary alcohol dehydrogenase (*secAdh*) converts acetone to isopropanol, such that this strain produces isopropanol rather than acetone. The acetone pathway is under the control of a P_{adhE1/E2} promoter and a P_{fer} promoter. The top panel shows metabolite production (ethanol, acetate, butanediol, and isopropanol). The bottom panel shows gas consumption and production (CO, CO₂, and H₂).

[0011] Fig. 5 is a bar graph depicting protein expression and acetone production levels of selected strains indicating generally low *ctfAB* expression particularly in strains with low acetone production. Metabolic modeling based on fermentation data also points to the *ctfAB* reaction being a limiting step in acetone/isopropanol production.

[0012] Figs. 6A-C are sets of graphs depicting a comparison of acetone and isopropanol plasmid strains in producing a desired product. The top panel shows increased acetone production in strains having an additional copy of *ctfAB*. The middle panel and bottom panel show increased isopropanol production in strains having an additional copy of *ctfAB*.

[0013] Fig. 7 is a graph depicting fermentation of a microorganism with additional expression of an acetone pathway (*thlA*, *ctfAB*, *adc*) on a plasmid, only additional expression *ctfAB*, or no additional pathway expression. The acetone pathway is under the control of a P_{fer} promoter.

[0014] Fig. 8 is a graph depicting integrated genome strains having an additional copy of *ctfAB* show an improvement in isopropanol production as compared to an integrated genome strain without the additional copy of *ctfAB*.

[0015] Fig. 9 is a set of bar graphs depicting the effect of an additional copy of *ctfAB* on acetone and isopropanol production. The left panel shows continuous acetone production and the effect of an additional copy of *ctfAB*. The right panel shows continuous isopropanol production with different *secAdh* variants and the effect of an additional copy of *ctfAB*.

DESCRIPTION

[0016] Acetolactate decarboxylase is a key step for formation of 2,3-butanediol (2,3-BDO) (Köpke, Appl Env Microbiol, 80: 3394-3405, 2014) and knocking out this enzyme has been demonstrated to abolish 2,3-BDO production (WO 2013/115659). In order to channel flux towards other heterologous products such as acetone or isopropanol, a knockout of acetolactate decarboxylase would be expected to increase production of those heterologous products.

[0017] However, the inventors have found that this is not necessarily the case. In particular, the inventors have discovered that knock-in of genes responsible for the production of heterologous products at the acetolactate decarboxylase locus is key for achieving stable fermentation and high product titers.

[0018] Moreover, it was previously speculated that the thiolase reaction serves as the bottleneck in the production pathway, as the condensation of two molecules of acetyl-CoA to acetoacetyl-CoA is the thermodynamically most unfavorable step in the pathway. However, the inventors have surprisingly discovered that the downstream CoA transferase step is most critical to improve flux to the target product.

[0019] The inventors have also discovered that plasmid expression or knock-in of genes responsible for the production of heterologous products at a bifunctional aldehyde-alcohol dehydrogenase gene locus while having a functional primary-secondary alcohol dehydrogenase gene increases isopropanol production. Similarly, plasmid expression or knock-in of genes responsible for the production of heterologous products at a bifunctional aldehyde-alcohol dehydrogenase gene locus and a knock-out of the primary-secondary alcohol dehydrogenase gene increases acetone production.

[0020] Provided is a genetically engineered microorganism comprising plasmid expression or knock-in of DNA at an acetolactate decarboxylase gene locus, or at both an acetolactate decarboxylase gene locus and at an aldehyde-alcohol dehydrogenase (*adhE1+adhE2*) gene

locus. In one embodiment, the DNA replaces the coding region of the acetolactate decarboxylase gene, either in its entirety or in part. In one embodiment, the DNA does not replace the acetolactate decarboxylase promoter. In one embodiment, DNA replaces the coding region of the acetolactate decarboxylase gene, either in its entirety or in part, and also
 5 DNA replaces the coding region of the aldehyde-alcohol dehydrogenase (*adhE1+adhE2*) gene, either in its entirety or in part.

[0021] In one embodiment, the acetolactate decarboxylase has the activity defined by EC 4.1.1.5, i.e., (S)-2-hydroxy-2-methyl-3-oxobutanoate $\leftrightarrow$ (R)-2-acetoin + CO₂. In one embodiment, the acetolactate decarboxylase is *budA*. In one embodiment, the *budA* comprises
 10 SEQ ID NO: 3.

[0022] After the knock-in is performed, the microorganism will typically not have a functional acetolactate decarboxylase gene, such that the microorganism will express acetolactate decarboxylase and will not produce products such as 2,3-butanediol.

[0023] In one embodiment, the knocked-in DNA encodes one or more enzymes. In one
 15 embodiment, these enzyme(s) are nonnative to the microorganism, i.e., not naturally present in the microorganism. In one embodiment these enzyme(s) are native to the microorganism, i.e., naturally present in the microorganism, and simply add another copy of the enzyme(s) into the genome of the microorganism.

[0024] In one embodiment, the enzyme(s) encoded by the knocked-in DNA are under the
 20 control of an acetolactate decarboxylase promoter, e.g., P_{budA}. In one embodiment, the DNA comprises a promoter, such as a P_{fer} promoter. In one embodiment, the enzyme(s) are under the control of both an acetolactate decarboxylase promoter and at least one other promoter. In one embodiment, the enzyme(s) are under the control of both P_{budA} and P_{fer}.

[0025] In one embodiment, an acetone pathway is knocked-in at the acetolactate
 25 decarboxylase gene locus. The acetone pathway may comprise a thiolase, a CoA transferase, and a decarboxylase. In one embodiment, the decarboxylase is acetoacetate decarboxylase or alpha-ketoisovalerate decarboxylase. For example, the acetone pathway may comprise *thlA*, *ctfAB*, and *adc* or *thlA*, *ctfAB*, and *kivd*. In one embodiment, the CoA transferase is a butyrate-acetoacetate CoA transferase, an acetate CoA transferase, a succinyl-CoA
 30 transferase, a 4-hydroxybutyryl CoA transferase, a ketoadipate-succinyl CoA transferase, or a propionate CoA transferase. In one embodiment, the CoA transferase is only the *ctfA* subunit. In one embodiment, the CoA transferase is only the *ctfB* subunit. In one embodiment the pathway may comprise *ctfA* but not *adc*. In one embodiment, the pathway may comprise *ctfB* but not *adc*. If present, a primary-secondary alcohol dehydrogenase, such as *secAdh*, will

convert acetone to isopropanol. Introduction of a disruptive mutation (e.g., a knock-out mutation) in a gene encoding this enzyme will result in the production of acetone whereas expression of this enzyme will result in the production of isopropanol. Accordingly, depending on the genetic background of the host microorganism, introduction of the acetone pathway will result in the production of either acetone or isopropanol. The engineering of microorganisms to produce acetone and isopropanol is described in WO 2012/115527. The engineering of microorganisms to knock-out primary-secondary alcohol dehydrogenase activity is described in WO 2015/085015.

[0026] In one embodiment, an acetone pathway is knocked-in at the acetolactate

decarboxylase gene locus, and also knocked-in at the aldehyde-alcohol dehydrogenase gene locus. In one embodiment, an acetone pathway is knocked-in at the acetolactate decarboxylase gene locus, and only a CoA transferase is knocked-in at the aldehyde-alcohol dehydrogenase gene locus. In one embodiment, the CoA transferase is a butyrate-acetoacetate CoA transferase, an acetate CoA transferase, a succinyl-CoA transferase, a 4-hydroxybutyryl CoA transferase, or a ketoadipate-succinyl CoA transferase. The acetone pathway may comprise *thlA*, *ctfAB*, and *adc* or *thlA*, *ctfAB*, and *kivd*. If present, a primary-secondary alcohol dehydrogenase, such as *secAdh*, will convert acetone to isopropanol. Introduction of a disruptive mutation (e.g., a knock-out mutation) in a gene encoding this enzyme will result in the production of acetone whereas expression of this enzyme will result in the production of isopropanol. Accordingly, depending on the genetic background of the host microorganism, introduction of the acetone pathway will result in the production of either acetone or isopropanol. The engineering of microorganisms to produce acetone and isopropanol is described in WO 2012/115527. The engineering of microorganisms to knock-out primary-secondary alcohol dehydrogenase activity is described in WO 2015/085015.

[0027] In one embodiment, the microorganism comprises an acetone pathway and also comprises a disruptive mutation in primary-secondary alcohol dehydrogenase gene, such that the microorganism produces acetone. In one embodiment, the microorganism comprises an acetone pathway and also comprises a functional primary-secondary alcohol dehydrogenase, such that the microorganism produces isopropanol.

[0028] In fact, the knocked-in DNA may encode essentially any enzyme or enzyme pathway. For example, the enzyme(s) encoded by the knocked-in DNA may enable production of 1-butanol, butyrate, butene, butadiene, methyl ethyl ketone, ethylene, acetone, isopropanol, lipids, 3-hydroxypropionate, terpenes, isoprene, fatty acids, 2-butanol, 1,2-propanediol, 1-propanol, 1-hexanol, 1-octanol, chorismate-derived products, 3-hydroxybutyrate,

1,3-butanediol, 2-hydroxyisobutyrate or 2-hydroxyisobutyric acid, isobutylene, adipic acid, keto-adipic acid, 1,3-hexanediol, 3-methyl-2-butanol, 2-buten-1-ol, isovalerate, isoamyl alcohol, or monoethylene glycol.

[0029] In one embodiment, the energetics for the enzyme pathways illustrated that thiolase would have been expected to require an additional copy since it is the only endergonic enzyme pathway. In one embodiment, the energetics are as follows, *thlA* (+26 kJ/mol), *ctfAB* (-5.7 kJ/mol), *Adc* (-41.9 kJ/mol), and *secAdh* (-16.2 kJ/mol NADH; -17.4 kJ/mol NADPH). In one embodiment, three copies of *thlA* were expressed on a plasmid demonstrating minimal improvement in desired product production. Unexpectedly, the inventors discovered that the downstream CoA transferase step is most critical to improve flux to the target product.

[0030] In one embodiment, a total of 247 strains harboring distinct acetone biosynthesis pathway designs, including all possible promoter-gene combinations for the reference enzymes, were obtained and screened. In one embodiment, varying promoters led to an 11-fold improvement in production, while combining promoters and the identified unique enzyme variants, resulted in another 2-fold improvement over the best-performing design using genes from the reference strains. In one embodiment, the best-performing designs were based on *thlA*, *ctfAB*, and *Adc*, and moved forward to continuous fermentation testing and genome integration. In one embodiment, after optimizing the acetone pathway, a library of engineered *C. autoethanogenum secAdh* enzymes in was screened for conversion of acetone to isopropanol. Several variants and wild-type showed nearly complete conversion (>97%) of 20g/L of fed acetone when grown on gas and were moved forward for testing in continuous fermentation and genome integration.

[0031] In one embodiment, by simulating changes to the expression level of each enzyme associated with central metabolism and the acetone pathway, the model predicted that over-expressing *ctfAB* would increase flux to acetone.

[0032] In one embodiment, the microorganism is a C1-fixing microorganism. In one embodiment, the microorganism is a Wood-Ljungdahl microorganism. In one embodiment, the microorganism is a bacterium. In one embodiment, the microorganism is a member of a genus selected from *Acetobacterium*, *Alkalibaculum*, *Blautia*, *Butyribacterium*, *Clostridium*, *Eubacterium*, *Moorella*, *Oxobacter*, *Sporomusa*, and *Thermoanaerobacter*.

[0033] Further provided is a method of increasing the production a product comprising culturing the microorganism in the presence of a gaseous substrate. In one embodiment, the gaseous substrate comprises a C1-carbon source comprising CO, CO₂, and/or H₂. In one embodiment, the gaseous substrate comprises syngas or industrial waste gas. In one

embodiment, the product is 1-butanol, butyrate, butene, butadiene, methyl ethyl ketone, ethylene, acetone, isopropanol, lipids, 3-hydroxypropionate, terpenes, isoprene, fatty acids, 2-butanol, 1,2-propanediol, 1-propanol, 1-hexanol, 1-octanol, chorismate-derived products, 3-hydroxybutyrate, 1,3-butanediol, 2-hydroxyisobutyrate or 2-hydroxyisobutyric acid, isobutylene, adipic acid, keto-adipic acid, 1,3-hexanediol, 3-methyl-2-butanol, 2-buten-1-ol, isovalerate, isoamyl alcohol, or monoethylene glycol.

[0034] The term “fermentation” should be interpreted as a metabolic process that produces chemical changes in a substrate. For example, a fermentation process receives one or more substrates and produces one or more products through utilization of one or more

microorganisms. The term “fermentation,” “gas fermentation” and the like should be interpreted as the process which receives one or more substrate, such as syngas produced by gasification and produces one or more product through the utilization of one or more C1-fixing microorganism. Preferably the fermentation process includes the use of one or more bioreactor. The fermentation process may be described as either “batch” or “continuous”.

“Batch fermentation” is used to describe a fermentation process where the bioreactor is filled with raw material, e.g. the carbon source, along with microorganisms, where the products remain in the bioreactor until fermentation is completed. In a “batch” process, after fermentation is completed, the products are extracted, and the bioreactor is cleaned before the next “batch” is started. “Continuous fermentation” is used to describe a fermentation process where the fermentation process is extended for longer periods of time, and product and/or metabolite is extracted during fermentation. Preferably the fermentation process is continuous.

[0035] The term “non-naturally occurring” when used in reference to a microorganism is intended to mean that the microorganism has at least one genetic modification not found in a naturally occurring strain of the referenced species, including wild-type strains of the referenced species. Non-naturally occurring microorganisms are typically developed in a laboratory or research facility.

[0036] The terms “genetic modification,” “genetic alteration,” or “genetic engineering” broadly refer to manipulation of the genome or nucleic acids of a microorganism by the hand of man. Likewise, the terms “genetically modified,” “genetically altered,” or “genetically engineered” refers to a microorganism containing such a genetic modification, genetic alteration, or genetic engineering. These terms may be used to differentiate a lab-generated microorganism from a naturally-occurring microorganism. 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.

[0037] Metabolic engineering of microorganisms, such as *Clostridia*, can tremendously

5 expand their ability to produce many important fuel and chemical molecules other than native metabolites, such as ethanol. However, until recently, *Clostridia* were considered genetically intractable and therefore generally off limits to extensive metabolic engineering efforts. In recent years several different methods for genome engineering for *Clostridia* have been developed including intron-based methods (ClosTron) (Kuehne, *Strain Eng: Methods and*
10 *Protocols*, 389-407, 2011), allelic exchange methods (ACE) (Heap, *Nucl Acids Res*, 40: e59, 2012; Ng, *PLoS One*, 8: e56051, 2013), Triple Cross (Liew, *Frontiers Microbiol*, 7: 694, 2016), methods mediated through I-SceI (Zhang, *Journal Microbiol Methods*, 108: 49-60, 2015), MazF (Al-Hinai, *Appl Environ Microbiol*, 78: 8112-8121, 2012), or others (Argyros, *Appl Environ Microbiol*, 77: 8288-8294, 2011), Cre-Lox (Ueki, *mBio*, 5: e01636-01614,
15 2014), and CRISPR/Cas9 (Nagaraju, *Biotechnol Biofuels*, 9: 219, 2016). However, it remains extremely challenging to iteratively introduce more than a few genetic changes, due to slow and laborious cycling times and limitations on the transferability of these genetic techniques across species. Furthermore, we do not yet sufficiently understand C1 metabolism in *Clostridia* to reliably predict modifications that will maximize C1 uptake, conversion, and
20 carbon/energy/redox flows towards product synthesis. Accordingly, introduction of target pathways in *Clostridia* remains a tedious and time-consuming process.

[0038] “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
25 material derived from multiple sources, such as two or more different strains or species of microorganisms.

[0039] “Wild type” refers to the typical form of an organism, strain, gene, or characteristic as it occurs in nature, as distinguished from mutant or variant forms.

[0040] “Endogenous” refers to a nucleic acid or protein that is present or expressed in the
30 wild-type or parental microorganism from which the microorganism of the disclosure 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 disclosure 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 originates outside the microorganism of the disclosure. For example, an exogenous gene or enzyme may be artificially or recombinantly created and introduced to or expressed in the microorganism of the disclosure. An exogenous gene or enzyme may also be isolated from a heterologous microorganism and introduced to or expressed in the microorganism of the disclosure. Exogenous nucleic acids may be adapted to integrate into the genome of the microorganism of the disclosure or to remain in an extra-chromosomal state in the microorganism of the disclosure, for example, in a plasmid.

[0042] “Heterologous” refers to a nucleic acid or protein that is not present in the wild-type or parental microorganism from which the microorganism of the disclosure is derived. For example, a heterologous gene or enzyme may be derived from a different strain or species and introduced to or expressed in the microorganism of the disclosure. The heterologous gene or enzyme may be introduced to or expressed in the microorganism of the disclosure in the form in which it occurs in the different strain or species. Alternatively, the heterologous gene or enzyme may be modified in some way, e.g., by codon-optimizing it for expression in the microorganism of the disclosure or by engineering it to alter function, such as to reverse the direction of enzyme activity or to alter substrate specificity.

[0043] The terms “polynucleotide,” “nucleotide,” “nucleotide sequence,” “nucleic acid,” and “oligonucleotide” are used interchangeably. They refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof.

Polynucleotides may have any three-dimensional structure, and may perform any function, known or unknown. The following are non-limiting examples of polynucleotides: coding or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, short interfering RNA (siRNA), short-hairpin RNA (shRNA), micro-RNA (miRNA), ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise one or more modified nucleotides, such as methylated nucleotides or nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component.

[0044] As used herein, “expression” refers to the process by which a polynucleotide is transcribed from a DNA template (such as into mRNA or other RNA transcript) and/or

the process by which a transcribed mRNA is subsequently translated into peptides, polypeptides, or proteins. Transcripts and encoded polypeptides may be collectively referred to as “gene products.”

[0045] The terms “polypeptide”, “peptide,” and “protein” are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation, such as conjugation with a labeling component. As used herein, the term “amino acid” includes natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics.

[0046] “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.

[0047] “Mutated” refers to a nucleic acid or protein that has been modified in the microorganism of the disclosure compared to the wild-type or parental microorganism from which the microorganism of the disclosure 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.

[0048] 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 any mutation that reduces, prevents, or blocks the biosynthesis of a product produced by an enzyme. The disruptive mutation may be a knockout (KO) mutation. The disruption may also be a knockdown (KD) mutation that reduces, but does not entirely eliminate, the expression or activity of a gene, protein, or enzyme. While KOs are generally effective in increasing product yields, they sometimes come with the penalty of growth defects or genetic instabilities that outweigh the benefits, particularly for non-growth coupled products. 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

5 be introduced using any method known in the art.

[0049] Introduction of a disruptive mutation results in a microorganism of the invention that produces no target product or substantially no target product or a reduced amount of target product compared to the parental microorganism from which the microorganism of the disclosure is derived. For example, the microorganism of the disclosure may produce no
10 target product or at least about 1%, 3%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 95% less target product than the parental microorganism. For example, the microorganism of the disclosure may produce less than about 0.001, 0.01, 0.10, 0.30, 0.50, or 1.0 g/L target product.

[0050] “Knock-in” refers to a genetic engineering method that involves the substitution of
15 DNA in a genetic locus or the insertion of new DNA in a genetic locus. Often, a knock-in will replace a gene with one or more different genes. For instance, an acetolactate decarboxylase (*budA*) gene may be replaced in whole or in part with one or more different genes. In one embodiment, only the coding region of the gene is replaced. In one embodiment, the entire operon for the gene is replaced, including any promoter regions.

[0051] “Codon optimization” refers to the mutation of a nucleic acid, such as a gene, for
20 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 an embodiment, the genes of the disclosure are codon optimized for expression in *Clostridium*, particularly *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. In another embodiment, the genes of the disclosure are codon optimized for
25 expression in *Clostridium autoethanogenum* LZ1561, which is deposited under DSMZ accession number DSM23693.

[0052] “Overexpressed” refers to an increase in expression of a nucleic acid or protein in the
30 microorganism of the disclosure compared to the wild-type or parental microorganism from which the microorganism of the disclosure 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.

[0053] 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 disclosure 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.

[0054] 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.

[0055] Nucleic acids may be delivered to a microorganism of the disclosure 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 an embodiment, nucleic acids are delivered to the microorganism of the disclosure 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.

[0056] 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.

[0057] A “microorganism” is a microscopic organism, especially a bacterium, archaeon, virus, or fungus. The microorganism of the disclosure is typically a bacterium. As used herein, recitation of “microorganism” should be taken to encompass “bacterium.”

[0058] A “parental microorganism” is a microorganism used to generate a microorganism of the disclosure. 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 disclosure 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 disclosure may be modified to contain one or more genes that were not contained by the parental microorganism. The microorganism of the disclosure 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 an embodiment, the parental microorganism is *Clostridium autoethanogenum* LZ1561, which was deposited with Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) located at Inhoffenstraße 7B, D-38124 Braunschweig, Germany on June 7, 2010 under the terms of the Budapest Treaty and accorded accession number DSM23693. This strain is described in International Patent Application No. PCT/NZ2011/000144, which published as WO 2012/015317.

[0059] 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 disclosure is derived from a parental microorganism. In one embodiment, the microorganism of the disclosure is derived

from *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. In an embodiment, the microorganism of the disclosure is derived from *Clostridium autoethanogenum* LZ1561, which is deposited under DSMZ accession number DSM23693.

[0060] The microorganism of the disclosure may be further classified based on functional

- 5 characteristics. For example, the microorganism of the disclosure may be or may be derived from a C1-fixing microorganism, an anaerobe, an acetogen, an ethanologen, a carboxydrotroph, and/or a methanotroph. Table 1 provides a representative list of microorganisms and identifies their functional characteristics.

Table 1

	Wood-Ljungdahl	C1-fixing	Anaerobe	Acetogen	Ethanologen	Autotroph	Carboxydrotroph
<i>Acetobacterium woodii</i>	+	+	+	+	+/- ¹	+	-
<i>Alkalibaculum bacchii</i>	+	+	+	+	+	+	+
<i>Blautia producta</i>	+	+	+	+	-	+	+
<i>Butyribacterium methylotrophicum</i>	+	+	+	+	+	+	+
<i>Clostridium aceticum</i>	+	+	+	+	-	+	+
<i>Clostridium autoethanogenum</i>	+	+	+	+	+	+	+
<i>Clostridium carboxidivorans</i>	+	+	+	+	+	+	+
<i>Clostridium coskatii</i>	+	+	+	+	+	+	+
<i>Clostridium drakei</i>	+	+	+	+	-	+	+
<i>Clostridium formicoaceticum</i>	+	+	+	+	-	+	+
<i>Clostridium ljungdahlii</i>	+	+	+	+	+	+	+
<i>Clostridium magnum</i>	+	+	+	+	-	+	+/- ²
<i>Clostridium ragsdalei</i>	+	+	+	+	+	+	+
<i>Clostridium scatologenes</i>	+	+	+	+	-	+	+
<i>Eubacterium limosum</i>	+	+	+	+	-	+	+
<i>Moorella thermautotrophica</i>	+	+	+	+	+	+	+
<i>Moorella thermoacetica</i> (formerly <i>Clostridium thermoaceticum</i>)	+	+	+	+	- ³	+	+
<i>Oxobacter pfennigii</i>	+	+	+	+	-	+	+
<i>Sporomusa ovata</i>	+	+	+	+	-	+	+/- ⁴

<i>Sporomusa silvacetica</i>	+	+	+	+	-	+	+/- ⁵
<i>Sporomusa sphaeroides</i>	+	+	+	+	-	+	+/- ⁶
<i>Thermoanaerobacter kivui</i>	+	+	+	+	-	+	-

¹ *Acetobacterium woodii* 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.

[0061] “Wood-Ljungdahl” refers to the Wood-Ljungdahl pathway of carbon fixation as described, e.g., by Ragsdale, *Biochim Biophys Acta*, 1784: 1873-1898, 2008. “Wood-

5 Ljungdahl microorganisms” refers, predictably, to microorganisms containing the Wood-Ljungdahl pathway. Generally, the microorganism of the disclosure contains a native Wood-Ljungdahl pathway. Herein, a Wood-Ljungdahl pathway may be a native, unmodified Wood-Ljungdahl pathway or it may be a Wood-Ljungdahl pathway with some degree of genetic modification (e.g., overexpression, heterologous expression, knockout, etc.) so long as it still
10 functions to convert CO, CO₂, and/or H₂ to acetyl-CoA.

[0062] “C1” refers to a one-carbon molecule, for example, CO, CO₂, CH₄, or CH₃OH. “C1-oxygenate” refers to a one-carbon molecule that also comprises at least one oxygen atom, for example, CO, CO₂, or CH₃OH. “C1-carbon source” refers a one carbon-molecule that serves as a partial or sole carbon source for the microorganism of the disclosure. For example, a C1-
15 carbon source may comprise one or more of CO, CO₂, CH₄, CH₃OH, or CH₂O₂. Preferably, the C1-carbon source comprises one or both of CO and CO₂. 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 disclosure is a C1-fixing bacterium. In an embodiment, the microorganism of the disclosure is derived from a C1-fixing microorganism
20 identified in Table 1.

[0063] 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. However, some anaerobes are capable of tolerating low levels of oxygen (e.g., 0.000001-5%

oxygen). Typically, the microorganism of the disclosure is an anaerobe. In an embodiment, the microorganism of the disclosure is derived from an anaerobe identified in Table 1.

[0064] “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). In particular, acetogens use the Wood-Ljungdahl pathway as a (1) mechanism for the reductive synthesis of acetyl-CoA from CO₂, (2) terminal electron-accepting, energy conserving process, (3) mechanism for the fixation (assimilation) of CO₂ 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 disclosure is an acetogen. In an embodiment, the microorganism of the disclosure is derived from an acetogen identified in Table 1.

[0065] An “ethanologen” is a microorganism that produces or is capable of producing ethanol. Typically, the microorganism of the disclosure is an ethanologen. In an embodiment, the microorganism of the disclosure is derived from an ethanologen identified in Table 1.

[0066] 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 CO₂. Typically, the microorganism of the disclosure is an autotroph. In an embodiment, the microorganism of the disclosure is derived from an autotroph identified in Table 1.

[0067] A “carboxydotroph” is a microorganism capable of utilizing CO as a sole source of carbon and energy. Typically, the microorganism of the disclosure is a carboxydotroph. In an embodiment, the microorganism of the disclosure is derived from a carboxydotroph identified in Table 1.

[0068] A “methanotroph” is a microorganism capable of utilizing methane as a sole source of carbon and energy. In certain embodiments, the microorganism of the disclosure is a methanotroph or is derived from a methanotroph. In other embodiments, the microorganism of the disclosure is not a methanotroph or is not derived from a methanotroph.

[0069] More broadly, the microorganism of the disclosure may be derived from any genus or species identified in Table 1. For example, the microorganism may be a member of a genus selected from the group consisting of *Acetobacterium*, *Alkalibaculum*, *Blautia*, *Butyribacterium*, *Clostridium*, *Eubacterium*, *Moorella*, *Oxobacter*, *Sporomusa*, and *Thermoanaerobacter*. In particular, the microorganism may be derived from a parental bacterium 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*
5 *thermautotrophica*, *Moorella thermoacetica*, *Oxobacter pfennigii*, *Sporomusa ovata*,
Sporomusa silvacetica, *Sporomusa sphaeroides*, and *Thermoanaerobacter kivui*.

[0070] In an embodiment, the microorganism of the disclosure 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,
10 *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*).

[0071] These three species have many similarities. In particular, these species are all C1
fixing, anaerobic, acetogenic, ethanologenic, and carboxydutrophic members of the genus
15 *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
20 (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
25 main fermentation products, and produce small amounts of 2,3-butanediol and lactic acid
under certain conditions.

[0072] 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.

30 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).

[0073] 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.

[0074] The microorganism of the disclosure 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), LZ1560 (DSM19630) (WO 2009/064200), and LZ1561 (DSM23693) (WO 2012/015317). 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).

[0075] “Substrate” refers to a carbon and/or energy source for the microorganism of the disclosure. Typically, the substrate is gaseous and comprises a C1-carbon source, for example, CO, CO₂, and/or CH₄. Preferably, the substrate comprises a C1-carbon source of CO or CO + CO₂. The substrate may further comprise other non-carbon components, such as H₂, N₂, or electrons.

[0076] The substrate generally comprises 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 disclosure 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.

[0077] The substrate may comprise some amount of H₂. For example, the substrate may comprise about 1, 2, 5, 10, 15, 20, or 30 mol% H₂. In some embodiments, the substrate may comprise a relatively high amount of H₂, such as about 60, 70, 80, or 90 mol% H₂. In further embodiments, the substrate comprises no or substantially no (< 1 mol%) H₂.

5 [0078] The substrate may comprise some amount of CO₂. For example, the substrate may comprise about 1-80 or 1-30 mol% CO₂. In some embodiments, the substrate may comprise less than about 20, 15, 10, or 5 mol% CO₂. In another embodiment, the substrate comprises no or substantially no (< 1 mol%) CO₂.

[0079] Although the substrate is typically gaseous, the substrate may also be provided in
10 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.

[0080] The substrate and/or C1-carbon source may be a waste gas obtained as a byproduct of an industrial process or from another source, such as combustion engine exhaust fumes,
15 biogas, landfill gas, direct air capture, or from electrolysis. The substrate and/or C1-carbon source may be syngas generated by pyrolysis, torrefaction, or gasification. In other words, carbon in waste material may be recycled by pyrolysis, torrefaction, or gasification to generate syngas which is used as the substrate and/or C1-carbon source. The substrate and/or C1-carbon source may be a gas comprising methane, and in certain embodiments the
20 substrate and/or C1-carbon source may be a non-waste gas.

[0081] In certain embodiments, the industrial process is selected from ferrous metal products manufacturing, such as a steel manufacturing, non-ferrous products manufacturing, petroleum refining, electric power production, carbon black production, paper and pulp manufacturing, ammonia production, methanol production, coke manufacturing, petrochemical production,
25 carbohydrate fermentation, cement making, aerobic digestion, anaerobic digestion, catalytic processes, natural gas extraction, cellulosic fermentation, oil extraction, industrial processing of geological reservoirs, processing fossil resources such as natural gas coal and oil, or any combination thereof. Examples of specific processing steps within an industrial process include catalyst regeneration, fluid catalyst cracking, and catalyst regeneration. Air separation
30 and direct air capture are other suitable industrial processes. Specific examples in steel and ferroalloy manufacturing include blast furnace gas, basic oxygen furnace gas, coke oven gas, direct reduction of iron furnace top-gas, and residual gas from smelting iron. Other general examples include flue gas from fired boilers and fired heaters, such as natural gas, oil, or coal

fired boilers or heaters, and gas turbine exhaust. 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 known method

[0082] The substrate and/or C1-carbon source may be synthesis gas known as syngas, which
5 may be obtained from reforming, partial oxidation, or gasification processes. Examples of gasification processes include gasification of coal, gasification of refinery residues, gasification of petroleum coke, gasification of biomass, gasification of lignocellulosic material, gasification of waste wood, gasification of black liquor, gasification of municipal solid waste, gasification of municipal liquid waste, gasification of industrial solid waste,
10 gasification of industrial liquid waste, gasification of refuse derived fuel, gasification of sewerage, gasification of sewerage sludge, gasification of sludge from wastewater treatment, gasification of biogas such as when biogas is added to enhance gasification of another material. Examples of reforming processes include, steam methane reforming, steam naphtha reforming, reforming of natural gas, reforming of biogas, reforming of landfill gas, naphtha
15 reforming, and dry methane reforming. Examples of partial oxidation processes include thermal and catalytic partial oxidation processes, catalytic partial oxidation of natural gas, partial oxidation of hydrocarbons. Examples of municipal solid waste include tires, plastics, and fibers such as in shoes, apparel, and textiles. Municipal solid waste may be simply landfill-type waste and may be sorted or unsorted. Examples of biomass may include
20 lignocellulosic material and microbial biomass. Lignocellulosic material may include agriculture waste and forest waste.

[0083] The substrate and/or C1-carbon source may be a gas stream comprising methane. Such a methane containing gas may be obtained from: fossil methane emissions such as during fracking, wastewater treatment, livestock, agriculture, and municipal solid waste
25 landfills. It is also envisioned that the methane may be burned to produce electricity or heat and the C1 byproducts may be used as the substrate or carbon source.

[0084] 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 (O₂) may reduce the efficiency of an anaerobic fermentation process. Depending on the composition of the
30 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.

[0085] In certain embodiments, the fermentation is performed in the absence of carbohydrate substrates, such as sugar, starch, lignin, cellulose, or hemicellulose.

[0086] The microorganism of the disclosure may be cultured with the gas stream to produce one or more products. For instance, the microorganism of the disclosure may produce or may be engineered to produce ethanol (WO 2007/117157), acetate (WO 2007/117157), 1-butanol (WO 2008/115080, WO 2012/053905, and WO 2017/066498), butyrate (WO 2008/115080), 2,3-butanediol (WO 2009/151342 and WO 2016/094334), 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), terpenes, including isoprene (WO 2013/180584), fatty acids (WO 2013/191567), 2-butanol (WO 2013/185123), 1,2-propanediol (WO 2014/036152), 1-propanol (WO 2017/066498), 1-hexanol (WO 2017/066498), 1-octanol (WO 2017/066498), chorismate-derived products (WO 2016/191625), 3-hydroxybutyrate (WO 2017/066498), 1,3-butanediol (WO 2017/066498), 2-hydroxyisobutyrate or 2-hydroxyisobutyric acid (WO 2017/066498), isobutylene (WO 2017/066498), adipic acid (WO 2017/066498), 1,3-hexanediol (WO 2017/066498), 3-methyl-2-butanol (WO 2017/066498), 2-buten-1-ol (WO 2017/066498), isovalerate (WO 2017/066498), isoamyl alcohol (WO 2017/066498), and monoethylene glycol (WO 2019/126400). In certain embodiments, microbial biomass itself may be considered a product. These products may be further converted to produce at least one component of diesel, jet fuel, and/or gasoline. Additionally, the microbial biomass may be further processed to produce a single cell protein (SCP).

[0087] 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.

[0088] “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 disclosure 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

disclosure. In one embodiment, the target product accounts for at least 10% of all fermentation products produced by the microorganism of the disclosure, such that the microorganism of the disclosure 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 disclosure, such that the microorganism of the disclosure has a selectivity for the target product of at least 30%.

[0089] 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.

[0090] 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.

EXAMPLES

[0091] The following examples further illustrate the disclosure but, of course, should not be construed to limit its scope in any way.

Example 1

[0092] This example describes a $\Delta budA\Delta secAdh$ microorganism.

[0093] Acetolactate decarboxylase is a key step for formation of 2,3-butanediol (2,3-BDO) (Köpke, Appl Env Microbiol, 80: 3394-3405, 2014) and knocking out this enzyme has been demonstrated to abolish 2,3-BDO production (WO 2013/115659). In order to channel flux towards other heterologous products such as acetone, a knockout of the respective *budA* gene was predicted to improve production.

[0094] A knock-out of *budA* in a strain of *C. autoethanogenum* that already contained a primary-secondary alcohol dehydrogenase (*secAdh*) gene knock-out ($\Delta secAdh$) (WO 2015/085015) to yield a $\Delta budA\Delta secAdh$ strain. Knock-out of *budA* was carried out as described earlier (WO 2013/115659).

[0095] Single colonies were isolated and restreaked to fresh appropriate selection plates and then screened for double crossover events. Confirmed double crossover events which had correct size of PCR product were re-streaked again to ensure plasmid loss. The correct colonies were picked into liquid medium and frozen stocks were prepared. Genotype was confirmed through whole genome sequencing and phenotype was checked through growth studies and continuous stirred tank reactor (CSTR) runs. As shown in Fig. 1, oscillations in metabolite production and gas consumption/production were observed.

Example 2

[0096] This example describes a $\Delta budA \Delta secAdh$ microorganism that expresses an acetone pathway (*thlA*, *ctfAB*, *adc*) from a plasmid.

[0097] The $\Delta budA \Delta secAdh$ microorganism of Example 1 was further modified to introduce a plasmid containing an acetone pathway (*thlA*, *ctfAB*, *adc*). This strain produced less acetone than a parent $\Delta secAdh$ strain with the same pathway and under the same growth conditions but without the knock-out of *budA*. This is surprising since knock-out of *budA* would have been expected to redirect carbon flux from 2,3-BDO to other metabolites such as acetone and/or ethanol.

[0098] Furthermore, this strain did not grow well or demonstrate stable acetone production in a CSTR with a gas mix: 50% CO 10% H₂, 30% CO₂, balance N₂ (Fig. 2). Again, an oscillation pattern was observed during growth: peaks and troughs of production, CO and hydrogen uptake in coordination with troughs and peaks of acetate and CO₂ production.

Example 3

[0099] This example describes a $\Delta secAdh$ microorganism with knock-in of an acetone pathway (*thlA*, *ctfAB*, *adc*) at an acetolactate decarboxylase (*budA*) gene locus.

[0100] The KI/KO plasmid for acetone pathway knock-in at the *budA* locus was constructed using the *budA* KO plasmid as the backbone and the acetone pathway was inserted in between the 5' and 3' *budA* KO homology arms of the *budA* KO plasmid. The acetone pathway contained *thlA*, *ctfAB*, and *adc* under the control of the P_{fer} promoter. The complete KI/KO plasmid was assembled using GeneArt Seamless Cloning and Assembly Kit (ThermoFisher Scientific). The correct KI/KO plasmid was PCR screened and confirmed by sequencing.

[0101] The process of obtaining the KI mutant was the same as previously described in constructing the *budA* KO strain, yielding a $\Delta budA \Delta secAdh$ strain with the acetone pathway introduced at the *budA* locus. PCR screening was carried out and colonies with correct size of

PCR product were grown up, genomic DNA isolated and subjected for whole genome DNA sequencing in order to confirm the genotype.

[0102] The strain was grown in CSTR with a gas mix: 50% CO 10% H₂, 30% CO₂, balance N₂. The strain grew well and produced high levels of acetone (Fig. 3).

5 *Example 4*

[0103] This comparative example describes a microorganism with a functional primary-secondary alcohol dehydrogenase (*secAdh*) and knock-in of an acetone pathway (*thlA*, *ctfAB*, *adc*) at a bifunctional aldehyde-alcohol dehydrogenase (*adhE1+adhE2*) gene locus. Primary-secondary alcohol dehydrogenase (*secAdh*) converts acetone to isopropanol, such that this strain produces isopropanol rather than acetone.

[0104] The acetone pathway was inserted at the *adhE1+adhE2* locus with similar steps as those described above. The homology arms were amplified via PCR and genotype was confirmed via sequencing.

[0105] The strain grew poorly in a CSTR and did not produce isopropanol or ethanol well (Fig. 4). It took about 6 days in the reactor to have enough cell biomass for appreciable production of ethanol and isopropanol. The fermentation was not stable over the two-week course of the experiment. Accordingly, knock-in at the *adhE1+adhE2* locus does not confer the same benefits as knock-in at the *budA* locus.

Example 5

[0106] This example describes integration of other genes or pathways at the *budA* locus.

[0107] Wood-Ljungdahl microorganisms have already been engineered to produce a variety of nonnative products, including 1-butanol (WO 2008/115080, WO 2012/053905, and WO 2017/066498), butyrate (WO 2008/115080), 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), terpenes, including isoprene (WO 2013/180584), fatty acids (WO 2013/191567), 2-butanol (WO 2013/185123), 1,2-propanediol (WO 2014/036152), 1-propanol (WO 2017/066498), 1-hexanol (WO 2017/066498), 1-octanol (WO 2017/066498), chorismate-derived products (WO 2016/191625), 3-hydroxybutyrate (WO 2017/066498), 1,3-butanediol (WO 2017/066498), 2-hydroxyisobutyrate or 2-hydroxyisobutyric acid (WO 2017/066498), isobutylene (WO 2017/066498), adipic acid (WO 2017/066498), 1,3-hexanediol (WO 2017/066498), 3-methyl-2-butanol (WO 2017/066498), 2-buten-1-ol (WO 2017/066498),

isovalerate (WO 2017/066498), isoamyl alcohol (WO 2017/066498), and monoethylene glycol (WO 2019/126400). Any of these genes or pathways could be knocked-in at the *budA* locus to yield a strain with improved performance.

[0108] In one embodiment, the knock-in DNA encodes a 3-hydroxybutyrate pathway comprising, e.g., *thlA* and *hbd*. In one embodiment, the knock-in DNA encodes an alternative 3-hydroxybutyrate pathway comprising, e.g., *thlA*, *ctfAB*, and *hbd*. In one embodiment, the knock-in DNA encodes a butanol pathway comprising, e.g., *thlA*, *hbd*, *bcd*, and *etfAB*. In one embodiment, the knock-in DNA comprises a mevalonate pathway comprising, e.g., *thlA*, *HMGS*, and *HMGR*. These pathways may be under the control of one or more promoters, e.g., P_{budA} and/or P_{fer} .

Example 6

[0109] This example describes omics and kinetic ensemble modeling guided flux optimization.

[0110] In one embodiment, proteomic measurements were conducted on eleven strains from a combinatorial library. These analyses included a collection of strains that had the same reference genes driven by different promoters with varied strengths, resulting in different acetone and 3-HB levels (Fig. 5). A total of 1916 distinct proteins were quantifiable across all strains, of which, the acetone pathway specific enzymes were abundant in all samples, falling within the top 3.2% of the dataset when ranked by median abundance. Statistical analysis showed that there were significant differences in the levels of these enzymes in all the combinatorial strains relative to the benchmark strains. Acetone production correlated well with the abundance of acetone pathway enzymes, with the lowest abundance found in the lowest producing strains. Consistently, the two subunits of *ctfAB* were found to have relatively lower expression levels compared to *thlA* and *Adc*, regardless of promoters used (Fig. 5). In one embodiment, the predicted flux to acetone and isopropanol was demonstrated by determining fold changes in *ctfAB*. In one embodiment, kinetic ensemble modeling predicted that total flux to acetone and isopropanol would increase as *ctfAB* expression increases.

Example 7

[0111] This comparative example describes testing a strain having a sequence selected with promoter pWL and a *ctfAB*.

[0112] In one embodiment, a sequence for promoter pWL and the *ctfAB* was cloned into a vector and was then transformed into the best performing acetone and isopropanol strains.

Growth experiments were performed to compare these strains. In one embodiment, a significant increase in desired product was seen along with a decrease in other metabolites, when an additional copy of *ctfAB* was present. These results were later confirmed in a CSTR, resulting in ~20g/L isopropanol (Figs. 6A-C).

5 *Example 8*

[0113] This comparative example describes testing an additional copy of the entire pathway (strain having *thlA*, *ctfAB*, and *Ade*).

[0114] In one embodiment, fermentation of a microorganism with additional expression of an acetone pathway (*thlA*, *ctfAB*, *adc*) on a plasmid, only additional expression *ctfAB*, or no
10 additional pathway expression (Fig. 7). More improvement was seen in the strain that had only the additional *ctfAB* than in the strain with an additional copy of each pathway gene. In one embodiment this is due to selecting a promoter and *ctfAB* variant with a known improvement in protein abundance.

Example 9

15 [0115] This comparative example describes integration of an extra copy of *ctfAB* into the genome.

[0116] In one embodiment, after confirming the improvement with the additional *ctfAB* copy, the sequence was integrated into the genome. While knocking in the genes, some genes were simultaneously knocked out. These were predicted to be beneficial knock-outs for both
20 acetone and isopropanol production. In each strain that was generated, an improvement in isopropanol production was seen, similar to the plasmid strains described previously, but without the extra copy of *ctfAB*. In one embodiment, integrated genome strains having an additional copy of *ctfAB* show an improvement in isopropanol production as compared to an integrated genome strain without the additional copy of *ctfAB* (Fig. 8).

25 *Example 10*

[0117] This example describes integration of an extra copy of *ctfAB* into the genome.

[0118] In one embodiment, omics measurements, kinetic modeling, and cell-free prototyping all suggest *ctfAB* as the limiting step for acetone production. It was discovered that a second copy of *ctfAB* by expressing the *ctfAB* variant and promoter combination registered the
30 highest proteomic abundance on a plasmid in the strain, which led to a further 40% increase in acetone selectivity (Fig. 9, left panel).

[0119] In one embodiment, an integrated strain led to a significant improvement in acetone selectivity and culture stability achieved with >4 weeks steady state production (Fig. 9, left panel).

[0120] In one embodiment, a variant exhibited the highest titer and stability for isopropanol production. In one embodiment integrated strain had a comparable level of performance (even with even slight improvements in selectivity after small adjustments in fermentation regime) as acetone integrated strains (Fig 9, right panel). For both acetone and isopropanol, expressing the extra copy of *ctfAB* further increased performance, achieving 90% selectivity (Fig 9.) at high rates of up to ~3 g/L/h with 85% gas utilization.

[0121] 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 endeavor in any country.

[0122] The use of the terms “a” and “an” and “the” and similar referents in the context of describing the disclosure (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. The term “consisting essentially of” limits the scope of a composition, process, or method to the specified materials or steps, or to those that do not materially affect the basic and novel characteristics of the composition, process, or method. The use of the alternative (e.g., “or”) should be understood to mean either one, both, or any combination thereof of the alternatives. As used herein, the term “about” means $\pm 20\%$ of the indicated range, value, or structure, unless otherwise indicated.

[0123] 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. For example, any concentration range, percentage range, ratio range, integer range, size range, or thickness range is to be understood to include the value of any integer within the recited range and, when appropriate, fractions thereof (such as one tenth and one hundredth of an integer), unless otherwise indicated.

[0124] 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 disclosure and does not pose a limitation on the scope of the disclosure unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the disclosure.

[0125] Embodiments of this disclosure are described herein. Variations of those 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 disclosure to be practiced otherwise than as specifically described herein. Accordingly, this disclosure 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 disclosure unless otherwise indicated herein or otherwise clearly contradicted by context.

[0126] In a first embodiment the disclosure is directed to a genetically engineered Wood-Ljungdahl microorganism comprising a first exogenous CoA transferase, and at least one additional exogenous CoA transferase.

[0127] The microorganism of the first embodiment, wherein the first exogenous CoA transferase replaces a coding region of an acetolactate decarboxylase gene.

[0128] The microorganism of the first embodiment, wherein the at least one additional exogenous CoA transferase replaces a coding region of an aldehyde-alcohol dehydrogenase gene.

[0129] The microorganism of the first embodiment, further comprising an exogenous thiolase and an exogenous decarboxylase selected from acetoacetate decarboxylase or alpha-ketoisovalerate decarboxylase, with the first exogenous CoA transferase.

[0130] The microorganism of the first embodiment, further comprising an exogenous thiolase and an exogenous decarboxylase selected from acetoacetate decarboxylase or alpha-ketoisovalerate decarboxylase, or any combination thereof.

[0131] The microorganism of the first embodiment, further comprising an exogenous thiolase and an exogenous decarboxylase selected from acetoacetate decarboxylase or alpha-ketoisovalerate decarboxylase, or any combination thereof, wherein the exogenous thiolase and the exogenous decarboxylase selected from acetoacetate decarboxylase or alpha-

ketoisovalerate decarboxylase, or any combination thereof, function with the first exogenous CoA transferase or function with the least one additional exogenous CoA transferase.

[0132] The microorganism of the first embodiment, wherein the exogenous CoA transferases are *Clostridium acetobutylicum* CtfA and CtfB, or *Clostridium beijerinckii* CtfA and CtfB.

5 [0133] The microorganism of the first embodiment, wherein the exogenous CoA transferases are *Clostridium acetobutylicum* CtfA and CtfB, or *Clostridium beijerinckii* CtfA and CtfB, and wherein the exogenous CoA transferases are nonnative to the microorganism.

[0134] The microorganism of the first embodiment, wherein the exogenous CoA transferases are *Clostridium acetobutylicum* CtfA and CtfB, or *Clostridium beijerinckii* CtfA and CtfB,
10 and wherein the exogenous CoA transferases are native to the microorganism.

[0135] The microorganism of the first embodiment, wherein the exogenous CoA transferases are *Clostridium acetobutylicum* CtfA and CtfB, or *Clostridium beijerinckii* CtfA and CtfB, and, wherein the exogenous CoA transferases are *Clostridium acetobutylicum* CtfA and CtfB, or *Clostridium beijerinckii* CtfA and CtfB wherein the exogenous CoA transferases are the
15 same.

[0136] The microorganism of the first embodiment, wherein the exogenous CoA transferases are *Clostridium acetobutylicum* CtfA and CtfB, or *Clostridium beijerinckii* CtfA and CtfB wherein the exogenous CoA transferases are different.

[0137] The microorganism of the first embodiment, wherein the exogenous CoA transferases
20 are *Clostridium acetobutylicum* CtfA and CtfB, or *Clostridium beijerinckii* CtfA and CtfB wherein the exogenous CoA transferases are under the control of both an acetolactate decarboxylase promoter and at least one other promoter.

[0138] The microorganism of the first embodiment, wherein the microorganism does not produce 2,3-butanediol.

25 [0139] The microorganism of the first embodiment, wherein the microorganism produces one or more of acetone and isopropanol.

[0140] The microorganism of the first embodiment, further comprising a disruptive mutation in a primary-secondary alcohol dehydrogenase gene, a thioesterase gene, a 3-hydroxybutyryl coA dehydrogenase gene, or any combination thereof.

30 [0141] The microorganism of the first embodiment wherein the exogenous CoA transferases are *Clostridium acetobutylicum* CtfA and CtfB, or *Clostridium beijerinckii* CtfA and CtfB, and wherein the exogenous CoA transferases enable production of 1-butanol, butyrate, butene, butadiene, methyl ethyl ketone, ethylene, acetone, isopropanol, lipids, 3-hydroxypropionate, terpenes, isoprene, fatty acids, 2-butanol, 1,2-propanediol, 1-propanol,

1-hexanol, 1-octanol, chorismate-derived products, 3-hydroxybutyrate, 1,3-butanediol, 2-hydroxyisobutyrate or 2-hydroxyisobutyric acid, isobutylene, adipic acid, keto-adipic acid, 1,3-hexanediol, 3-methyl-2-butanol, 2-buten-1-ol, isovalerate, isoamyl alcohol, or monoethylene glycol.

5 **[0142]** The microorganism of the first embodiment, wherein the microorganism is a C1-fixing microorganism.

[0143] The microorganism of the first embodiment, further comprising an exogenous thiolase, an exogenous decarboxylase selected from acetoacetate decarboxylase or alpha-ketoisovalerate decarboxylase, or any combination thereof, with the at least one additional
10 exogenous CoA transferase.

[0144] The microorganism of the first embodiment, further comprising an exogenous thiolase, an exogenous decarboxylase selected from acetoacetate decarboxylase or alpha-ketoisovalerate decarboxylase, or any combination thereof.

[0145] The microorganism of the first embodiment, wherein the microorganism is a
15 bacterium.

[0146] The microorganism of the first embodiment, wherein the microorganism is a member of a genus selected from *Acetobacterium*, *Alkalibaculum*, *Blautia*, *Butyribacterium*, *Clostridium*, *Eubacterium*, *Moorella*, *Oxobacter*, *Sporomusa*, or *Thermoanaerobacter*.

[0147] A method for increasing production of a product comprising culturing the
20 microorganism of the first embodiment in the presence of a gaseous substrate.

[0148] The method for increasing production of a product comprising culturing the microorganism of the first embodiment in the presence of a gaseous substrate, wherein the gaseous substrate comprises a C1-carbon source comprising one or more of CO, CO₂, and H₂.

[0149] The method for increasing production of a product comprising culturing the
25 microorganism of the first embodiment in the presence of a gaseous substrate, wherein the gaseous substrate comprises syngas or industrial waste gas.

[0150] The method for increasing production of a product comprising culturing the microorganism of the first embodiment in the presence of a gaseous substrate, wherein the product is one or more of acetone and isopropanol.

30

CLAIMS:

1. A genetically engineered Wood-Ljungdahl microorganism comprising a first exogenous CoA transferase, and at least one additional exogenous CoA transferase.
- 5 2. The microorganism of claim 1, wherein the first exogenous CoA transferase replaces a coding region of an acetolactate decarboxylase gene.
3. The microorganism of claim 1, wherein the at least one additional exogenous CoA transferase replaces a coding region of an aldehyde-alcohol dehydrogenase gene.
4. The microorganism of claim 1, further comprising an exogenous thiolase and an
10 exogenous decarboxylase selected from acetoacetate decarboxylase or alpha-ketoisovalerate decarboxylase, or any combination thereof.
5. The microorganism of claim 4, wherein the exogenous thiolase and the exogenous decarboxylase selected from acetoacetate decarboxylase or alpha-ketoisovalerate decarboxylase, or any combination thereof, function with the first exogenous CoA transferase
15 or function with the least one additional exogenous CoA transferase.
6. The microorganism of claim 1, wherein the exogenous CoA transferases are *Clostridium acetobutylicum* CtfA and CtfB, or *Clostridium beijerinckii* CtfA and CtfB.
7. The microorganism of claim 6, wherein the exogenous CoA transferases are nonnative to the microorganism.
- 20 8. The microorganism of claim 6, wherein the exogenous CoA transferases are native to the microorganism.
9. The microorganism of claim 6, wherein the exogenous CoA transferases are the same.
10. The microorganism of claim 6, wherein the exogenous CoA transferases are different.
11. The microorganism of claim 6, wherein the exogenous CoA transferases are under the
25 control of both an acetolactate decarboxylase promoter and at least one other promoter.
12. The microorganism of claim 1, wherein the microorganism does not produce 2,3-butanediol.
13. The microorganism of claim 1, wherein the microorganism produces one or more of acetone and isopropanol.

14. The microorganism of claim 1, further comprising a disruptive mutation in a primary-secondary alcohol dehydrogenase gene, a thioesterase gene, a 3-hydroxybutyryl coA dehydrogenase gene, or any combination thereof.
15. The microorganism of claim 6, wherein the exogenous CoA transferases enable
5 production of 1-butanol, butyrate, butene, butadiene, methyl ethyl ketone, ethylene, acetone, isopropanol, lipids, 3-hydroxypropionate, terpenes, isoprene, fatty acids, 2-butanol, 1,2-propanediol, 1-propanol, 1-hexanol, 1-octanol, chorismate-derived products, 3-hydroxybutyrate, 1,3-butanediol, 2-hydroxyisobutyrate or 2-hydroxyisobutyric acid, isobutylene, adipic acid, keto-adipic acid, 1,3-hexanediol, 3-methyl-2-butanol, 2-buten-1-ol,
10 isovalerate, isoamyl alcohol, or monoethylene glycol.
16. The microorganism of claim 1, wherein the microorganism is a C1-fixing microorganism.
17. The microorganism of claim 1, wherein the microorganism is a bacterium.
18. The microorganism of claim 1, wherein the microorganism is a member of a genus
15 selected from *Acetobacterium*, *Alkalibaculum*, *Blautia*, *Butyribacterium*, *Clostridium*, *Eubacterium*, *Moorella*, *Oxobacter*, *Sporomusa*, or *Thermoanaerobacter*.
19. A method for increasing production of a product comprising culturing the microorganism of claim 1 in the presence of a gaseous substrate.
20. The method of claim 19, wherein the gaseous substrate comprises a C1-carbon source
20 comprising one or more of CO, CO₂, and H₂.
21. The method of claim 19, wherein the gaseous substrate comprises syngas or industrial waste gas.
22. The method of claim 19, wherein the product is one or more of acetone and isopropanol.

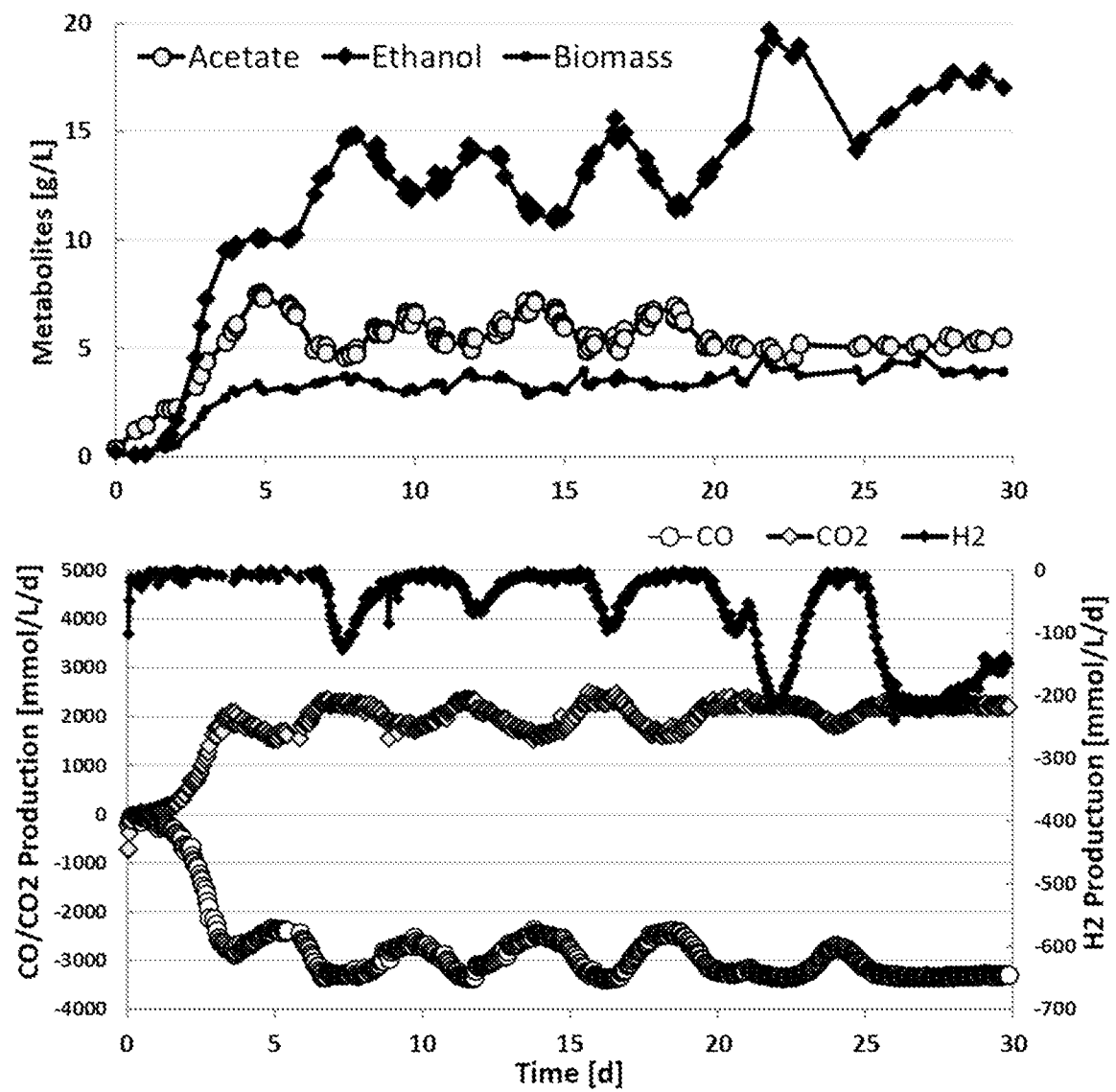


Fig. 1

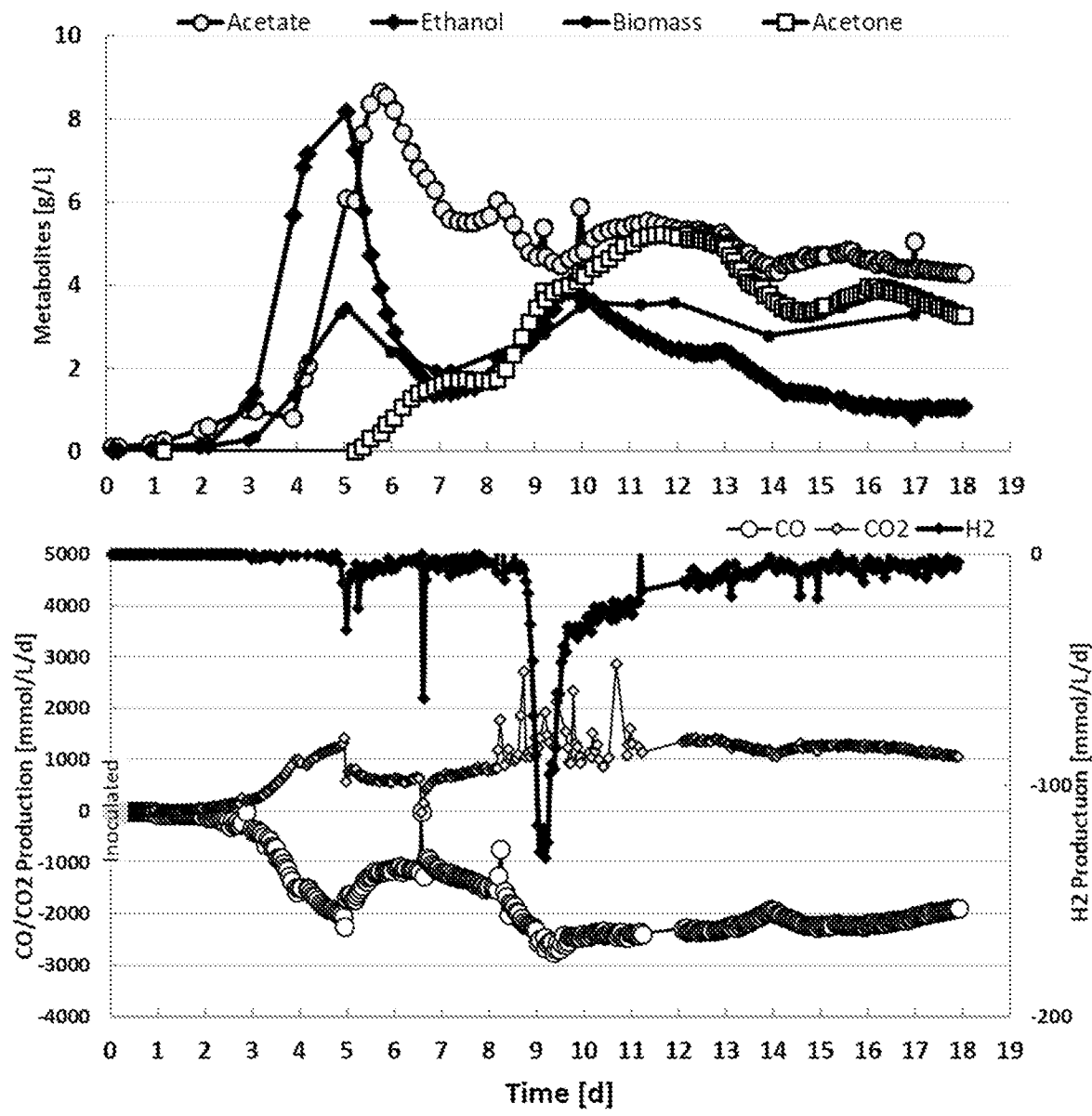


Fig. 2

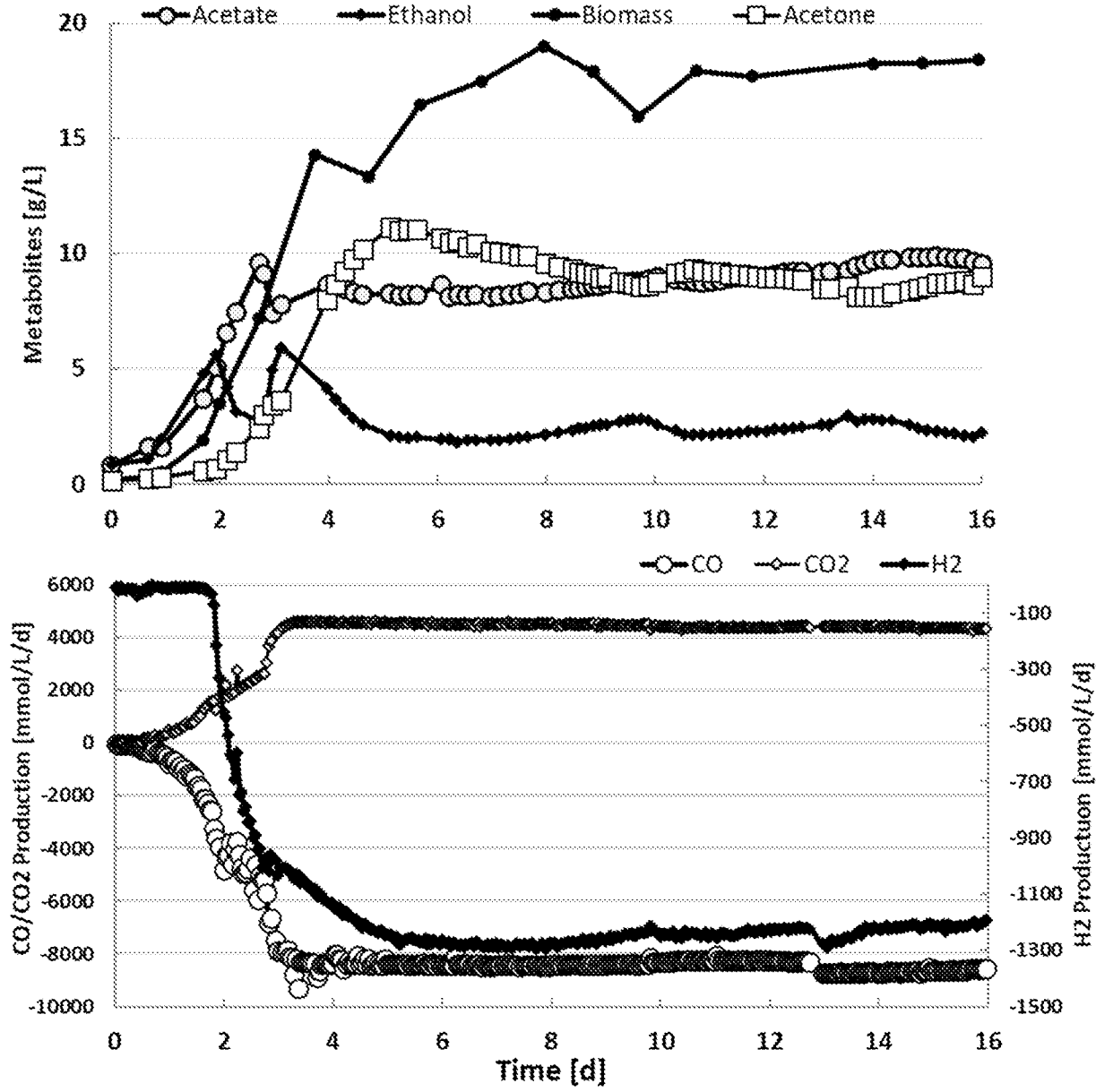


Fig. 3

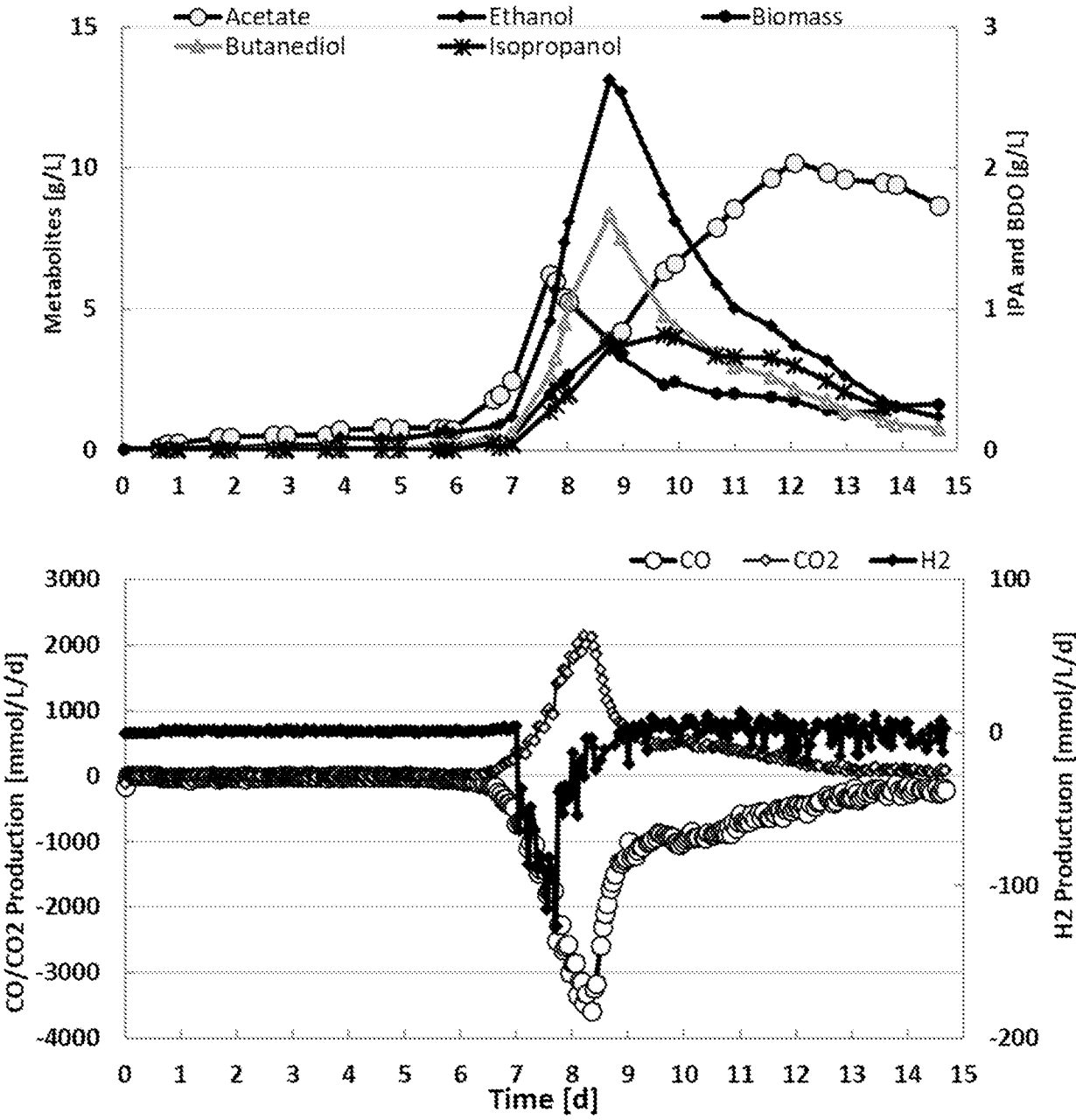


Fig. 4

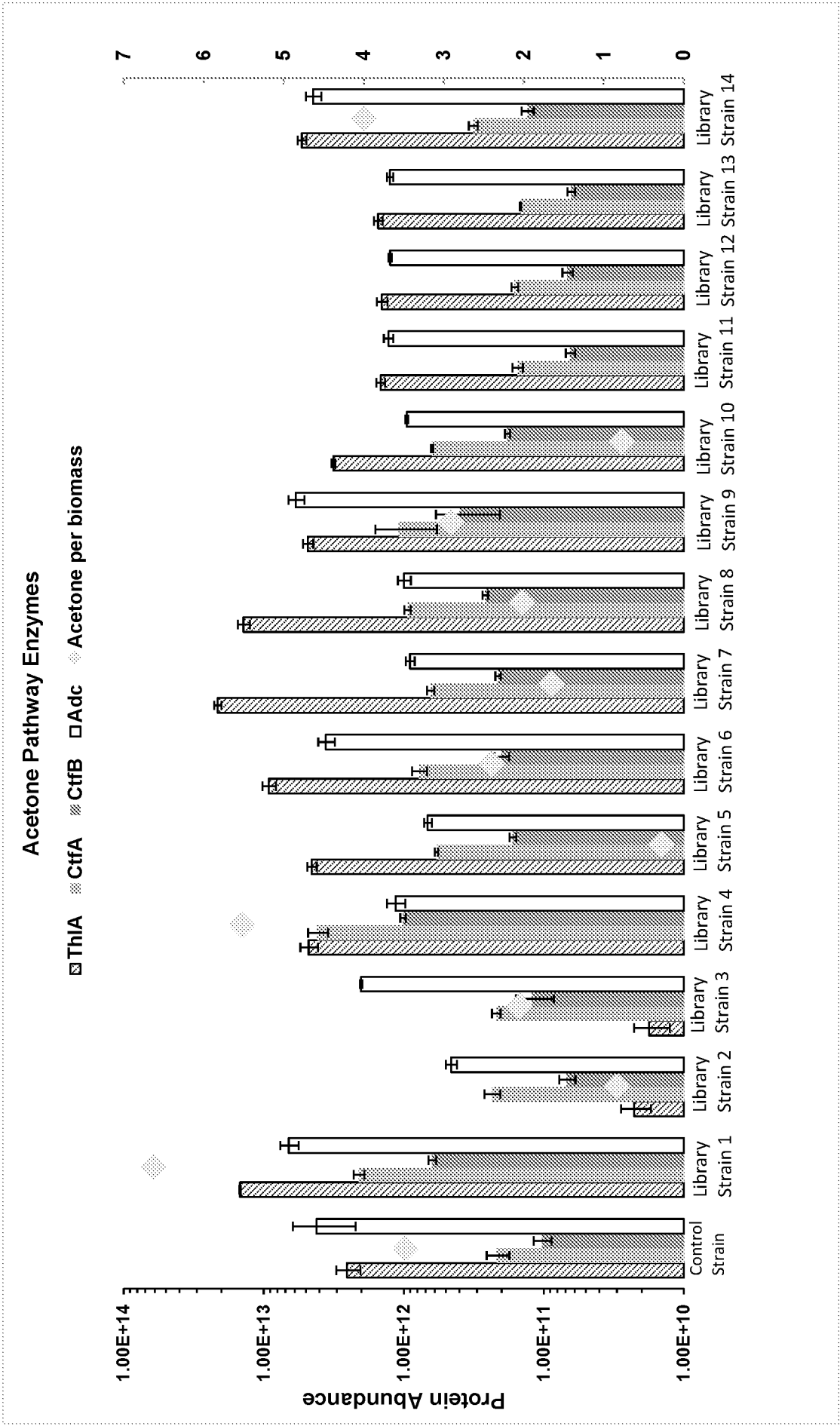


Fig. 5

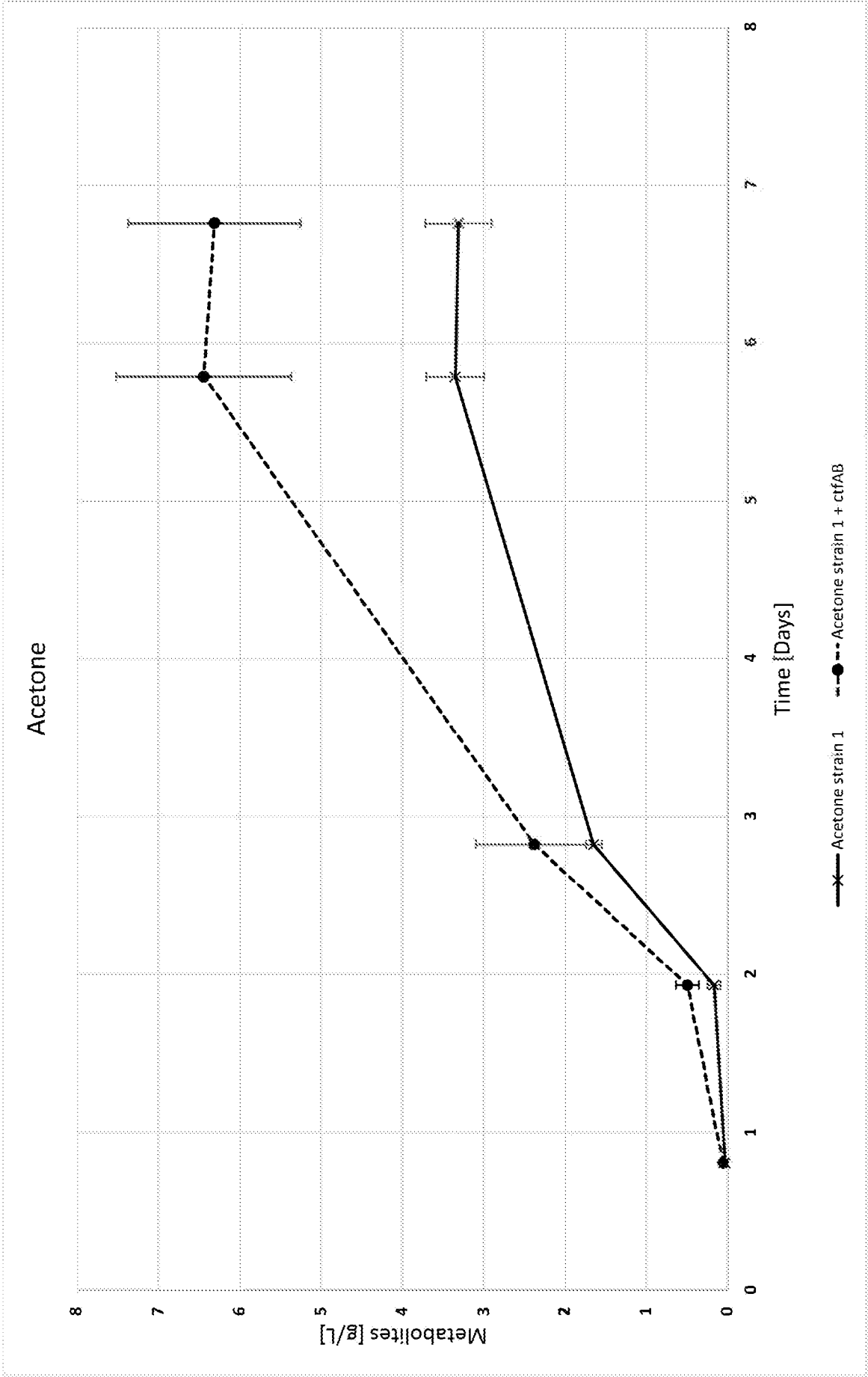


Fig. 6A

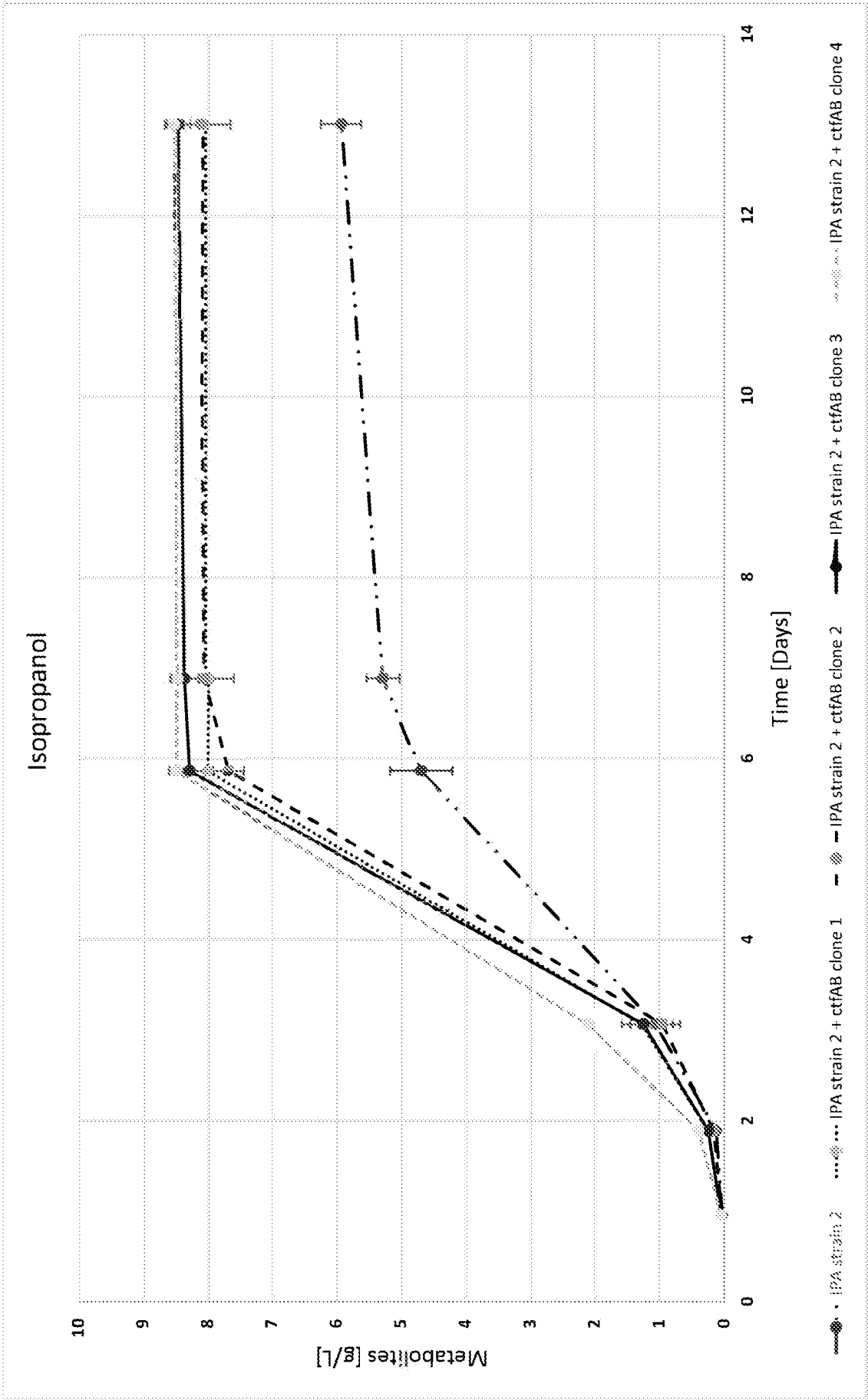


Fig. 6B

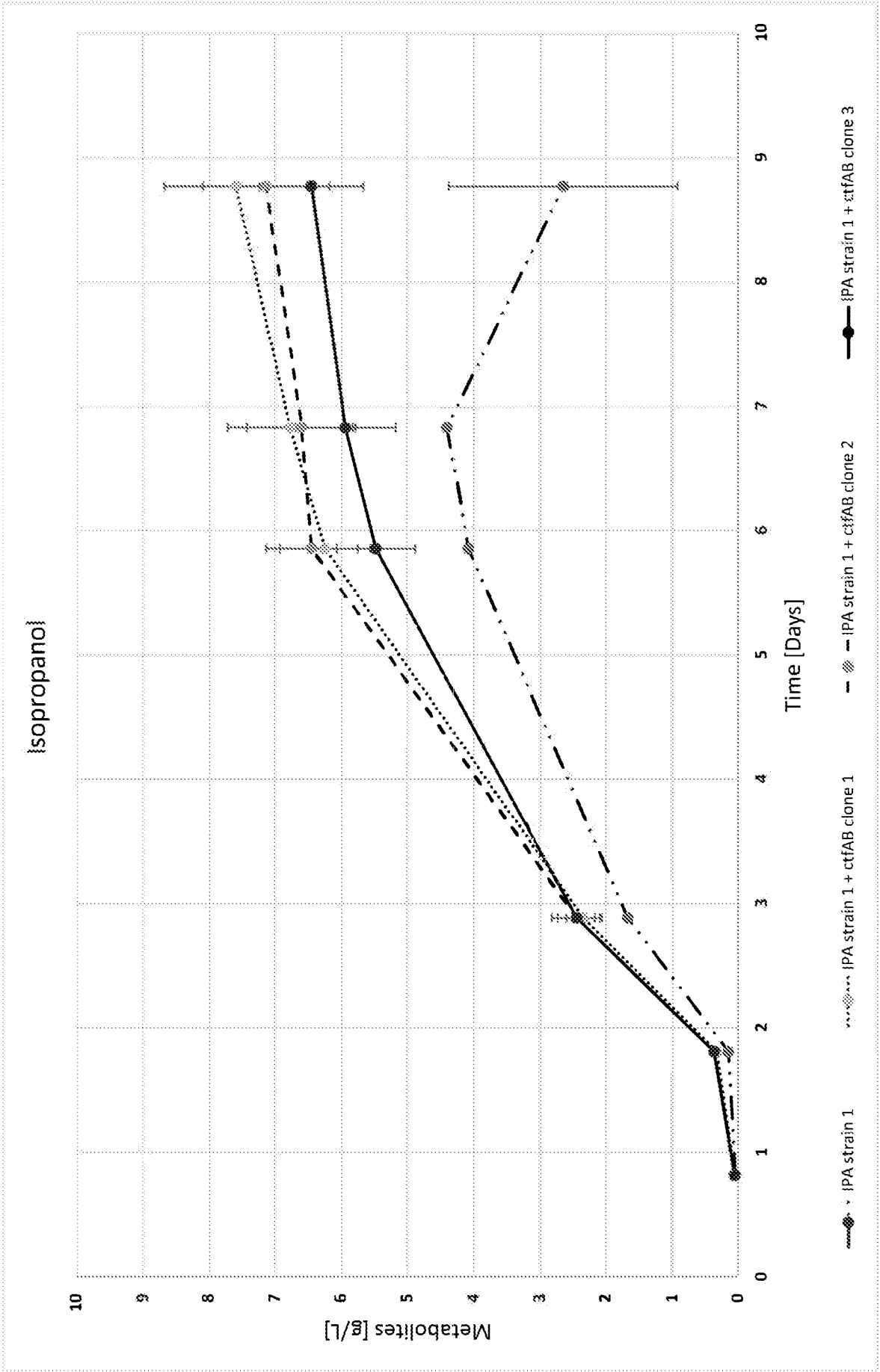


Fig. 6C

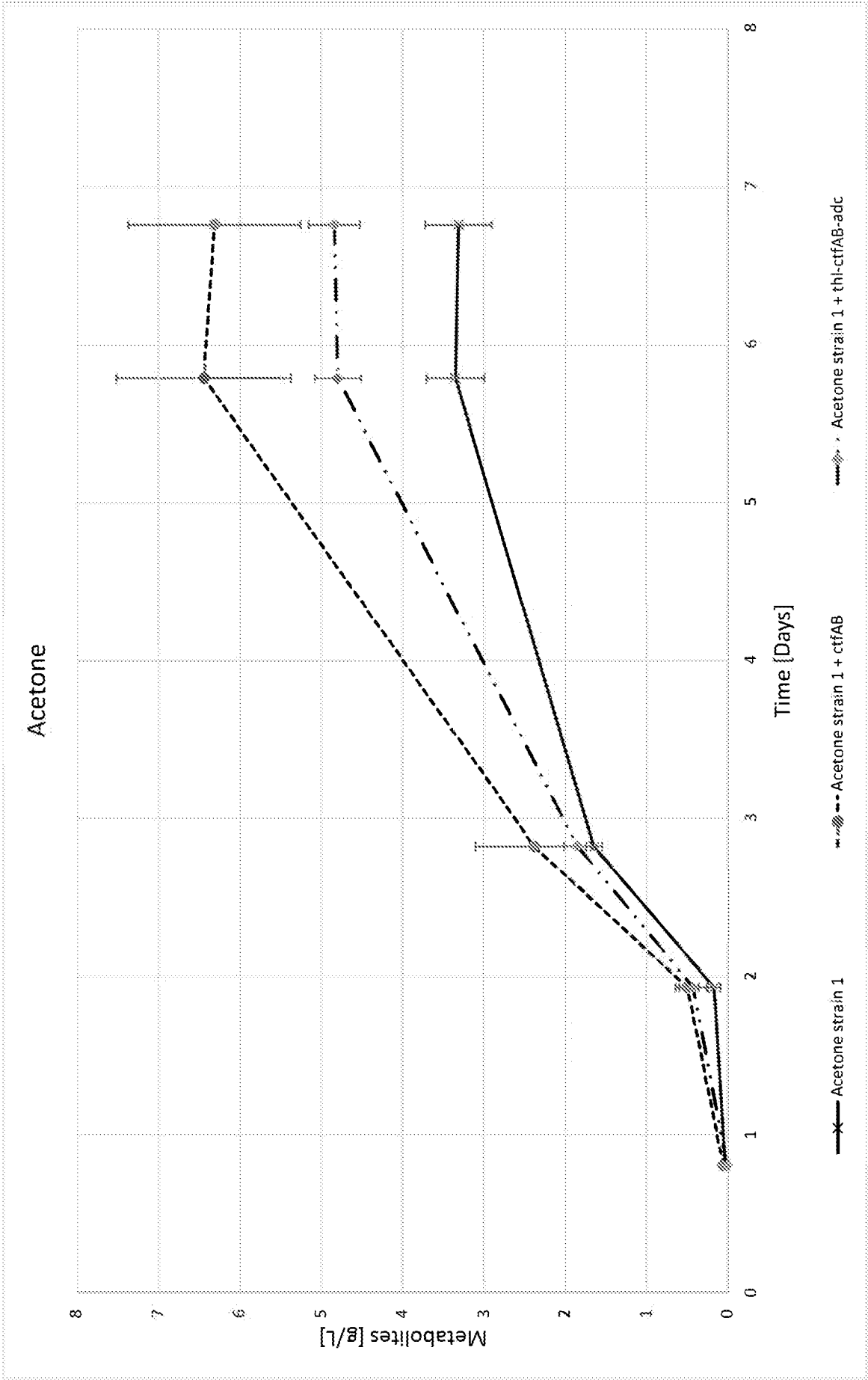


Fig. 7

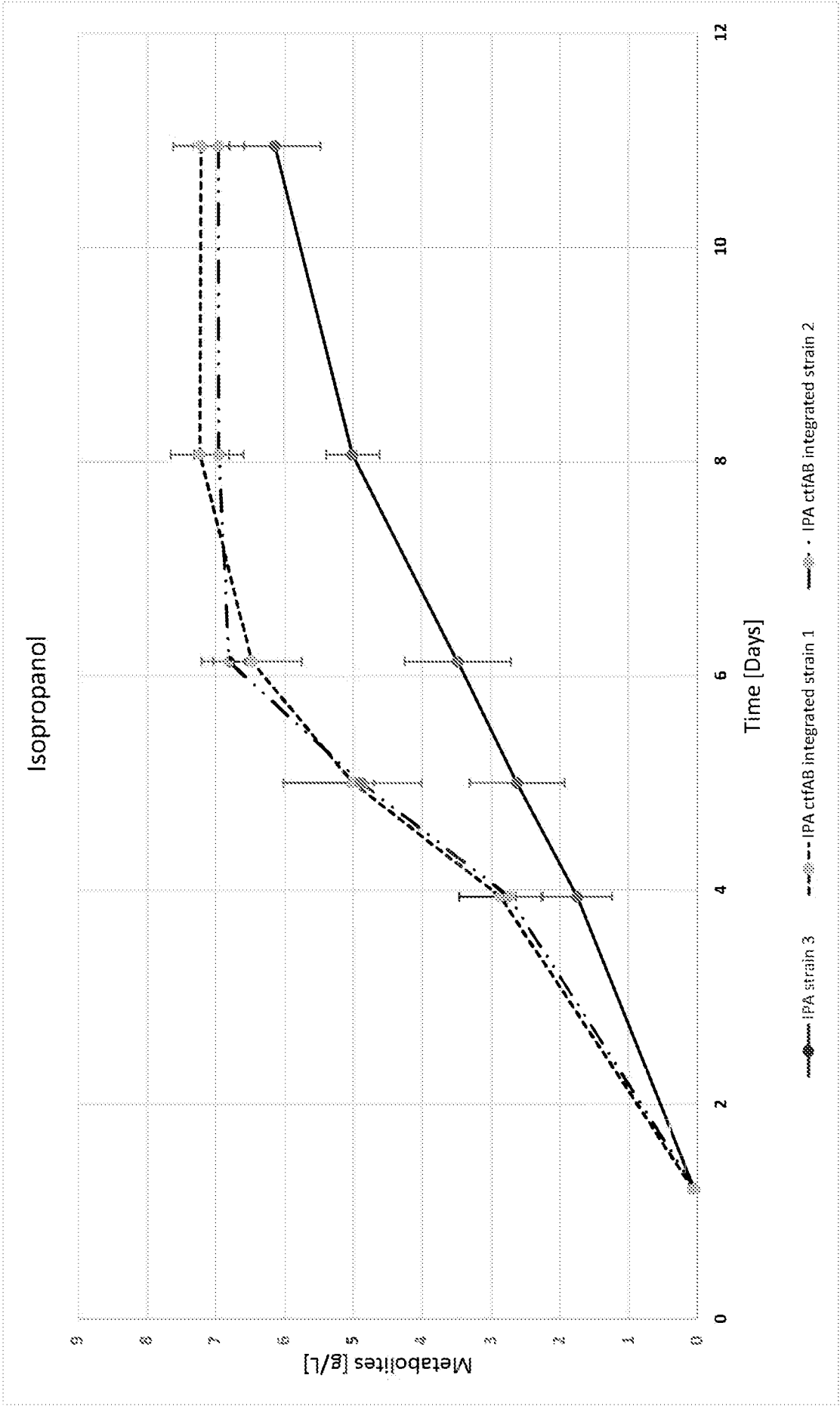


Fig. 8

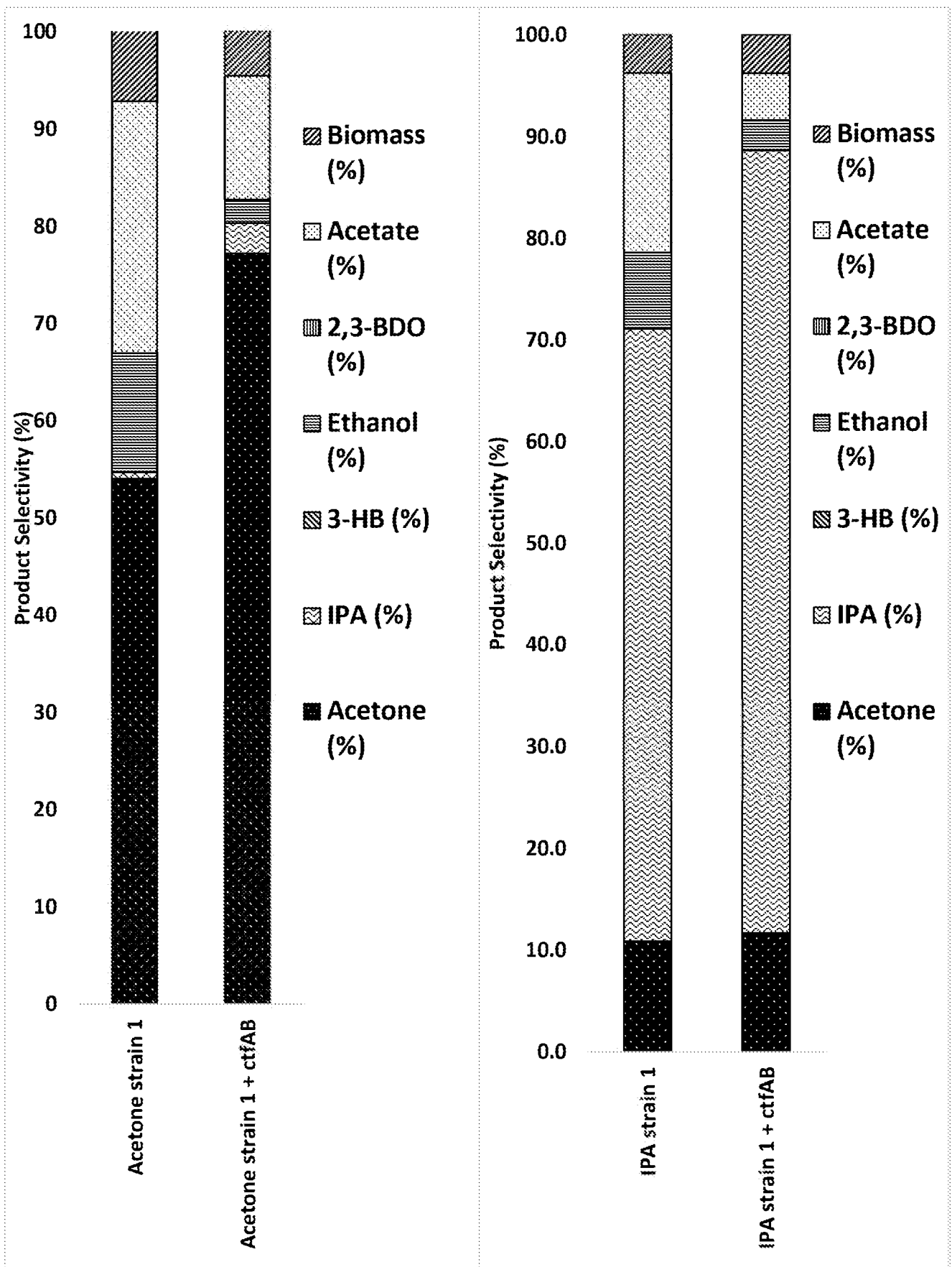


Fig. 9

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2021/051888

A. CLASSIFICATION OF SUBJECT MATTER C12N 15/74(2006.01)i; C12N 9/10(2006.01)i; C12P 7/26(2006.01)i; C12P 7/04(2006.01)i According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C12N 15/74(2006.01); C12N 15/00(2006.01); C12N 9/10(2006.01); C12P 7/06(2006.01); C12P 7/18(2006.01); C12P 7/30(2006.01); C12P 7/42(2006.01) 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: Wood-Ljungdahl, microorganism, exogenous CoA transferase, promoter, acetone, isopropanol		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 3401405 A1 (LANZATECH NEW ZEALAND LIMITED) 14 November 2018 (2018-11-14) abstract; claims 1-2, 7-8, 10, 14; paragraphs [0025], [0072], [0085], [0139], [0240]; figs. 2, 4	1,4-22
A		2-3
Y	SRIRANGAN, K. 'Manipulating the Sleeping Beauty Mutase Operon in Engineered Escherichia coli for Controlled Biosynthesis of 1-Propanol and Other Value-Added Chemicals', uwspace, 2016, https://uwspace.uwaterloo.ca/handle/10012/10436 pages 107-108	1,4-22
Y	WO 2017-066498 A1 (LANZATECH NEW ZEALAND LIMITED et al.) 20 April 2017 (2017-04-20) claims 10, 16; paragraph [0239]	14
A	US 2019-0264242 A1 (GENOMATICA, INC.) 29 August 2019 (2019-08-29) 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 "D" document cited by the applicant in the international application "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 13 January 2022		Date of mailing of the international search report 13 January 2022
Name and mailing address of the ISA/KR Korean Intellectual Property Office 189 Cheongsa-ro, Seo-gu, Daejeon 35208, Republic of Korea Facsimile No. +82-42-481-8578		Authorized officer HEO, Joo Hyung Telephone No. +82-42-481-5373

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International application No.

PCT/US2021/051888

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 9297026 B2 (LANZATECH NEW ZEALAND LIMITED) 29 March 2016 (2016-03-29) the whole document	1-22

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2021/051888

Box No. I **Nucleotide and/or amino acid sequence(s) (Continuation of item 1.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 13*ter*.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 13*ter*.1(a)).
- ☐ on paper or in the form of an image file (Rule 13*ter*.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
Information on patent family members

International application No.

PCT/US2021/051888

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)		Publication date (day/month/year)	
EP	3401405	A1	14 November 2018	AU	2012-221176	A1	09 May 2013
				AU	2012-221176	B2	19 March 2015
				BR	112013021524	A2	13 June 2017
				CA	2825267	A1	30 August 2012
				CA	2825267	C	03 April 2018
				CA	2995872	A1	30 August 2012
				CA	2995872	C	17 September 2019
				CN	103502435	A	08 January 2014
				CN	103502435	B	18 May 2018
				CN	108486169	A	04 September 2018
				DK	2678432	T3	26 November 2018
				EP	2678432	A2	01 January 2014
				EP	2678432	B1	01 August 2018
				ES	2702621	T3	04 March 2019
				JP	2014-506478	A	17 March 2014
				JP	6199747	B2	20 September 2017
				KR	10-2014-0012106	A	29 January 2014
				KR	10-2015672	B1	29 August 2019
				KR	10-2019-0097292	A	20 August 2019
				KR	10-2121886	B1	15 June 2020
				MY	168208	A	15 October 2018
				NZ	614459	A	25 September 2015
				PL	2678432	T3	31 May 2019
				PL	2678432	T4	31 May 2019
				TW	201309795	A	01 March 2013
				TW	I541348	B	11 July 2016
				US	2012-0252083	A1	04 October 2012
				US	2013-0224838	A1	29 August 2013
				US	2015-0284692	A9	08 October 2015
				US	2016-0376615	A1	29 December 2016
				US	9365868	B2	14 June 2016
				US	9410130	B2	09 August 2016
				US	9834795	B2	05 December 2017
				WO	2012-115527	A2	30 August 2012
				WO	2012-115527	A3	10 January 2013
WO	2017-066498	A1	20 April 2017	AU	2016-339989	A1	10 May 2018
				AU	2021-203008	A1	10 June 2021
				BR	112018007338	A2	23 October 2018
				CA	3001596	A1	20 April 2017
				CA	3001596	C	17 September 2019
				CA	3051234	A1	20 April 2017
				CA	3051234	C	15 September 2020
				CA	3051235	A1	20 April 2017
				CN	108884467	A	23 November 2018
				CN	113322220	A	31 August 2021
				EA	201890952	A1	28 September 2018
				EP	3362567	A1	22 August 2018
				EP	3362567	A4	27 March 2019
				EP	3901267	A1	27 October 2021
				EP	3901267	A4	27 October 2021

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/US2021/051888

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)		Publication date (day/month/year)
				JP	2018-530342 A	18 October 2018
				JP	2021-166530 A	21 October 2021
				JP	6905518 B2	21 July 2021
				KR	10-2018-0056785 A	29 May 2018
				KR	10-2021-0037002 A	05 April 2021
				MX	2018004642 A	15 April 2019
				US	10316337 B2	11 June 2019
				US	2017-0101631 A1	13 April 2017
				US	2018-0105847 A1	19 April 2018
				US	2018-0208952 A1	26 July 2018
				US	9738875 B2	22 August 2017
				US	9957531 B1	01 May 2018
				ZA	201802346 B	18 December 2019
				ZA	201904455 B	27 January 2021
US	2019-0264242	A1	29 August 2019	US	10087470 B2	02 October 2018
				US	2011-0201089 A1	18 August 2011
				US	2011-0207189 A1	25 August 2011
				US	2011-0212507 A1	01 September 2011
				US	2014-0011249 A1	09 January 2014
				US	2016-0145652 A1	26 May 2016
				US	8048661 B2	01 November 2011
				US	8445244 B2	21 May 2013
				US	8637286 B2	28 January 2014
				WO	2012-099621 A1	26 July 2012
US	9297026	B2	29 March 2016	AU	2013-215706 A1	10 October 2013
				AU	2013-215706 B2	27 March 2014
				BR	112014018844 A2	18 August 2020
				CA	2862790 A1	08 August 2013
				CA	2862790 C	27 September 2016
				CN	104395455 A	04 March 2015
				CN	104395455 B	19 May 2020
				EA	031093 B1	30 November 2018
				EA	201491418 A1	27 February 2015
				EP	2710117 A2	26 March 2014
				EP	2710117 B1	27 April 2016
				ES	2583203 T3	19 September 2016
				JP	2015-505471 A	23 February 2015
				JP	2019-088292 A	13 June 2019
				JP	6512825 B2	15 May 2019
				JP	6898365 B2	07 July 2021
				KR	10-1511639 B1	16 April 2015
				KR	10-2013-0138866 A	19 December 2013
				MY	165103 A	28 February 2018
				US	2014-0206901 A1	24 July 2014
				US	2015-0299737 A1	22 October 2015
				US	9057071 B2	16 June 2015
				WO	2013-115659 A2	08 August 2013
				WO	2013-115659 A3	03 October 2013
				ZA	201405858 B	31 August 2016