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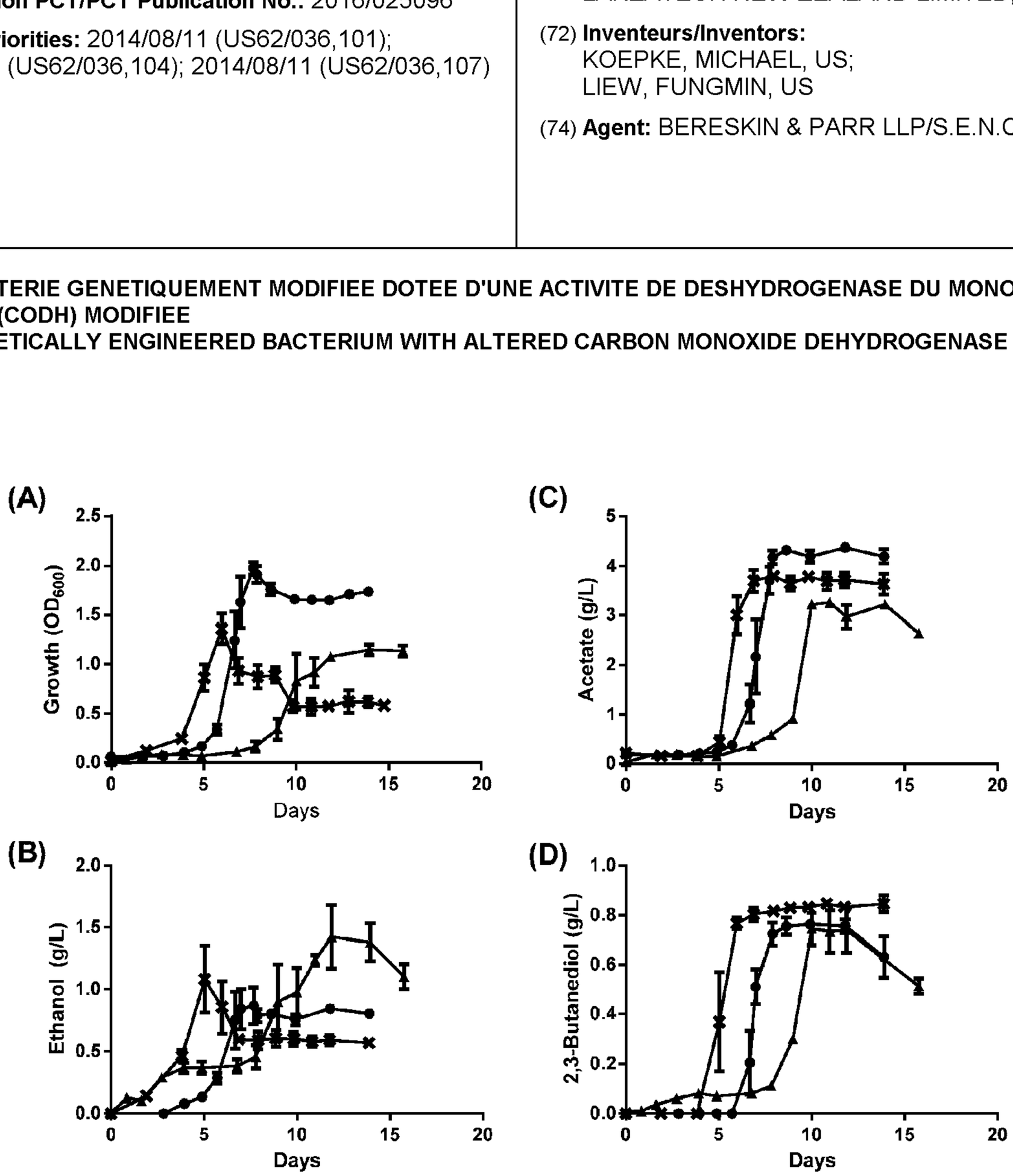
(54) **Titre : BACTERIE GENETIQUEMENT MODIFIEE DOTEE D'UNE ACTIVITE DE DESHYDROGENASE DU MONOXYDE DE CARBONE (CODH) MODIFIEE**
(54) **Title: GENETICALLY ENGINEERED BACTERIUM WITH ALTERED CARBON MONOXIDE DEHYDROGENASE (CODH) ACTIVITY**



Figs. 1A-1D

(57) **Abrégé/Abstract:**
The invention provides genetically engineered microorganisms with altered carbon monoxide dehydrogenase (CODH) activity and methods related thereto. In particular, the invention provides a genetically engineered carboxydotrophic acetogenic bacterium

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(57) **Abrégé(suite)/Abstract(continued):**
having decreased or eliminated activity of CODH1 and/or CODH2. In certain embodiments, the bacterium may also have increased activity of CODH/ACS. The invention further provides a method for producing a product by culturing the bacterium in the presence of a gaseous substrate comprising one or more of carbon monoxide, carbon dioxide, and hydrogen.

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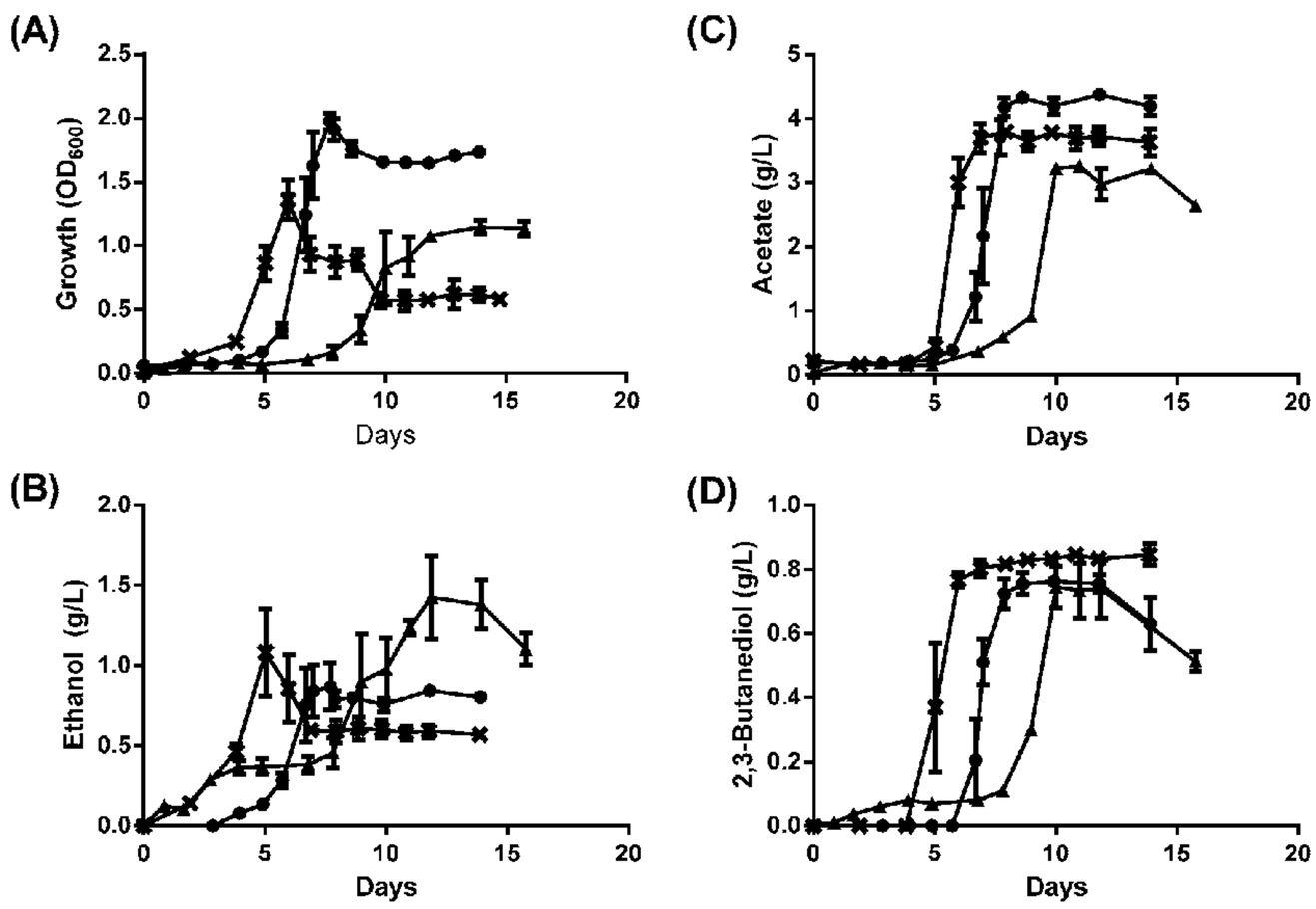
(54) **Title**: GENETICALLY ENGINEERED BACTERIUM WITH ALTERED CARBON MONOXIDE DEHYDROGENASE (CODH) ACTIVITY



Figs. 1A-1D

(57) **Abstract**: The invention provides genetically engineered microorganisms with altered carbon monoxide dehydrogenase (CODH) activity and methods related thereto. In particular, the invention provides a genetically engineered carboxydotrophic acetogenic bacterium having decreased or eliminated activity of CODH1 and/or CODH2. In certain embodiments, the bacterium may also have increased activity of CODH/ACS. The invention further provides a method for producing a product by culturing the bacterium in the presence of a gaseous substrate comprising one or more of carbon monoxide, carbon dioxide, and hydrogen.

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GENETICALLY ENGINEERED BACTERIUM WITH ALTERED CARBON MONOXIDE DEHYDROGENASE (CODH) ACTIVITY

CROSS REFERENCE TO RELATED APPLICATIONS

0001 This application claims the benefit of U.S. Provisional Patent Application 62/036,101 filed August 11, 2014, U.S. Provisional Patent Application 62/036,104 filed August 11, 2014, and U.S. Provisional Patent Application 62/036,107 filed August 11, 2014, the entirety of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

0002 Certain microorganisms can produce fuels, such as ethanol, and other chemicals, such as 2,3-butanediol, by fermentation of gaseous substrates comprising one or more of carbon monoxide (CO), carbon dioxide (CO_2), and hydrogen (H_2). However, efficient production of such fuels and chemicals may be limited diversion of carbon substrates into undesired byproducts or by slow microorganism growth. Accordingly, there remains a need for genetically engineered microorganisms having improved product and/or growth profiles.

SUMMARY OF THE INVENTION

0003 The invention provides genetically engineered microorganisms with altered carbon monoxide dehydrogenase (CODH) activity and methods related thereto. In particular, the invention provides a genetically engineered carboxydotrophic acetogenic bacterium having decreased or eliminated activity of CODH1 and/or CODH2. The invention further provides a method for producing a product by culturing the bacterium in the presence of a gaseous substrate comprising one or more of CO, CO_2, and H_2.

0004 The bacterium may be modified to comprise at least one disruptive mutation in a *CODH1* gene and/or *CODH2* gene, which results in decreased or eliminated activity of CODH1 and/or CODH2. Specifically, the disruptive mutation(s) may reduce or eliminate expression of a *CODH1* gene and/or a *CODH2* gene. In one embodiment, the disruptive mutation is a knockout mutation.

0005 Furthermore, the bacterium may be modified to have increased activity of CODH/ACS. In one embodiment, the bacterium may overexpresses a *CODH/ACS* gene, which results in increased activity of CODH/ACS.

0006 The bacterium may produce a number of products or byproducts, including ethanol, 2,3-butanediol, acetate, and/or lactate. In a preferred embodiment, the bacterium produces one or more of ethanol and 2,3-butanediol. The bacterium may also have altered growth characteristics compared to a parental bacterium, such as decreased lag phase or increased growth rate. Preferably, the bacterium produces a higher amount of ethanol, produces a higher amount of 2,3-butanediol, produces a lower amount of acetate, has a shorter lag phase, and/or has a higher growth rate compared to a parental bacterium.

0007 The bacterium generally consumes a gaseous substrate, such as a gaseous substrate comprising one or more of CO, CO_2, and H_2. The gaseous substrate may be derived from syngas or an industrial process, for example.

0008 In a preferred embodiment, the bacterium is derived from a parental bacterium of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*.

BRIEF DESCRIPTION OF THE DRAWINGS

0009 Figs. 1A-1D are graphs showing growth and metabolite profiles of a CODH1 mutant (triangles), a CODH2 mutant (crosses), and WT *C. autoethanogenum* DSM10061 (circles) on 30 psi CO. In particular, Fig. 1A shows growth, Fig. 1B shows ethanol production, Fig. 1C shows acetate production, and Fig. 1D shows 2,3-butanediol production. N = 3. Error bar = standard error of mean.

0010 Figs. 2A-2C are graphs showing growth and metabolite profiles of a CODH1 mutant (triangles) and WT *C. autoethanogenum* DSM10061 (circles) on steel mill gas. In particular, Fig. 2A shows growth, Fig. 2B shows ethanol production, and Fig. 2C shows acetate production. N = 3. Error bar = standard error of mean.

0011 Figs. 3A-3C are graphs showing growth and metabolite profiles of a CODH1 mutant (triangles), a CODH2 mutant (crosses), and WT *C. autoethanogenum* DSM10061 (circles) under H_2 + CO_2 conditions. In particular, Fig. 3A shows growth, Fig. 3B shows ethanol production, and Fig. 3C shows acetate production. N = 3. Error bar = standard error of mean.

0012 Fig. 4 is a diagram showing a plasmid map of pMTL83157-CODH/ACS.

0013 Figs. 5A-5E are graphs showing the effect of CODH/ACS overexpression on the growth and metabolite profiles of CODH/ACS-overexpressing (pMTL83157-CODH/ACS) (square) and plasmid control pMTL83157 (circle) *C. autoethanogenum* DSM10061 on 100%

CO. In particular, Fig. 5A shows growth, Fig. 5B shows acetate production, Fig. 5C shows ethanol production, Fig. 5C shows ethanol production, Fig. 5D shows 2,3-butanediol production, and Fig. 5E shows lactate production. N = 3. Error bar = standard error of mean.

0014 Figs. 6A-6B are graphs showing the growth of CODH/ACS-inactivated (squares) and WT (circles) *C. autoethanogenum* DSM10061. Fig. 6A shows failure of the CODH/ACS-KO mutant to grown on CO. Fig. 6B shows failure of the CODH/ACS-KO mutant to grow on $CO_2 + H_2$.

0015 Figs. 7A-7D are graphs showing the effect of CODH/ACS inactivation on the growth and metabolite profiles of a CODH/ACS KO mutant (squares), a CODH/ACS KO mutant complemented with plasmid pMTL83157-CODH/ACS (triangles), and WT (circles) *C. autoethanogenum* DSM10061 on fructose. In particular, Fig. 7A shows growth, Fig. 7B shows acetate production, Fig. 7C shows ethanol production, and Fig. 7D shows 2,3-butanediol production.

0016 Fig. 8 is a diagram showing that CODH/ACS inactivation may prevent the Wood-Ljungdahl pathway from serving as a sink for reducing equivalents generated during glycolysis so that excessive reducing equivalents generate driving force for ethanol and 2,3-butanediol production.

DETAILED DESCRIPTION OF THE INVENTION

0017 The invention provides, *inter alia*, novel genetically engineered microorganisms with altered carbon monoxide dehydrogenase (CODH) activity and methods related thereto.

0018 CODH enzymes (EC 1.2.99.2) are oxidoreductases that catalyze the reversible oxidation of CO to CO_2 and generate reducing equivalents according to the equation: $CO + H_2O \leftrightarrow CO_2 + 2H^+ + 2e^-$. CODHs are well known in nature and have been described in various organisms, including carboxydotrophic acetogens.

0019 CODHs can be broadly categorized into two classes: (i) the aerobic Cox-type Mo-Cu-Se CODH from carboxydobacteria, which comprises a highly conserved molybdenum active site and uses oxygen (sometimes nitrate) as terminal electron acceptor; and (ii) the anaerobic-type Ni-CODH, which transfer the electrons liberated from CO oxidation to a range of physiological electron acceptors including ferredoxin, cytochromes, flavodoxin, rubredoxin, and $NAD(P)^+$. The reducing equivalents can then be harnessed in several pathways including acetogenesis, methanogenesis, sulphate reduction, hydro genogenesis, and metal reduction.

0020 The O_2-sensitive Ni-CODH can be further divided into two groups: (i) Mono-functional CODH which functions physiologically in CO oxidation; and (ii) CODH as part of a bi-functional CODH/ACS complex that couples the reduction of CO_2 into CO moiety to acetyl-CoA biosynthesis.

0021 *C. autoethanogenum*, for example, is able to grow autotrophically using CO as the sole source of carbon and energy. Genome sequencing uncovered three putative Ni-CODH in this acetogen: CAETHG_3005 (*CODH1*), CAETHG_3899 (*CODH2*), and CAETHG_1620-1621 (*AcsA,* which encodes the CODH component of the bifunctional CODH/ACS complex). *CODH1* is genetically colocalized upstream of a putative 4Fe-4S ferredoxin Fe-S binding protein and ferredoxin-NAD(+) reductase, while *CODH2* appears to be an orphan. Similarly, carboxydotrophic acetogens *C. ljungdahlii* and *C. carboxidivorans* are also described to have three CODHs, one bifunctional CODH/ACS and two additional mono-functional CODHs. Additionally, at least CODH1 is found in all sequenced carboxydotrophic acetogens, including *C. ljungdahlii*, *C. ragsdalei*, *C. difficile*, and *A. woodii*.

0022 The prior art generally accepts that CODHs, including CODH1 and CODH2, are involved in CO utilization. For example, US 2010/0151543 describes how overexpression of CODH within the acetogenic *Clostridia* may increase electron flow from syngas components to the oxidized nucleotide cofactors NAD^+ and $NADP^+$, whereby the nucleotide cofactors (NADH and NADPH) then stimulate generation of intermediate compounds in Wood-Ljungdahl pathway.

0023 However, the inventors have surprisingly identified that disrupting CODH1 and/or CODH2 in a carboxydotrophic acetogenic microorganism does *not* negatively affect gas utilization. In fact, the inventors have discovered that disrupting CODH1 and/or CODH2 in a carboxydotrophic acetogenic microorganism results in a microorganism that produces a higher amount of ethanol, produces a lower amount of acetate, has a shorter lag phase, and/or has a higher growth rate compared to an unmodified parental microorganism.

0024 The invention provides a genetically engineered carboxydotrophic acetogenic bacterium having decreased or eliminated activity of CODH1 and/or CODH2. The invention further provides a method for producing a product by culturing the bacterium in the presence of a gaseous substrate comprising one or more of CO, CO_2, and H_2.

Microorganisms

0025 The microorganism of the invention is genetically engineered, i.e., non-naturally occurring. The term "genetic modification" or "genetic engineering" broadly refers to manipulation of the genome or nucleic acids of a microorganism. Likewise, the term "genetically engineered" refers to a microorganism comprising a manipulated genome or nucleic acids. Methods of genetic modification of include, for example, heterologous gene expression, gene or promoter insertion or deletion, nucleic acid mutation, altered gene expression or inactivation, enzyme engineering, directed evolution, knowledge-based design, random mutagenesis methods, gene shuffling, and codon optimization.

0026 A "microorganism" is a microscopic organism, especially a bacterium, archaea, virus, or fungus. The microorganism of the invention is typically a bacterium. As used herein, recitation of "microorganism" should be taken to encompass "bacterium."

0027 A "parental microorganism" is a microorganism used to generate a microorganism of the invention. The parental microorganism may be a naturally-occurring microorganism (i.e., a wild-type microorganism) or a microorganism that has been previously modified (i.e., a mutant or recombinant microorganism). The microorganism of the invention may be modified to express or overexpress one or more enzymes that were not expressed or overexpressed in the parental microorganism. Similarly, the microorganism of the invention may be modified to contain one or more genes that were not contained by the parental microorganism. In one embodiment, the parental microorganism is *C. autoethanogenum*, *C. ljungdahlii*, or *C. ragsdalei*. In a preferred embodiment, the parental microorganism is *C. autoethanogenum* LZ1561, which is deposited under DSMZ accession DSM23693.

0028 The term "derived from" indicates that a nucleic acid, protein, or microorganism is modified or adapted from a different (e.g., a parental or wild-type) nucleic acid, protein, or microorganism, so as to produce a new nucleic acid, protein, or microorganism. Such modifications or adaptations typically include insertion, deletion, mutation, or substitution of nucleic acids or genes. Generally, the microorganism of the invention is derived from a parental microorganism. In one embodiment, the microorganism of the invention is derived from *C. autoethanogenum*, *C. ljungdahlii*, or *C. ragsdalei*. In a preferred embodiment, the microorganism of the invention is derived from *C. autoethanogenum* LZ1561, which is deposited under DSMZ accession DSM23693.

0029 The microorganism of the invention may be further classified based on functional characteristics. For example, the microorganism of the invention may be or may be derived from a C1-fixing microorganism, an anaerobe, an acetogen, an ethanologen, a carboxydotroph, and/or a combination thereof. Table 1 provides a representative list of microorganisms and identifies their functional characteristics.

Table 1

	C1-fixing	Anaerobe	Acetogen	Ethanologen	Autotroph	Carboxydotroph
Acetobacterium woodii	+	+	+	+/- [1]	-	+/- [2]
Alkalibaculum bacchii	+	+	+	+	+	+
Blautia producta	+	+	+	-	+	+
Butyribacterium methylotrophicum	+	+	+	+	+	+
Clostridium aceticum	+	+	+	-	+	+
Clostridium autoethanogenum	+	+	+	+	+	+
Clostridium carboxidivorans	+	+	+	+	+	+
Clostridium coskatii	+	+	+	+	+	+
Clostridium drakei	+	+	+	-	+	+
Clostridium formicoaceticum	+	+	+	-	+	+
Clostridium ljungdahlii	+	+	+	+	+	+
Clostridium magnum	+	+	+	-	+	+/- [3]
Clostridium ragsdalei	+	+	+	+	+	+
Clostridium scatologenes	+	+	+	-	+	+
Eubacterium limosum	+	+	+	-	+	+
Moorella thermautotrophica	+	+	+	+	+	+
Moorella thermoacetica (formerly *Clostridium thermoaceticum*)	+	+	+	- [4]	+	+
Oxobacter pfennigii	+	+	+	-	+	+
Sporomusa ovata	+	+	+	-	+	+/- [5]
Sporomusa silvacetica	+	+	+	-	+	+/- [6]
Sporomusa sphaeroides	+	+	+	-	+	+/- [7]
Thermoanaerobacter kiuvi	+	+	+	-	+	-

[1] *Acetobacterium woodi* can produce ethanol from fructose, but not from gas.
[2] It has been reported that *Acetobacterium woodi* can grow on CO, but the methodology is questionable.
[3] It has not been investigated whether *Clostridium magnum* can grow on CO.
[4] One strain of *Moorella thermoacetica, Moorella* sp. HUC22-1, has been reported to produce ethanol from gas.
[5] It has not been investigated whether *Sporomusa ovata* can grow on CO.
[6] It has not been investigated whether *Sporomusa silvacetica* can grow on CO.
[7] It has not been investigated whether *Sporomusa sphaeroides* can grow on CO.

0030 "C1" refers to a one-carbon molecule, for example, CO, CO_2, CH_4, or CH_3OH. "C1-oxygenate" refers to a one-carbon molecule that also comprises at least one oxygen atom, for example, CO, CO_2, or CH_3OH. "C1-carbon source" refers a one carbon-molecule that serves as a partial or sole carbon source for the microorganism of the invention. For example, a C1-carbon source may comprise one or more of CO, CO_2, CH_4, CH_3OH, or CH_2O_2. Preferably, the C1-carbon source comprises one or both of CO and CO_2. A "C1-fixing microorganism" is a microorganism that has the ability to produce one or more products from a C1-carbon source. Typically, the microorganism of the invention is a C1-fixing bacterium. In a preferred embodiment, the microorganism of the invention is derived from a C1-fixing microorganism identified in Table 1.

0031 An "anaerobe" is a microorganism that does not require oxygen for growth. An anaerobe may react negatively or even die if oxygen is present above a certain threshold. Typically, the microorganism of the invention is an anaerobe. In a preferred embodiment, the microorganism of the invention is derived from an anaerobe identified in Table 1.

0032 An "acetogen" is a microorganism that produces or is capable of producing acetate (or acetic acid) as a product of anaerobic respiration. Typically, acetogens are obligately anaerobic bacteria that use the Wood-Ljungdahl pathway as their main mechanism for energy conservation and for synthesis of acetyl-CoA and acetyl-CoA-derived products, such as acetate (Ragsdale, *Biochim Biophys Acta*, 1784: 1873-1898, 2008). Acetogens use the acetyl-CoA pathway as a (1) mechanism for the reductive synthesis of acetyl-CoA from CO_2, (2) terminal electron-accepting, energy conserving process, (3) mechanism for the fixation (assimilation) of CO_2 in the synthesis of cell carbon (Drake, Acetogenic Prokaryotes, In: The Prokaryotes, 3rd edition, p. 354, New York, NY, 2006). All naturally occurring acetogens are C1-fixing, anaerobic, autotrophic, and non-methanotrophic. Typically, the microorganism of the invention is an acetogen. In a preferred embodiment, the microorganism of the invention is derived from an acetogen identified in Table 1.

0033 An "ethanologen" is a microorganism that produces or is capable of producing ethanol. Typically, the microorganism of the invention is an ethanologen. In a preferred embodiment, the microorganism of the invention is derived from an ethanologen identified in Table 1.

0034 An "autotroph" is a microorganism capable of growing in the absence of organic carbon. Instead, autotrophs use inorganic carbon sources, such as CO and/or CO_2. Typically,

the microorganism of the invention is an autotroph. In a preferred embodiment, the microorganism of the invention is derived from an autotroph identified in Table 1.

0035 A "carboxydotroph" is a microorganism capable of utilizing CO as a sole source of carbon. Typically, the microorganism of the invention is a carboxydotroph. In a preferred embodiment, the microorganism of the invention is derived from a carboxydotroph identified in Table 1.

0036 More broadly, the microorganism of the invention may be derived from any genus or species identified in Table 1.

0037 In a preferred embodiment, the microorganism of the invention is derived from the cluster of *Clostridia* comprising the species *C. autoethanogenum*, *C. ljungdahlii*, and *C. ragsdalei*. These species were first reported and characterized by Abrini, *Arch Microbiol*, 161: 345-351, 1994 (*C. autoethanogenum*), Tanner, *Int J System Bacteriol*, 43: 232-236, 1993 (*C. ljungdahlii*), and Huhnke, WO 2008/028055 (*C. ragsdalei*).

0038 These three species have many similarities. In particular, these species are all C1-fixing, anaerobic, acetogenic, ethanologenic, and carboxydotrophic members of the genus *Clostridium*. These species have similar genotypes and phenotypes and modes of energy conservation and fermentative metabolism. Moreover, these species are clustered in clostridial rRNA homology group I with 16S rRNA DNA that is more than 99% identical, have a DNA G + C content of about 22-30 mol%, are gram-positive, have similar morphology and size (logarithmic growing cells between 0.5-0.7 x 3-5 μm), are mesophilic (grow optimally at 30-37 °C), have similar pH ranges of about 4-7.5 (with an optimal pH of about 5.5-6), lack cytochromes, and conserve energy via an Rnf complex. Also, reduction of carboxylic acids into their corresponding alcohols has been shown in these species (Perez, *Biotechnol Bioeng*, 110:1066-1077, 2012). Importantly, these species also all show strong autotrophic growth on CO-containing gases, produce ethanol and acetate (or acetic acid) as main fermentation products, and produce small amounts of 2,3-butanediol and lactic acid under certain conditions.

0039 However, these three species also have a number of differences. These species were isolated from different sources: *C. autoethanogenum* from rabbit gut, *C. ljungdahlii* from chicken yard waste, and *C. ragsdalei* from freshwater sediment. These species differ in utilization of various sugars (e.g., rhamnose, arabinose), acids (e.g., gluconate, citrate), amino acids (e.g., arginine, histidine), and other substrates (e.g., betaine, butanol). Moreover, these

species differ in auxotrophy to certain vitamins (e.g., thiamine, biotin). These species have differences in nucleic and amino acid sequences of Wood-Ljungdahl pathway genes and proteins, although the general organization and number of these genes and proteins has been found to be the same in all species (Köpke, *Curr Opin Biotechnol*, 22: 320-325, 2011).

0040 Thus, in summary, many of the characteristics of *C. autoethanogenum*, *C. ljungdahlii*, or *C. 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.

0041 The microorganism of the invention may also be derived from an isolate or mutant of *C. autoethanogenum*, *C. ljungdahlii*, or *C. ragsdalei*. Isolates and mutants of *C. autoethanogenum* include JA1-1 (DSM10061) (Abrini, *Arch Microbiol*, 161: 345-351, 1994), LBS1560 (DSM19630) (WO 2009/064200), and LZ1561 (DSM23693). Isolates and mutants of *C. 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 *C. ljungdahlii*, PhD thesis, North Carolina State University, 2010). Isolates and mutants of *C. ragsdalei* include PI 1 (ATCC BAA-622, ATCC PTA-7826) (WO 2008/028055).

Enzymes

0042 "CODH1" refers to CODH which catalyzes the reversible oxidation of CO to CO_2 and generates reducing equivalents according to the equation: $CO + H_2O \leftrightarrow CO_2 + 2H^+ + 2e^-$. Reference to "CODH1" herein should be taken to include reference to functionally equivalent variants thereof. The CODH1 may be, for example, CODH1 of *C. autoethanogenum* (SEQ ID NO: 1), *C. ragsdalei* (SEQ ID NO: 5), *C. ljungdahlii* (ADK13979.1), *C. difficile* (YP_001086644.1), or *A. woodii* (YP_005269573).

0043 "CODH2" refers to CODH which catalyzes the reversible oxidation of CO to CO_2 and generates reducing equivalents according to the equation: $CO + H_2O \leftrightarrow CO_2 + 2H^+ + 2e^-$. Reference to "CODH2" herein should be taken to include reference to functionally equivalent variants thereof. The CODH2 may be, for example, CODH2 of *C. autoethanogenum* (SEQ

ID NO: 3), *C. ragsdalei* (SEQ ID NO: 7), *C. ljungdahlii* (ADK14854.1), *C. scatologenes* (SEQ ID NO: 9), *C. acetobutylicum* (AAK78101.1 and AAK80452.1), *C. carboxidivorans* "P7" (ZP_05390164.1), *C. hydrogenoformans* (ABB14220.1, ABB14432.1 and ABB15066.1), or *C. beijerinckii* (YP_001310115.1). Furthermore, CODH2 homologs can be found in *C. botulinum (*CBO_2218; A5I3Y9), but not in *C. perfringens, C. thermocellum*, *C. pasteurianum*, or *C. kluyveri*.

0044 The bifunctional "CODH/ACS" is unique to acetogenic bacteria and, in addition to the reversible oxidation of CO, also catalyzes the synthesis of acetyl-CoA from CO, a methyl group, and CoA. The CODH/ACS enzyme complex consists of multiple subunits: CODH subunit (AcsA); ACS subunit (AcsB); corrinoid iron-sulfur protein large subunit (AcsC); corrinoid iron-sulfur protein small subunit (AcsD); methyltransferase subunit (AcsE); and, CODH accessory protein (CooC). The inventors have discovered that increasing the level of activity of CODH/ACS improves growth and/or product formation. Surprisingly, overexpression of a single CODH subunit of the CODH/ACS complex is sufficient to increase activity of the complex.

0045 The AcsB subunit of CODH/ACS may be, for example, AcsB of *C. autoethanogenum* (CAETHG_1608 gene, WP_023162339.1 protein), *C. ljungdahlii* (CLJU_c37550 gene, WP_013240359.1 protein), *C. ragsdalei* (HQ876032.1 gene, AEI90761.1 protein), *C. carboxidivorans* (Ccar3245 gene, WP_007061841.1), *C. scatalogenes* (WP_029162953.1 protein), *C. difficile* (CD0728 gene, WP_021369307.1 protein), and *A. woodii* (Awo_c10760 gene, WP_014357691.1 protein).

0046 The AcsC subunit of CODH/ACS may be, for example, AcsC of *C. autoethanogenum* (CAETHG_1610 gene, WP_023162341.1 protein), *C. ljungdahlii* (CLJU_c37570 gene, WP_013240361.1 protein), *C. ragsdalei* (HQ876032.1 gene, AEI90763.1 protein), *C. carboxidivorans* (Ccar3247 gene, WP_007061843.1 protein), *C. scatalogenes* (WP_029162955.1 protein), *C. difficile* (CD0730 gene, WP_021369309.1 protein), or *A. woodii* (Awo_c10720 gene, WP_014357687.1 protein).

0047 The AcsD subunit of CODH/ACS may be, for example, AcsD of *C. autoethanogenum* (CAETHG_1611 gene, WP_023162342.1 protein), *C. ljungdahlii* (CLJU_c37580 gene, WP_013240362.1 protein), *C. ragsdalei* (HQ876032.1 gene, AEI90764.1 protein), *C. carboxidivorans* (Ccar3248 gene, WP_007061844.1 protein), *C. scatalogenes*

(WP_029162956.1 protein), *C. difficile* (CD0731 gene, WP_021369310.1 protein), or *A. woodii* (Awo_c10710 gene, WP_014357686.1 protein).

0048 The AcsE subunit of CODH/ACS may be, for example, AcsE of *C. autoethanogenum* (CAETHG_1609 gene, WP_023162340.1 protein), *C. ljungdahlii* (CLJU_c37560 gene, WP_013240360.1 protein), *C. ragsdalei* (HQ876032.1 gene, AEI90762.1 protein), *C. carboxidivorans* (Ccar3246 gene, WP_007061842.1 protein), *C. scatalogenes* (WP_029162954.1 protein), *C. difficile* (CD0729 gene, WP_021369308.1 protein), or *A. woodii* (Awo_c10730 gene, WP_014357688.1 protein).

0049 The CooC accessory protein of CODH/ACS may be, for example, CooC of *C. autoethanogenum* (CAETHG_1612 gene, WP_023162343.1 protein), *C. ljungdahlii* (CLJU_c37590 gene, WP_013240363.1 protein), *C. ragsdalei* (HQ876032.1 gene, AEI90765.1 protein), *C. carboxidivorans* (Ccar3249 gene, WP_007061845.1 protein), *C. scatalogenes* (WP_029162957.1 protein), *C. difficile* (CD0732 gene, WP_021369311.1 protein), or *A. woodii* (Awo_c10709 gene, WP_014357685.1 protein).

0050 Sequence information is provided for CODH1, CODH2, and CODH/ACS to identify exemplary sequences applicable to the invention and to allow a skilled person to practice specific embodiments of the invention without undue experimentation. It should be appreciated that nucleic acid and amino acid sequences for CODH1, CODH2, and CODH/ACS may differ from one microorganism to another. Accordingly, the invention should not be construed as being limited to these specific sequences and embodiments, but rather to extend to functionally equivalent variants of any specific CODH1, CODH2, or CODH/ACS referred to herein, including homologs in other strains and species.

0051 The term "variants" includes nucleic acids and proteins whose sequence varies from the sequence of a reference nucleic acid and protein, such as a sequence of a reference nucleic acid and protein disclosed in the prior art or exemplified herein. The invention may be practiced using variant nucleic acids or proteins that perform substantially the same function as the reference nucleic acid or protein. For example, a variant protein may perform substantially the same function or catalyze substantially the same reaction as a reference protein. A variant gene may encode the same or substantially the same protein as a reference gene. A variant promoter may have substantially the same ability to promote the expression of one or more genes as a reference promoter.

0052 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 *C. acetobutylicum, C. beijerinckii,* or *C. ljungdahlii*, the details of which are publicly available on websites such as Genbank or NCBI. Functionally equivalent variants also 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. For example, enzyme assays of use in assessing the activity of CODH1, CODH2, CODH/ACS and variants thereof include anaerobic purification of CODH followed by spectrophotometric measurement of change in absorbance at 604 nm using methyl viologens as electron acceptors (Ragsdale, *J Biol Chem*, 258: 2364-2369, 1983).

0053 The microorganism of the invention has altered CODH1, CODH2, and/or CODH/ACS activity. "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" or "reducing" 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. In one embodiment, the function or activity of CODH1 and/or CODH2 is decreased. In another embodiment, the function or activity of CODH1 and/or CODH2 is eliminated or substantially eliminated. In another embodiment, the function or activity of CODH/ACS is increased. In a related embodiment, the function or activity of one or more subunits or accessory proteins of CODH/ACS is increased, particularly the function or activity of the CODH subunit.

0054 As one approach, a change in enzyme activity may be achieved by mutating a gene encoding a protein. "Mutated" refers to a nucleic acid or protein that has been modified in the microorganism of the invention compared to the wild-type or parental microorganism from which the microorganism of the invention is derived. In one embodiment, the mutation may be a deletion, insertion, or substitution in a gene encoding an enzyme. In another embodiment, the mutation may be a deletion, insertion, or substitution of one or more amino acids in an enzyme.

0055 In particular, a "disruptive mutation" is a mutation that reduces or eliminates (i.e., "disrupts") the expression or activity of a gene or enzyme. The disruptive mutation may partially inactivate, fully inactivate, or delete the gene or enzyme. The disruptive mutation may be a knockout (KO) mutation, whereby the gene or protein is made inoperative. The disruptive mutation may be any mutation that reduces, prevents, or blocks the biosynthesis of a product produced by an enzyme. The disruptive mutation may include, for example, a mutation in a gene encoding an enzyme, a mutation in a genetic regulatory element involved in the expression of a gene encoding an enzyme, the introduction of a nucleic acid which produces a protein that reduces or inhibits the activity of an enzyme, or the introduction of a nucleic acid (e.g., antisense RNA, siRNA, CRISPR) or protein which inhibits the expression of an enzyme. The microorganism of the invention typically comprises at least one disruptive mutation in a *CODH1* gene and/or *CODH2* gene. Such a mutation may decrease or eliminate expression of the *CODH1* gene and/or the *CODH2* gene compared to a parental microorganism.

0056 The disruptive mutation may be introduced using any method known in the art. In particular, the disruptive mutation may be introduced by permanently inactivating a gene by targeted insertion of foreign DNA into the coding sequence. A genetic tool known as ClosTron can be used to stably insert an intron (1.8 kb) into a specified locus. Specifically, ClosTron utilizes the specificity of mobile group II intron Ll.*ltrB* from *L. lactis* to propagate into the specified site via a RNA-mediated, retro-homing mechanism (Heap, *J Microbiol Meth*, 80: 49-55, 2010). Another approach involves the transfer of plasmid with homology arms to permanently delete part or whole gene by employing homologous recombination. For instance, a genetic method termed "ACE", or allele-coupled exchange (Heap, *Nucl Acids Res*, 40: e59, 2012) can be used to carry out this deletion without relying on the use of a counter selectable marker.

0057 In some embodiments, the microorganism of the invention has increased activity of CODH/ACS in combination with decreased or eliminated activity of CODH1 and/or CODH2. In particular, the microorganism may overexpress a *CODH/ACS* gene. Herein, "*CODH/ACS* gene" refers to any gene encoding any subunit or accessory protein of the CODH/ACS enzyme complex. In a preferred embodiment, the microorganism expresses a gene encoding the CODH subunit of the CODH/ACS enzyme complex. "Overexpressed" refers to an increase in expression of a nucleic acid or protein in the microorganism of the invention compared to the wild-type or parental microorganism from which the microorganism of the invention is derived. Overexpression may be achieved by any means known in the art, including modifying gene copy number, gene transcription rate, gene translation rate, or enzyme degradation rate.

0058 Nucleic acids may be delivered to a microorganism of the invention using any method known in the art. For example, nucleic acids may be delivered as naked nucleic acids or may be formulated with one or more agents, such as liposomes. The nucleic acids may be DNA, RNA, cDNA, or combinations thereof, as is appropriate. Restriction inhibitors may be used in certain embodiments. Additional vectors may include plasmids, viruses, bacteriophages, cosmids, and artificial chromosomes. In a preferred embodiment, nucleic acids are delivered to the microorganism of the invention using a plasmid. By way of example, transformation (including transduction or transfection) may be achieved by electroporation, ultrasonication, polyethylene glycol-mediated transformation, chemical or natural competence, protoplast transformation, prophage induction, or conjugation. In certain embodiments having active restriction enzyme systems, it may be necessary to methylate a nucleic acid before introduction of the nucleic acid into a microorganism.

0059 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.

0060 Nucleic acids of the invention may be codon optimized for expression in a particular strain or species, particularly *C. autoethanogenum* (including *C. autoethanogenum* LZ1561), *C. ljungdahlii*, or *C. ragsdalei*. "Codon optimization" refers to the mutation of a nucleic acid,

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

Growth and Products

0061 The microorganism of the invention has an altered growth and/or metabolic profile compared to the parental microorganism from which it is derived. For instance, the microorganism may produce a higher amount of ethanol, produce a higher amount of 2,3-butanediol, produce a lower amount of acetate, have a shorter lag phase, and/or have a higher growth rate compared to the parental microorganism.

0062 The microorganism of the invention may have an altered lag phase. "Lag phase" or "growth lag phase" refers to the amount of time a culture or population of microorganisms takes to reach the early log growth phase or log/exponential growth phase after inoculation. In one embodiment, the microorganism has a shorter lag phase compared to a parental microorganism. For example, the microorganism may have a lag phase that is about 20%, 25%, or 30% shorter than the lag phase of the parental microorganism. In one embodiment, the microorganism has a lag phase that is about 25% to 30% shorter than the lag phase of the parental microorganism. In other embodiments, the microorganism may have a lag phase that is about 3, 5, or 8 times shorter than the lag phase of the parental microorganism. In one embodiment, the lag phase may be about 7.8 to 8 days shorter than the lag phase of the parental microorganism. In another embodiment, the lag phase may be about 1-4 days or less or about 2.9 days or less. In some instances, the microorganism may have a dramatically shorter lag phase than the parental microorganism. For example, the microorganism may have a lag phase that is about 10, 50, 100, or 200 times shorter than the lag phase of the parental microorganism. In one embodiment, the lag phase may be about 0.1 days or less.

0063 The microorganism of the invention may have an altered growth rate. "Growth rate" or "rate of growth" refers to the rate at which a culture or population of microorganisms increases with time. Growth rates are typically expressed herein using the units h^{-1}. In one embodiment, the microorganism has an increased or higher growth rate compared to the parental microorganism. For example, the microorganism may have a growth rate that is about 20%, 40%, 60%, 80%, or 100% higher than the growth rate of the parental microorganism. In certain embodiments, the microorganism has a growth rate that is about 2, 3, 4, or 5 times higher than the growth rate of the parental microorganism.

0064 The microorganism of the invention may produce an altered amount of biomass. "Biomass" refers to the collective population of microorganisms generated from a growth or fermentation process. In one embodiment, fermentation of the microorganism produces an increased or higher amount of biomass compared to fermentation of the parental microorganism. For example, fermentation of the microorganism may produce about 20%, 30%, 40%, 50%, 80%, 100%, 120%, 150%, 180%, 200% or 220% more biomass compared to fermentation of the parental microorganism. In one embodiment, fermentation of the microorganism produces about 200% to 220% more biomass compared to fermentation of the parental microorganism.

0065 The microorganism of the invention may produce an altered amount of ethanol. In one embodiment, the microorganism produces an increased or higher amount of ethanol compared to a parental microorganism. For example, the microorganism may produce about 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 110%, or 120% more ethanol compared to the parental microorganism. In one embodiment, the microorganism produces about 20% to 113% more ethanol compared to the parental microorganism.

0066 The microorganism of the invention may produce an altered amount of 2,3-butanediol. In one embodiment, the microorganism produces an increased or higher amount of 2,3-butanediol compared to a parental microorganism. For example, the microorganism may produce about 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 120%, 140%, 160%, 180%, 200%, 220%, 240%, 260%, 270%, 280%, 300%, 320%, or 340% more 2,3-butanediol compared to the parental microorganism. In one embodiment, the microorganism produces about 220% to 230% more 2,3-butanediol compared to the parental microorganism. In another embodiment, the microorganism produces at least about 330% more 2,3-butanediol compared to the parental microorganism. In a further embodiment, the microorganism produces about 300% to 330% more 2,3-butanediol compared to the parental microorganism. In an additional embodiment, the microorganism produces about 0.5-20 g/L 2,3-butanediol.

0067 The microorganism of the invention may produce an altered amount of acetate. The term "acetate" includes both acetate salt alone and a mixture of molecular or free acetic acid and acetate salt. In one embodiment, the microorganism produces a decreased or lower amount of acetate compared to a parental microorganism. For example, the microorganism may produce about 10%, 20%, 30%, 40%, or 50% less acetate compared to the parental

microorganism. In one embodiment, the microorganism produces about 18% to 37% less acetate compared to the parental microorganism. In another embodiment, the microorganism produces about 0-5 g/L acetate.

0068 The microorganism of the invention may produce an altered amount of lactate. In one embodiment, the microorganism produces a decreased or lower amount of lactate compared to a parental microorganism.

0069 In a particularly preferred embodiment, the microorganism of the invention produces an increased amount of ethanol and/or 2,3-butanediol and a decreased amount of acetate compared to a parental microorganism.

0070 The microorganism and methods described herein may be used to increase the efficiency of a fermentation process. "Increasing the efficiency," "increased efficiency," and the like include, but are not limited to, increasing microorganism growth rate, product production rate or volume, product volume per volume of substrate consumed, or product selectivity. Efficiency may be measured relative to the performance of a parental microorganism from which the microorganism of the invention is derived.

0071 The microorganism of the invention may also produce one or more additional products. For instance, *Clostridium autoethanogenum* produces or can be engineered to produce ethanol (WO 2007/117157), acetate (WO 2007/117157), butanol (WO 2008/115080 and WO 2012/053905), butyrate (WO 2008/115080), 2,3-butanediol (WO 2009/151342), lactate (WO 2011/112103), butene (WO 2012/024522), butadiene (WO 2012/024522), methyl ethyl ketone (2-butanone) (WO 2012/024522 and WO 2013/185123), ethylene (WO 2012/026833), acetone (WO 2012/115527), isopropanol (WO 2012/115527), lipids (WO 2013/036147), 3-hydroxypropionate (3-HP) (WO 2013/180581), isoprene (WO 2013/180584), fatty acids (WO 2013/191567), 2-butanol (WO 2013/185123), 1,2-propanediol (WO 2014/0369152), and 1-propanol (WO 2014/0369152). In certain embodiments, microbial biomass itself may be considered a product.

0072 The invention further provides methods for producing one or more products, such as ethanol and/or 2,3-butanediol, by culturing a microorganism of the invention. The invention also provides methods for reducing total atmospheric carbon emissions from an industrial process by using a microorganism of the invention to convert CO, CO_2 and/or H_2 in an industrial waste gas to useful products.

Substrate

0073 "Substrate" refers to a carbon and/or energy source for the microorganism of the invention. Typically, the substrate is gaseous and comprises a C1-carbon source, for example, CO, CO_2, and/or CH_4. Preferably, the substrate comprises a C1-carbon source of CO or CO + CO_2. The substrate may further comprise other non-carbon components, such as H_2, N_2, or electrons.

0074 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 invention typically converts at least a portion of the CO in the substrate to a product. In some embodiments, the substrate comprises no or substantially no CO.

0075 The substrate may comprise some amount of H_2. For example, the substrate may comprise about 1, 2, 5, 10, 15, 20, or 30 mol% H_2. In some embodiments, the substrate may comprise a relatively high amount of H_2, such as about 60, 70, 80, or 90 mol% H_2. In further embodiments, the substrate comprises no or substantially no H_2. H_2-rich gas streams may be produced, for example, via steam reformation of hydrocarbons, particularly steam reformation of natural gas, partial oxidation of coal or hydrocarbons, electrolysis of water, and capture byproducts from electrolytic cells used to produce chlorine and from refinery or chemical streams.

0076 The substrate may comprise some amount of CO_2. For example, the substrate may comprise about 1-80 or 1-30 mol% CO_2. In some embodiments, the substrate may comprise less than about 20, 15, 10, or 5 mol% CO_2. In another embodiment, the substrate comprises no or substantially no CO_2. CO_2-rich gas streams include, for example, exhaust gasses from hydrocarbon combustion, such as natural gas or oil combustion, byproducts from the production of ammonia, lime, or phosphate, and natural carbon dioxide wells.

0077 Although the substrate is typically gaseous, the substrate may also be provided in alternative forms. For example, the substrate may be dissolved in a liquid saturated with a

CO-containing gas using a microbubble dispersion generator. By way of further example, the substrate may be adsorbed onto a solid support.

0078 The substrate and/or C1-carbon source may be a waste gas obtained as a byproduct of an industrial process or from some other source, such as from automobile exhaust fumes or biomass gasification. In certain embodiments, the industrial process is selected from the group consisting of ferrous metal products manufacturing, such as a steel mill manufacturing, non-ferrous products manufacturing, petroleum refining processes, coal gasification, electric power production, carbon black production, ammonia production, methanol production, and coke manufacturing. In these embodiments, the substrate and/or C1-carbon source may be captured from the industrial process before it is emitted into the atmosphere, using any convenient method.

0079 The substrate and/or C1-carbon source may be syngas, such as syngas obtained by gasification of coal or refinery residues, gasification of biomass or lignocellulosic material, or reforming of natural gas. In another embodiment, the syngas may be obtained from the gasification of municipal solid waste or industrial solid waste.

0080 The composition of the substrate may have a significant impact on the efficiency and/or cost of the reaction. For example, the presence of oxygen (O_2) may reduce the efficiency of an anaerobic fermentation process. Depending on the composition of the substrate, it may be desirable to treat, scrub, or filter the substrate to remove any undesired impurities, such as toxins, undesired components, or dust particles, and/or increase the concentration of desirable components.

Effect of Substrate and Genetic Modifications

0081 The composition of the substrate may affect the growth and/or metabolic profile of the microorganism of the invention. For instance, a microorganism grown on CO may have a different growth and/or metabolic profile than a microorganism grown on $CO_2 + H_2$. Additionally, the particular combination of genetic modifications may affect the growth and/or metabolic profile of the microorganism of the invention. For instance, a microorganism comprising a disruptive mutation in a *CODH1* gene may have a different growth and/or metabolic profile than a microorganism comprising a disruptive mutation in a *CODH2* gene, which may have a different growth and/or metabolic profile than a microorganism comprising a disruptive mutation in both a *CODH1* gene and a *CODH2* gene. CODH/ACS overexpression in any of these microorganisms may further alter the growth

and/or metabolic profile of the microorganisms. Strategically combining genetic modifications and growing microorganisms on particular substrates may yield growth and/or metabolic profiles tailored to specific applications or production goals.

0082 Growing a *CODH1* knockout strain on CO generally results in decreased biomass production, decreased acetate production, increased ethanol production, and similar 2,3-butanediol production. Growing a *CODH1* knockout strain on $CO_2 + H_2$ generally results in a decreased lag phase and faster growth. For example, a *CODH1* knockout strain grown on $CO_2 + H_2$ may have no lag phase and may produce about 0.4 g/L biomass.

0083 Growing a *CODH2* knockout strain on CO generally results in decreased lag phase, decreased ethanol production, decreased acetate production, and increased or similar 2,3-butanediol production. For example, a *CODH2* knockout strain grown on CO may have a lag phase of 2-4 days and may produce about 0.1-4 g/L acetate. Growing a *CODH2* knockout strain on $CO_2 + H_2$ generally results in decreased lag phase and faster growth. For example, a *CODH2* knockout strain grown on $CO_2 + H_2$ may have a lag phase of 4 days.

0084 Growing a CODH/ACS overexpression strain on CO generally results in decreased lag phase, increased ethanol production, similar acetate production, and increased lactate production.

Fermentation

0085 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.

0086 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.

0087 The culture/fermentation should desirably be carried out under appropriate conditions for production of the target product. Typically, the culture/fermentation is performed under anaerobic conditions. Reaction conditions to consider include pressure (or partial pressure), temperature, gas flow rate, liquid flow rate, media pH, media redox potential, agitation rate (if using a continuous stirred tank reactor), inoculum level, maximum gas substrate concentrations to ensure that gas in the liquid phase does not become limiting, and maximum product concentrations to avoid product inhibition. In particular, the rate of introduction of the substrate may be controlled to ensure that the concentration of gas in the liquid phase does not become limiting, since products may be consumed by the culture under gas-limited conditions.

0088 Operating a bioreactor at elevated pressures allows for an increased rate of gas mass transfer from the gas phase to the liquid phase. Accordingly, it is generally preferable to perform the culture/fermentation at pressures higher than atmospheric pressure. Also, since a given gas conversion rate is, in part, a function of the substrate retention time and retention time dictates the required volume of a bioreactor, the use of pressurized systems can greatly reduce the volume of the bioreactor required and, consequently, the capital cost of the culture/fermentation equipment. This, in turn, means that the retention time, defined as the liquid volume in the bioreactor divided by the input gas flow rate, can be reduced when bioreactors are maintained at elevated pressure rather than atmospheric pressure. The optimum reaction conditions will depend partly on the particular microorganism used. However, in general, it is preferable to operate the fermentation at a pressure higher than atmospheric pressure. Also, since a given gas conversion rate is in part a function of substrate retention time and achieving a desired retention time in turn dictates the required volume of a bioreactor, the use of pressurized systems can greatly reduce the volume of the bioreactor required, and consequently the capital cost of the fermentation equipment.

0089 Target products may be separated or purified from a fermentation broth using any method or combination of methods known in the art, including, for example, fractional distillation, evaporation, pervaporation, gas stripping, phase separation, and extractive fermentation, including for example, liquid-liquid extraction. In certain embodiments, target products are recovered from the fermentation broth by continuously removing a portion of the broth from the bioreactor, separating microbial cells from the broth (conveniently by filtration), and recovering one or more target products from the broth. Alcohols and/or acetone may be recovered, for example, by distillation. Acids may be recovered, for

example, by adsorption on activated charcoal. Separated microbial cells are preferably returned to the bioreactor. The cell-free permeate remaining after target products have been removed is also preferably returned to the bioreactor. Additional nutrients (such as B vitamins) may be added to the cell-free permeate to replenish the medium before it is returned to the bioreactor.

EXAMPLES

0090 The following examples further illustrate the invention but, of course, should not be construed to limit its scope in any way.

Example 1

0091 This example describes group II intron-based insertional inactivation of CODH1 and CODH2 genes involved in carbon fixation in *C. autoethanogenum* DSM10061.

0092 *C. autoethanogenum* DSM10061 was obtained from the DSMZ, the German Collection of Microorganisms and Cell Cultures, Inhoffenstraße 7B, 38124 Braunschweig, Germany. *E. coli* conjugation strain CA434 was kindly provided by Professor Nigel Minton (University of Nottingham, UK).

0093 The genome of *C. autoethanogenum* DSM10061 encodes the carbon monoxide dehydrogenases (CODHs) CODH1 (SEQ ID NOs: 1 and 2) and CODH2 (SEQ ID NOs: 3 and 4). These CODHs were inactivated using ClosTron group II intron mediated gene disruption tool (Heap, *J Microbiol Meth*, 80: 49-55, 2010). The Perutka algorithm hosted on the ClosTron website was used to identify the group II intron target site between bases 600/601 and 528/529 on the sense strand of the CODH1 and CODH2 genes, respectively. The same algorithm was used to design the intron targeting regions for CODH1 (SEQ ID NO: 15) and CODH2 (SEQ ID NO: 16) which were commercially synthesized by DNA2.0 Inc. (CA) and delivered in pTML007C-E2 vector (HQ263410.1). The final vectors, pMTL007C-E2-CODH1-600!601s and pMTL007C-E2-CODH2-528!529s contained a retro-transposition-activated *ermB* marker (RAM) which confered resistance to antibiotic clarithromycin upon insertion into the target site.

0094 The pMTL007C-E2-CODH1-600!601s and pMTL007C-E2-CODH2-528!529s plasmids were introduced into *C. autoethanogenum* DSM10061 as described above and in WO 2012/053905. The transformation mixture was spotted on YTF agar media and incubated at 37 °C inside anaerobic workstation. After 24 hours, the cells were scraped and

resuspended in 500 µL PBS and spread on YTF agar media supplemented with 7.5 µg/mL thiamphenicol (Sigma). Transformants were selected using 7.5 µg/mL thiamphenicol. Colonies were observed after 3 days of incubation.

0095 Streaks of single colonies were made sequentially first on YTF media supplemented with 7.5 µg/mL thiamphenicol and 10 µg/mL trimethoprim followed by YTF media containing 6 µg/mL clarithromycin. > 8 colonies were randomly screened for group II insertion by PCR (Maxime PCR PreMix kit) using flanking oligonucleotides.

Primer name	Target gene	WT amplicon size (bp)	Mutant amplicon size (bp)
CODH1-601s-F	CODH1	377	2177
CODH1-601s-R			
CODH2-529s-F	CODH2	425	2225
CODH2-529s-R			
Univ-0027-F	16s rRNA	1600	Not applicable
Univ-1492-R			

0096 Amplification of clarithromycin-resistant colonies using flanking oligonucleotides and gel electrophoresis analysis showed the presence of the larger ClosTron band (> 2 kb) instead of the smaller wild-type band (< 520 bp), which indicated that the ClosTron group II intron had successfully inserted into the specified CODH sites (CODH1::CTermB-601s and CODH2::CTermB-529s). These amplicons were purified using QIAquick PCR purification kit (Qiagen) and sequence validated by Sanger sequencing (Source Bioscience, UK).

0097 As a final validation step, PCR-verified clones were subjected to Southern blot analysis to confirm single ClosTron insertion. Genomic DNA of the ClosTron mutants were isolated according to Bertram, *Arch Microbiol*, 151: 551-557, 1989 and then digested with restriction enzyme HindIII. Digests were subjected to Southern blot analysis using a random labelled DIG probe (Roche) and was performed according to the manufacturer's instructions. Oligonucleotides EBS2 (SEQ ID NO: 27) and Intron-SalI-R1 (SEQ ID NO: 28) were used to generate the probe, using plasmid pMTL007C-E2 as a template. The resulting probe hybridized to the group II intron. Southern blot analysis detected a single band per mutant clone, indicating single event of group II intron insertion into the genome of *C. autoethanogenum DSM10061*. These validated mutants were termed CODH1::CTermB-601s (or "CODH1 mutant") and CODH2::CTermB-529s (or "CODH2 mutant").

Example 2

0098 This example demonstrates the effect of inactivation of CODH1 in *C. autoethanogenum* DSM10061 cultured under CO conditions.

0099 The ability of CODH1 mutant to grow autotrophically with 100% CO was tested in triplicates of 250 mL serum bottles containing 50 mL PETC media pressurized with 30 psi CO. 0.5 OD600 equivalent of active culture was inoculated into each serum bottle and liquid phase samples were harvested for OD measurements at a wavelength of 600nm and metabolite analysis by HPLC.

0100 Analysis of metabolites were performed by HPLC using an Agilent 1100 Series HPLC system equipped with a RID operated at 35 °C (Refractive Index Detector) and an Alltech IOA-2000 Organic acid column (150 x 6.5 mm, particle size 5 μm) kept at 60 °C. Slightly acidified water was used (0.005 M H_2SO_4) as mobile phase with a flow rate of 0.7 ml/min. To remove proteins and other cell residues, 400 μl samples were mixed with 100 μl of a 2 % (w/v) 5-sulfosalicylic acid and centrifuged at 14,000 x g for 3 min to separate precipitated residues. 10 μl of the supernatant were then injected into the HPLC for analyses.

0101 As shown in Figs. 1A-1D, the CODH1 mutant exhibited favorable metabolite profiles in the form of enhanced ethanol at the expense of biomass (42% less) and acetate formation. The CODH1 mutant produced 64% more ethanol (Fig. 1B), 25% less acetate (Fig. 1C), and similar 2,3-butanediol (Fig. 1D) as WT.

0102 A similar pattern was also observed when the CODH1 mutant and WT were grown in steel mill gas comprising 51.24% CO, 31.22% N_2, 11.98% CO_2, and 3.05% H_2 from a steel mill in Glenbrook, New Zealand. The experiment was conducted in triplicates of 250 mL serum bottles containing 100 mL PETC media and pressurized to 30 psi with steel mill gas. In terms of growth on CO (in steel mill gas), the CODH1 mutant produced 113% more ethanol (Fig. 2B), again at the expense of biomass (17% less) (Fig. 2A) and acetate (18% less) (Fig. 2C) than WT.

Example 3

0103 This example demonstrates the effect of inactivation of CODH2 in *C. autoethanogenum* DSM10061 cultured under CO conditions.

0104 The ability of the CODH2 mutant to grow autotrophically in 100% CO was tested under the same conditions as the CODH1 mutant, described above. In comparison to WT,

the CODH2 mutant displayed lag phase reduction of 1 day while utilizing 100% CO as substrate (Fig. 1A). The early exponential phase of the CODH2 mutant occurred at day 3.8, in comparison to exponential phase of WT at day 4.8 (Fig. 1A). The CODH2 mutant produced 27% less acetate (Fig. 1C) and 27% less ethanol than WT (Fig. 1B). However, the peak 2,3-butanediol production of the CODH2 mutant was higher than WT (Fig. 1D).

Example 4

0105 This example demonstrates the effect of inactivation of CODH1 or CODH2 in *C. autoethanogenum* DSM10061 cultured under H_2 + CO_2 conditions.

0106 To test the ability of the CODH1 and CODH2 mutants to grow in hydrogen and carbon dioxide, WT and the CODH mutants were separately inoculated into 50mL PETC media (without fructose) in 250 mL serum bottles in triplicates, and the headspace was exchanged with 20 psi H_2 + 10 psi CO_2. The cultures were allowed to grow at 37 °C with agitation and samples were harvested for OD600 measurements and HPLC analysis.

0107 Under H_2 + CO_2 conditions, the CODH1 mutant displayed a markedly improved growth profile than WT. WT experienced a lag phase of 6 days before reaching a max OD600 of 0.184 on day 22.7, whereas the CODH1 mutant was able to grow without apparent lag phase and reached a max OD600 of 0.40 on day 1.6 (Fig. 3A). The CODH2 mutant displayed a shorter lag phase and faster growth than WT and reached a peak OD600 of 0.20 (Fig. 3A). HPLC analysis showed that very similar levels of acetate and ethanol were produced by the CODH1 mutant, the CODH2 mutant, and WT under H_2 + CO_2 conditions (Figs. 3B-5C).

Example 5

0108 This example describes the expected effect of combined inactivation of CODH1 and CODH2 in *C. autoethanogenum* DSM10061 cultured under CO conditions.

0109 Given the desirable metabolite profile of the CODH1 mutant under CO conditions and the reduced lag phase of the CODH1 and CODH2 mutants under both CO and H_2 + CO_2 conditions, the combined inactivation of CODH1 and CODH2 may result in a strain that has superior growth and metabolite profiles under autotrophic conditions. While not wishing to be bound by any particular theory, inactivation of these two CODHs may increase the availability of CO and/or CO_2 for reaction with the CODH/ACS and result in more efficient formation of acetyl-CoA.

0110 For example, allele-coupled exchange or ACE (Heap, *Nucl Acids Res*, 40: e59, 2012) may be used to generate a double CODH (i.e., CODH1 and CODH2) disruption. Using this technique, the *pyrE* gene (SEQ ID NO: 19) of *C. autoethanogenum* DSM10061 may be deleted so that *pyrE* can be used as a positive and negative selectable marker for later stages of genetic manipulation. Mutants with deleted *pyrE* are auxotrophic to uracil auxotrophic and resistant to pro-drug 5`-fluoroorotic acid. As a next step, a ClosTron plasmid targeting one of the CODH may be introduced into *pyrE* deletion mutant, and clarithromycin resistant colonies may be verified by PCR, sequencing, and Southern Blot. Once ClosTron inactivation of one CODH has been confirmed in this *pyrE* deletion mutant, an ACE deletion plasmid containing *pyrE* as a negative selectable marker may be introduced to delete the other CODH. As a final step, an ACE plasmid with the *pyrE* gene may be introduced to restore *pyrE* integrity, resulting in a combined CODH1 and CODH2 disruption mutant in a WT background with functional *pyrE* gene.

Example 6

0111 This example demonstrates the construction and introduction of CODH/ACS overexpression plasmid into *C. autoethanogenum* DSM10061.

0112 *C. autoethanogenum* DSM 10061 was obtained from the DSMZ, the German Collection of Microorganisms and Cell Cultures, Inhoffenstraße 7B, 38124 Braunschweig, Germany. *E. coli* strains DH5α-T1^R and XL1-Blue MRF' were purchased from Invitrogen and Stratagene, respectively.

0113 The DNA sequences of Wood-Ljungdahl promoter (P_{WL}) (SEQ ID NO: 18) and bi-functional carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) subunits AcsA (SEQ ID NO: 12) and AcsB (SEQ ID NO: 14), both from *C. autoethanogenum* DSM10061, were obtained from genome sequencing. The Wood-Ljungdahl cluster of *C. autoethanogenum* was found to be highly expressed under autotrophic conditions (Köpke, *Curr Opin Biotechnol*, 22: 320-325, 2011) so P_{WL} was used for expression of CODH/ACS.

0114 Genomic DNA from *C. autoethanogenum* DSM10061 was isolated using a modified method by Bertram, *Arch Microbiol*, 151: 551-557, 1989. A 100 ml overnight culture was harvested (6,000 x g, 15 min, 4 °C), washed with potassium phosphate buffer (10 mM, pH 7.5) and suspended in 1.9 ml STE buffer (50 mM Tris-HCl, 1 mM EDTA, 200 mM sucrose; pH 8.0). 300 µl lysozyme (~100,000 U) was added and the mixture was incubated at 37 °C for 30 min, followed by addition of 280 µl of a 10 % (w/v) SDS solution and another

incubation for 10 min. RNA was digested at room temperature by addition of 240 µl of an EDTA solution (0.5 M, pH 8), 20 µl Tris-HCl (1 M, pH 7.5), and 10 µl RNase A (Fermentas). Then, 100 µl Proteinase K (0.5 U) was added and proteolysis took place for 1-3 h at 37 °C. Finally, 600 µl of sodium perchlorate (5 M) was added, followed by a phenol-chloroform extraction and an isopropanol precipitation. DNA quantity and quality was inspected spectrophotometrically.

0115 The CODH/ACS gene and P_{WL} were amplified by PCR using Phusion High Fidelity DNA Polymerase (New England Biolabs). The amplified 573 bp P_{WL} was cloned into the *E. coli-Clostridium* shuttle vector pMTL 83151 (GenBank accession number FJ797647; Nigel Minton, University of Nottingham; Heap, *J Microbiol Meth*, 78: 79-85, 2009) using *Not*I and *Nde*I restriction sites and strain DH5α-T1^R (Invitrogen), resulting in plasmid pMTL83157. Since the coding sequence of CODH/ACS contains one internal *NdeI* site, splice overlapping (SOE) PCR (Warrens, *Gene*, 186: 29-35, 1997) was used to remove this *NdeI* site without alteration of the codon. Both the 1946bp PCR product of CODH/ACS and plasmid pMTL83157 were digested with *NdeI* and *SacI*, and ligated to produce plasmid pMTL83157-CODH/ACS (Fig. 4) (SEQ ID NO: 20).

0116 The insert of the expression plasmid pMTL83157-CODH/ACS was completely sequenced using oligonucleotides CODH/ACS-NdeI-F (SEQ ID NO: 31) and CODH/ACS-SacI-R (SEQ ID NO: 32). Sanger sequencing using primers primers CODH/ACS-NdeI-F and CODH/ACS-SacI-R confirmed that the internal *NdeI* site of CODH/ACS was successfully altered and free of mutations.

Target	Oligonucleotide
P_{WL}	P_{WL}-NotI-F
P_{WL}	P_{WL}-NdeI-R
CODH/ACS	CODH/ACS-NdeI-F
CODH/ACS	CODH/ACS-SacI-R
CODH/ACS	CODH/ACS-SOE-B
CODH/ACS	CODH/ACS-SOE-C

0117 The plasmids pMTL83157 and pMTL83157-CODH/ACS were introduced into *C. autoethanogenum* DSM10061 by conjugating with donor *E. coli* strain CA434 as donor. Donor strains were grown overnight in LB media supplemented with 25 µg/mL chloramphenicol and 100 µg/mL spectinomycin. Cells from 1.5 mL culture were harvested by centrifugation and washed in phosphate buffered saline (PBS). Inside an anaerobic

workstation, the donor cell pellet was resuspended in 200 µL of exponentially growing recipient *C. autoethanogenum* DSM10061. The conjugation mixture was spotted on YTF agar media and incubated at 37 °C inside an anaerobic workstation. After 24 hours, the cells were scrapped and resuspended in 500 µL PBS and spread on YTF agar media supplemented with 7.5 µg/mL thiamphenicol (Sigma) and 10 µg/mL trimethoprim (Sigma). *C. autoethanogenum* transconjugants were selected using 7.5 µg/mL thiamphenicol whereas *E. coli* CA434 strain was counter-selected using 10 µg/mL trimethoprim. Colonies were observed after 3 days of incubation and they were re-streaked onto the same selective agar media for purification.

0118 Likewise, the plasmid could be introduced into other carboxydotrophic acetogens, such as *C. ljungdahlii* or *C. ragsdalei*, using similar protocols.

0119 To check the identity of the transconjugants, the 16s rRNA was amplified and Sanger sequenced using oligonucleotides Univ-0027-F (SEQ ID NO: 25) and Univ-1492-R (SEQ ID NO: 26). Plasmid DNA was extracted from *C. autoethanogenum* transconjugants and transformed into *E. coli* XL1-Blue MRF' (Stratagene) before plasmid restriction digest analysis was carried out. This is commonly referred to as 'plasmid rescue' because plasmids isolated from *Clostridia* are not of sufficient quality for restriction digest analysis. Gel electrophoresis of *PmeI* and *FseI* restriction digested plasmids rescued from pMTL83157 transconjugants showed the presence of the expected fragments (2600 bp and 2424 bp). Gel electrophoresis of *NdeI* and *SacI* restriction digested plasmids rescued from pMTL83157-CODH/ACS transconjugants showed the presence of the expected fragments (4995 bp and 1932 bp).

Example 7

0120 This example demonstrates the effect of overexpression of CODH/ACS in *C. autoethanogenum* DSM10061 cultured under CO conditions.

0121 The effect of overexpression of CODH/ACS against a plasmid control (pMTL83157) was compared in batch growth experiments with CO as sole carbon and energy source. Under 100% CO, the CODH/ACS overexpression strain showed a reduction in lag phase of growth by 4.2 days, produced 21% more ethanol, and produced 2.7-fold higher lactate titres while generating similar amounts of acetate as the plasmid control (Figs. 5A-5E).

0122 Both strains were grown autotrophically in 100% CO and were tested in triplicates of 250 mL serum bottles containing 50 mL PETC media and pressurized with 30 psi CO. Thiamphenicol was supplemented to a final concentration 7.5 μg/mL. OD_{600} of 0.5 worth of active culture was inoculated into each serum bottle and liquid phase samples were harvested for OD measurements at a wavelength of 600nm and metabolite analysis by HPLC.

0123 Analysis of metabolites was performed using Varian ProStar HPLC system equipped with a RID (Refractive Index Detector) operated at 35 °C and a Biorad Aminex HPX-87H column (1300 x 7.8 mm, particle size 9 μm) kept at 35 °C. Slightly acidified water was used (0.005 M H_2SO_4) as mobile phase with a flow rate of 0.5 ml/min. To remove proteins and other cell residues, samples were centrifuged at 14000 rpm for 5 minutes and the supernatant was filtered with Spartan 13/0.2 RC filters. 20 μl of the supernatant was then injected into the HPLC for analyses.

Example 8

0124 This example describes the expected effect of overexpression of CODH/ACS in *C. ljungdahlii* cultured under CO conditions.

0125 The CODH/ACS overexpression plasmid described above may also be introduced into *C. ljungdahlii*. *C. ljungdahlii* may be grown on 100% CO. Under these conditions, the CODH/ACS overexpressing *C. ljungdahlii* should show reduced lag phase of growth while improving ethanol and lactate production by at least 20%.

Example 9

0126 This example describes the expected effect of overexpression of CODH/ACS in *C. autoethanogenum* cultured under CO_2 + H_2 conditions.

0127 The CODH/ACS overexpression strain and plasmid control strain of *C. autoethanogenum* may be grown on PETC-MES media with 80% CO_2 and 20% H_2 as sole sources of carbon and energy. Under these conditions, the CODH/ACS overexpressing *C. autoethanogenum* should show reduced lag phase of growth and increased ethanol and lactate production by at least 20%.

Example 10

0128 This example demonstrates inactivation of CODH/ACS in *C. autoethanogenum* DSM10061.

0129 The upstream CODH/ACS (CAETHG_1621) of *C. autoethanogenum* DSM10061 was inactivated using ClosTron group II intron mediated gene disruption tool (Heap, *J Microbiol Meth*, 80: 49-55, 2010). The Perutka algorithm hosted at ClosTron website was used to identify the group II intron target site between bases 142/143 on the sense strand of CAETHG_1621. The same algorithm was used to design the intron targeting region (SEQ ID NO: 17) which was commercially synthesized by DNA2.0 Inc. (CA) and delivered in pTML007C-E2 vector (GenBank Accession Number HQ263410.1). The final vector, pMTL007C-E2-CODH/ACS-142!143s, contained a retro-transposition-activated *ermB* marker (RAM) which conferred resistance to antibiotic clarithromycin upon insertion into the target site.

0130 The pMTL007C-E2-CODH/ACS-142!143s plasmid was conjugated into *C. autoethanogenum* DSM10061 as described above. *C. autoethanogenum* transconjugants were selected using 7.5 μg/mL thiamphenicol whereas *E. coli* CA434 strain was counter-selected using 10 μg/mL trimethoprim. Colonies were observed after 3 days of incubation. Streaks of single colonies were made sequentially first on YTF media supplemented with 7.5 μg/mL thiamphenicol and 10 μg/mL trimethoprim followed by YTF media containing 6 μg/mL clarithromycin. > 8 colonies were randomly screened for group II insertion by PCR (Maxime PCR PreMix kit) using flanking oligonucleotides.

Primer	Target gene	WT amplicon size (bp)	Mutant amplicon size (bp)
CODHACS-143s-F	CODH/ACS	517	2317
CODHACS-143s-R			

0131 Amplification of clarithromycin resistant colonies using flanking oligonucleotides and gel electrophoresis analysis showed the presence of the larger ClosTron band (> 2 kb) instead of the smaller wild-type band (< 520 bp), which indicated that the ClosTron group II intron successfully inserted into the specified CODH/ACS site. These amplicons were purified using QIAquick PCR purification kit (Qiagen) and sequence validated by Sanger sequencing (Source Bioscience, UK).

0132 As a final validation step, PCR-verified clones were subjected to Southern blot analysis to confirm single ClosTron insertion. Genomic DNA of the ClosTron mutants were isolated according to Bertram, *Arch Microbiol*, 151: 551-557, 1989 and then digested with restriction enzyme *Hind*III. Digests were subjected to Southern blot analysis using a random labelled DIG probe (Roche). Oligonucleotides EBS2 (SEQ ID NO: 27) and Intron-SalI-R1

(SEQ ID NO: 28) were used to generate the probe, using plasmid pMTL007C-E2 as a template. The resulting probe hybridized to the group II intron. Southern blot analysis detected a single band per mutant clones, indicating single event of group II intron insertion into the genome of *C. autoethanogenum* DSM10061. The validated mutant was termed CODH/ACS::CTermB-143s (or "CODH/ACS KO mutant"). For complementation assay, the overexpression plasmid pMTL83157-CODH/ACS was conjugated into the CODH/ACS KO mutant.

0133 Accordingly, CODH/ACS is required for autotrophic growth (CO or $H_2 + CO_2$) of *C. autoethanogenum*.

Example 11

0134 This example demonstrates the effect of inactivation of CODH/ACS in *C. autoethanogenum* DSM10061 grown on fructose.

0135 While *C. autoethanogenum* is unable to grow on CO (Fig. 6A) or CO_2 and H_2 (Fig. 6B) after inactivation of the CODH/ACS enzyme, the strain is still able to grow on sugars, such as fructose. Surprisingly, it was found that under these conditions, the CODH/ACS inactivated strain stops producing acetate. This is especially surprising as acetate formation is typically a hallmark feature of acetogens. During heterotrophic growth, acetogens typically fix CO_2 (produced during sugar metabolism) in the presence of H_2 into biomass and products via the actions of CODH/ACS and other genes from the Wood-Ljungdahl pathway, also known as the reductive acetyl-CoA pathway.

0136 The CODH/ACS inactivation mutant, the complemented strain, and WT *C. autoethanogenum* DSM10061 were grown in triplicates in 250mL serum bottles containing 50 mL of PETC media supplemented with 10 g/L fructose (final concentration) under N_2 atmosphere. 0.5 OD_{600} equivalent of active culture was inoculated into each serum bottle and liquid phase samples were harvested for OD measurements at a wavelength of 600nm and metabolite analysis by HPLC.

0137 Inactivation of CODH/ACS significantly reduced peak OD_{600} by 61% from WT level of 4.53 to 1.77 (Fig. 7A). This was also accompanied by an increase in growth lag phase in the CODH/ACS KO mutant (Fig. 7A). The complementation of CODH/ACS activity by plasmid expression of pMTL83157-CODH/ACS in KO mutant increased peak OD_{600} to 3.11 and also shortened the growth lag phase closer to WT level (Fig. 7A).

0138 One striking feature of CODH/ACS KO mutant is the lack of acetate production, as only 2.61 mM acetate was momentarily detected on day 2.8 (Fig. 7B). In contrast, the WT produced up to 85.96 mM acetate on day 3.0 (Fig. 7B).

0139 Without significant acetate production, most of the carbon from fructose was diverted towards reduced products ethanol and 2,3-butanediol in the CODH/ACS KO mutant. The inactivation of CODH/ACS increased peak ethanol levels by 113% from WT level of 48.3 mM to 102.7 mM (Fig. 7C). Furthermore, the peak 2,3-butanediol level of the CODH/ACS KO mutant were also 138% higher than WT (10.95 mM vs 4.61 mM) (Fig. 7D). The expression of complementation plasmid pMTL83157-CODH/ACS in the CODH/ACS KO mutant successfully restored acetate, ethanol, and 2,3-butanediol levels closer to WT levels (Fig. 7B-7D), confirming the role of CODH/ACS in *C. autoethanogenum* during heterotrophic growth.

0140 Without wishing to be bound to any particular theory, it appears that CODH/ACS inactivation prevents the Wood-Ljungdahl pathway from serving as a sink for reducing equivalents generated during glycolysis so that excessive reducing equivalents generate driving force for ethanol and 2,3-butanediol production (Fig. 8).

DESCRIPTION OF THE SEQUENCES

0141 The nucleic acid and amino acid sequences referenced herein are briefly summarized as follows.

SEQ ID NO:	Sequence type	Description	Species
1	amino acid	CODH1	*Clostridium autoethanogenum*
2	nucleic acid	CODH1	*Clostridium autoethanogenum*
3	amino acid	CODH2	*Clostridium autoethanogenum*
4	nucleic acid	CODH2	*Clostridium autoethanogenum*
5	amino acid	CODH1	*Clostridium ragsdalei*
6	nucleic acid	CODH1	*Clostridium ragsdalei*
7	amino acid	CODH2	*Clostridium ragsdalei*
8	nucleic acid	CODH2	*Clostridium ragsdalei*
9	amino acid	CODH2	*Clostridium scatologenes*
10	nucleic acid	CODH2	*Clostridium scatologenes*
11	amino acid	AcsA1	*Clostridium autoethanogenum*
12	nucleic acid	AcsA1	*Clostridium autoethanogenum*
13	amino acid	AcsA2	*Clostridium autoethanogenum*
14	nucleic acid	AcsA2	*Clostridium autoethanogenum*

15	nucleic acid	Intron targeting region for *Clostridium autoethanogenum* CODH1	Synthetic
16	nucleic acid	Intron targeting region for *Clostridium autoethanogenum* CODH2	Synthetic
17	nucleic acid	Intron targeting region for *Clostridium autoethanogenum* CODH/ACS	Synthetic
18	nucleic acid	Promotor region of Wood-Ljungdahl cluster	*Clostridium autoethanogenum*
19	nucleic acid	pyrE	*Clostridium autoethanogenum*
20	nucleic acid	pMTL83157-CODH/ACS	Synthetic
21	nucleic acid	CODH1-601s-F	Synthetic
22	nucleic acid	CODH1-601s-R	Synthetic
23	nucleic acid	CODH2-529s-F	Synthetic
24	nucleic acid	CODH2-529s-R	Synthetic
25	nucleic acid	Univ-0027-F	Synthetic
26	nucleic acid	Univ-1492-R	Synthetic
27	nucleic acid	EBS2	Synthetic
28	nucleic acid	Intron-SalI-R1	Synthetic
29	nucleic acid	P_{WL}-NotI-F	Synthetic
30	nucleic acid	P_{WL}-NdeI-R	Synthetic
31	nucleic acid	CODH/ACS-NdeI-F	Synthetic
32	nucleic acid	CODH/ACS-SacI-R	Synthetic
33	nucleic acid	CODH/ACS-SOE-B	Synthetic
34	nucleic acid	CODH/ACS-SOE-C	Synthetic
35	nucleic acid	CODHACS-143s-F	Synthetic
36	nucleic acid	CODHACS-143s-R	Synthetic

0142 All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein. The reference to any prior art in this specification is not, and should not be taken as, an acknowledgement that that prior art forms part of the common general knowledge in the field of endeavour in any country.

0143 The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (i.e., meaning "including, but not limited to") unless otherwise noted. Recitation of ranges of values herein are merely

intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

0144 Preferred embodiments of this invention are described herein. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

CLAIMS

1. A genetically engineered carboxydotrophic acetogenic bacterium having decreased or eliminated activity of CODH1 and/or CODH2 compared to a parental bacterium.

2. The bacterium of claim 1, wherein the bacterium comprises at least one disruptive mutation in a *CODH1* gene and/or *CODH2* gene.

3. The bacterium of claim 2, wherein the disruptive mutation decreases or eliminates expression of the *CODH1* gene and/or the *CODH2* gene compared to a parental bacterium.

4. The bacterium of claim 2, wherein the disruptive mutation is a knockout mutation.

5. The bacterium of claim 1, wherein the bacterium additionally has increased activity of CODH/ACS compared to the parental bacterium.

6. The bacterium of claim 5, wherein the bacterium overexpresses a *CODH/ACS* gene compared to the parental bacterium.

7. The bacterium of claim 1, wherein the bacterium produces one or more of ethanol and 2,3-butanediol.

8. The bacterium of claim 1, wherein the bacterium produces a higher amount of ethanol, produces a lower amount of acetate, has a shorter lag phase, and/or has a higher growth rate compared to the parental bacterium.

9. The bacterium of claim 1, wherein the bacterium consumes a gaseous substrate comprising one or more of CO, CO_2, and H_2.

10. The bacterium of claim 1, wherein the parental bacterium is *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*.

11. A method for producing a product, comprising culturing the bacterium of claim 1 in the presence of a gaseous substrate comprising one or more of CO, CO_2, and H_2, whereby the bacterium produces a product.

12. The method of claim 11, wherein the bacterium comprises at least one disruptive mutation in a *CODH1* gene and/or *CODH2* gene.

13. The method of claim 12, wherein the disruptive mutation decreased or eliminates expression of the *CODH1* gene and/or the *CODH2* gene compared to a parental bacterium.

14. The method of claim 12, wherein the disruptive mutation is a knockout mutation.

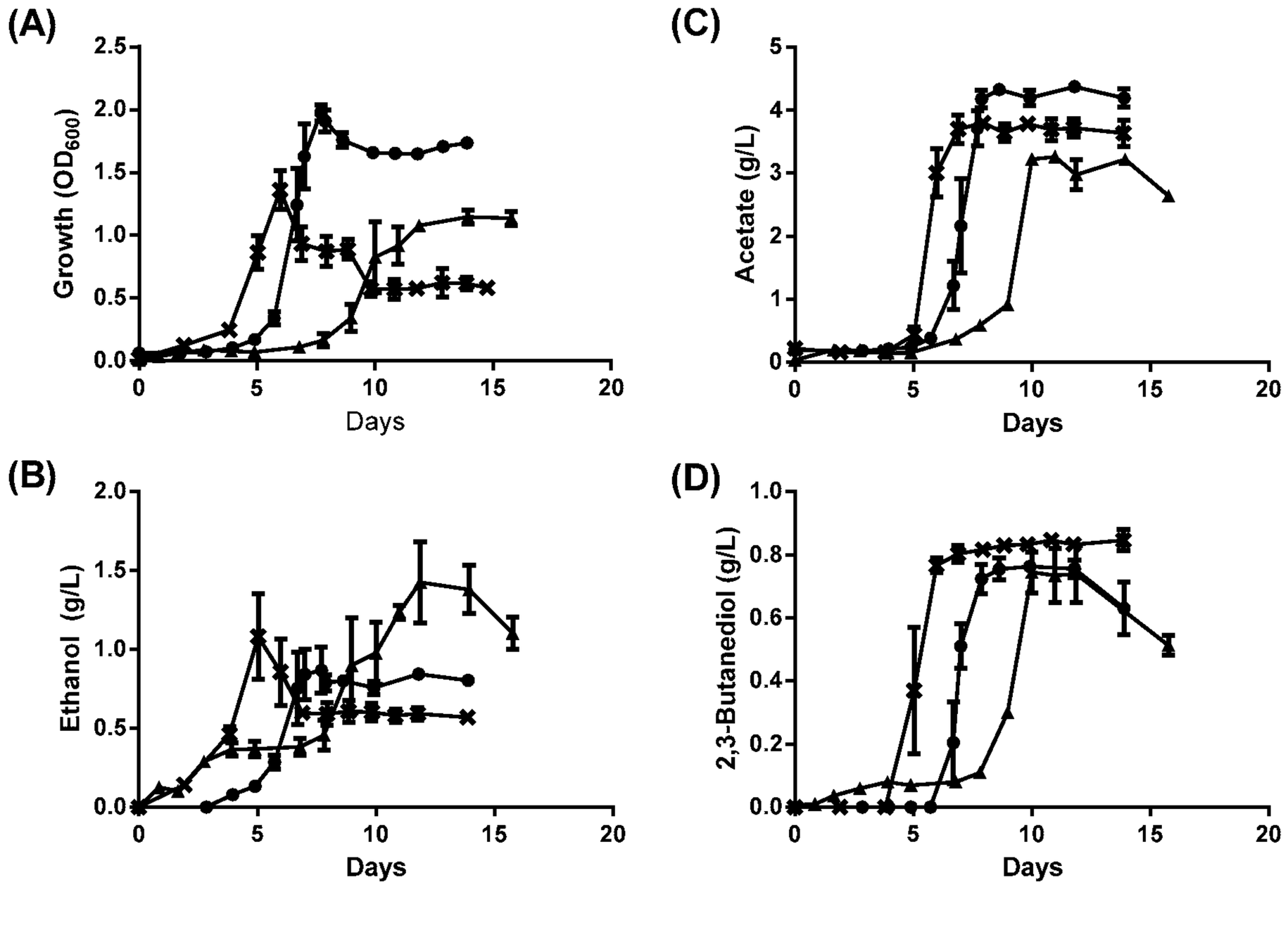
15. The method of claim 11, wherein the bacterium additionally has increased activity of CODH/ACS compared to the parental bacterium.

16. The method of claim 15, wherein the bacterium overexpresses a *CODH/ACS* gene compared to the parental bacterium.

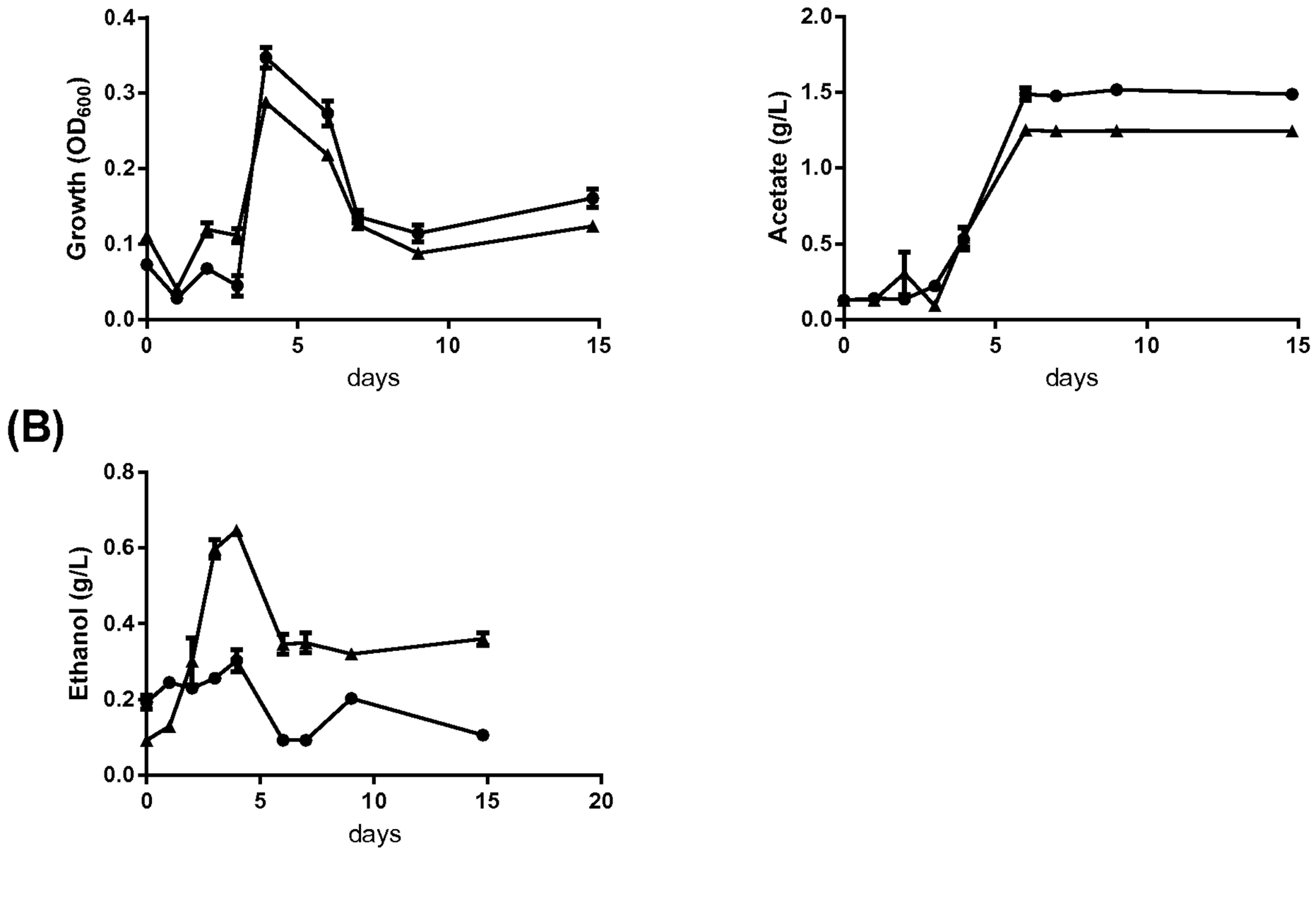
17. The method of claim 11, wherein the product comprises one or more of ethanol and 2,3-butanediol.

18. The method of claim 11, wherein the bacterium produces a higher amount of ethanol, produces a lower amount of acetate, has a shorter lag phase, and/or has a higher growth rate compared to the parental bacterium.

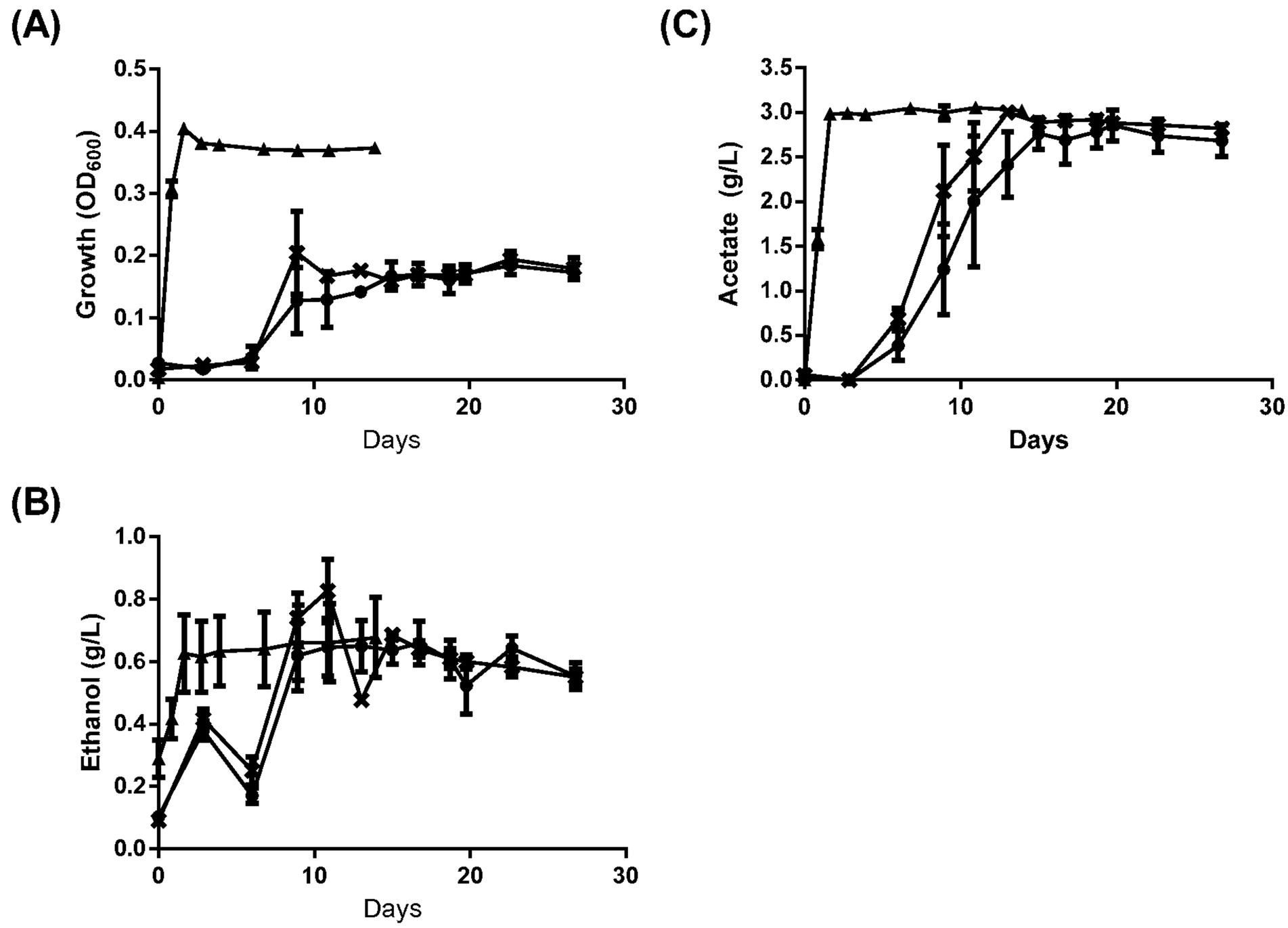
19. The method of claim 11, wherein the parental bacterium is *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*.


(A)
Growth (OD_{600})
Days
(B)
Ethanol (g/L)
Days
(C)
Acetate (g/L)
Days
(D)
2,3-Butanediol (g/L)
Days

Figs. 1A-1D


(A)
Growth (OD_{600})
0.4
0.3
0.2
0.1
0.0
0
5
10
15
days
(C)
Acetate (g/L)
2.0
1.5
1.0
0.5
0.0
0
5
10
15
days
(B)
Ethanol (g/L)
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
days

Figs. 2A-2C



Figs. 3A-3C

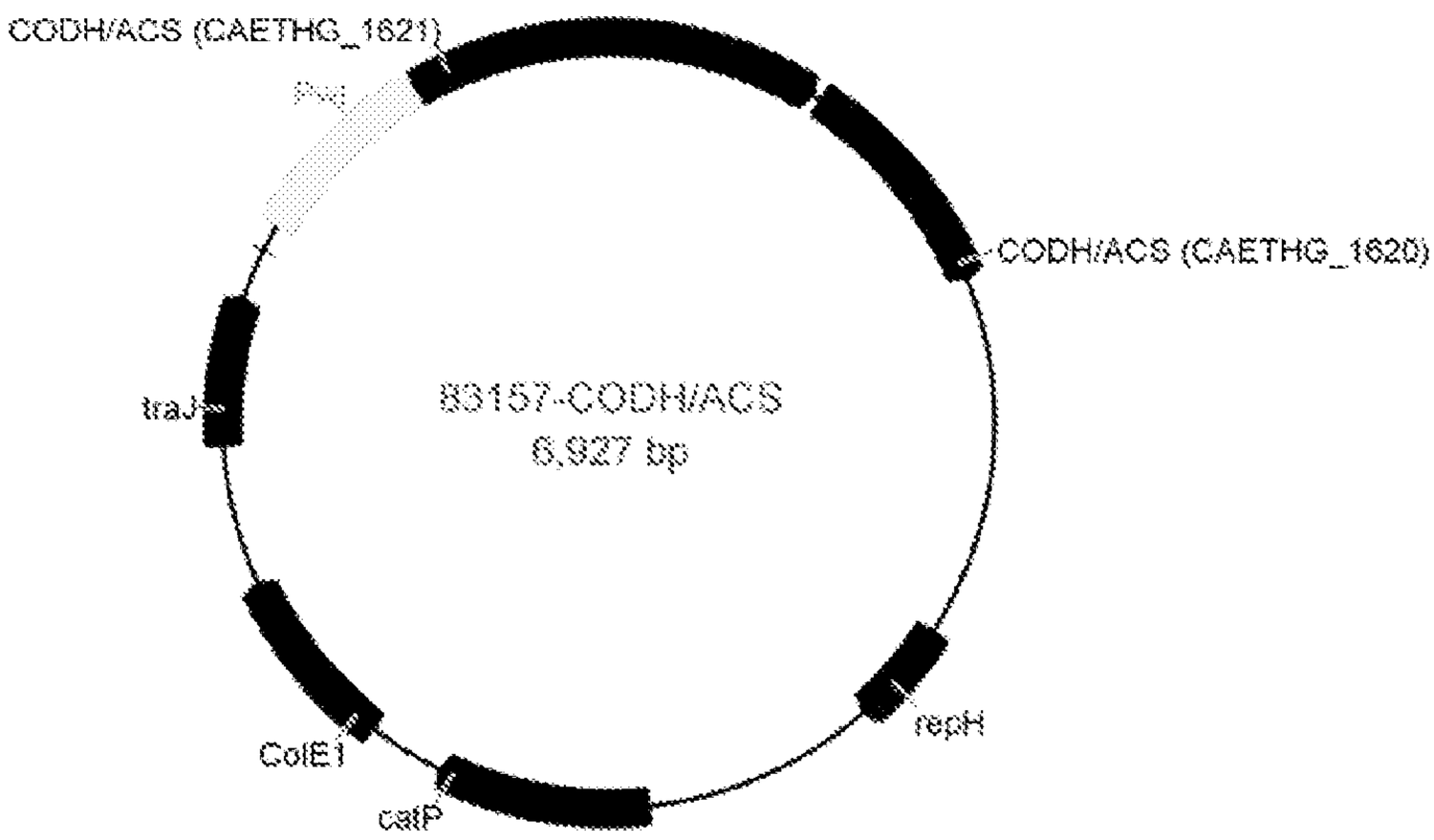
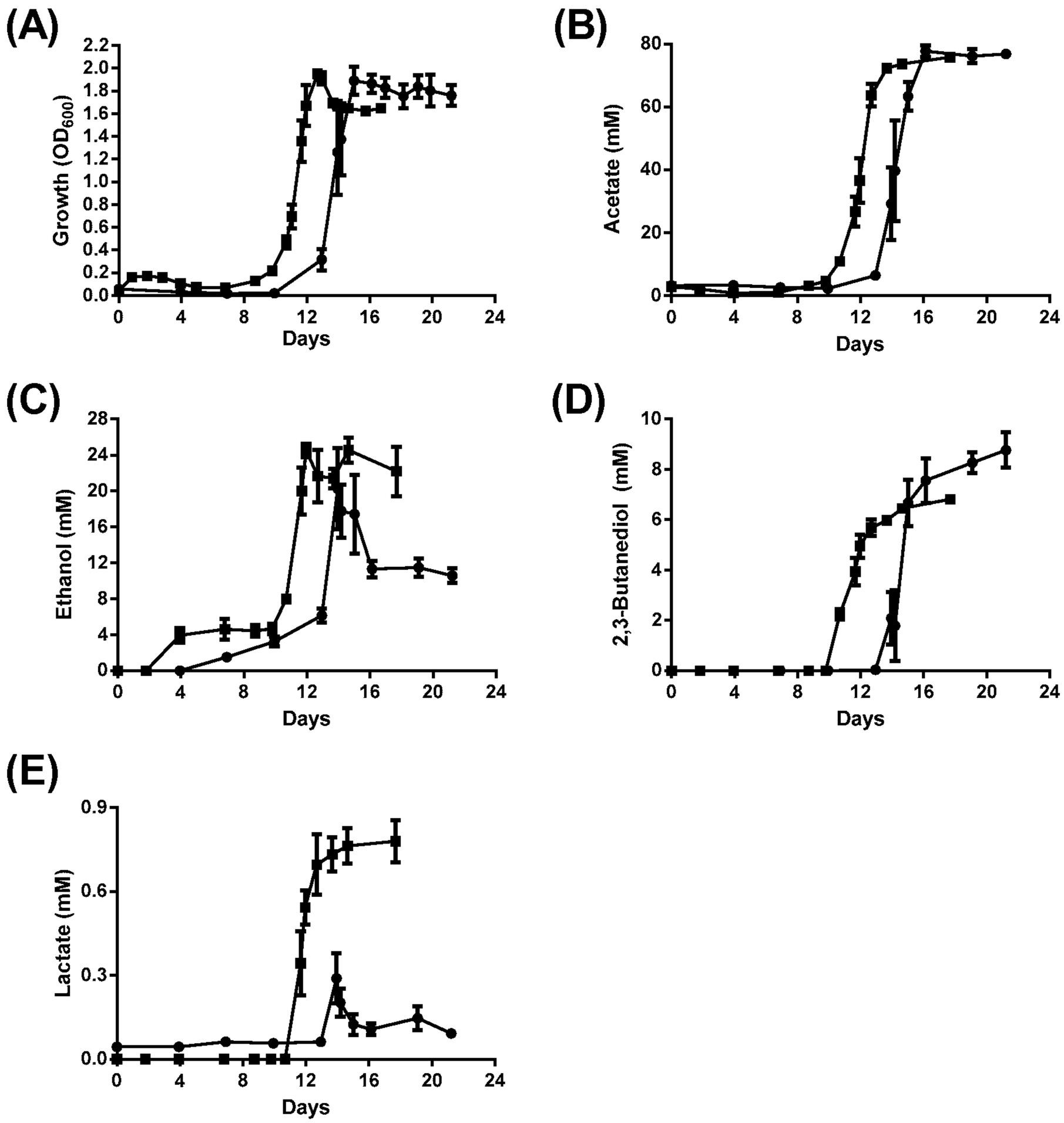
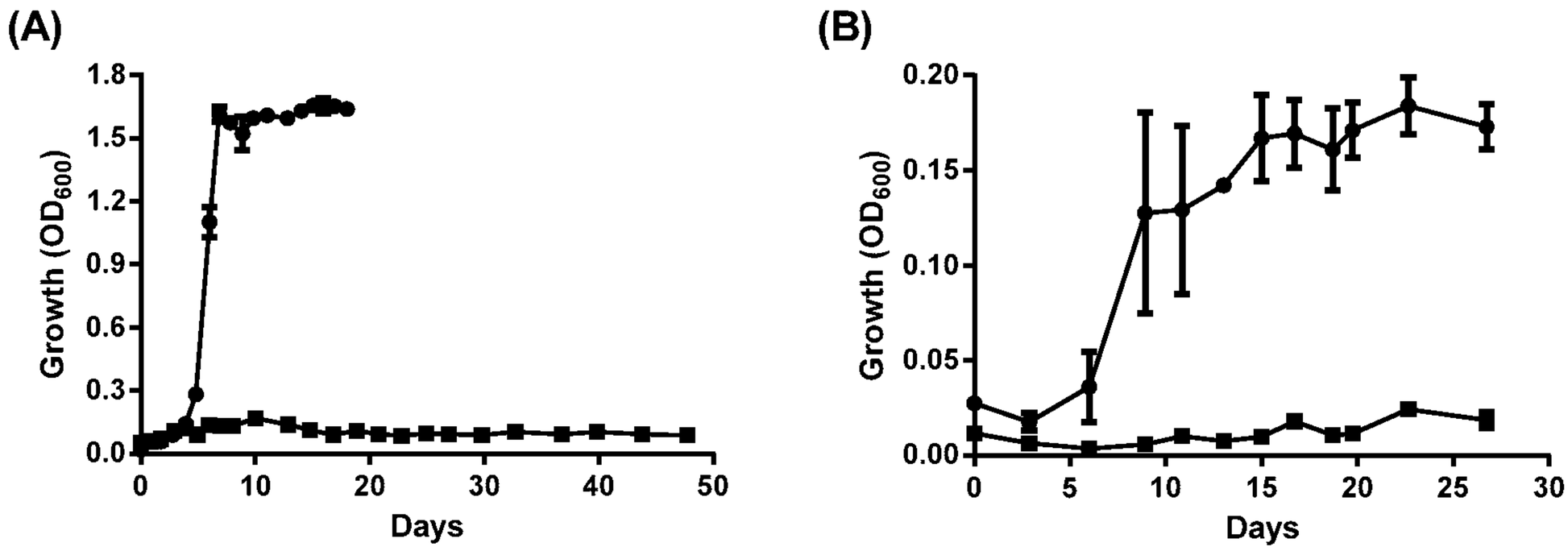


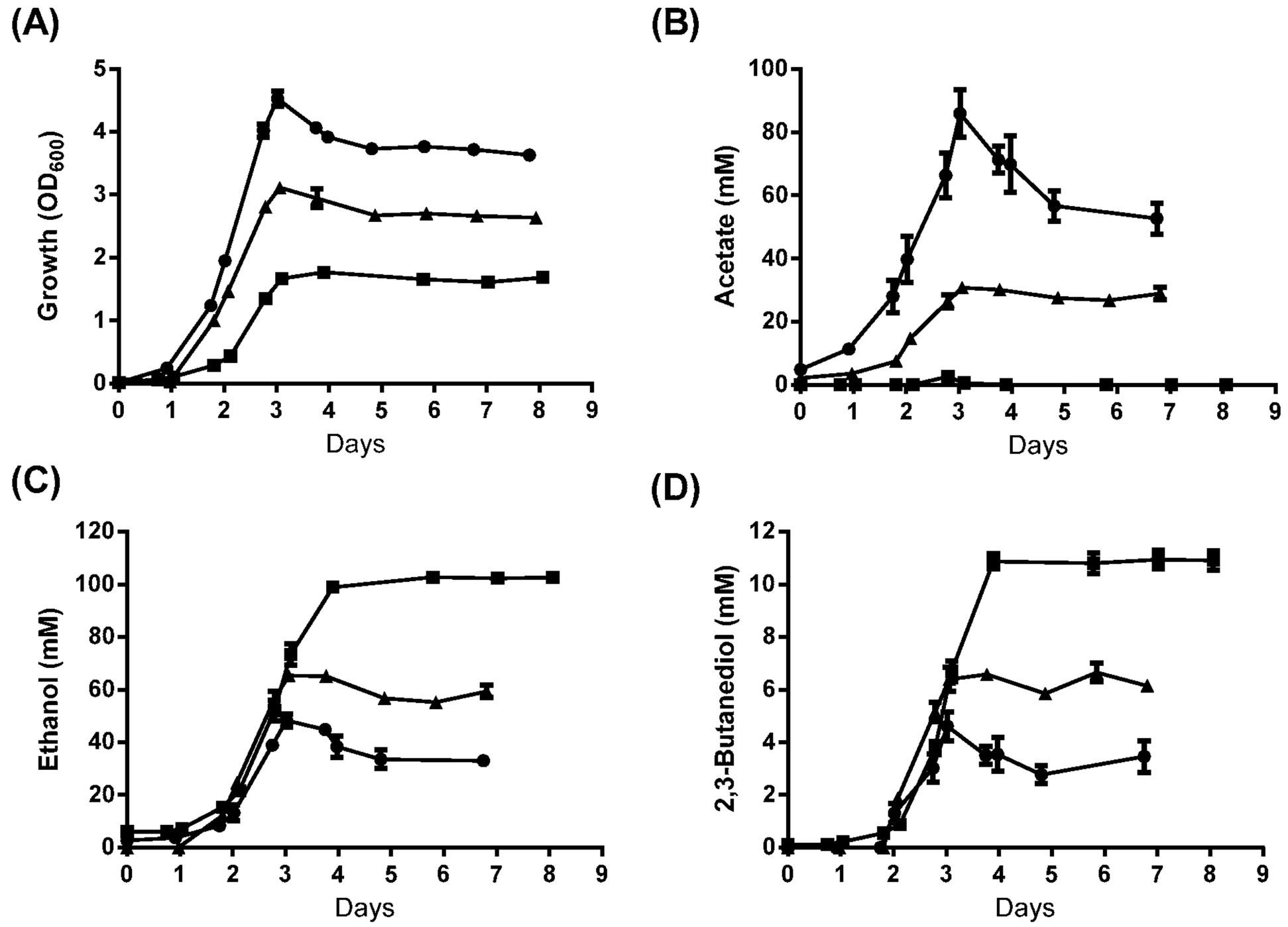
Fig. 4


(A)
Growth (OD_{600})
Days
(B)
Acetate (mM)
Days
(C)
Ethanol (mM)
Days
(D)
2,3-Butanediol (mM)
Days
(E)
Lactate (mM)
Days

Figs. 5A-5E



Figs. 6A-6B



Figs. 7A-7D

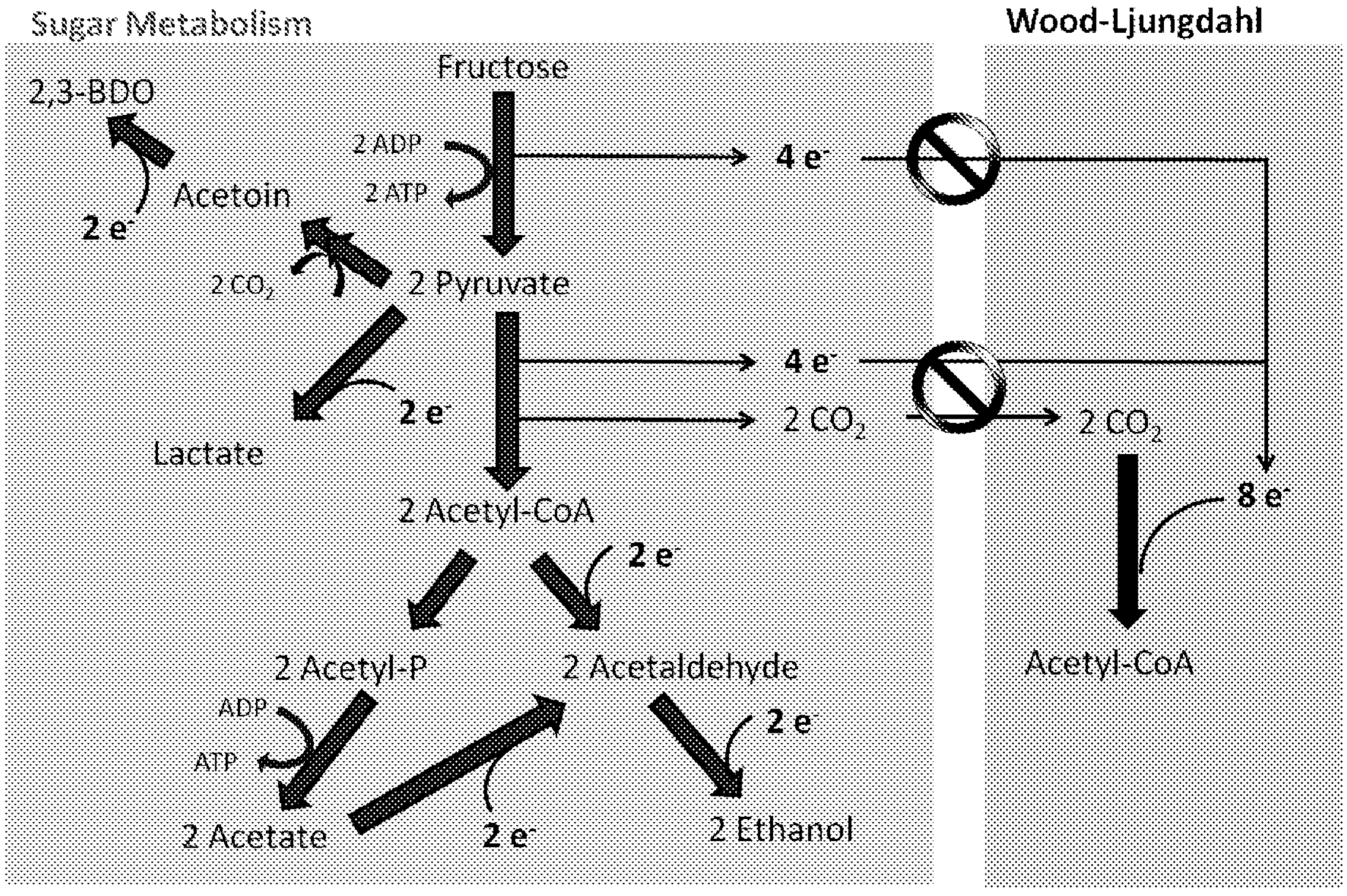
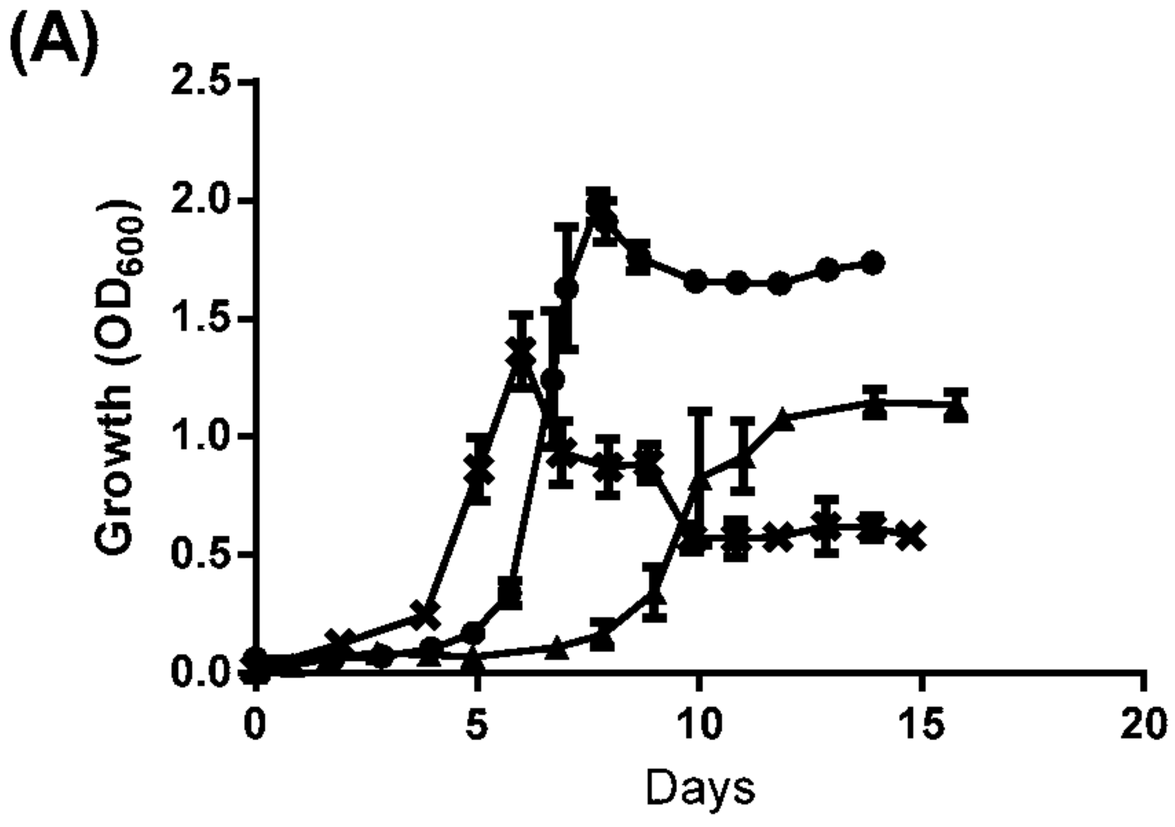
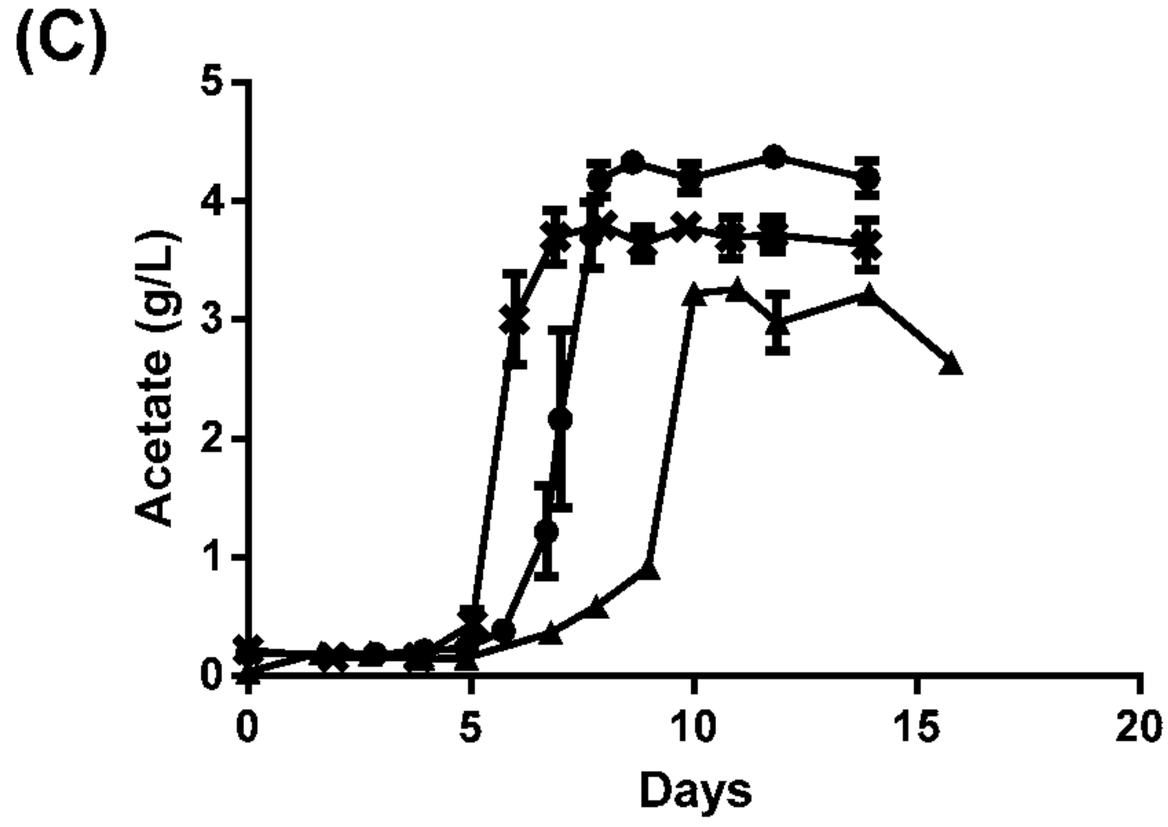
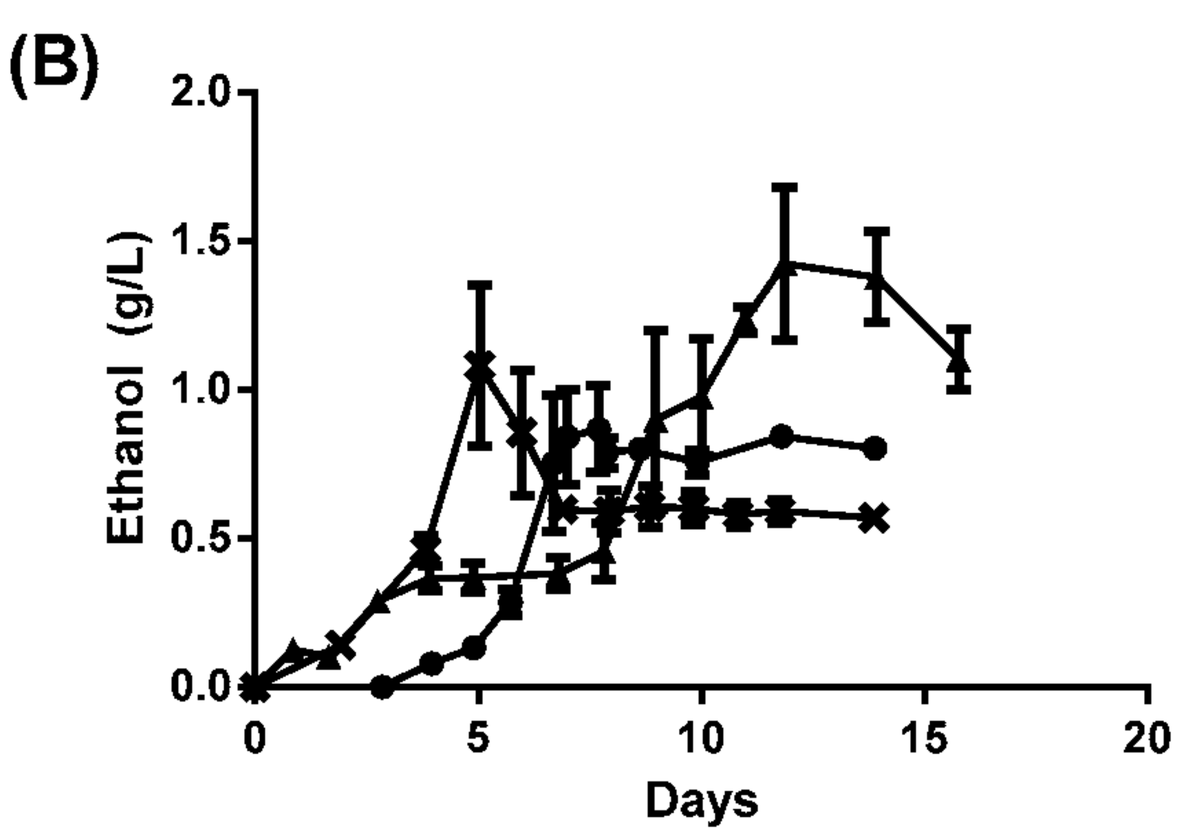
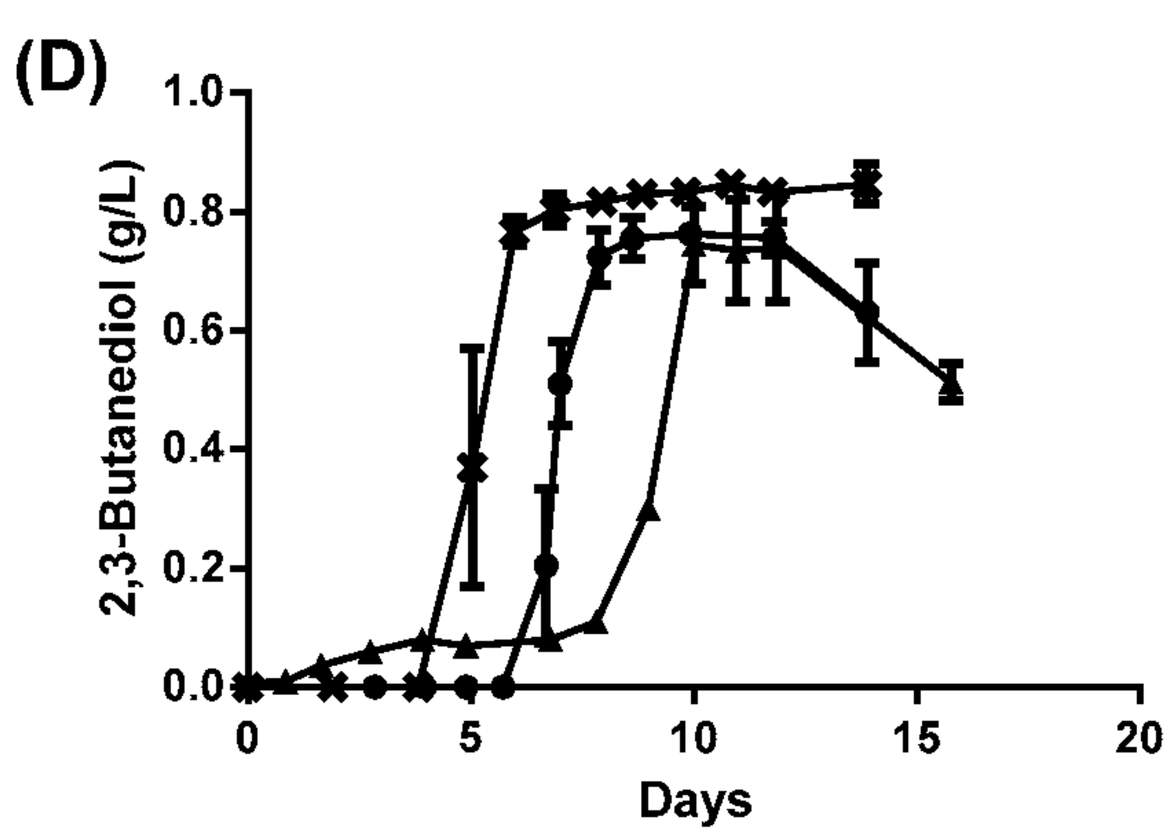

Sugar Metabolism
Wood-Ljungdahl
Fructose
2,3-BDO
2 ADP
2 ATP
4 e-
Acetoin
2 e-
2 CO2
2 Pyruvate
2 e-
4 e-
2 CO2
2 CO2
Lactate
2 Acetyl-CoA
8 e-
2 e-
2 Acetyl-P
2 Acetaldehyde
Acetyl-CoA
ADP
ATP
2 e-
2 Acetate
2 e-
2 Ethanol

Fig. 8


(A)
Growth (OD_{600})
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
Days


(C)
Acetate (g/L)
5
4
3
2
1
0
0
5
10
15
20
Days


(B)
Ethanol (g/L)
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
Days


(D)
2,3-Butanediol (g/L)
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
Days

Figs. 1A-1D