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(54) Titre : BACTERIE GENETIQUEMENT MODIFIEE COMPRENANT UNE VOIE DE FERMENTATION A PRODUCTION D'ENERGIE

(54) Title: GENETICALLY ENGINEERED BACTERIUM COMPRISING ENERGY-GENERATING FERMENTATION PATHWAY

(57) Abrégé/Abstract:
The invention relates to a genetically engineered bacterium comprising an energy-generating fermentation pathway and methods related thereto. In particular, the invention provides a bacterium comprising a phosphate butyryltransferase (Ptb) and a butyrate kinase (Buk) (Ptb-Buk) that act on non-native substrates to produce a wide variety of products and intermediates. In certain embodiments, the invention relates to the introduction of Ptb-Buk into a C1-fixing microoorgansim capable of producing products from a gaseous substrate.

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(54) Title: GENETICALLY ENGINEERED BACTERIUM COMPRISING ENERGY-GENERATING FERMENTATION PATHWAY

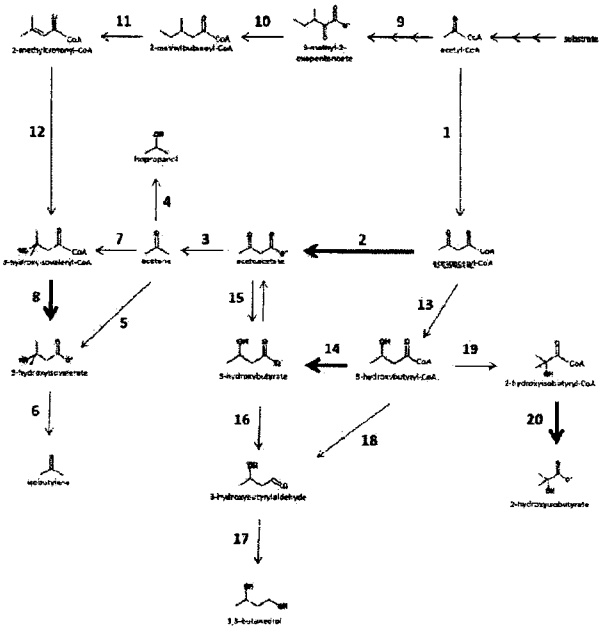

FIG. 1

(57) Abstract: The invention relates to a genetically engineered bacterium comprising an energy-generating fermentation pathway and methods related thereto. In particular, the invention provides a bacterium comprising a phosphate butyryltransferase (Ptb) and a butyrate kinase (Buk) (Ptb-Buk) that act on non-native substrates to produce a wide variety of products and intermediates. In certain embodiments, the invention relates to the introduction of Ptb-Buk into a C1-fixing microorganism capable of producing products from a gaseous substrate.

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GENETICALLY ENGINEERED BACTERIUM COMPRISING ENERGY-GENERATING FERMENTATION PATHWAY

BACKGROUND OF THE INVENTION

0002 With recent advances in fermentation and metabolic engineering, fermentation routes to various products have been identified and developed (Clomburg, *Appl Microbiol Biotechnol*, 86: 419-434, 2010; Peralta-Yahya, *Biotechnol J*, 5: 147-162, 2010; Cho, *Biotechnol Adv*, pii: S0734-9750(14)00181-5, 2014. However, all of these fermentation routes are energy (ATP)-consuming or, at best, energy (ATP)-neutral, which restricts product yield in energy-limited systems and uncouples product production from microorganism growth. The present invention provides energy (ATP)-generating pathways that overcome these limitations by providing novel fermentation routes and pathways to a variety of products, including acids, alkenes, aldehydes, alcohols, and diols. These pathways are directly coupled to microorganism growth and offer high product yields.

0003 In particular, the invention relates to fermentation pathways involving Ptb-Buk. Phosphate butyryltransferase (Ptb) (EC 2.3.1.19) natively catalyzes the reaction of butanoyl-CoA and phosphate to form CoA and butanoyl phosphate. Butyrate kinase (Buk) (EC 2.7.2.7) natively catalyzes the reaction of butanoyl phosphate and ADP to form butyrate (butanoate) and ATP. Accordingly, these enzymes together (Ptb-Buk) natively catalyze the conversion of butanoyl-CoA to butyrate and generate one ATP via substrate level phosphorylation (SLP).

0004 The inventors have discovered that Ptb is promiscuous and is capable of accepting a variety of acyl-CoAs and enoyl-CoAs as substrates, such that Ptb-Buk may be used to convert a number of acyl-CoAs and enoyl-CoAs to their corresponding acids or alkenates, respectively, while simultaneously generating ATP via substrate level phosphorylation.

0005 Furthermore, in combination with an aldehyde:ferredoxin oxidoreductase (AOR) and an alcohol dehydrogenase, acids formed via the Ptb-Buk system can be further converted to their respective aldehydes, alcohols, or diols. AOR (EC 1.2.7.5) catalyzes the reaction of an acid and reduced ferredoxin (which can, for example, be generated from oxidation of CO or hydrogen) to form an aldehyde and oxidized ferredoxin. Alcohol dehydrogenase (EC 1.1.1.1 and EC 1.1.1.2) can convert an aldehyde and NAD(P)H to an alcohol and NAD(P).

0006 Introduction of Ptb-Buk and/or AOR into a heterologous species, therefore, provides a novel, alternate route to the formation of native and non-native products, such as as acids, alkenes, ketones, aldehydes, alcohols, and diols at high yields, thus overcoming limitations of the current state of the art.

SUMMARY OF THE INVENTION

0007 The invention provides a genetically engineered bacterium comprising exogenous phosphate butyryltransferase (Ptb) and exogenous butyrate kinase (Buk) (Ptb-Buk). Generally, the Ptb-Buk acts on a non-native substrate, e.g., a substrate other than butanoyl-CoA and/or butanoyl phosphate, and produces a non-native product, e.g., a product other than butanoyl phosphate or butyrate. In certain embodiments, the Ptb-Buk converts acetoacetyl-CoA to acetoacetate, 3-hydroxyisovaleryl-CoA to 3-hydroxyisovalerate, 3-hydroxybutyryl-CoA to 3-hydroxybutyrate, or 2-hydroxyisobutyryl-CoA to 2-hydroxyisobutyrate.

0008 The bacterium may produce one or more of an acid, an alkene, a ketone, an aldehyde, an alcohol, or a diol. More specifically, the bacterium may produce one or more of acetone or a precursor thereof, isopropanol or a precursor thereof, isobutylene or a precursor thereof, 3-hydroxybutyrate or a precursor thereof, 1,3-butanediol or a precursor thereof, 2-hydroxyisobutyrate or a precursor thereof, adipic acid or a precursor thereof, 1,3-hexanediol or a precursor thereof, 3-methyl-2-butanol or a precursor thereof, 2-buten-1-ol or a precursor thereof, isovalerate or a precursor thereof, or isoamyl alcohol or a precursor thereof. The bacterium does not typically produce butanol.

0009 The bacterium may further comprise a disruptive mutation in a phosphotransacetylase (Pta) and an acetate kinase (Ack). The bacterium may further comprise a disruptive mutation in a thioesterase. In another embodiment, the invention provides a genetically engineered bacterium comprising exogenous Ptb-Buk and exogenous or endogenous aldehyde:ferredoxin oxidoreductase.

0010 The invention further provides a method of producing a product comprising culturing the bacterium of any of the aforementioned embodiments in the presence of a substrate. The product may be, for example, acetone or a precursor thereof, isopropanol or a precursor thereof, isobutylene or a precursor thereof, 3-hydroxybutyrate or a precursor thereof, 1,3-butanediol or a precursor thereof, 2-hydroxyisobutyrate or a precursor thereof, adipic acid or a precursor thereof, 1,3-hexanediol or a precursor thereof, 3-methyl-2-butanol or a precursor thereof, 2-buten-1-ol or a precursor thereof, isovalerate or a precursor thereof, or isoamyl alcohol or a precursor thereof. Typically, the substrate is a gaseous substrate comprising, for example, one or more of CO, CO_2, and H_2. In one embodiment, the gaseous substrate is syngas. In another embodiment, the gaseous substrate is an industrial waste gas.

BRIEF DESCRIPTION OF THE DRAWINGS

0011 Fig. 1 is a diagram of metabolic pathways for the production of various products, including acetone, isopropanol, isobutylene, 3-hydroxybutyrate, 1,3-butanediol, and 2-hydroxyisobutyrate from acetyl-CoA. Acetyl-CoA may be generated from any suitable substrate, such as a carbohydrate (e.g., sugar) substrate or a gaseous substrate. In the present invention, acetyl-CoA is often generated from a gaseous substrate. Bold arrows indicate steps that may be catalyzed by Ptb-Buk.

0012 Fig. 2 is a diagram showing the reactions natively catalyzed by Ptb-Buk, namely the conversion of butanoyl-CoA to butyrate and the generation of one ATP.

0013 Fig. 3 is a diagram comparing the activities of CoA-transferase, thioesterase, and Ptb-Buk.

0014 Fig. 4 is a graph showing average acetone production in *E. coli* BL21 (D3) modified with plasmids comprising exogenous genes. This data demonstrates the ability of Ptb-Buk to convert acetoacetyl-CoA to acetoacetate in *E. coli in vivo*.

0015 Fig. 5 is a graph showing the effect of induction of *E. coli* BL21 (DE3) carrying both the pACYC-ptb-buk and pCOLA-thlA-adc plamids (expressing thiolase, Ptb-Buk, and acetoacate decarboxylase).

0016 Fig. 6 is a diagram of a pathway designed to use Ptb-Buk for acetone production, while recycling the reducing equivalents produced in the production of (R)-3-hydroxybutyryl-CoA and the ATP generated by Ptb-Buk.

0017 Fig. 7 is a diagram showing the role of aldehyde:ferredoxin oxidoreductase (AOR), ferredoxin, and Adh in the production of 1,3-butanediol in *C. autoethanogenum*. More generally, AOR may be used to catalyze the conversion of an acid to an aldehyde and Adh may be used to catalyze the conversion of the aldehyde to an alcohol/diol.

0018 Fig. 8 is a diagram showing the stereospecificity of Ptb-Buk for the production of (R)-3-hydroxybutyrate and 2-hydroxyisobutyrate. The term "native" in Fig. 8 refers to native thioesterase.

0019 Fig. 9 is a diagram showing the production of isobutene via Ptb-Buk conversion of 3-hydroxyisovaleryl-CoA and 3-hydroxyisovalerate using alternative pathway 1.

0020 Fig. 10 is a diagram showing the production of isobutene via Ptb-Buk conversion of 3-hydroxyisovaleryl-CoA and 3-hydroxyisovalerate using alternative pathway 2.

0021 Fig. 11 is a diagram showing the production of 1,3-butanediol via 3-butyraldehyde dehydrogenase (Bld).

0022 Fig. 12 is a graph showing isopropanol production in *C. autoethanogenum* using the Ptb-Buk system over a control. ○ pMTL85147-thlA-adc, ● pMTL85147-thlA-ptb-buk-adc.

0023 Figs. 13A-F are graphs showing production of 3-hydroxybutyrate, acetate, ethanol, and acetone with modular plasmids in *E. coli* with different concentrations of inducer IPTG (0, 50, 100 μM). Fig. 13A: pACYC-ptb-buk, pCOLA-thlA-adc, pCDF-phaB. Fig. 13B: pACYC-ptb-buk, pCOLA-thlA-adc, pCDF-phaB-bdh1. Fig. 13C: pCOLA-thlA-adc, pCDF-phaB-bdh1. Fig. 13D: pCOLA-thlA-adc. Fig. 13E: pCDF-phaB-bdh1. Fig. 13F: pCDF-phaB.

0024 Fig. 14 is a plasmid map of plasmid pMTL8225-budA::thlA-phaB.

0025 Fig. 15 is a gel image of PCR verification of replacement of acetolactate synthase (*budA*) genes with thiolase (*thlA*) and 3-hydroxybutyryl-CoA dehydrogenase (*phaB*) genes in

C. autoethanogenum for 4 clones (1, 4, 7, 9) compared to wild-type (W). All clones are positive as seen by a larger PCR fragment size compared to wild-type.

0026 Fig. 16 is a graph showing fermentation profile of a batch fermentation *C. autoethanogenum* budA::thlAphaB strain and demonstrating 3-hydroxybutyrate and 1,3-butanediol formation from gas.

0027 Fig. 17A is a graph showing production of 1,3-BDO via thiolase, 3-hydroxybutyryl-CoA dehydrogenase (Bld), and butyraldehyde dehydrogenase. Fig. 17B is a graph showing the impact of *bld* expression on growth.

0028 Fig. 18A is a graph showing the formation of 3-hydroxybutyrate and 1,3-butanediol from gaseous substrate in *C. autoethanogenum* pMTL8315-Pfdx-hbd1-thlA. Fig. 18B is a graph showing the reduction of acetate to ethanol in the same culture.

0029 Fig. 19 is a graph showing the fermentation profile for strain *C. autoethanogenum* pMTL8315-Pfdx-hbd1-thlA demonstrating formation of 3-hydroxybutyrate and 1,3-butanediol from gaseous substrate in continuous culture (where indicated, media was replenished continuously with given dilution rate D).

0030 Fig. 20A and Fig. 20B are graphs showing increased CoA hydrolysis activity on a range of acyl-CoAs (acetoacetyl-CoA, 3-hydroxybutyryl-CoA and 2-hydroxyisobutyryl-CoA) in *C. autoethanogenum* expressing the Ptb-Buk system from plasmid pMTL82256-ptb-buk compared to wild-type (WT).

0031 Fig. 21A and Fig. 21B are graphs showing reduced acyl-CoA hydrolysis activity of *C. autoethanogenum* strains with inactivated thioesterases (CT2640 = thioesterase 1, CT 1524 = thioesterase 2, CT1780 = thioesterase 3) compared to activity found in *C. autoethanogenum* LZ1560 or LZ1561.

0032 Fig. 22 is a graph showing increased specific isopropanol production in a *C. autoethanogenum* strain with disrupted thioesterase 3 CAETHG_1780 compared to wild-type *C. autoethanogenum*.

0033 Figs. 23A-D are graphs showing growth (Fig. 23A) and isopropanol (Fig. 23B), acetate (Fig. 23C), and ethanol (Fig. 23D) production profiles of *C. autoethanogenum* wild-type and strain with disrupted thioesterase 3 (CAETHG_1780) compared to wild-type *C. autoethanogenum*.

0034 Fig. 24 is a plasmid map of pMTL8225-pta-ack::ptb-buk.

0035 Fig. 25 is a gel image indicating the replacement of *pta* and *ack* genes replaced with *ptb* and *buk* genes and ermB cassette.

0036 Fig. 26 is a graph showing increased conversion 3-hydroxybutyrate to 1,3-BDO by overexpression of the aldehyde:ferredoxin oxidoreductase gene *aor1*.

0037 Fig. 27 is a graph showing the activity of thioesterase TesB, Pta-Ack, and Ptb-Buk system on CoA hydrolysis of acetoacetyl-CoA, 3-hydroxybutyryl-CoA and 2-hydroxyisobutyryl-CoA compared to control (BL21 strain). Ptb-Buk shows highest activity, while Pta-Ack shows no activity.

0038 Figs. 28A and 28B are graphs showing production of 3-hydroxybutyrate via Ptb-Buk in combination with an (S)-specific (Hbd) (Fig. 28A) or (R)-specific 3-hydroxybutyrate (PhaB) (Fig. 28B) dehydrogenase.

0039 Figs. 29A-D are graphs showing LC-MS/MS detection of 2-hydroxyisobutyric acid (2-HIB) and 2-hydroxybutyrate (2-HB). Fig. 29A: 1 mM 2-HIB standard. Fig. 29B: 1 mM 2-HB standard. Fig. 29C: 0.5 mM 2-HB and 2-HIB standard. Fig. 29D: duplicate of *C. autoethanogenum* sample showing 2-HIB and 2-HB production from gas.

0040 Fig. 30 is a set of graphs showing GC-MS confirmation of 2-hydroxyisobutyric acid (8.91 min) production. First panel: *C. autoethanogenum* + pMTL83155-thlA-hbd-Pwl-meaBhcmA-hcmB + pMTL82256-tesB. Second panel: *C. autoethanogenum* + pMTL83155-thlA-hbd-Pwl-meaBhcmA-hcmB + pMTL82256-ptb-buk (spectrum). Third panel: *E. coli* + pMTL83155-thlA-hbd-Pwl-meaBhcmA-hcmB + pMTL82256-tesB. Fourth panel: *E. coli* + pMTL83155-thlA-hbd-Pwl-meaBhcmA-hcmB + pMTL82256-ptb-buk.

0041 Fig. 31 is a set of graphs of real time PCR showing expression of genes of the 2-HIBA pathway (*thlA, hba, meaBhcmA, hcmB* from pta-ack promoter and respectively Wood-Ljungdahl operon promoter) in *E. coli*, *C. autoethanogenum* LZ1561 at 30 °C, and *C. autoethanogenum* LZ1561 at 37 °C.

0042 Fig. 32 is a diagram showing the production of various products in a microorganism comprising Ptb-Buk, AOR, and Adh.

0043 Fig. 33 is a diagram showing the coupling firefly luciferase (Luc) to the Ptb-Buk system to characterize Ptb-Buk variants.

0044 Fig. 34 is a diagram of metabolic pathways for the production of various products, including adipic acid. Bold arrows indicate steps that may be catalyzed by Ptb-Buk.

0045 Fig. 35 is a diagram of metabolic pathways for the production of various products, including 1,3-hexanediol, 2-methyl-2-butanol, and 2-buten-1-ol. Bold arrows indicate steps that may be catalyzed by Ptb-Buk.

0046 Fig. 36 is a diagram of metabolic pathways for the production of various products, including isovalerate and isoamyl alcohol. Bold arrows indicate steps that may be catalyzed by Ptb-Buk.

0047 Fig. 37 is a graph of 3-HB production in *C. autoethanogenum* containing plasmid pMTL82256-thlA-ctfAB at various points of growth.

0048 Fig. 38A is a graph showing the growth and ethanol and 2,3-butanediol production profile of strain *C. autoethanogenum* pta-ack::ptb-buk + pMTL85147-thlA-ptb-buk-adc. Fig. 38B is a graph

showing the isopropanol and 3-HB production profile of strain *C. autoethanogenum* pta-ack::ptb-buk + pMTL85147-thlA-ptb-buk-adc.

0049 Fig. 39 is a diagram of a pathway scheme for producing a range of C_4, C_6, C_8, C_{10}, C_{12}, C14 alcohols, ketones, enols or diols via combining known chain elongation pathway (Hbd, Crt, Bcd-EtfAB, Thl) with Ptb-Buk + AOR/Adc-Adh.

0050 Fig. 40 is a graph showing production of 3-HB and 1,3-BDO by *C. autoethanogenum* transformed with plasmid pMTL83159-phaB-thlA at various points of growth.

0051 Fig. 41 is a graph showing production of 3-HB and 1,3-BDO by *C. autoethanogenum* comprising budA knockout and pMTL-HBD-ThlA at various points of growth.

0052 Fig. 42A is a graph showing production of 3-HB in a *C. autoethanogenum* pMTL83159-phaB-thlA + pMTL82256 fermentation. Fig. 42B is a graph showing production of 3-HB in a *C. autoethanogenum* pMTL83159-phaB-thlA + pMTL82256-buk-ptb fermentation.

0053 Fig. 43 is a graph showing the production of 3-HB in a *C. autoethanogenum* strain with thioesterase knockout (ΔCAETHG_1524) expressing plasmid pMTL83156-phaB-thlA with and without Ptb-Buk expression plasmid pMTL82256-buk-ptb.

0054 Fig. 44 is a graph showing showing ethanol and 1,3-BDO production in a *C. autoethanogenum* strain expressing plasmid pMTL82256-hbd-thlA (2pf) with and without AOR overexpression plasmid pMTL83159-aor1 (+aor1).

DETAILED DESCRIPTION OF THE INVENTION

Metabolic Pathways of Figs. 1 and 34-36

0055 Figs. 1 and 34-36 are diagrams of metabolic pathways for the production of various acid, alkene, ketone, aldehyde, alcohol, and diol products, including acetone, isopropanol, isobutylene, 3-hydroxybutyrate (R- and S-isomers), 1,3-butanediol, 2-hydroxyisobutyrate, adipic acid, 1,3-hexanediol, 2-methyl-2-butanol, 2-buten-1-ol, isovalerate, and isoamyl alcohol from a substrate. Bold arrows indicate steps that may be catalyzed by Ptb-Buk. Exemplary enzymes are provided for each of the steps and enzymatic pathways detailed in Figs. 1 and 34-36. However, additional suitable enzymes may be known to a person of ordinary skill in the art.

0056 Step 1 shows the conversion of acetyl-CoA to acetoacetyl-CoA. This step may be catalyzed by thiolase (i.e., acetyl-CoA acetyltransferase) (EC 2.3.1.9). The thiolase may be, for example, ThlA from *Clostridium acetobutylicum* (WP_010966157.1) (SEQ ID NO: 1), PhaA from *Cupriavidus necator* (WP_013956452.1) (SEQ ID NO: 2), BktB from *Cupriavidus necator* (WP_011615089.1) (SEQ ID NO: 3), or AtoB from *Escherichia coli* (NP_416728.1) (SEQ ID NO: 4). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. *Escherichia coli* has native activity for this step.

0057 Step 2 shows the conversion of acetoacetyl-CoA to acetoacetate. This step may be catalyzed by CoA-transferase (i.e., acetyl-CoA:acetoacetyl-CoA transferase) (EC 2.8.3.9). The CoA-transferase may be, for example, CtfAB, a heterodimer comprising subunits CtfA and CtfB, from *Clostridium beijerinckii* (CtfA, WP_012059996.1) (SEQ ID NO: 5) (CtfB, WP_012059997.1) (SEQ ID NO: 6). This step may also be catalyzed by thioesterase (EC 3.1.2.20). The thioesterase may be, for example, TesB from *Escherichia coli* (NP_414986.1) (SEQ ID NO: 7). This step may also be catalyzed by a putative thioesterase, e.g., from *Clostridium autoethanogenum* or *Clostridium ljungdahlii*. In particular, three putative thioesterases have been identified in *Clostridium autoethanogenum*: (1) "thioesterase 1" (AGY74947.1; annotated as palmitoyl-CoA hydrolase; SEQ ID NO: 8), (2) "thioesterase 2" (AGY75747.1; annotated as 4-hydroxybenzoyl-CoA thioesterase; SEQ ID NO: 9), and (3) "thioesterase 3" (AGY75999.1; annotated as putative thioesterase; SEQ ID NO: 10). Three putative thioesterases have also been identified in *Clostridium ljungdahlii*: (1) "thioesterase 1" (ADK15695.1; annotated as predicted acyl-CoA thioesterase 1; SEQ ID NO: 11), (2) "thioesterase 2" (ADK16655.1; annotated as predicted thioesterase; SEQ ID NO: 12), and (3) "thioesterase 3" (ADK16959.1; annotated as predicted thioesterase; SEQ ID NO: 13). This step may also be catalyzed by phosphate butyryltransferase (EC 2.3.1.19) + butyrate kinase (EC 2.7.2.7). Exemplary sources for phosphate butyryltransferase and butyrate kinase are described elsewhere in this application. Native enzymes in *Clostridium autoethanogenum, Clostridium ljungdahlii*, and *Clostridium ragsdalei* (or *Escherichia coli*), such as thioesterases from *Clostridium autoethanogenum*, may catalyze this step and result in the production of some amount of downstream products. However, introduction of an exogenous enzyme or overexpression of an endogenous enzyme may be required to produce downstream products at desirable levels. Additionally, in certain embodiments, a disruptive mutation may be introduced to an endogenous enzyme, such as an endogenous thioesterase, to reduce or eliminate competition with introduced Ptb-Buk.

0058 Step 3 shows the conversion of acetoacetate to acetone. This step may be catalyzed by an acetoacetate decarboxylase (EC 4.1.1.4). The acetoacetate decarboxylase may be, for example, Adc from *Clostridium beijerinckii* (WP_012059998.1) (SEQ ID NO: 14). This step may also be catalyzed by an alpha-ketoisovalerate decarboxylase (EC 4.1.1.74). The alpha-ketoisovalerate decarboxylase may be, for example, KivD from *Lactococcus lactis* (SEQ ID NO: 15). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. Additionally, *Escherichia coli* does not have known native activity for this step. Rarely, conversion of acetoacetate to acetone may occur spontaneously. However, spontaneous conversion is highly inefficient and unlikely to result in the production of downstream products at desirable levels.

0059 Step 4 shows the conversion of acetone to isopropanol. This step may be catalyzed by a primary:secondary alcohol dehydrogenase (EC 1.1.1.2). The primary:secondary alcohol dehydrogenase may be, for example, SecAdh from *Clostridium autoethanogenum* (AGY74782.1) (SEQ ID NO: 16), SecAdh from *Clostridium ljungdahlii* (ADK15544.1) (SEQ ID NO: 17), SecAdh

from *Clostridium ragsdalei* (WP_013239134.1) (SEQ ID NO: 18), or SecAdh from *Clostridium beijerinckii* (WP_026889046.1) (SEQ ID NO: 19). This step may also be catalyzed by a primary:secondary alcohol dehydrogenase (EC 1.1.1.80), such as SecAdh from *Thermoanaerobacter brokii* (3FSR_A) (SEQ ID NO: 20). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* have native activity for this step (Köpke, *Appl Environ Microbiol*, 80: 3394-3403, 2014). However, *Escherichia coli* does not have known native activity for this step. Knocking down or knocking out this enzyme in *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei* results in the production and accumulation of acetone rather than isopropanol (WO 2015/085015).

0060 Step 5 shows the conversion of acetone to 3-hydroxyisovalerate. This step may be catalyzed by a hydroxyisovalerate synthase, such as hydroxymethylglutaryl-CoA synthase (HMG-CoA synthase) (EC 2.3.3.10) from *Mus musculus* (SEQ ID NO: 21) (US 2012/0110001). The hydroxymethylglutaryl-CoA synthase may be engineered to improve activity. *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step.

0061 Step 6 shows the conversion of 3-hydroxyisovalerate to isobutylene (isobutene). This step may be catalyzed by a hydroxyisovalerate phosphorylase/decarboxylase. This step may also be catalyzed by mevalonate diphosphate decarboxylase (hydroxyisovalerate decarboxylase) (EC 4.1.1.33). The mevalonate diphosphate decarboxylase may be, for example, Mdd from *Saccharomyces cerevisiae* (CAA96324.1) (SEQ ID NO: 22) or Mdd from *Picrophilus torridus* (WP_011178157.1) (SEQ ID NO: 23) (US 2011/0165644; van Leeuwen, *Appl Microbiol Biotechnol*, 93: 1377-1387, 2012). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step

0062 Step 7 shows the conversion of acetone to 3-hydroxyisovaleryl-CoA. This step may be catalyzed by a 3-hydroxyisovaleryl-CoA synthase. *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step

0063 Step 8 shows the conversion of 3-hydroxyisovaleryl-CoA to 3-hydroxyisovalerate. This step may be catalyzed by CoA-transferase (i.e., acetyl-CoA:acetoacetyl-CoA transferase) (EC 2.8.3.9). The CoA-transferase may be, for example, CtfAB, a heterodimer comprising subunits CtfA and CtfB, from *Clostridium beijerinckii* (CtfA, WP_012059996.1) (SEQ ID NO: 5) (CtfB, WP_012059997.1) (SEQ ID NO: 6). This step may also be catalyzed by thioesterase (EC 3.1.2.20). The thioesterase may be, for example, TesB from *Escherichia coli* (NP_414986.1) (SEQ ID NO: 7). This step may also be catalyzed by a putative thioesterase, e.g., from *Clostridium autoethanogenum* or *Clostridium ljungdahlii*. In particular, three putative thioesterases have been identified in *Clostridium autoethanogenum*: (1) "thioesterase 1" (AGY74947.1; annotated as palmitoyl-CoA hydrolase; SEQ

ID NO: 8), (2) "thioesterase 2" (AGY75747.1; annotated as 4-hydroxybenzoyl-CoA thioesterase; SEQ ID NO: 9), and (3) "thioesterase 3" (AGY75999.1; annotated as putative thioesterase; SEQ ID NO: 10). Three putative thioesterases have also been identified in *Clostridium ljungdahlii*: (1) "thioesterase 1" (ADK15695.1; annotated as predicted acyl-CoA thioesterase 1; SEQ ID NO: 11), (2) "thioesterase 2" (ADK16655.1; annotated as predicted thioesterase; SEQ ID NO: 12), and (3) "thioesterase 3" (ADK16959.1; annotated as predicted thioesterase; SEQ ID NO: 13). This step may also be catalyzed by phosphate butyryltransferase (EC 2.3.1.19) + butyrate kinase (EC 2.7.2.7). Exemplary sources for phosphate butyryltransferase and butyrate kinase are described elsewhere in this application. Native enzymes in *Clostridium autoethanogenum, Clostridium ljungdahlii*, and *Clostridium ragsdalei* (or *Escherichia coli*), such as thioesterases from *Clostridium autoethanogenum*, may catalyze this step and result in the production of some amount of downstream products. However, introduction of an exogenous enzyme or overexpression of an endogenous enzyme may be required to produce downstream products at desirable levels. Additionally, in certain embodiments, a disruptive mutation may be introduced to an endogenous enzyme, such as an endogenous thioesterase, to reduce or eliminate competition with introduced Ptb-Buk.

0064 Step 9 shows the conversion of acetyl-CoA to 3-methyl-2-oxopentanoate. This step encompasses a number of enzymatic reactions involved in the isoleucine biosynthesis pathway, which is natively present in many bacteria, including *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* (and *Escherichia coli*). Enzymes involved in the conversion of acetyl-CoA to 3-methyl-2-oxopentanoate may include citramalate synthase (EC 2.3.1.182), 3-isopropylmalate dehydratase (EC 4.2.1.35), 3-isopropylmalate dehydrogenase (EC 1.1.1.85), acetolactate synthase (EC 2.2.1.6), ketol-acid reductoisomerase (EC 1.1.1.86), and/or dihydroxyacid dehydratase (EC 4.2.1.9). The citramalate synthase may be, for example, CimA from *Clostridium autoethanogenum* (AGY76958.1) (SEQ ID NO: 24) or CimA from *Methanocaldococcus jannaschii* (NP_248395.1) (SEQ ID NO: 25). The 3-isopropylmalate dehydratase may be, for example, LeuCD from *Clostridium autoethanogenum* (WP_023162955.1, LeuC; AGY77204.1, LeuD) (SEQ ID NOs: 26 and 27, respectively) or LeuCD from *Escherichia coli* (NP_414614.1, LeuC; NP_414613.1, LeuD) (SEQ ID NOs: 28 and 29, respectively). The 3-isopropylmalate dehydrogenase may be, for example, LeuB from *Clostridium autoethanogenum* (WP_023162957.1) (SEQ ID NO: 30) or LeuB from *Escherichia coli* (NP_414615.4) (SEQ ID NO: 31). The acetolactate synthase may be, for example, IlvBN from *Clostridium autoethanogenum* (AGY74359.1, IlvB; AGY74635.1, IlvB; AGY74360.1, IlvN) (SEQ ID NOs: 32, 33, and 34, respectively) or IlvBN from *Escherichia coli* (NP_418127.1, IlvB; NP_418126.1, IlvN) (SEQ ID NOs: 35 and 36, respectively). The ketol-acid reductoisomerase may be, for example, IlvC from *Clostridium autoethanogenum* (WP_013238693.1) (SEQ ID NO: 37) or IlvC from *Escherichia coli* (NP_418222.1) (SEQ ID NO: 38). The dihydroxyacid dehydratase may be, for example, IlvD from *Clostridium autoethanogenum* (WP_013238694.1) (SEQ ID NO: 39) or IlvD from *Escherichia coli* (YP_026248.1) (SEQ ID NO: 40). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* have native activity for this step.

0065 Step 10 shows the conversion of 3-methyl-2-oxopentoate to 2-methylbutanoyl-CoA. This step may be catalyzed by ketoisovalerate oxidoreductase (EC 1.2.7.7). The ketoisovalerate oxidoreductase may be, for example, the VorABCD from *Methanothermobacter thermautotrophicus* (WP_010876344.1, VorA; WP_010876343.1, VorB; WP_010876342.1, VorC; WP_010876341.1, VorD) (SEQ ID NOs: 41-44, respectively) or VorABCD from *Pyrococcus furiosus* (WP_011012106.1, VorA; WP_011012105.1, VorB; WP_011012108.1, VorC; WP_011012107.1, VorD) (SEQ ID NOs: 45-48, respectively). VorABCD is a 4-subunit enzyme. *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step.

0066 Step 11 shows the conversion of 2-methylbutanoyl-CoA to 2-methylcrotonyl-CoA. This step may be catalyzed by 2-methylbutanoyl-CoA dehydrogenase (EC 1.3.99.12). The 2-methylbutanoyl-CoA dehydrogenase may be, for example, AcdH from *Streptomyces avermitilis* (AAD44196.1 or BAB69160.1) (SEQ ID NO: 49) or AcdH from *Streptomyces coelicolor* (AAD44195.1) (SEQ ID NO: 50). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step.

0067 Step 12 shows the conversion of 2-methylcrotonyl-CoA to 3-hydroxyisovaleryl-CoA. This step may be catalyzed by crotonase/3-hydroxybutyryl-CoA dehydratase (EC 4.2.1.55). The crotonase/3-hydroxybutyryl-CoA dehydratase may be, for example, Crt from *Clostridium beijerinckii* (ABR34202.1) (SEQ ID NO: 51), Crt from *Clostridium acetobutylicum* (NP_349318.1) (SEQ ID NO: 52), or LiuC from *Myxococcus xanthus* (WP_011553770.1). This step may also be catalyzed by crotonyl-CoA carboxylase-reductase (EC 1.3.1.86). The crotonyl-CoA carboxylase-reductase may be, for example, Ccr from *Treponema denticola* (NP_971211.1) (SEQ ID NO: 53). This step may also be catalyzed by crotonyl-CoA reductase (EC 1.3.1.44). The crotonyl-CoA reductase may be, for example, Ter from *Euglena gracilis* (AAW66853.1) (SEQ ID NO: 54). This step may also be catalyzed by a 3-hydroxypropionyl-CoA dehydratase (EC 4.2.1.116). This 3-hydroxypropionyl-CoA dehydratase may be, for example, Msed_2001 from *Metallosphaera sedula* (WP_012021928.1). This step may also be catalyzed by a enoyl-CoA hydratase. This enoyl-CoA hydratase (4.2.1.17) may be, for example, YngF from *Bacillus anthracis* (WP_000787371.1). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step.

0068 Step 13 shows the conversion of acetoacetyl-CoA to 3-hydroxybutyryl-CoA. This step may be catalyzed by 3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157). The 3-hydroxybutyryl-CoA dehydrogenase may be, for example, Hbd from *Clostridium beijerinckii* (WP_011967675.1) (SEQ ID NO: 55), Hbd from *Clostridium acetobutylicum* (NP_349314.1) (SEQ ID NO: 56), or Hbd1 from *Clostridium kluyveri* (WP_011989027.1) (SEQ ID NO: 57). This step may also be catalyzed by acetoacetyl-CoA reductase (EC 4.2.1.36). The acetoacetyl-CoA reductase may be, for example, PhaB from *Cupriavidus necator* (WP_010810131.1) (SEQ ID NO: 58). This step may also be catalyzed by acetoacetyl-CoA hydratase (EC 4.2.1.119). Of note, PhaB is R-specific and Hbd is S-specific.

Additionally, Hbd1 from *Clostridium kluyveri* is NADPH-dependent and Hbd from *Clostridium acetobutylicum* and *Clostridium beijerinckii* are NADH-dependent. *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step.

0069 Step 14 shows the conversion of 3-hydroxybutyryl-CoA to 3-hydroxybutyrate. This step may be catalyzed by thioesterase (EC 3.1.2.20). The thioesterase may be, for example, TesB from *Escherichia coli* (NP_414986.1) (SEQ ID NO: 7). This step may also be catalyzed by a putative thioesterase, e.g., from *Clostridium autoethanogenum* or *Clostridium ljungdahlii*. In particular, three putative thioesterases have been identified in *Clostridium autoethanogenum*: (1) "thioesterase 1" (AGY74947.1; annotated as palmitoyl-CoA hydrolase; SEQ ID NO: 8), (2) "thioesterase 2" (AGY75747.1; annotated as 4-hydroxybenzoyl-CoA thioesterase; SEQ ID NO: 9), and (3) "thioesterase 3" (AGY75999.1; annotated as putative thioesterase; SEQ ID NO: 10). Three putative thioesterases have also been identified in *Clostridium ljungdahlii*: (1) "thioesterase 1" (ADK15695.1; annotated as predicted acyl-CoA thioesterase 1; SEQ ID NO: 11), (2) "thioesterase 2" (ADK16655.1; annotated as predicted thioesterase; SEQ ID NO: 12), and (3) "thioesterase 3" (ADK16959.1; annotated as predicted thioesterase; SEQ ID NO: 13). This step may also be catalyzed by phosphate butyryltransferase (EC 2.3.1.19) + butyrate kinase (EC 2.7.2.7). Exemplary sources for phosphate butyryltransferase and butyrate kinase are described elsewhere in this application. Native enzymes in *Clostridium autoethanogenum, Clostridium ljungdahlii*, and *Clostridium ragsdalei* (or *Escherichia coli*), such as thioesterases from *Clostridium autoethanogenum*, may catalyze this step and result in the production of some amount of downstream products. However, introduction of an exogenous enzyme or overexpression of an endogenous enzyme may be required to produce downstream products at desirable levels. Additionally, in certain embodiments, a disruptive mutation may be introduced to an endogenous enzyme, such as an endogenous thioesterase, to reduce or eliminate competition with introduced Ptb-Buk.

0070 Step 15 shows the conversion of 3-hydroxybutyrate to acetoacetate. This step may be catalyzed by 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30). The 3-hydroxybutyrate dehydrogenase may be, for example, Bdh1 from *Ralstonia pickettii* (BAE72684.1) (SEQ ID NO: 60) or Bdh2 from *Ralstonia pickettii* (BAE72685.1) (SEQ ID NO: 61). The reverse reaction, the conversion of acetoacetate to 3-hydroxybutyrate, may be catalyzed by different 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) enzymes. For example, the conversion of acetoacetate to 3-hydroxybutyrate may be catalyzed by Bdh from *Clostridium autoethanogenum* (AGY75962) (SEQ ID NO: 62). *Clostridium ljungdahlii* and *Clostridium ragsdalei* likely have enzymes with similar activity. *Escherichia coli* does not have known native activity for this step.

0071 Step 16 shows the conversion of 3-hydroxybutyrate to 3-hydroxybutyrylaldehyde. This step may be catalyzed by aldehyde:ferredoxin oxidoreductase (EC 1.2.7.5). The aldehyde:ferredoxin oxidoreductase (AOR) may be, for example, AOR from *Clostridium autoethanogenum* (WP_013238665.1; WP_013238675.1) (SEQ ID NOs: 63 and 64, respectively) or AOR from

Clostridium ljungdahlii (ADK15073.1; ADK15083.1) (SEQ ID NOs: 65 and 66, respectively). In further embodiments, the aldehyde:ferredoxin oxidoreductase may be or may be derived, for example, from any of the following sources, the sequences of which are publically available:

Description	Microrganism	Accession	GeneID
aldehyde:ferredoxin oxidoreductase	*Acidilobus saccharovorans 345-15*	NC_014374.1	9498931
aldehyde:ferredoxin oxidoreductase	*Acidilobus saccharovorans 345-15*	NC_014374.1	9499504
aldehyde:ferredoxin oxidoreductase	*Acidilobus saccharovorans 345-15*	NC_014374.1	9499550
aldehyde:ferredoxin oxidoreductase	*Acidilobus saccharovorans 345-15*	NC_014374.1	9498997
aldehyde:ferredoxin oxidoreductase	*Aciduliprofundum boonei T469*	NC_013926.1	8828075
aldehyde:ferredoxin oxidoreductase	*Aciduliprofundum boonei T469*	NC_013926.1	8828511
aldehyde:ferredoxin oxidoreductase	*Aciduliprofundum boonei T469*	NC_013926.1	8828305
aldehyde:ferredoxin oxidoreductase	*Aciduliprofundum boonei T469*	NC_013926.1	8827762
aldehyde:ferredoxin oxidoreductase	*Aciduliprofundum boonei T469*	NC_013926.1	8827370
aldehyde:ferredoxin oxidoreductase	*Aciduliprofundum sp. MAR08-339*	NC_019942.1	14306579
aldehyde:ferredoxin oxidoreductase	*Aciduliprofundum sp. MAR08-339*	NC_019942.1	14306982
aldehyde:ferredoxin oxidoreductase	*Aciduliprofundum sp. MAR08-339*	NC_019942.1	14306639
aldehyde:ferredoxin oxidoreductase	*Aciduliprofundum sp. MAR08-339*	NC_019942.1	14307339
aldehyde:ferredoxin oxidoreductase	*Aeropyrum pernix K1*	NC_000854.2	1444491
aldehyde:ferredoxin oxidoreductase	*Archaeoglobus fulgidus DSM 4304*	NC_000917.1	1483287
aldehyde:ferredoxin oxidoreductase	*Archaeoglobus fulgidus DSM 4304*	NC_000917.1	1483233
aldehyde:ferredoxin oxidoreductase	*Archaeoglobus fulgidus DSM 4304*	NC_000917.1	1483554
aldehyde:ferredoxin oxidoreductase	*Archaeoglobus fulgidus DSM 4304*	NC_000917.1	1485513
aldehyde:ferredoxin oxidoreductase	*Archaeoglobus profundus DSM 5631*	NC_013741.1	8738726
aldehyde:ferredoxin oxidoreductase	*Archaeoglobus profundus DSM 5631*	NC_013741.1	8740019
aldehyde:ferredoxin oxidoreductase	*Archaeoglobus sulfaticallidus PM70-1*	NC_021169.1	15392228
aldehyde:ferredoxin oxidoreductase	*Archaeoglobus sulfaticallidus PM70-1*	NC_021169.1	15393814
aldehyde:ferredoxin oxidoreductase	*Archaeoglobus sulfaticallidus PM70-1*	NC_021169.1	15391826
aldehyde:ferredoxin oxidoreductase	*Archaeoglobus sulfaticallidus PM70-1*	NC_021169.1	15393763
aldehyde:ferredoxin oxidoreductase	*Archaeoglobus sulfaticallidus PM70-1*	NC_021169.1	15393491
aldehyde:ferredoxin oxidoreductase	*Archaeoglobus veneficus SNP6*	NC_015320.1	10393142
aldehyde:ferredoxin oxidoreductase	*Archaeoglobus veneficus SNP6*	NC_015320.1	10395048
aldehyde:ferredoxin oxidoreductase	*Caldisphaera lagunensis DSM 15908*	NC_019791.1	14212403
aldehyde:ferredoxin oxidoreductase	*Caldisphaera lagunensis DSM 15908*	NC_019791.1	14211524
aldehyde:ferredoxin oxidoreductase	*Caldisphaera lagunensis DSM 15908*	NC_019791.1	14212092
aldehyde:ferredoxin oxidoreductase	*Caldisphaera lagunensis DSM 15908*	NC_019791.1	14212561
aldehyde:ferredoxin oxidoreductase	*Caldivirga maquilingensis IC-167*	NC_009954.1	5710116
aldehyde:ferredoxin oxidoreductase	*Caldivirga maquilingensis IC-167*	NC_009954.1	5710117
aldehyde:ferredoxin oxidoreductase	*Caldivirga maquilingensis IC-167*	NC_009954.1	5709088
aldehyde:ferredoxin oxidoreductase	*Caldivirga maquilingensis IC-167*	NC_009954.1	5708891
aldehyde:ferredoxin oxidoreductase	*Caldivirga maquilingensis IC-167*	NC_009954.1	5710478

aldehyde:ferredoxin oxidoreductase	*Caldivirga maquilingensis IC-167*	NC_009954.1	5710457
aldehyde:ferredoxin oxidoreductase	*Caldivirga maquilingensis IC-167*	NC_009954.1	5709696
aldehyde:ferredoxin oxidoreductase	*Candidatus Caldiarchaeum subterraneum*	NC_022786.1	17602865
aldehyde:ferredoxin oxidoreductase	*Candidatus Korarchaeum cryptofilum OPF8*	NC_010482.1	6094361
aldehyde:ferredoxin oxidoreductase	*Candidatus Korarchaeum cryptofilum OPF8*	NC_010482.1	6094198
aldehyde:ferredoxin oxidoreductase	*Candidatus Korarchaeum cryptofilum OPF8*	NC_010482.1	6093546
aldehyde:ferredoxin oxidoreductase	*Candidatus Korarchaeum cryptofilum OPF8*	NC_010482.1	6093319
aldehyde:ferredoxin oxidoreductase	*Candidatus Korarchaeum cryptofilum OPF8*	NC_010482.1	6094057
aldehyde:ferredoxin oxidoreductase	*Candidatus Korarchaeum cryptofilum OPF8*	NC_010482.1	6093563
aldehyde:ferredoxin oxidoreductase	*Chloroflexus aurantiacus J-10-fl*	NC_010175.1	5828639
aldehyde:ferredoxin oxidoreductase	*Clostridium acetobutylicum ATCC 824*	NC_003030.1	1118201
aldehyde:ferredoxin oxidoreductase	*Clostridium botulinum A str. ATCC 3502*	NC_009495.1	5187636
aldehyde:ferredoxin oxidoreductase	*Clostridium botulinum A str. Hall*	NC_009698.1	5400593
aldehyde:ferredoxin oxidoreductase	*Desulfovibrio vulgaris str. Hildenborough*	NC_002937.3	2796664
aldehyde:ferredoxin oxidoreductase	*Desulfovibrio vulgaris str. Hildenborough*	NC_002937.3	2795337
aldehyde:ferredoxin oxidoreductase	*Desulfurococcus fermentans DSM 16532*	NC_018001.1	13061477
aldehyde:ferredoxin oxidoreductase	*Desulfurococcus fermentans DSM 16532*	NC_018001.1	13061068
aldehyde:ferredoxin oxidoreductase	*Desulfurococcus fermentans DSM 16532*	NC_018001.1	13062247
aldehyde:ferredoxin oxidoreductase	*Desulfurococcus kamchatkensis 1221n*	NC_011766.1	7171099
aldehyde:ferredoxin oxidoreductase	*Desulfurococcus kamchatkensis 1221n*	NC_011766.1	7171759
aldehyde:ferredoxin oxidoreductase	*Desulfurococcus kamchatkensis 1221n*	NC_011766.1	7170725
aldehyde:ferredoxin oxidoreductase	*Desulfurococcus mucosus DSM 2162*	NC_014961.1	10152801
aldehyde:ferredoxin oxidoreductase	*Ferroglobus placidus DSM 10642*	NC_013849.1	8778536
aldehyde:ferredoxin oxidoreductase	*Ferroglobus placidus DSM 10642*	NC_013849.1	8779007
aldehyde:ferredoxin oxidoreductase	*Ferroglobus placidus DSM 10642*	NC_013849.1	8778940
aldehyde:ferredoxin oxidoreductase	*Ferroglobus placidus DSM 10642*	NC_013849.1	8779639
aldehyde:ferredoxin oxidoreductase	*Ferroglobus placidus DSM 10642*	NC_013849.1	8778820
aldehyde:ferredoxin oxidoreductase	*Ferroglobus placidus DSM 10642*	NC_013849.1	8778745
aldehyde:ferredoxin oxidoreductase	*Ferroglobus placidus DSM 10642*	NC_013849.1	8779874
aldehyde:ferredoxin oxidoreductase	*Fervidicoccus fontis Kam940*	NC_017461.1	12449263
aldehyde:ferredoxin oxidoreductase	*Fervidicoccus fontis Kam940*	NC_017461.1	12449994
aldehyde:ferredoxin oxidoreductase	*Fervidicoccus fontis Kam940*	NC_017461.1	12449294
aldehyde:ferredoxin oxidoreductase	*Fervidicoccus fontis Kam940*	NC_017461.1	12449682
aldehyde:ferredoxin oxidoreductase	*Geobacter sulfurreducens PCA*	NC_002939.5	2685730
aldehyde:ferredoxin oxidoreductase	*Geobacter sulfurreducens PCA*	NC_002939.5	2687039
aldehyde:ferredoxin oxidoreductase	*Halalkalicoccus jeotgali B3*	NC_014297.1	9418623
aldehyde:ferredoxin oxidoreductase	*Halalkalicoccus jeotgali B3*	NC_014297.1	9418760

aldehyde:ferredoxin oxidoreductase	*Halalkalicoccus jeotgali B3*	NC_014297.1	9420819
aldehyde:ferredoxin oxidoreductase	*Halalkalicoccus jeotgali B3*	NC_014297.1	9418748
aldehyde:ferredoxin oxidoreductase	*Haloarcula hispanica ATCC 33960*	NC_015948.1	11051410
aldehyde:ferredoxin oxidoreductase	*Haloarcula hispanica ATCC 33960*	NC_015948.1	11050783
aldehyde:ferredoxin oxidoreductase	*Haloarcula hispanica ATCC 33960*	NC_015948.1	11051433
aldehyde:ferredoxin oxidoreductase	*Haloarcula hispanica N601*	NC_023013.1	23805333
aldehyde:ferredoxin oxidoreductase	*Haloarcula hispanica N601*	NC_023013.1	23805138
aldehyde:ferredoxin oxidoreductase	*Haloarcula hispanica N601*	NC_023013.1	23804665
aldehyde:ferredoxin oxidoreductase	*Haloarcula marismortui ATCC 43049*	NC_006396.1	3127969
aldehyde:ferredoxin oxidoreductase	*Haloarcula marismortui ATCC 43049*	NC_006396.1	3129232
aldehyde:ferredoxin oxidoreductase	*Haloferax mediterranei ATCC 33500*	NC_017941.2	13028168
aldehyde:ferredoxin oxidoreductase	*Haloferax mediterranei ATCC 33500*	NC_017941.2	13028399
aldehyde:ferredoxin oxidoreductase	*Haloferax volcanii DS2*	NC_013964.1	8919329
aldehyde:ferredoxin oxidoreductase	*Haloferax volcanii DS2*	NC_013964.1	8919033
aldehyde:ferredoxin oxidoreductase	*Haloferax volcanii DS2*	NC_013967.1	8926544
aldehyde:ferredoxin oxidoreductase	*Halogeometricum borinquense DSM 11551*	NC_014735.1	9989054
aldehyde:ferredoxin oxidoreductase	*Halogeometricum borinquense DSM 11551*	NC_014729.1	9994424
aldehyde:ferredoxin oxidoreductase	*Halogeometricum borinquense DSM 11551*	NC_014729.1	9992444
aldehyde:ferredoxin oxidoreductase	*halophilic archaeon DL31*	NC_015954.1	11095016
aldehyde:ferredoxin oxidoreductase	*halophilic archaeon DL31*	NC_015954.1	11095541
aldehyde:ferredoxin oxidoreductase	*halophilic archaeon DL31*	NC_015954.1	11094595
aldehyde:ferredoxin oxidoreductase	*halophilic archaeon DL31*	NC_015954.1	11096497
aldehyde:ferredoxin oxidoreductase	*halophilic archaeon DL31*	NC_015954.1	11094563
aldehyde:ferredoxin oxidoreductase	*halophilic archaeon DL31*	NC_015954.1	11095602
aldehyde:ferredoxin oxidoreductase	*Halopiger xanaduensis SH-6*	NC_015666.1	10799161
aldehyde:ferredoxin oxidoreductase	*Halopiger xanaduensis SH-6*	NC_015658.1	10795465
aldehyde:ferredoxin oxidoreductase	*Halopiger xanaduensis SH-6*	NC_015666.1	10798686
aldehyde:ferredoxin oxidoreductase	*Halopiger xanaduensis SH-6*	NC_015666.1	10796679
aldehyde:ferredoxin oxidoreductase	*Halorubrum lacusprofundi ATCC 49239*	NC_012029.1	7400122
aldehyde:ferredoxin oxidoreductase	*Halorubrum lacusprofundi ATCC 49239*	NC_012029.1	7400291
aldehyde:ferredoxin oxidoreductase	*Halorubrum lacusprofundi ATCC 49239*	NC_012029.1	7400689
aldehyde:ferredoxin oxidoreductase	*Haloterrigena turkmenica DSM 5511*	NC_013744.1	8744461
aldehyde:ferredoxin oxidoreductase	*Haloterrigena turkmenica DSM 5511*	NC_013744.1	8744695
aldehyde:ferredoxin oxidoreductase	*Haloterrigena turkmenica DSM 5511*	NC_013743.1	8740954
aldehyde:ferredoxin oxidoreductase	*Haloterrigena turkmenica DSM 5511*	NC_013745.1	8745418
aldehyde:ferredoxin oxidoreductase	*Haloterrigena turkmenica DSM 5511*	NC_013743.1	8742968
aldehyde:ferredoxin oxidoreductase	*Haloterrigena turkmenica DSM 5511*	NC_013743.1	8741246

aldehyde:ferredoxin oxidoreductase	*Haloterrigena turkmenica DSM 5511*	NC_013743.1	8741269
aldehyde:ferredoxin oxidoreductase	*Haloterrigena turkmenica DSM 5511*	NC_013745.1	8745313
aldehyde:ferredoxin oxidoreductase	*Hyperthermus butylicus DSM 5456*	NC_008818.1	4781896
aldehyde:ferredoxin oxidoreductase	*Hyperthermus butylicus DSM 5456*	NC_008818.1	4782266
aldehyde:ferredoxin oxidoreductase	*Hyperthermus butylicus DSM 5456*	NC_008818.1	4782804
aldehyde:ferredoxin oxidoreductase	*Hyperthermus butylicus DSM 5456*	NC_008818.1	4781774
aldehyde:ferredoxin oxidoreductase	*Ignicoccus hospitalis KIN4/I*	NC_009776.1	5562477
aldehyde:ferredoxin oxidoreductase	*Ignicoccus hospitalis KIN4/I*	NC_009776.1	5562774
aldehyde:ferredoxin oxidoreductase	*Ignisphaera aggregans DSM 17230*	NC_014471.1	9716798
aldehyde:ferredoxin oxidoreductase	*Methanocaldococcus jannaschii DSM 2661*	NC_000909.1	1452083
aldehyde:ferredoxin oxidoreductase	*Methanocella arvoryzae MRE50*	NC_009464.1	5142690
aldehyde:ferredoxin oxidoreductase	*Methanocella arvoryzae MRE50*	NC_009464.1	5143773
aldehyde:ferredoxin oxidoreductase	*Methanocella conradii HZ254*	NC_017034.1	11972399
aldehyde:ferredoxin oxidoreductase	*Methanocella conradii HZ254*	NC_017034.1	11971349
aldehyde:ferredoxin oxidoreductase	*Methanocella paludicola SANAE*	NC_013665.1	8680711
aldehyde:ferredoxin oxidoreductase	*Methanocella paludicola SANAE*	NC_013665.1	8680676
aldehyde:ferredoxin oxidoreductase	*Methanocorpusculum labreanum Z*	NC_008942.1	4795790
aldehyde:ferredoxin oxidoreductase	*Methanoculleus marisnigri JR1*	NC_009051.1	4847673
aldehyde:ferredoxin oxidoreductase	*Methanohalobium evestigatum Z-7303*	NC_014253.1	9347460
aldehyde:ferredoxin oxidoreductase	*Methanohalobium evestigatum Z-7303*	NC_014253.1	9347022
aldehyde:ferredoxin oxidoreductase	*Methanolobus psychrophilus R15*	NC_018876.1	13845119
aldehyde:ferredoxin oxidoreductase	*Methanomethylovorans hollandica DSM 15978*	NC_019977.1	14408029
aldehyde:ferredoxin oxidoreductase	*Methanosaeta harundinacea 6Ac*	NC_017527.1	12511443
aldehyde:ferredoxin oxidoreductase	*Methanosaeta thermophila PT*	NC_008553.1	4462364
aldehyde:ferredoxin oxidoreductase	*Methanosalsum zhilinae DSM 4017*	NC_015676.1	10822365
aldehyde:ferredoxin oxidoreductase	*Methanosarcina acetivorans C2A*	NC_003552.1	1475882
aldehyde:ferredoxin oxidoreductase	*Methanosarcina acetivorans C2A*	NC_003552.1	1474856
aldehyde:ferredoxin oxidoreductase	*Methanosarcina acetivorans C2A*	NC_003552.1	1473602
aldehyde:ferredoxin oxidoreductase	*Methanosarcina barkeri str. Fusaro*	NC_007355.1	3625763
aldehyde:ferredoxin oxidoreductase	*Methanosarcina mazei Go1*	NC_003901.1	1479263
aldehyde:ferredoxin oxidoreductase	*Methanosarcina mazei Go1*	NC_003901.1	1481668
aldehyde:ferredoxin oxidoreductase	*Methanosarcina mazei Go1*	NC_003901.1	1480987
aldehyde:ferredoxin oxidoreductase	*Methanosarcina mazei Tuc01*	NC_020389.1	14656065
aldehyde:ferredoxin oxidoreductase	*Methanosarcina mazei Tuc01*	NC_020389.1	14656771
aldehyde:ferredoxin oxidoreductase	*Methanosarcina mazei Tuc01*	NC_020389.1	14654304
aldehyde:ferredoxin oxidoreductase	*Methanosphaerula palustris E1-9c*	NC_011832.1	7271108
aldehyde:ferredoxin oxidoreductase	*Methanospirillum hungatei JF-1*	NC_007796.1	3924565
aldehyde:ferredoxin oxidoreductase	*Methylomicrobium alcaliphilum 20Z*	NC_016112.1	11361147
aldehyde:ferredoxin oxidoreductase	*Moorella thermoacetica ATCC 39073*	NC_007644.1	3831332
aldehyde:ferredoxin oxidoreductase	*Moorella thermoacetica ATCC 39073*	NC_007644.1	3830998
aldehyde:ferredoxin oxidoreductase	*Moorella thermoacetica ATCC 39073*	NC_007644.1	3831866
aldehyde:ferredoxin oxidoreductase	*Natrialba magadii ATCC 43099*	NC_013922.1	8824961

aldehyde:ferredoxin oxidoreductase	*Natrialba magadii ATCC 43099*	NC_013922.1	8823392
aldehyde:ferredoxin oxidoreductase	*Natrialba magadii ATCC 43099*	NC_013923.1	8826737
aldehyde:ferredoxin oxidoreductase	*Natrialba magadii ATCC 43099*	NC_013922.1	8825516
aldehyde:ferredoxin oxidoreductase	*Natrinema pellirubrum DSM 15624*	NC_019962.1	14335278
aldehyde:ferredoxin oxidoreductase	*Natrinema pellirubrum DSM 15624*	NC_019962.1	14333050
aldehyde:ferredoxin oxidoreductase	*Natrinema pellirubrum DSM 15624*	NC_019962.1	14333754
aldehyde:ferredoxin oxidoreductase	*Natrinema sp. J7-2*	NC_018224.1	13349954
aldehyde:ferredoxin oxidoreductase	*Natronobacterium gregoryi SP2*	NC_019792.1	14210296
aldehyde:ferredoxin oxidoreductase	*Natronobacterium gregoryi SP2*	NC_019792.1	14207133
aldehyde:ferredoxin oxidoreductase	*Natronobacterium gregoryi SP2*	NC_019792.1	14209682
aldehyde:ferredoxin oxidoreductase	*Natronobacterium gregoryi SP2*	NC_019792.1	14207576
aldehyde:ferredoxin oxidoreductase	*Natronobacterium gregoryi SP2*	NC_019792.1	14206941
aldehyde:ferredoxin oxidoreductase	*Natronobacterium gregoryi SP2*	NC_019792.1	14206532
aldehyde:ferredoxin oxidoreductase	*Natronococcus occultus SP4*	NC_019974.1	14403316
aldehyde:ferredoxin oxidoreductase	*Natronococcus occultus SP4*	NC_019974.1	14405255
aldehyde:ferredoxin oxidoreductase	*Natronococcus occultus SP4*	NC_019974.1	14403781
aldehyde:ferredoxin oxidoreductase	*Natronococcus occultus SP4*	NC_019974.1	14402014
aldehyde:ferredoxin oxidoreductase	*Natronomonas moolapensis 8.8.11*	NC_020388.1	14651997
aldehyde:ferredoxin oxidoreductase	*Natronomonas moolapensis 8.8.11*	NC_020388.1	14652892
aldehyde:ferredoxin oxidoreductase	*Natronomonas moolapensis 8.8.11*	NC_020388.1	14651999
aldehyde:ferredoxin oxidoreductase	*Natronomonas pharaonis DSM 2160*	NC_007427.1	3694680
aldehyde:ferredoxin oxidoreductase	*Natronomonas pharaonis DSM 2160*	NC_007426.1	3702508
aldehyde:ferredoxin oxidoreductase	*Natronomonas pharaonis DSM 2160*	NC_007426.1	3702507
aldehyde:ferredoxin oxidoreductase	*Natronomonas pharaonis DSM 2160*	NC_007426.1	3702509
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum aerophilum str. IM2*	NC_003364.1	1464236
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum aerophilum str. IM2*	NC_003364.1	1464102
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum aerophilum str. IM2*	NC_003364.1	1465126
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum aerophilum str. IM2*	NC_003364.1	1465445
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum arsenaticum DSM 13514*	NC_009376.1	5055904
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum arsenaticum DSM 13514*	NC_009376.1	5055700
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum arsenaticum DSM 13514*	NC_009376.1	5054881
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum arsenaticum DSM 13514*	NC_009376.1	5054644
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum arsenaticum DSM 13514*	NC_009376.1	5054547
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum calidifontis JCM 11548*	NC_009073.1	4910224
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum calidifontis JCM 11548*	NC_009073.1	4908822
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum calidifontis JCM 11548*	NC_009073.1	4909927
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum calidifontis JCM 11548*	NC_009073.1	4910099
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum islandicum DSM 4184*	NC_008701.1	4617364
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum islandicum DSM 4184*	NC_008701.1	4616724
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum islandicum DSM 4184*	NC_008701.1	4617494
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum neutrophilum V24Sta*	NC_010525.1	6165427
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum neutrophilum V24Sta*	NC_010525.1	6164958

aldehyde:ferredoxin oxidoreductase	*Pyrobaculum neutrophilum V24Sta*	NC_010525.1	6164976
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum oguniense TE7*	NC_016885.1	11853778
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum oguniense TE7*	NC_016885.1	11854024
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum oguniense TE7*	NC_016885.1	11856490
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum oguniense TE7*	NC_016885.1	11856176
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum oguniense TE7*	NC_016885.1	11854908
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum sp. 1860*	NC_016645.1	11594868
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum sp. 1860*	NC_016645.1	11596631
aldehyde:ferredoxin oxidoreductase	*Pyrobaculum sp. 1860*	NC_016645.1	11594049
aldehyde:ferredoxin oxidoreductase	*Pyrococcus abyssi GE5*	NC_000868.1	1496313
aldehyde:ferredoxin oxidoreductase	*Pyrococcus abyssi GE5*	NC_000868.1	1495669
aldehyde:ferredoxin oxidoreductase	*Pyrococcus abyssi GE5*	NC_000868.1	1496580
aldehyde:ferredoxin oxidoreductase	*Pyrococcus abyssi GE5*	NC_000868.1	1495287
aldehyde:ferredoxin oxidoreductase	*Pyrococcus furiosus COM1*	NC_018092.1	13302148
aldehyde:ferredoxin oxidoreductase	*Pyrococcus furiosus COM1*	NC_018092.1	13301806
aldehyde:ferredoxin oxidoreductase	*Pyrococcus furiosus COM1*	NC_018092.1	13301219
aldehyde:ferredoxin oxidoreductase	*Pyrococcus furiosus COM1*	NC_018092.1	13300785
aldehyde:ferredoxin oxidoreductase	*Pyrococcus furiosus DSM 3638*	NC_003413.1	1468181
aldehyde:ferredoxin oxidoreductase	*Pyrococcus furiosus DSM 3638*	NC_003413.1	1469073
aldehyde:ferredoxin oxidoreductase	*Pyrococcus furiosus DSM 3638*	NC_003413.1	1469843
aldehyde:ferredoxin oxidoreductase	*Pyrococcus horikoshii OT3*	NC_000961.1	1443218
aldehyde:ferredoxin oxidoreductase	*Pyrococcus horikoshii OT3*	NC_000961.1	1443341
aldehyde:ferredoxin oxidoreductase	*Pyrococcus horikoshii OT3*	NC_000961.1	1443932
aldehyde:ferredoxin oxidoreductase	*Pyrococcus horikoshii OT3*	NC_000961.1	1443598
aldehyde:ferredoxin oxidoreductase	*Pyrococcus sp. NA2*	NC_015474.1	10555029
aldehyde:ferredoxin oxidoreductase	*Pyrococcus sp. NA2*	NC_015474.1	10554020
aldehyde:ferredoxin oxidoreductase	*Pyrococcus sp. NA2*	NC_015474.1	10555341
aldehyde:ferredoxin oxidoreductase	*Pyrococcus sp. ST04*	NC_017946.1	13022107
aldehyde:ferredoxin oxidoreductase	*Pyrococcus sp. ST04*	NC_017946.1	13022436
aldehyde:ferredoxin oxidoreductase	*Pyrococcus sp. ST04*	NC_017946.1	13021314
aldehyde:ferredoxin oxidoreductase	*Pyrococcus yayanosii CH1*	NC_015680.1	10837518
aldehyde:ferredoxin oxidoreductase	*Pyrococcus yayanosii CH1*	NC_015680.1	10837112
aldehyde:ferredoxin oxidoreductase	*Pyrococcus yayanosii CH1*	NC_015680.1	10837264
aldehyde:ferredoxin oxidoreductase	*Pyrolobus fumarii 1A*	NC_015931.1	11138144
aldehyde:ferredoxin oxidoreductase	*Pyrolobus fumarii 1A*	NC_015931.1	11138776
aldehyde:ferredoxin oxidoreductase	*Pyrolobus fumarii 1A*	NC_015931.1	11139127
aldehyde:ferredoxin oxidoreductase	*Rhodospirillum rubrum ATCC 11170*	NC_007643.1	3833668
aldehyde:ferredoxin oxidoreductase	*Staphylothermus hellenicus DSM 12710*	NC_014205.1	9234557
aldehyde:ferredoxin oxidoreductase	*Staphylothermus hellenicus DSM 12710*	NC_014205.1	9233414
aldehyde:ferredoxin oxidoreductase	*Staphylothermus hellenicus DSM 12710*	NC_014205.1	9234134
aldehyde:ferredoxin oxidoreductase	*Staphylothermus hellenicus DSM 12710*	NC_014205.1	9234110
aldehyde:ferredoxin oxidoreductase	*Staphylothermus marinus F1*	NC_009033.1	4907444
aldehyde:ferredoxin oxidoreductase	*Staphylothermus marinus F1*	NC_009033.1	4907343
aldehyde:ferredoxin oxidoreductase	*Thermanaerovibrio acidaminovorans DSM 6589*	NC_013522.1	8630284

aldehyde:ferredoxin oxidoreductase	*Thermanaerovibrio acidaminovorans DSM 6589*	NC_013522.1	8630027
aldehyde:ferredoxin oxidoreductase	*Thermanaerovibrio acidaminovorans DSM 6589*	NC_013522.1	8630623
aldehyde:ferredoxin oxidoreductase	*Thermoanaerobacter wiegelii Rt8.B1*	NC_015958.1	11082596
aldehyde:ferredoxin oxidoreductase	*Thermococcus barophilus MP*	NC_014804.1	10041639
aldehyde:ferredoxin oxidoreductase	*Thermococcus barophilus MP*	NC_014804.1	10041106
aldehyde:ferredoxin oxidoreductase	*Thermococcus barophilus MP*	NC_014804.1	10042460
aldehyde:ferredoxin oxidoreductase	*Thermococcus cleftensis*	NC_018015.1	13037745
aldehyde:ferredoxin oxidoreductase	*Thermococcus cleftensis*	NC_018015.1	13038896
aldehyde:ferredoxin oxidoreductase	*Thermococcus cleftensis*	NC_018015.1	13037242
aldehyde:ferredoxin oxidoreductase	*Thermococcus gammatolerans EJ3*	NC_012804.1	7988317
aldehyde:ferredoxin oxidoreductase	*Thermococcus gammatolerans EJ3*	NC_012804.1	7987451
aldehyde:ferredoxin oxidoreductase	*Thermococcus kodakarensis KOD1*	NC_006624.1	3233851
aldehyde:ferredoxin oxidoreductase	*Thermococcus kodakarensis KOD1*	NC_006624.1	3233735
aldehyde:ferredoxin oxidoreductase	*Thermococcus litoralis DSM 5473*	NC_022084.1	16550741
aldehyde:ferredoxin oxidoreductase	*Thermococcus litoralis DSM 5473*	NC_022084.1	16548761
aldehyde:ferredoxin oxidoreductase	*Thermococcus litoralis DSM 5473*	NC_022084.1	16550885
aldehyde:ferredoxin oxidoreductase	*Thermococcus onnurineus NA1*	NC_011529.1	7018383
aldehyde:ferredoxin oxidoreductase	*Thermococcus onnurineus NA1*	NC_011529.1	7016739
aldehyde:ferredoxin oxidoreductase	*Thermococcus onnurineus NA1*	NC_011529.1	7017051
aldehyde:ferredoxin oxidoreductase	*Thermococcus onnurineus NA1*	NC_011529.1	7017476
aldehyde:ferredoxin oxidoreductase	*Thermococcus sibiricus MM 739*	NC_012883.1	8096638
aldehyde:ferredoxin oxidoreductase	*Thermococcus sibiricus MM 739*	NC_012883.1	8096005
aldehyde:ferredoxin oxidoreductase	*Thermococcus sibiricus MM 739*	NC_012883.1	8096629
aldehyde:ferredoxin oxidoreductase	*Thermococcus sibiricus MM 739*	NC_012883.1	8095463
aldehyde:ferredoxin oxidoreductase	*Thermococcus sibiricus MM 739*	NC_012883.1	8096131
aldehyde:ferredoxin oxidoreductase	*Thermococcus sibiricus MM 739*	NC_012883.1	8096636
aldehyde:ferredoxin oxidoreductase	*Thermococcus sp. 4557*	NC_015865.1	11015504
aldehyde:ferredoxin oxidoreductase	*Thermococcus sp. 4557*	NC_015865.1	11015249
aldehyde:ferredoxin oxidoreductase	*Thermococcus sp. 4557*	NC_015865.1	11015571
aldehyde:ferredoxin oxidoreductase	*Thermococcus sp. AM4*	NC_016051.1	7419050
aldehyde:ferredoxin oxidoreductase	*Thermococcus sp. AM4*	NC_016051.1	7418514
aldehyde:ferredoxin oxidoreductase	*Thermococcus sp. AM4*	NC_016051.1	7420292
aldehyde:ferredoxin oxidoreductase	*Thermodesulfovibrio yellowstonii DSM 11347*	NC_011296.1	6941429
aldehyde:ferredoxin oxidoreductase	*Thermodesulfovibrio yellowstonii DSM 11347*	NC_011296.1	6943174
aldehyde:ferredoxin oxidoreductase	*Thermodesulfovibrio yellowstonii DSM 11347*	NC_011296.1	6941905
aldehyde:ferredoxin oxidoreductase	*Thermofilum pendens Hrk 5*	NC_008698.1	4602054
aldehyde:ferredoxin oxidoreductase	*Thermofilum pendens Hrk 5*	NC_008698.1	4601386
aldehyde:ferredoxin oxidoreductase	*Thermofilum pendens Hrk 5*	NC_008698.1	4600878
aldehyde:ferredoxin oxidoreductase	*Thermofilum pendens Hrk 5*	NC_008698.1	4600730
aldehyde:ferredoxin oxidoreductase	*Thermofilum sp. 1910b*	NC_022093.1	16572780
aldehyde:ferredoxin oxidoreductase	*Thermofilum sp. 1910b*	NC_022093.1	16572926
aldehyde:ferredoxin oxidoreductase	*Thermofilum sp. 1910b*	NC_022093.1	16573009
aldehyde:ferredoxin oxidoreductase	*Thermofilum sp. 1910b*	NC_022093.1	16574342
aldehyde:ferredoxin oxidoreductase	*Thermogladius cellulolyticus 1633*	NC_017954.1	13012904

aldehyde:ferredoxin oxidoreductase	*Thermoplasma acidophilum DSM 1728*	NC_002578.1	1456355
aldehyde:ferredoxin oxidoreductase	*Thermoplasma acidophilum DSM 1728*	NC_002578.1	1456646
aldehyde:ferredoxin oxidoreductase	*Thermoplasma volcanium GSS1*	NC_002689.2	1441901
aldehyde:ferredoxin oxidoreductase	*Thermoplasma volcanium GSS1*	NC_002689.2	1441379
aldehyde:ferredoxin oxidoreductase	*Thermoproteus tenax Kra 1*	NC_016070.1	11262174
aldehyde:ferredoxin oxidoreductase	*Thermoproteus tenax Kra 1*	NC_016070.1	11262275
aldehyde:ferredoxin oxidoreductase	*Thermoproteus tenax Kra 1*	NC_016070.1	11262652
aldehyde:ferredoxin oxidoreductase	*Thermoproteus tenax Kra 1*	NC_016070.1	11262926
aldehyde:ferredoxin oxidoreductase	*Thermoproteus uzoniensis 768-20*	NC_015315.1	10361668
aldehyde:ferredoxin oxidoreductase	*Thermoproteus uzoniensis 768-20*	NC_015315.1	10361250
aldehyde:ferredoxin oxidoreductase	*Thermoproteus uzoniensis 768-20*	NC_015315.1	10360972
aldehyde:ferredoxin oxidoreductase	*Thermosphaera aggregans DSM 11486*	NC_014160.1	9165115
aldehyde:ferredoxin oxidoreductase	*Thermosphaera aggregans DSM 11486*	NC_014160.1	9165462
aldehyde:ferredoxin oxidoreductase	*Thermus thermophilus HB8*	NC_006461.1	3168554
aldehyde:ferredoxin oxidoreductase	*Thermus thermophilus HB8*	NC_006461.1	3168612
aldehyde:ferredoxin oxidoreductase	*Vulcanisaeta distributa DSM 14429*	NC_014537.1	9753145
aldehyde:ferredoxin oxidoreductase	*Vulcanisaeta distributa DSM 14429*	NC_014537.1	9750947
aldehyde:ferredoxin oxidoreductase	*Vulcanisaeta distributa DSM 14429*	NC_014537.1	9750989
aldehyde:ferredoxin oxidoreductase	*Vulcanisaeta distributa DSM 14429*	NC_014537.1	9753486
aldehyde:ferredoxin oxidoreductase	*Vulcanisaeta distributa DSM 14429*	NC_014537.1	9751414
aldehyde:ferredoxin oxidoreductase	*Vulcanisaeta moutnovskia 768-28*	NC_015151.1	10288238
aldehyde:ferredoxin oxidoreductase	*Vulcanisaeta moutnovskia 768-28*	NC_015151.1	10288894
aldehyde:ferredoxin oxidoreductase	*Vulcanisaeta moutnovskia 768-28*	NC_015151.1	10288574
aldehyde:ferredoxin oxidoreductase	*Vulcanisaeta moutnovskia 768-28*	NC_015151.1	10288827
aldehyde:ferredoxin oxidoreductase	*Vulcanisaeta moutnovskia 768-28*	NC_015151.1	10288607
aldehyde:ferredoxin oxidoreductase	*Vulcanisaeta moutnovskia 768-28*	NC_015151.1	10288523
aldehyde:ferredoxin oxidoreductase	*Vulcanisaeta moutnovskia 768-28*	NC_015151.1	10288815

0072 AOR catalyzes the reaction of an acid and reduced ferredoxin to form an aldehyde and oxidized ferredoxin. In acetogens, this reaction can be coupled to oxidation CO (via CO dehydrogenase, EC 1.2.7.4) or hydrogen (via ferredoxin-dependent hydrogenase, EC 1.12.7.2 or 1.12.1.4) that both yield reduced ferredoxin (Köpke, Curr Opin Biotechnol 22: 320-325, 2011; Köpke, *PNAS USA*, 107: 13087-13092, 2010). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* have native activity for this step. However, overexpression of endogenous AOR or introduction of an exogenous AOR in *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei* may be desirable to enhance product yields. Alternatively, exogenous AOR may be introduced into a microorganism that does not natively comprise AOR, e.g., *E. coli*. In particular, the co-expression of Ptb-Buk and AOR (and, optionally, Adh) may enable such a microorganism to produce new non-native products.

0073 Step 17 shows the conversion of 3-hydroxybutyrylaldehyde to 1,3-butanediol. This step may be catalyzed by alcohol dehydrogenase (EC 1.1.1.1. or 1.1.1.2.). Alcohol dehydrogenase can convert

an aldehyde and NAD(P)H to an alcohol and NAD(P). The alcohol dehydrogenase may be, for example, Adh from *Clostridium autoethanogenum* (AGY76060.1) (SEQ ID NO: 67), *Clostridium ljungdahlii* (ADK17019.1) (SEQ ID NO: 68), or *Clostridium ragsdalei*, BdhB from *Clostridium acetobutylicum* (NP_349891.1) (SEQ ID NO: 69), Bdh from *Clostridium beijerinckii* (WP_041897187.1) (SEQ ID NO: 70), Bdh1 from *Clostridium ljungdahlii* (YP_003780648.1) (SEQ ID NO: 71), Bdh1 from *Clostridium autoethanogenum* (AGY76060.1) (SEQ ID NO: 72), Bdh2 from *Clostridium ljungdahlii* (YP_003782121.1) (SEQ ID NO: 73), Bdh2 from *Clostridium autoethanogenum* (AGY74784.1) (SEQ ID NO: 74), AdhE1 from *Clostridium acetobutylicum* (NP_149325.1) (SEQ ID NO: 75), AdhE2 from *Clostridium acetobutylicum* (NP_149199.1) (SEQ ID NO: 76), AdhE from *Clostridium beijerinckii* (WP_041893626.1) (SEQ ID NO: 77), AdhE1 from *Clostridium autoethanogenum* (WP_023163372.1) (SEQ ID NO: 78), or AdhE2 from *Clostridium autoethanogenum* (WP_023163373.1) (SEQ ID NO: 79). *Clostridium autoethanogenum, Clostridium ljungdahlii*, and *Clostridium ragsdalei* have native activity for this step. However, overexpression of endogenous alcohol dehydrogenase or introduction of an exogenous alcohol dehydrogenase in *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei* may be desirable to enhance product yields. *Escherichia coli* likely does not have native activity for this step.

0074 Step 18 shows the conversion of 3-hydroxybutyryl-CoA to 3-hydroxybutyrylaldehyde. This step may be catalyzed by butyraldehyde dehydrogenase (EC 1.2.1.57). The butyraldehyde dehydrogenase may be, for example, Bld from *Clostridium saccharoperbutylacetonicum* (AAP42563.1) (SEQ ID NO: 80). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step.

0075 Step 19 shows the conversion of 3-hydroxybutyryl-CoA to 2-hydroxyisobutyryl-CoA. This step may be catalyzed by 2-hydroxyisobutyryl-CoA mutase (EC 5.4.99.-). The 2-hydroxyisobutyryl-CoA mutase may be, for example, HcmAB from *Aquincola tertiaricarbonis* (AFK77668.1, large subunit; AFK77665.1, small subunit) (SEQ ID NOs: 81 and 82, respectively) or HcmAB from *Kyrpidia tusciae* (WP_013074530.1, large subunit; WP_013074531.1, small subunit) (SEQ ID NOs: 83 and 84, respectively). Chaperone MeaB (AFK77667.1, *Aquincola tertiaricarbonis*; WP_013074529.1, *Kyrpidia tusciae*) (SEQ ID NOs: 85 and 86, respectively) has been described to improve activity of HcmAB by reactivating HcmAB, although MeaB is not required for HcmAB function (Yaneva, *J Biol Chem*, 287: 15502-15511, 2012). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step.

0076 Step 20 shows the conversion of 2-hydroxyisobutyryl-CoA to 2-hydroxyisobutyrate. This step may be catalyzed by phosphate butyryltransferase (EC 2.3.1.19) + butyrate kinase (EC 2.7.2.7). Exemplary sources for phosphate butyryltransferase and butyrate kinase are described elsewhere in this application. *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei*

do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step.

0077 Step 21 shows the conversion of acetyl-CoA to succinyl-CoA. This step encompasses a number of enzymatic reactions involved in the reductive TCA pathway, which is natively present in many bacteria, including *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* (and *Escherichia coli*) (Brown, *Biotechnol Biofuels*, 7: 40, 2014; U.S. Patent 9,297,026). Enzymes involved in the conversion of acetyl-CoA to succinyl-CoA may include pyruvate:ferredoxin oxidoreductase (PFOR) (EC 1.2.7.1), pyruvate carboxylase (PYC) (EC 6.4.1.1), malic enzyme/malate dehydrogenase (EC 1.1.1.38, EC 1.1.1.40), pyruvate phosphate dikinase (PPDK) (EC:2.7.9.1), PEP carboxykinase (PCK) (EC 4.1.1.49), fumarate hydratase/fumerase (EC 4.2.1.2), fumarate reductase (EC 1.3.5.1)/succinate dehydrogenase (EC 1.3.5.4), and succinyl-CoA synthetase (EC 6.2.1.5). The pyruvate:ferredoxin oxidoreductase may be, for example, from *Clostridium autoethanogenum* (AGY75153, AGY77232) or *Escherichia coli* (NP_415896). The pyruvate carboxylase may be, for example, from *Clostridium autoethanogenum* (AGY75817). The malic enzyme/malate dehydrogenase may be, for example, from *Clostridium autoethanogenum* (AGY76687) or *Escherichia coli* (NP_416714, NP_417703). The pyruvate phosphate dikinase (PPDK) may be, for example, from *Clostridium autoethanogenum* (AGY76274, AGY77114). The PEP carboxykinase (PCK) may be, for example, from *Clostridium autoethanogenum* (AGY76928) or *Escherichia coli* (NP_417862). The fumarate hydratase/fumerase may be, for example, from *Clostridium autoethanogenum* (AGY76121, AGY76122) or *Escherichia coli* (NP_416128, NP_416129, NP_418546). The fumarate reductase/succinate dehydrogenase may be, for example, from *Clostridium autoethanogenum* (AGY74573, AGY74575, AGY75257, AGY77166) or *Escherichia coli* (NP_415249, NP_415250, NP_415251, NP_415252, NP_418575, NP_418576, NP_418577, NP_418578). The succinyl-CoA synthetase may be, for example, from *Escherichia coli* (NP_415256, NP_415257).

0078 Step 22 shows shows the conversion of acetyl-CoA and succinyl-CoA to 3-oxo-adipyl-CoA. This step may be catalyzed by β-ketoadipyl-CoA thiolase (EC 2.3.1.16). The ketoisovalerate oxidoreductase may be, for example, PaaJ from *Escherichia coli* (WP_001206190.1). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step.

0079 Step 23 shows the conversion of 3-oxo-adipyl-CoA to 3-hydroxyadipyl-CoA. This step may be catalyzed by 3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157) or acetoacetyl-CoA hydratase (EC 4.2.1.119). The 3-hydroxybutyryl-CoA dehydrogenase or acetoacetyl-CoA hydratase may be, for example, Hbd from *Clostridium beijerinckii* (WP_011967675.1) (SEQ ID NO: 55), Hbd from *Clostridium acetobutylicum* (NP_349314.1) (SEQ ID NO: 56), Hbd1 from *Clostridium kluyveri* (WP_011989027.1) (SEQ ID NO: 57), or PaaH1 from *Cupriavidus necator* (WP_010814882.1). Of note, PhaB is R-specific and Hbd is S-specific. Additionally, Hbd1 from *Clostridium kluyveri* is NADPH-dependent and Hbd from *Clostridium acetobutylicum* and *Clostridium beijerinckii* are NADH-dependent. *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium*

ragsdalei do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step.

0001 Step 24 shows the conversion of 3-hydroxyadipyl-CoA to 2,3-dehydroadipyl-CoA. This step may be catalyzed by an enoyl-CoA hydratase (EC: 4.2.1.17) or enoyl-CoA reductase (EC: 1.3.1.38). The enoyl-CoA hydratase or enoyl-CoA reductase may be, for example, Crt from *C. acetobutylicum* (NP_349318.1) or PhaJ from *Aeromonas caviae* (O32472) (Seq. ID No. 52). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step.

0002 Step 25 shows the conversion of 2,3-dehydroadipyl-CoA to adipyl-CoA. This step may be catalyzed by trans-2-enoyl-CoA reductase (EC 1.3.8.1, EC 1.3.1.86, EC 1.3.1.85, EC 1.3.1.44). The trans-2-enoyl-CoA reductase may be, for example, Bcd from *C. acetobutylicum* (NP_349317.1) that forms a complex with electron flavoproteins EtfAB (NP_349315, NP_349316), Ccr from *Streptomyces collinus* (AAA92890), Ccr from *Rhodobacter sphaeroides* (YP_354044.1), Ter from *Treponema denticola* (NP_971211.1), or Ter from *Euglena gracilis* (AY741582.1). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step.

0003 Step 26 shows the conversion of adipyl-CoA to adipic acid. This step may be catalyzed by phosphate butyryltransferase (EC 2.3.1.19) + butyrate kinase (EC 2.7.2.7). Exemplary sources for phosphate butyryltransferase and butyrate kinase are described elsewhere in this application. Native enzymes in *Clostridium autoethanogenum, Clostridium ljungdahlii*, and *Clostridium ragsdalei* (or *Escherichia coli*), such as thioesterases from *Clostridium autoethanogenum*, may catalyze this step and result in the production of some amount of downstream products. However, introduction of an exogenous enzyme or overexpression of an endogenous enzyme may be required to produce downstream products at desirable levels. Additionally, in certain embodiments, a disruptive mutation may be introduced to an endogenous enzyme, such as an endogenous thioesterase, to reduce or eliminate competition with introduced Ptb-Buk.

0004 Step 27 shows the conversion of shows the conversion of 3-hydroxbutyryl-CoA to crotonyl-CoA. This step may be catalyzed by a crotonyl-CoA hydratase (crotonase) (EC 4.2.1.17) or crotonyl-CoA reductase (EC 1.3.1.38). The crotonyl-CoA hydratase (crotonase) or crotonyl-CoA reductase may be, for example, Crt from *C. acetobutylicum* (NP_349318.1) (SEQ ID NO: 52) or PhaJ from *Aeromonas caviae* (O32472). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step.

0005 Step 28 shows the conversion of crotonyl-CoA to crotonate. This step may be catalyzed by phosphate butyryltransferase (EC 2.3.1.19) + butyrate kinase (EC 2.7.2.7). Exemplary sources for phosphate butyryltransferase and butyrate kinase are described elsewhere in this application. Native enzymes in *Clostridium autoethanogenum, Clostridium ljungdahlii*, and *Clostridium ragsdalei* (or

Escherichia coli), such as thioesterases from *Clostridium autoethanogenum*, may catalyze this step and result in the production of some amount of downstream products. However, introduction of an exogenous enzyme or overexpression of an endogenous enzyme may be required to produce downstream products at desirable levels. Additionally, in certain embodiments, a disruptive mutation may be introduced to an endogenous enzyme, such as an endogenous thioesterase, to reduce or eliminate competition with introduced Ptb-Buk.

0006 Step 29 shows the conversion of crotonate to crotonaldehyde. This step may be catalyzed by aldehyde:ferredoxin oxidoreductase (EC 1.2.7.5). Exemplary sources for aldehyde:ferredoxin oxidoreductases are described elsewhere in this application. AOR catalyzes the reaction of an acid and reduced ferredoxin to form an aldehyde and oxidized ferredoxin. In acetogens, this reaction can be coupled to oxidation CO (via CO dehydrogenase, EC 1.2.7.4) or hydrogen (via ferredoxin-dependent hydrogenase, EC 1.12.7.2 or 1.12.1.4) that both yield reduced ferredoxin (Köpke, Curr Opin Biotechnol 22: 320-325, 2011; Köpke, *PNAS USA*, 107: 13087-13092, 2010). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* have native activity for this step. However, overexpression of endogenous AOR or introduction of an exogenous AOR in *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei* may be desirable to enhance product yields. AOR of *Pyrococcus furiosus* has been demonstrated activity converting crotonaldehyde and crotonate (Loes, *J Bacteriol*, 187: 7056-7061, 2005). Alternatively, exogenous AOR may be introduced into a microorganism that does not natively comprise AOR, e.g., *E. coli*. In particular, the co-expression of Ptb-Buk and AOR (and, optionally, Adh) may enable such a microorganism to produce new non-native products.

0007 Step 30 shows the conversion of crotonaldehyde to 2-buten-1-ol. This step may be catalyzed by alcohol dehydrogenase (EC 1.1.1.1. or 1.1.1.2.). Alcohol dehydrogenase can convert an aldehyde and NAD(P)H to an alcohol and NAD(P). The alcohol dehydrogenase may be, for example, Adh from *Clostridium autoethanogenum* (AGY76060.1) (SEQ ID NO: 67), *Clostridium ljungdahlii* (ADK17019.1) (SEQ ID NO: 68), or *Clostridium ragsdalei*, BdhB from *Clostridium acetobutylicum* (NP_349891.1) (SEQ ID NO: 69), Bdh from *Clostridium beijerinckii* (WP_041897187.1) (SEQ ID NO: 70), Bdh1 from *Clostridium ljungdahlii* (YP_003780648.1) (SEQ ID NO: 71), Bdh1 from *Clostridium autoethanogenum* (AGY76060.1) (SEQ ID NO: 72), Bdh2 from *Clostridium ljungdahlii* (YP_003782121.1) (SEQ ID NO: 73), Bdh2 from *Clostridium autoethanogenum* (AGY74784.1) (SEQ ID NO: 74), AdhE1 from *Clostridium acetobutylicum* (NP_149325.1) (SEQ ID NO: 75), AdhE2 from *Clostridium acetobutylicum* (NP_149199.1) (SEQ ID NO: 76), AdhE from *Clostridium beijerinckii* (WP_041893626.1) (SEQ ID NO: 77), AdhE1 from *Clostridium autoethanogenum* (WP_023163372.1) (SEQ ID NO: 78), or AdhE2 from *Clostridium autoethanogenum* (WP_023163373.1) (SEQ ID NO: 79). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* have native activity for this step. However, overexpression of endogenous alcohol dehydrogenase or introduction of an exogenous alcohol dehydrogenase in *Clostridium*

autoethanogenum, *Clostridium ljungdahlii*, or *Clostridium ragsdalei* may be desirable to enhance product yields. *Escherichia coli* likely does not have native activity for this step.

0008 Step 31 shows the conversion of crotonyl-CoA to butyryl-CoA. This step may be catalyzed by butyryl-CoA dehydrogenase or trans-2-enoyl-CoA reductase (EC 1.3.8.1, EC 1.3.1.86, EC 1.3.1.85, EC 1.3.1.44). The butyryl-CoA dehydrogenase or trans-2-enoyl-CoA reductase may be, for example, Bcd from *C. acetobutylicum* (NP_349317.1) that forms a complex with electron flavoproteins EtfAB (NP_349315, NP_349316), Ccr from *Streptomyces collinus* (AAA92890), Ccr from *Rhodobacter sphaeroides* (YP_354044.1), Ter from *Treponema denticola* (NP_971211.1), or Ter from *Euglena gracilis* (AY741582.1). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step.

0009 Step 32 shows the conversion of butyryl-CoA to acetobutyryl-CoA. This step may be catalyzed by thiolase or acyl-CoA acetyltransferase (EC 2.3.1.9). The thiolase may be, for example, ThlA from *Clostridium acetobutylicum* (WP_010966157.1) (SEQ ID NO: 1), ThlA1 from *Clostridium kluyveri* (EDK35681), ThlA2 from *Clostridium kluyveri* (EDK35682), ThlA3 from *Clostridium kluyveri* (EDK35683), PhaA from *Cupriavidus necator* (WP_013956452.1) (SEQ ID NO: 2), BktB from *Cupriavidus necator* (WP_011615089.1) (SEQ ID NO: 3), or AtoB from *Escherichia coli* (NP_416728.1) (SEQ ID NO: 4). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. *Escherichia coli* has native activity for this step.

0010 Step 33 shows the conversion of acetobutyryl-CoA to acetobutyrate. This step may be catalyzed by phosphate butyryltransferase (EC 2.3.1.19) + butyrate kinase (EC 2.7.2.7). Exemplary sources for phosphate butyryltransferase and butyrate kinase are described elsewhere in this application. Native enzymes in *Clostridium autoethanogenum, Clostridium ljungdahlii*, and *Clostridium ragsdalei* (or *Escherichia coli*), such as thioesterases from *Clostridium autoethanogenum*, may catalyze this step and result in the production of some amount of downstream products. However, introduction of an exogenous enzyme or overexpression of an endogenous enzyme may be required to produce downstream products at desirable levels. Additionally, in certain embodiments, a disruptive mutation may be introduced to an endogenous enzyme, such as an endogenous thioesterase, to reduce or eliminate competition with introduced Ptb-Buk.

0011 Step 34 shows the conversion of acetobutyrate to acetylacetone. This step may be catalyzed by an acetoacetate decarboxylase (EC 4.1.1.4). The acetoacetate decarboxylase may be, for example, Adc from *Clostridium beijerinckii* (WP_012059998.1) (SEQ ID NO: 14). This step may also be catalyzed by an alpha-ketoisovalerate decarboxylase (EC 4.1.1.74). The alpha-ketoisovalerate decarboxylase may be, for example, KivD from *Lactococcus lactis* (SEQ ID NO: 15). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. Additionally, *Escherichia coli* does not have known native activity for this step.

Rarely, conversion of acetoacetate to acetone may occur spontaneously. However, spontaneous conversion is highly inefficient and unlikely to result in the production of downstream products at desirable levels.

0012 Step 35 shows the conversion of acetylacetone to 3-methyl-2-butanol. This step may be catalyzed by a primary:secondary alcohol dehydrogenase (EC 1.1.1.2). The primary:secondary alcohol dehydrogenase may be, for example, SecAdh from *Clostridium autoethanogenum* (AGY74782.1) (SEQ ID NO: 16), SecAdh from *Clostridium ljungdahlii* (ADK15544.1) (SEQ ID NO: 17), SecAdh from *Clostridium ragsdalei* (WP_013239134.1) (SEQ ID NO: 18), or SecAdh from *Clostridium beijerinckii* (WP_026889046.1) (SEQ ID NO: 19). This step may also be catalyzed by a primary:secondary alcohol dehydrogenase (EC 1.1.1.80), such as SecAdh from *Thermoanaerobacter brokii* (3FSR_A) (SEQ ID NO: 20). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* have native activity for this step (Köpke, *Appl Environ Microbiol*, 80: 3394-3403, 2014). However, *Escherichia coli* does not have known native activity for this step. Knocking down or knocking out this enzyme in *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei* results in the production and accumulation of acetylacetone rather than 3-methyl-2-butanol (WO 2015/085015).

0013 Step 36 shows the conversion of acetobutyryl-CoA to 3-hydroxyhexanoyl-CoA. This step may be catalyzed by 3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157) or acetoacetyl-CoA hydratase (EC 4.2.1.119). The 3-hydroxybutyryl-CoA dehydrogenase or acetoacetyl-CoA hydratase may be, for example, Hbd from *Clostridium beijerinckii* (WP_011967675.1) (SEQ ID NO: 55), Hbd from *Clostridium acetobutylicum* (NP_349314.1) (SEQ ID NO: 56), Hbd1 from *Clostridium kluyveri* (WP_011989027.1) (SEQ ID NO: 57), Hbd2 from *Clostridium kluyveri* (EDK34807), or PaaH1 from *Cupriavidus necator* (WP_010814882.1). Of note, PhaB is R-specific and Hbd is S-specific. Additionally, Hbd1 from *Clostridium kluyveri* is NADPH-dependent and Hbd from *Clostridium acetobutylicum* and *Clostridium beijerinckii* are NADH-dependent. *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step.

0014 Step 37 shows the conversion of 3-hydroxyhexanoyl-CoA to 3-hydroxyhexanoate. This step may be catalyzed by phosphate butyryltransferase (EC 2.3.1.19) + butyrate kinase (EC 2.7.2.7). Exemplary sources for phosphate butyryltransferase and butyrate kinase are described elsewhere in this application. Native enzymes in *Clostridium autoethanogenum, Clostridium ljungdahlii*, and *Clostridium ragsdalei* (or *Escherichia coli*), such as thioesterases from *Clostridium autoethanogenum*, may catalyze this step and result in the production of some amount of downstream products. However, introduction of an exogenous enzyme or overexpression of an endogenous enzyme may be required to produce downstream products at desirable levels. Additionally, in certain embodiments, a disruptive mutation may be introduced to an endogenous enzyme, such as an endogenous thioesterase, to reduce or eliminate competition with introduced Ptb-Buk.

0015 Step 38 shows the conversion of 3-hydroxyhexanoate to 1,3-hexaldehyde. This step may be catalyzed by aldehyde:ferredoxin oxidoreductase (EC 1.2.7.5). Exemplary sources for aldehyde:ferredoxin oxidoreductases are described elsewhere in this application. AOR catalyzes the reaction of an acid and reduced ferredoxin to form an aldehyde and oxidized ferredoxin. In acetogens, this reaction can be coupled to oxidation CO (via CO dehydrogenase, EC 1.2.7.4) or hydrogen (via ferredoxin-dependent hydrogenase, EC 1.12.7.2 or 1.12.1.4) that both yield reduced ferredoxin (Köpke, Curr Opin Biotechnol 22: 320-325, 2011; Köpke, *PNAS USA*, 107: 13087-13092, 2010). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* have native activity for this step. However, overexpression of endogenous AOR or introduction of an exogenous AOR in *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei* may be desirable to enhance product yields. Alternatively, exogenous AOR may be introduced into a microorganism that does not natively comprise AOR, e.g., *E. coli*. In particular, the co-expression of Ptb-Buk and AOR (and, optionally, Adh) may enable such a microorganism to produce new non-native products.

0016 Step 39 shows the conversion of 1,3-hexaldehyde to 1,3-hexanediol. This step may be catalyzed by alcohol dehydrogenase (EC 1.1.1.1. or 1.1.1.2.). Alcohol dehydrogenase can convert an aldehyde and NAD(P)H to an alcohol and NAD(P). The alcohol dehydrogenase may be, for example, Adh from *Clostridium autoethanogenum* (AGY76060.1) (SEQ ID NO: 67), *Clostridium ljungdahlii* (ADK17019.1) (SEQ ID NO: 68), or *Clostridium ragsdalei*, BdhB from *Clostridium acetobutylicum* (NP_349891.1) (SEQ ID NO: 69), Bdh from *Clostridium beijerinckii* (WP_041897187.1) (SEQ ID NO: 70), Bdh1 from *Clostridium ljungdahlii* (YP_003780648.1) (SEQ ID NO: 71), Bdh1 from *Clostridium autoethanogenum* (AGY76060.1) (SEQ ID NO: 72), Bdh2 from *Clostridium ljungdahlii* (YP_003782121.1) (SEQ ID NO: 73), Bdh2 from *Clostridium autoethanogenum* (AGY74784.1) (SEQ ID NO: 74), AdhE1 from *Clostridium acetobutylicum* (NP_149325.1) (SEQ ID NO: 75), AdhE2 from *Clostridium acetobutylicum* (NP_149199.1) (SEQ ID NO: 76), AdhE from *Clostridium beijerinckii* (WP_041893626.1) (SEQ ID NO: 77), AdhE1 from *Clostridium autoethanogenum* (WP_023163372.1) (SEQ ID NO: 78), or AdhE2 from *Clostridium autoethanogenum* (WP_023163373.1) (SEQ ID NO: 79). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* have native activity for this step. However, overexpression of endogenous alcohol dehydrogenase or introduction of an exogenous alcohol dehydrogenase in *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei* may be desirable to enhance product yields. *Escherichia coli* likely does not have native activity for this step.

0017 Step 40 shows the conversion of acetoacetyl-CoA to 3-hydroxy-3-methylglutaryl-CoA. This step may be catalyzed by a hydroxymethylglutaryl-CoA synthase (HMG-CoA synthase) (EC 2.3.3.10). HMG-CoA synthases are widespread across many genera and kingdoms of life and includ, e.g., MvaS from *Staphylococcus aureus* (WP_053014863.1), ERG13 from *Saccharomyces cerevisiae* (NP_013580.1), HMGCS2 from *Mus musculus* (NP_032282.2), and many other members of the EC 2.3.3.10 group of enzymes. *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and

Clostridium ragsdalei do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step.

0018 Step 41 shows the conversion of 3-hydroxy-3-methylglutanoyl-CoA to 3-methylgluconyl-CoA. This step may be catalyzed by a 3-hydroxybutyryl-CoA dehydratase (EC 4.2.1.55). The 3-hydroxybutyryl-CoA dehydratase may be, for example, LiuC from *Myxococcus xanthus* (WP_011553770.1). This step may also be catalyzed by a short-chain-enoyl-CoA hydratase (EC 4.2.1.150) or an enoyl-CoA hydratase (EC 4.2.1.17). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step.

0019 Step 42 shows the conversion of 3-methylgluconyl-CoA to 2-methylcrotonyl-CoA. This step may be catalyzed by a methylcrotonyl-CoA decarboxylase (with high structural similarity to glutaconate-CoA transferase (EC 2.8.3.12)), e.g., aibAB from *Myxococcus xanthus* (WP_011554267.1 and WP_011554268.1). This step may also be catalyzed by a methylcrotonoyl-CoA carboxylase (EC 6.4.1.4), e.g., LiuDB from *Pseudomonas aeruginosa* (NP_250702.1 and NP_250704.1) or MCCA and MCCB from *Arabidopsis thaliana* (NP_563674.1 and NP_567950.1). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step.

0020 Step 43 shows the conversion of methylcrotonyl-CoA to isovaleryl-CoA. This step may be catalyzed by an oxidoreductase, zinc-binding dehydrogenase. This oxidoreductase, zinc-binding dehydrogenase may be, for example, AibC from *Myxococcus xanthus* (WP_011554269.1). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not have known native activity for this step. *Escherichia coli* does not have known native activity for this step.

0021 Step 44 shows the conversion of isovaleryl-CoA to isovalerate. This step may be catalyzed by CoA-transferase (i.e., acetyl-CoA:acetoacetyl-CoA transferase) (EC 2.8.3.9). The CoA-transferase may be, for example, CtfAB, a heterodimer comprising subunits CtfA and CtfB, from *Clostridium beijerinckii* (CtfA, WP_012059996.1) (SEQ ID NO: 5) (CtfB, WP_012059997.1) (SEQ ID NO: 6). This step may also be catalyzed by thioesterase (EC 3.1.2.20). The thioesterase may be, for example, TesB from *Escherichia coli* (NP_414986.1) (SEQ ID NO: 7). This step may also be catalyzed by a putative thioesterase, e.g., from *Clostridium autoethanogenum* or *Clostridium ljungdahlii*. In particular, three putative thioesterases have been identified in *Clostridium autoethanogenum*: (1) "thioesterase 1" (AGY74947.1; annotated as palmitoyl-CoA hydrolase; SEQ ID NO: 8), (2) "thioesterase 2" (AGY75747.1; annotated as 4-hydroxybenzoyl-CoA thioesterase; SEQ ID NO: 9), and (3) "thioesterase 3" (AGY75999.1; annotated as putative thioesterase; SEQ ID NO: 10). Three putative thioesterases have also been identified in *Clostridium ljungdahlii*: (1) "thioesterase 1" (ADK15695.1; annotated as predicted acyl-CoA thioesterase 1; SEQ ID NO: 11), (2) "thioesterase 2" (ADK16655.1; annotated as predicted thioesterase; SEQ ID NO: 12), and (3) "thioesterase 3" (ADK16959.1; annotated as predicted thioesterase; SEQ ID NO: 13). This step may also be

catalyzed by phosphate butyryltransferase (EC 2.3.1.19) + butyrate kinase (EC 2.7.2.7). Exemplary sources for phosphate butyryltransferase and butyrate kinase are described elsewhere in this application. Native enzymes in *Clostridium autoethanogenum, Clostridium ljungdahlii*, and *Clostridium ragsdalei* (or *Escherichia coli*), such as thioesterases from *Clostridium autoethanogenum*, may catalyze this step and result in the production of some amount of downstream products. However, introduction of an exogenous enzyme or overexpression of an endogenous enzyme may be required to produce downstream products at desirable levels. Additionally, in certain embodiments, a disruptive mutation may be introduced to an endogenous enzyme, such as an endogenous thioesterase, to reduce or eliminate competition with introduced Ptb-Buk.

0022 Step 45 shows the conversion of isovalerate to isovaleraldehyde. This step may be catalyzed by aldehyde:ferredoxin oxidoreductase (EC 1.2.7.5). The aldehyde:ferredoxin oxidoreductase (AOR) may be, for example, AOR from *Clostridium autoethanogenum* (WP_013238665.1; WP_013238675.1) (SEQ ID NOs: 63 and 64, respectively) or AOR from *Clostridium ljungdahlii* (ADK15073.1; ADK15083.1) (SEQ ID NOs: 65 and 66, respectively). Further exemplary sources for aldehyde:ferredoxin oxidoreductases are described elsewhere in this application. *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* have native activity for this step. However, overexpression of endogenous AOR or introduction of an exogenous AOR in *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei* may be desirable to enhance product yields. Alternatively, exogenous AOR may be introduced into a microorganism that does not natively comprise AOR, e.g., *E. coli*. In particular, the co-expression of Ptb-Buk and AOR (and, optionally, Adh) may enable such a microorganism to produce new non-native products.

0023 Step 46 shows the conversion of isovaleraldehyde to isoamyl alcohol. This step may be catalyzed by alcohol dehydrogenase (EC 1.1.1.1. or 1.1.1.2.). Alcohol dehydrogenase can convert an aldehyde and NAD(P)H to an alcohol and NAD(P). The alcohol dehydrogenase may be, for example, Adh from *Clostridium autoethanogenum* (AGY76060.1) (SEQ ID NO: 67), *Clostridium ljungdahlii* (ADK17019.1) (SEQ ID NO: 68), or *Clostridium ragsdalei*, BdhB from *Clostridium acetobutylicum* (NP_349891.1) (SEQ ID NO: 69), Bdh from *Clostridium beijerinckii* (WP_041897187.1) (SEQ ID NO: 70), Bdh1 from *Clostridium ljungdahlii* (YP_003780648.1) (SEQ ID NO: 71), Bdh1 from *Clostridium autoethanogenum* (AGY76060.1) (SEQ ID NO: 72), Bdh2 from *Clostridium ljungdahlii* (YP_003782121.1) (SEQ ID NO: 73), Bdh2 from *Clostridium autoethanogenum* (AGY74784.1) (SEQ ID NO: 74), AdhE1 from *Clostridium acetobutylicum* (NP_149325.1) (SEQ ID NO: 75), AdhE2 from *Clostridium acetobutylicum* (NP_149199.1) (SEQ ID NO: 76), AdhE from *Clostridium beijerinckii* (WP_041893626.1) (SEQ ID NO: 77), AdhE1 from *Clostridium autoethanogenum* (WP_023163372.1) (SEQ ID NO: 78), or AdhE2 from *Clostridium autoethanogenum* (WP_023163373.1) (SEQ ID NO: 79). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* have native activity for this step. However, overexpression of endogenous alcohol dehydrogenase or introduction of an exogenous alcohol dehydrogenase in *Clostridium*

autoethanogenum, *Clostridium ljungdahlii*, or *Clostridium ragsdalei* may be desirable to enhance product yields. *Escherichia coli* likely does not have native activity for this step.

0024 Step 47 shows the conversion of isovaleryl-CoA to isovaleraldehyde. This step may be catalyzed by butyraldehyde dehydrogenase (EC 1.2.1.57). The butyraldehyde dehydrogenase may be, for example, Bld from Clostridium saccharoperbutylacetonicum (AAP42563.1) (SEQ ID NO: 80). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* likely do not have native activity for this step. *Escherichia coli* does not have known native activity for this step.

Overview of Ptb-Buk

0025 The invention provides new pathways utilizing the Ptb-Buk enzyme system. In nature, this enzyme system is found in a range of butyrate producing microorganisms, such as butyrate-producing *Clostridia* or *Butyrivibrio*. In particular, phosphate butyryltransferase (Ptb) (EC 2.3.1.19) natively catalyzes the reaction of butanoyl-CoA + phosphate to form CoA + butanoyl phosphate and butyrate kinase (Buk) (EC 2.7.2.7) natively catalyzes the reaction of butanoyl phosphate and ADP to form butyrate (butanoate) and ATP. Accordingly, these enzymes together (Ptb-Buk) natively catalyze the conversion of butanoyl-CoA to butyrate and generate one ATP via substrate level-phosphorylation (Fig. 2). However, the inventors have discovered that Ptb is promiscuous and is capable of accepting a variety of acyl-CoAs and enoyl-CoAs as substrates, such that Ptb-Buk may be used to convert a number of acyl-CoAs and enoyl-CoAs to their corresponding acids or alkenates, respectively, while simultaneously generating ATP. It has been reported Ptb is active on a range of acyl-CoAs including acetoacetyl-CoA, *in vitro* (Thompson, *Appl Environ Microbiol*, 56: 607-613, 1990). It has not previously been shown that acetoacetyl-phosphate could be a substrate for Buk. Although Buk is known to accept a broad substrate range (Liu, *Appl Microbiol Biotechnol*, 53: 545-552, 2000), no activity has been shown *in vivo*.

0026 Additionally, the inventors have discovered that the introduction of exogenous Ptb-Buk enables certain microorganisms to produce useful products, including acetone, isopropanol, isobutylene, 3-hydroxybutyrate, 1,3-butanediol, and 2-hydroxyisobutyrate, as well as other products such as propionate, caproate, and octonate.

0027 New pathways that rely on Ptb-Buk offer several major advantages over other known and existing pathway routes for production of products that rely on a CoA-transferase – as in the classic *Clostridial* acetone-butanol-ethanol (ABE) fermentation pathway – or a thioesterase (Jones, *Microbiol Rev*, 50: 484-524, 1986; Matsumoto, *Appl Microbiol Biotechnol*, 97: 205-210, 2013; May, *Metabol Eng*, 15: 218-225, 2013) (Fig. 3). In particular, these new pathways (1) are not dependent on the presence or production of particular molecules, such as organic acids, e.g., butyrate or acetate, required for the CoA-transferase reaction and (2) allow for generation of ATP via substrate level phosphorylation that would not be conserved in a thioesterase or CoA-transferase reaction. The same advantages also apply when using the Ptb-Buk system for other reactions, such as the conversion of 3-hydroxybutyryl-CoA to 3-hydroxybutyrate. Thus, these new pathways have the potential to yield

much higher production titers and rates by generating additional energy and producing target products without co-production of undesired byproducts, such as acetate.

0028 Particularly on a commercial scale, it is not desirable for microorganisms to produce acetate (or other organic acids required for the CoA transferase reaction) as byproduct, since acetate diverts carbon away from target products and thus affects the efficiency and yield of target products. Additionally, acetate may be toxic to microorganisms and/or may serve as a substrate for the growth of contaminating microorganisms. Furthermore, the presence of acetate makes it more difficult to recover and separate target products and to control fermentation conditions to favor the production of target products.

0029 ATP generation through substrate level phosphorylation can be used as a driving force for product synthesis, especially in ATP-limited systems. In particular, acetogenic bacteria are known to live on the thermodynamic edge of life (Schuchmann, *Nat Rev Microbiol*, 12: 809-821, 2014). As such, all acetogenic microorganisms isolated to date have been described to produce acetate (Drake, Acetogenic Prokaryotes, In: *The Prokaryotes*, 3rd edition, pages 354-420, New York, NY, Springer, 2006) since the production of acetate provides the microorganism with an option to directly generate ATP from substrate level phosphorylation via Pta (phosphotransacetylase) (EC 2.3.1.8) and Ack (acetate kinase) (EC 2.7.2.1). Although mechanisms such as membrane gradients and electro bifurcation enzymes coupled to ion or proton translocating systems, e.g., the Rnf complex (Schuchmann, *Nat Rev Microbiol*, 12: 809-821, 2014), conserve ATP in these microorganisms, direct ATP generation remains critical for their survival. As a result, when introducing heterologous pathways that do not allow for ATP generation, acetate is produced as a byproduct (Schiel-Bengelsdorf, *FEBS Lett*, 586: 2191-2198, 2012). The Ptb-Buk pathways described herein, however, provide an alternative mechanism for the microorganism to generate ATP via substrate level phosphorylation and, therefore, avoid acetate production. In particular, acetate-forming enzymes, such as Pta-Ack, that would otherwise be essential (Nagarajan, *Microb Cell Factories*, 12: 118, 2013) can be replaced with Ptb-Buk as an alternative means of ATP generation. Since the microorganism can then rely on ATP generation via Ptb-Buk, this system provides a driving force that ensures maximum flux through the new pathways that use Ptb-Buk. The generation of ATP may also be crucial for downstream pathways that require ATP. For example, fermentative production of isobutylene from acetone requires ATP. While the complete pathway from acetyl-CoA to isobutylene is ATP-consuming when using a CoA-transferase or a thioesterase, the pathway is energy neutral when using Ptb-Buk.

0030 Exemplary sources for Ptb and Buk are provided. However, it should be appreciated that other suitable sources for Ptb and Buk may be available. Additionally, Ptb and Buk may be engineered to improve activity and/or genes encoding Ptb-Buk may be codon-optimized for expression in particular host microorganisms.

0031 The phosphate butyryltransferase may be or may be derived, for example, from any of the following sources, the sequences of which are publically available:

Description	Microorganism	Accession
phosphate butyryltransferase	*Clostridium sp.*	EKQ52186
phosphate butyryltransferase	*Clostridium sp.*	WP_009167896
phosphate butyryltransferase	*Clostridium saccharoperbutylacetonicum*	WP_015390396
phosphate butyryltransferase	*Clostridium saccharobutylicum*	WP_022743598
phosphate butyryltransferase	*Clostridium beijerinckii*	WP_026886639
phosphate butyryltransferase	*Clostridium beijerinckii*	WP_041893500
phosphate butyryltransferase	*Clostridium butyricum*	WP_003410761
phosphate butyryltransferase	*Clostridium sp.*	CDB14331
phosphate butyryltransferase	*Clostridium botulinum*	WP_049180512
phosphate butyryltransferase	*Clostridium sp.*	CDB74819
phosphate butyryltransferase	*Clostridium paraputrificum*	WP_027098882
phosphate butyryltransferase	*Clostridium sp.*	WP_024615655
phosphate butyryltransferase	*Clostridium celatum*	WP_005211129
phosphate butyryltransferase	*Clostridium baratii*	WP_039312969
phosphate butyryltransferase	*Clostridium intestinale*	WP_021800215
phosphate butyryltransferase	*Clostridium sp.*	WP_042402499
phosphate butyryltransferase	*Clostridium sp.*	WP_032117069
phosphate butyryltransferase	*Clostridium perfringens*	ABG85761
phosphate butyryltransferase	*Clostridium botulinum*	WP_003374233
phosphate butyryltransferase	*Clostridium perfringens*	WP_004460499
phosphate butyryltransferase	*Clostridium perfringens*	WP_003454254
phosphate butyryltransferase	*Clostridium perfringens*	WP_041707926
phosphate butyryltransferase	*Clostridium perfringens*	BAB82054
phosphate butyryltransferase	*Clostridium sp.*	WP_008681116
phosphate butyryltransferase	*Clostridium chauvoei*	WP_021876993
phosphate butyryltransferase	*Clostridium colicanis*	WP_002598839
phosphate butyryltransferase	*Clostridium cadaveris*	WP_027637778
phosphate butyryltransferase	*Clostridium acetobutylicum*	WP_010966357
phosphate butyryltransferase	*Clostridium pasteurianum*	WP_015617430
phosphate butyryltransferase	*Clostridium arbusti*	WP_010238988
phosphate butyryltransferase	*Clostridium pasteurianum*	WP_003445696
phosphate butyryltransferase	*Clostridium scatologenes*	WP_029160341
phosphate butyryltransferase	*Clostridium sp.*	WP_032120461
phosphate butyryltransferase	*Clostridium drakei*	WP_032078800
phosphate butyryltransferase	*Clostridium sp.*	WP_021281241
phosphate butyryltransferase	*Clostridium argentinense*	WP_039635970
phosphate butyryltransferase	*Clostridium akagii*	WP_026883231
phosphate butyryltransferase	*Clostridium sp.*	WP_053242611
phosphate butyryltransferase	*Clostridium carboxidivorans*	WP_007063154
phosphate butyryltransferase	*Clostridium sp.*	WP_035292411

phosphate butyryltransferase	*Clostridium sulfidigenes*	WP_035133394
phosphate butyryltransferase	*Clostridium tetanomorphum*	WP_035147564
phosphate butyryltransferase	*Clostridium hydrogeniformans*	WP_027633206
phosphate butyryltransferase	*Clostridium sp.*	WP_040212965
phosphate butyryltransferase	*Candidatus Clostridium*	WP_040327613
phosphate butyryltransferase	*Clostridium sp.*	WP_040192242
phosphate butyryltransferase	*Clostridium sp.*	WP_050606427
phosphate butyryltransferase	*Clostridium lundense*	WP_027625137
phosphate butyryltransferase	*Clostridium algidicarnis*	WP_029451333
phosphate butyryltransferase	*Clostridium sp.*	WP_035306567
phosphate butyryltransferase	*Clostridium acetobutylicum*	AAA75486
phosphate butyryltransferase	*Clostridium botulinum*	WP_025775938
phosphate butyryltransferase	*Clostridium botulinum*	WP_045541062
phosphate butyryltransferase	*Clostridium botulinum*	WP_003357252
phosphate butyryltransferase	*Clostridium botulinum*	WP_030037192
phosphate butyryltransferase	*Clostridium bornimense*	WP_044039341
phosphate butyryltransferase	*Clostridium botulinum*	WP_041346554
phosphate butyryltransferase	*Clostridium sp.*	WP_053468896
phosphate butyryltransferase	*Clostridiales bacterium*	WP_034572261
phosphate butyryltransferase	*Clostridium tetani*	WP_023439553
phosphate butyryltransferase	*Clostridiales bacterium*	ERI95297
phosphate butyryltransferase	*Clostridium botulinum*	WP_047403027
phosphate butyryltransferase	*Clostridium tetani*	WP_011100667
phosphate butyryltransferase	*Clostridium tetani*	WP_035111554
phosphate butyryltransferase	*Clostridium senegalense*	WP_010295062
phosphate butyryltransferase	*Caloramator sp.*	WP_027307587
phosphate butyryltransferase	*Thermobrachium celere*	WP_018661036
phosphate butyryltransferase	*Clostridium cellulovorans*	WP_010073683
phosphate butyryltransferase	*Coprococcus comes*	CDB84786
phosphate butyryltransferase	*Coprococcus comes*	WP_008371924
phosphate butyryltransferase	*Eubacterium sp.*	CCZ03827
phosphate butyryltransferase	*Clostridium sp.*	CCZ05442
phosphate butyryltransferase	*Caloramator australicus*	WP_008907395
phosphate butyryltransferase	*Clostridium sp.*	CCY59505
phosphate butyryltransferase	*Lachnospiraceae bacterium*	WP_035626368
phosphate butyryltransferase	*Lachnospiraceae bacterium*	WP_027440767
phosphate butyryltransferase	*Fervidicella metallireducens*	WP_035381340
phosphate butyryltransferase	*Clostridium sp.*	CCX89274
phosphate butyryltransferase	*Eubacterium xylanophilum*	WP_026834525
phosphate butyryltransferase	*Roseburia sp.*	CDF44203
phosphate butyryltransferase	*Butyrivibrio crossotus*	WP_005600912
phosphate butyryltransferase	*Lachnospiraceae bacterium*	WP_027117626
phosphate butyryltransferase	*Clostridium sp.*	CDA68345
phosphate butyryltransferase	*Peptostreptococcaceae bacterium*	WP_026899905

phosphate butyryltransferase	*Butyrivibrio crossotus*	CCY77124
phosphate butyryltransferase	*Clostridium sp.*	CDE44914
phosphate butyryltransferase	*Coprococcus eutactus*	WP_004853197
phosphate butyryltransferase	*Firmicutes bacterium*	CCY23248
phosphate butyryltransferase	*Lachnospiraceae bacterium*	WP_027111007
phosphate butyryltransferase	*Lachnospiraceae bacterium*	WP_016293387
phosphate butyryltransferase	*Clostridium sp.*	WP_046822491

0032 In a preferred embodiment, the phosphate butyryltransferase is Ptb from *Clostridium acetobutylicum* (WP_010966357; SEQ ID NO: 87) or *Clostridium beijerinckii* (WP_026886639; SEQ ID NO: 88) (WP_041893500; SEQ ID NO: 89). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not natively contain phosphate butyryltransferase.

0033 The butyrate kinase may be or may be derived, for example, from any of the following sources, the sequences of which are publically available:

Description	Microorganism	Accession
butyrate kinase	*Clostridium pasteurianum*	ALB48406
butyrate kinase	*Clostridium sp.*	CDB14330
butyrate kinase	*Clostridium sp.*	CDB74820
butyrate kinase	*Clostridium sp.*	EKQ52187
butyrate kinase	*Clostridium perfringens*	Q0SQK0
butyrate kinase	*Clostridium sp.*	WP_002582660
butyrate kinase	*Clostridium colicanis*	WP_002598838
butyrate kinase	*Clostridium botulinum*	WP_003371719
butyrate kinase	*Clostridium perfringens*	WP_003454444
butyrate kinase	*Clostridium perfringens*	WP_004459180
butyrate kinase	*Clostridium celatum*	WP_005211128
butyrate kinase	*Clostridium sp.*	WP_008681112
butyrate kinase	*Clostridium sp.*	WP_008681114
butyrate kinase	*Clostridium sp.*	WP_009167897
butyrate kinase	*Clostridium perfringens*	WP_011010889
butyrate kinase	*Clostridium beijerinckii*	WP_011967556
butyrate kinase	*Clostridium botulinum*	WP_012422882
butyrate kinase	*Clostridium botulinum*	WP_012450845
butyrate kinase	*Clostridium saccharoperbutylacetonicum*	WP_015390397
butyrate kinase	*Clostridium beijerinckii*	WP_017209677
butyrate kinase	*Clostridium botulinum*	WP_017825911
butyrate kinase	*Clostridium chauvoei*	WP_021876994
butyrate kinase	*Clostridium saccharobutylicum*	WP_022743599
butyrate kinase	*Clostridium sp.*	WP_024615656
butyrate kinase	*Clostridium perfringens*	WP_025648345
butyrate kinase	*Clostridium beijerinckii*	WP_026886638
butyrate kinase	*Clostridium paraputrificum*	WP_027098883

butyrate kinase	*Clostridium sp.*	WP_032117070
butyrate kinase	*Clostridium botulinum*	WP_035786166
butyrate kinase	*Clostridium baratii*	WP_039312972
butyrate kinase	*Clostridium diolis*	WP_039772701
butyrate kinase	*Clostridium botulinum*	WP_041082388
butyrate kinase	*Clostridium beijerinckii*	WP_041893502
butyrate kinase	*Clostridium sp.*	WP_042402497
butyrate kinase	*Clostridium baratii*	WP_045725505
butyrate kinase	*Clostridium perfringens*	WP_049039634
butyrate kinase	*Clostridium botulinum*	WP_049180514
butyrate kinase	*Clostridium botulinum*	WP_053341511
butyrate kinase	*Clostridium butyricum*	ABU40948
butyrate kinase	*Clostridium sp.*	CDE44915
butyrate kinase	*Clostridium senegalense*	WP_010295059
butyrate kinase	*Clostridium intestinale*	WP_021800216
butyrate kinase	*Eubacterium ventriosum*	WP_005363839
butyrate kinase	*Clostridiales bacterium*	WP_021657038
butyrate kinase	*Clostridium sp.*	WP_021281242
butyrate kinase	*Clostridium sporogenes*	WP_045520059
butyrate kinase	*Clostridium sp.*	WP_050606428
butyrate kinase	*Clostridium botulinum*	WP_012048334
butyrate kinase	*Clostridium botulinum*	WP_012343352
butyrate kinase	*Clostridium botulinum*	WP_003401518
butyrate kinase	*Clostridium argentinense*	WP_039635972
butyrate kinase	*Clostridium botulinum*	WP_003357547
butyrate kinase	*Clostridium hydrogeniformans*	WP_027633205
butyrate kinase	*Clostridium botulinum*	WP_033066487
butyrate kinase	*Roseburia sp.*	CDF44202
butyrate kinase	*Lachnospiraceae bacterium*	WP_027111008
butyrate kinase	*Clostridium sp.*	CDA68344
butyrate kinase	*Lachnospiraceae bacterium*	WP_022782491
butyrate kinase	*Clostridium botulinum*	WP_012101111
butyrate kinase	*Clostridium carboxidivorans*	WP_007063155
butyrate kinase	*Clostridium botulinum*	WP_041346556
butyrate kinase	*Clostridium drakei*	WP_032078801
butyrate kinase	*Clostridium sp.*	WP_032120462
butyrate kinase	*Clostridium sp.*	WP_053468897
butyrate kinase	*Firmicutes bacterium*	CCZ27888
butyrate kinase	*Clostridium sp.*	WP_035306569
butyrate kinase	*Coprococcus comes*	CDB84787
butyrate kinase	*Clostridium sp.*	WP_035292410
butyrate kinase	*Clostridium sp.*	CCX89275
butyrate kinase	*Clostridium sp.*	WP_040212963
butyrate kinase	*Clostridium pasteurianum*	WP_003445697

butyrate kinase	*Clostridium sp.*	WP_053242610
butyrate kinase	*Lachnospiraceae bacterium*	WP_016299320
butyrate kinase	*Lachnospiraceae bacterium*	WP_022785085
butyrate kinase	*Lachnospiraceae bacterium*	WP_016281561
butyrate kinase	*Eubacterium sp.*	CDA28786
butyrate kinase	*Clostridium scatologenes*	WP_029160342
butyrate kinase	*Lachnospiraceae bacterium*	WP_016228168
butyrate kinase	*Clostridium pasteurianum*	WP_015617429
butyrate kinase	*Clostridium algidicarnis*	WP_029451332
butyrate kinase	*Lachnospiraceae bacterium*	WP_016293388
butyrate kinase	*Clostridium sulfidigenes*	WP_035133396
butyrate kinase	*Clostridium tetani*	WP_011100666
butyrate kinase	*Clostridium tetanomorphum*	WP_035147567
butyrate kinase	*Subdoligranulum variabile*	WP_007045828
butyrate kinase	*Eubacterium sp.*	CCZ03826
butyrate kinase	*Firmicutes bacterium*	CDF07483
butyrate kinase	*Eubacterium sp.*	CDB13677
butyrate kinase	*Clostridium sp.*	WP_008400594
butyrate kinase	*Clostridium tetani*	WP_023439552
butyrate kinase	*Clostridiales bacterium*	WP_022787536
butyrate kinase	*Lachnospiraceae bacterium*	WP_027434709
butyrate kinase	*Firmicutes bacterium*	CCY23249
butyrate kinase	*Clostridium acetobutylicum*	WP_010966356

0034 In a preferred embodiment, the butyrate kinase is Buk from *Clostridium acetobutylicum* (WP_010966356; SEQ ID NO: 90) or *Clostridium beijerinckii* (WP_011967556; SEQ ID NO: 91) (WP_017209677; SEQ ID NO: 92) (WP_026886638; SEQ ID NO: 93) (WP_041893502; SEQ ID NO: 94). *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* do not natively contain butyrate kinase.

0035 Since Ptb-Buk has been shown to function on a broad range of substrates it is reasonable to assume that if Ptb-Buk does not exhibit any activity and a desired substrate it can be engineered to achieve activity on the substrate in question. A strategy could be (but would not be limited to) rational design based on available crystal structures of Ptb and Buk with and without a bound substrate where the binding pocket would be changed to accommodate the new substrate or through saturation mutagenesis. When activity is obtained, it can be further improved through iterative cycles of enzyme engineering. These engineering efforts would be combined with assays to test enzyme activity. These types of strategies have previously proven effective (see, e.g., Huang, *Nature*, 537: 320-327, 2016; Khoury, *Trends Biotechnol*, 32: 99-109, 2014; Packer, *Nature Rev Genetics*, 16: 379-394, 2015; Privett, *PNAS USA*, 109: 3790-3795, 2012).

0036 To improve substrate specificity of Ptb-Buk towards a specific acyl-CoA substrate, Ptb-Buk variants from public databases or generated Ptb-Buk mutants (for example, from directed evolution) can be screened using a high throughput assay, namely overexpressing Ptb-Buk enzyme pairs in *E. coli*, adding a test substrate, and screening for ATP production with a bioluminescence assay. The assay can use the well-established practice of correlating ATP concentration with firefly luciferase enzyme bioluminescence. The amenability of this assay to multi-well plate formats would facilitate efficient screening of substrate preference across new Ptb-Buk combinations (Fig. 33).

0037 By screening for ATP production rather than depletion of substrate or accumulation of product, the assay avoids measuring spontaneous hydrolysis of the CoA group. However, an alternative approach described in literature, is to use free CoA can be measured using the established assay using Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB) (Thompson, Appl Environ Microbiol, 56: 607-613, 1990.) in order to estimate the coupling efficiency of the Ptb-Buk reactions (Fig. 33). Acyl-CoAs and corresponding free acids and phospho-intermediates can also be measured during the validation phase using LC-MS/MS.

0038 In a high-throughput screening approach, it is difficult gather kinetic data due to the labor involved in protein quantification. Instead, for each preparation of *E. coli* lysate containing Ptb-Buk enzymes, the activity against each substrate of interest (measured as luminescence per unit time) can be compared to the activity against the positive control substrate (butyryl-CoA) and against acetyl-CoA (the physiological substrate that will likely provide the greatest competition for enzyme active sites against target acyl-CoA).

0039 In order to ensure that the assay is not biased due to native phosphotransacetylase (Pta) and/or acetate kinase (Ack) activity, the assay can also be evaluated in an *E. coli* strain where *pta* and/or *ack* genes have been knocked out.

Production of Acetone and Isopropanol

0040 Acetone and isopropanol are important industrial solvents with a combined market size of 8 million tons and a global market value of $8.5-11 billion. In addition, acetone and isopropanol are precursors to valuable downstream products, including polymethyl methacrylate (PMMA), which has a global market value of $7 billion, isobutylene, which has a global market value of $25-29 billion, and propylene, which has a global market value of $125 billion. Additionally, a route from acetone to jet fuel has recently been reported. Currently, industrial acetone production is directly linked to petrochemical phenol production, as it is a by-product of the cumene process. Around 92% of acetone output by volume is a co-product of phenol production from cumene. This has significant implications on both environment and market. In the cumene process, per mol phenol produced one mol of sodium sulfite accumulates posing a serious waste management problem and a challenge to natural environments and human health. The world market demand for phenol is expected to stagnate or decline, while the demand for acetone is predicted to rise. Alternative phenol production routes from

direct oxidation of benzene are in development and expected to commercialize soon; this could result in a complete elimination of acetone production.

0041 Acetone has been produced at industrial scale for almost 100 years, as a by-product of butanol in ABE fermentation. While industrial ABE fermentation declined in the second half of the 20th century due to low oil prices and high sugar costs, it has recently revived, with several commercial plants built during the last few years. Multiple groups have also demonstrated acetone production from sugar in heterologous hosts that express the corresponding enzymes from ABE fermentation organisms, in particular *E. coli* and yeast through metabolic engineering and synthetic biology approaches by several academic groups. However, low yields and high costs associated the pre-treatment needed to release the polysaccharide-component of biomass make the production of acetone via standard fermentation uneconomic as current biochemical conversion technologies do not utilize the lignin component of biomass, which can constitute up to 40% of this material.

0042 The invention provides a microorganism capable of producing acetone or precursors thereof from a substrate. The invention further provides a method of producing acetone or precursors thereof by culturing such a microorganism in the presence of a substrate. In preferred embodiments, the microorganism is derived from a parental microorganism selected from the group consisting of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. However, the microorganism may also be derived from an entirely different microorganism, e.g., *Eschericia coli*. The enzymatic pathways described for the production of acetone may comprise endogenous enzymes and, where endogenous enzyme activity is absent or low, exogenous enzymes.

0043 Acetone via steps 1, 2, and 3: In one embodiment, the invention provides a microorganism comprising enzymes for steps 1, 2, and 3, whereby the microorganism is capable of producing acetone or precursors thereof from a substrate, such as a gaseous substrate. Typically, at least one of the enzymes in this pathway is exogenous to the microorganism. In a preferred embodiment, step 2 is catalyzed by Ptb-Buk. Exemplary types and sources of enzymes for steps 1, 2, and 3 are described elsewhere in this application. If the microorganism is derived from a parental microorganism that natively contains a primary:secondary alcohol dehydrogenase capable of converting acetone to isopropanol (step 4) (e.g., *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*), the microorganism may be modified to knock down or knock out the expression of primary:secondary alcohol dehydrogenase (e.g., by disrupting the gene encoding the primary:secondary alcohol dehydrogenase), such that the microorganism produces acetone without converting it to isopropanol (WO 2015/085015).

0044 Acetone via steps 1, 13, 14, 15, and 3: In one embodiment, the invention provides a microorganism comprising exogenous enzymes for steps 1, 13, 14, 15, and 3, whereby the microorganism is capable of producing acetone or precursors thereof from a substrate, such as a gaseous substrate. Typically, at least one of the enzymes in this pathway is exogenous to the microorganism. In a preferred embodiment, step 14 is catalyzed by Ptb-Buk. Exemplary types and

sources of enzymes for steps 1, 13, 14, 15, and 3 are described elsewhere in this application. If the microorganism is derived from a parental microorganism that natively contains a primary:secondary alcohol dehydrogenase capable of converting acetone to isopropanol (step 4) (e.g., *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*), the microorganism may be modified to knock down or knock out the expression of primary:secondary alcohol dehydrogenase (e.g., by disrupting the gene encoding the primary:secondary alcohol dehydrogenase), such that the microorganism produces acetone without converting it to isopropanol (WO 2015/085015).

0045 In one embodiment, the microorganism may comprise more than one pathway for the production of acetone.

0046 The invention provides a microorganism capable of producing isopropanol or precursors thereof from a substrate. The invention further provides a method of producing isopropanol or precursors thereof by culturing such a microorganism in the presence of a substrate. In preferred embodiments, the microorganism is derived from a parental microorganism selected from the group consisting of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. However, the microorganism may also be derived from an entirely different microorganism, e.g., *Escherichia coli*. The enzymatic pathways described for the production of isopropanol may comprise endogenous enzymes and, where endogenous enzyme activity is absent or low, exogenous enzymes.

0047 Isopropanol via steps 1, 2, 3, and 4: In one embodiment, the invention provides a microorganism comprising enzymes for steps 1, 2, 3, and 4, whereby the microorganism is capable of producing isopropanol or precursors thereof from a substrate, such as a gaseous substrate. Typically, at least one of the enzymes in this pathway is exogenous to the microorganism. In a preferred embodiment, step 2 is catalyzed by Ptb-Buk. Exemplary types and sources of enzymes for steps 1, 2, 3, and 4 are described elsewhere in this application. If the microorganism is derived from a parental microorganism that natively contains a primary:secondary alcohol dehydrogenase capable of converting acetone to isopropanol (step 4) (e.g., *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*), introduction of an exogenous enzyme for step 4 is not required to produce isopropanol. However, modification of the microorganism, for example, to overexpress a native primary:secondary alcohol dehydrogenase may result in enhanced production of isopropanol.

0048 Isopropanol via steps 1, 13, 14, 15, 3, and 4: In one embodiment, the invention provides a microorganism comprising enzymes for steps 1, 13, 14, 15, 3, and 4, whereby the microorganism is capable of producing isopropanol or precursors thereof from a substrate, such as a gaseous substrate. Typically, at least one of the enzymes in this pathway is exogenous to the microorganism. In a preferred embodiment, step 14 is catalyzed by Ptb-Buk. Exemplary types and sources of enzymes for steps 1, 13, 14, 15, 3, and 4 are described elsewhere in this application. If the microorganism is derived from a parental microorganism that natively contains a primary:secondary alcohol dehydrogenase capable of converting acetone to isopropanol (step 4) (e.g., *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*), introduction of an exogenous

enzyme for step 4 is not required to produce isopropanol. However, modification of the microorganism, for example, to overexpress a native primary:secondary alcohol dehydrogenase may result in enhanced production of isopropanol.

0049 In one embodiment, the microorganism may comprise more than one pathway for the production of isopropanol.

Production of Isobutylene

0050 Isobutylene is a major chemical building block with a market size of over 15 million tons and a global market value of $ 25-29 billion. Beyond its use in chemistry and as a fuel additive (15 Mt/yr), isobutylene may be converted to isooctane, a high performance, drop-in fuel for gasoline cars. Global Bioenergies has filed patent applications on the fermentative production of isobutene (i.e., isobutylene) from acetone, but none of the disclosed routes involve Ptb-Buk (WO 2010/001078; EP 2295593; WO 2011/076691; van Leeuwen, *Appl Microbiol Biotechnol*, 93: 1377-1387, 2012).

0051 The invention provides a microorganism capable of producing isobutylene or precursors thereof from a substrate. The invention further provides a method of producing isobutylene or precursors thereof by culturing such a microorganism in the presence of a substrate. In preferred embodiments, the microorganism is derived from a parental microorganism selected from the group consisting of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. However, the microorganism may also be derived from an entirely different microorganism, e.g., *Eschericia coli*. The enzymatic pathways described for the production of isobutylene may comprise endogenous enzymes and, where endogenous enzyme activity is absent or low, exogenous enzymes.

0052 Fig. 1 shows two alternative routes to isobutylene. The first involves the production of isobutylene via steps 1, 2, 3, 5, and 6. The second involves the production of isobutylene via steps 1, 2, 3, 7, 8, and 6. Steps 2 and 8 may be catalyzed by Ptb-Buk. Accordingly, each route may involve Ptb-Buk.

0053 Isobutylene via steps 1, 2, 3, 5, and 6: In one embodiment, the invention provides a microorganism comprising enzymes for steps 1, 2, 3, 5, and 6, whereby the microorganism is capable of producing isobutylene or precursors thereof from a substrate, such as a gaseous substrate. Typically, at least one of the enzymes in this pathway is exogenous to the microorganism. In a preferred embodiment, step 2 is catalyzed by Ptb-Buk. Exemplary types and sources of enzymes for steps 1, 2, 3, 5, and 6 are described elsewhere in this application. If the microorganism is derived from a parental microorganism that natively contains a primary:secondary alcohol dehydrogenase capable of converting acetone to isopropanol (step 4) (e.g., *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*), the microorganism may be modified to knock down or knock out the expression of primary:secondary alcohol dehydrogenase (e.g., by disrupting the gene encoding the primary:secondary alcohol dehydrogenase) to prevent the conversion of acetone to isopropanol and maximize the conversion of acetone to isobutylene.

0054 Isobutylene via steps 1, 2, 3, 7, 8, and 6: In one embodiment, the invention provides a microorganism comprising enzymes for steps 1, 2, 3, 7, 8, and 6, whereby the microorganism is capable of producing isobutylene or precursors thereof from a substrate, such as a gaseous substrate. Typically, at least one of the enzymes in this pathway is exogenous to the microorganism. In a preferred embodiment, step 2 and/or step 8 are catalyzed by Ptb-Buk. Exemplary types and sources of enzymes for steps 1, 2, 3, 7, 8, and 6 are described elsewhere in this application. If the microorganism is derived from a parental microorganism that natively contains a primary:secondary alcohol dehydrogenase capable of converting acetone to isopropanol (step 4) (e.g., *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*), the microorganism may be modified to knock down or knock out the expression of primary:secondary alcohol dehydrogenase (e.g., by disrupting the gene encoding the primary:secondary alcohol dehydrogenase) to prevent the conversion of acetone to isopropanol and maximize the conversion of acetone to isobutylene.

Production of 3-Hydroxybutyrate

0055 3-Hydroxybutyrate (3-HB) is a four carbon carboxylic acid in the family of betahydroxy acids. 3-hydroxybutyrate is a cosmetic ingredient for oily skin clarification, an intermediate for anti-aging cream formulations, an intermediate for polyhydroxybutyrate (PHB), a biodegradable polymer resin, and co-monomer with other polyhydroxy acids for novel bioplastics. Additionally, 3-hydroxybutyrate has specialty applications in biocompatible and biodegradable nanocomposites, particularly for medical implants, intermediate for C3/C4 chemicals, chiral building blocks, and fine chemicals. Although the production of (R)- and (S)-3-hydroxybutyrate by recombinant *E. coli* grown on glucose, the production of 3-hydroxybutyrate has not been demonstrated from microorganisms grown on gaseous substrates (Tseng, *Appl Environ Microbiol*, 75: 3137-3145, 2009). Notably, the system previously demonstrated in *E. coli* was not directly transferrable to acetogens, including *C. autoethanogenum*, due to the presence of native thioesterases in acetogens. Although *E. coli* also has a thioesterase TesB that can act on 3-HB-CoA, Tseng showed that background activity is minimal (< 0.1 g/L). While in *E. coli* production of stereopure isomers were reported, the inventors surprisingly found that a mix of isomers were produced in *C. autoethanogenum*. Without being bound to this theory, this is likely a result of native isomerase activivty. This enables the combination of an (S)-specific 3-hydroxybutyryl-CoA dehydrogenase (Hbd) to be combined with the (R)-specific Ptb-Buk for optimized production. To produce stereopure isomers, this activity can be knocked-out. Taken together, it this invention enables to produce several g/L of 3-HB compared to low production in *E. coli* and using Ptb-Buk any combination of (R)- or (S)-specific 3-hydroxybutyryl-CoA dehydrogenase and native *Clostridium autoethanogenum* thioesterase.

0056 The invention provides a microorganism capable of producing 3-hydroxybutyrate or precursors thereof from a substrate. The invention further provides a method of producing 3-hydroxybutyrate or precursors thereof by culturing such a microorganism in the presence of a substrate. In preferred embodiments, the microorganism is derived from a parental microorganism selected from the group consisting of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or

Clostridium ragsdalei. However, the microorganism may also be derived from an entirely different microorganism, e.g., *Eschericia coli*. The enzymatic pathways described for the production of 3-hydroxybutyrate may comprise endogenous enzymes and, where endogenous enzyme activity is absent or low, exogenous enzymes.

0057 Fig. 1 shows two alternative routes to 3-hydroxybutyrate. The first involves the production of 3-hydroxybutyrate via steps 1, 2, and 15. The second involves the production of 3-hydroxybutyrate via steps 1, 13, and 14. Steps 2 and 14 may be catalyzed by Ptb-Buk. Accordingly, each route may involve Ptb-Buk. In one embodiment, the microorganism may comprise more than one pathway for the production of 3-hydroxybutyrate, wherein Ptb-Buk may catalyze more than one step (e.g., steps 2 and 14).

0058 3-Hydroxybutyrate via steps 1, 2, and 15: In one embodiment, the invention provides a microorganism comprising enzymes for steps 1, 2, and 15, whereby the microorganism is capable of producing 3-hydroxybutyrate or precursors thereof from a substrate, such as a gaseous substrate. Typically, at least one of the enzymes in this pathway is exogenous to the microorganism. In a preferred embodiment, step 2 is catalyzed by Ptb-Buk. Exemplary types and sources of enzymes for steps 1, 2, and 15 are described elsewhere in this application.

0059 3-Hydroxybutyrate via steps 1, 13, and 14: In one embodiment, the invention provides a microorganism comprising enzymes for steps 1, 13, and 14, whereby the microorganism is capable of producing 3-hydroxybutyrate or precursors thereof from substrate, such as a gaseous substrate. Typically, at least one of the enzymes in this pathway is exogenous to the microorganism. In a preferred embodiment, step 14 is catalyzed by Ptb-Buk. Exemplary types and sources of enzymes for steps 1, 13, and 14 are described elsewhere in this application.

Production of 1,3-Butanediol

0060 1,3-Butanediol (1,3-BDO) is commonly used as a solvent for food flavoring agents and is a co-monomer used in certain polyurethane and polyester resins. More importantly, 1,3-butanediol may be catalytically converted to 1,3-butadiene (Makshina, *Chem Soc Rev*, 43: 7917-7953, 2014). Butadiene is used to produce rubber, plastics, lubricants, latex, and other products. While much of the butadiene produced today is used for the rubber in automobile tires, it can also be used to produce adiponitrile, which can be used in the manufacture of nylon 6,6. Global demand for butadiene is on the rise. In 2011, there was an estimated 10.5 million tons of demand, valued at $40 billion.

0061 The invention provides a microorganism capable of producing 1,3-butanediol or precursors thereof from a substrate. The invention further provides a method of producing 1,3-butanediol or precursors thereof by culturing such a microorganism in the presence of substrate. In preferred embodiments, the microorganism is derived from a parental microorganism selected from the group consisting of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. However, the microorganism may also be derived from an entirely different microorganism, e.g., *Eschericia coli*. The enzymatic pathways described for the production of 1,3-butanediol may

comprise endogenous enzymes and, where endogenous enzyme activity is absent or low, exogenous enzymes.

0062 In certain embodiments, the microorganism may produce 1,3-butanediol without co-production of ethanol (or with production of only a small amount of ethanol, e.g., less than 0.1-1.0 g/L ethanol or less than 1-10 g/L ethanol).

0063 Fig. 1 shows three alternative routes to 1,3-butanediol. The first involves the production of 1,3-butanediol via steps 1, 2, 15, 16, and 17. The second involves the production of 1,3-butanediol via steps 1, 13, 14, 16, and 17. The third involves the production of 1,3-butanediol via steps 1, 13, 18, and 17. Steps 2 and 14 may be catalyzed by Ptb-Buk. Accordingly, at least the first and second routes may involve Ptb-Buk. In one embodiment, the microorganism may comprise more than one pathway for the production of 1,3-butanediol. In a related embodiment, the Ptb-Buk may catalyze more than one step (e.g., steps 2 and 14).

0064 1,3-Butanediol via steps 1, 2, 15, 16, and 17: In one embodiment, the invention provides a microorganism comprising enzymes for steps 1, 2, 15, 16, and 17, whereby the microorganism is capable of producing 1,3-butanediol or precursors thereof from a substrate, such as a gaseous substrate. Typically, at least one of the enzymes in this pathway is exogenous to the microorganism. In a preferred embodiment, step 2 is catalyzed by Ptb-Buk. Exemplary types and sources of enzymes for steps 1, 2, 15, 16, and 17 are described elsewhere in this application.

0065 1,3-Butanediol via steps 1, 13, 14, 16, and 17: In one embodiment, the invention provides a microorganism comprising enzymes for steps 1, 13, 14, 16, and 17, whereby the microorganism is capable of producing 1,3-butanediol or precursors thereof from a substrate, such as a gaseous substrate. Typically, at least one of the enzymes in this pathway is exogenous to the microorganism. In a preferred embodiment, step 14 is catalyzed by Ptb-Buk. Exemplary types and sources of enzymes for steps 1, 13, 14, 16, and 17 are described elsewhere in this application.

0066 1,3-Butanediol via steps 1, 13, 18, and 17: In one embodiment, the invention provides a microorganism comprising enzymes for steps 1, 13, 18, and 17, whereby the microorganism is capable of producing 1,3-butanediol or precursors thereof from a substrate, such as a gaseous substrate (Fig. 11). Typically, at least one of the enzymes in this pathway is exogenous to the microorganism. Exemplary types and sources of enzymes for steps 1, 13, 18, and 17 are described elsewhere in this application. A similar route has been demonstrated in *E. coli*, but not in acetogens such as *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* (Kataoka, *J Biosci Bioeng*, 115: 475-480, 2013). Although the use of Ptb-Buk results in the production of (R)-1,3-butanediol, this route, which does not require the use of Ptb-Buk, may result in the production of (S)-1,3-butanediol.

Production of 2-Hydroxyisobutyrate

0067 2-Hydroxyisobutyrate (2-HIB) is a four carbon carboxylic acid that may serve as a building block for many types of polymers. The methyl ester of methacrylic acid, which can be synthesized by dehydration of 2-hydroxyisobutyrate or via the corresponding amide, is polymerized to polymethylmethacrylate (PMMA) for the production of acrylic glass, durable coatings, and inks. For this compound alone, the global market exceeds 3 million tons. Other branched C4 carboxylic acids, e.g., chloro- and amino- derivatives of 2-hydroxyisobutyrate, as well as isobutylene glycol and its oxide, are also used in polymers and for many other applications.

0068 The stereospecificity of the Ptb-Buk system is particularly useful in overcoming the limitations of the current state of art with respect to the production of 2-hydroxyisobutyrate. Both Ptb-Buk and thioesterases are promiscuous, such that side activity with 3-hydroxybutyryl-CoA may divert resources away from target pathways for the production of 2-hydroxyisobutyryl-CoA (see, e.g., Fig. 1 and Fig. 8). However, Ptb-Buk is able to distinguish between stereoisomers and will act on (R)-3-hydroxybutyryl-CoA, but not on (S)-3-hydroxybutyryl-CoA. In contrast, thioesterases are not able to distinguish between 3-hydroxybutyryl-CoA stereoisomers. In a preferred embodiment, an (S)-specific acetoacetyl-CoA hydratase (EC 4.2.1.119) (step 13) is chosen in combination with the Ptb-Buk (step 20) to avoid losses to 3-hydroxybutyrate and maximize 2-hydroxyisobutyrate yield (Fig. 8). The (S)-specific form of 3-hydroxybutyryl-CoA is also the preferred substrate for the 2-hydroxyisobutyryl-CoA mutase (EC 5.4.99.-) (step 19) (Yaneva, *J Biol Chem*, 287: 15502-15511, 2012).

0069 The invention provides a microorganism capable of producing 2-hydroxyisobutyrate or precursors thereof from a substrate. The invention further provides a method of producing 2-hydroxyisobutyrate or precursors thereof by culturing such a microorganism in the presence of a substrate. In preferred embodiments, the microorganism is derived from a parental microorganism selected from the group consisting of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. However, the microorganism may also be derived from an entirely different microorganism, e.g., *Eschericia coli*. The enzymatic pathways described for the production of 2-hydroxyisobutyrate may comprise endogenous enzymes and, where endogenous enzyme activity is absent or low, exogenous enzymes.

0070 2-Hydroxyisobutyrate via steps 1, 13, 19, and 20: In one embodiment, the invention provides a microorganism comprising enzymes for steps 1, 13, 19, and 20, whereby the microorganism is capable of producing 2-hydroxyisobutyrate or precursors thereof from a substrate, such as a gaseous substrate. Typically, at least one of the enzymes in this pathway is exogenous to the microorganism. In a preferred embodiment, step 20 is catalyzed by Ptb-Buk. Exemplary types and sources of enzymes for steps 1, 13, 19, and 20 are described elsewhere in this application.

0071 In certain embodiments, the invention also provides a microorganism capable of producing 2-hydroxybutyrate (2-HB) or precursors thereof from a substrate. The invention further provides a

method of producing 2-hydroxybutyrate or precursors thereof by culturing such a microorganism in the presence of a substrate. Without wishing to be bound by any particular theory, the inventors believe the observed production of 2-hydroxybutyrate is attributable to nonspecific mutase activity in microorganisms such as *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei*.

Production of Adipic Acid

0072 Adipic acid is the most important dicarboxylic acid with an estimated market of greater US $4.5 billion with about 2.5 billion kgs produced annually. Over 60% of produced adipic acid is being used as monomer precursor for the production of nylon and the global market for adipic acid is expected to reach US $7.5 billion by 2019. Currently, adipic acid is almost excusively produced petrochemically, e.g. by carbonylation of butadiene.

0073 The invention provides a microorganism capable of producing adipic acid or precursors thereof from a substrate (Fig. 34). The invention further provides a method of producing adipic acid or precursors thereof by culturing such a microorganism in the presence of a substrate. In preferred embodiments, the microorganism is derived from a parental microorganism selected from the group consisting of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. However, the microorganism may also be derived from an entirely different microorganism, e.g., *Eschericia coli*. The enzymatic pathways described for the production of adipic acid may comprise endogenous enzymes and, where endogenous enzyme activity is absent or low, exogenous enzymes.

0074 Adipic acid via steps 22, 23, 24, 25, and 26: In one embodiment, the invention provides a microorganism comprising enzymes for steps 22, 23, 24, 25, and 26, whereby the microorganism is capable of producing adipic acid or precursors thereof from a substrate, such as a gaseous substrate. Typically, at least one of the enzymes in this pathway is exogenous to the microorganism. In a preferred embodiment, step 26 is catalyzed by Ptb-Buk. Exemplary types and sources of enzymes for steps 22, 23, 24, 25, and 26 are described elsewhere in this application.

0075 Adipic acid via steps 21, 22, 23, 24, 25, and 26: In one embodiment, the invention provides a microorganism comprising enzymes for steps 21, 22, 23, 24, 25, and 26, whereby the microorganism is capable of producing adipic acid or precursors thereof from a substrate, such as a gaseous substrate. Typically, at least one of the enzymes in this pathway is exogenous to the microorganism. In a preferred embodiment, step 26 is catalyzed by Ptb-Buk. Exemplary types and sources of enzymes for steps 21, 22, 23, 24, 25, and 26 are described elsewhere in this application.

0076 In one embodiment, the microorganism may comprise more than one pathway for the production of adipic acid.

Production of 1,3-Hexanediol

0077 The invention provides a microorganism capable of producing 1,3-hexanediol or precursors thereof from a substrate (Fig. 35). The invention further provides a method of producing 1,3-

hexanediol or precursors thereof by culturing such a microorganism in the presence of a substrate. In preferred embodiments, the microorganism is derived from a parental microorganism selected from the group consisting of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. However, the microorganism may also be derived from an entirely different microorganism, e.g., *Escherichia coli*. The enzymatic pathways described for the production of 1,3-hexanediol may comprise endogenous enzymes and, where endogenous enzyme activity is absent or low, exogenous enzymes.

0078 The pathways depicted in Fig. 35 begin with 3-hydroxybutyryl-CoA, which may be produced via steps 1 and 13, as depicted in Fig. 1.

0079 1,3-Hexanediol via steps 1, 13, 27, 31, 32, 36, 37, 38, and 39: In one embodiment, the invention provides a microorganism comprising enzymes for steps 1, 13, 27, 31, 32, 36, 37, 38, and 39, whereby the microorganism is capable of producing 1,3-hexanediol or precursors thereof from a substrate, such as a gaseous substrate. Typically, at least one of the enzymes in this pathway is exogenous to the microorganism. In a preferred embodiment, step 37 is catalyzed by Ptb-Buk. Exemplary types and sources of enzymes for steps 1, 13, 27, 31, 32, 36, 37, 38, and 39 are described elsewhere in this application.

Production of 3-Methyl-2-butanol

0080 The invention provides a microorganism capable of producing 3-methyl-2-butanol or precursors thereof from a substrate (Fig. 35). The invention further provides a method of producing 3-methyl-2-butanol or precursors thereof by culturing such a microorganism in the presence of a substrate. In preferred embodiments, the microorganism is derived from a parental microorganism selected from the group consisting of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. However, the microorganism may also be derived from an entirely different microorganism, e.g., *Escherichia coli*. The enzymatic pathways described for the production of 3-methyl-2-butanol may comprise endogenous enzymes and, where endogenous enzyme activity is absent or low, exogenous enzymes.

0081 The pathways depicted in Fig. 35 begin with 3-hydroxybutyryl-CoA, which may be produced via steps 1 and 13, as depicted in Fig. 1.

0082 3-Methyl-2-butanol via steps 1, 13, 27, 31, 32, 33, 34, and 35: In one embodiment, the invention provides a microorganism comprising enzymes for steps 1, 13, 27, 31, 32, 33, 34, and 35, whereby the microorganism is capable of producing 3-methyl-2-butanol or precursors thereof from a substrate, such as a gaseous substrate. Typically, at least one of the enzymes in this pathway is exogenous to the microorganism. In a preferred embodiment, step 33 is catalyzed by Ptb-Buk. Exemplary types and sources of enzymes for steps 1, 13, 27, 31, 32, 33, 34, and 35 are described elsewhere in this application.

Production of 2-Buten-1-ol

0083 The invention provides a microorganism capable of producing 2-buten-1-ol or precursors thereof from a substrate (Fig. 35). The invention further provides a method of producing 2-buten-1-ol or precursors thereof by culturing such a microorganism in the presence of a substrate. In preferred embodiments, the microorganism is derived from a parental microorganism selected from the group consisting of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. However, the microorganism may also be derived from an entirely different microorganism, e.g., *Eschericia coli*. The enzymatic pathways described for the production of 2-buten-1-ol may comprise endogenous enzymes and, where endogenous enzyme activity is absent or low, exogenous enzymes.

0084 The pathways depicted in Fig. 35 begin with 3-hydroxybutyryl-CoA, which may be produced via steps 1 and 13, as depicted in Fig. 1.

0085 2-Buten-1-ol via steps 1, 13, 27, 28, 29, and 30: In one embodiment, the invention provides a microorganism comprising enzymes for steps 1, 13, 27, 28, 29, and 30, whereby the microorganism is capable of producing 2-buten-1-ol or precursors thereof from a substrate, such as a gaseous substrate. Typically, at least one of the enzymes in this pathway is exogenous to the microorganism. In a preferred embodiment, step 28 is catalyzed by Ptb-Buk. Exemplary types and sources of enzymes for steps 1, 13, 27, 28, 29, and 30 are described elsewhere in this application.

Production of Isovalerate

0086 The invention provides a microorganism capable of producing isovalerate or precursors thereof from a substrate (Fig. 36). The invention further provides a method of producing isovalerate or precursors thereof by culturing such a microorganism in the presence of a substrate. In preferred embodiments, the microorganism is derived from a parental microorganism selected from the group consisting of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. However, the microorganism may also be derived from an entirely different microorganism, e.g., *Eschericia coli*. The enzymatic pathways described for the production of isovalerate may comprise endogenous enzymes and, where endogenous enzyme activity is absent or low, exogenous enzymes.

0087 Isovalerate via steps 1, 40, 41, 42, 43, and 44: In one embodiment, the invention provides a microorganism comprising enzymes for steps 1, 40, 41, 42, 43, and 44, whereby the microorganism is capable of producing isovalerate or precursors thereof from a substrate, such as a gaseous substrate. Typically, at least one of the enzymes in this pathway is exogenous to the microorganism. In a preferred embodiment, step 44 is catalyzed by Ptb-Buk. Exemplary types and sources of enzymes for steps 1, 40, 41, 42, 43, and 44 are described elsewhere in this application.

Production of Isoamyl Alcohol

0088 The invention provides a microorganism capable of producing isoamyl alcohol or precursors thereof from a substrate (Fig. 36). The invention further provides a method of producing isoamyl alcohol or precursors thereof by culturing such a microorganism in the presence of a substrate. In

preferred embodiments, the microorganism is derived from a parental microorganism selected from the group consisting of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. However, the microorganism may also be derived from an entirely different microorganism, e.g., *Eschericia coli*. The enzymatic pathways described for the production of isoamyl alcohol may comprise endogenous enzymes and, where endogenous enzyme activity is absent or low, exogenous enzymes.

0089 Isoamyl alcohol via steps 1, 40, 41, 42, 43, 44, 45, and 46: In one embodiment, the invention provides a microorganism comprising enzymes for steps 1, 40, 41, 42, 43, 44, 45, and 46, whereby the microorganism is capable of producing isoamyl alcohol or precursors thereof from a substrate, such as a gaseous substrate. Typically, at least one of the enzymes in this pathway is exogenous to the microorganism. In a preferred embodiment, step 44 is catalyzed by Ptb-Buk. Exemplary types and sources of enzymes for steps 1, 40, 41, 42, 43, 44, 45, and 46 are described elsewhere in this application.

0090 Isoamyl alcohol via steps 1, 40, 41, 42, 43, 47 and 46: In one embodiment, the invention provides a microorganism comprising enzymes for steps 1, 40, 41, 42, 43, 47 and 46, whereby the microorganism is capable of producing isoamyl alcohol or precursors thereof from a substrate, such as a gaseous substrate. Typically, at least one of the enzymes in this pathway is exogenous to the microorganism. Exemplary types and sources of enzymes for steps 1, 40, 41, 42, 43, 47 and 46 are described elsewhere in this application.

0091 In one embodiment, the microorganism may comprise more than one pathway for the production of isoamyl alcohol.

Production of Additional Products

0092 The invention provides a microorganism comprising exogenous Ptb-Buk and exogenous or endogenous aldehyde:ferredoxin oxidoreductase (AOR). Such a microorganism may produce, for example, 1-propanol, 1-butanol, 1-hexanol, and 1-octanol or precursors thereof from acetyl-CoA generated, for example, from a gaseous substrate (Fig. 32). The invention further provides a method of producing 1-propanol, 1-butanol, 1-hexanol, and 1-octanol or precursors thereof by culturing such a microorganism in the presence of a gaseous substrate. *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei* natively comprise AOR. However, AOR may be overexpressed in such microorganisms in combination with expression of exogenous Ptb-Buk. Alternatively, exogenous AOR and exogenous Ptb-Buk may be expressed in a microorganism other than *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei*, such as *Escherichia coli*.

Production of Precursors and Intermediates

0093 The pathways depicted in Figs. 1, 34, 35, and 36 may be modified to produce precursors or intermediates of the aforementioned products. In particular, partial enzymatic pathways for any of the

pathways described herein may be inserted in a host microorganism to obtain production of precursors or intermediates.

Definitions and Background

0094 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.

0095 "Recombinant" indicates that a nucleic acid, protein, or microorganism is the product of genetic modification, engineering, or recombination. Generally, the term "recombinant" refers to a nucleic acid, protein, or microorganism that contains or is encoded by genetic material derived from multiple sources, such as two or more different strains or species of microorganisms. As used herein, the term "recombinant" may also be used to describe a microorganism that comprises a mutated nucleic acid or protein, including a mutated form of an endogenous nucleic acid or protein.

0096 "Endogenous" refers to a nucleic acid or protein that is present or expressed in the wild-type or parental microorganism from which the microorganism of the invention is derived. For example, an endogenous gene is a gene that is natively present in the wild-type or parental microorganism from which the microorganism of the invention is derived. In one embodiment, the expression of an endogenous gene may be controlled by an exogenous regulatory element, such as an exogenous promoter.

0097 "Exogenous" refers to a nucleic acid or protein that is not present in the wild-type or parental microorganism from which the microorganism of the invention is derived. In one embodiment, an exogenous gene or enzyme may be derived from a heterologous (i.e., different) strain or species and introduced to or expressed in the microorganism of the invention. In another embodiment, an exogenous gene or enzyme may be artificially or recombinantly created and introduced to or expressed in the microorganism of the invention. Exogenous nucleic acids may be adapted to integrate into the genome of the microorganism of the invention or to remain in an extra-chromosomal state in the microorganism of the invention, for example, in a plasmid.

0098 "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.

0099 With respect to enzyme activity, a "substrate" is a molecule upon which an enzyme acts and a "product" is a molecule produced by the action of an enzyme. A "native substrate," therefore, is a molecule upon which an enzyme natively acts in a wild-type microorganism and a "native product" is a molecule natively produced by the action of the enzyme in the wild-type microorganism. For example, butanoyl-CoA is the native substrate of Ptb and butanoyl phosphate and is the native substrate of Buk. Additionally, butanoyl phosphate is the native product of Ptb and butyrate (butanoate) is the native product of Buk. Likewise, a "non-native substrate" is a molecule upon which an enzyme does not natively act in a wild-type microorganism and a "non-native product" is a molecule not natively produced by the action of the enzyme in the wild-type microorganism. An enzyme that is capable of acting on multiple different substrates, whether native or non-native, is typically referred to as a "promiscuous" enzyme. The inventors have discovered that Ptb is promiscuous and is capable of accepting a variety of acyl-CoAs and enoyl-CoAs as substrates, such that Ptb-Buk may be used to convert a number of acyl-CoAs and enoyl-CoAs to their corresponding acids or alkenates, respectively, while simultaneously generating ATP. Thus, in preferred embodiments, the Ptb-Buk of the invention acts on non-native substrates (i.e., substrates other than butanoyl-CoA and/or butanoyl phosphate) to produce non-native products (i.e., products other than butanoyl phosphate and/or butyrate (butanoate)).

0100 The term "butyryl-CoA" may be used interchangeably herein with "butanoyl-CoA."

0101 The term "energy-generating" or the like may be used interchangeably herein with "energy-conserving" or the like. Both of these terms are commonly used in the literature.

0102 "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.

0103 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. The disruptive mutation may be any mutation that reduces, prevents, or blocks the biosynthesis of a product produced by an enzyme. The disruptive mutation may include, for example, a mutation in a gene encoding an enzyme, a mutation in a genetic regulatory element involved in the expression of a gene encoding an enzyme, the introduction of a nucleic acid which produces a protein that reduces or inhibits the activity of an enzyme, or the introduction of a nucleic acid (e.g., antisense RNA, siRNA, CRISPR) or protein which inhibits the expression of an enzyme. The disruptive mutation may be introduced using any method known in the art.

0104 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 invention is derived. For example, the microorganism of the invention may produce no 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 invention may produce less than about 0.001, 0.01, 0.10, 0.30, 0.50, or 1.0 g/L target product.

0105 "Codon optimization" refers to the mutation of a nucleic acid, such as a gene, for optimized or improved translation of the nucleic acid in a particular strain or species. Codon optimization may result in faster translation rates or higher translation accuracy. In a preferred embodiment, the genes of the invention are codon optimized for expression in *Clostridium*, particularly *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. In a further preferred embodiment, the genes of the invention are codon optimized for expression in *Clostridium autoethanogenum* LZ1561, which is deposited under DSMZ accession number DSM23693.

0106 "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.

0107 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.

0108 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.

0109 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.

0110 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.

0111 A "microorganism" is a microscopic organism, especially a bacterium, archea, virus, or fungus. The microorganism of the invention is typically a bacterium. As used herein, recitation of "microorganism" should be taken to encompass "bacterium."

0112 A "parental microorganism" is a microorganism used to generate a microorganism of the invention. The parental microorganism may be a naturally-occurring microorganism (i.e., a wild-type microorganism) or a microorganism that has been previously modified (i.e., a mutant or recombinant microorganism). The microorganism of the invention may be modified to express or overexpress one or more enzymes that were not expressed or overexpressed in the parental microorganism. Similarly, the microorganism of the invention may be modified to contain one or more genes that were not contained by the parental microorganism. The microorganism of the invention may also be modified to not express or to express lower amounts of one or more enzymes that were expressed in the parental microorganism. In one embodiment, the parental microorganism is *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. In a preferred embodiment, the parental microorganism is *Clostridium autoethanogenum* LZ1561, which is deposited under DSMZ accession number DSM23693.

0113 The term "derived from" indicates that a nucleic acid, protein, or microorganism is modified or adapted from a different (e.g., a parental or wild-type) nucleic acid, protein, or microorganism, so

as to produce a new nucleic acid, protein, or microorganism. Such modifications or adaptations typically include insertion, deletion, mutation, or substitution of nucleic acids or genes. Generally, the microorganism of the invention is derived from a parental microorganism. In one embodiment, the microorganism of the invention is derived from *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. In a preferred embodiment, the microorganism of the invention is derived from *Clostridium autoethanogenum* LZ1561, which is deposited under DSMZ accession number DSM23693.

0114 The microorganism of the invention may be further classified based on functional characteristics. For example, the microorganism of the invention may be or may be derived from a C1-fixing microorganism, an anaerobe, an acetogen, an ethanologen, a carboxydotroph, and/or a methanotroph. Table 1 provides a representative list of microorganisms and identifies their functional characteristics.

Table 1

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

[1] *Acetobacterium woodi* can produce ethanol from fructose, but not from gas.
[2] It has not been investigated whether *Clostridium magnum* can grow on CO.

[3] One strain of *Moorella thermoacetica, Moorella* sp. HUC22-1, has been reported to produce ethanol from gas.
[4] It has not been investigated whether *Sporomusa ovata* can grow on CO.
[5] It has not been investigated whether *Sporomusa silvacetica* can grow on CO.
[6] It has not been investigated whether *Sporomusa sphaeroides* can grow on CO.

0115 "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.

0116 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.

0117 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.

0118 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.

0119 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.

0120 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.

0121 A "methanotroph" is a microorganism capable of utilizing methane as a sole source of carbon and energy. In certain embodiments, the microorganism of the invention is derived from a methanotroph.

0122 More broadly, the microorganism of the invention may be derived from any genus or species identified in Table 1.

0123 In a preferred embodiment, the microorganism of the invention is derived from the cluster of *Clostridia* comprising the species *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, and *Clostridium ragsdalei*. These species were first reported and characterized by Abrini, *Arch Microbiol*, 161: 345-351, 1994 (*Clostridium autoethanogenum*), Tanner, *Int J System Bacteriol*, 43: 232-236, 1993 (*Clostridium ljungdahlii*), and Huhnke, WO 2008/028055 (*Clostridium ragsdalei*).

0124 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.

0125 However, these three species also have a number of differences. These species were isolated from different sources: *Clostridium autoethanogenum* from rabbit gut, *Clostridium ljungdahlii* from chicken yard waste, and *Clostridium ragsdalei* from freshwater sediment. These species differ in utilization of various sugars (e.g., rhamnose, arabinose), acids (e.g., gluconate, citrate), amino acids (e.g., arginine, histidine), and other substrates (e.g., betaine, butanol). Moreover, these species differ in auxotrophy to certain vitamins (e.g., thiamine, biotin). These species have differences in nucleic and amino acid sequences of Wood-Ljungdahl pathway genes and proteins, although the general organization and number of these genes and proteins has been found to be the same in all species (Köpke, *Curr Opin Biotechnol*, 22: 320-325, 2011).

0126 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.

0127 The microorganism of the invention may also be derived from an isolate or mutant of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. Isolates and mutants of *Clostridium autoethanogenum* include JA1-1 (DSM10061) (Abrini, *Arch Microbiol*, 161: 345-351, 1994), LBS1560 (DSM19630) (WO 2009/064200), and LZ1561 (DSM23693). Isolates and mutants of *Clostridium ljungdahlii* include ATCC 49587 (Tanner, *Int J Syst Bacteriol*, 43: 232-236, 1993), PETCT (DSM13528, ATCC 55383), ERI-2 (ATCC 55380) (US 5,593,886), C-01 (ATCC 55988) (US 6,368,819), O-52 (ATCC 55989) (US 6,368,819), and OTA-1 (Tirado-Acevedo, Production of bioethanol from synthesis gas using *Clostridium ljungdahlii*, PhD thesis, North Carolina State University, 2010). Isolates and mutants of *Clostridium ragsdalei* include PI 1 (ATCC BAA-622, ATCC PTA-7826) (WO 2008/028055).

0128 In some embodiments, however, the microorganism of the invention is a microorganism other than *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, or *Clostridium ragsdalei*. For example, the microorganism may be selected from the group consisting of *Escherichia coli, Saccharomyces cerevisiae, Clostridium acetobutylicum, Clostridium beijerinckii, Clostridium saccharbutyricum, Clostridium saccharoperbutylacetonicum, Clostridium butyricum, Clostridium diolis, Clostridium kluyveri, Clostridium pasterianium, Clostridium novyi, Clostridium difficile, Clostridium thermocellum, Clostridium cellulolyticum, Clostridium cellulovorans, Clostridium phytofermentans, Lactococcus lactis, Bacillus subtilis, Bacillus licheniformis, Zymomonas mobilis, Klebsiella oxytoca, Klebsiella pneumonia, Corynebacterium glutamicum, Trichoderma reesei, Cupriavidus necator, Pseudomonas putida, Lactobacillus plantarum,* and *Methylobacterium extorquens*.

0129 "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.

0130 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.

0131 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.

0132 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.

0133 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.

0134 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.

0135 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.

0136 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.

0137 The microorganism of the invention may be cultured to produce one or more products. For instance, *Clostridium autoethanogenum* produces or can be engineered to produce ethanol (WO 2007/117157), acetate (WO 2007/117157), butanol (WO 2008/115080 and WO 2012/053905), butyrate (WO 2008/115080), 2,3-butanediol (WO 2009/151342), lactate (WO 2011/112103), butene (WO 2012/024522), butadiene (WO 2012/024522), methyl ethyl ketone (2-butanone) (WO 2012/024522 and WO 2013/185123), ethylene (WO 2012/026833), acetone (WO 2012/115527), isopropanol (WO 2012/115527), lipids (WO 2013/036147), 3-hydroxypropionate (3-HP) (WO 2013/180581), isoprene (WO 2013/180584), fatty acids (WO 2013/191567), 2-butanol (WO 2013/185123), 1,2-propanediol (WO 2014/0369152), and 1-propanol (WO 2014/0369152). In

addition to one or more target products, the microorganism of the invention may also produce ethanol, acetate, and/or 2,3-butanediol. In certain embodiments, microbial biomass itself may be considered a product.

0138 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.

0139 The terms "intermediate" and "precursor," which may be referred to interchangeably herein, refer to a molecular entity in an enzymatic pathway upstream of an observed or target product.

0140 "Selectivity" refers to the ratio of the production of a target product to the production of all fermentation products produced by a microorganism. The microorganism of the invention may be engineered to produce products at a certain selectivity or at a minimum selectivity. In one embodiment, a target product account for at least about 5%, 10%, 15%, 20%, 30%, 50%, or 75% of all fermentation products produced by the microorganism of the invention. In one embodiment, the target product accounts for at least 10% of all fermentation products produced by the microorganism of the invention, such that the microorganism of the invention has a selectivity for the target product of at least 10%. In another embodiment, the target product accounts for at least 30% of all fermentation products produced by the microorganism of the invention, such that the microorganism of the invention has a selectivity for the target product of at least 30%.

0141 "Increasing the efficiency," "increased efficiency," and the like include, but are not limited to, increasing growth rate, product production rate or volume, product volume per volume of substrate consumed, or product selectivity. Efficiency may be measured relative to the performance of parental microorganism from which the microorganism of the invention is derived.

0142 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.

0143 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.

0144 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.

0145 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.

0146 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

0147 The following examples further illustrate the invention but, of course, should not be construed to limit its scope in any way.

Example 1

0148 This example demonstrates the ability of Ptb-Buk to convert acetoacetyl-CoA to acetoacetate in *E. coli in vivo* and its use in production of acetone, isopropanol, 3-hydroxybutyrate, and isobutylene

0149 Pathways that rely on the Ptb-Buk system for acetoacetate production from acetoacetyl-CoA were designed and constructed. This was done in a modular fashion using a pDUET vector system (Novagen). One module contained *ptb-buk* genes from *C. beijerinckii* NCIMB8052 (GenBank NC_009617, position 232027..234147; Cbei_0203-204; NCBI-GeneID 5291437-38) on plasmid pACYC. Another module contained the thiolase gene *thlA* of *C. acetobutylicum* (Genbank NC_001988, position 82040..83218; CA_P0078; NCBI-GeneID 1116083) and the acetoacetate decarboxylase gene *adc* of *C. beijerinckii* NCIMB8052 (Genbank NC_009617, position 4401916..4402656; Cbei_3835; NCBI-GeneID 5294996) on plasmid pCOLA. *Ptb* and *buk* genes were amplified from genomic DNA of *C. beijerinckii* NCIMB8052 and *thlA* and *adc* genes from an existing acetone plasmid pMTL85147-thlA-ctfAB-adc (WO 2012/115527) and cloned under control of the T7 promoter present in the pDUET vectors via restriction independent cloning with the circular polymerase extension cloning (CPEC) method (Quan, *PloS One*, 4:e6441, 2009).

0150 Oligonucleotides used for amplification of *ptb* and *buk* genes:

SEQ ID NO:	Name	Sequence	Direction
95	pACYCDuet-ptb-buk - pACYC-ptb-R1	AAGTTTTTACTCATATGTATATC TCCTTCTTATACTTAAC	reverse
96	pACYCDuet-ptb-buk - ptb-pACYC-F1	AGAAGGAGATATACATATGAGT AAAAACTTTGATGAGTTA	forward
97	pACYCDuet-ptb-buk - buk-pACYC-R1	ACCAGACTCGAGGGTACCTAGT AAACCTTAGCTTGTTC	reverse
98	pACYCDuet-ptb-buk - pACYC-buk-F1	TAAGGTTTACTAGGTACCCTCG AGTCTGGTAAAGAAAC	forward

0151 Oligonucleotides used for amplification of *thlA* and *adc* genes:

SEQ ID NO:	Name	Sequence	Direction
99	pCOLADuet-thlA-adc - thlA-adc-R1	ACATATGTATATCTCCTTCTTAC TAGCACTTTTCTAGCAATATTG	reverse
100	pCOLADuet-thlA-adc - adc-ThlA-F1	AGTAAGAAGGAGATATACATAT GTTAGAAAGTGAAGTATCTAAA C	forward
101	pCOLADuet-thlA-adc - adc-pCOLA-R1	CAGACTCGAGGGTACCTTATTT TACTGAAAGATAATCATGTAC	reverse
102	pCOLADuet-thlA-adc - pCOLA-adc-F1	TCTTTCAGTAAAATAAGGTACC CTCGAGTCTGGTAAAGAAAC	forward
103	pCOLADuet-thlA-adc - thlA-pCOLA-F1	GAAGGAGATATACATATGAAA GAAGTTGTAATAGCTAGTG	forward
104	pCOLADuet-thlA-adc - pCOLA-thlA-R1	ACAACTTCTTTCATATGTATATC TCCTTCTTATACTTAAC	reverse

0152 After the plasmids pACYC-ptb-buk (SEQ ID NO: 105) and pCOLA-thlA-adc (SEQ ID NO: 106) were constructed, they were transformed individually and together into *E. coli* BL21 (DE3)

(Novagen) and growth experiments carried out in quadruplicates in 1.5 mL cultures in 12-well plates at 28 °C with 160 rpm orbital shaking using M9 minimal medium (Sambrook, Molecular Cloning: A Laboratory Manual, Vol 3, Cold Spring Harbour Press, 1989) with glucose (Fig. 4). The cultures were inoculated at an OD600nm of 0.1 and induced with different concentrations of IPTG (0, 50, 100 μM) after 2 h of growth (Fig. 5). The plates were sealed using plate tape strips and each well was pierced with a green tipped needle to provide micro-aerobic conditions. Growth was carried out for another 64 h of induction. The experiment was repeated in triplicate.

0153 Acetone concentrations, as well as the concentrations of other metabolites such as isobutylene, were measured using gas chromatography (GC) analysis, employing an Agilent 6890N headspace GC equipped with a Supelco polyethylene glycol (PEG) 60-μm solid-phase microextraction fiber, a Restek Rtx-1 (30 m × 0.32 μm × 5 μm) column, and a flame ionization detector (FID). Samples (4 ml) were transferred into a 20-ml headspace vial, upon which the fiber was incubated (exposed) for 10 min at 50 °C. The sample was desorbed in the injector at 250 °C for 9 min. Chromatography was performed with an oven program of 40 °C (5-min hold) and 10 °C/min to 200°C, followed by a 5-min hold at 220 °C. The column flow rate was 1 ml/min, with hydrogen as the carrier gas. The FID was kept at 250 °C, with hydrogen at 40 ml/min, air at 450 ml/min, and nitrogen at 15 ml/min as the makeup gas.

0154 It was immediately obvious that acetone was produced in the strain carrying both the pACYC-ptb-buk and pCOLA-thlA-adc plamids (expressing thiolase, Ptb-Buk, and acetoacetate decarboxylase). Average final acetone production of 0.19 g/L was measured, whereas no acetone was produced in a no plasmid control, media control, and single plasmid controls pACYC-ptb-buk (expressing Ptb-Buk) or pCOLA-thlA-adc plamid (expressing thiolase and acetoacetate decarboxylase) (below reliable detection limit). The uninduced culture of the strain carrying both the pACYC-ptb-buk and pCOLA-thlA-adc plamids (expressing thiolase, Ptb-Buk, and acetoacetate decarboxylase) did not produce appreciable amounts of acetone.

Average acetone production in *E. coli* BL21 (DE3):

Strain	Acetone (g/L)
Thl+Ptb-Buk+Adc [*E. coli* BL21 (DE3) + pACYC-ptb-buk + pCOLA-thlA-adc]	0.19±0.04
Thl+Adc alone [*E. coli* BL21 (DE3) + pCOLA-thlA-adc]	0.04±0.01
Ptb-Buk alone [*E. coli* BL21 (DE3) + pACYC-ptb-buk]	0.03±0.01
No plasmid control [*E. coli* BL21 (DE3)]	0.04±0.01
Media control	0.03±0.01

0155 This experiment clearly demonstrates that Ptb-Buk is able to perform the conversion of acetoacetyl-CoA to acetoacetate can be used in place of a CoA-transferase or a thioesterase for the production of acetone, exemplified using a route that comprises steps 1, 2, and 3 of Fig. 1.

0156 It is well known that isopropanol can be produced from acetone by addition of a primary:secondary alcohol dehydrogenase (Köpke, *Appl Environ Microbiol*, 80: 3394-3403, 2014) (step 4 in Fig. 1) and that isobutylene can be produced from acetone via addition of a hydroxyisovalerate synthase (step 5 in Fig. 1) and decarboxylase (step 6 in Fig. 1) (van Leeuwen, *Appl Microbiol Biotechnol*, 93: 1377-1387, 2012). A pathway can be constructed that includes the above-demonstrated acetone route via Ptb-Buk with the genes *thlA*, *ptb-buk*, and *adc* and a primary:secondary alcohol dehydrogenase gene (e.g., Genbank accession number NC_022592, pos. 609711..610766; CAETHG_0553; NCBI-GeneID: 17333984) that would allow isopropanol production via the Ptb-Buk system in *E. coli* comprising steps 1, 2, 3, and 4 of Fig. 1. Similarly, a pathway can be constructed that includes the above-demonstrated acetone route via Ptb-Buk conversion of acetoacetyl-CoA to acetoacetate with the genes *thlA*, *ptb-buk*, *and adc* and genes for a hydroxyisovalerate synthase and decarboxylase that would allow isobutylene production via the Ptb-Buk system in *E. coli* comprising of steps 1, 2, 3, 5, and 6 of Fig. 1. Acetoacetete can also be converted to 3-hydroxybutyrate via a 3-hydroxybutyrate dehydrogenase Bdh. This can be combined with Ptb-Buk conversion of acetoacetyl-CoA to acetoacetate for 3-hydroxybutyrate production in a strain expressing genes *thlA*, *ptb-buk*, and *bdh* resulting in a pathway comprising steps 1, 2, and 15 of Fig. 1.

Example 2

0157 This example demonstrates the ability of Ptb-Buk to convert acetoacetyl-CoA to acetoacetate in *C. autoethanogenum in vivo* and the use of Ptb-Buk in the production of acetone, isopropanol, 3-hydroxybutyrate, and isobutylene from a gaseous substrate.

0158 To demonstrate that the Ptb-Buk system also allows acetone, isopropanol, or isobutylene synthesis from gaseous substrates, a plasmid was constructed that contains the same genes as in Example 1, *thl* + *ptb-buk* + *adc* under control of a clostridial promoter on a shuttle vector that allows expression in acetogens such as *C. autoethanogenum, C. ljungdahlii* or *C. ragsdalei.*

0159 The pMTL plasmid is a shuttle plasmid system for introducing circular dna into Clostridia via *E. coli* conjugation (Heap, *J Microbiol Methods*, 78: 79-85, 2009. The genes of interest (i.e., hbd, phaB, thlA, ptb, buk, and aor1) were cloned into the lacZ region of the plasmids using common techniques in molecular biology including dna restriction digestion followed by ligation, and the golden gate dna assembly technology when more than one pieces of dna fragments were to be cloned simultaneously into the plasmid. The constructed plasmids are verified by DNA sequencing.

0160 Production of acetone and isopropanol was previously demonstrated in *C. autoethanogenum* using a plasmid pMTL85147-thlA-ctfAB-adc encoding *thl*+ *ctfAB* + *adc* (WO 2012/115527) under the control of a clostridial promoter from the Wood-Ljungdahl gene cluster. In this plasmid the *ctfAB* genes encoding the CoA transferase were replaced directly with *ptb-buk* genes encoding the Ptb-Buk system. This was done as described in Example 1 using the CPEC method. The resulting plasmid is pMTL85147-thlA-ptb-buk-adc.

0161 Oligonucleotides used for the amplification of ptb-buk and cloning into pMTL8317-thl-ptb-buk-adc are described below.

SEQ ID NO:	Name	Sequence	Direction
107	thlA-ptb-R1	ATTTCCTCCCTTTCTAGCACTTT TCTAGCAATATTG	reverse
108	adc-buk-F1	TAAGGTTTACTAAGGAGGTTGT TTTATGTTAGAAAG	forward
109	thlA-ptb-F1	GCTAGAAAAGTGCTAGAAAGG GAGGAAATGAACATG	forward
110	Buk-adc-R1	AAAACAACCTCCTTAGTAAACC TTAGCTTGTTCTTC	reverse

0162 *C. autoethanogenum* DSM10061 and DSM23693 (a derivate of DSM10061) were sourced from DSMZ (The German Collection of Microorganisms and Cell Cultures, Inhoffenstraße 7 B, 38124 Braunschweig, Germany).

0163 Strains were grown at 37 °C in PETC medium at pH 5.6 using standard anaerobic techniques (Hungate, *Meth Microbiol*, 3B: 117-132, 1969; Wolfe, *Adv Microb Physiol*, 6: 107-146, 1971). 30 psi CO-containing steel mill gas (collected from New Zealand Steel site in Glenbrook, NZ) or a synthetic gas blend with same composition of 44% CO, 32% N_2, 22% CO_2, 2% H_2 was used as substrate for autotrophic growth. For solid media, 1.2 % bacto agar (BD, Franklin Lakes, NJ 07417, USA) was added.

0164 The construct was synthesized and then transformed into *C. autoethanogenum* via conjugation. For this, the expression vector was first introduced into the conjugative donor strain *E. coli* HB101+R702 (CA434) (Williams, *J Gen Microbiol*, 1136: 819-826, 1990) (the donor) using standard heat shock transformation. Donor cells were recovered in SOC medium (Sambrook, Molecular Cloning: A Laboratory Manual, Vol 3, Cold Spring Harbour Press, 1989) at 37 °C for 1 h before being plated on to LB medium (Sambrook, Molecular Cloning: A Laboratory Manual, Vol 3, Cold Spring Harbour Press, 1989) plates containing 100 μg/ml spectinomycin and 25 μg/ml chloramphenicol. LB plates were incubated at 37 °C overnight. The next day, 5 ml LB aliquots containing 100 μg/ml spectinomycin and 25 μg/ml chloramphenicol were inoculated with several donor colonies and incubated at 37 °C, shaking for approximately 4 h, or until the culture was visibly dense but had not yet entered stationary phase. 1.5 ml of the donor culture was harvested in a microcentrifuge tube at room temperature by centrifugation at 4000 rpm for 2 min, and the supernatant was discarded. The donor cells were gently resuspended in 500 μl sterile PBS buffer (Sambrook, Molecular Cloning: A Laboratory Manual, Vol 3, Cold Spring Harbour Press, 1989) and centrifuged at 4000 rpm for 2 min and the PBS supernatant was discarded. The pellet was introduced into an anaerobic chamber and gently resuspended in 200 μl during late exponential phase *C. autoethanogenum* culture (the recipient). The conjugation mixture (the mix of donor and recipient cells) was spotted onto PETC-MES + fructose agar plates and left to dry. When the spots were no longer visibly wet, the plates were introduced into a pressure jar, pressurized with syngas to 25-30 psi

and incubated at 37 °C for ~24 h. After 24 h incubation, the conjugation mixture was removed from the plates by gently scraping it off using a 10 µl inoculation loop. The removed mixture was suspended in 200-300 µl PETC medium. 100 µl aliquots of the conjugation mixture were plated on to PETC medium agar plates supplemented 15 µg/ml thiamphenicol to select for transformants bearing the plasmid, which confers resistance to thiamphenicol via expression of chloramphenicol acetyl-transferase.

0165 Three distinct colonies of *C. autoethanogenum* bearing the pMTL85147-thlA-ptb-buk-adc plasmid were inoculated into 2 mL of PETC-MES medium with 15 µg/ml thiamphenicol and grown autotrophically at 37 °C with 100 rpm orbital shaking for three days. Cultures were diluted to $OD_{600\ nm}$ = 0.05 in 10 mL PETC-MES medium with 15 µg/ml thiamphenicol in serum bottles and grown autotrophically at 37 °C with 100 rpm orbital shaking for five days, sampling daily to measure biomass and metabolites. In parallel a control strain was examined where the expression plasmid encoded only *thl* and *adc* under the control of the Wood-Ljungdahl cluster promoter, with no *ctfAB* or *ptb-buk* genes to catalyse the formation of acetoacetate from acetoacetyl-CoA (pMTL85147-thlA-adc). Cultures were sampled for five days in order to monitor metabolites and biomass accumulation.

0166 Isopropanol concentrations as well as concentrations of ethanol, acetic acid, 2,3-butanediol and lactic acid were measured by high-performance liquid chromatography (HPLC) on an Agilent LC with refractive index (RI) detection at 35°C. Samples were prepared by diluting 400 µL with 100 µL of 5-sulfosalicylic acid solution (1% w/v in 1 M sulphuric acid), followed by a 3 minute centrifugation at 14,000 rpm; the supernatant was transferred to a glass vial for analysis. Separation was carried out with a 10 µL injection on to an Alltech IOA-2000 column (150 mm x 6.5 mm x 8 µm) at 0.7 mL/min and 65°C under isocratic conditions, using 5 mM sulphuric acid mobile phase.

0167 In some instances, a longer HPLC method was used to improve peak separation. In this method, isopropanol, ethanol, acetate, 2,3-butanediol, and also 3-hydroxybutyrate (which is not separated using the shorter method) concentrations were measured by high-performance liquid chromatography (HPLC) on an Agilent 1260 Infinity LC with refractive index (RI) detection at 35°C. Samples were prepared by diluting 400 µL with 100 µL of 5-sulfosalicylic acid solution (1% w/v in 1 M sulphuric acid), followed by a 3 minute centrifugation at 14,000 rpm; the supernatant was transferred to a glass vial for analysis. Separation was carried out with a 10 µL injection on to an Aminex HPX-87H column (300 mm x 7.8 mm x 9 µm) at 0.6 mL/min and 35°C under isocratic conditions, using 5 mM sulphuric acid mobile phase.

0168 *C. autoethanogenum* bearing the pMTL85147-thlA-ptb-buk-adc produced isopropanol up to 0.804 g IPA/g of biomass, whereas control strain *C. autoethanogenum* with pMTL85147-thlA-adc that does not contain Ptb-Buk produced no IPA (Fig. 12).

0169 This experiment clearly demonstrates that Ptb-Buk is able to perform the conversion of acetoacetyl-CoA to acetoacetate in the isopropanol pathway when using a gaseous substrate. Ptb-Buk

can be used in place of a CoA transferase or a thioesterase in a gas-fermenting acetogen such as *C. autoethanogenum*, exemplified using a route that comprises steps 1, 2, 3, and 4 of Fig. 1.

0170 *C. autoethanogenum* contains a native primary:secondary alcohol dehydrogenase that converts acetone to isopropanol (Köpke, *Appl Environ Microbiol*, 80: 3394-3403, 2014). It has been demonstrated that knock-out of this gene eliminates conversion of acetone to isopropanol in *C. autoethanogenum* (WO 2015/085015). In background of this knock-out, it becomes possible to produce acetone (rather than isopropanol) via the Ptb-Buk system from a gaseous feedstock, using the same genes comprising steps 1, 2, and 3 of Fig. 1. Addition of hydroxyisovalerate synthase and decarboxylase genes (van Leeuwen, *Appl Microbiol Biotechnol*, 93: 1377-1387, 2012) to this strain would enable isobutylene production from gas in *C. autoethanogenum* or similar bacteria comprising of steps 1, 2, 3, 5, and 6 of Fig. 1.

0171 Acetoacetate can also be converted to 3-hydroxybutyrate via a 3-hydroxybutyrate dehydrogenase Bdh. A 3-hydroxybutyrate dehydrogenase was identified in the genome of *C. autoethanogenum* (AGY75962) and other acetogens as *C. ljungdahlii* (ADK16920.1). This activity can be combined with Ptb-Buk (or CoA transferase) conversion of acetoacetyl-CoA to acetoacetate for 3-hydroxybutyrate production in a strain expressing genes *thlA*, *ptb-buk* (or *ctfAB*) and *bdh* resulting a pathway comprising steps 1, 2, and 15 of Fig. 1. Low levels of 3-hydroxybutyrate formation (up to 2 g/L) via this route have been demonstrated in *C. autoethanogenum*. These levels could be enhanced by overexpressing the Bdh gene that is only expressed in at low levels natively.

0172 In one experiment, *C. autoethanogenum* was transformed with plasmid pMTL82256-thlA-ctfAB as described in Example 2. The production was monitored for 10 days from six biological replicates under autotrophic conditions as described in Example 2. The average of 3-HB after 10 days was 1.86 ± 0.14 g/L. At day 10, 1,3-butanediol was produced (from 3-HB) at an average titer of 0.38 ± 0.05 g/L (Fig. 37). No acetone or isopropanol was formed. This demonstrates that 3-HB can be produced efficiently via acetoacetate through native enzymes.

0173 In certain embodiments, it may be desirable to knock out or knock down expression of 3-hydroxybutyrate dehydrogenases, such as Bdh, to prevent carbon drain to 3-HB and therefore boost production of products such as acetone, isopropanol, and isobutylene.

Example 3

0174 This example demonstrates the ability of Ptb-Buk to convert (R)-3-hydroxybutyryl-CoA to (R)-3-hydroxybutyryrate in *E. coli in vivo* for production of (R)-hydroxybutyrate, acetone, isopropanol, or isobutylene.

0175 Pathways were designed and constructed that rely on the Ptb-Buk system for (R)-3-hydroxybutyrate production from (R)-3-hydroxybutyryl-CoA. Additionally, a 3-hydroxybutyrate dehydrogenase (Bdh) was utilized for conversion of (R)-3-HB to acetoacetate. It has been reported that *Ralstonia pickettii* have two 3-hydroxybutyrate dehydrogenases Bdh1 and Bdh2 that are able to

convert 3-hydroxybutyrate to acetoacetate *in vitro* (Takanashi, *J Biosci Bioeng*, 101: 501-507, 2006). One pathway was designed making use of this enzyme for acetone production (steps 1, 13, 14, 15, 3 of Fig. 1), while recycling the reducing equivalents produced in the production of (R)-3-hydroxybutyryl-CoA and the ATP generated by Ptb-Buk (Fig. 6).

0176 The pathways were constructed in a modular fashion using the pDUET vector system (Novagen). The two modules described in example above (pACYC-ptb-buk for expression of Ptb-Buk and pCOLA-thlA-adc for expression of thiolase and acetoacetate decarboxylase) were used together with two additional modules containing either (R)-specific 3-hydroxybutyrate dehydrogenase *phaB* of *Cupravidus necator* (WP_010810131.1) alone (pCDF-phaB) and one with 3-hydroxybutyrate dehydrogenase *bdh1* gene of *Rasltonia pickettii* (BAE72684.1) (pCDF-phaB-bdh1) in vector pCDF. Both *phaB* and *bdh1* gene were synthesized from GeneArt and cloned under control of the T7 promoter present in via restriction independent cloning with the circular polymerase extension cloning (CPEC) method (Quan, PloS One, 4:e6441, 2009).

0177 Oligonucleotides used for amplification of *bdh1* gene:

SEQ ID NO:	Name	Sequence	Direction
111	pDuet-insert2-R1	CATATGTATATCTCCTTCTTATA CTTAAC	forward
112	insert2-pDuet-F1	GTTAAGTATAAGAAGGAGATAT ACATATG	forward
113	pDuet-insert2-F1	CCTCGAGTCTGGTAAAGAAAC	forward
114	insert2-pDuet-R1	GTTTCTTTACCAGACTCGAGG	forward

0178 Oligonucleotides used for amplification of *phaB* gene:

SEQ ID NO:	Name	Sequence	Direction
115	pCDF-phaB - pACYC-phaB-R1	CTATTCTTTGTGTCATGGTATAT CTCCTTATTAAAG	forward
116	pCDF-phaB - phaB-pACYC-F1	ATAAGGAGATATACCATGACAC AAAGAATAGCATAC	forward
117	pCDF-phaB - pACYC-phaB-F1	TGGTTTACACATGGGATAAGAT CCGAATTCGAGCTC	forward
118	pCDF-phaB - phaB-pACYC-R1	AGCTCGAATTCGGATCTTATCC CATGTGTAAACCAC	forward

0179 After the plasmids pACYC-ptb-buk (SEQ ID NO: 105), pCOLA-thlA-adc (SEQ ID NO: 106), pCDF-phaB (SEQ ID NO: 119) and pCDF-phaB-bdh1 (SEQ ID NO: 120) were constructed, they were transformed individually and in combinations into *E. coli* BL21 (DE3) (Novagen) and growth experiments were carried out in quadruplicate in 1.5 mL cultures in 12-well plates at 28 °C with 160 rpm orbital shaking using M9 minimal medium with glucose. The cultures were inoculated at an OD600nm of 0.1 and after 2 h of growth induced with different concentrations of IPTG (0, 50, 100 μM). The plates were sealed using BioRad plate tape strips and each well pierced with a green tipped needle to provide micro-aerobic conditions. Growth was carried out for another 64 h of

induction. The experiment was repeated 3 times. Metabolites were measured as described in previous examples.

0180 Cultures containing a combination of plasmids pACYC-ptb-buk, pCOLA-thlA-adc and pCDF-phaB produced between 1.65-2.4 g/L (R)-3-hydroxybutyrate (depending on level of inducer), with only very small amounts of byproducts (Figs. 13A-F), demonstrating the efficiency of the Ptb-Buk system to convert (R)-3-hydroxybutyryl-CoA to (R)-3-hydroxybutyryrate and support growth (Fig. 13A-F). In cultures that also expressed bdh1 (containing a combination of plasmids pACYC-ptb-buk, pCOLA-thlA-adc, and pCDF-phaB-bdh1) only small amounts of (R)-3-hydroxybutyryrate were found in the culture media, while between 0.89-1.16 g/L acetone was found (depending on level of inducer), indicating that bdh1 gene is efficient in converting (R)-3-hydroxybutyrate to acetoacctate and further to acetone. In all plasmid combinations that lack Ptb-Buk, no 3-hydroxybutyrate or acetone was found (Fig. 13A-F). In these cultures, acetate levels were significantly higher.

0181 This experiment clearly demonstrates that Ptb-Buk is able to perform the conversion of (R)-3-hydroxybutyrate-CoA to 3-hydroxybutyrate and also that Bdh1 is able *in vivo* to convert 3-hydroxybutyrate further to acetoacetate by recycling the reducing equivalents produced in the production of (R)-3-hydroxybutyryl-CoA. The experiment also highlights that Ptb-Buk is able to support growth and therefore acetate production becomes unnecessary. Production of (R)-3-hydroxybutyrate formation was exemplified in a strain that comprises steps 1, 13, and 14 of Fig.1. Production of acetone was exemplified via a route that comprises steps 1, 13, 14, 15, and 3 of Fig. 1.

0182 It is well known that isopropanol can be produced from acetone by addition of a primary:secondary alcohol dehydrogenase (step 4 in Fig. 1) (Köpke, *Appl Environ Microbiol*, 80: 3394-3403, 2014) and that isobutylene can be produced from acetone via addition of a hydroxyisovalerate synthase (step 5 in Fig. 1) and decarboxylase (step 6 in Fig. 1) (van Leeuwen, *Appl Microbiol Biotechnol*, 93: 1377-1387, 2012). A pathway can be constructed that includes the above-demonstrated acetone route via Ptb-Buk with the genes *thlA*, *ptb-buk*, and *adc* and a primary:secondary alcohol dehydrogenase gene (e.g., Genbank NC_022592, pos. 609711..610766; CAETHG_0553; NCBI-GeneID: 17333984) that would allow isopropanol production via the Ptb-Buk system in *E. coli* (steps 1, 13, 14, 15, 3, and 4 of Fig. 1). Similarly, a pathway can be constructed that includes the above-demonstrated acetone route via Ptb-Buk with the genes *thlA*, *ptb-buk*, and *adc* and genes for a hydroxyisovalerate synthase and decarboxylase that would allow isobutylene production via the Ptb-Buk system in *E. coli* (steps 1, 13, 14, 15, 3, 5, and 6 of Fig. 1).

Example 4

0183 This example demonstrates the production of (R)-3-hydroxybutyrate and 1,3-butanediol in *C. autoethanogenum*. It also demonstrates production of 1,3-butanediol in absence of 2,3-butanediol.

0184 A strain of *C. autoethanogenum* was constructed in which the native pathway for 2,3-butanediol production was inactivated and replaced with genes for (R)-3-hydroxybutyryl-CoA formation. This was achieved by replacing the acetolactate decarboxylase gene (*budA*) on genome of

C. autoethanogenum with genes for thiolase (*thlA* of *C. acetobutylicum*; GenBank NC_001988, position 82040..83218; CA_P0078; NCBI-GeneID 1116083) and (R)-specific 3-hydroxybutyrate dehydrogenase (*phaB* of *Cupravidus necator*; GenBank WP_010810131.1) resulting in strain *C. autoethanogenum* budA::thlAphaB.

0185 To replace *budA* gene with *thlA* and *phaB* genes a plasmid, pMTL8225-budA::thlA-phaB (Fig. 14), with *E. coli* toxin gene *mazF* under tet3n0 tetracycline inducible promoter (for counter selection), ~1kb upstream homology arm of *budA* gene, *thlA, phaB, ermB* cassette flanked by *loxP* sites and ~1kb downstream homology arm of *budA* gene were assembled on plasmid pMTL-tet3no.

0186 The ~1kb upstream and downstream homology arms of *budA* were PCR amplified from *C. autoethanogenum* with primers SN01/ SN02 and SN07/ SN08. *thlA* and *phaB* genes were PCR amplified from genomic DNA of *Cupriavidus necator* using primers SN03/ SN04mod. The *ermB* cassette flanked with *loxP* sites was PCR amplified using primers SN05mod/ SN06. tet3no promoter flanked by FseI and PmeI was synthesized and treated with restriction enzymes FseI and PmeI and cleaned. The PCR products and digested vector were assembled using GeneArt Seamless cloning kit from Life Technologies and plasmid pMTL8225-budA::thlA-phaB (SEQ ID NO: 121) with no mutations in the inserted fragments was used to transform *C. autoethanogenum* by conjugation as described in previous examples.

0187 Following conjugation and selection on trimethoprim and clarithromycin, 9 colonies were streaked twice on PETC-MES agar plates with clarithromycin and anhydrotetracycline to induce the expression of mazF genes. The colonies from clarithromycin and anhydrotetracycline should have the *budA* genes replaced with *thlA* and *phaB* genes and *ermB* cassette. This was verified by PCR using primers Og31f/ Og32r flanking the homology arms and KAPA polymerase (Fig. 15).

0188 While a band of ~3.3 kb is amplified from the wild type strain, bands of ~5.7 kb were amplified from colonies 1,4, 7 and 9 indicating the replacement of *budA* gene with *thlA*, *phaB* and *ermB* cassette. The above event was further confirmed by sequencing the PCR products of all 4 clones. With the resulting modification the expression of *thlA* and *phaB* genes is driven by the promoter upstream of *budA* gene.

SEQ ID NO:	Description	Sequence
122	SN01	ATTTACAAATTCGGCCGGCCTACCTCCTCGTATAAATAAGATG
123	SN02	CTAGCTATTACAACTTCTTTCATATTACATTCACCTCTATGTC
124	SN03	GACATAGAGGTGAATGTAATATGAAAGAAGTTGTAATAGCTAG
125	SN04mod	GTATAGCATACATTATACGAACGGTATTATCCCATGTGTAAACC ACCGT
126	SN05mod	TTCGTATAATGTATGCTATACGAAGTTATCCTTAGAAGCAAACT TAAG
127	SN06	GTCTAGTGTTTTTTTCTATCAATACTCTAGATACCGTTCGTATAG C
128	SN07	TGTATGCTATACGAACGGTAAGTATTGATAGAAAAAAACACTA GAC
129	SN08	CAAAAAGGAGTTTAAACAAAAAGTCATAAACCTGGATAAC

130	Og31f	CCGTTTCTCACAACAACAATACCAG
131	Og32r	AAACCACCTTGACGATGAAACCATA

0189 A fermentation with *C. autoethanogenum* budA::thlA-phaB strain was carried out. The culture was grown at 37 °C under synthetic gas (50% CO, 18% CO_2, 2 % H_2, and 30% N_2) that was continuously fed into the bioreactor. The gas flow was initially set at 50 ml/min, increasing to 400 ml/min over the course of the experiment, while the agitation was increased from 200 rpm to 500 rpm. The fermentation was carried out for close to 5 days. Metabolites were measured as described in examples above.

0190 The concentration of 1,3-butanediol and other metabolites, such as 2-hydroxyisobutyric acid, were measured using gas chromatography (GC) analysis, employing an Agilent 6890N GC equipped a Agilent CP-SIL 5CB-MS (50 m × 0.25 μm × 0.25 μm) column, autosampler and a flame ionization detector (FID). Samples were prepared by diluting 400 μL of sample with 400 μL of acetonitrile, followed by a 3 minute centrifugation at 14,000 rpm; the supernatant was transferred to a glass vial and the sample was dried in a Thermo SpeedVac. Once dry, the samples were then suspended in a solution of 400 μL of N,O-Bistrifluoroacetamide (BSTFA) and pyridine (3:1 ratio) and heated in a sealed glass vial for 60 minutes at 60 °C. Samples were transferred to an autosampler for analysis using a 1 μL injection, a split ration of 30 to 1, and an inlet temperature of 250 °C. Chromatography was performed with an oven program of 70 °C (no hold) to a ramp of 3 °C/min to 110 °C to a ramp of 15 °C/min to 230 °C, followed by a final ramp of 40 °C/min to 310 °C with a 3-min hold. The column flow rate was 1.8 ml/min, with helium as the carrier gas. The FID was kept at 320 °C, with hydrogen at 40 ml/min, air at 400 ml/min, and helium at 20 ml/min as the makeup gas.

0191 Surprisingly, up to 1.55 g/L 3-hydroxybutyrate was produced from gas in a *C. autoethanogenum* budA::thlA-phaB strain expressing *thlA* and *phaB* (Fig. 16). A native thioesterase may convert the formed 3-hydroxybutyryl-CoA to 3-hydroxybutyrate. In the genome sequence, three putative thioesterases were identified.

0192 Even more surprising, it was also found that, along 3-hydroxybutyrate formation, there was also 1,3-butanediol formation of up to 150 mg/L (Fig. 16). This may be due to native aldehyde:ferredoxin oxidoreductase (AOR) and alcohol dehydrogenase activity. Two AOR genes and several alcohol dehydrogenases are present in the genome of *C. autoethanogenum* (Mock, *J Bacteriol*, 197: 2965-2980, 2015). This reduction of 3-hydroxybutyrate is powered by reduced ferredoxin and thus can be directly coupled to CO oxidation, which provides reduced ferredoxin ($CO + Fd_{ox} \rightarrow CO_2 + Fd_{red}$) (Fig. 7).

0193 1,3-BDO production was also demonstrated from gas via an alternative route using a butyraldehyde dehydrogenase Bld from *Clostridium saccharoperbutylacetonicum* (AAP42563.1) (SEQ ID NO: 80). The *bld* gene was synthesized and cloned together with the same thiolase (*thlA* of *C. acetobutylicum*) and (R)-specific 3-hydroxybutyrate dehydrogenase (*phaB* of *Cupravidus necator*) into a plasmid pMTL8315-Pfdx-thlA-phaB-bld (SEQ ID NO: 132). *Bld* and *phaB* genes were

amplified from the above plasmid via primers in table below and cloned into existing plasmid pMTL85147-thlA (WO 2012/115527).

SEQ ID NO:	Primer	Sequence	Direction
133	bld-phaB-F1	ACATGGGATAAGAAGGAGATATACATATGAT AAAAG	forward
134	bld-pMTL-R1	CGTCGACTCTAGATTAACCTGCTAAAACACAT CTTC	forward
135	pMTL-bld-F1	GTGTTTTAGCAGGTTAATCTAGAGTCGACGTC ACGC	forward

0194 The resulting construct was transformed into *C. autoethanogenum* as described above and a growth experiment was conducted in serum bottles with 50-mL PETC media and pressurized at 30 psi with CO-containing steel mill gas (collected from New Zealand Steel site in Glenbrook, NZ) or a synthetic gas blend with same composition of 44% CO, 32% N_2, 22% CO_2, 2% H_2.

0195 1,3-BDO production was demonstrated via this route from gas (Fig. 17A), but production was less (up to 67 mg/L 1,3-BDO) than via the AOR route and, in contrast to the AOR route, growth was impacted when expressing the *bld* gene comparing to the *C. autoethanogenum* wild-type (Fig. 17B).

0196 In another experiment, *C. autoethanogenum* transformed with plasmid pMTL83159-phaB-thlA as described in Example 2 produced 0.33 and 0.46 g/L of 3-HB and 1,3-BDO, respectively, in a bottle experiment under autotrophic conditions as described in Example 2 (Fig. 40).

Example 5

0197 This example demonstrates the production of (S)-3-hydroxybutyrate and 1,3-butanediol in *C. autoethanogenum*.

0198 A plasmid was constructed that expresses a thiolase (*thlA* from *C. acetobutylicum*; SEQ ID NO: 136) and an (S)-specific 3-hydroxybutyrate dehydrogenase (*hbd1* from *C. kluyveri*; SEQ ID NO: 137) under either a ferredoxin promoter (P_{fdx} isolated from *C. autoethanogenum*; SEQ ID NO: 138) or a pyruvate-ferredoxin oxidoreductase promoter (P_{pfor} isolated from *C. autoethanogenum*; SEQ ID NO: 139). The plasmid was constructed as follows: P-hbd1-rbs2-thlA and pieced together and cloned into the pMTL83151 vector (Heap, *J Microbiol Meth*, 78: 79-85, 2009) by routine methods in molecular cloning, including restrictive enzyme digestion followed by ligation, overlap extension polymerase chain reaction, seamless cloning (Thermo Fisher Scientific), and GeneArt Type IIs (Thermo Fisher Scientific). The operon P – hbd1 – rbs2 – thlA was cloned in between restriction sites NotI and XhoI found in the multiple cloning region of the plasmid. P is the constitutive promoter which contains an intact ribosome binding site (rbs). rbs2 (SEQ ID NO: 140) is the ribosome binding site for expressing thlA. The stepwise procedures were amplification of the P, hbd1, and thlA from existing templates with primers listed below.

SEQ ID NO:	Name	Sequence	Direction
141	Pfdx-F1	AAAGGTCTCCGGCCGCGCTCACTATCTGCG GAACC	forward
142	Pfdx-R1	TTTGGTCTCGAATTCTGTAACACCTCCTTAA TTTTTAG	reverse
143	Ppfor-F1	AAAGGTCTCCGGCCGCAAAATAGTTGATAA TAATGCAGAG	forward
144	Ppfor-R1	TTTGGTCTCGAATTCCTCTCCTTTTCAAGCAT ATA	reverse
145	hbd1-F1	AAAGGTCTCGAATTCAAAGATCTATGTCTAT TAAATCAGTTGCAG	forward
146	hbd1-R1	TTTGGTCTCCCTCCTTTCTATTTCTAATATGC GAAAAATCCTTTACC	reverse
147	thlA-F1	AAAGGTCTCAGGAGGTGTTACATATGAAAG AAGTTGTAATAGCTAGTGC	forward
148	thlA-R1	TTTGGTCTCCTCGAGTATGGATCCCTAGCAC TTTTCTAGCAATATTGC	reverse

0199 The polymerase chain reactions were performed as follow using Kapa Taq PCR Kit (Kapa Biosystems). Set annealing temperature at 56 °C, and extension for 1 minute. Repeat PCR reaction for 30 cycles. Afterwards, PCR products were desalted using the DNA Clean & Concentrator Kit (Zymo Research Corporation).

0200 pMTL83151 plasmid backbone was prepared by carrying out the NotI/XhoI double digestion using the FastDigest NotI and FastDigest XhoI (Thermo Fisher Scientific) following the protocol provided, followed by treatment with alkaline phosphate, using the FastAP Alkaline Phosphatase (Thermo Fisher Scientific) and the protocols provided. The digested backbone was then desalted with the DNA Clean & Concentrator Kit (Zymo Research Corporation).

0201 The assembly of the PCR products and the plasmid backbone was carried out using the GeneArt Type IIs Kit (Thermo Fisher Scientific). The resulting plasmid was then isolated from the *E. coli* plasmid expression host using the QIAprep Spin Miniprep Kit (Qiagen).

0202 To introduce the assembled plasmids pMTL8315-Pfdx-hbd1-thlA and pMTL8315-Ppfor-hbd1-thlA consisting of the operons, the plasmid was first introduced into the *E. coli* CA434 strain by chemical transformation. Afterwards, conjugation was performed by mixing the transformed CA434 strain with a *C. autoethanogenum* production host on a solid LB-agar media, and incubation in an anaerobic environment under pressure with a mix consisting of carbon monoxide and hydrogen as described in Example 2. *C. autoethanogenum*, after conjugation, was selected by successive growth on the solid media containing the proper antibiotic and trimethroprim to remove the remaining *E. coli* CA434 strain, under the anaerobic conditions.

0203 The *C. autoethanogenum* strains carrying the introduced pMTL8315-Pfdx-hbd1-thlA or pMTL8315-Ppfor-hbd1-thlA plasmids consisting of the operon P-hbd1-rbs2-thlA were grown in a 10-mL PETC media in a 250-mL Schott bottle, sealed tight with rubber septum and cap, and pressurized

at 30psi with CO-containing steel mill gas (collected from New Zealand Steel site in Glenbrook, NZ) or a synthetic gas blend with same composition of 44% CO, 32% N_2, 22% CO_2, 2% H_2. Metabolites were measured as described in previous examples.

0204 Surprisingly, there was 3-hydroxybutyrate produced from gas in *C. autoethanogenum* cultures expressing *thlA* and *hbd1* (Fig. 18A). A native thioesterase may convert the formed 3-hydroxybutyryl-CoA to 3-hydroxybutyrate. In the genome sequence, three putative thioesterases were identified. In the strain carrying pMTL8315-Pfdx-hbd1-thlA up to 2.55 g/L 3-hydroxybutyrate was found (Fig. 18A).

0205 Even more surprising, it was also found that 3-hydroxybutyrate is over time converted to 1,3-butanediol, at the end of growth up to 1.1 g/L 1,3-butanediol was produced in strain carrying plasmid pMTL8315-Pfdx-hbd1-thlA (Fig. 18A). This may be due to native aldehyde:ferredoxin oxidoreductase (AOR) and alcohol dehydrogenase activity. Two AOR genes and several alcohol dehydrogenases are present in the genome of *C. autoethanogenum* (Mock, *J Bacteriol*, 197: 2965-2980, 2015). This reduction of 3-hydroxybutyrate (and reduction of acetate to ethanol; Fig. 18B) is powered by reduced ferredoxin and thus can be directly coupled to CO oxidation, which provides reduced ferredoxin ($CO + Fd_{ox} \rightarrow CO_2 + Fd_{red}$) (Fig. 7).

0206 The same strain of *C. autoethanogenum* carrying plasmid pMTL8315-Pfdx-hbd1-thlA was also tested in continuous fermentation. Fermentation was carried out as described in previous example, but the culture was turned continious with a dilution rate with fresh media of around 0.05 at day 2 and then increased to 1.0 at day 3. High 3-hydroxybutyrate production of up to 7 g/L was observed with 1,3-BDO production of 0.5 g/L.

0207 To improve production of (S)-3-hydroxybutyrate and 1,3-butanediol and avoid synthesis of another form of butanediol (2,3-butanediol), plasmid pMTL-HBD-ThlA was introduced into a strain that has an inactivated 2,3-butanediol pathway where the acetolactate decarboxylase gene BudA has been deleted (U.S. 9,297,026). This budA knockout eliminated the major pathway to 2,3-BDO, increasing the specificity for 3-HB and 1,3-BDO production. When pMTL-HBD-ThlA was expressed in the budA deletion strain, a total of 15% C-mol was achieved for both 3-HB and 1,3-BDO (Fig. 41).

	Selectivity (C-mol%)
Acetate	14.7
Ethanol	64.9
2,3-BDO	1.3
Biomass	3.7
3-HB	10.4
1,3-BDO	5.0

0208 As a comparison, in a strain expressing the same plasmid, pMTL83159-hbd-thlA without budA knockout, the total specificity for the production of 3-HB and 1,3-BDO at the steady state was only 6.9%

	Selectivity (C-mol%)
Acetate	0.4
Ethanol	84.3
2,3-BDO	6.2
Biomass	2.2
3-HB	3.5
1,3-BDO	3.4

Example 6

0209 This example demonstrates that the Ptb-Buk system is efficient in *C. autoethanogenum* on a range of acyl-CoAs including acetoacetyl-CoA, 3-hydroxybutyryl-CoA, and 2-hydroxyisobutyryl-CoA

0210 The Ptb-Buk system was expressed from a plasmid in *C. autoethanogenum* and its activity measured using a CoA hydrolysis assay. For this, *ptb-buk* genes from *C. beijerinckii* NCIMB8052 (GenBank NC_009617, position 232027..234147; Cbei_0203-204; NCBI-GeneID 5291437-38) were amplified from genomic DNA of *C. beijerinckii* NCIMB8052 and cloned under control of a pyruvate-ferredoxin oxidoreductase promoter (P_{pfor} isolated from *C. autoethanogenum*; SEQ ID NO: 139) into pMTL82251 vector ((Heap, *J Microbiol Meth*, 78: 79-85, 2009) by routine methods in molecular cloning, including restrictive enzyme digestion followed by ligation, overlap extension polymerase chain reaction, seamless cloning (Thermo Fisher Scientific), and GeneArt Type IIs (Thermo Fisher Scientific) as described in Example 5. Oligonucleotides are described below.

SEQ ID NO:	Name	Sequence	Direction
149	Ppfor-F2	aaacagctatgaccgcGGCCGCAAAATAGT	forward
150	Ppfor-R2	ttactcatTGGATTCCTCTCCTTT	reverse
151	Ptb-Buk-F2	ggaatccaATGAGTAAAAACTTTGATGAG	forward
152	Ptb-Buk-R2	caggcctcgagatctcCTAGTAAACCTTAGCTTGTTC	reverse

0211 The resulting plasmid pMTL82256-ptb-buk (SEQ ID NO: 153) was introduced into *C. autoethanogenum* as described in previous examples.

0212 Acyl-CoA hydrolysis assays were performed as follows. *C. autoethanogenum* cells were harvested at OD 2 (late exponential phase) by centrifugation (14,000 rpm for 1 min at 4 °C). Cells

were re-suspended in 500 µl lysis buffer (potassium phosphate buffer, pH 8). Cells were lysed using a freeze thaw cycle (optional), sonication 6 x 30 s at amplitude 20 on ice. Samples were centrifuged for 10 min at 14,000 rpm at 4 °C and the supernatant with soluble proteins was removed. The protein concentration was measured, e.g., with a Bradford assay.

0213 The assay mix contained: 484 µl of potassium phosphate buffer pH 8.0, 1 µl of DTNB (final concentration of 0.1 mM), 10 µl of cell lysate, and 5 µl of CoA (final concentration of 500 µM). All the components were mixed in a quartz cuvette (1 ml cuvette with a read length of 1 cm) except the protein. The assay was started by adding the cell lysate and followin the reaction in a spectrophotometer at 405 nm, 30 °C for 3 min. A control without lysate was run to measure autolysis of the acyl-CoA.

0214 To determine activity, slope on the linear part of the curve (usually in the first 30 s), was calculated. The protein amount was normalized and slope was divided by protein amount. An extinction coefficient (14,150 M^{-1} cm^{-1}) was used to calculate the specific activity in M/s/mg. The activity of the negative control was subtracted.

0215 The assay was performed with acetoacetyl-CoA, a racemic mix of 3-hydroxybutyryl-CoA (3-HB-CoA) and 2-hydroxyisobutyryl-CoA (2-HIB-CoA). The possibility of artificially low hydrolysis rates for 3-HB-CoA and 2-HIB-CoA due to potential substrate limitation was addressed by repeating the hydrolysis assays for *C. autoethanogenum* lysates using different concentrations of acyl-CoA, 500 µM and 200 µM.

0216 The results of the assay show significantly increased CoA hydrolysis in lysates of *C. autoethanogenum* carrying plasmid pMTL82256-ptb-buk expressing the Ptb-Buk system on a range of acyl-CoAs including acetoacetyl-CoA, 3-hydroxybutyryl-CoA and 2-hydroxyisobutyryl-CoA (Figs. 20A-B). Notably, there is also CoA hydrolysis for acyl-CoAs as 2-hydroxyisobutyryl-CoA that are not hydrolysed by the *C. autoethanogenum* wild-type. With acetoacetyl-CoA and 3-hydroxybutyryl-CoA some native CoA hydrolysis activity was observed.

Example 7

0217 This example demonstrates the disruption of identified native thioesterase genes improve efficiency of the Ptb-Buk and CoA transferase system by increasing the pool of available acyl-CoAs such as acetoacetyl-CoA, 3-hydroxybutyryl-CoA or 2-hydroxyisobutyryl-CoA.

0218 In contrast to the Ptb-Buk system, where energy is conserved in the form of ATP during conversion of acyl-CoAs to their respective acids, no energy is conserved if the CoAs are simply hydrolyzed.

0219 In hydrolase assays it was found that there is native hydrolysis activity for acetoacetyl-CoA and 3-hydroxybutyryl-CoA in *C. autoethanogenum.*

0220 Acyl-CoA hydrolysis assays with acetoacetyl-CoA, a racemic mix of 3-hydroxybutyryl-CoA (3-HB-CoA) and 2-hydroxyisobutyryl-CoA (2-HIB-CoA were performed as described in previous

example. The results of the assay show cleavage of acetoacetyl-CoA and 3-HB-CoA, but not 2-HIB-CoA, and confirm native activity is present in *C. autoethanogenum* (Fig. 11).

0221 An analysis of the genome of *C. autoethanogenum* led to identification of three putative CoA-thioesterases (thioester-hydrolases) that could be responsible for to the cleavage of acetoacetyl-CoA or 3-hydroxybutyryl-CoA thioester bond. These are also present in other acetogens such as *C. ljungdahlii*.

Description	Annotation	*C. autoethanogenum*	SEQ ID NO:	*C. ljungdahlii*	SEQ ID NO:
thioesterase 1 (CAETHG_0718)	Palmitoyl-CoA hydrolase	AGY74947.1	154	ADK15695.1	157
thioesterase 2 (CAETHG_1524)	4-Hydroxybenzoyl-CoA thioesterase	AGY75747.1	155	ADK16655.1	158
thioesterase 3 (CAETHG_1780)	Putative Thioesterase	AGY75999.1	156	ADK16959.1	159

0222 Inactivation of these three putative CoA-thioesterases lead to higher product titers, improving efficiency of the Ptb-Buk system. The three putative thioesterases were inactivated using ClosTron technology. In brief, the targeting domain of the type II Ltr was reprogrammed using the ClosTron website and the retargeted ClosTron plasmids were ordered from DNA 2.0. The ClosTron knock out vectors pMTL007C-E2-Cau-2640-571s targeting the thioesterase 1(CAETHG_0718), pMTL007C-E2-PBor3782-166s targeting the thioesterase 2 (CAETHG_1524), and pMTL007C-E2-PBor4039-199s targeting the thioesterase 3(CAETHG_1780) were introduced into *C. autoethanogenum* using conjugation.

0223 Selection for integration was done by selecting PETC supplemented with 5 μg/ml clarithromycin and successful inactivation by integration of the type II intron was confirmed by PCR across the insertion site.

0224 The CoA hydrolase activity on acetoacetyl-CoA of both wild type *C. autoethanogenum* and each of the *C. autoethanogenum* with one of the putative genes inactivated was measured using the assay described above. It was shown that all three strains with the inactivated putative thioesterases showed less hydrolysis activity on acetoacetyl-CoA and 3-hydroxybutyryl-CoA (Figs. 21A-B).

0225 To demonstrate that the decreased CoA hydrolase activity, and thus an increased pool in acetoacetyl-CoA, is beneficial for production of acetoacetyl-CoA derived products, the isopropanol plasmid pMTL85147-thlA-ctfAB-adc encoding *thl+ ctfAB + adc* (WO 2012/115527) was introduced into the *C. autoethanogenum* wild-type strain and the strain with inactivated thioesterase 1. A growth experiment was carried out 40 ml PETC medium in 1L Schott bottles in technical triplicates with Co gas at 37 °C at 110 rpm shaking. Synthetic gas (50% CO, 18% CO_2, 2 % H_2, and 30% N_2) was used

as sole energy and carbon source. Headspace exchanged once and gassed to 21 psi (1.5 bar) at 37 °C under synthetic gas (50% CO, 18% CO_2, 2 % H_2, and 30% N_2). Samples for OD and analytics were taken twice a day.

0226 The strain with inactivated thioesterase 3 CAETHG_1780 produced significantly higher levels of isopropanol than the wild-type (Fig. 22 and Figs. 23A-D).

0227 Similarly, knockout of thioesterases in *C. autoethanogenum* would increase the pool of 3-hydroxybutyryl-CoA, allowing more efficient utilization of 3-hydroxybutyryl-CoA by Ptb-Buk and leading to higher production of acetone, isopropanol, isobutylene, (R)-3-hydroxybutyrate, 1,3-butanediol, and/or 2-hydroxyisobutyric acid. When plasmid pMTL8315-Pfdx-hbd1-thlA of Example 5 was introduced into *C. autoethanogenum* strain with interrupted thioesterase 2 CAETHG_1524, 3-hydroxybutyrate synthesis was abolished (compared to the up to 2.55 g/L 3-hydroxybutyrate that were found when expressing this plasmid in the *C. autoethanogenum* wild type strain). No competing activity for 3-hydroxybutyryl-CoA is present in this strain.

0228 These results demonstrate that by reducing thioesterase activity, a higher CoA pool for the Ptb-Buk system and product synthesis is available.

0229 Additionally, the production of 3-HB and 1,3-BDO can be increased by overexpression of ptb-buk. In a control experiment, whereby *C. autoethanogenum* as described in Example 2 was transformed with plasmids pMTL83159-phaB-thlA from Example 4 plus pMTL82256 (Heap, *J Microbiol Methods*, 78: 79-85, 2009), in which the latter is an empty plasmid used as a background control, the fermentation of such strain resulted in a production of 3-HB with highest titer at 1.68 g/L at day 10 (Fig. 42A). When pMTL82256-buk-ptb, instead of the empty plasmid pMTL82256, was coexpressed with pMTL83159-phaB-thlA in *C. autoethanogenum*, the fermentation resulted in a higher titter of 3-HB, at 4.76 g/L, at an earlier time, day 4 (Fig. 42B).

0230 Deletion of native thioesterases enhances the efficiency of the ptb-buk system, which has preference for (R)-3-HB-CoA. The locus of the thioesterase gene in the genome was deleted and replaced with the buk-ptb dna fragment via the common molecular biology technique known as homologous recombination. The substitution of the thioesterase gene by the buk-ptb was confirmed by PCR, followed by agarose gel electrophoresis and dna sequencing.

0231 In a bottle experiment, when pMTL83156-phaB-thlA was expressed without ptb-buk in the thioesterase deletion mutant, described above, the average maximum titer of 3-HB produced was 0.50 ± 0.05 g/L, similar to the titer obtained using an unmodified *C. autoethanogenum* strain. When pMTL82256-buk-ptb was coexpressed with the pMTL83156-phaB-thlA plasmid in a thioesterase knockout strain, the production of 3-HB increased to 1.29 ± 0.10 g/L (Fig. 43).

Example 8

0232 This example demonstrates that it is possible to eliminate acetate production system in an acetogen *C. autoethanogenum* with the Ptb-buk system.

0233 All acetogenic microorganisms are described to produce acetate (Drake, Acetogenic Prokaryotes, In: *The Prokaryotes*, 3rd edition, pages 354-420, New York, NY, Springer, 2006) as the production of acetate provides the microorganism with an option to directly generate ATP from substrate level phosphorylation via Pta (phosphotransacetylase) and Ack (phosphotransacetylase-acetate kinase). Native acetate-forming enzymes such as Pta-Ack are therefore considered to be essential in acetogens (Nagarajan, *Microb Cell Factories*, 12: 118, 2013). Since Ptb-Buk provides an alternative means for energy generation, it becomes possible to replace the native Pta-Ack system with Ptb-Buk.

0234 The *pta* and *ack* genes in *C. autoethanogenum* are in one operon. To replace pta and ack genes with *ptb* and *buk* genes a plasmid, pMTL8225-pta-ack::ptb-buk (Fig. 24), with *mazF* counter selection marker that is under tetracycline inducible promoter, ~1kb upstream homology arm, *ptb, buk, ermB* cassette flanked by *loxP* sites and ~1kb downstream homology arm was assembled (SEQ ID NO: 160).

0235 The ~1kb upstream and downstream homology arms were PCR amplified from *C. autoethanogenum* with primers SN22f/ SN23r and SN28f/ SN29r. *Ptb* and *buk* genes were PCR amplified from pIPA_16 plasmid using primers SN24f/ SN25r. The ermB cassette with loxP sites was PCR amplified using primers SN26f/ SN27r. The plasmid backbone was PCR amplified with primers SN30f/ SN31r. KAPA polymerase was used for all PCR amplifications. The PCR products were assembled using GeneArt Seamless cloning kit from Life Technologies and plasmid with no mutations in the insert fragments was used to transform *C. autoethanogenum* by conjugation as described earlier.

0236 Following conjugation and selection on trimethoprim and clarithromycin, 7 colonies were streaked twice on PETC-MES agar plates with clarithromycin and anhydrotetracycline to induce the expression of mazF genes. The colonies from clarithromycin and anhydrotetracycline should have the *pta* and *ack* genes replaced with *ptb* and *buk* genes and ermB cassette. This was verified by PCR using primers Og29f/ Og30r flanking the homology arms and KAPA polymerase (Fig. 25). While a band of ~4.6 kb is amplified from the wildtype strain, bands of ~5.7 kb was amplified from colonies 1 and 4-7, indicating the replacement of *pta* and *ack* genes replaced with *ptb* and *buk* genes and ermB cassette. The above event was further confirmed by sequencing the PCR products from clones 4-7.

0237 With the resulting modification the expression of ptb and buk genes is driven by the promoter upstream of pta gene.

SEQ ID NO:	Name	Sequence
161	SN22f	TTTACAAATTCGGCCGGCCAAAGATTGCTCTATGTTTAAGCT
162	SN23r	CATCAAAGTTTTTACTCATCAATTTCATGTTCATTTCCTCCCT
163	SN24f	AGGGAGGAAATGAACATGAAATTGATGAGTAAAAACTTTGAT GAGT

164	SN25r	GTATAGCATACATTATACGAACGGTACTAGTAAACCTTAGCTT GTTCTTC
165	SN26f	GAAGAACAAGCTAAGGTTTACTAGTACCGTTCGTATAATGTAT GCTATAC
166	SN27r	AGAGATGAGCATTAAAAGTCAAGTCTACCGTTCGTATAGCATA CA
167	SN28f	TGTATGCTATACGAACGGTAGACTTGACTTTTAATGCTCATCTC T
168	SN29r	CATGAGATTATCAAAAAGGAGTTTAAATATCTATTTTGTCCTTA GGA
169	SN30f	TCCTAAGGACAAAATAGATATTTAAACTCCTTTTTGATAATCTC ATG
170	SN31r	AGCTTAAACATAGAGCAATCTTTGGCCGGCCGAATTTGTAAA
171	Og29f	AGCCACATCCAGTAGATTGAACTTT
172	Og30r	AATTCGCCCTACGATTAAAGTGGAA

0238 The resulting strain *C. autoethanogenum* pta-ack::ptb-buk, in which the pta-ack operon was replaced by the ptb-buk operon was transformed as described above with the isopropanol production plasmid pMTL85147-thlA-adc from Example 2. A growth study was carried out under autotrophic conditions and analyzed for metabolic end products. No acetate production was observed, while isopropanol (up to 0.355 g/L) and 3-HB (up to 0.29 g/L) was still produced alongside ethanol and 2,3-butanediol (Figs. 39A and 39B). This demonstrates that it is possible to produce isopropanol and 3-HB without acetate production from gaseous substrates CO and/or CO_2 and H_2 using the Ptb-Buk system.

0239 If acetone rather than isopropanol is the target product, the primary:secondary alcohol dehydrogenase gene (SEQ ID NO: 17) can be further knocked out this strain *C. autoethanogenum* pta-ack::ptb-buk using methods described above and in detail in WO 2015/085015. Introducing plasmid pMTL85147-thlA-adc into this strain results in production of acetone at similar levels as described above for isopropanol without co-production of acetate. Ethanol, 2,3-butanediol and 3-HB may be further products.

0240 By further knock-outs it is possible to eliminate these products as well, e.g., knock-out of the acetolactate decarboxylase gene BudA results in a strain unable to produce 2,3-butanediol (U.S. 9,297,026). 3-HB production may be reduced or eliminated by deletion of 3-hydroxybutyrate dehydrogenase gene Bdh (SEQ ID NO: 62).

Example 9

0241 This example demonstrates improvement of conversion of 3-hydroxybutyrate to 1,3-BDO by overexpression of the aldehyde:ferredoxin oxidoreductase gene *aor1*.

0242 The pMTL82251 plasmid backbone was used for overexpression of the *C. autoethanogenum aor1* gene. The pMTL82251 plasmid was selected since it has a different replication origin and antibiotic marker, but could be co-expressed with, the plasmid used in Example 5 that contained *hbd1* and *thlA*. Preparation of the plasmid backbone and the assembly reaction were carried out following the procedures listed above, first generating plasmid pMTL82256 by introducing the *C. autoethanogenum* ferredoxin promoter into plasmid pMTL82251 and then adding the *aor1* genes to form plasmid pMTL82256-aor1. The following primers were used.

SEQ ID NO:	Name	Sequence	Direction
173	Pfdx-F1	AAAGGTCTCCGGCCGCGCTCACTATCTGCGGAACC	forward
174	Pfdx-R1	TTTGGTCTCGAATTCTGTAACACCTCCTTAATTTTTAG	reverse
175	aor1-F1	AAAGGTCTCGAATTCAAAGATCTATGTATGGTTATGATGGTAAAGTATTAAG	forward
176	aor1-R1	TTTGGTCTCCTCGAGTATGGATCCCTAGAACTTACCTATATATTCATCTAATCC	reverse

0243 After transforming the resulting plasmid pMTL82256-aor1 into the *E. coli* CA434 strain, conjugation was performed on the previous *C. autoethanogenum* 1,3-BDO production host. Thus, the resulting *C. autoethanogenum* strain carried two plasmids, one for overexpressing *hbd1* and *thlA*, and another for *aor1*, under different replication origins and selection marker. The production for 1,3-BDO was characterized and quantified following the procedures above.

0244 The results clearly show that 1,3-BDO production can be improved by overexpressing *aor1*. Likewise other aldehyde:ferredoxin oxidoreductase genes could be expressed in *C. autoethanogenum* to facilitate convserion of 3-hydroxybutyrate to 1,3-butanediol.

0245 To improve of 1,3-BDO production, AOR was overexpressed to improve conversion of 3-HB to 3-HB-aldehyde. To do this, pMTL82256-hbd-thlA and pMTL83159-aor1 were coexpressed in *C. autoethanogenum*. As compared to the strain that carried pMTL82256-hbd-thlA alone, the aor1-coexpressed strain produced higher ethanol and 1,3-BDO (Fig. 44).

Example 10

0246 This example demonstrates the stereospecificity of Ptb-Buk that allows for the production of 2-hydroxyisobutyric acid without the production of unwanted byproducts.

0247 2-hydroxyisobutyic acid can be produced in *E. coli* and *C. autoethanogenum* by introduction of a thiolase and a 3-hydroxybutyryl-CoA dehydrogenase to convert acetyl-CoA to 3-hydroxybutyryl-CoA, a 2-hydroxyisobutyryl-CoA mutase enzyme for conversion of 3-hydroxybutyryl-CoA to 2-hydroxyisobutyryl-CoA and an enzyme that can hydrolyse the CoA to form 2-hydroxyisobutyric acid. The 3-hydroxybutyryl-CoA dehydrogenase can either be (R)- or (S)-specific and the enzyme

converting 2-hydroxyisobutyryl-CoA to 2-hydroxybutyrate according to steps 1, 13, 19, and 20 of Fig. 1. This last step can either be done via a thioesterase or the Ptb-Buk system.

0248 Three potential candidate genes, *E. coli* thioesterase type II TesB, the *C. autoethanogenum* phosphate acetyltransferase / acetate kinase pair and the *C. beijerinckii* butyryltransferase / butyrate kinase pair were cloned into *E. coli* pDUET T7 expression vectors via methods described above and primers below.

SEQ ID NO:	Primer	Sequence
177	pETDuet-pta-ack - ack-DuetI2-R1	GGGTACCTTATTTATTTTCAACTATTTCTTTTGTATC
178	pETDuet-pta-ack - DuetI2-ack-F1	TTGAAAATAAATAAGGTACCCTCGAGTCTGGTAAAG
179	pETDuet-pta-ack - DuetI2-pta-R1	TTTTTTCCATATGTATATCTCCTTCTTATACTTAAC
180	pETDuet-pta-ack - pta-DuetI2-F1	AGGAGATATACATATGGAAAAAATTTGGAGTAAGGC
181	pETDuet-tesB - DuetI2-tesB-F1	GAAATCATAATTAAGGTACCCTCGAGTCTGGTAAAG
182	pETDuet-tesB - DuetI2-tesB-R1	CCTGACTCATATGTATATCTCCTTCTTATACTTAAC
183	pETDuet-tesB - tesB-DuetI2-F1	AAGAAGGAGATATACATATGAGTCAGGCACTTAAAA
184	pETDuet-tesB - testB-DuetI2-R1	AGGGTACCTTAATTATGATTTCTCATAACACCTTC

0249 The obtained plasmids pDUET-pta-ack (SEQ ID NO: 185), pDUET-ptb-buk (SEQ ID NO: 186), pDUET-tesB (SEQ ID NO: 187) and introduced into *E. coli* BL21(DE3) for expression and then assayed for their activity on acetoacetyl-CoA, 3-hydroxybutyryl-CoA and 2-hydroxyisobutyryl-CoA. The results are shown in Fig. 27. *E. coli* BL21 has a small but measurable amount of activity on all three substrates. Pta-Ack resulted in no activity above background, while both thioesterase TesB and Ptb-Buk showed high activity on all three substrates, including 2-hydroxyisobutyryl-CoA.

0250 The activity of both thioesterase TesB and Ptb-Buk was higher on linear acetoacetyl-CoA, 3-hydroxybutyryl-CoA than on branched 2-hydroxyisobutyryl-CoA. This creates a problem in the pathway as it results in early termination of the pathway at 3-hydroxybutyryl-CoA, in particular as activities are higher than activities on the 2-hydroxyisobutyryl-CoA mutase enzyme.

0251 However, Ptb-Buk in contrast to thioesterases is able to distinguish between stereoisomers and will only (or preferentially) act on (R)-3-hydroxybutyryl-CoA but not on (S)-3-hydroxybutyryl-CoA. This was demonstrated by expressing the Ptb-Buk system either with ThlA and (S)-specific Hbd (Fig. 28A) or (R)-specific phaB (Fig. 28B) in the pDuet system in *E. coli*. The constructs were

constructed as described in Examples 1 and 3. Growth studies confirmed that appreciable amounts of 3-hydroxybutyrate were only formed when Ptb-Buk was expressed in combination with the (S)-specific Hbd but not the (R)-specific phaB.

0252 Therefore, a route via an (S)-specific 3-hydroxybutyryl-CoA dehydrogenase and the Ptb-Buk provides significant advantages, as the Ptb-Buk system (unlike thioesterases) is not active on (S)-3-hydroxybutyryl-CoA but (S)-3-hydroxybutyryl-CoA is also the preferred isomer of the 2-hydroxyisobutyryl-CoA mutase (Yaneva, *J Biol Chem*, 287: 15502-15511, 2012). The produced 2-hydroxyisobutyryl-CoA can then be used via the Ptb-Buk to produce 2-hydroxyisobutyric acid and (unlike thioesterases) 2-hydroxyisobutyryl-CoA hydrolysis provides additional energy (Fig. 8).

0253 Modular constructs were designed to compare performance of the pathway. A gene cassette containing the Wood-Ljungdahl promoter in front of the genes *meaB*, *hcmA* and *hcmB* was codon optimized and synthesized (SEQ ID NO: 188). *HcmA* and *hcmB* encode a 2-hydroxyisobutyryl-CoA mutase and *meaB* a chaperon from *Aquincola tertiaricarbonis*, in the construct *hcmA* and *meaB* genes were fused together as one protein as described (SEQ ID NO: 189) (Yaneva, *J Biol Chem*, 287: 15502-15511, 2012). The gene cassette was cloned into either a plasmid containing thiolase (*thlA* from *C. acetobutylicum*; SEQ ID NO: 136) and an (S)-specific 3-hydroxybutyrate dehydrogenase (*hbd* from *C. acetobutylicum*; SEQ ID NO: 190) (pMTL83155-thlA-hbd) or an (R)-specific 3-hydroxybutyrate dehydrogenase (*phaB* from *R. eutropha*) (pMTL83155-thlA-phaB) using the restriction enzymes KpnI and NcoI to form plasmids pMTL83155-thlA-hbd-Pwl-meaBhcmA-hcmB (SEQ ID NO: 191) and pMTL83155-thlA-phaB-Pwl-meaBhcmA-hcmB (SEQ ID NO: 192), respectively. Sub-cloning of the codon optimized 2-hydroxyisobutyryl-CoA mutase casette in *E. coli* Top-10 was only successful after some initial cloning complications; it was found that the 2-hydroxyisobutyryl-CoA mutase casette could only be cloned into the plasmid at a lower temperature (28 °C).

0254 Vector pMTL83155-thlA-hbd and pMTL83155-thlA-phaB were created by first amplifying a promoter region of the phosphate acetyltransferase of *C. autoethanogenum* (SEQ ID NO: 193) and cloning into vector pMTL83151 (FJ797647.1; Heap, *J Microbiol Meth*, 78: 79-85, 2009) using NotI and NdeI restriction sites before introducing genes *thlA* and *hbd* or respectively *phaB* via *NdeI* and *KpnI* in a double ligation reaction.

0255 In addition, compatible plasmid modules for expressing *ptb-buk* or *tesB* were built. For this, the respective genes were amplified from genomic DNA and introduced into plasmid pMTL82256 described in Example 9 and then introducing either *ptb-buk* or *phaB* using NdeI and NcoI and Seamless Cloning kit (Life technologies) to form plasmids pMTL82256-ptb-buk (SEQ ID NO: 194) and pMTL82256-tesB (SEQ ID NO: 195).

0256 Plasmids pMTL83155-thlA-hbd-Pwl-meaBhcmA-hcmB, pMTL83155-thlA-phaB-Pwl-meaBhcmA-hcmB, pMTL82256-ptb-buk and pMTL82256-tesB were introduced into *E. coli* Top-10 (all steps at 28 °C) and *C. autoethanogenum* by transformation as described in previous examples in

the following combinations: pMTL83155-thlA-hbd-Pwl-meaBhcmA-hcmB + pMTL82256-ptb-buk, pMTL83155-thlA-hbd-Pwl-meaBhcmA-hcmB + pMTL82256-tcsB, pMTL83155-thlA-phaB-Pwl-meaBhcmA-hcmB + pMTL82256-ptb-buk and pMTL83155-thlA-phaB-Pwl-meaBhcmA-hcmB + pMTL82256-tcsB.

0257 Growth experiments were carried out with *E. coli* in LB medium at 30 °C for 4 days and *C. autoethanogenum* in PETC medium with 30 psi CO-containing steel mill gas (collected from New Zealand Steel site in Glenbrook, NZ) at 30 °C and 37 °C for 6 days. Metabolites were measured as described above. In addition to measurement by GC-MS, 2-Hydroxyisobutyric acid poroduction was also confirmed using liquid chromatography tandem mass spectrometry (LC-MS/MS) and ^{1}H nuclear magnetic resonance (NMR) spectroscopy.

0258 Liquid chromatography tandem mass spectrometry (LC-MS/MS) data was acquired on a Dionex UltiMate 3000 liquid chromatography system (Dionex, California, USA) coupled to an ABSciex 4000 QTRAP mass spectrometer (ABSciex, Concord, Canada). The liquid chromatography system was controlled by Chromeleon software (Dionex), and chromatographic separation was achieved by injecting 10µL onto a Gemini-NX C18 150 mm × 2 mm I.D., 3µm 110 Å particle column (Phenomenex, Aschaffenburg, Germany) equipped with a pre-column Security Guard Gemini-NX C18 4mm x 2 mm I.D. cartridge. The column oven temperature was controlled and maintained at 55°C throughout the acquisition and the mobile phases were as follows: 7.5mM aqueous tributylamine adjusted to pH 4.95 (±0.05) with glacial acetic acid (eluent A) and acetonitrile (eluent B). The mobile phase flow rate was maintained at 300 µL/min throughout a gradient profile and was introduced directly into the mass spectrometer with no split. The mass spectrometer was controlled by Analyst 1.5.2 software (ABSciex) and was equipped with a TurboV electrospray source operated in negative ionisation mode. The following previously optimized (and therefore general) parameters were used to acquire scheduled Multiple Reaction Monitoring (MRM) data: ionspray voltage −4500V, nebulizer (GS1), auxiliary (GS2), curtain (CUR) and collision (CAD) gases were 60, 60, 20 and medium (arbitrary units), respectively, generated via a N300DR nitrogen generator (Peak Scientific, Massachusetts, USA). The auxiliary gas temperature was maintained at 350 °C. The entrance potential (EP) was -10 volts. This method is also able to detect and separate 2-hydroxybutyric acid.

0259 ^{1}H nuclear magnetic resonance (NMR) spectroscopy at a field strength of 400 MHz. Samples were prepared by diluting 400 µL of sample with 400 µL of 20 mM phosphate buffer prepared with D_2O and containing trimethylsilyl proprionic acid (TMSP) as internal standard (pH of 7). The samples were then transferred glass NMR tube (5mm x 8 inches) and analysed by ^{1}H NMR using presaturation for water suppression with a 30° excitation pulse, 15 second relaxation delay and 64 scans at a temperature of 27°C. Once acquired the spectrum was transformed, flattened and integrated using Agilent VnmrJ software. The known concentration of TMSP was used for quantitation of 2-hydroxyisobutyric using the resonance at 1.36 ppm (singlet).

0260 In both *E. coli* growing heterotrophically as well as *C. autoethanogenum* growing autotrophically, 2-hydroxyisobutyric acid could be detected in constructs pMTL83155-thlA-hbd-Pwl-meaBhcmA-hcmB + pMTL82256-tesB (1.5 mg/L in LC-MS/MS method and 8 mg/L in GC-MS in *C. autoethanogenum*; 0.5 mg/L in LC-MS/MS method and 2 mg/L in GC-MS in *E. coli*) and pMTL83155-thlA-phaB-Pwl-meaBhcmA-hcmB + pMTL82256-ptb-buk (15 mg/L in LC-MS/MS method and 75 mg/L in GC-MS in *C. autoethanogenum;* 1.1 mg/L in LC-MS/MS method and 8.5 mg/L in GC-MS in *E. coli*), but not in constructs all other constructs including the control. By far the highest production occurred in strain carrying plasmid pMTL83155-thlA-hbd-Pwl-meaBhcmA-hcmB + pMTL82256-ptb-buk (10x higher than all other routes), that has the optimal pathway with thiolase, (S)-specific (S)-specific 3-hydroxybutyryl-CoA dehydrogenase, the 2-hydroxyisobutyryl-CoA mutase, and the Ptb-Buk system (Figs. 29A-D). Surprisingly, also production of 2-hydroxybutyrate (2-HB) (up to 64 mg/L by LC-MS/MS and 50 mg/L by GC-MS in *C. autoethanogenum*; 12 mg/L by LC-MS/MS and 9.5 mg/L by GC-MS in *E. coli*) was found in this strain, indicating unspecific mutase activity (Fig. 30). This was also found in the tesB strain, but again at significant lower levels (18 mg/L in LC-MS-MS and 9 mg/L in GC-MS in *C. autoethanogenum*). Production of 2-hydroxyisobutyric acid was also confirmed by NMR.

0261 In addition, also qRT-PCR was carried out to confirm expression of the genes *thlA, hbd, meaBhcmA* and *hcmB* (Fig. 31).

0262 The RT-PCR graphs show that *thlA* gene product is expressed to slightly higher levels with the $P_{pta\text{-}ack}$ promoter than *hbd* (as expected with a second gene in an operon) and that *hmcB* shows slightly lower expression levels than *meaBhcmA*. Also there is lower expression in *C. autoethanogenum* at 30° C than at 37 °C and *E. coli* at 30°C. For specific cycle numbers see below.

Condition	Target	Cq Mean	Cq Std Dev
E. coli / 30 °C	*thlA*	18.26	0.243
	hbd	20.6	0.603
	meaBhcmA	16.20	0.108
	hmcB	18.30	0.666
C. autoethanogenum / 30 °C	*thlA*	26.10	0.169
	Hbd	27.54	0.415
	meaBhcmA	20.63	0.604
	hmcB	22.64	0.697
C. autoethanogenum / 37 °C	*thlA*	18.48	0.069
	hbd	21.85	0.222
	meaBhcmA	16.72	0.119
	hmcB	19.62	0.173

0263 The ratio of (S)-3-hydroxybutyric acid to (R)-3-hydroxybutyric acid was measured by high-performance liquid chromatography (HPLC) on an Agilent 1260 Infinity LC with UV detection at 210 nm. Samples were prepared by centrifugation at 14,000 rpm for 3 minutes, followed by evaporation of 200 µL of supernatant to dryness. The pellet was then re-suspended in 100% Isopropanol and sonicated under heat for 1 hour. Centrifugation was repeated and the supernatant transferred to an HPLC vial for analysis. Separation was achieved with a 5 µL injection on to a TCI Chiral MB-S column (250 mm x 4.6 mm x 3 µm) at 1.5 mL/min and 40°C under isocratic conditions, using 95-5 hexane-isopropanol mobile phase containing 0.1% trifluoracetic acid.

0264 A stereospecific analysis of produce 3-HB has been performed. Surprisingly it was found that in *C. autoethanogenum*, a mix of isomers was produced. Enzymes Hbd and PhaB are described to be stereospecific, PhaB is R-specific and Hbd is S-specific and when expressing these enzymes in *E. coli* a stereopure product has been observed (Tseng, *Appl Environ Microbiol*, 75: 3137-3145, 2009).

0265 The following table indicates the distribution of (R)- and (S)-form of 3-HB at equilibrium produced via three different routes in *C. autoethanogenum*. These data suggest the presence of isomerase in the *C. autoethanogenum*.

Route	% R-form	% S-form
ThlA – PhaB	55 ± 7	53 ± 5
ThlA – HBD	12 ± 3	88 ± 3
ThlA – ctfAB	16 ± 7	84 ± 7

0266 Knockout of native isomerases may prevent interconversion of (R) and (S) forms of 3-HB. Alternatively, expression or overexpression of isomerases could enable new ptb-buk routes. For example, Hbd could be used to generate (S)-3-HB, isomerase could convert (S)-3-HB to (R)-3-HB, and ptb-buk could act on (R)-3-HB to produce products of interest.

Example 11

0267 This example demonstrates the production of isobutylene via Ptb-Buk conversion of 3-hydroxyisovaleryl-CoA and 3-hydroxyisovalerate.

0268 Different routes for production of isobutylene have been described, for example the conversion of acetone to isobutylene via a hydroxyisovalerate synthase and decarboxylase (van Leeuwen, *Appl Microbiol Biotechnol*, 93: 1377-1387, 2012). However, the hydroxyisovalerate decarboxylase step is an ATP requiring step and kinetics of this enzyme may not be ideal. Two alternative routes to isobutylene using the Ptb-Buk system have been identified through 3-hydroxyisovaleryl-CoA which has been shown *in vitro* to be a viable substrate for the Ptb-Buk system (Liu, *Appl Microbiol Biotechnol*, 53: 545-552, 2000).

0269 Alternative pathway 1 consists of a synthase that converts acetone into 3-hydroxyisovaleryl-CoA (Fig. 9).

0270 Alternative pathway 2 proceeds via known intermediate 3-methyl-2-oxopentanoate of the isoleucine biosynthesis that is common to bacteria such as *E. coli* or *C. autoethanogenum* (Fig. 10).

Example 12

0271 This example describes methods for characterizing Ptb-Buk variants.

0272 Given the substrate promiscuity of Ptb-Buk, it is likely that Ptb-Buk systems of varying amino acid sequences will possess varying preferences for given substrates. In order to identify a Ptb-Buk system that favors a desired substrate (e.g. acetoacetyl-CoA, 3-hydroxybutyryl-CoA, 2-hydroxyisobutyryl-CoA, acetyl-CoA, and/or butyryl-CoA), a high-throughput screen is desirable. Such a screen can be accomplished by coupling firefly luciferase (Luc) to the Ptb-Buk system (Fig. 33). Luc reacts with D-luciferin, generating oxyluciferin, carbon dioxide, and light. In addition to magnesium and molecular oxygen, Luc requires ATP for the reaction to proceed. ATP is a product generated by Ptb-Buk when provided an appropriate acyl-CoA or enoyl-CoA substrate. Therefore, Ptb-Buk reaction rates and preferences can be compared for varying substrates by quantifying the amount of light generated by a reaction containing Ptb-Buk, Luc, d-luciferin, magnesium, molecular oxygen, phosphate, ADP, and an acyl-CoA or enoyl-CoA.

Example 13

0273 This example uses genome-scale modeling to demonstrate that high non-native product selectivities can be achieved using Ptb-Buk. Furthermore, it shows that the use of Ptb-Buk could permit the coupling of cellular growth with product production, allowing the construction of stable and high-yielding fermentation strains.

0274 A genome-scale metabolic model of *C. autoethanogenum* similar to the one described by Marcellin, *Green Chem*, 18: 3020-3028, 2006 was utilized. Variants of this model were created that incorporate additional metabolic reactions, each one representing a different genetically modified microorganism for non-native product formation. Three model versions were created for each non-native product pathway, incorporating either a thioesterase, acetate CoA-transferase or Ptb-Buk reaction.

0275 Maximum selectivities were calculated using flux balance analysis (FBA), using scripts from the COBRA Toolbox v2.0 in MATLAB R2014a (The Mathworks, Inc.) with Gurobi version 6.0.4 as the solver (Gurobi Optimization, Inc.). Exchange reactions were constrained to represent a chemically defined minimal growth medium with CO as the source of carbon and energy. An evolutionary algorithm was used to search for the existence of strain designs incorporating up to ten gene knockouts that couple target non-native chemical production with growth.

0276 FBA predicts that pathways using Ptb-Buk or CoA transferase offer the highest product selectivities due to ATP gain through substrate level phosphorylation. The results are illustrated in

Table 2. However, it should be noted that one limitation of Genome-scale models and FBA analysis is that enzyme kinetics are not captured. The CoA transferase reaction requires a certain basal level of acetate for functionality, therefore in reality the maximum selectivity using a CoA transferase would be less than 100% due to a basal level of acetate required to be present.

Non-native product	Maximum selectivity % (C in target product/C in all fermentation products)		
	Thioesterase	CoA-transferase	Ptb-Buk
Acetone	82.0	100	100
Isopropanol	82.1	100	100
Isobutylene	55.9	80.2	80.2
3-Hydroxybutyrate	86.0	100	100
1,3-Butanediol	88.6	100	100
2-Hydroxyisobutyrate	86.0	100	100

0277 Table 2. Flux balance analysis (FBA) showing the maximum possible non-native product selectivities in *C. autoethanogenum* for a set of products and candidate enzymes.

0278 It is desirable to construct strains where the target non-native chemical must be produced for cell growth. FBA predicts that in most cases it would be difficult to couple target chemical production with growth when using a thioesterase or a CoA transferase; instead, native products acetate and ethanol would be favored. However, when using Ptb-Buk, many growth-coupled chemical production strain designs exist, often incorporating a disruption of the phosphotransacetylase-acetate kinase reactions. Table 3 summarizes the growth coupling ability of each strain.

Non-native product	Ability to couple non-native chemical production with growth		
	Thioesterase	CoA-transferase	Ptb-Buk
Acetone	No	No	Yes
Isopropanol	No	No	Yes
Isobutylene	No	No	No
3-Hydroxybutyrate	No	No	Yes
1,3-Butanediol	No	Yes	Yes

2-Hydroxyisobutyrate	No	No	Yes

0279 Table 3. Potential to couple non-native chemical production with growth in *C. autoethanogenum* during growth on CO when reconfiguring the metabolic network with up to ten gene knockouts.

0280 While both Ptb-Buk and CoA transferase can support high selectivities, flux balance analysis predicts that in most cases, only Ptb-Buk would allow the construction of stable, high-yielding fermentation strains that couple non-native chemical production with growth.

Example 14

0281 This example demonstrates the production of adipic acid via Ptb-Buk from gaseous feedstock.

0282 Production of adipic acid in *E. coli* from sugar has been described by a pathway utilizing Ptb-Buk (Yu, *Biotechnol Bioeng*, 111: 2580-2586, 2014). However production was low, in the μg/L range. Without wishing to be bound by any particular theory, the inventors believe that this is likely a function of lacking driving force in forms of reducing power and surplus ATP. Using a reduced gaseous substrate as CO and H_2 and an acetogenic bacterium such as *C. autoethanogenum*, this current limitation can be overcome. CO and H_2 oxidation provide sufficient driving force for reduction of 3-oxo-adipyl-CoA to 3-hydroxyadipyl-CoA by 3-hydroxybutyryl-CoA dehydrogenase or acetoacetyl-CoA hydratase and 2,3-dehydroadipyl-CoA to adipyl-CoA by enoyl-CoA hydrolase or enoyl-CoA reductase (Fig. 34, steps 23 and 25), in contrast to *E. coli* growing heterotrophically on more oxidized sugars. Acetogenic bacteria live on the energetic limit of life and therefore ATP generating reactions like the Ptb-Buk system have a strong driving force, ensuring efficient conversion of adipyl-CoA to adipic acid (Fig. 34, step 26), in contrast to *E. coli* growing heterotrophically on sugars generating surplus ATP from glycolysis.

0283 To produce adipic acid from gas in *C. autoethanogenum*, genes encoding a succinyl-CoA synthetase from *E. coli* (NP_415256, NP_415257), a ketoisovalerate oxidoreductase PaaJ from *E. coli* (WP_001206190.1), a 3-hydroxybutyryl-CoA dehydrogenase Hbd from *Clostridium beijerinckii* (WP_011967675.1), a trans-2-enoyl-CoA reductase Crt from *C. acetobutylicum* (NP_349318.1), trans-2-enoyl-CoA reductase Bcd from *C. acetobutylicum* (NP_349317.1) and electron flavoproteins EtfAB (NP_349315, NP_349316) are cloned on an expression plasmid and then transformed as described above in *C. autoethanogenum* strains pta-ack::ptb-buk or CAETHG_1524::ptb-buk from previous examples. Adipic acid is produce according to the steps depicted in Fig. 34.

Example 15

0284 This example demonstrates the production of various products including 2-buten-1-ol, 3-methyl-2-butanol, 1,3-hexanediol (HDO) via Ptb-Buk and AOR.

0285 As demonstrated in Example 6, Ptb-Buk is highly promiscuous and acts on a wide range of CoAs as substrates or can be engineered to use a range of non-natural CoAs as substrates. Likewise AOR enzyme has been shown to act on a wide range of substrates. Together these two enzymes can convert a wide range of CoAs via their acids into aldehydes, which then can be further converted to alcohols, ketones or enols via alcohol dehdydrogeneses, for which a wide variety exists in nature. While under standard conditions the reduction of acids with ferredoxin to aldehydes via the AOR is endergonic (Thauer, *Bacteriol Rev*, 41: 100-180, 1977) and as such not feasible, it surprisingly is in carboxydotrophic acetogens such as *C. autoethanogenum* that operate at low pH and with CO or H2 as substrate (Mock, *J Bacteriol*, 197: 2965-2980, 2015). One common limitation working with acetogens is that they are ATP-limited, living on the thermodynamic edge of life (Schuchmann, *Nat Rev Microbiol*, 12: 809-821, 2014), which can be overcome by coupling this acid reduction to ATP-linked formation of acids from CoAs via the Ptb-Buk system.

0286 The Ptb-Buk system and AOR system has been demonstrated in above examples for several different products, but can be extended to further products, for example production of 2-buten-1-ol, 3-methyl-2-butanol, 1,3-hexanediol (HDO). 2-Buten-1-ol can be produced via Ptb-Buk, AOR and an alcohol dehydrogenase from crotonyl-CoA (Fig. 35). 1,3-Hexanediol can be produced via Ptb-Buk, AOR and an alcohol dehydrogenase from 3-hydroxy-hexanoyl-CoA (Fig. 35). By combining Ptb-Buk, Adc and an alcohol dehydrogenase (such as native primary:secondary alcohol dehydrogenase), 3-methyl-2-butanol can be formed from acetobutyryl-CoA.

0287 All of these precursors, crotonyl-CoA, 3-hydroxy-hexanoyl-CoA, or acetobutyryl-CoA can be formed by reduction and elongation of acetyl-CoA, acetoacetyl-CoA and 3-HB-CoA which are described in previous examples via known fermentation pathways of, for example, *Clostridium kluyveri* (Barker, *PNAS USA*, 31: 373-381, 1945; Seedorf, *PNAS USA*, 105: 2128-2133, 2008) and other Clostridia. Involved enzymes include crotonyl-CoA hydratase (crotonase) or crotonyl-CoA reductase, butyryl-CoA dehydrogenase or trans-2-enoyl-CoA reductase, thiolase or acyl-CoA acetyltransferase and 3-hydroxybutyryl-CoA dehydrogenase or acetoacetyl-CoA hydratase (Fig. 35). Respective genes from *C. kluyveri* or other Clostridia have be cloned on an expression plasmid (U.S. 2011/0236941) and and then transformed as described above in *C. autoethanogenum* strains pta-ack::ptb-buk or CAETHG_1524::ptb-buk from previous examples for production of 2-buten-1-ol, 3-methyl-2-butanol, 1,3-hexanediol (HDO). 2-Buten-1-ol, 3-methyl-2-butanol, and 1,3-hexanediol (HDO) may be precursors for further downstream products.

0288 While these are only a few examples, it should be clear that this pathway can be further extended using the same enzymes or engineered variants thereof that have specificity for higher chain length to produce a range of C4, C6, C8, C10, C12, C14 alcohols, ketones, enols or diols (Fig. 39). Different type of molecules can be obtained also by using primer or extender units different than acetyl-CoA in the thiolase step as been described elsewhere (Cheong, *Nature Biotechnol*, 34: 556-561, 2016).

0289

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.

0290 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.

0291 Preferred embodiments of this invention are described herein. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

CLAIMS

1. A genetically engineered C1-fixing bacterium comprising:

(a) an exogenous enzyme that converts acetyl-CoA to acetoacetyl-CoA selected from thiolase (EC 2.3.1.9),

(b) an exogenous enzyme that converts acetoacetyl-CoA to 3-hydroxybutyryl-CoA selected from 3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157) and acetoacetyl-CoA reductase (EC 4.2.1.36), and

(c) an exogenous or endogenous enzyme that converts 3-hydroxybutyryl-CoA to 3-hydroxybutyrate selected from thioesterase (EC 3.1.2.20), phosphate butyryltransferase (EC 2.3.1.19), butyrate kinase (EC 2.7.2.7), and CoA-transferase (EC 2.8.3.9).

2. The bacterium of claim 1, wherein the exogenous or endogenous enzyme that converts 3-hydroxybutyryl-CoA to 3-hydroxybutyrate is stereospecific.

3. The bacterium of claim 1, wherein the 3-hydroxybutyrate is (R)-3-hydroxybutyrate, (S)-3-hydroxybutyrate, or a combination thereof.

4. The bacterium of claim 1, wherein the bacterium further comprises an isomerase that interconverts (R)-3-hydroxybutyrate and (S)-3-hydroxybutyrate.

5. The bacterium of claim 1, wherein the bacterium further comprises an enzyme that converts 3-hydroxybutyrate to 3-hydroxybutyryaldehyde selected from aldehyde:ferredoxin oxidoreductase (EC 1.2.7.5).

6. The bacterium of claim 5, wherein the bacterium further comprises an enzyme that converts 3-hydroxybutyryaldehyde to 1,3-butanediol selected from alcohol dehydrogenase (EC 1.1.1.1. or 1.1.1.2).

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

8. The bacterium of claim 1, wherein the bacterium further comprises exogenous or endogenous aldehyde:ferredoxin oxidoreductase (AOR).

9. The bacterium of claim 1, wherein the bacterium further comprises a disruptive mutation in a phosphotransacetylase (Pta) and an acetate kinase (Ack).

10. The bacterium of claim 1, wherein the bacterium further comprises a disruptive mutation in a thioesterase.

11. A method of producing 3-hydroxybutyrate 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 3-hydroxybutyrate.

12. The method of claim 11, wherein the gaseous substrate comprises syngas or industrial waste gas.

13. A method of producing 3-hydroxybutyryaldehyde comprising culturing the bacterium of claim 8 in the presence of a gaseous substrate comprising one or more of CO, CO_2, and H_2, whereby the bacterium produces 3-hydroxybutyryaldehyde.

14. A method of producing 1,3-butanediol comprising culturing the bacterium of claim 10 in the presence of a gaseous substrate comprising one or more of CO, CO_2, and H_2, whereby the bacterium produces 1,3-butanediol.

15. A process comprising:

obtaining an industrial waste gas comprising one or more of CO, CO_2, and H_2; or gasifying coal, refinery residues, biomass, lignocellulosic material, municipal solid waste or industrial solid waste to generate syngas comprising one or more of CO, CO_2, and H_2;

contacting the industrial waste gas or the syngas, with a genetically engineered C1-fixing bacterium comprising:

(a) an exogenous enzyme that converts acetyl-CoA to acetoacetyl-CoA selected from thiolase (EC 2.3.1.9),

(b) an exogenous enzyme that converts acetoacetyl-CoA to 3-hydroxybutyryl-CoA selected from 3- hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157) and acetoacetyl-CoA reductase (EC 4.2.1.36), and

(c) an exogenous or endogenous enzyme that converts 3-hydroxybutyryl-CoA to 3-hydroxybutyrate selected from thioesterase (EC 3.1.2.20), phosphate butyryltransferase (EC 2.3.1.19), butyrate kinase (EC 2.7.2.7), and CoA-transferase (EC 2.8.3.9)

to culture and produce a 3-hydroxybutyrate product;

and

using the 3-hydroxybuterate as a cosmetic ingredient, or

converting the 3-hydroxybutyrate to polyhydroxybutyrate; or

using the 3-hydroxybutyrate as a comonomer with polyhydroxy acids.

16. The process of claim 15, wherein the bacterium further comprises an enzyme that converts 3-hydroxybutyrate to 3-hydroxybutyryaldehyde selected from aldehyde:ferredoxin oxidoreductase (EC 1.2.7.5).

17. The process of claim 16, wherein the bacterium further comprises an enzyme that converts 3-hydroxybutyryaldehyde to 1,3-butanediol selected from alcohol dehydrogenase (EC 1.1.1.1. or 1.1.1.2) to culture and produce a 1, 3-butane diol product.

18. The process of claim 17, comprising converting, catalytically, the 1,3-butane diol product to butadiene and using the butadiene to produce rubber, plastics, lubricants, latex or adiponitrile.

19. The process of claim 18, further comprising using the rubber to manufacture tires.

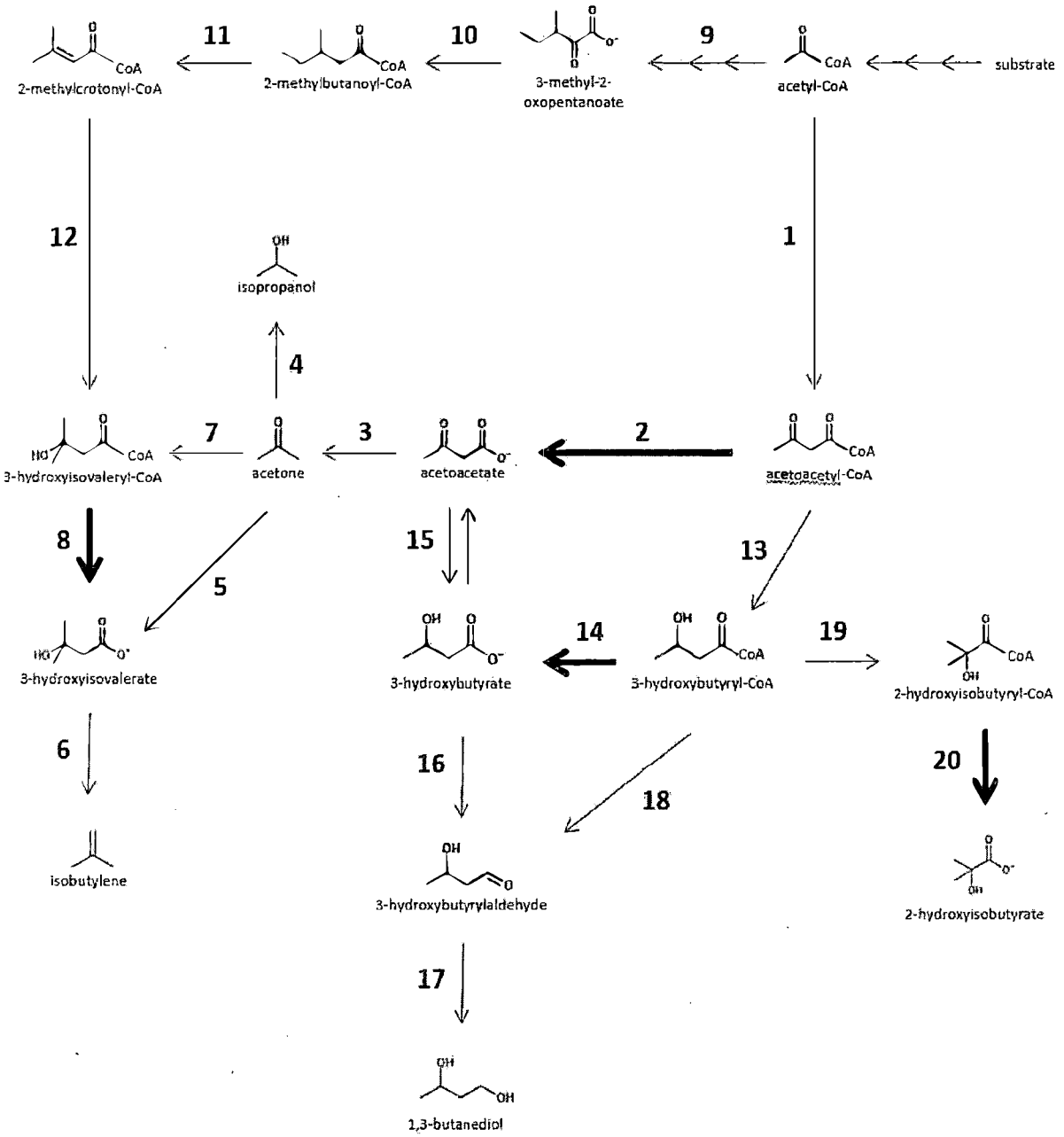
20. The process of claim 18, further comprising using the adiponitrile to manufacture nylon.

21. The process of claim 15, further comprising separating the product using fractional distillation, evaporation, pervaporation, gas stripping, phase separation, extractive fermentation, adsorption, or liquid-liquid extraction.

22. The process of claim 15, wherein the bacterium is derived from a parental bacterium selected from the group consisting of *Clostridium autoethanogenum, Clostridium ljungdahlii, Clostridium ragsdalei, Escherichia coli, Saccharomyces cerevisiae, Clostridium acetobutylicum, Clostridium beijerinckii, Clostridium saccharbutyricum, Clostridium saccharoperbutylacetonicum, Clostridium butyricum, Clostridium diolis, Clostridium kluyveri, Clostridium pasterianium, Clostridium novyi, Clostridium difficile, Clostridium thermocellum, Clostridium cellulolyticum, Clostridium cellulovorans, Clostridium phytofermentans, Lactococcus lactis, Bacillus subtilis, Bacillus licheniformis, Zymomonas mobilis, Klebsiella oxytoca, Klebsiella pneumonia, Corynebacterium glutamicum, Trichoderma reesei, Cupriavidus necator, Pseudomonas putida, Lactobacillus plantarum*, and *Methylobacterium extorquens.*

23. The process of claim 15, wherein the syngas is generated from biomass or municipal solid waste and is contacted with the genetically engineered bacterium and the product is 1,3-butane diol, which is catalytically converted to butadiene and used to produce rubber, and the rubber is used to manufacture tires.

24. The process of claim 15, wherein the syngas is generated from biomass or municipal solid waste and is contacted with the genetically engineered bacterium and the product is 1,3-butane diol which is converted to butadiene, the butadiene is converted to adiponitrile, and the adiponitrile is used to produce nylon.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2-methylcrotonyl-CoA
acetoacetyl-CoA
acetoacetate
acetone
isopropanol
3-hydroxyisovaleryl-CoA
3-hydroxyisovalerate
3-hydroxybutyryl-CoA
3-hydroxybutyrate
2-hydroxyisobutyryl-CoA
2-hydroxyisobutyrate
isobutylene
3-hydroxybutyrylaldehyde
1,3-butanediol
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20

FIG. 1

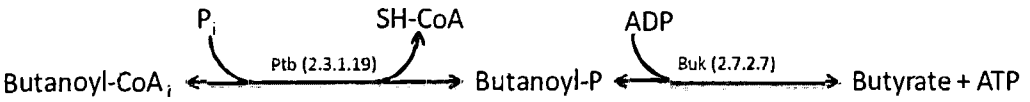
P$_i$
SH-CoA
ADP
Ptb (2.3.1.19)
Buk (2.7.2.7)
Butanoyl-CoA
Butanoyl-P
Butyrate + ATP

FIG. 2

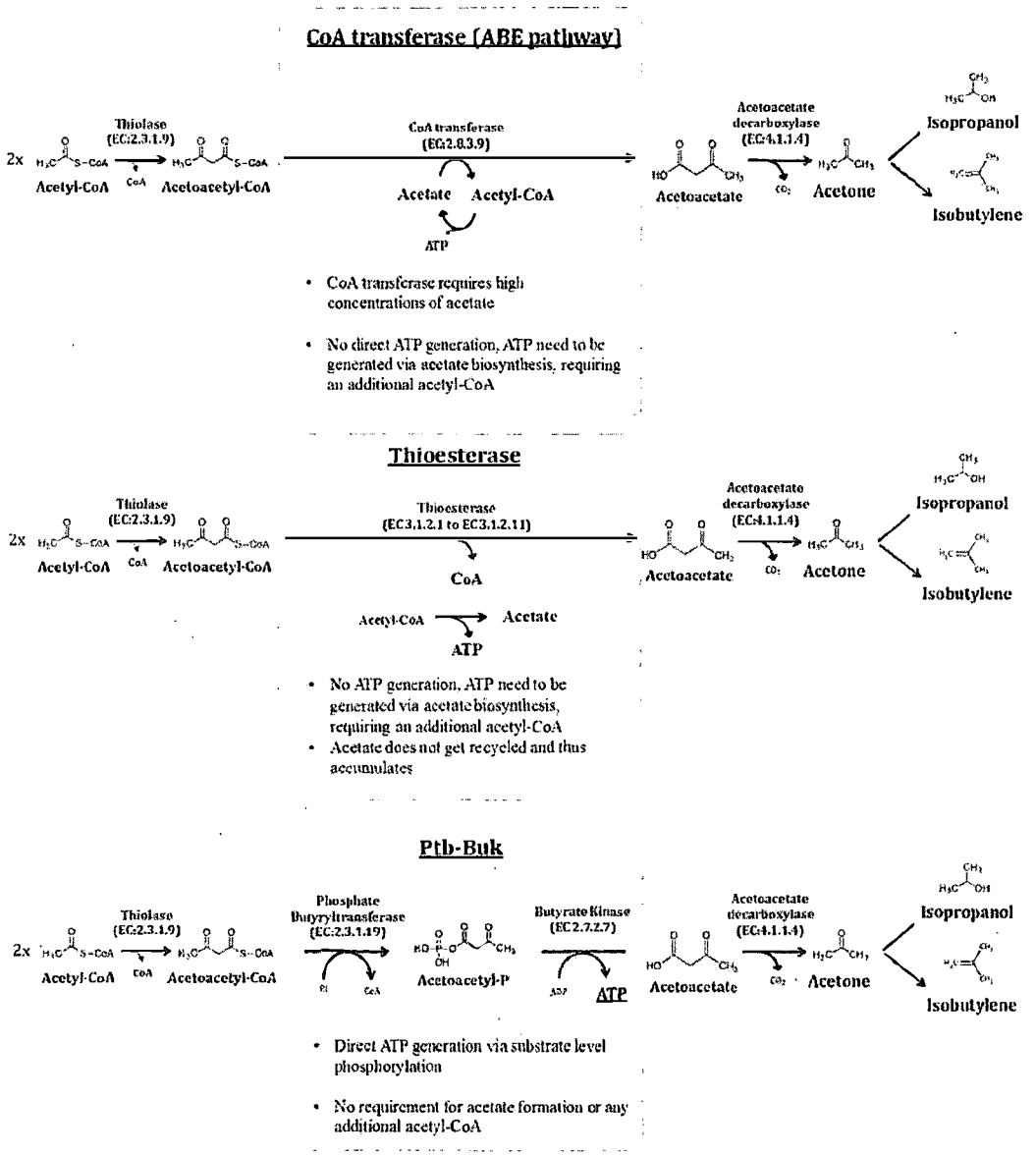
CoA transferase (ABE pathway)
Thiolase (EC:2.3.1.9)
2x
Acetyl-CoA
CoA
Acetoacetyl-CoA
CoA transferase (EC:2.8.3.9)
Acetate
Acetyl-CoA
ATP
Acetoacetate decarboxylase (EC:4.1.1.4)
Acetoacetate
CO2
Acetone
Isopropanol
Isobutylene
• CoA transferase requires high concentrations of acetate
• No direct ATP generation, ATP need to be generated via acetate biosynthesis, requiring an additional acetyl-CoA
Thioesterase
Thiolase (EC:2.3.1.9)
Thioesterase (EC3.1.2.1 to EC3.1.2.11)
CoA
Acetyl-CoA
Acetate
ATP
Acetoacetate decarboxylase (EC:4.1.1.4)
Isopropanol
Isobutylene
• No ATP generation, ATP need to be generated via acetate biosynthesis, requiring an additional acetyl-CoA
• Acetate does not get recycled and thus accumulates
Ptb-Buk
Thiolase (EC:2.3.1.9)
Phosphate Butyryltransferase (EC:2.3.1.19)
Acetoacetyl-P
Butyrate Kinase (EC 2.7.2.7)
ATP
Acetoacetate decarboxylase (EC:4.1.1.4)
Isopropanol
Isobutylene
• Direct ATP generation via substrate level phosphorylation
• No requirement for acetate formation or any additional acetyl-CoA

FIG. 3

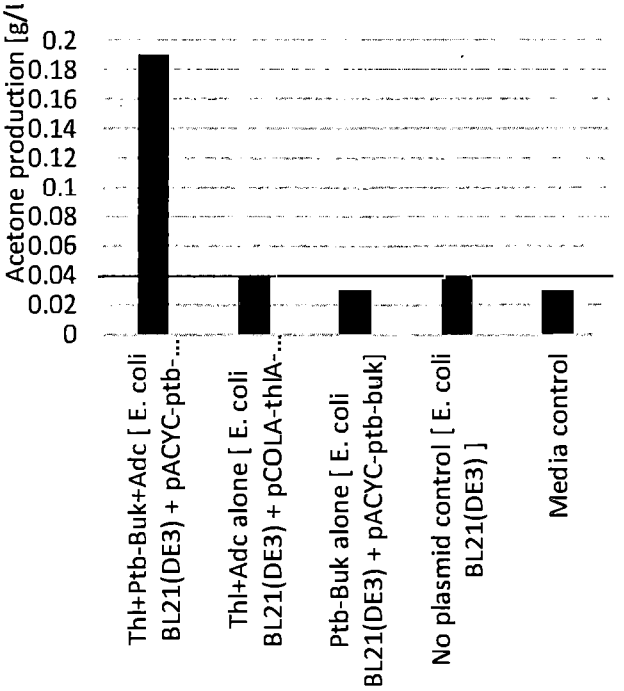
Acetone production [g/l
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Thl+Ptb-Buk+Adc [E. coli BL21(DE3) + pACYC-ptb-...
Thl+Adc alone [E. coli BL21(DE3) + pCOLA-thlA-...
Ptb-Buk alone [E. coli BL21(DE3) + pACYC-ptb-buk]
No plasmid control [E. coli BL21(DE3)]
Media control

FIG. 4

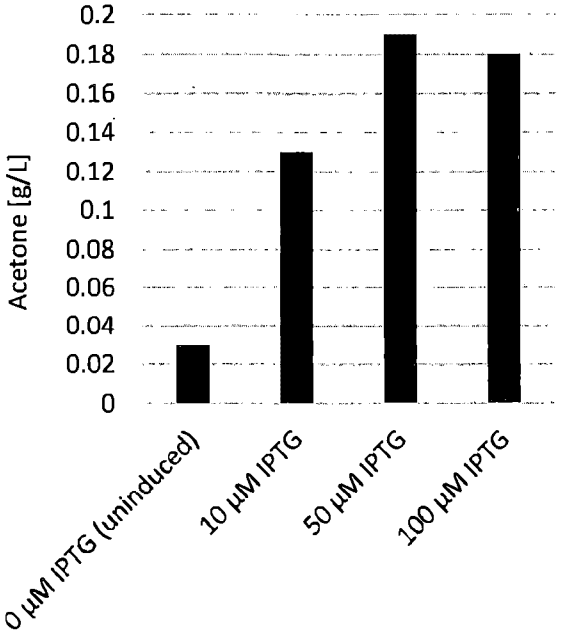
Acetone [g/L]
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0 µM IPTG (uninduced)
10 µM IPTG
50 µM IPTG
100 µM IPTG

FIG. 5

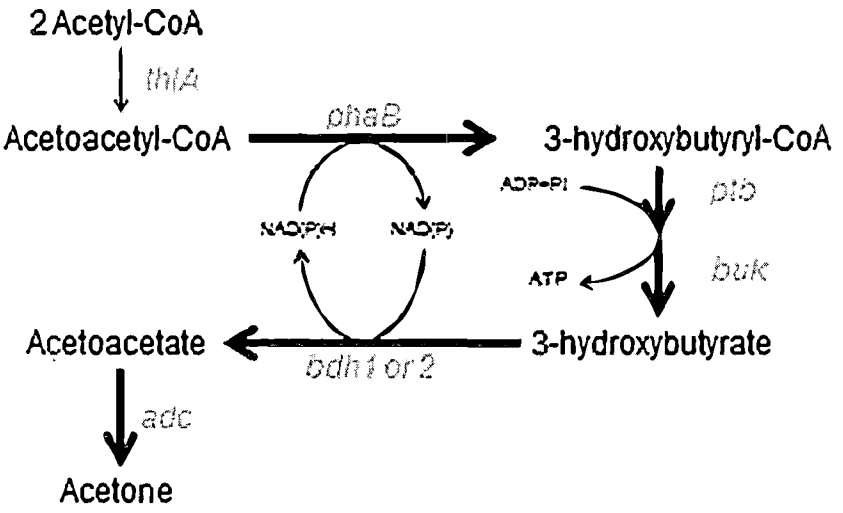
2 Acetyl-CoA
thlA
Acetoacetyl-CoA
phaB
3-hydroxybutyryl-CoA
ADP-Pi
ptb
NAD(P)H
NAD(P)
ATP
buk
Acetoacetate
3-hydroxybutyrate
bdh1 or 2
adc
Acetone

FIG. 6

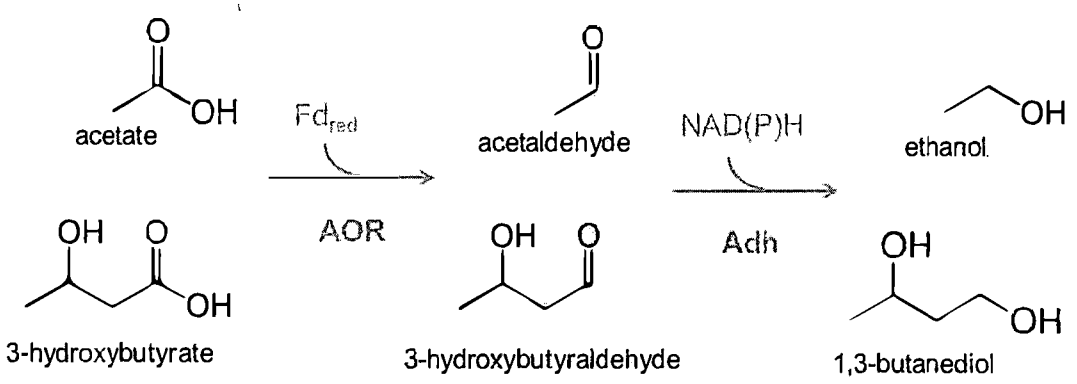
acetate
Fd$_{red}$
acetaldehyde
NAD(P)H
ethanol
AOR
Adh
3-hydroxybutyrate
3-hydroxybutyraldehyde
1,3-butanediol

FIG. 7

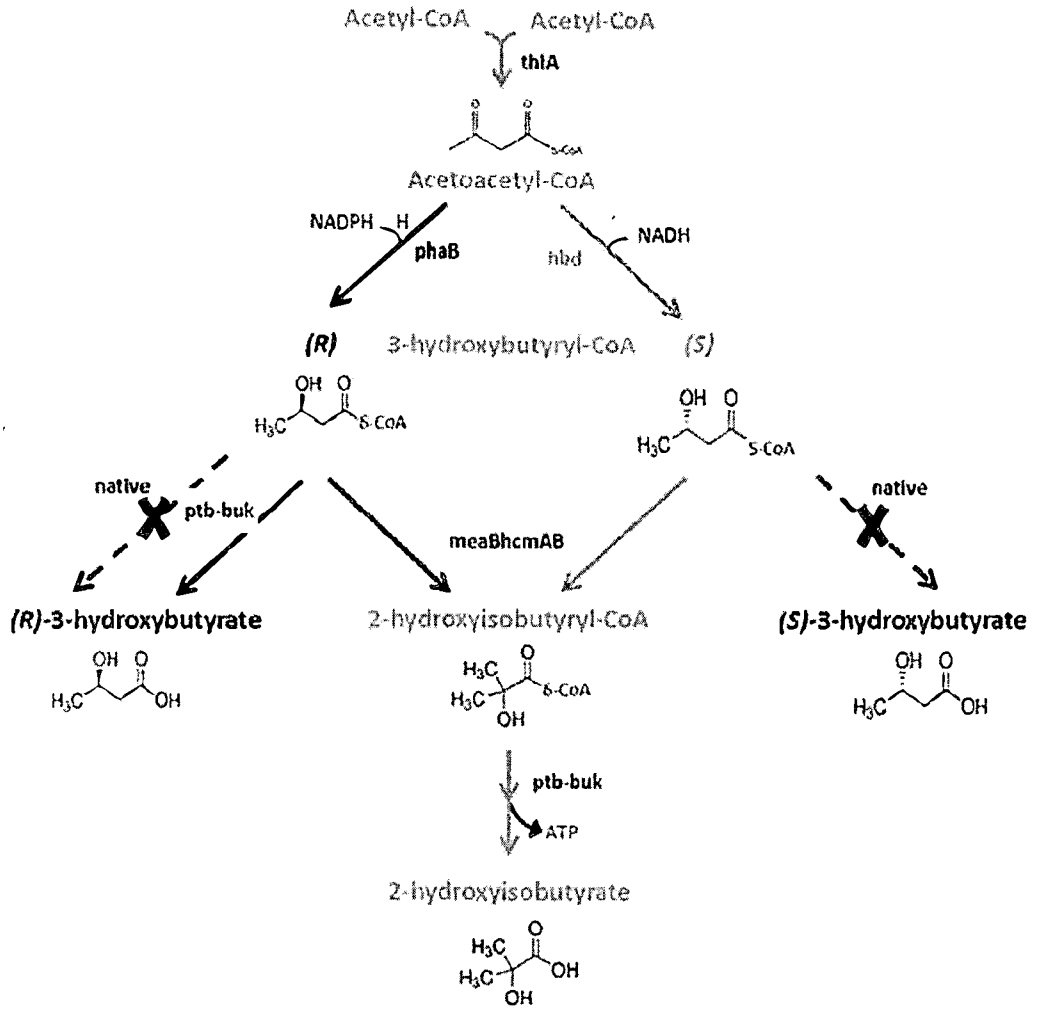
Acetyl-CoA
Acetyl-CoA
thlA
Acetoacetyl-CoA
NADPH H
phaB
hbd
NADH
(R)
3-hydroxybutyryl-CoA
(S)
native
ptb-buk
meaBhcmAB
native
(R)-3-hydroxybutyrate
2-hydroxyisobutyryl-CoA
(S)-3-hydroxybutyrate
ptb-buk
ATP
2-hydroxyisobutyrate

FIG. 8

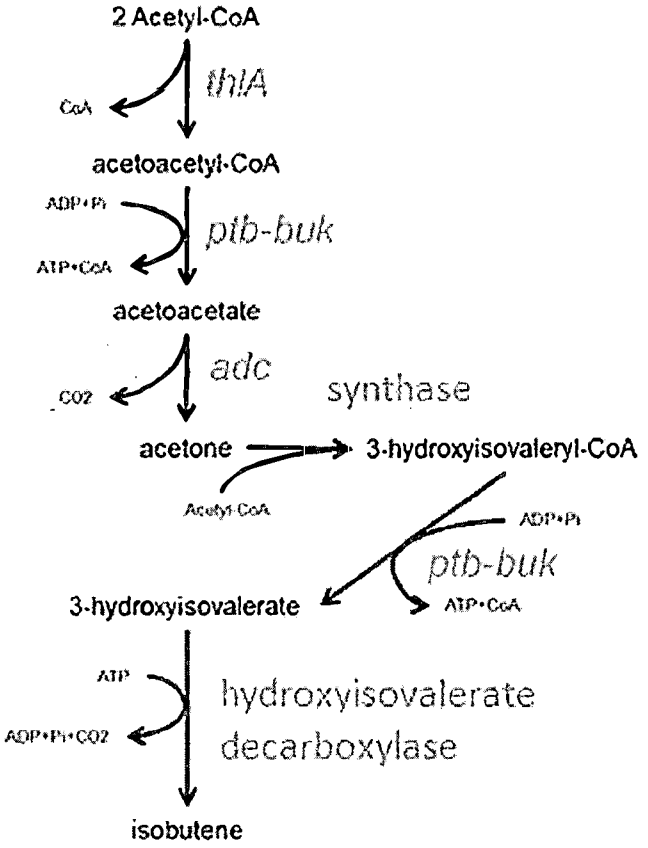
2 Acetyl-CoA
thlA
CoA
acetoacetyl-CoA
ADP•Pi
ptb-buk
ATP•CoA
acetoacetate
adc
synthase
CO2
acetone
3-hydroxyisovaleryl-CoA
Acetyl-CoA
ADP•Pi
ptb-buk
3-hydroxyisovalerate
ATP•CoA
ATP
hydroxyisovalerate
decarboxylase
ADP•Pi•CO2
isobutene

FIG. 9

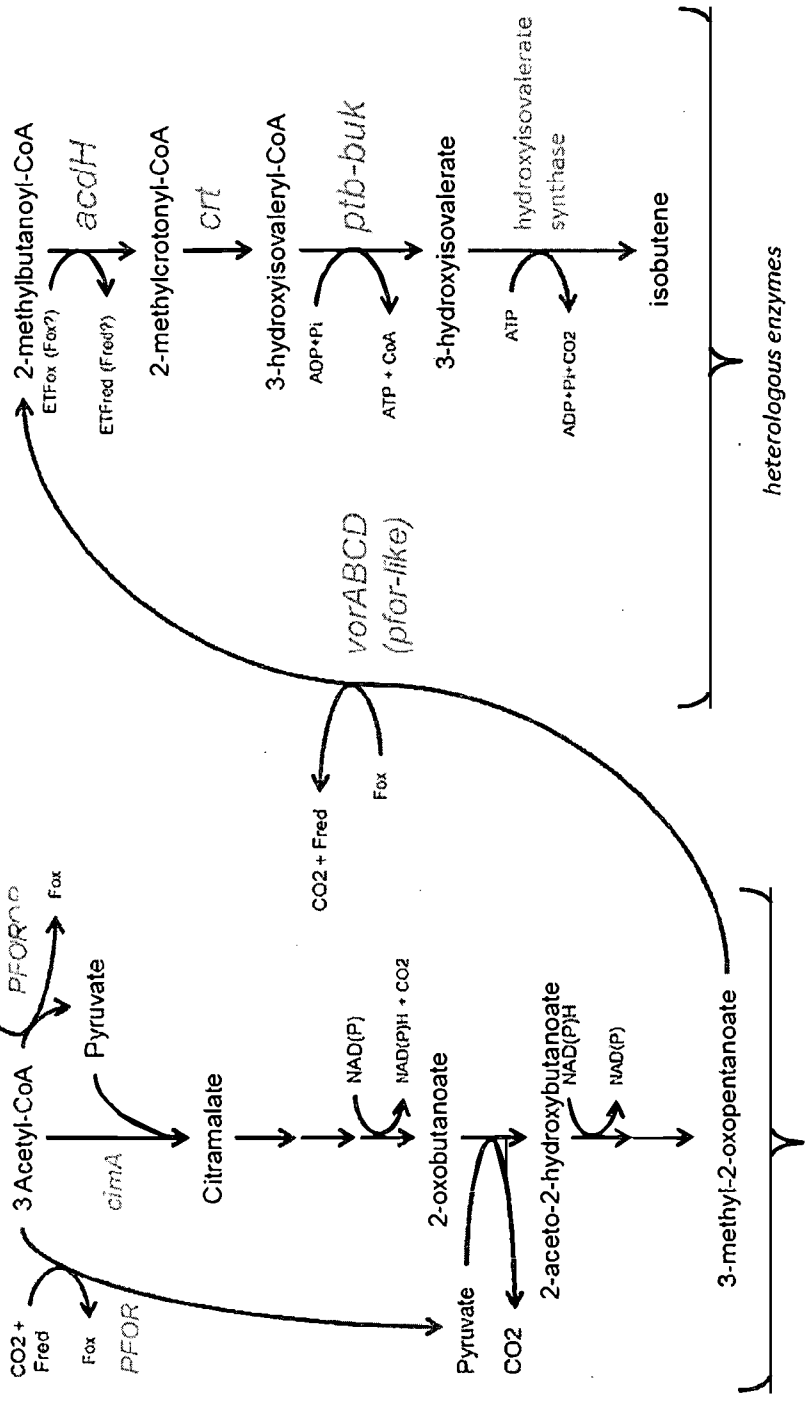
2-methylbutanoyl-CoA
ETFox (Fox?)
acdH
ETFred (Fred?)
2-methylcrotonyl-CoA
crt
3-hydroxyisovaleryl-CoA
ADP+Pi
ptb-buk
ATP + CoA
3-hydroxyisovalerate
ATP
hydroxyisovalerate synthase
ADP+Pi+CO2
isobutene
heterologous enzymes
vorABCD (pfor-like)
CO2 + Fred
Fox
3 Acetyl-CoA
dimA
Citramalate
NAD(P)
NAD(P)H + CO2
2-oxobutanoate
2-aceto-2-hydroxybutanoate
NAD(P)H
NAD(P)
3-methyl-2-oxopentanoate
Isoleucine biosynthesis
CO2 + Fred
Fox
PFOR
Pyruvate
CO2
Pyruvate
PFOR
Fox

FIG. 10

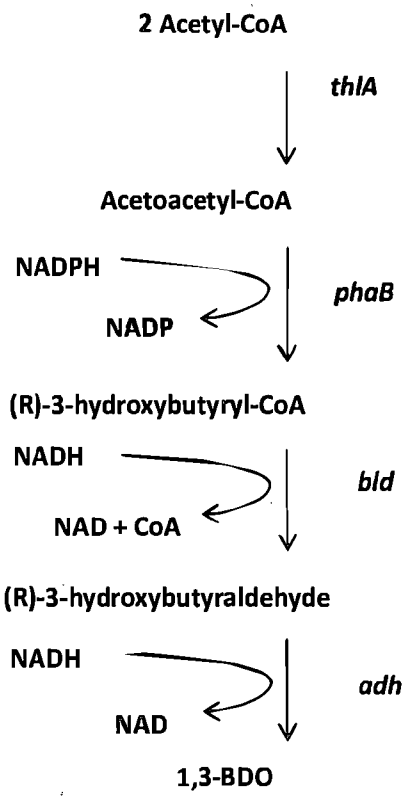
2 Acetyl-CoA
thlA
Acetoacetyl-CoA
NADPH
NADP
phaB
(R)-3-hydroxybutyryl-CoA
NADH
NAD + CoA
bld
(R)-3-hydroxybutyraldehyde
NADH
NAD
adh
1,3-BDO

FIG. 11

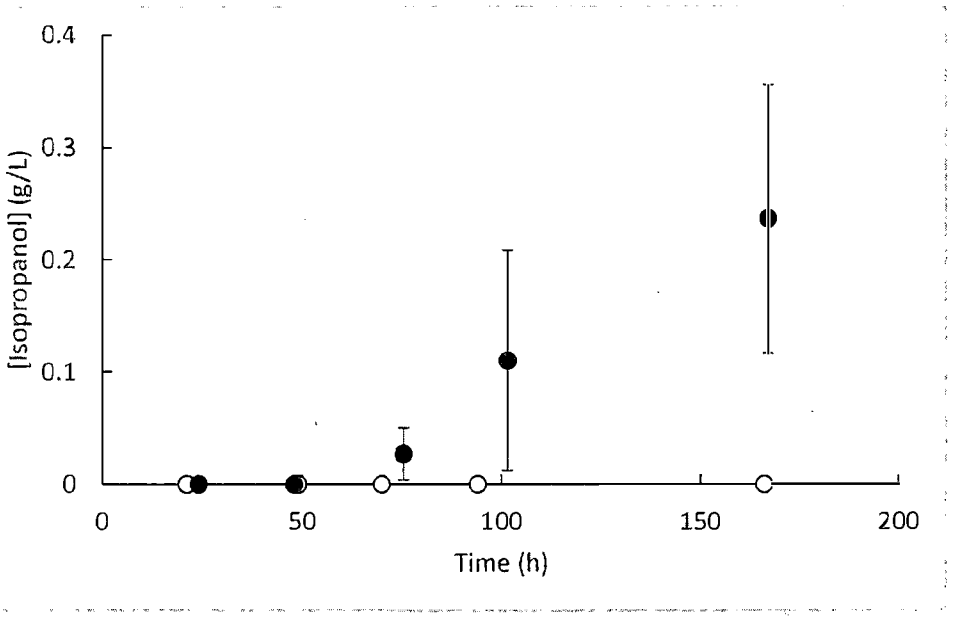

FIG. 12

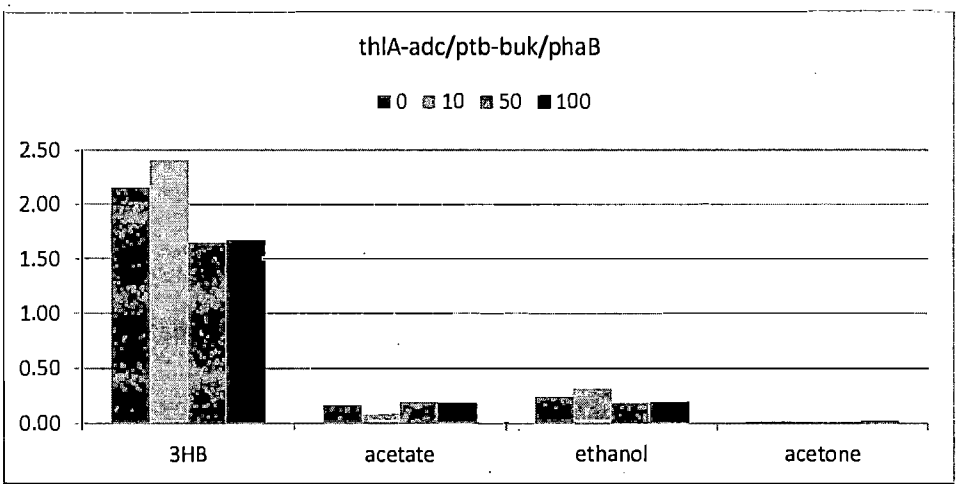

FIG. 13A

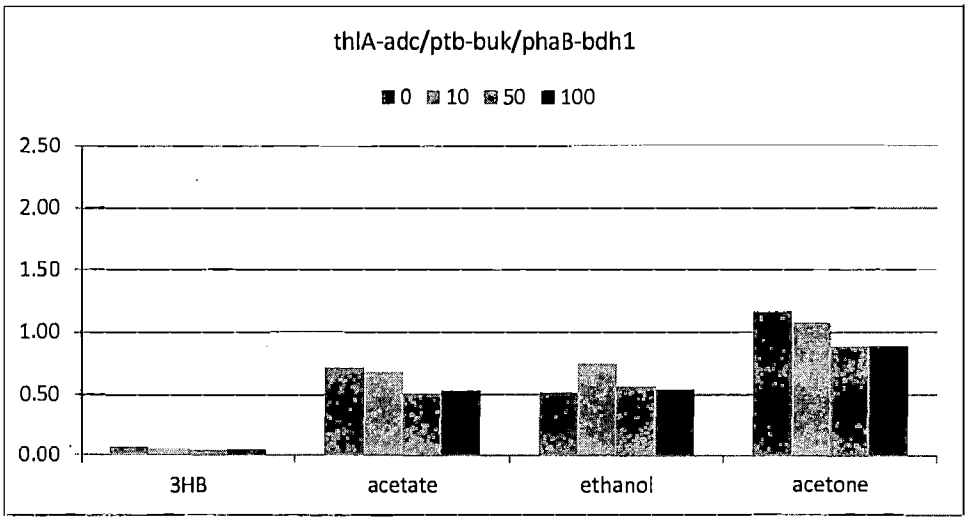

FIG. 13B

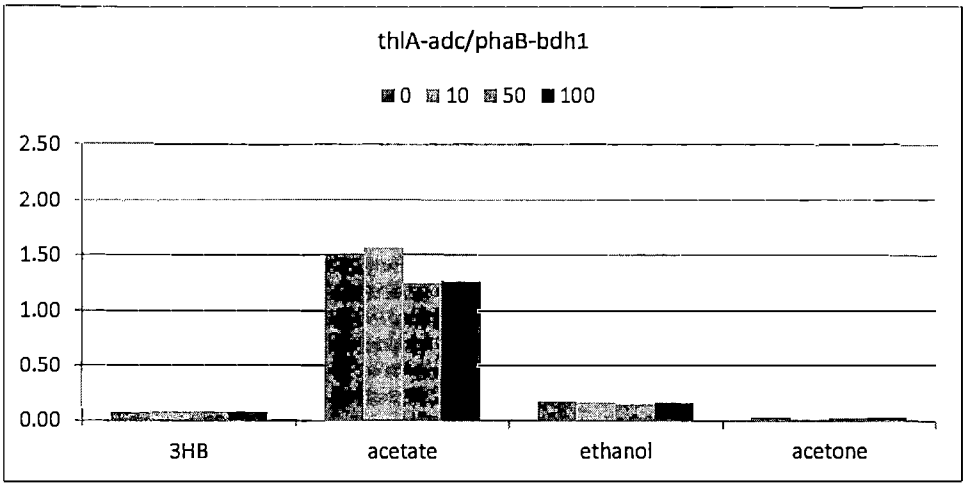

FIG. 13C

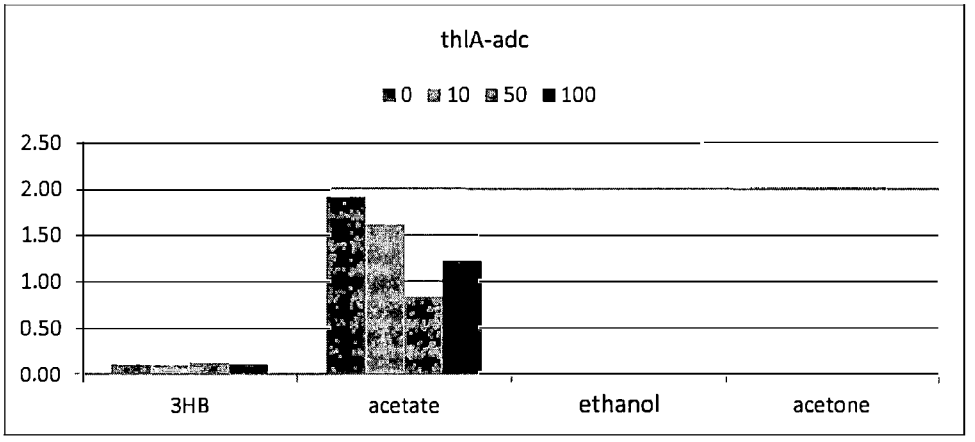

FIG. 13D

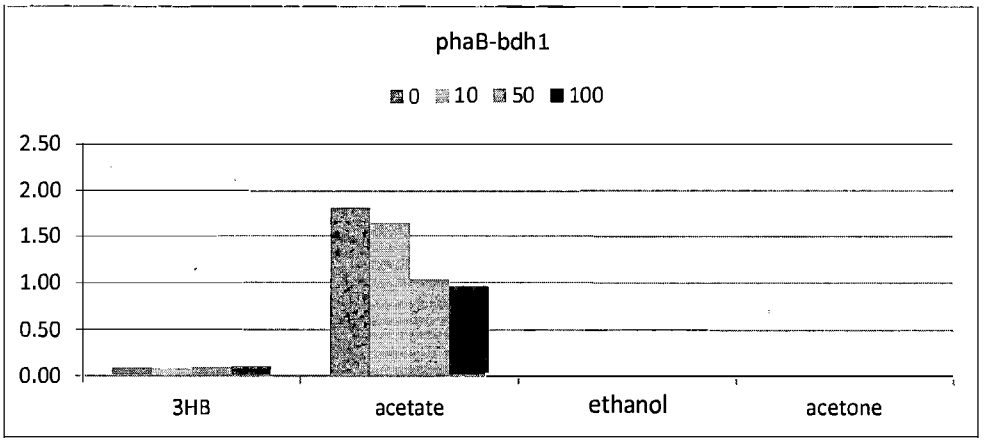

FIG. 13E

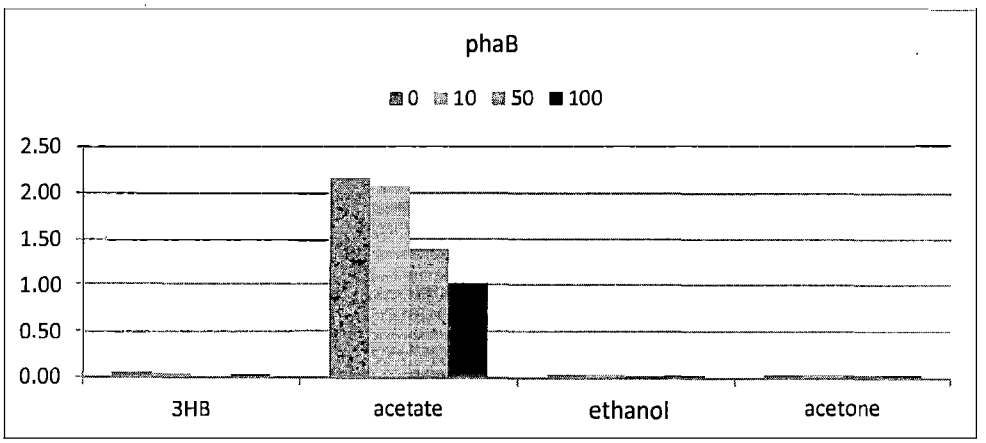

FIG. 13F

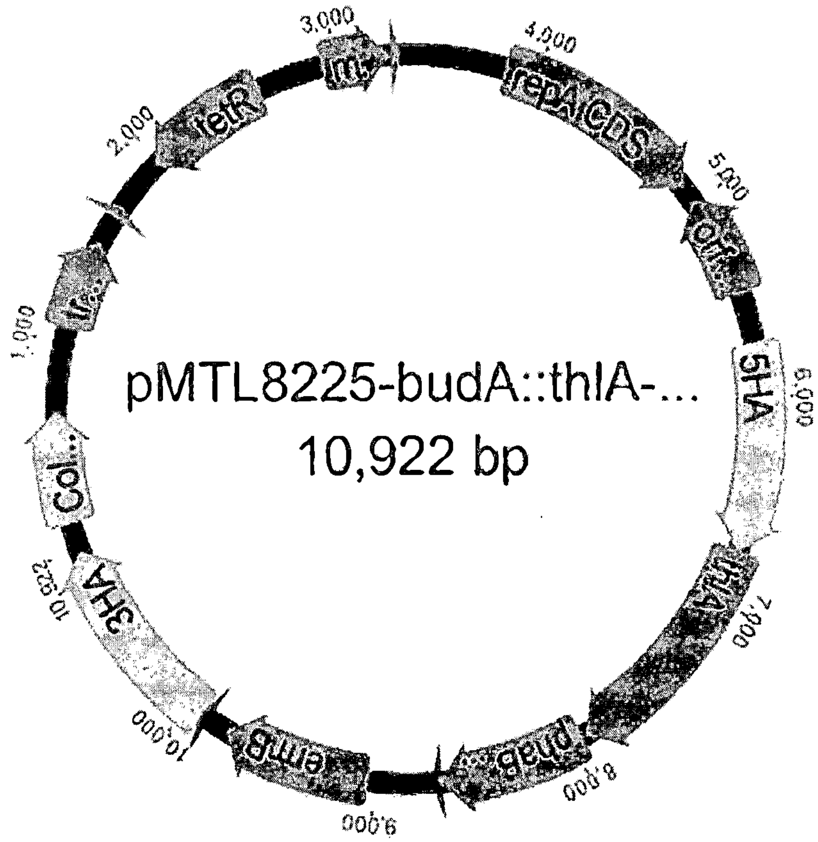
pMTL8225-budA::thlA-...
10,922 bp
3,000
4,000
5,000
6,000
7,000
8,000
9,000
10,000
10,922
1,000
2,000
tetR
repA CDS
5HA
3HA
Col...
phaB...
ermB

FIG. 14

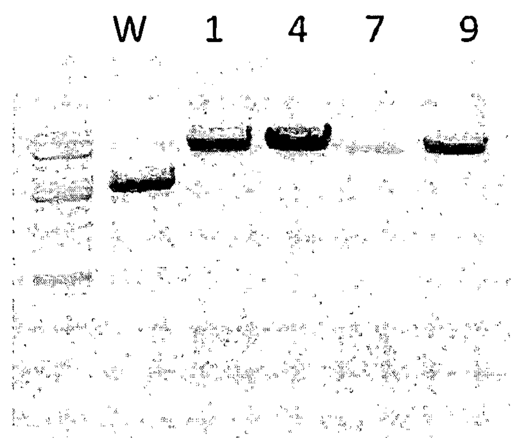
W
1
4
7
9

FIG. 15

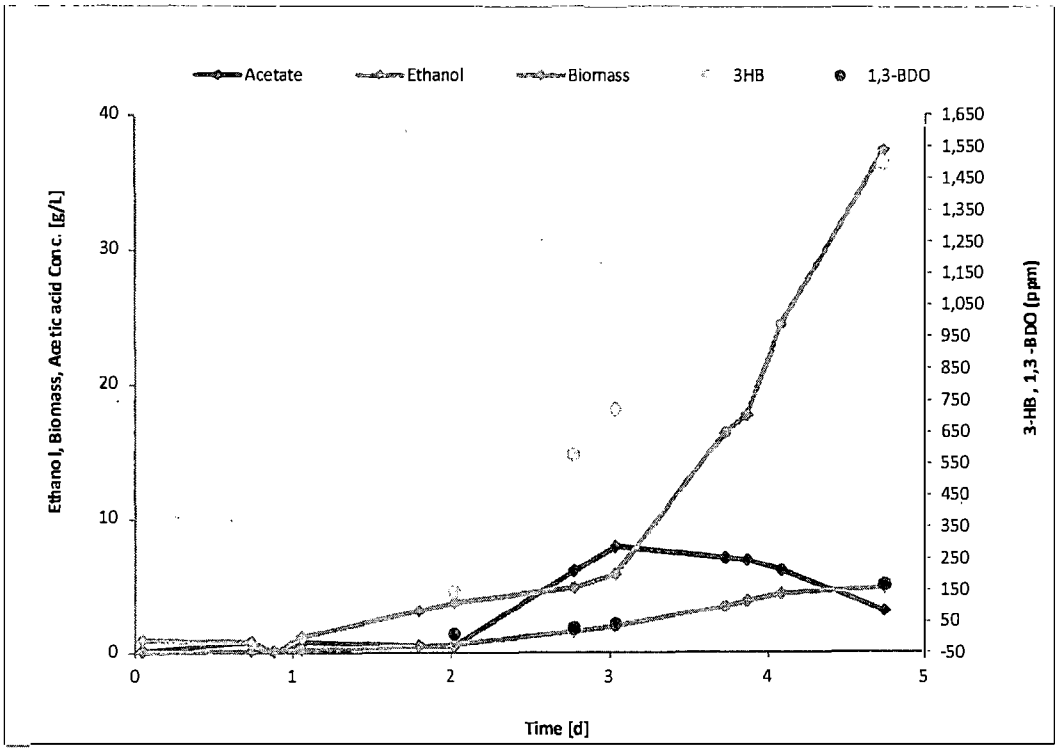
Acetate
Ethanol
Biomass
3HB
1,3-BDO
Ethanol, Biomass, Acetic acid Conc. [g/L]
3-HB, 1,3-BDO (ppm)
Time [d]
0
10
20
30
40
-50
50
150
250
350
450
550
650
750
850
950
1,050
1,150
1,250
1,350
1,450
1,550
1,650
0
1
2
3
4
5

FIG. 16

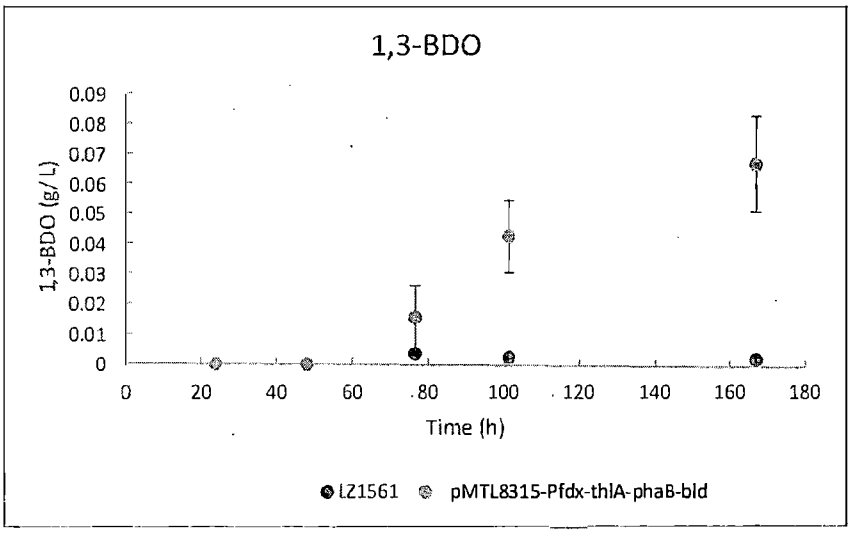

FIG. 17A

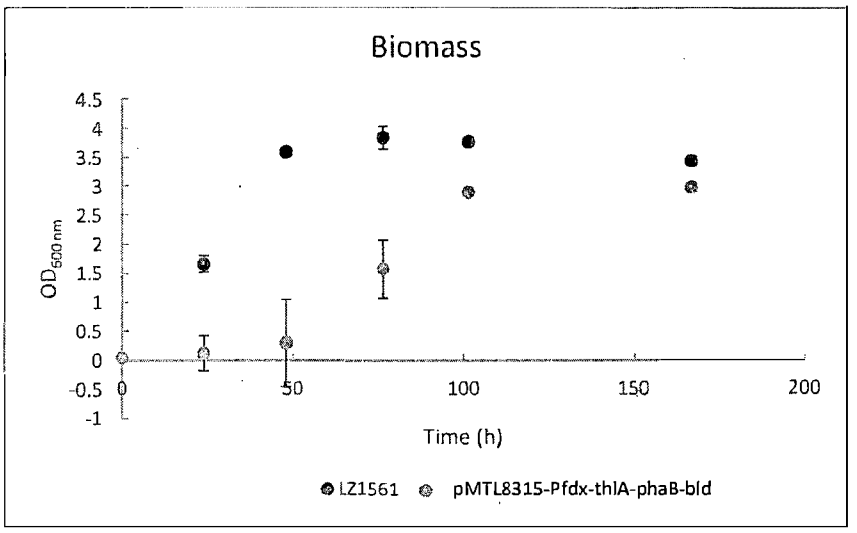

FIG. 17B

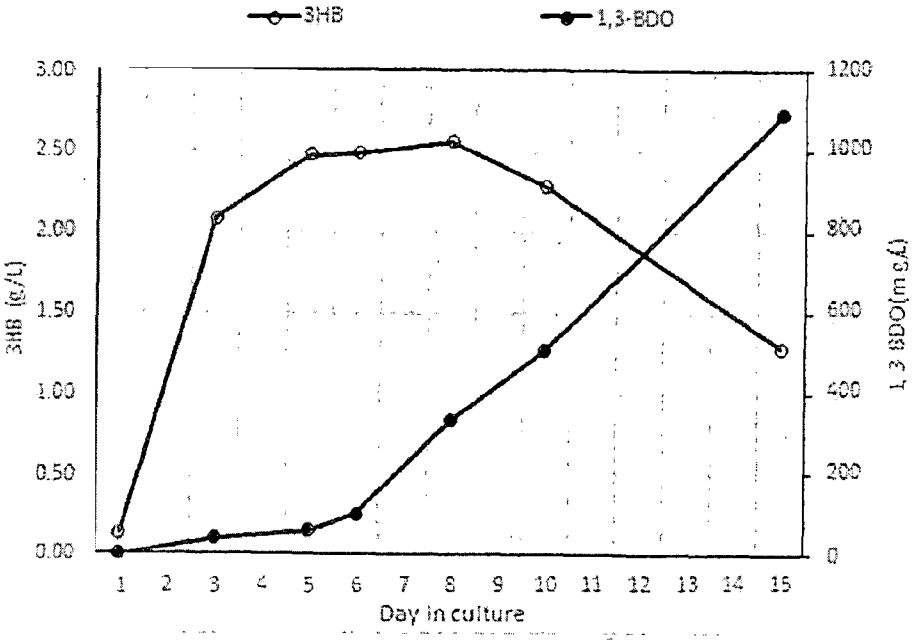

FIG. 18A

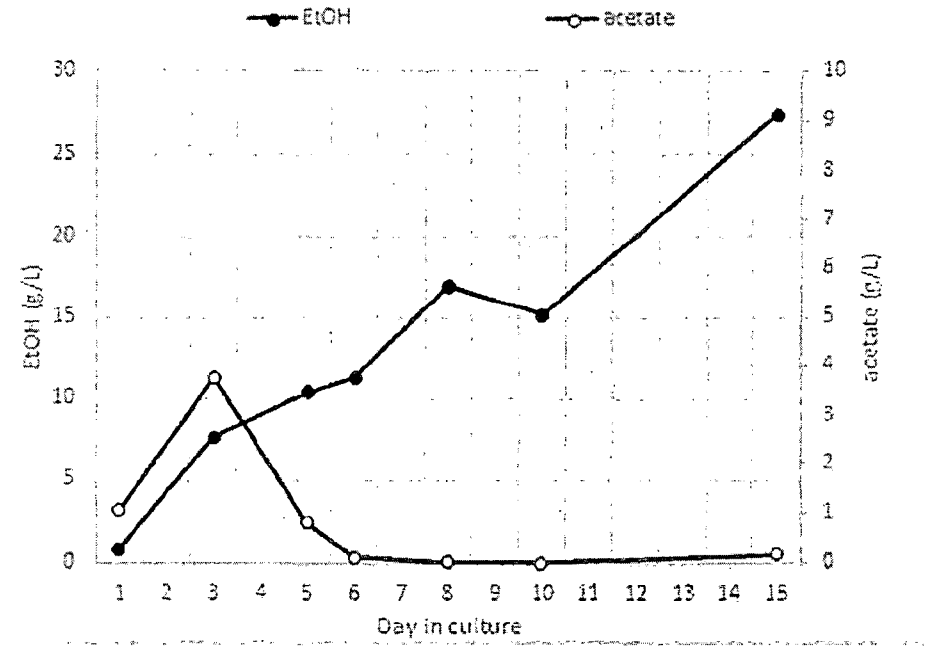

FIG. 18B

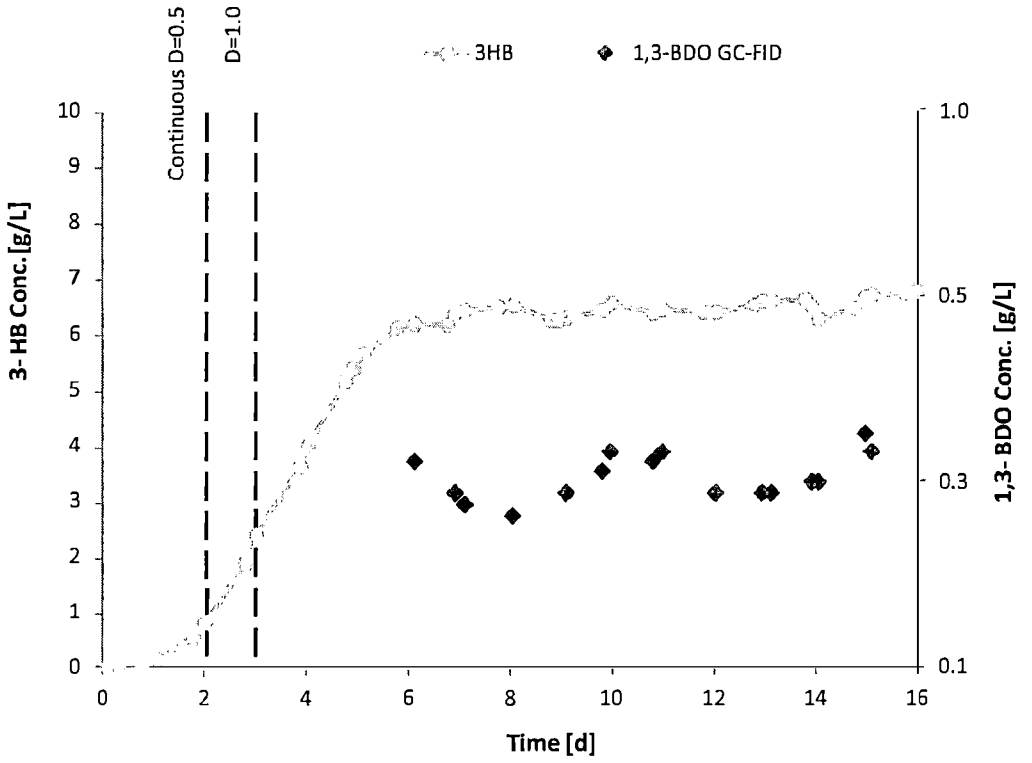
Continuous D=0.5
D=1.0
3HB
1,3-BDO GC-FID
3- HB Conc. [g/L]
1,3- BDO Conc. [g/L]
Time [d]
0
1
2
3
4
5
6
7
8
9
10
0.1
0.3
0.5
1.0
0
2
4
6
8
10
12
14
16

FIG. 19

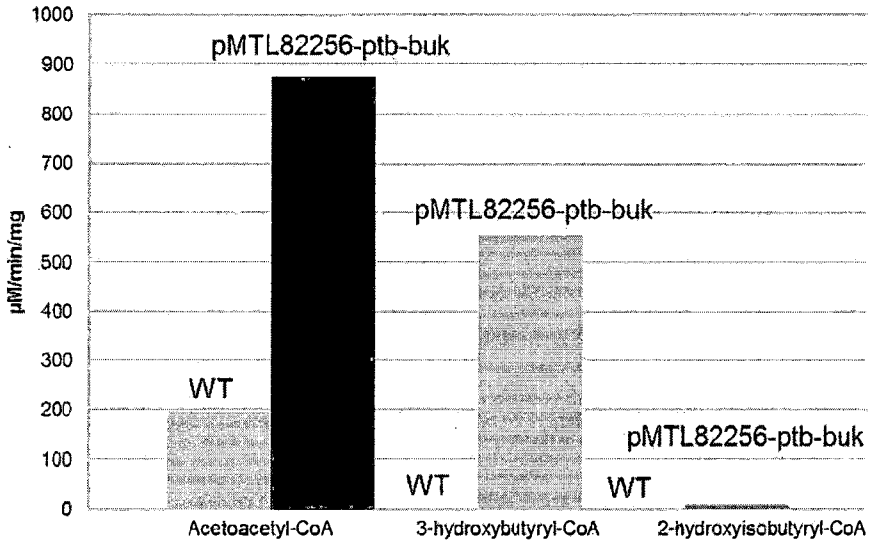

FIG. 20A

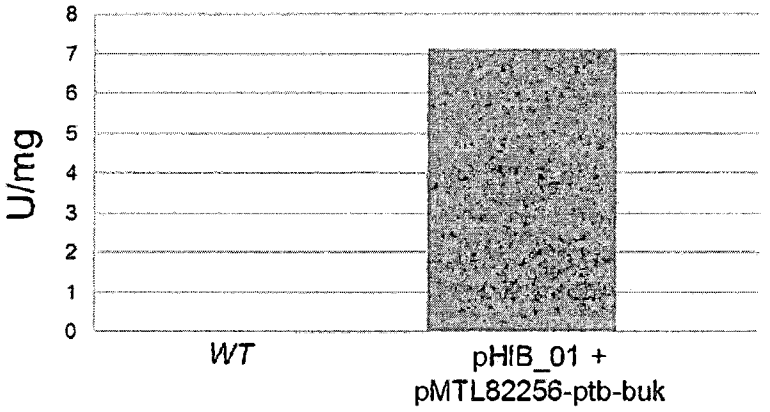

FIG. 20B

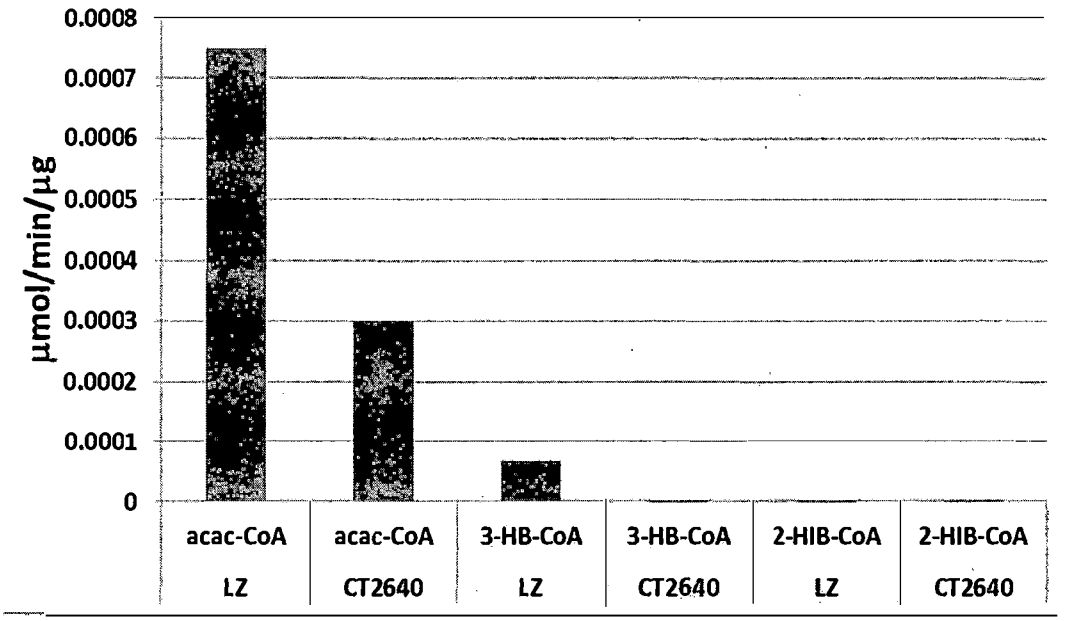

FIG. 21A

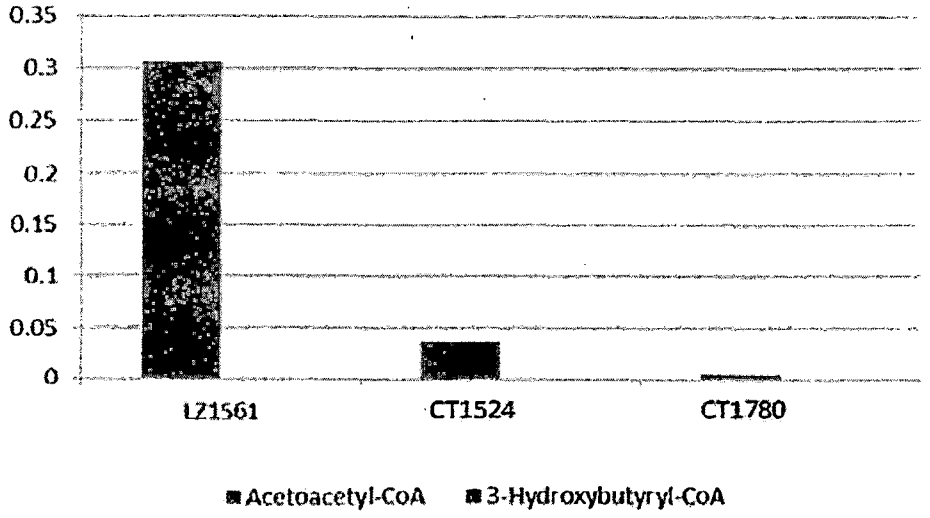

FIG. 21B

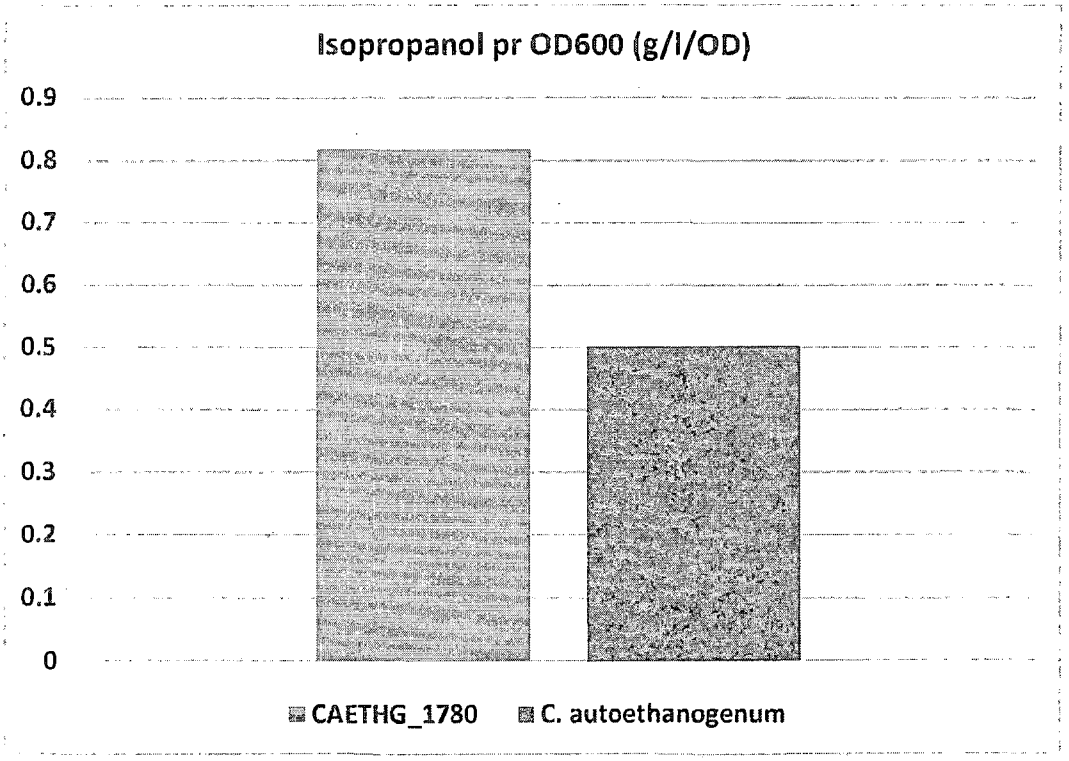
Isopropanol pr OD600 (g/l/OD)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
CAETHG_1780
C. autoethanogenum

FIG. 22

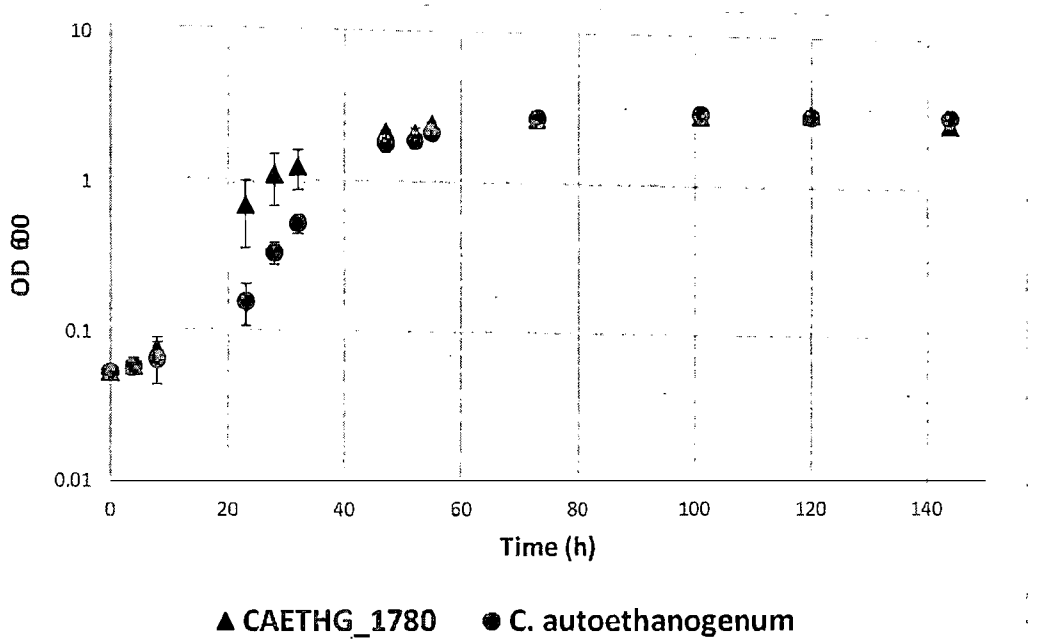

FIG. 23A

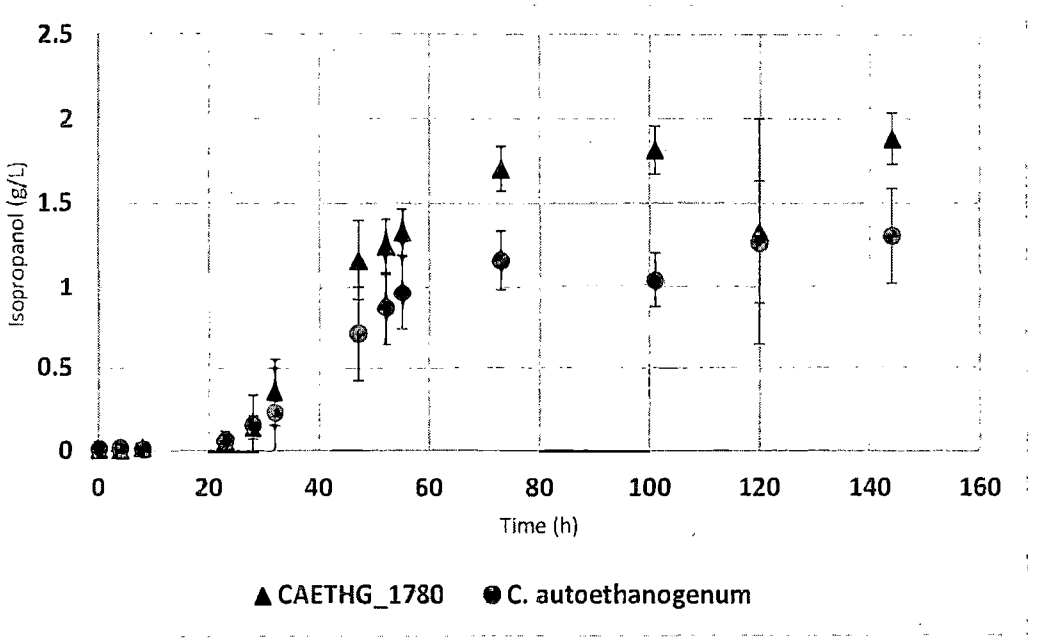

FIG. 23B

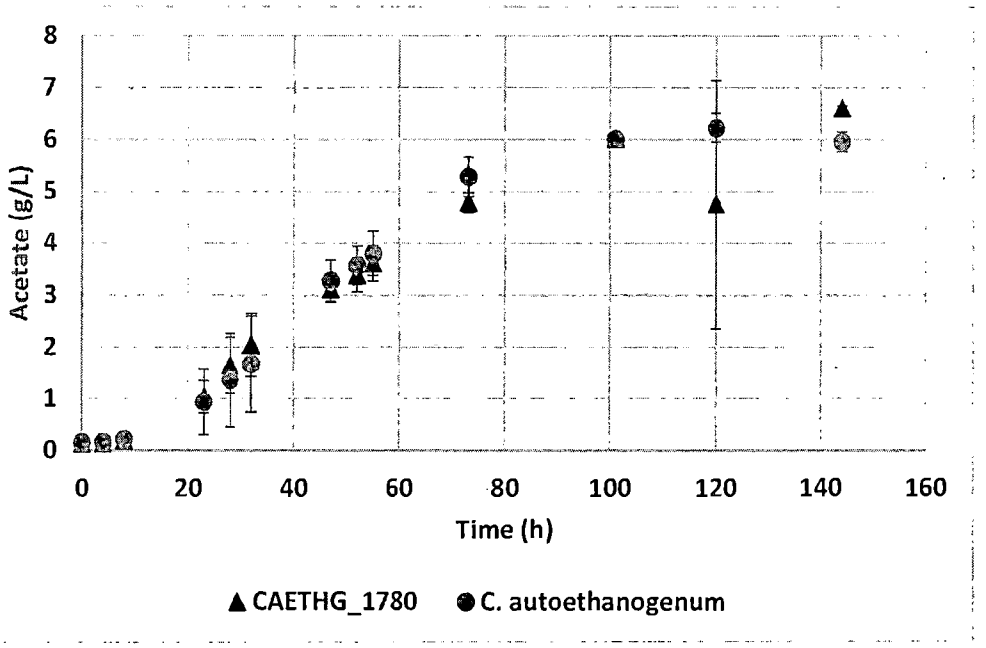

FIG. 23C

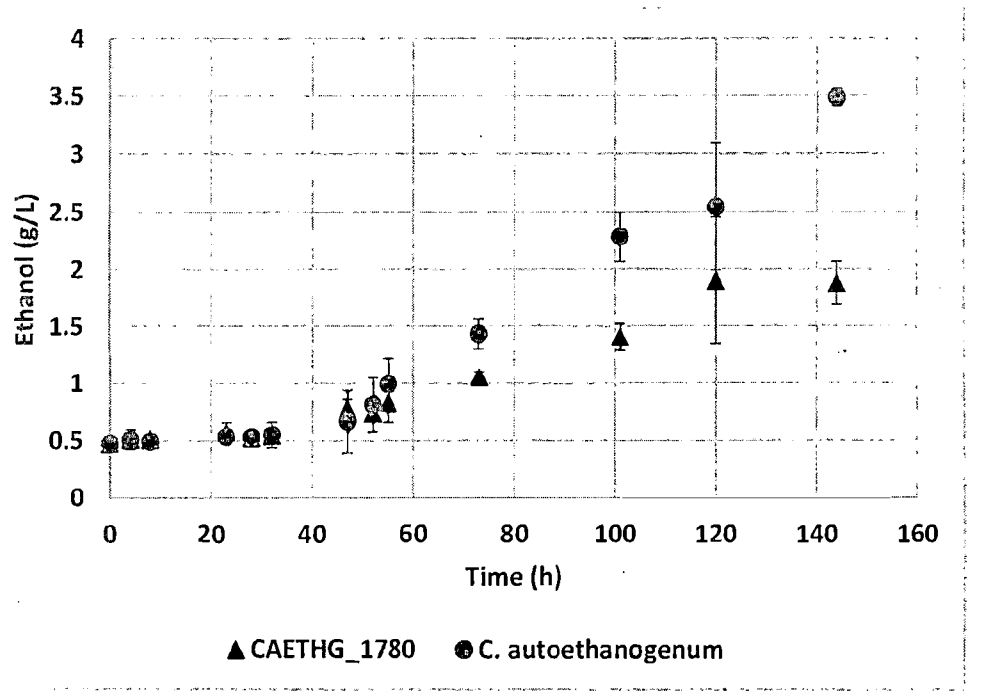

FIG. 23D

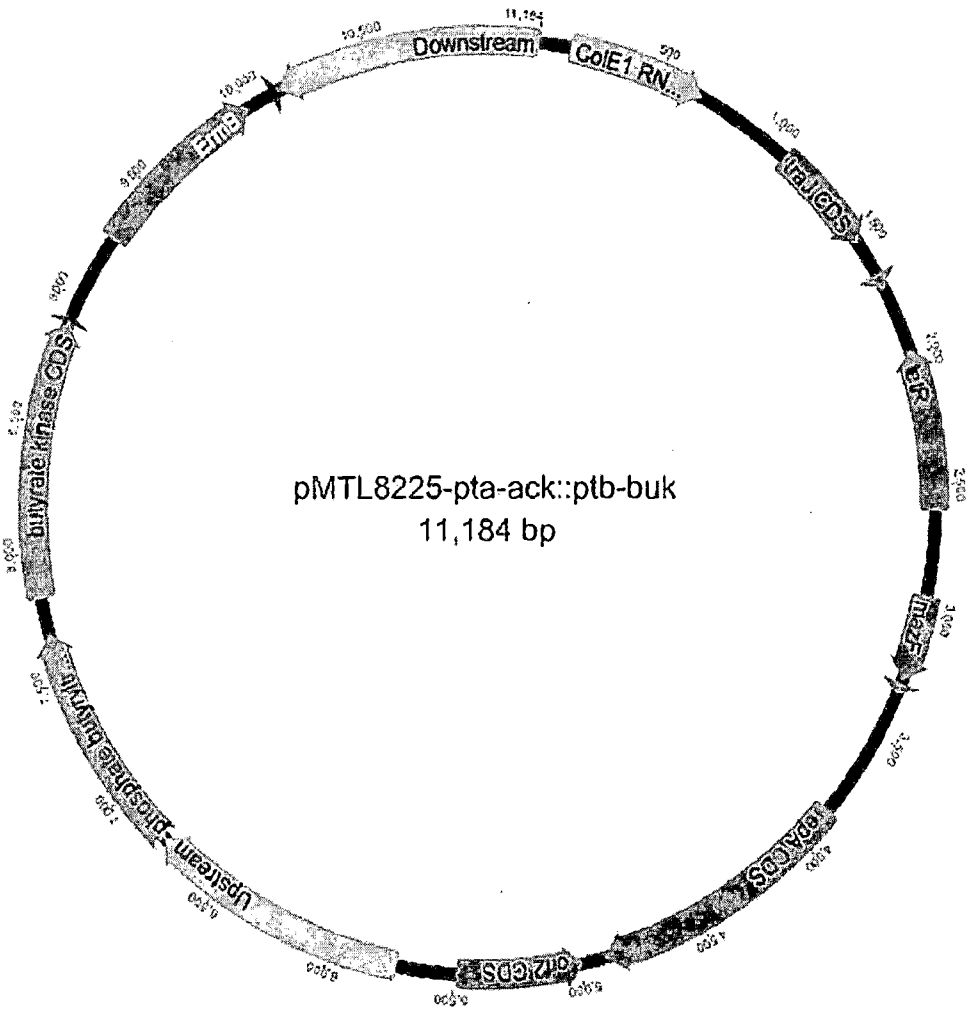
pMTL8225-pta-ack::ptb-buk
11,184 bp
Downstream
ColE1 RN...
traJ CDS
ER
lacZ
repA CDS
ori2 CDS
Upstream
phosphate butyryltr...
butyrate kinase CDS
ermB

FIG. 24

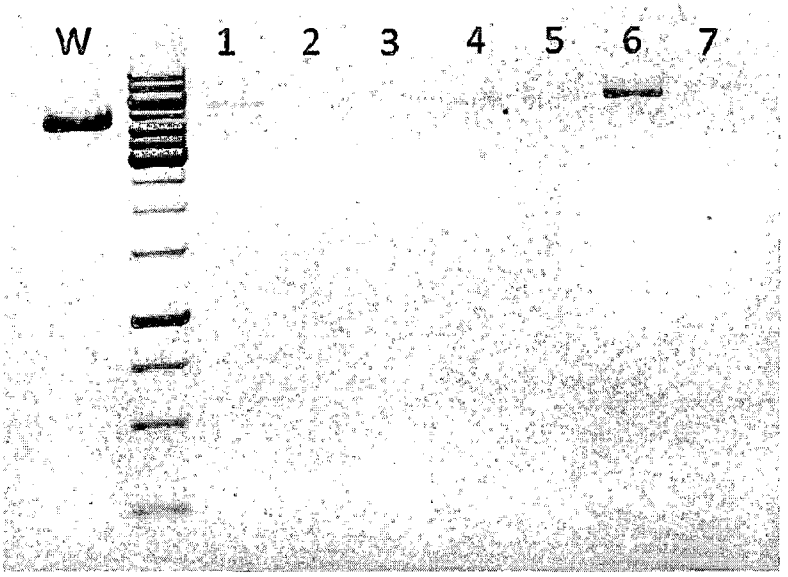

FIG. 25

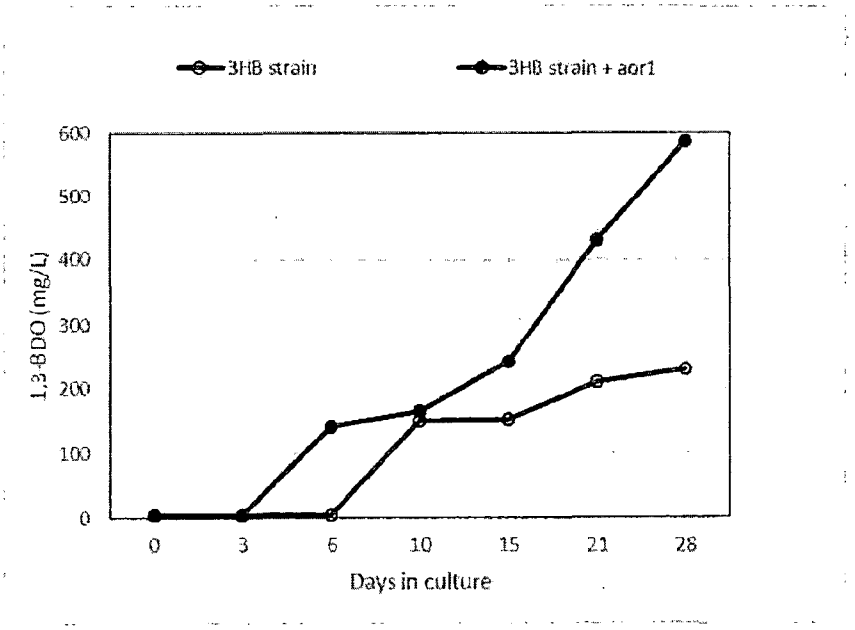
3HB strain
3HB strain + aor1
1,3-BDO (mg/L)
600
500
400
300
200
100
0
0
3
6
10
15
21
28
Days in culture

FIG. 26

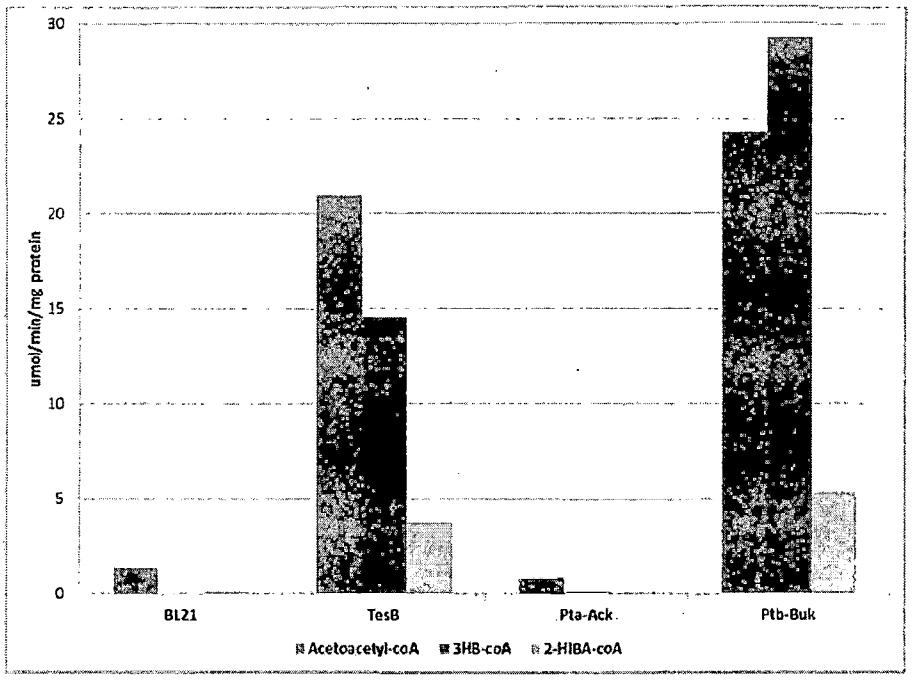
30
25
20
15
10
5
0
umol/min/mg protein
BL21
TesB
Pta-Ack
Ptb-Buk
Acetoacetyl-coA
3HB-coA
2-HIBA-coA

FIG. 27

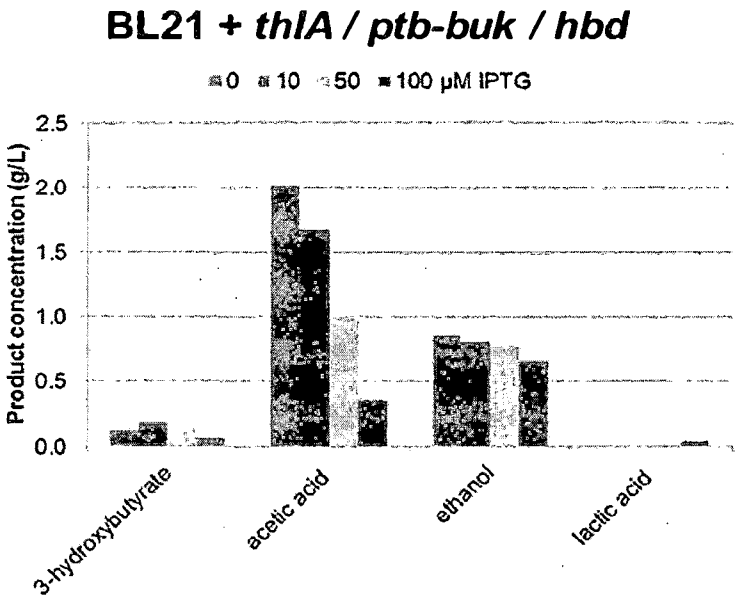

FIG. 28A

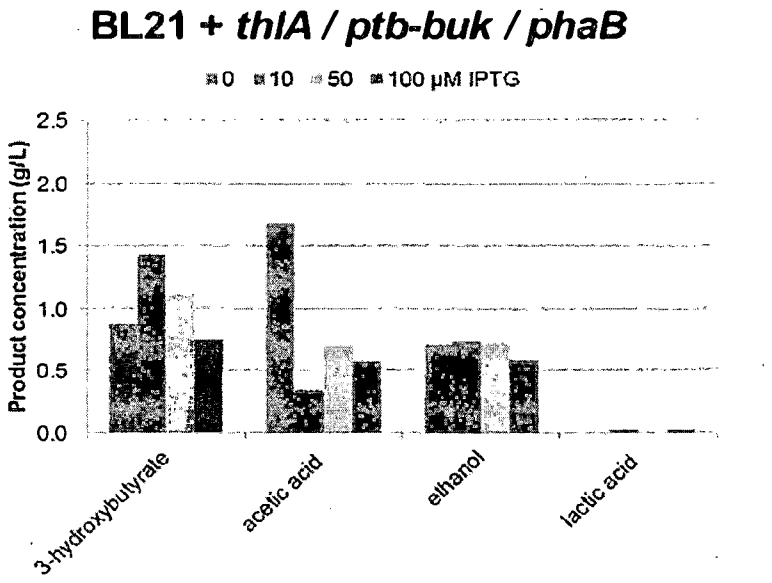

FIG. 28B

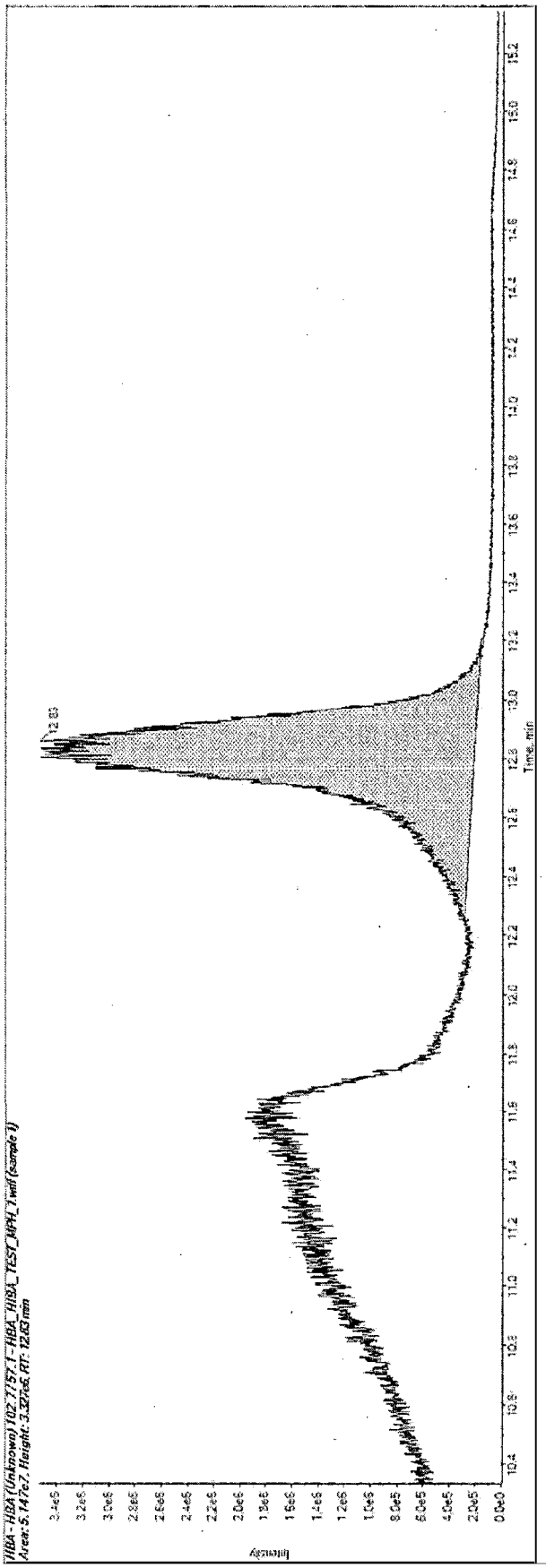
HBA - HBA (Unknown) 102.7157.1 - HBA_HBA_TEST_MPL_1.wiff (sample 1)
Area: 5.147e7, Height: 3.327e6, RT: 12.63 min
12.63
Intensity
Time, min

FIG. 29A

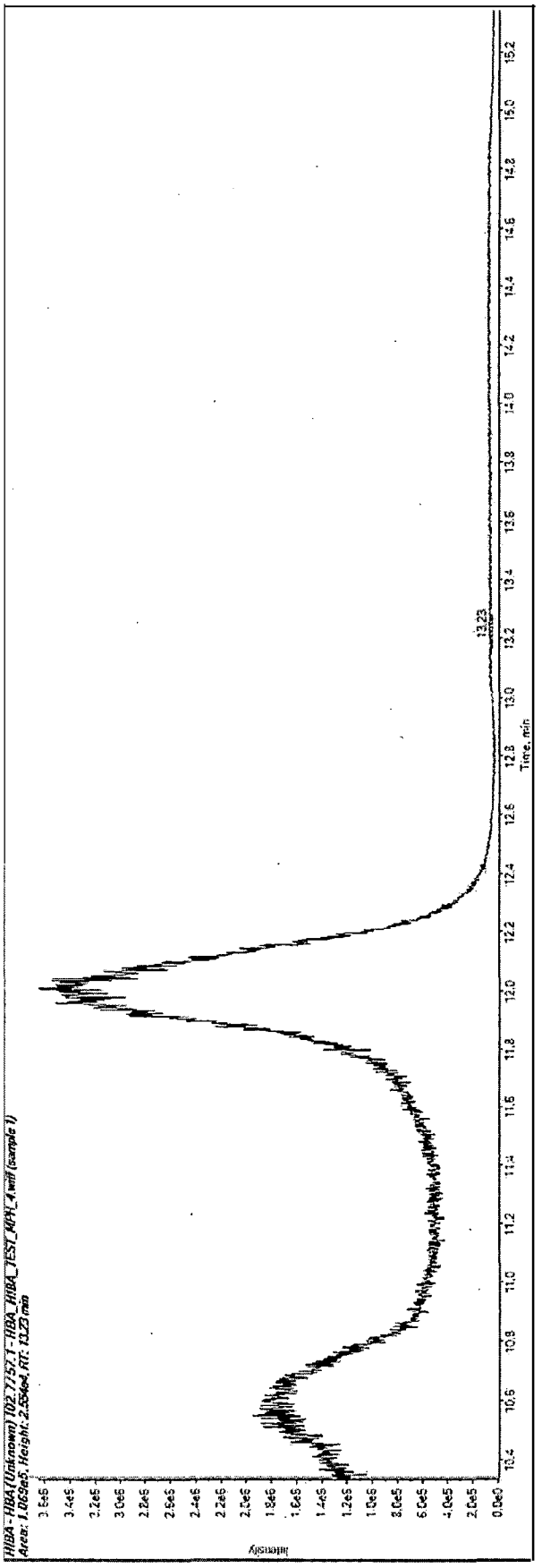
HIBA - HIBA (Unknown) 102.7157.1 - HIBA_HIBA_TEST_NPY(_4.wiff (sample 1)
Area: 1.069e5, Height: 2.554e4, RT: 13.23 min
Intensity
Time, min
13.23

FIG. 29B

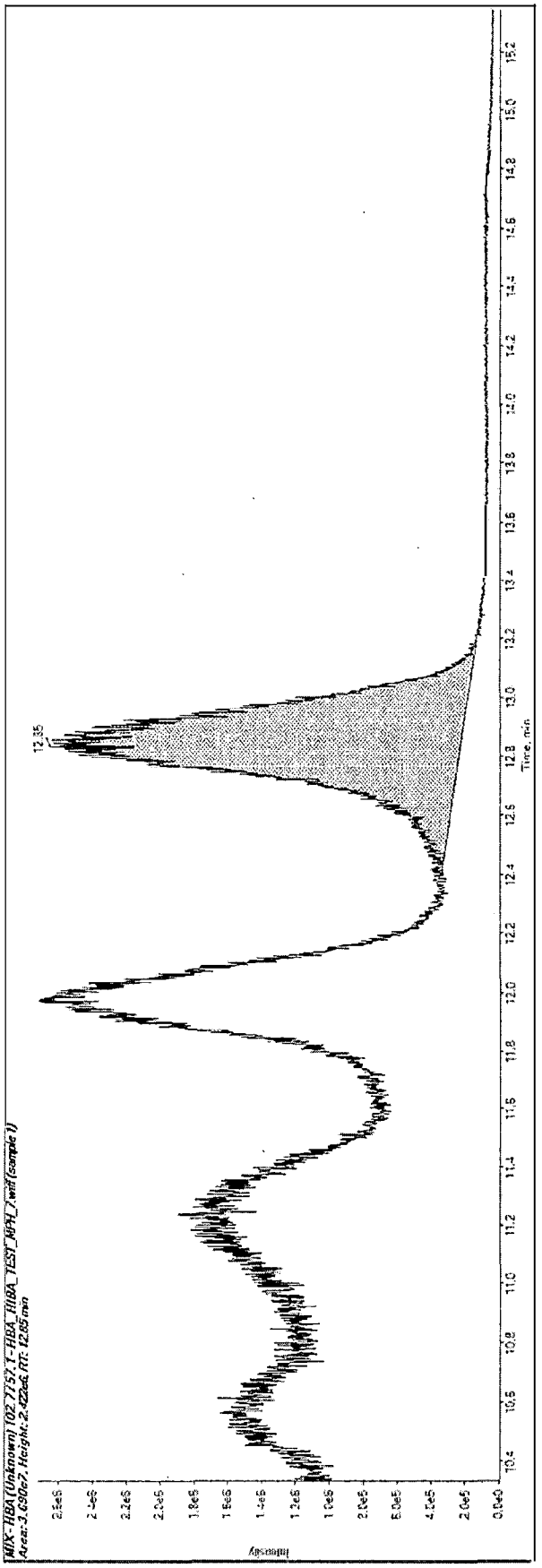
MIX-HBA (Unknown) 102.7757.T-HBA_HIBA_TEST_H6H_2.wiff (sample 1)
Area: 3.690e7, Height: 2.422e6, RT: 12.85 min
12.85
Time, min
Intensity

FIG. 29C

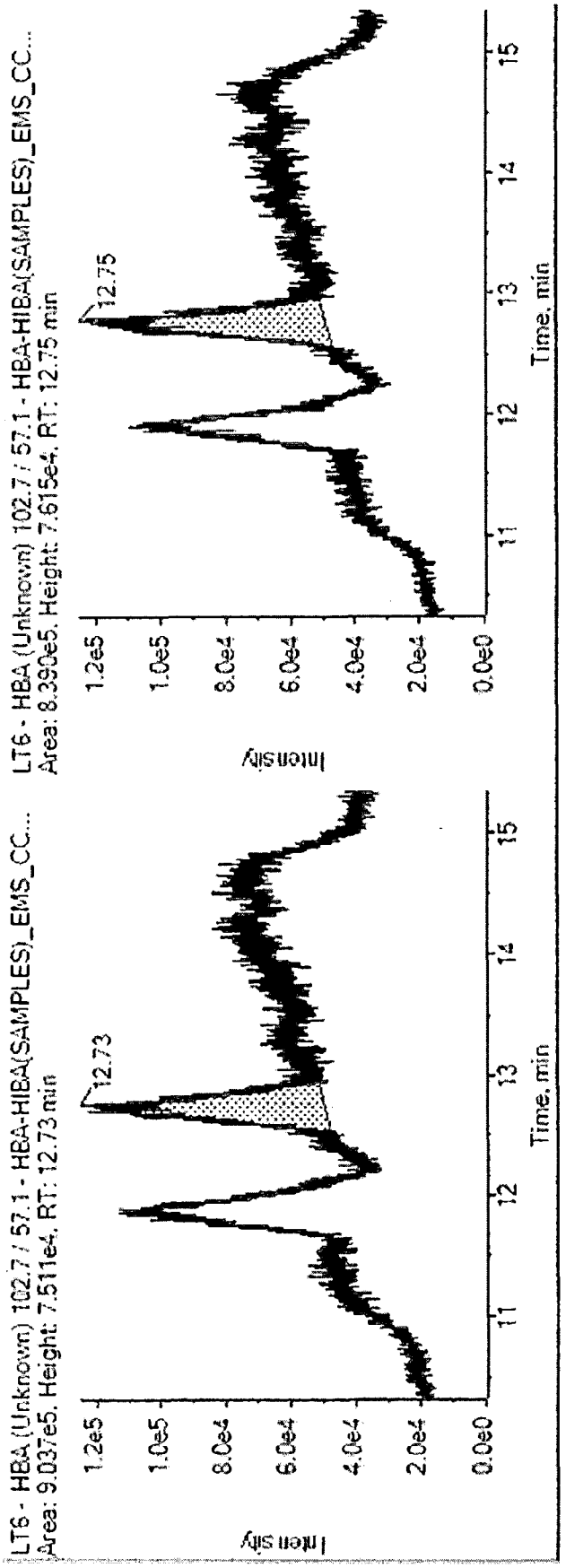
LT6 - HBA (Unknown) 102.7 / 57.1 - HBA-HIBA(SAMPLES)_EMS_CC...
Area: 9.037e5, Height: 7.511e4, RT: 12.73 min
12.73
LT6 - HBA (Unknown) 102.7 / 57.1 - HBA-HIBA(SAMPLES)_EMS_CC...
Area: 8.390e5, Height: 7.615e4, RT: 12.75 min
12.75
Intensity
1.2e5
1.0e5
8.0e4
6.0e4
4.0e4
2.0e4
0.0e0
11
12
13
14
15
Time, min

FIG. 29D

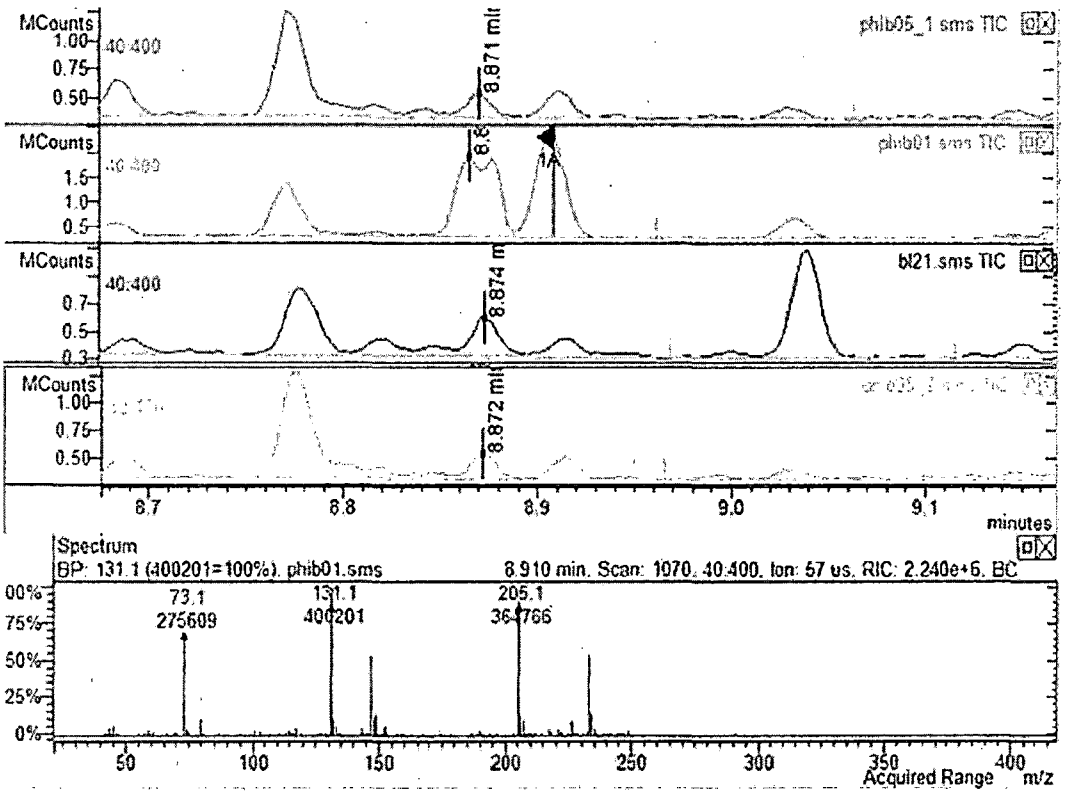
MCounts
40:400
8.871 min
phlb05_1.sms TIC
phlb01.sms TIC
8.874 min
bl21.sms TIC
8.872 min
8.7
8.8
8.9
9.0
9.1
minutes
Spectrum
BP: 131.1 (400201=100%), phlb01.sms
8.910 min, Scan: 1070, 40:400, Ion: 57 us, RIC: 2.240e+6, BC
73.1
275609
131.1
400201
205.1
36.1766
Acquired Range
m/z

FIG. 30

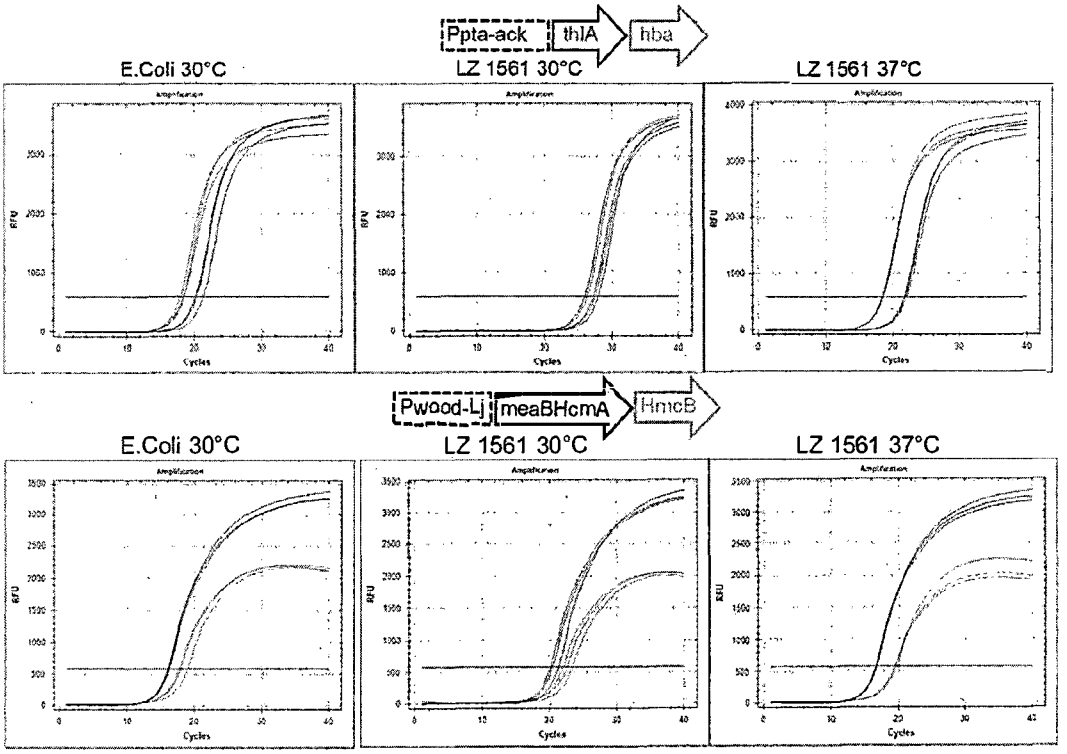
Ppta-ack
thlA
hba
E.Coli 30°C
LZ 1561 30°C
LZ 1561 37°C
Pwood-Lj
meaBHcmA
HmcB
E.Coli 30°C
LZ 1561 30°C
LZ 1561 37°C

FIG. 31

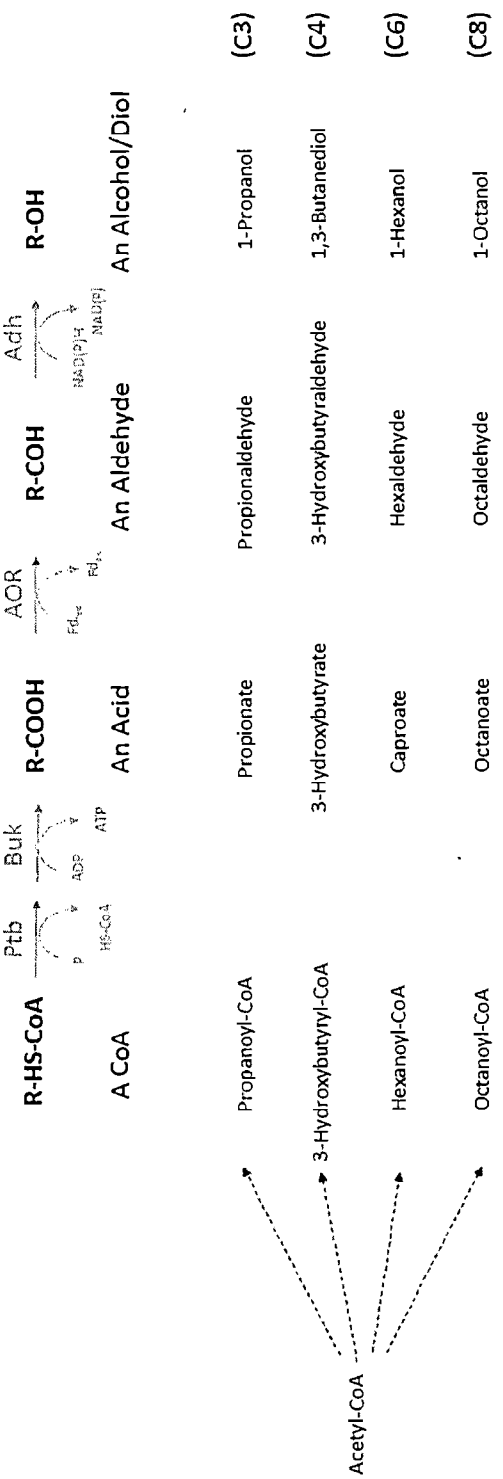
R-HS-CoA
Ptb
Buk
R-COOH
AOR
R-COH
Adh
R-OH
A CoA
An Acid
An Aldehyde
An Alcohol/Diol
Propanoyl-CoA
Propionate
Propionaldehyde
1-Propanol
(C3)
3-Hydroxybutyryl-CoA
3-Hydroxybutyrate
3-Hydroxybutyraldehyde
1,3-Butanediol
(C4)
Hexanoyl-CoA
Caproate
Hexaldehyde
1-Hexanol
(C6)
Octanoyl-CoA
Octanoate
Octaldehyde
1-Octanol
(C8)
Acetyl-CoA

FIG. 32

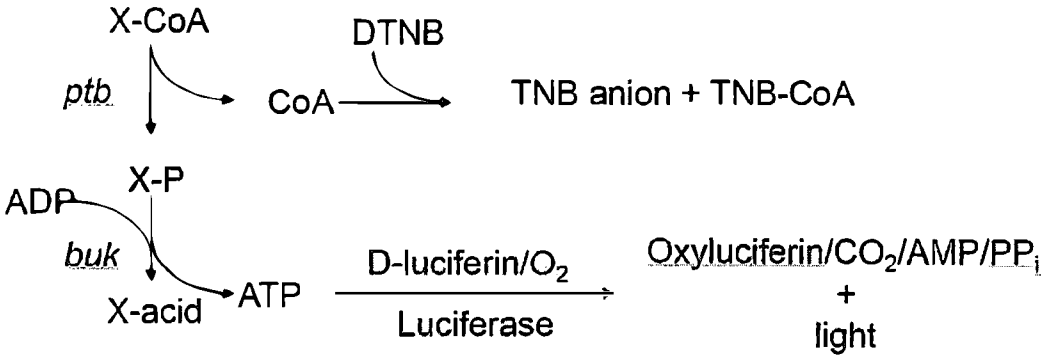
X-CoA
DTNB
ptb
CoA
TNB anion + TNB-CoA
X-P
ADP
buk
ATP
X-acid
D-luciferin/O2
Luciferase
Oxyluciferin/CO2/AMP/PPi
+
light

FIG. 33

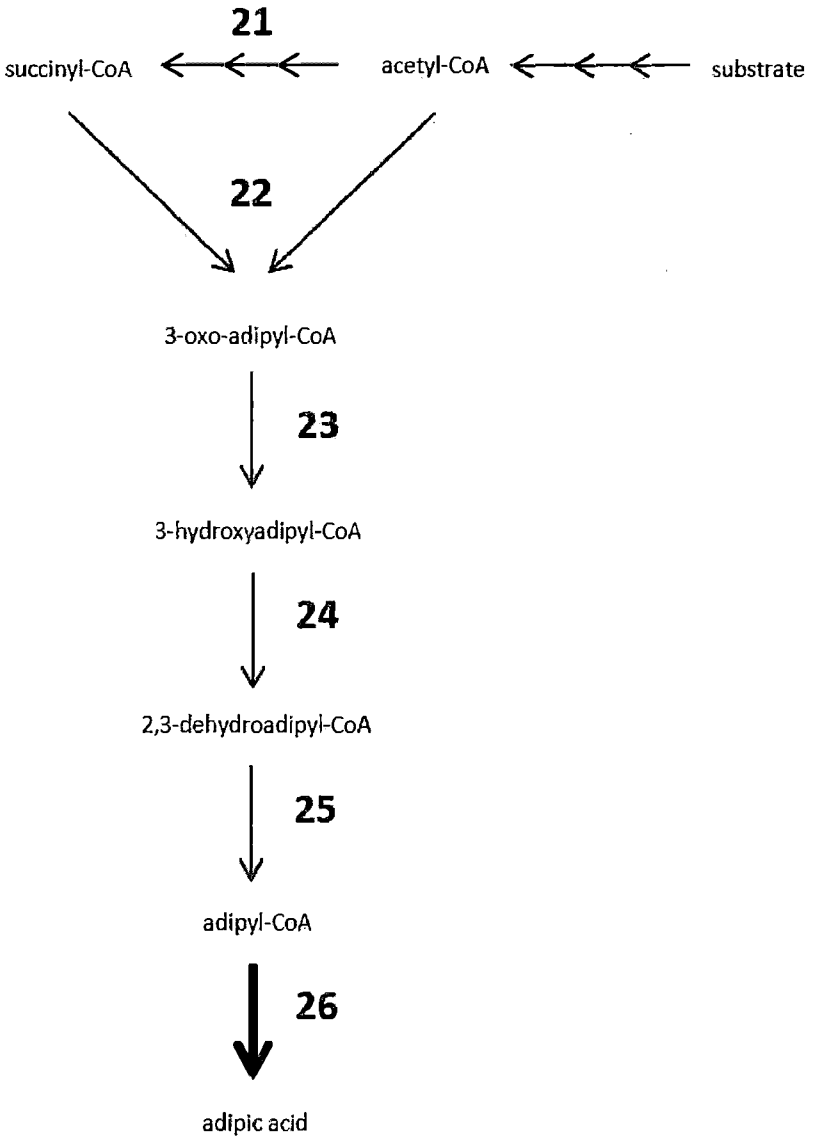
21
succinyl-CoA
acetyl-CoA
substrate
22
3-oxo-adipyl-CoA
23
3-hydroxyadipyl-CoA
24
2,3-dehydroadipyl-CoA
25
adipyl-CoA
26
adipic acid

FIG. 34

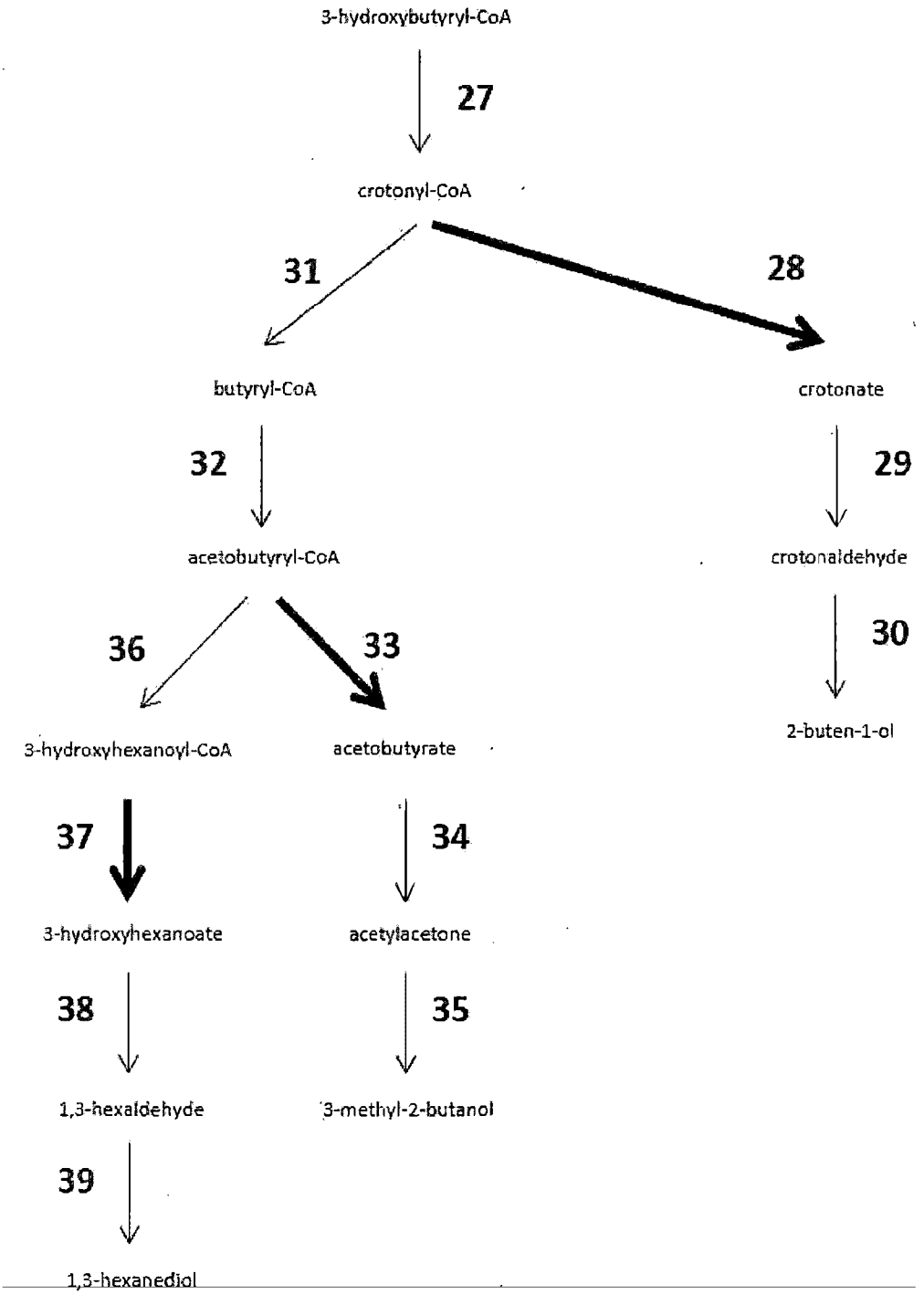
3-hydroxybutyryl-CoA
27
crotonyl-CoA
31
28
butyryl-CoA
crotonate
32
29
acetobutyryl-CoA
crotonaldehyde
36
33
30
3-hydroxyhexanoyl-CoA
acetobutyrate
2-buten-1-ol
37
34
3-hydroxyhexanoate
acetylacetone
38
35
1,3-hexaldehyde
3-methyl-2-butanol
39
1,3-hexanediol

FIG. 35

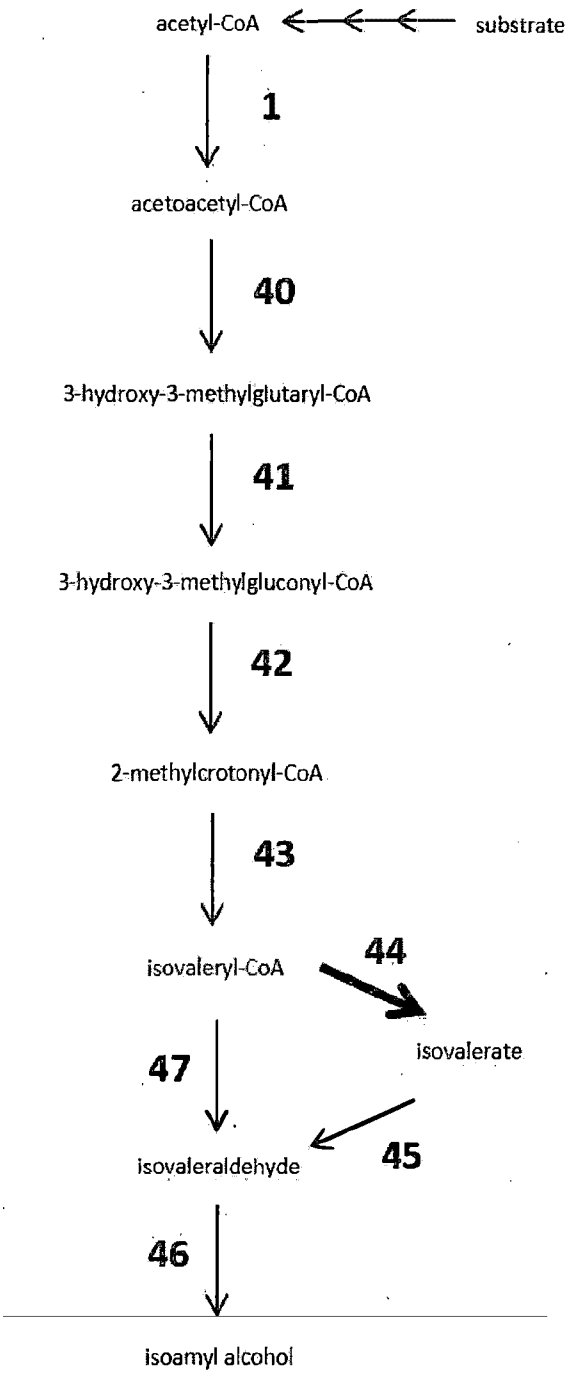
acetyl-CoA
substrate
1
acetoacetyl-CoA
40
3-hydroxy-3-methylglutaryl-CoA
41
3-hydroxy-3-methylgluconyl-CoA
42
2-methylcrotonyl-CoA
43
isovaleryl-CoA
44
isovalerate
47
45
isovaleraldehyde
46
isoamyl alcohol

FIG. 36

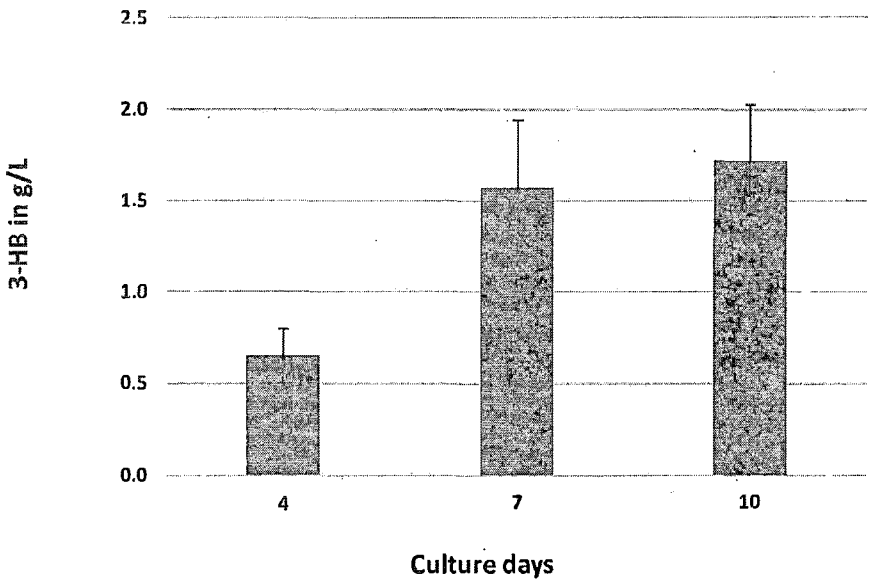

FIG. 37

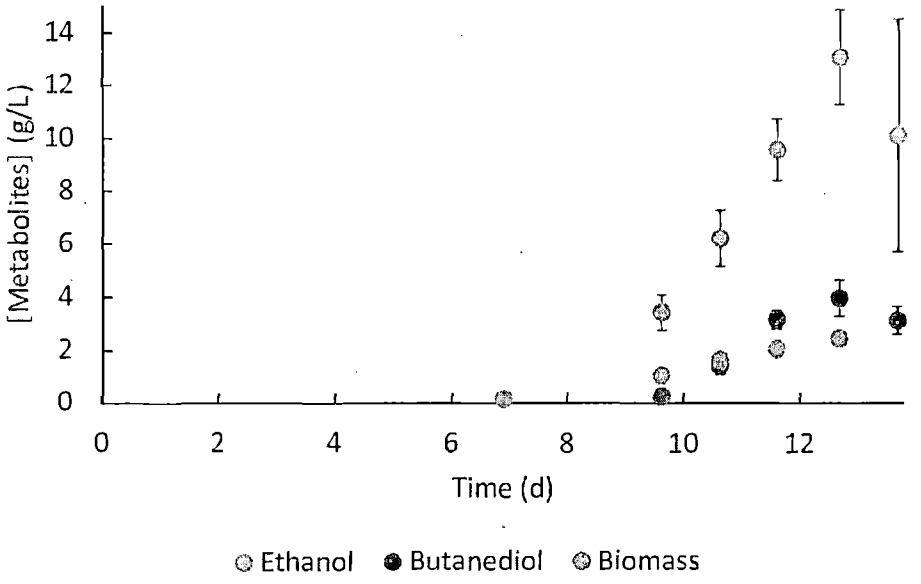

FIG. 38A

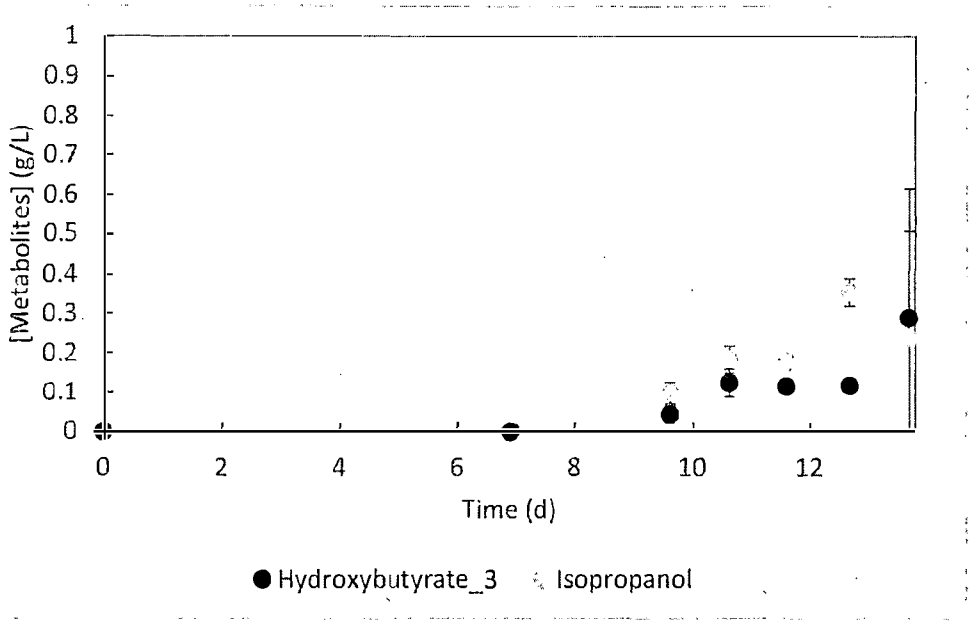

FIG. 38B

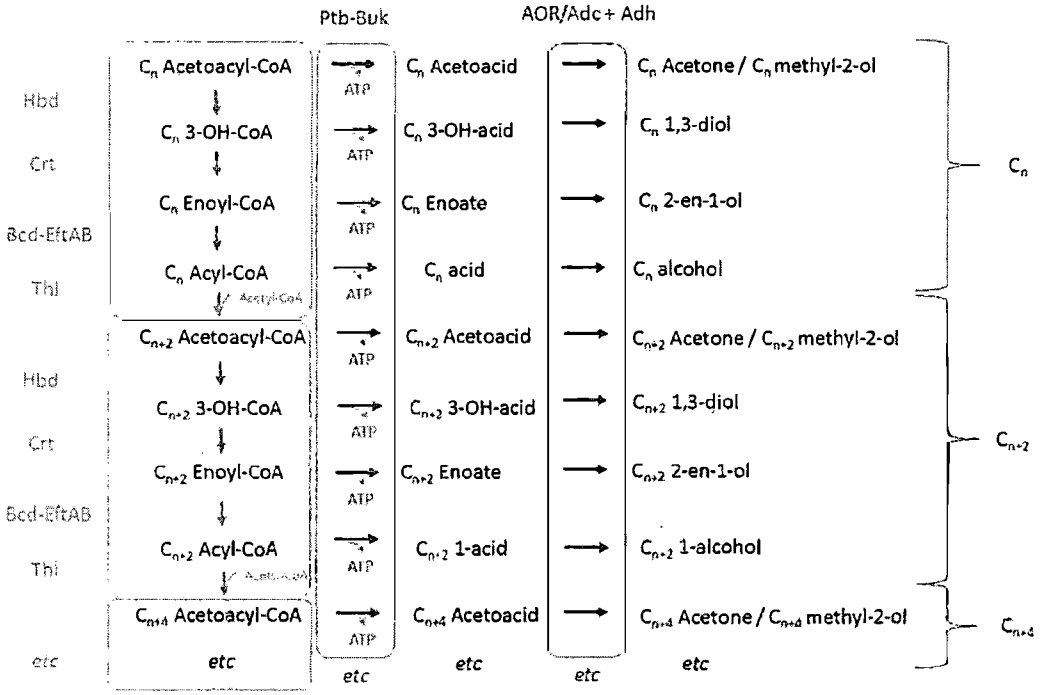
Ptb-Buk
AOR/Adc+ Adh
Hbd
Crt
Bcd-EftAB
Thl
Hbd
Crt
Bcd-EftAB
Thl
etc
ATP
C_n Acetoacyl-CoA
C_n 3-OH-CoA
C_n Enoyl-CoA
C_n Acyl-CoA
C_{n+2} Acetoacyl-CoA
C_{n+2} 3-OH-CoA
C_{n+2} Enoyl-CoA
C_{n+2} Acyl-CoA
C_{n+4} Acetoacyl-CoA
etc
C_n Acetoacid
C_n 3-OH-acid
C_n Enoate
C_n acid
C_{n+2} Acetoacid
C_{n+2} 3-OH-acid
C_{n+2} Enoate
C_{n+2} 1-acid
C_{n+4} Acetoacid
etc
C_n Acetone / C_n methyl-2-ol
C_n 1,3-diol
C_n 2-en-1-ol
C_n alcohol
C_{n+2} Acetone / C_{n+2} methyl-2-ol
C_{n+2} 1,3-diol
C_{n+2} 2-en-1-ol
C_{n+2} 1-alcohol
C_{n+4} Acetone / C_{n+4} methyl-2-ol
etc
C_n
C_{n+2}
C_{n+4}

FIG. 39

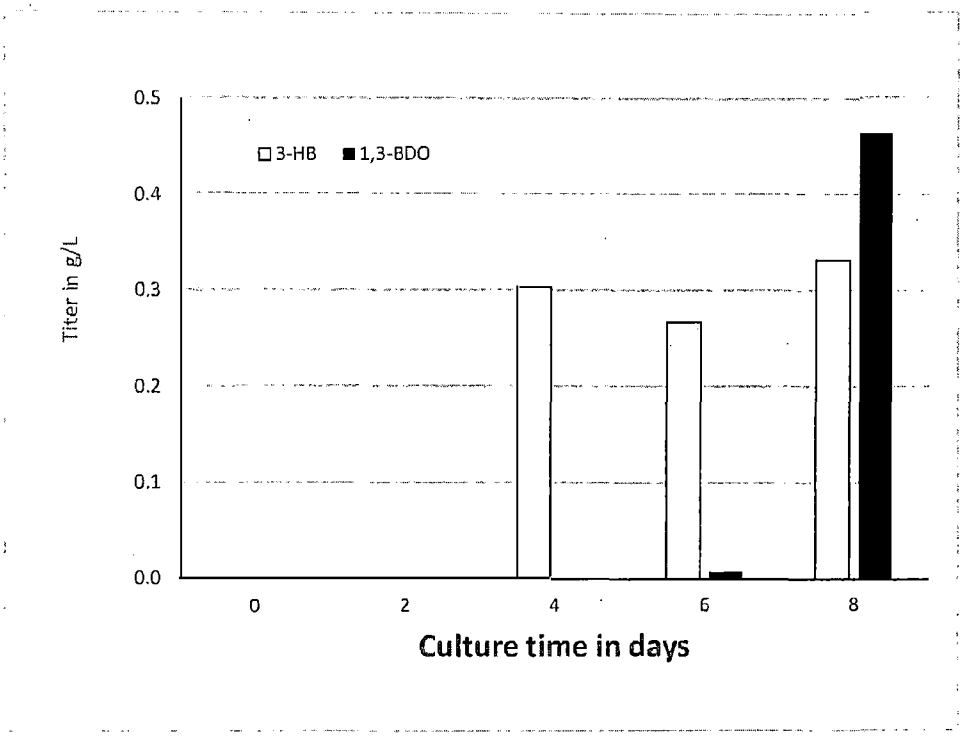
0.5
0.4
0.3
0.2
0.1
0.0
□3-HB ■1,3-BDO
Titer in g/L
0
2
4
6
8
Culture time in days

FIG. 40

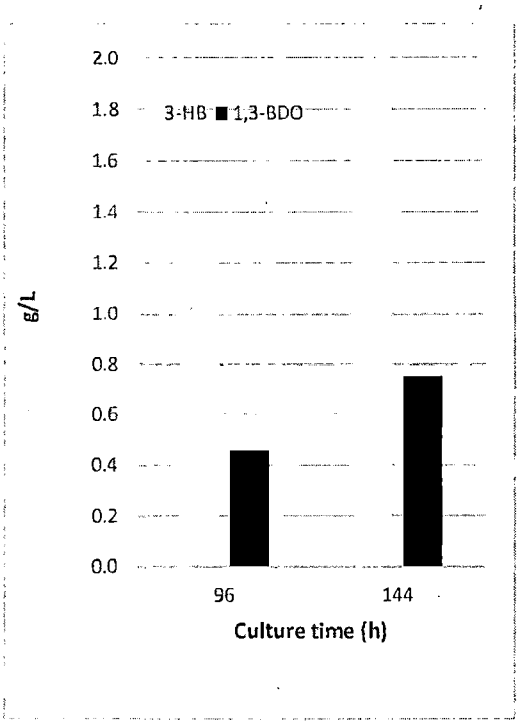
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
3-HB ■1,3-BDO
g/L
96
144
Culture time (h)

FIG. 41

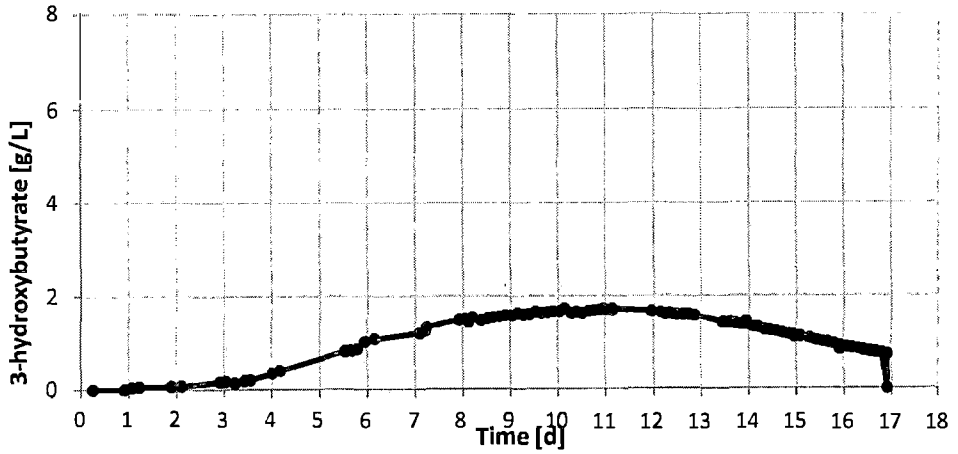

FIG. 42A

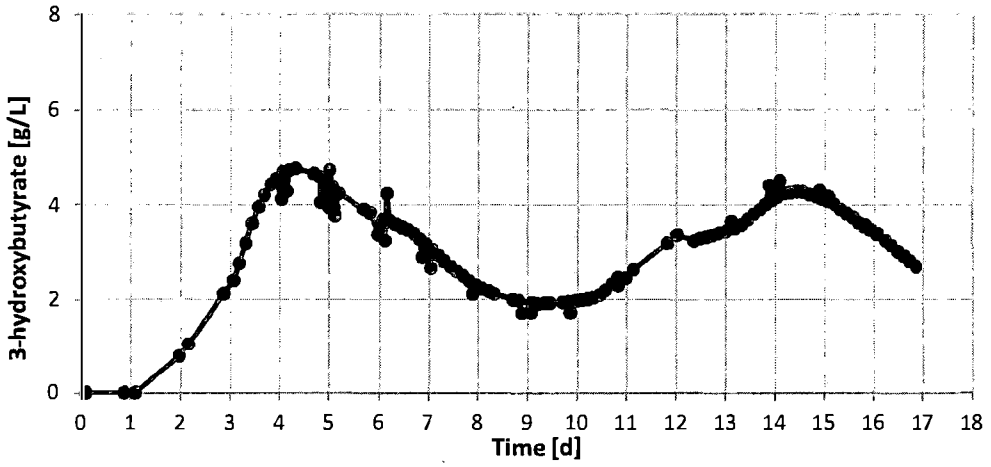

FIG. 42B

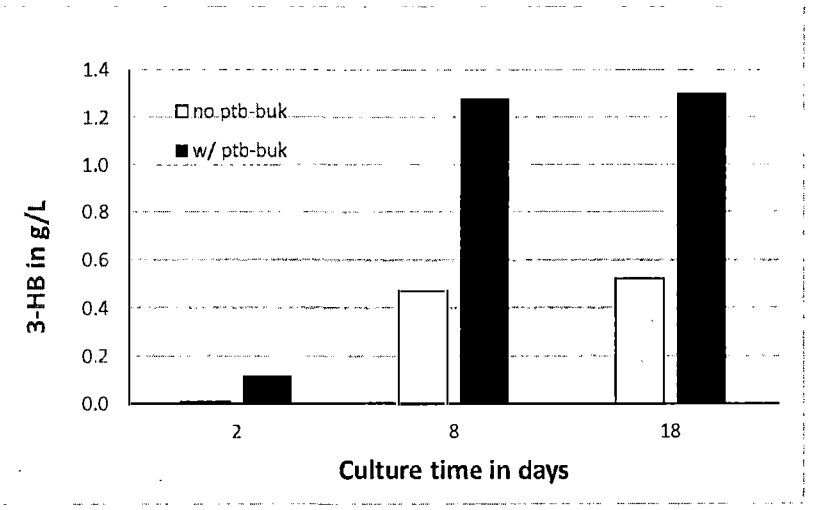
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
3-HB in g/L
no ptb-buk
w/ ptb-buk
2
8
18
Culture time in days

FIG. 43

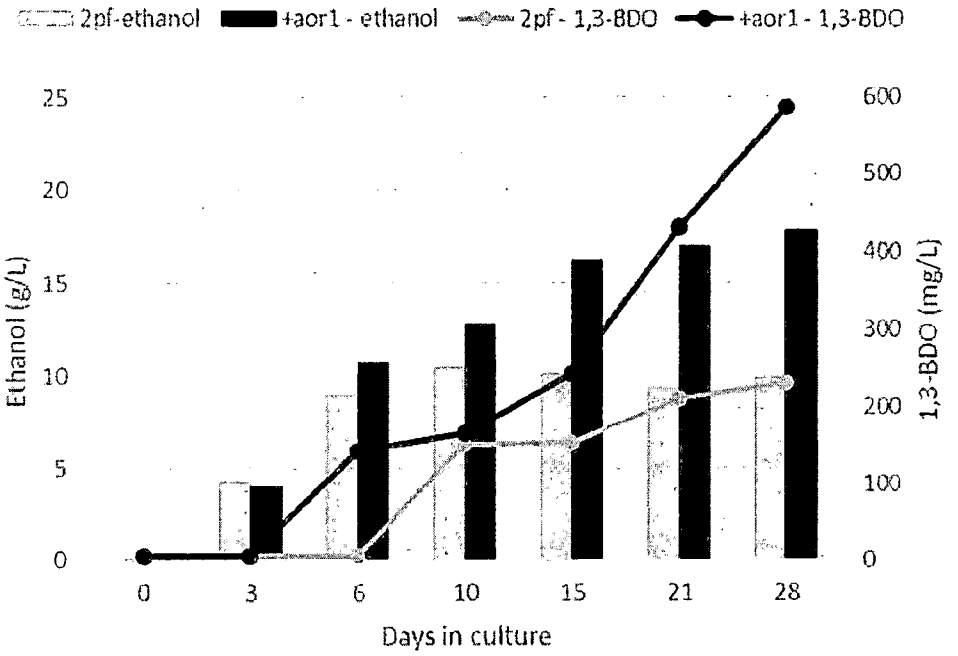
2pf-ethanol
+aor1 - ethanol
2pf - 1,3-BDO
+aor1 - 1,3-BDO
25
20
15
10
5
0
Ethanol (g/L)
600
500
400
300
200
100
0
1,3-BDO (mg/L)
0
3
6
10
15
21
28
Days in culture

FIG. 44