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(54) **Title**: MICROORGANISMS AND METHODS FOR CONVERSION OF SYNGAS AND OTHER CARBON SOURCES TO USEFUL PRODUCTS

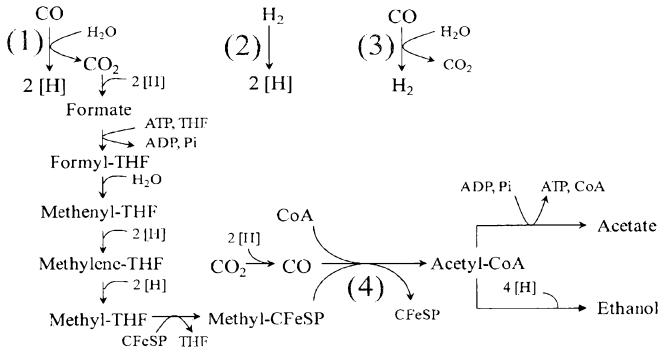

Figure 1

(57) **Abstract**: An exemplary non-naturally occurring microbial organism having an isopropanol pathway includes at least one exogenous nucleic acid encoding an isopropanol pathway enzyme expressed in a sufficient amount to produce isopropanol and includes a succinyl-CoA:3-ketoacid-CoA transferase. Another organism having a 4-hydroxybutryate pathway includes at least one exogenous nucleic acid including an acetoacetyl-CoA thiolase, a 3-hydroxybutyryl-CoA dehydrogenase, a crotonase, a crotonyl-CoA hydratase, a 4-hydroxybutyryl-CoA transferase, a phosphotrans-4-hydroxybutyrylase, and a 4-hydroxybutyrate kinase. An exemplary organism having a 1,4-butanediol pathway includes an acetoacetyl-CoA thiolase, a 3-Hydroxybutyryl-CoA dehydrogenase, a crotonase, a crotonyl-CoA hydratase, a 4-hydroxybutyryl-CoA reductase (alcohol forming), a 4-hydroxybutyryl-CoA reductase (aldehyde forming), and a 1,4-butanediol dehydrogenase. Such an organism further comprising an acetyl-CoA pathway. Any of the aforementioned organisms are cultured to produce isopropanol, 4-hydroxybutryate, or 1,4- butanediol.

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MICROORGANISMS AND METHODS FOR CONVERSION OF SYNGAS AND OTHER CARBON SOURCES TO USEFUL PRODUCTS

This application claims the benefit of priority of U.S. Provisional Application serial No. 61/138,108, filed December 16, 2007, the entire contents of which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

The present invention relates generally to biosynthetic processes and organisms capable of converting methanol, synthesis gas and other gaseous carbon sources into higher-value chemicals. More specifically, the invention relates to non-naturally occurring organisms that can produce the commodity chemicals, isopropanol, 4-hydroxybutryate, and 1,4-butanediol.

Increasing the flexibility of cheap and readily available feedstocks and minimizing the environmental impact of chemical production are beneficial for a sustainable chemical industry. Feedstock flexibility relies on the introduction of methods that can access and use a wide range of materials as primary feedstocks for chemical manufacturing.

Isopropanol (IPA) is a colorless, flammable liquid that mixes completely with most solvents, including water. The largest use for IPA is as a solvent, including its well known yet small use as "rubbing alcohol," which is a mixture of IPA and water. As a solvent, IPA is found in many everyday products such as paints, lacquers, thinners, inks, adhesives, general-purpose cleaners, disinfectants, cosmetics, toiletries, de-icers, and pharmaceuticals. Low-grade IPA is also used in motor oils. The second largest use is as a chemical intermediate for the production of isopropylamines (e.g. in agricultural products), isopropylethers, and isopropyl esters.

Isopropanol is manufactured by two petrochemical routes. The predominant process entails the hydration of propylene either with or without sulfuric acid catalysis. Secondarily, IPA is produced via hydrogenation of acetone, which is a by-product formed in the production of phenol and propylene oxide. High-priced propylene is currently driving costs up and margins down throughout the chemical industry motivating the need for an expanded range of low cost feedstocks.

4-hydroxybutanoic acid (4-hydroxybutanoate, 4-hydroxybutyrate, 4-HB) is a 4-carbon carboxylic acid that has industrial potential as a building block for various commodity and specialty chemicals. In particular, 4-HB has the potential to serve as a new entry point into the 1,4-butanediol family of chemicals, which includes solvents, resins, polymer precursors, and specialty chemicals.

BDO is a valuable chemical for the production of high performance polymers, solvents, and fine chemicals. It is the basis for producing other high value chemicals such as tetrahydrofuran (THF) and gamma-butyrolactone (GBL). Uses of BDO include (1) polymers, (2) THF derivatives, and (3) GBL derivatives. In the case of polymers, BDO is a co-monomer for polybutylene terephthalate (PBT) production. PBT is a medium performance engineering thermoplastic made by companies such as DuPont and General Electric finding use in automotive, electrical, water systems, and small appliance applications. When converted to THF, and subsequently to polytetramethylene ether glycol (PTMEG), the Spandex and Lycra fiber and apparel industries are added to the markets served. PTMEG is also combined with BDO in the production of specialty polyester ethers (COPE). COPEs are high modulus elastomers with excellent mechanical properties and oil/environmental resistance, allowing them to operate at high and low temperature extremes. PTMEG and BDO also make thermoplastic polyurethanes processed on standard thermoplastic extrusion, calendaring, and molding equipment, and are characterized by their outstanding toughness and abrasion resistance. The GBL produced from BDO provides the feedstock for making pyrrolidones, as well as serving agrochemical market applications itself. The pyrrolidones are used as high performance solvents for extraction processes of increasing use in the electronics industry as well as use in pharmaceutical production.

BDO is produced by two main petrochemical routes with a few additional routes also in commercial operation. One route involves reacting acetylene with formaldehyde, followed by hydrogenation. More recently BDO processes involving butane or butadiene oxidation to maleic anhydride, followed by hydrogenation have been introduced. BDO is used almost exclusively as an intermediate to synthesize other chemicals and polymers.

Synthesis gas (syngas) is a mixture of primarily H_2 and CO that can be obtained via gasification of any organic feedstock, such as coal, coal oil, natural gas, biomass, or waste organic matter. Numerous gasification processes have been developed, and most designs are based on partial oxidation, where limiting oxygen avoids full combustion, of organic materials at high temperatures (500-1500°C) to provide syngas as, for example, 0.5:1-3:1 H_2/CO mixture. Steam is sometimes added to increase the hydrogen content, typically with increased CO_2 production through the water gas shift reaction.

Today, coal is the main substrate used for industrial production of syngas, which is usually used for heating and power and as a feedstock for Fischer-Tropsch synthesis of methanol

and liquid hydrocarbons. Many large chemical and energy companies employ coal gasification processes on large scale and there is experience in the industry using this technology.

Overall, technology now exists for cost-effective production of syngas from a plethora of materials, including coal, biomass, wastes, polymers, and the like, at virtually any location in the world. Biomass gasification technologies are being practiced commercially, particularly for heat and energy generation.

Despite the availability of organisms that utilize syngas, such organisms are generally poorly characterized and are not well-suited for commercial development. For example, *Clostridium* and related bacteria are strict anaerobes that are intolerant to high concentrations of certain products such as butanol, thus limiting titers and commercialization potential. The *Clostridia* also produce multiple products, which presents separations issues in isolating a desired product. Finally, development of facile genetic tools to manipulate clostridial genes is in its infancy, therefore, they are not currently amenable to rapid genetic engineering to improve yield or production characteristics of a desired product.

Thus, there exists a need to develop microorganisms and methods of their use to utilize syngas or other gaseous carbon sources for the production of desired chemicals and fuels. More specifically, there exists a need to develop microorganisms for syngas utilization that also have existing and efficient genetic tools to enable their rapid engineering to produce valuable products at useful rates and quantities. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

In some aspects, the present invention provides a non-naturally occurring microbial organism having an isopropanol pathway that includes at least one exogenous nucleic acid encoding an isopropanol pathway enzyme expressed in a sufficient amount to produce isopropanol. The isopropanol pathway enzyme includes a succinyl-CoA:3-ketoacid-CoA transferase.

In other aspects, the present invention provides a non-naturally occurring microbial organism having a 4-hydroxybutryate pathway that includes at least one exogenous nucleic acid encoding an 4-hydroxybutryate pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutryate. The 4-hydroxybutryate pathway enzyme includes an acetoacetyl-CoA thiolase, a 3-hydroxybutyryl-CoA dehydrogenase, a crotonase, a crotonyl-CoA hydratase, a 4-

hydroxybutyryl-CoA transferase, a phosphotrans-4-hydroxybutyrylase, and a 4-hydroxybutyrate kinase.

In still other aspects, the present invention provides a non-naturally occurring microbial organism having a 1,4-butanediol pathway that includes at least one exogenous nucleic acid encoding a 1,4-butanediol pathway enzyme expressed in a sufficient amount to produce 1,4-butanediol. The 1,4-butanediol pathway enzyme includes an acetoacetyl-CoA thiolase, a 3-Hydroxybutyryl-CoA dehydrogenase, a crotonase, a crotonyl-CoA hydratase, a 4-hydroxybutyryl-CoA reductase (alcohol forming), a 4-hydroxybutyryl-CoA reductase (aldehyde forming), and a 1,4-butanediol dehydrogenase. Such an organism also includes an acetyl-CoA pathway having at least one exogenous nucleic acid encoding an acetyl-CoA pathway enzyme expressed in a sufficient amount to produce acetyl-CoA. The acetyl-CoA pathway enzyme includes a corrinoid protein, a methyltetrahydrofolate:corrinoid protein methyltransferase, a corrinoid iron-sulfur protein, a nickel-protein assembly protein, a ferredoxin, an acetyl-CoA synthase, a carbon monoxide dehydrogenase, a pyruvate ferredoxin oxidoreductase, and a hydrogenase.

In yet other aspects, the present invention provides a non-naturally occurring microbial organism having a 1,4-butanediol pathway that includes at least one exogenous nucleic acid encoding a 1,4-butanediol pathway enzyme expressed in a sufficient amount to produce 1,4-butanediol. The 1,4-butanediol pathway enzyme includes an acetoacetyl-CoA thiolase, a 3-Hydroxybutyryl-CoA dehydrogenase, a crotonase, a crotonyl-CoA hydratase, a 4-hydroxybutyryl-CoA reductase (alcohol forming), a 4-hydroxybutyryl-CoA reductase (aldehyde forming), and a 1,4-butanediol dehydrogenase. Such an organism also includes an acetyl-CoA pathway having at least one exogenous nucleic acid encoding an acetyl-CoA pathway enzyme expressed in a sufficient amount to produce acetyl-CoA. The acetyl-CoA pathway enzyme includes an acetyl-CoA synthase, a formate dehydrogenase, a formyltetrahydrofolate synthetase, a methenyltetrahydrofolate cyclohydrolase, a methylenetetrahydrofolate dehydrogenase, and a methylenetetrahydrofolate reductase.

In still further aspects, the present invention provides a non-naturally occurring microbial organism having an isopropanol pathway that includes at least one exogenous nucleic acid encoding an isopropanol pathway enzyme expressed in a sufficient amount to produce isopropanol. The isopropanol pathway enzyme includes an acetoacetyl-CoA thiolase, an acetoacetyl-CoA:acetate:CoA transferase, an acetoacetate decarboxylase, and an isopropanol dehydrogenase. Such an organism also includes at least one exogenous nucleic acid encoding an

acetyl-CoA enzyme expressed in a sufficient amount to produce acetyl-CoA. The acetyl-CoA pathway enzyme includes a methanol methyl transferase, a corrinoid protein, a methyltetrahydro-folate:corrinoid protein methyltransferase, a corrinoid iron-sulfur protein, a nickel-protein assembly protein, a ferredoxin, an acetyl-CoA synthase, a carbon monoxide dehydrogenase, a pyruvate ferredoxin oxidoreductase,and a hydrogenase.

In still further aspects, the present invention provides a method for producing isopropanol that includes culturing a non-naturally occurring microbial organism having an isopropanol pathway. The pathway includes at least one exogenous nucleic acid encoding an isopropanol pathway enzyme expressed in a sufficient amount to produce isopropanol under conditions and for a sufficient period of time to produce isopropanol. The isopropanol pathway includes a succinyl-CoA:3-ketoacid-CoA transferase.

In yet still further aspects, the present invention provides a method for producing 4-hydroxybutyrate that includes culturing a non-naturally occurring microbial organism having an 4-hydroxybutyrate pathway. The pathway includes at least one exogenous nucleic acid encoding an 4-hydroxybutyrate pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutyrate under conditions and for a sufficient period of time to produce 4-hydroxybutyrate. The 4-hydroxybutyrate pathway includes an acetoacetyl-CoA thiolase, a 3-hydroxybutyryl-CoA dehydrogenase, a crotonase, a crotonyl-CoA hydratase, a 4-hydroxybutyryl-CoA transferase, a phosphotrans-4-hydroxybutyrylase, and a 4-hydroxybutyrate kinase.

In still other aspects, the present invention provides a method for producing 1,4-butanediol that includes culturing a non-naturally occurring microbial organism having an 1,4-butanediol pathway. The pathway includes at least one exogenous nucleic acid encoding an 1,4-butanediol pathway enzyme expressed in a sufficient amount to produce 1,4-butanediol under conditions and for a sufficient period of time to produce 1,4-butanediol. The 1,4-butanediol pathway includes an acetoacetyl-CoA thiolase, a 3-hydroxybutyryl-CoA dehydrogenase, a crotonase, a crotonyl-CoA hydratase, a 4-hydroxybutyryl-CoA reductase (alcohol forming), a 4-hydroxybutyryl-CoA reductase (aldehyde forming), a 1,4-butanediol dehydrogenase. Such an organism also includes an acetyl-CoA pathway comprising at least one exogenous nucleic acid encoding an acetyl-CoA pathway enzyme expressed in a sufficient amount to produce acetyl-CoA. The acetyl-CoA pathway enzyme includes a corrinoid protein, a methyltetrahydro-folate:corrinoid protein methyltransferase, a corrinoid iron-sulfur protein, a nickel-protein

assembly protein, a ferredoxin, an acetyl-CoA synthase, a carbon monoxide dehydrogenase, a pyruvate ferredoxin oxidoreductase,and a hydrogenase.

Finally, in some aspects, the present invention provides a method for producing 1,4-butanediol that includes culturing a non-naturally occurring microbial organism having an 1,4-butanediol pathway. The pathway includes at least one exogenous nucleic acid encoding an 1,4-butanediol pathway enzyme expressed in a sufficient amount to produce 1,4-butanediol under conditions and for a sufficient period of time to produce 1,4-butanediol. The 1,4-butanediol pathway includes an acetoacetyl-CoA thiolase, a 3-hydroxybutyryl-CoA dehydrogenase, a crotonase, a crotonyl-CoA hydratase, a 4-hydroxybutyryl-CoA reductase (alcohol forming), a 4-hydroxybutyryl-CoA reductase (aldehyde forming), and a 1,4-butanediol dehydrogenase. Such an organism also includes an acetyl-CoA pathway having at least one exogenous nucleic acid encoding an acetyl-CoA pathway enzyme expressed in a sufficient amount to produce acetyl-CoA. The acetyl-CoA pathway enzyme includes an acetyl-CoA synthase, a formate dehydrogenase, a formyltetrahydrofolate synthetase, a methenyltetrahydrofolate cyclohydrolase, a methylenetetrahydrofolate dehydrogenase, and a methylenetetrahydrofolate reductase.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a diagram depicting the Wood-Ljungdahl pathway and formation routes for acetate and ethanol. The transformations that are typically unique to organisms capable of growth on synthesis gas are 1) CO dehydrogenase, 2) hydrogenase, 3) energy-conserving hydrogenase (ECH), and 4) bi-functional CO dehydrogenase/acetyl-CoA synthase. Oxidation of hydrogen to 2 [H] or of CO with H_2O to CO_2 and 2 [H] provides reducing equivalents for the reduction of CO_2 to formate, of methenyl-tetrahydrofolate (methenyl-THF) to methylene-tetrahydrofolate (methylene-THF), of methylene-THF to methyltetrahydrofolate (methyl-THF), and of CO_2 to CO.

Figure 2A shows the complete Wood-Ljungdahl pathway for the conversion of gases including CO, CO_2, and/or H_2 to acetyl-CoA which is subsequently converted to cell mass and products such as ethanol or acetate. Figure 2B shows a synthetic metabolic pathway for the conversion of gases including CO, CO_2, and/or H_2, and methanol to acetyl-CoA and further to isopropanol. Figure 2C shows a synthetic metabolic pathway for the conversion of gases including CO, CO_2, and/or H_2, and methanol to acetyl-CoA and further to 4-hydroxybutyrate. Figure 2D shows a synthetic metabolic pathway for the conversion of gases including CO, CO_2, and/or H_2, and methanol to acetyl-CoA and further to 1,4-butanediol. In Figures 2A-D, the specific enzymatic transformations that can be engineered into a production host are numbered.

Abbreviations: 10FTHF: 10-formyltetrahydrofolate, 5MTHF: 5-methyltetrahydrofolate, ACTP: acetyl phosphate, CFeSp: corrinoid iron sulfur protein, FOR: formate, MeOH: methanol, METHF: methyltetrahydrofolate, MLTHF: metheneyltetrahydrofolate, THF: tetrahydrofolate.

Figure 3A shows a synthetic metabolic pathway for the conversion of gases including CO, CO_2, and/or H_2 to acetyl-CoA, and further to isopropanol. Figure 3B shows a synthetic metabolic pathway for the conversion of gases including CO, CO_2, and/or H_2 to acetyl-CoA, and further to 4-hydroxybutyrate. Figure 3C shows a synthetic metabolic pathway for the conversion of gases including CO, CO_2, and/or H_2 to acetyl-CoA, and further to 1,4-butanediol. In Figures 3A-C the specific enzymatic transformations that can be engineered into a production host are numbered. Abbreviations: 10FTHF: 10-formyltetrahydrofolate, 5MTHF: 5-methyltetrahydrofolate, ACTP: acetyl phosphate, CFeSp: corrinoid iron sulfur protein, FOR: formate, MeOH: methanol, METHF: methyltetrahydrofolate, MLTHF: metheneyltetrahydrofolate, THF: tetrahydrofolate.

Figure 4 shows Western blots of 10 micrograms ACS90 (lane 1), ACS91 (lane2), Mta98/99 (lanes 3 and 4) cell extracts with size standards (lane 5) and controls of *M. thermoacetica* CODH (Moth_1202/1203) or Mtr (Mo th_1197) proteins (50, 150, 250, 350, 450, 500, 750, 900, and 1000 ng).

Figure 5 shows cuvettes used in a methyl viologen assay. A blank is on the right and a cuvette with reduced methyl viologen is on the left. Stoppers and vacuum grease on top of each are used to keep the reactions anaerobic.

Figure 6 shows a spectrogram of ACS90 cell extracts assayed for transfer of CH_3 from added CH_3-THF to purified *M. thermoacetica* corrinoid protein.

Figure 7 shows anaerobic growth of recombinant *E. coli* MG1655 in N2 and CO for 36 hr at 37C. From left to right: Empty vector, ACS90, and ACS91 are shown.

DETAILED DESCRIPTION OF THE INVENTION

This invention is directed, in part, to non-naturally occurring microorganisms that express genes encoding enzymes that catalyze the carbonyl-branch of the Wood-Ljungdahl pathway in conjunction with a MtaABC-type methyltransferase system. Such organisms are capable converting methanol, a relatively inexpensive organic feedstock that can be derived from synthesis gas, and gasses including CO, CO_2, and/or H_2 into acetyl-CoA, cell mass, and products such as isopropanol (IPA), 4-hydroxybutyrate (4-HB), and 1,4-butanediol (BDO). The invention

is also directed, in part, to non-naturally occurring microorganisms that express genes encoding enzymes that catalyze the carbonyl and methyl-branches of the Wood-Ljungdahl pathway. Such organisms are capable converting gasses including CO, CO_2, and/or H_2 into acetyl-CoA, cell mass, and products such as IPA, 4-HB, and BDO.

In one embodiment, the invention provides non-naturally occurring microbial organisms capable of producing isopropanol, 4-hydroxybutyrate, or 1,4-butanediol from methanol and gaseous feedstocks such as mixtures of syngas. In other embodiments, the invention provides non-naturally occurring microbial organisms capable of producing isopropanol, 4-hydroxybutyrate, or 1,4-butanediol from mixtures of syngas, without the need for methanol.

In another embodiment, the invention provides methods for producing isopropanol, 4-hydroxybutyrate, or 1,4-butanediol through culturing of these non-naturally occurring microbial organisms.

Biotechnological processes utilizing these organisms will provide operational flexibility for isopropanol, 4-hydroxybutyrate, and 1,4-butanediol manufacturers to assure the lowest operating costs based on diversified feedstocks and optionally to offset volatility in market-driven commodity pricing of current feedstocks such as oil and natural gas. Furthermore, these processes will deliver sustainable manufacturing practices that utilize renewable feedstocks, reduce energy intensity and lower greenhouse gas emissions.

Acetogens, such as *Moorella thermoacetica*, *C. ljungdahlii* and *C. carboxidivorans*, can grow on a number of carbon sources ranging from hexose sugars to carbon monoxide. Hexoses, such as glucose, are metabolized first via Embden-Meyerhof-Parnas (EMP) glycolysis to pyruvate, which is then converted to acetyl-CoA via pyruvate:ferredoxin oxidoreductase (PFOR). Acetyl-CoA can be used to build biomass precursors or can be converted to acetate which produces energy via acetate kinase and phosphotransacetylase. The overall conversion of glucose to acetate, energy, and reducing equivalents is given by equation 1:

$$C_6H_{12}O_6 + 4\ ADP + 4\ Pi \rightarrow 2\ CH_3COOH + 2\ CO_2 + 4\ ATP + 8\ [H] \quad \text{equation 1}$$

Acetogens extract even more energy out of the glucose to acetate conversion while also maintaining redox balance by further converting the released CO_2 to acetate via the Wood-Ljungdahl pathway

$$2\ CO_2 + 8\ [H] + n\ ADP + n\ Pi \rightarrow CH_3COOH + n\ ATP \quad \text{equation 2}$$

The coefficient n in the above equation signify that this conversion is an energy generating endeavor, as many acetogens can grow in the presence of CO_2 via the Wood-Ljungdahl pathway even in the absence of glucose as long as hydrogen is present to supply the necessary reducing equivalents.

$$2\ CO_2 + 4\ H_2 + n\ ADP + n\ Pi \rightarrow CH_3COOH + 2\ H_2O + n\ ATP \quad \text{equation 3}$$

The Wood-Ljungdahl pathway, illustrated in Figure 1, is coupled to the creation of Na^+ or H^+ ion gradients that can generate ATP via an Na^+- or H^+- dependant ATP synthase, respectively (Muller, V. Appl Environ Microbiol 69:6345-6353 (2003)). Based on these known transformations, acetogens also have the capacity to utilize CO as the sole carbon and energy source. Specifically, CO can be oxidized to produce reducing equivalents and CO_2, or directly assimilated into acetyl-CoA which is subsequently converted to either biomass or acetate.

$$4\ CO + 2\ H_2O \rightarrow CH_3COOH + 2\ CO_2 \quad \text{equation 4}$$

Even higher acetate yields, however, can be attained when enough hydrogen is present to satisfy the requirement for reducing equivalents.

$$2\ CO + 2\ H_2 \rightarrow CH_3COOH \quad \text{equation 5}$$

Following from Figure 1, the production of acetate via acetyl-CoA generates one ATP molecule, whereas the production of ethanol from acetyl-CoA does not and requires two reducing equivalents. Thus one might speculate that ethanol production from syngas will not generate sufficient energy for cell growth in the absence of acetate production. However, under certain conditions, *Clostridium ljungdahlii* produces mostly ethanol from synthesis gas (Klasson et al., Fuel 72:1673-1678 (1993)). indicating that some combination of the pathways

$$2\ CO_2 + 6\ H_2 \rightarrow CH_3CH_2OH + 3\ H_2O \quad \text{equation 6}$$
$$6\ CO + 3\ H_2O \rightarrow CH_3CH_2OH + 4\ CO_2 \quad \text{equation 7}$$
$$2\ CO + 4\ H_2 \rightarrow CH_3CH_2OH + H_2O \quad \text{equation 8}$$

does indeed generate enough energy to support cell growth. Hydrogenic bacteria such as *R. rubrum* can also generate energy from the conversion of CO and water to hydrogen (see Figure 1) (Simpma et al., Critical Reviews in Biotechnology 26:41-65 (2006)). One important mechanism is the coordinated action of an energy converting hydrogenase (ECH) and CO dehydrogenase. The CO dehydrogenase supplies electrons from CO which are then used to reduce protons to H_2 by ECH, whose activity is coupled to energy-generating proton translocation. The net result is the generation of energy via the water-gas shift reaction.

Embodiments of the present invention describe the combination of (1) pathways for the conversion of synthesis gases with and without methanol to acetyl-CoA and (2) pathways for the conversion of acetyl-CoA to isopropanol, 4-hydroxybutyrate, or 1,4-butanediol. As such, this invention provides production organisms and conversion routes with inherent yield advantages over organisms engineered to produce isopropanol, 4-hydroxybutyrate, or 1,4-butanediol from carbohydrate feedstocks. For example, the maximum theoretical yields of isopropanol, 4-hydroxybutyrate, and 1,4-butanediol from glucose are 1 mole per mole using the metabolic pathways proceeding from acetyl-CoA as described herein. Specifically, 2 moles of acetyl-CoA are derived per mole of glucose via glycolysis and 2 moles of acetyl-CoA are required per mole of isopropanol, 4-hydroxybutyrate, or 1,4-butanediol. The net conversions are described by the following stoichiometric equations:

Isopropanol: $C_6H_{12}O_6 + 1.5\ O_2 \rightarrow C_3H_8O + 3\ CO_2 + 2\ H_2O$

4-Hydroxybutyate: $C_6H_{12}O_6 + 1.5\ O_2 \rightarrow C_4H_8O_3 + 2\ CO_2 + 2\ H_2O$

1,4-Butanediol: $C_6H_{12}O_6 \rightarrow C_4H_{10}O_2 + CH_2O_2 + CO_2$

On the other hand, gasification of glucose to its more simpler components, CO and H_2, followed by their conversion to isopropanol, 4-hydroxybutyrate, and 1,4-butanediol using the pathways described herein results in the following maximum theoretical yields:

Isopropanol: $6\ CO + 6\ H_2 \rightarrow 1.333\ C_3H_8O + 2\ CO_2 + 0.667\ H_2O$

4-Hydroxybutyate: $6\ CO + 6\ H_2 \rightarrow 1.333\ C_4H_8O_3 + 0.667\ CO_2 + 0.667\ H_2O$

1,4-Butanediol: $6\ CO + 6\ H_2 \rightarrow 1.091\ C_4H_{10}O_2 + 1.636\ CO_2 + 0.545\ H_2O$

Note that the gasification of glucose can at best provide 6 moles of CO and 6 moles of H_2. The maximum theoretical yields of isopropanol, 4-hydroxybutyrate, and 1,4-butanediol from synthesis gases can be further enhanced by the addition of methanol as described below:

Isopropanol: $CH_4O + 6\ CO + 6\ H_2 \rightarrow 1.667\ C_3H_8O + 2\ CO_2 + 1.333\ H_2O$

4-Hydroxybutyate: $CH_4O + 6\ CO + 6\ H_2 \rightarrow 1.667\ C_4H_8O_3 + 0.333\ CO_2 + 1.333\ H_2O$

1,4-Butanediol: $CH_4O + 6\ CO + 6\ H_2 \rightarrow 1.364\ C_4H_{10}O_2 + 1.545\ CO_2 + 1.182\ H_2O$

Isopropanol: $2\ CH_4O + 6\ CO + 6\ H_2 \rightarrow 2\ C_3H_8O + 2\ CO_2 + 2\ H_2O$

4-Hydroxybutyate: $2\ CH_4O + 6\ CO + 6\ H_2 \rightarrow 2\ C_4H_8O_3 + 2\ H_2O$

1,4-Butanediol: $2\ CH_4O + 6\ CO + 6\ H_2 \rightarrow 1.636\ C_4H_{10}O_2 + 1.455\ CO_2 + 1.818\ H_2O$

Thus it is clear that the organisms and conversion routes described herein provide an efficient means of converting carbohydrates to isopropanol, 4-hydroxybutyrate, or 1,4-butanediol.

As used herein, the term "non-naturally occurring" when used in reference to a microbial organism or microorganism of the invention is intended to mean that the microbial organism has at least one genetic alteration not normally found in a naturally occurring strain of the referenced species, including wild-type strains of the referenced species. Genetic alterations include, for example, modifications introducing expressible nucleic acids encoding metabolic polypeptides, other nucleic acid additions, nucleic acid deletions and/or other functional disruption of the microbial genetic material. Such modifications include, for example, coding regions and functional fragments thereof, for heterologous, homologous or both heterologous and homologous polypeptides for the referenced species. Additional modifications include, for example, non-coding regulatory regions in which the modifications alter expression of a gene or operon. Exemplary metabolic polypeptides include enzymes or proteins within an isopropanol, 4-hydroxybutyrate, or 1,4-butanediol biosynthetic pathway.

A metabolic modification refers to a biochemical reaction that is altered from its naturally occurring state. Therefore, non-naturally occurring microorganisms can have genetic modifications to nucleic acids encoding metabolic polypeptides or, functional fragments thereof. Exemplary metabolic modifications are disclosed herein.

As used herein, the term "isolated" when used in reference to a microbial organism is intended to mean an organism that is substantially free of at least one component as the referenced microbial organism is found in nature. The term includes a microbial organism that is removed from some or all components as it is found in its natural environment. The term also includes a microbial organism that is removed from some or all components as the microbial organism is found in non-naturally occurring environments. Therefore, an isolated microbial organism is partly or completely separated from other substances as it is found in nature or as it is grown, stored or subsisted in non-naturally occurring environments. Specific examples of isolated microbial organisms include partially pure microbes, substantially pure microbes and microbes cultured in a medium that is non-naturally occurring.

As used herein, the terms "microbial," "microbial organism" or "microorganism" is intended to mean any organism that exists as a microscopic cell that is included within the

domains of archaea, bacteria or eukarya. Therefore, the term is intended to encompass prokaryotic or eukaryotic cells or organisms having a microscopic size and includes bacteria, archaea and eubacteria of all species as well as eukaryotic microorganisms such as yeast and fungi. The term also includes cell cultures of any species that can be cultured for the production of a biochemical.

As used herein, the term "CoA" or "coenzyme A" is intended to mean an organic cofactor or prosthetic group (nonprotein portion of an enzyme) whose presence is required for the activity of many enzymes (the apoenzyme) to form an active enzyme system. Coenzyme A functions in certain condensing enzymes, acts in acetyl or other acyl group transfer and in fatty acid synthesis and oxidation, pyruvate oxidation and in other acetylation.

As used herein, the term "substantially anaerobic" when used in reference to a culture or growth condition is intended to mean that the amount of oxygen is less than about 10% of saturation for dissolved oxygen in liquid media. The term also is intended to include sealed chambers of liquid or solid medium maintained with an atmosphere of less than about 1% oxygen.

"Exogenous" as it is used herein is intended to mean that the referenced molecule or the referenced activity is introduced into the host microbial organism. The molecule can be introduced, for example, by introduction of an encoding nucleic acid into the host genetic material such as by integration into a host chromosome or as non-chromosomal genetic material such as a plasmid. Therefore, the term as it is used in reference to expression of an encoding nucleic acid refers to introduction of the encoding nucleic acid in an expressible form into the microbial organism. When used in reference to a biosynthetic activity, the term refers to an activity that is introduced into the host reference organism. The source can be, for example, a homologous or heterologous encoding nucleic acid that expresses the referenced activity following introduction into the host microbial organism. Therefore, the term "endogenous" refers to a referenced molecule or activity that is present in the host. Similarly, the term when used in reference to expression of an encoding nucleic acid refers to expression of an encoding nucleic acid contained within the microbial organism. The term "heterologous" refers to a molecule or activity derived from a source other than the referenced species whereas "homologous" refers to a molecule or activity derived from the host microbial organism. Accordingly, exogenous expression of an encoding nucleic acid of the invention can utilize either or both a heterologous or homologous encoding nucleic acid.

The non-naturally occurring microbal organisms of the invention can contain stable genetic alterations, which refers to microorganisms that can be cultured for greater than five generations without loss of the alteration. Generally, stable genetic alterations include modifications that persist greater than 10 generations, particularly stable modifications will persist more than about 25 generations, and more particularly, stable genetic modifications will be greater than 50 generations, including indefinitely.

Those skilled in the art will understand that the genetic alterations, including metabolic modifications exemplified herein, are described with reference to a suitable host organism such as *E. coli* and their corresponding metabolic reactions or a suitable source organism for desired genetic material such as genes for a desired metabolic pathway. However, given the complete genome sequencing of a wide variety of organisms and the high level of skill in the area of genomics, those skilled in the art will readily be able to apply the teachings and guidance provided herein to essentially all other organisms. For example, the *E. coli* metabolic alterations exemplified herein can readily be applied to other species by incorporating the same or analogous encoding nucleic acid from species other than the referenced species. Such genetic alterations include, for example, genetic alterations of species homologs, in general, and in particular, orthologs, paralogs or nonorthologous gene displacements.

An ortholog is a gene or genes that are related by vertical descent and are responsible for substantially the same or identical functions in different organisms. For example, mouse epoxide hydrolase and human epoxide hydrolase can be considered orthologs for the biological function of hydrolysis of epoxides. Genes are related by vertical descent when, for example, they share sequence similarity of sufficient amount to indicate they are homologous, or related by evolution from a common ancestor. Genes can also be considered orthologs if they share three-dimensional structure but not necessarily sequence similarity, of a sufficient amount to indicate that they have evolved from a common ancestor to the extent that the primary sequence similarity is not identifiable. Genes that are orthologous can encode proteins with sequence similarity of about 25% to 100% amino acid sequence identity. Genes encoding proteins sharing an amino acid similarity less that 25% can also be considered to have arisen by vertical descent if their three-dimensional structure also shows similarities. Members of the serine protease family of enzymes, including tissue plasminogen activator and elastase, are considered to have arisen by vertical descent from a common ancestor.

Orthologs include genes or their encoded gene products that through, for example, evolution, have diverged in structure or overall activity. For example, where one species

encodes a gene product exhibiting two functions and where such functions have been separated into distinct genes in a second species, the three genes and their corresponding products are considered to be orthologs. For the production of a biochemical product, those skilled in the art will understand that the orthologous gene harboring the metabolic activity to be introduced or disrupted is to be chosen for construction of the non-naturally occurring microorganism. An example of orthologs exhibiting separable activities is where distinct activities have been separated into distinct gene products between two or more species or within a single species. A specific example is the separation of elastase proteolysis and plasminogen proteolysis, two types of serine protease activity, into distinct molecules as plasminogen activator and elastase. A second example is the separation of mycoplasma 5'-3' exonuclease and *Drosophila* DNA polymerase III activity. The DNA polymerase from the first species can be considered an ortholog to either or both of the exonuclease or the polymerase from the second species and vice versa.

In contrast, paralogs are homologs related by, for example, duplication followed by evolutionary divergence and have similar or common, but not identical functions. Paralogs can originate or derive from, for example, the same species or from a different species. For example, microsomal epoxide hydrolase (epoxide hydrolase I) and soluble epoxide hydrolase (epoxide hydrolase II) can be considered paralogs because they represent two distinct enzymes, co-evolved from a common ancestor, that catalyze distinct reactions and have distinct functions in the same species. Paralogs are proteins from the same species with significant sequence similarity to each other suggesting that they are homologous, or related through co-evolution from a common ancestor. Groups of paralogous protein families include HipA homologs, luciferase genes, peptidases, and others.

A nonorthologous gene displacement is a nonorthologous gene from one species that can substitute for a referenced gene function in a different species. Substitution includes, for example, being able to perform substantially the same or a similar function in the species of origin compared to the referenced function in the different species. Although generally, a nonorthologous gene displacement will be identifiable as structurally related to a known gene encoding the referenced function, less structurally related but functionally similar genes and their corresponding gene products nevertheless will still fall within the meaning of the term as it is used herein. Functional similarity requires, for example, at least some structural similarity in the active site or binding region of a nonorthologous gene product compared to a gene encoding the function sought to be substituted. Therefore, a nonorthologous gene includes, for example, a paralog or an unrelated gene.

Therefore, in identifying and constructing the non-naturally occurring microbial organisms of the invention having isopropanol, 4-hydroxybutyrate, or 1,4-butanediol biosynthetic capability, those skilled in the art will understand with applying the teaching and guidance provided herein to a particular species that the identification of metabolic modifications can include identification and inclusion or inactivation of orthologs. To the extent that paralogs and/or nonorthologous gene displacements are present in the referenced microorganism that encode an enzyme catalyzing a similar or substantially similar metabolic reaction, those skilled in the art also can utilize these evolutionally related genes.

Orthologs, paralogs and nonorthologous gene displacements can be determined by methods well known to those skilled in the art. For example, inspection of nucleic acid or amino acid sequences for two polypeptides will reveal sequence identity and similarities between the compared sequences. Based on such similarities, one skilled in the art can determine if the similarity is sufficiently high to indicate the proteins are related through evolution from a common ancestor. Algorithms well known to those skilled in the art, such as Align, BLAST, Clustal W and others compare and determine a raw sequence similarity or identity, and also determine the presence or significance of gaps in the sequence which can be assigned a weight or score. Such algorithms also are known in the art and are similarly applicable for determining nucleotide sequence similarity or identity. Parameters for sufficient similarity to determine relatedness are computed based on well known methods for calculating statistical similarity, or the chance of finding a similar match in a random polypeptide, and the significance of the match determined. A computer comparison of two or more sequences can, if desired, also be optimized visually by those skilled in the art. Related gene products or proteins can be expected to have a high similarity, for example, 25% to 100% sequence identity. Proteins that are unrelated can have an identity which is essentially the same as would be expected to occur by chance, if a database of sufficient size is scanned (about 5%). Sequences between 5% and 24% may or may not represent sufficient homology to conclude that the compared sequences are related. Additional statistical analysis to determine the significance of such matches given the size of the data set can be carried out to determine the relevance of these sequences.

Exemplary parameters for determining relatedness of two or more sequences using the BLAST algorithm, for example, can be as set forth below. Briefly, amino acid sequence alignments can be performed using BLASTP version 2.0.8 (Jan-05-1999) and the following parameters: Matrix: 0 BLOSUM62; gap open: 11; gap extension: 1; x_dropoff: 50; expect: 10.0; wordsize: 3; filter: on. Nucleic acid sequence alignments can be performed using BLASTN version 2.0.6 (Sept-16-1998) and the following parameters: Match: 1; mismatch: -2; gap open:

5; gap extension: 2; x_dropoff: 50; expect: 10.0; wordsize: 11; filter: off. Those skilled in the art will know what modifications can be made to the above parameters to either increase or decrease the stringency of the comparison, for example, and determine the relatedness of two or more sequences.

Escherichia coli is a common organism with a well-studied set of available genetic tools. Engineering the capability to convert synthesis gas into acetyl-CoA, the central metabolite from which cell mass components and many valuable products can be derived, into a foreign host such as *E. coli* can be accomplished following the expression of exogenous genes that encode various proteins of the Wood-Ljungdahl pathway. This pathway is active in acetogenic organisms such as *Moorella thermoacetica (formerly, Clostridium thermoaceticum)*, which has been the model organism for elucidating the Wood-Ljungdahl pathway since its isolation in 1942 (Fontaine et al., J Bacteriol. 43:701-715 (1942)). The Wood-Ljungdahl pathway includes two branches: the Eastern (or methyl) branch that enables the conversion of CO_2 to methyltetrahydrofolate (Me-THF) and the Western (or carbonyl) branch that enables the conversion of methyl-THF, CO, and Coenzyme-A into acetyl-CoA as shown in Figure 2A. In some embodiments, the present invention provides a non-naturally occurring microorganism expressing genes encoding enzymes that catalyze the carbonyl-branch of the Wood-Ljungdahl pathway in conjunction with a MtaABC-type methyltransferase system. Such an organism is capable converting methanol, a relatively inexpensive organic feedstock that can be derived from synthesis gas, and gasses including CO, CO_2, and/or H_2 into acetyl-CoA, cell mass, and products.

In some embodiments organisms of the present invention has the following capabilities as depicted in Figure 2B: 1) a functional methyltransferase system enabling the production of 5-methyl-tetrahydrofolate (Me-THF) from methanol and THF, 2) the ability to combine CO, Coenzyme A, and the methyl group of Me-THF to form acetyl-CoA, and 3) the ability to synthesize IPA from acetyl-CoA. In other embodiments, organisms of the present invention have a functional methyltransferase system, the ability to synthesize acetyl-CoA, and the ability to synthesize 4-HB from acetyl-CoA as depicted in Figure 2C. Still other organisms described herein have a functional methyltransferase system, the ability to synthesize acetyl-CoA, and the ability to synthesize BDO from acetyl-CoA depicted in Figure 2D.

In some embodiments, organisms of the present invention are able to 'fix' carbon from exogenous CO and/or CO_2 and methanol to synthesize acetyl-CoA, cell mass, and products. The direct conversion of synthesis gas to acetate is an energetically neutral process (see Figures 1 and 2A). Specifically, one ATP molecule is consumed during the formation of formyl-THF by

formyl-THF synthase and one ATP molecule is produced during the production of acetate via acetate kinase. ATP consumption is circumvented by ensuring that the methyl group on the methyl branch product, methyl-THF, is obtained from methanol rather than CO_2. This thereby ensures that acetate formation has a positive ATP yield that can help support cell growth and maintenance. A host organism engineered with these capabilities that also naturally possesses the capability for anapleurosis (e.g., *E. coli*) can grow on the methanol and syngas-generated acetyl-CoA in the presence of a suitable external electron acceptor such as nitrate. This electron acceptor is used to accept electrons from the reduced quinone formed via succinate dehydrogenase. One advantage of adding an external electron acceptor is that additional energy for cell growth, maintenance, and product formation can be generated from respiration of acetyl-CoA. In other embodiments, a pyruvate ferredoxin oxidoreductase (PFOR) enzyme can be inserted into the strain to provide the synthesis of biomass precursors in the absence of an external electron acceptor. A further characteristic of organisms of the present invention is the capability of extracting reducing equivalents from molecular hydrogen. This enables a high yield of reduced products such as ethanol, butanol, isobutanol, isopropanol, 1,4-butanediol, succinic acid, fumaric acid, malic acid, 4-hydroxybutyric acid, 3-hydroxypropionic acid, lactic acid, adipic acid, 3-hydoxyisobutyric acid, 2-hydroxyisobutyric acid, methacrylic acid, and acrylic acid.

Organisms of the present invention can produce acetyl-CoA, cell mass, and targeted chemicals, more specifically IPA, 4-HB, or BDO, from: 1) methanol and CO, 2) methanol, CO_2, and H_2, 3) methanol, CO, CO_2, and H_2, 4) methanol and synthesis gas comprising CO and H_2, and 5) methanol and synthesis gas comprising CO, CO_2, and H_2.

Successfully engineering pathways into an organism involves identifying an appropriate set of enzymes, cloning their corresponding genes into a production host, optimizing the stability and expression of these genes, optimizing fermentation conditions, and assaying for product formation following fermentation. A number of enzymes catalyze each step of the pathways for the conversion of synthesis gas and methanol to acetyl-CoA, and further to isopropanol, 4-hydroxybutyrate, or 1,4-butanediol. To engineer a production host for the utilization of syngas and methanol, one or more exogenous DNA sequence(s) encoding the enzymes can be expressed in the microorganism.

In some embodiments, the present invention provides a non-naturally occurring microbial organism having an isopropanol pathway that includes at least one exogenous nucleic acid encoding an isopropanol pathway enzyme expressed in a sufficient amount to produce

isopropanol. The isopropanol pathway enzyme includes an acetoacetyl-CoA thiolase, an acetoacetyl-CoA:acetate:CoA transferase, an acetoacetate decarboxylase, and an isopropanol dehydrogenase. An additional isopropanol pathway of the present invention includes a succinyl-CoA:3-ketoacid-CoA transferase (SCOT), an acetoacetate decarboxylase, and an isopropanol dehydrogenase.

Such organisms may also include at least one enzyme or polypeptide such as a corrinoid protein, a methyltetrahydrofolate:corrinoid protein methyltransferase, a corrinoid iron-sulfur protein, a nickel-protein assembly protein, a ferredoxin, an acetyl-CoA synthase, a carbon monoxide dehydrogenase, a pyruvate ferredoxin oxidoreductase,and a hydrogenase.

In some embodiments, organisms having an isopropanol pathway have a methanol methyltransferase. In such embodiments, the organisms utilize a feedstock such as 1) methanol and CO, 2) methanol, CO_2, and H_2, 3) methanol, CO, CO_2, and H_2, 4) methanol and synthesis gas comprising CO and H_2, and 5) methanol and synthesis gas comprising CO, CO_2, and H_2.

In other embodiments, organisms of the present invention have a formate dehydrogenase, a formyltetrahydrofolate synthetase, a methenyltetrahydrofolate cyclohydrolase, a methylenetetrahydrofolate dehydrogenase, and a methylenetetrahydrofolate reductase. Such organisms utilize a feedstock selected from the group consisting of: 1) CO, 2) CO_2 and H_2, 3) CO and CO_2, 4) synthesis gas comprising CO and H_2, and 5) synthesis gas comprising CO, CO_2, and H_2.

The invention also provides a non-naturally occurring microbial organism having a 4-hydroxybutryate pathway that includes at least one exogenous nucleic acid encoding an 4-hydroxybutryate pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutryate. The 4-hydroxybutryate pathway enzyme includes an acetoacetyl-CoA thiolase, a 3-hydroxybutyryl-CoA dehydrogenase, a crotonase, a crotonyl-CoA hydratase, a 4-hydroxybutyryl-CoA transferase, a phosphotrans-4-hydroxybutyrylase, and a 4-hydroxybutyrate kinase.

Such organisms can also include at least one enzyme or polypeptide such as a corrinoid protein, a methyltetrahydrofolate:corrinoid protein methyltransferase, a corrinoid iron-sulfur protein, a nickel-protein assembly protein, a ferredoxin, an acetyl-CoA synthase, a carbon monoxide dehydrogenase, a pyruvate ferredoxin oxidoreductase,and a hydrogenase.

In some embodiments, organisms that have a 4-hydroxybutyrate pathway can include a methanol methyltransferase. Such organisms utilize a feedstock such as 1) methanol and CO, 2)

methanol, CO_2, and H_2, 3) methanol, CO, CO_2, and H_2, 4) methanol and synthesis gas comprising CO and H_2, and 5) methanol and synthesis gas comprising CO, CO_2, and H_2.

Other organisms that have a 4-hydroxybutyrate pathway can have a formate dehydrogenase, a formyltetrahydrofolate synthetase, a methenyltetrahydrofolate cyclohydrolase, a methylenetetrahydrofolate dehydrogenase, and a methylenetetrahydrofolate reductase. Such organisms utilize a feedstock such as 1) CO, 2) CO_2 and H_2, 3) CO and CO_2, 4) synthesis gas comprising CO and H_2, and 5) synthesis gas comprising CO, CO_2, and H_2.

The present invention also provides a non-naturally occurring microbial organism having a 1,4-butanediol pathway that includes at least one exogenous nucleic acid encoding a 1,4-butanediol pathway enzyme expressed in a sufficient amount to produce 1,4-butanediol. The 1,4-butanediol pathway enzyme include, for example, an acetoacetyl-CoA thiolase, a 3-Hydroxybutyryl-CoA dehydrogenase, a crotonase, a crotonyl-CoA hydratase, a 4-hydroxybutyryl-CoA reductase (alcohol forming), a 4-hydroxybutyryl-CoA reductase (aldehyde forming), and a 1,4-butanediol dehydrogenase.

Such organisms can also includ at least one enzyme or polypeptide such as a corrinoid protein, a methyltetrahydrofolate:corrinoid protein methyltransferase, a corrinoid iron-sulfur protein, a nickel-protein assembly protein, a ferredoxin, an acetyl-CoA synthase, a carbon monoxide dehydrogenase, a pyruvate ferredoxin oxidoreductase,and a hydrogenase.

In some embodiments, an organism having a 1,4-butanediol pathway can include a methanol methyltransferase. Such organisms utilize a feedstock such as 1) methanol and CO, 2) methanol, CO_2, and H_2, 3) methanol, CO, CO_2, and H_2, 4) methanol and synthesis gas comprising CO and H_2, and 5) methanol and synthesis gas comprising CO, CO_2, and H_2.

In other embodiments, an organism having a 1,4-butanediol pathway can include a formate dehydrogenase, a formyltetrahydrofolate synthetase, a methenyltetrahydrofolate cyclohydrolase, a methylenetetrahydrofolate dehydrogenase, and a methylenetetrahydrofolate reductase. Such organisms utilize a feedstock selected from the group consisting of: 1) CO, 2) CO_2 and H_2, 3) CO and CO_2, 4) synthesis gas comprising CO and H_2, and 5) synthesis gas comprising CO, CO_2, and H_2.

Also disclosed herein is a non-naturally occurring microbial organism having an acetyl-CoA pathway that includes at least one exogenous nucleic acid encoding an acetyl-CoA pathway enzyme expressed in a sufficient amount to produce acetyl-CoA. The acetyl-CoA pathway enzyme includes a methanol methyltransferase and an acetyl-CoA synthase.

In still further embodiments, the present invention provides a non-naturally occurring microbial organism having an isopropanol pathway comprising at least one exogenous nucleic acid encoding an isopropanol pathway enzyme expressed in a sufficient amount to produce isopropanol, said isopropanol pathway enzyme comprising a methanol methyltransferase, a corrinoid protein, a methyltetrahydrofolate:corrinoid protein methyltransferase, a corrinoid iron-sulfur protein, a nickel-protein assembly protein, a ferredoxin, an acetyl-CoA synthase, a carbon monoxide dehydrogenase, a pyruvate ferredoxin oxidoreductase,and a hydrogenase.

In some embodiments, such an organism can include an exogenous polypeptide or enzyme such as an acetoacetyl-CoA thiolase, an acetoacetyl-CoA:acetate:CoA transferase, an acetoacetate decarboxylase, and an isopropanol dehydrogenase. In additional embodiments, such an organism can include an exogenous polypeptide or enzyme such as a succinyl-CoA:3-ketoacid-CoA transferase (SCOT), an acetoacetate decarboxylase, and an isopropanol dehydrogenase.

Expression of the modified Wood-Ljungdahl pathway in a foreign host (see Figure 2B) requires a set of methyltransferases to utilize the carbon and hydrogen provided by methanol and the carbon provided by CO and/or CO_2. A complex of 3 methyltransferase proteins, denoted MtaA, MtaB, and MtaC, perform the desired methanol methyltransferase activity (Naidu and Ragsdale, J Bacteriol. 183:3276-3281 (2001); Ragsdale, S. W., Crit Rev.Biochem.Mol.Biol 39:165-195 (2004); Sauer et al., Eur.J Biochem. 243:670-677 (1997); Tallant and Krzycki, J Bacteriol. 178:1295-1301 (1996); Tallant and Krzycki, J Bacteriol. 179:6902-6911 (1997); Tallant et al., J Biol Chem. 276:4485-4493 (2001)).

MtaB is a zinc protein that catalyzes the transfer of a methyl group from methanol to MtaC, a corrinoid protein. Exemplary genes encoding MtaB and MtaC can be found in methanogenic archaea such as *Methanosarcina barkeri* (Maeder et al., J Bacteriol. 188:7922-7931 (2006)) and *Methanosarcina acetivorans* (Galagan et al., Genome Res 12:532-542 (2002)), as well as the acetogen, *Moorella thermoacetica* (Das et al., Proteins 67:167-176 (2007)). In general, the MtaB and MtaC genes are adjacent to one another on the chromosome as their activities are tightly interdependent. The protein sequences of various MtaB and MtaC encoding genes in *M. barkeri*, *M. acetivorans*, and *M. thermoaceticum* can be identified by their following GenBank accession numbers.

Table 1.

Protein	GenBank ID	Organism
MtaB1	YP_304299	*Methanosarcina barkeri*
MtaC1	YP_304298	*Methanosarcina barkeri*
MtaB2	YP_307082	*Methanosarcina barkeri*
MtaC2	YP_307081	*Methanosarcina barkeri*
MtaB3	YP_304612	*Methanosarcina barkeri*
MtaC3	YP_304611	*Methanosarcina barkeri*
MtaB1	NP_615421	*Methanosarcina acetivorans*
MtaB1	NP_615422	*Methanosarcina acetivorans*
MtaB2	NP_619254	*Methanosarcina acetivorans*
MtaC2	NP_619253	*Methanosarcina acetivorans*
MtaB3	NP_616549	*Methanosarcina acetivorans*
MtaC3	NP_616550	*Methanosarcina acetivorans*
MtaB	YP_430066	*Moorella thermoacetica*
MtaC	YP_430065	*Moorella thermoacetica*

The MtaB1 and MtaC1 genes, YP_304299 and YP_304298, from *M. barkeri* were cloned into *E. coli* and sequenced (Sauer et al., Eur.J Biochem. 243:670-677 (1997)). The crystal structure of this methanol-cobalamin methyltransferase complex is also available (Hagemeier et al.,. Proc Natl Acad Sci U S.A 103:18917-18922 (2006)). The MtaB genes, YP_307082 and YP_304612, in *M. barkeri* were identified by sequence homology to YP_304299. In general, homology searches are an effective means of identifying methanol methyltransferases because MtaB encoding genes show little or no similarity to methyltransferases that act on alternative substrates such as trimethylamine, dimethylamine, monomethylamine, or dimethylsulfide. The MtaC genes, YP_307081 and YP_304611, were identified based on their proximity to the MtaB genes and also their homology to YP_304298. The three sets of MtaB and MtaC genes from *M. acetivorans* have been genetically, physiologically, and biochemically characterized (Pritchett and Metcalf, Mol.Microbiol 56:1183-1194 (2005)). Mutant strains lacking two of the sets were able to grow on methanol, whereas a strain lacking all three sets of MtaB and MtaC genes sets could not grow on methanol. This indicates that each set of genes plays a role in methanol utilization. The *M. thermoacetica* MtaB gene was identified based on homology to the methanogenic MtaB genes and also by its adjacent chromosomal proximity to the methanol-induced corrinoid protein, MtaC, which has been crystallized (Zhou et al., Acta Crystallogr.Sect.F Struct.Biol Cryst.Commun. 61:537-540 (2005)) and further characterized by Northern hybridization and Western Blotting (Das et al., Proteins 67:167-176 (2007)).

MtaA is zinc protein that catalyzes the transfer of the methyl group from MtaC to either Coenzyme M in methanogens or methyltetrahydrofolate in acetogens. MtaA can also utilize methylcobalamin as the methyl donor. Exemplary genes encoding MtaA can be found in methanogenic archaea such as *Methanosarcina barkeri* (Maeder et al., J Bacteriol. 188:7922-7931 (2006)) and *Methanosarcina acetivorans* (Galagan et al., Genome Res 12:532-542 (2002)), as well as the acetogen, *Moorella thermoacetica* (Das et al., Proteins 67:167-176 (2007)). In general, MtaA proteins that catalyze the transfer of the methyl group from CH_3-MtaC are difficult to identify bioinformatically as they share similarity to other corrinoid protein methyltransferases and are not oriented adjacent to the MtaB and MtaC genes on the chromosomes. Nevertheless, a number of MtaA encoding genes have been characterized. The protein sequences of these genes in *M. barkeri* and *M. acetivorans* can be identified by the following GenBank accession numbers.

Table 2.

Protein	GenBank ID	Organism
MtaA	YP_304602	*Methanosarcina barkeri*
MtaA1	NP_619241	*Methanosarcina acetivorans*
MtaA2	NP_616548	*Methanosarcina acetivorans*

The MtaA gene, YP_304602, from *M. barkeri* was cloned, sequenced, and functionally overexpressed in *E. coli* (Harms and Thauer, Eur.J Biochem. 235:653-659 (1996)). In *M. acetivorans*, MtaA1 is required for growth on methanol, whereas MtaA2 is dispensable even though methane production from methanol is reduced in MtaA2 mutants (Bose et al., J Bacteriol. 190:4017-4026 (2008)). There are multiple additional MtaA homologs in *M. barkeri* and *M. acetivorans* that are as yet uncharacterized, but can also catalyze corrinoid protein methyltransferase activity.

Putative MtaA encoding genes in *M. thermoacetica* were identified by their sequence similarity to the characterized methanogenic MtaA genes. Specifically, three *M. thermoacetica* genes show high homology (>30% sequence identity) to YP_304602 from *M. barkeri*. Unlike methanogenic MtaA proteins that naturally catalyze the transfer of the methyl group from CH_3-MtaC to Coenzyme M, an *M. thermoacetica* MtaA is likely to transfer the methyl group to methyltetrahydrofolate given the similar roles of methyltetrahydrofolate and Coenzyme M in methanogens and acetogens, respectively. The protein sequences of putative MtaA encoding genes from *M. thermoacetica* can be identified by the following GenBank accession numbers.

Table 3.

Protein	GenBank ID	Organism
MtaA	YP_430937	*Moorella thermoacetica*
MtaA	YP_431175	*Moorella thermoacetica*
MtaA	YP_430935	*Moorella thermoacetica*

ACS/CODH is the central enzyme of the carbonyl branch of the Wood-Ljungdahl pathway. It catalyzes the reversible reduction of carbon dioxide to carbon monoxide and also the synthesis of acetyl-CoA from carbon monoxide, Coenzyme A, and the methyl group from a methylated corrinoid-iron-sulfur protein. The corrinoide-iron-sulfur-protein is methylated by methyltetrahydrofolate via a methyltransferase. Expression of ACS/CODH in a foreign host can be done by introducing one or more of the following proteins and their corresponding activities: Methyltetrahydrofolate:corrinoid protein methyltransferase (AcsE), Corrinoid iron-sulfur protein (AcsD), Nickel-protein assembly protein (AcsF), Ferredoxin (Orf7), Acetyl-CoA synthase (AcsB and AcsC), Carbon monoxide dehydrogenase (AcsA) or Nickel-protein assembly protein (CooC).

The genes used for carbon-monoxide dehydrogenase/acetyl-CoA synthase activity typically reside in a limited region of the native genome that may be an extended operon (Morton et al., J Biol Chem. 266:23824-23828 (1991); Ragsdale, S. W., Crit Rev.Biochem.Mol.Biol 39:165-195 (2004); Roberts et al., Proc Natl Acad Sci U S.A 86:32-36 (1989)). Each of the genes in this operon from the acetogen, *M. thermoacetica*, has already been cloned and expressed actively in *E. coli* (Lu et al., J Biol Chem. 268:5605-5614 (1993); Roberts et al., Proc Natl Acad Sci U S.A 86:32-36 (1989)). The protein sequences of these genes can be identified by the following GenBank accession numbers.

Table 4.

Protein	GenBank ID	Organism
AcsE	YP_430054	*Moorella thermoacetica*
AcsD	YP_430055	*Moorella thermoacetica*
AcsF	YP_430056	*Moorella thermoacetica*
Orf7	YP_430057	*Moorella thermoacetica*
AcsC	YP_430058	*Moorella thermoacetica*
AcsB	YP_430059	*Moorella thermoacetica*
AcsA	YP_430060	*Moorella thermoacetica*
CooC	YP_430061	*Moorella thermoacetica*

The hydrogenogenic bacterium, *Carboxydothermus hydrogenoformans*, can utilize carbon monoxide as a growth substrate by means of acetyl-CoA synthase (Wu et al., PLoS Genet.1:e65 (1995)). In strain Z-2901, the acetyl-CoA synthase enzyme complex lacks carbon

monoxide dehydrogenase due to a frameshift mutation (Wu et al., PLoS Genet.1:e65 (1995)), whereas in strain DSM 6008, a functional unframeshifted full-length version of this protein has been purified (Svetlitchnyi et al., Proc Natl Acad Sci U S.A 101:446-451 (2004)). The protein sequences of the *C. hydrogenoformans* genes from strain Z-2901 are identified by the following GenBank accession numbers. Sequences for *Carboxydothermus hydrogenoformans* DSM 6008 are not yet accessible in publicly available databases.

Table 5.

Protein	GenBank ID	Organism
AcsE	YP_360065	*Carboxydothermus hydrogenoformans*
AcsD	YP_360064	*Carboxydothermus hydrogenoformans*
AcsF	YP_360063	*Carboxydothermus hydrogenoformans*
Orf7	YP_360062	*Carboxydothermus hydrogenoformans*
AcsC	YP_360061	*Carboxydothermus hydrogenoformans*
AcsB	YP_360060	*Carboxydothermus hydrogenoformans*
CooC	YP_360059	*Carboxydothermus hydrogenoformans*

The methanogenic archaeon, *Methanosarcina acetivorans*, can also grow on carbon monoxide, exhibits acetyl-CoA synthase/carbon monoxide dehydrogenase activity, and produces both acetate *and* formate (Lessner et al., Proc Natl Acad Sci U S.A 103:17921-17926 (2006)). This organism contains two sets of genes that encode ACS/CODH activity (Rother and Metcalf Proc Natl Acad Sci U S.A 101:16929-16934 (2004)). The protein sequences of both sets of *M. acetivorans* genes can be identified by the following GenBank accession numbers.

Table 6.

Protein	GenBank ID	Organism
AcsC	NP_618736	*Methanosarcina acetivorans*
AcsD	NP_618735	*Methanosarcina acetivorans*
AcsF, CooC	NP_618734	*Methanosarcina acetivorans*
AcsB	NP_618733	*Methanosarcina acetivorans*
AcsEps	NP_618732	*Methanosarcina acetivorans*
AcsA	NP_618731	*Methanosarcina acetivorans*
AcsC	NP_615961	*Methanosarcina acetivorans*
AcsD	NP_615962	*Methanosarcina acetivorans*
AcsF, CooC	NP_615963	*Methanosarcina acetivorans*
AcsB	NP_615964	*Methanosarcina acetivorans*
AcsEps	NP_615965	*Methanosarcina acetivorans*
AcsA	NP_615966	*Methanosarcina acetivorans*

The AcsC, AcsD, AcsB, AcsEps, and AcsA proteins are commonly referred to as the gamma, delta, beta, epsilon, and alpha subunits of the methanogenic CODH/ACS. Homologs to the epsilon encoding genes are not present in acetogens such as *M. thermoacetica* or hydrogenogenic bacteria such as *C. hydrogenoformans*. Hypotheses for the existence of two active CODH/ACS operons in *M. acetivorans* include catalytic properties (i.e., K_m, V_{max}, k_{cat}) that favor carboxidotrophic or aceticlastic growth or differential gene regulation enabling various stimuli to induce CODH/ACS expression (Rother et al., Arch.Microbiol 188:463-472 (2007)).

In both *M. thermoacetica* and *C. hydrogenoformans*, additional CODH encoding genes are located outside of the ACS/CODH operons. These enzymes provide a means for extracting electrons (or reducing equivalents) from the conversion of carbon monoxide to carbon dioxide. The reducing equivalents are then passed to accepters such as oxidized ferredoxin, NADP+, water, or hydrogen peroxide to form reduced ferredoxin, NADPH, H_2, or water, respectively. In some cases, hydrogenase encoding genes are located adjacent to a CODH. In *Rhodospirillum rubrum*, the encoded CODH/hydrogenase proteins form a membrane-bound enzyme complex that is proposed to be a site where energy, in the form of a proton gradient, is generated from the conversion of CO to CO_2 and H_2 (Fox et al., J Bacteriol. 178:6200-6208 (1996)). The CODH-I of *C. hydrogenoformans* and its adjacent genes have been proposed to catalyze a similar functional role based on their similarity to the *R. rubrum* CODH/hydrogenase gene cluster (Wu et al., PLoS Genet. 1:e65 (2005)). The *C. hydrogenoformans* CODH-I was also shown to exhibit intense CO oxidation and CO_2 reduction activities when linked to an electrode (Parkin et al., J Am.Chem.Soc. 129:10328-10329 (2007)). The genes encoding the *C. hydrogenoformans* CODH-II and CooF, a neighboring protein, were cloned and sequenced (Gonzalez and Robb, FEMS Microbiol Lett. 191:243-247 (2000)). The resulting complex was membrane-bound,

although cytoplasmic fractions of CODH-II were shown to catalyze the formation of NADPH suggesting an anabolic role (Svetlitchnyi et al., J Bacteriol. 183:5134-5144 (2001)). The crystal structure of the CODH-II is also available (Dobbek et al., Science 293:1281-1285 (2001)). The protein sequences of exemplary CODH and hydrogenase genes can be identified by the following GenBank accession numbers.

Table 7.

Protein	GenBank ID	Organism
CODH (putative)	YP_430813	*Moorella thermoacetica*
CODH-I (CooS-I)	YP_360644	*Carboxydothermus hydrogenoformans*
CooF	YP_360645	*Carboxydothermus hydrogenoformans*
HypA	YP_360646	*Carboxydothermus hydrogenoformans*
CooH	YP_360647	*Carboxydothermus hydrogenoformans*
CooU	YP_360648	*Carboxydothermus hydrogenoformans*
CooX	YP_360649	*Carboxydothermus hydrogenoformans*
CooL	YP_360650	*Carboxydothermus hydrogenoformans*
CooK	YP_360651	*Carboxydothermus hydrogenoformans*
CooM	YP_360652	*Carboxydothermus hydrogenoformans*
CooM	AAC45116	*Rhodospirillum rubrum*
CooK	AAC45117	*Rhodospirillum rubrum*
CooL	AAC45118	*Rhodospirillum rubrum*
CooX	AAC45119	*Rhodospirillum rubrum*
CooU	AAC45120	*Rhodospirillum rubrum*
CooH	AAC45121	*Rhodospirillum rubrum*
CooF	AAC45122	*Rhodospirillum rubrum*
CODH (CooS)	AAC45123	*Rhodospirillum rubrum*
CooC	AAC45124	*Rhodospirillum rubrum*
CooT	AAC45125	*Rhodospirillum rubrum*
CooJ	AAC45126	*Rhodospirillum rubrum*
CODH-II (CooS-II)	YP_358957	*Carboxydothermus hydrogenoformans*
CooF	YP_358958	*Carboxydothermus hydrogenoformans*

Anaerobic growth on synthesis gas and methanol in the absence of an external electron acceptor is conferred upon the host organism with MTR and ACS/CODH activity by providing pyruvate synthesis via pyruvate ferredoxin oxidoreductase (PFOR). The PFOR from *Desulfovibrio africanus* has been cloned and expressed in *E. coli* resulting in an active recombinant enzyme that was stable for several days in the presence of oxygen (Pieulle et al., J Bacteriol. 179:5684-5692 (1997)). Oxygen stability is relatively uncommon in PFORs and is reported to be conferred by a 60 residue extension in the polypeptide chain of the *D. africanus* enzyme. The *M. thermoacetica* PFOR is also well characterized (Menon and Ragsdale, Biochemistry 36:8484-8494 (1997)) and was shown to have high activity in the direction of

pyruvate synthesis during autotrophic growth (Furdui and Ragsdale, J Biol Chem. 275:28494-28499 (2000)). Further, *E. coli* possesses an uncharacterized open reading frame, *ydbK*, that encodes a protein that is 51% identical to the *M. thermoacetica* PFOR. Evidence for pyruvate oxidoreductase activity in *E. coli* has been described (Blaschkowski et al., Eur.J Biochem. 123:563-569 (1982)). The protein sequences of these exemplary PFOR enzymes are identified by the following GenBank accession numbers shown in Table 8 below. Several additional PFOR enzymes are described in Ragsdale, S. W., Chem.Rev. 103:2333-2346 (2003).

Table 8.

Protein	GenBank ID	Organism
Por	CAA70873.1	*Desulfovibrio africanus*
Por	YP_428946.1	*Moorella thermoacetica*
YdbK	NP_415896.1	*Escherichia coli*

Unlike the redox neutral conversion of CO and MeOH to acetyl-CoA or acetate, the production of more highly reduced products such as ethanol, butanol, isobutanol, isopropanol, 1,4-butanediol, succinic acid, fumaric acid, malic acid, 4-hydroxybutyric acid, 3-hydroxypropionic acid, lactic acid, adipic acid, 3-hydroxyisobutyric acid, 2-hydroxyisobutyric acid, methacrylic acid, and acrylic acid at the highest possible yield requires the extraction of additional reducing equivalents from both CO and H_2 (for example, see ethanol formation in Figure 2A). Specifically, reducing equivalents (e.g., 2 [H] in Figure 2) are obtained by the conversion of CO and water to CO_2 via carbon monoxide dehydrogenase or directly from the activity of a hydrogen-utilizing hydrogenase which transfers electrons from H_2 to an acceptor such as ferredoxin, flavodoxin, FAD^+, NAD^+, or $NADP^+$.

Native to *E. coli* and other enteric bacteria are multiple genes encoding up to four hydrogenases (Sawers, G., Antonie Van Leeuwenhoek 66:57-88 (1994); Sawers et al., J Bacteriol. 164:1324-1331 (1985); Sawers and Boxer, Eur.J Biochem. 156:265-275 (1986); Sawers et al., J Bacteriol. 168:398-404 (1986)). Given the multiplicity of enzyme activities *E. coli* or another host organism can provide sufficient hydrogenase activity to split incoming molecular hydrogen and reduce the corresponding acceptor. Among the endogenous hydrogen-lyase enzymes of *E. coli* are hydrogenase 3, a membrane-bound enzyme complex using ferredoxin as an acceptor, and hydrogenase 4 that also uses a ferredoxin acceptor. Hydrogenase 3 and 4 are encoded by the *hyc* and *hyf* gene clusters, respectively. Hydrogenase activity in *E. coli* is also dependent upon the expression of the *hyp* genes whose corresponding proteins are involved in the assembly of the hydrogenase complexes (Jacobi et al., Arch.Microbiol 158:444-451 (1992); Rangarajan et al., J Bacteriol. 190:1447-1458 (2008)). The *M. thermoacetica*

hydrogenases are suitable for a host that lacks sufficient endogenous hydrogenase activity. *M. thermoacetica* can grow with CO_2 as the exclusive carbon source indicating that reducing equivalents are extracted from H_2 to enable acetyl-CoA synthesis via the Wood-Ljungdahl pathway (Drake, H. L., J Bacteriol. 150:702-709 (1982); Drake and Daniel, Res Microbiol 155:869-883 (2004); Kellum and Drake, J Bacteriol. 160:466-469 (1984)) (see Figure 2A). *M. thermoacetica* has homologs to several *hyp*, *hyc*, and *hyf* genes from *E. coli*. These protein sequences encoded for by these genes are identified by the following GenBank accession numbers. In addition, several gene clusters encoding hydrogenase functionality are present in *M. thermoacetica* and their corresponding protein sequences are also provided below in Table 9.

Table 9.

Protein	GenBank ID	Organism
HypA	NP_417206	*Escherichia coli*
HypB	NP_417207	*Escherichia coli*
HypC	NP_417208	*Escherichia coli*
HypD	NP_417209	*Escherichia coli*
HypE	NP_417210	*Escherichia coli*
HypF	NP_417192	*Escherichia coli*

Proteins in *M. thermoacetica* whose genes are homologous to the *E. coli hyp* genes are shown below in Table 10. Hydrogenase 3 proteins are listed in Table 11. Hydrogenase 4 proteins are shown in Table 12.

Table 10.

Protein	GenBank ID	Organism
Moth_2175	YP_431007	*Moorella thermoacetica*
Moth_2176	YP_431008	*Moorella thermoacetica*
Moth_2177	YP_431009	*Moorella thermoacetica*
Moth_2178	YP_431010	*Moorella thermoacetica*
Moth_2179	YP_431011	*Moorella thermoacetica*
Moth_2180	YP_431012	*Moorella thermoacetica*
Moth_2181	YP_431013	*Moorella thermoacetica*

Table 11.

Protein	GenBank ID	Organism
HycA	NP_417205	*Escherichia coli*
HycB	NP_417204	*Escherichia coli*
HycC	NP_417203	*Escherichia coli*
HycD	NP_417202	*Escherichia coli*
HycE	NP_417201	*Escherichia coli*
HycF	NP_417200	*Escherichia coli*
HycG	NP_417199	*Escherichia coli*
HycH	NP_417198	*Escherichia coli*
HycI	NP_417197	*Escherichia coli*

Table 12.

Protein	GenBank ID	Organism
HyfA	NP_416976	*Escherichia coli*
HyfB	NP_416977	*Escherichia coli*
HyfC	NP_416978	*Escherichia coli*
HyfD	NP_416979	*Escherichia coli*
HyfE	NP_416980	*Escherichia coli*
HyfF	NP_416981	*Escherichia coli*
HyfG	NP_416982	*Escherichia coli*
HyfH	NP_416983	*Escherichia coli*
HyfI	NP_416984	*Escherichia coli*
HyfJ	NP_416985	*Escherichia coli*
HyfR	NP_416986	*Escherichia coli*

Proteins in *M. thermoacetica* whose genes are homologous to the *E. coli hyc* and/or *hyf* genes are shown in Table 13 below. Additional hydrogenase-encoding gene clusters in *M. thermoacetica* are shown in Table 14.

Table 13.

Protein	GenBank ID	Organism
Moth_2182	YP_431014	*Moorella thermoacetica*
Moth_2183	YP_431015	*Moorella thermoacetica*
Moth_2184	YP_431016	*Moorella thermoacetica*
Moth_2185	YP_431017	*Moorella thermoacetica*
Moth_2186	YP_431018	*Moorella thermoacetica*
Moth_2187	YP_431019	*Moorella thermoacetica*
Moth_2188	YP_431020	*Moorella thermoacetica*
Moth_2189	YP_431021	*Moorella thermoacetica*
Moth_2190	YP_431022	*Moorella thermoacetica*
Moth_2191	YP_431023	*Moorella thermoacetica*
Moth_2192	YP_431024	*Moorella thermoacetica*

Table 14.

Protein	GenBank ID	Organism
Moth_0439	YP_429313	*Moorella thermoacetica*
Moth_0440	YP_429314	*Moorella thermoacetica*
Moth_0441	YP_429315	*Moorella thermoacetica*
Moth_0442	YP_429316	*Moorella thermoacetica*
Moth_0809	YP_429670	*Moorella thermoacetica*
Moth_0810	YP_429671	*Moorella thermoacetica*
Moth_0811	YP_429672	*Moorella thermoacetica*
Moth_0812	YP_429673	*Moorella thermoacetica*
Moth_0813		*Moorella thermoacetica*
Moth_0814	YP_429674	*Moorella thermoacetica*
Moth_0815	YP_429675	*Moorella thermoacetica*
Moth_0816	YP_429676	*Moorella thermoacetica*
Moth_1193	YP_430050	*Moorella thermoacetica*
Moth_1194	YP_430051	*Moorella thermoacetica*
Moth_1195	YP_430052	*Moorella thermoacetica*
Moth_1196	YP_430053	*Moorella thermoacetica*
Moth_1717	YP_430562	*Moorella thermoacetica*
Moth_1718	YP_430563	*Moorella thermoacetica*
Moth_1719	YP_430564	*Moorella thermoacetica*
Moth_1883	YP_430726	*Moorella thermoacetica*
Moth_1884	YP_430727	*Moorella thermoacetica*
Moth_1885	YP_430728	*Moorella thermoacetica*
Moth_1886	YP_430729	*Moorella thermoacetica*
Moth_1887	YP_430730	*Moorella thermoacetica*
Moth_1888	YP_430731	*Moorella thermoacetica*
Moth_1452	YP_430305	*Moorella thermoacetica*
Moth_1453	YP_430306	*Moorella thermoacetica*
Moth_1454	YP_430307	*Moorella thermoacetica*

Isopropanol production is achieved in recombinant *E. coli* following expression of two heterologous genes from *C. acetobutylicum* (*thl* and *adc* encoding acetoacetyl-CoA thiolase and acetoacetate decarboxylase, respectively) and one from *C. beijerinckii* (*adh* encoding a secondary alcohol dehydrogenase), along with the increased expression of the native *atoA* and *atoD* genes which encode acetoacetyl-CoA:acetate:CoA transferase activity (Hanai et al., Appl Environ Microbiol 73:7814-7818 (2007)).

Acetoacetyl-CoA thiolase converts two molecules of acetyl-CoA into one molecule each of acetoacetyl-CoA and CoA. Exemplary acetoacetyl-CoA thiolase enzymes include the gene products of *atoB* from *E. coli* (Martin et al., Nat.Biotechnol 21:796-802 (2003)), *thlA and thlB*

from *C. acetobutylicum* (Hanai et al., Appl Environ Microbiol 73:7814-7818 (2007); Winzer et al., J.Mol.Microbiol Biotechnol 2:531-541 (2000), and *ERG10* from *S. cerevisiae* Hiser et al., J.Biol.Chem. 269:31383-31389 (1994)).

Table 15.

Protein	GenBank ID	Organism
AtoB	NP_416728	*Escherichia coli*
ThlA	NP_349476.1	*Clostridium acetobutylicum*
ThlB	NP_149242.1	*Clostridium acetobutylicum*
ERG10	NP_015297	*Saccharomyces cerevisiae*

Acetoacetyl-CoA:acetate:CoA transferase converts acetoacetyl-CoA and acetate to acetoacetate and acetyl-CoA. Exemplary enzymes include the gene products of *atoAD* from *E. coli* (Hanai et al., Appl Environ Microbiol 73:7814-7818 (2007), *ctfAB* from *C. acetobutylicum* (Jojima et al., Appl Microbiol Biotechnol 77:1219-1224 (2008), and *ctfAB* from *Clostridium saccharoperbutylacetonicum* (Kosaka et al., Biosci.Biotechnol Biochem. 71:58-68 (2007)) are shown below in Table 16. A succinyl-CoA:3-ketoacid CoA transferase (SCOT) can also catalyze the conversion of the 3-ketoacyl-CoA, acetoacetyl-CoA, to the 3-ketoacid, acetoacetate. As opposed to acetoacetyl-CoA:acetate:CoA transferase, SCOT employs succinate as the CoA acceptor instead of acetate. Exemplary succinyl-CoA:3:ketoacid-CoA transferases are present in *Helicobacter pylori* (Corthesy-Theulaz, et al., J Biol Chem 272:25659-25667 (1997)), *Bacillus subtilis* (Stols, L., et al., Protein Expr Purif 53:396-403 (2007)), and *Homo sapiens* (Fukao, T., et al., Genomics 68:144-151 (2000); Tanaka, H., et al., Mol Hum Reprod 8:16-23 (2002)). Yet another transferase capable of this conversion is butyryl-CoA:acetoacetate CoA-transferase. Exemplary enzymes can be found in *Fusobacterium nucleatum* (Barker H.A., et al., J Bacteriol 152(1):201-7 (1982)), *Clostridium* SB4 (Barker H.A., et al., J Biol Chem 253(4):1219-25 (1978)), and *Clostridium acetobutylicum* (Wiesenborn D.P., et al., Appl Environ Microbiol 55(2):323-9 (1989)). Note many transferases have broad specificity and thus may utilize CoA acceptors as diverse as acetate, succinate, propionate, butyrate, 2-methylacetoacetate, 3-ketohexanoate, 3-ketopentanoate, valerate, crotonate, 3-mercaptopropionate, propionate, vinylacetate, butyrate, among others. Alternatively, an acetoacetyl-CoA hydrolase can be employed to convert acetoacetyl-CoA to acetoacetate. Such enzymes are common in various mammalian species (Patel, T.B., et al., Biochem J, 176 951-958 (1978); Rous, S., Biochem Biophys Res Commun 69 74-78 (1976); Baird, G.D., et al., Biochem J 117 703-709 (1970)).

Table 16.

Protein	GenBank ID	Organism
AtoA	NP_416726.1	*Escherichia coli*
AtoD	NP_416725.1	*Escherichia coli*
CtfA	NP_149326.1	*Clostridium acetobutylicum*
CtfB	NP_149327.1	*Clostridium acetobutylicum*
CtfA	AAP42564.1	*Clostridium saccharoperbutylacetonicum*
CtfB	AAP42565.1	*Clostridium saccharoperbutylacetonicum*
HPAG1_0676	YP_627417	*Helicobacter pylori*
HPAG1_0677	YP_627418	*Helicobacter pylori*
ScoA	NP_391778	*Bacillus subtilis*
ScoB	NP_391777	*Bacillus subtilis*
OXCT1	NP_000427	*Homo sapiens*
OXCT2	NP_071403	*Homo sapiens*

Acetoacetate decarboxylase converts acetoacetate into carbon dioxide and acetone. Exemplary acetoacetate decarboxylase enzymes are encoded by the gene products of *adc* from *C. acetobutylicum* (Petersen and Bennett, Appl Environ.Microbiol 56:3491-3498 (1990)) and *adc* from *Clostridium saccharoperbutylacetonicum* (Kosaka, et al., Biosci.Biotechnol Biochem. 71:58-68 (2007)). The enzyme from *C. beijerinkii* can be inferred from sequence similarity. These proteins are identified below in Table 17.

Table 17.

Protein	GenBank ID	Organism
Adc	NP_149328.1	*Clostridium acetobutylicum*
Adc	AAP42566.1	*Clostridium saccharoperbutylacetonicum*
Adc	YP_001310906.1	*Clostridium beijerinckii*

The final step in the isopropanol synthesis pathway involves the reduction of acetone to isopropanol. Exemplary alcohol dehydrogenase enzymes capable of this transformation include *adh* from *C. beijerinckii* (Hanai et al., Appl Environ Microbiol 73:7814-7818 (2007); Jojima et al., Appl Microbiol Biotechnol 77:1219-1224 (2008)) and *adh* from *Thermoanaerobacter brockii* (Hanai et al., Appl Environ Microbiol 73:7814-7818 (2007); Peretz et al., Anaerobe 3:259-270 (1997)). Additional characterized enzymes include alcohol dehydrogenases from *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) (Steinbuchel and Schlegel et al., Eur.J.Biochem. 141:555-564 (1984)) and *Phytomonas* species (Uttaro and Opperdoes et al., Mol.Biochem.Parasitol. 85:213-219 (1997)).

Table 18.

Protein	GenBank ID	Organism
Adh	P14941.1	*Thermoanaerobobacter brockii*
Adh	AAA23199.2	*Clostridium beijerinckii*
Adh	YP_299391.1	*Ralstonia eutropha*
iPDH	AAP39869.1	*Phtomonas sp.*

Exemplary 3-hydroxyacyl dehydrogenases which convert acetoacetyl-CoA to 3-hydroxybutyryl-CoA include *hbd* from *C. acetobutylicum* (Boynton et al., Journal of Bacteriology 178:3015-3024 (1996)), *hbd* from *C. beijerinckii* (Colby and Chen et al., Appl Environ.Microbiol 58:3297-3302 (1992)), and a number of similar enzymes from *Metallosphaera sedula* (Berg et al., 2007 Science 318:1782-1786 (2007)).

Table 19.

Protein	GenBank ID	Organism
hbd	NP_349314.1	*Clostridium acetobutylicum*
hbd	AAM14586.1	*Clostridium beijerinckii*
Msed_1423	YP_001191505	*Metallosphaera sedula*
Msed_0399	YP_001190500	*Metallosphaera sedula*
Msed_0389	YP_001190490	*Metallosphaera sedula*
Msed_1993	YP_001192057	*Metallosphaera sedula*

The gene product of *crt* from *C. acetobutylicum* catalyzes the dehydration of 3-hydroxybutyryl-CoA to crotonyl-CoA (Atsumi et al., Metab Eng (2007); Boynton et al., Journal of Bacteriology 178:3015-3024 (1996)). Further, enoyl-CoA hydratases are reversible enzymes and thus suitable candidates for catalyzing the dehydration of 3-hydroxybutyryl-CoA to crotonyl-CoA. The enoyl-CoA hydratases, *phaA* and *phaB*, of *P. putida* are believed to carry out the hydroxylation of double bonds during phenylacetate catabolism (Olivera et al., Proc Nat Acad Sci U.S.A. 95:6419-6424 (1998)). The *paaA* and *paaB* from *P. fluorescens* catalyze analogous transformations (Olivera et al., Proc Nat Acad Sci U.S.A. 95:6419-6424 (1998)). Lastly, a number of *Escherichia coli* genes have been shown to demonstrate enoyl-CoA hydratase functionality including *maoC*, *paaF*, and *paaG* (Ismail et al., European Journal of Biochemistry 270:3047-3054 (2003); Park and Lee, J Bacteriol. 185:5391-5397 (2003); Park and Lee, Appl Biochem.Biotechnol 113-116:335-346 (2004); Park and Yup, Biotechnol Bioeng 86:681-686 (2004)).

Table 20.

Protein	GenBank ID	Organism
crt	NP_349318.1	*Clostridium acetobutylicum*
paaA	NP_745427.1	*Pseudomonas putida*
paaB	NP_745426.1	*Pseudomonas putida*
phaA	ABF82233.1	*Pseudomonas fluorescens*
phaB	ABF82234.1	*Pseudomonas fluorescens*
maoC	NP_415905.1	*Escherichia coli*
paaF	NP_415911.1	*Escherichia coli*
paaG	NP_415912.1	*Escherichia coli*

Several enzymes that naturally catalyze the reverse reaction (i.e., the dehydration of 4-hydroxybutyryl-CoA to crotonoyl-CoA) *in vivo* have been identified in numerous species. This transformation is used for 4-aminobutyrate fermentation by *Clostridium aminobutyricum* (Scherf and Buckel, Eur.J Biochem. 215:421-429 (1993)) and succinate-ethanol fermentation by *Clostridium kluyveri* (Scherf et al., Arch.Microbiol 161:239-245 (1994)). The transformation is also a step in Archaea, for example, *Metallosphaera sedula*, as part of the 3-hydroxypropionate/4-hydroxybutyrate autotrophic carbon dioxide assimilation pathway (Berg et al., Science 318:1782-1786 (2007)). This pathway uses the hydration of crotonoyl-CoA to form 4-hydroxybutyryl-CoA. The reversibility of 4-hydroxybutyryl-CoA dehydratase is well-documented (Friedrich et al., Angew.Chem.Int.Ed.Engl. 47:3254-3257 (2008); Muh et al., Eur.J.Biochem. 248:380-384 (1997); Muh et al., Biochemistry 35:11710-11718 (1996)) and the equilibrium constant has been reported to be about 4 on the side of crotonoyl-CoA (Scherf and Buckel, Eur.J Biochem. 215:421-429 (1993)). This indicates that the downstream 4-hydroxybutyryl-CoA dehydrogenase keeps the 4-hydroxybutyryl-CoA concentration low so as to not create a thermodynamic bottleneck at crotonyl-CoA.

Table 21.

Protein	GenBank ID	Organism
AbfD	CAB60035	*Clostridium aminobutyricum*
AbfD	YP_001396399	*Clostridium kluyveri*
Msed_1321	YP_001191403	*Metallosphaera sedula*
Msed_1220	YP_001191305	*Metallosphaera sedula*

4-Hydroxybutyryl-CoA transferase transfers the CoA moiety from 4-hydroxybutyryl-CoA to acetate, in turn, forming 4-hydroxybutyrate and acetyl-CoA. One exemplary 4-hydroxybutyryl-CoA transferase is encoded by the *cat2* gene of *Clostridium kluyveri* (Seedorf et al., Proc.Natl.Acad.Sci.U S.A. 105:2128-2133 (2008); Sohling and Gottschalk J Bacteriol. 178:871-880 (1996)). The *abfT-2* gene from *Porphyromonas gingivalis* was also shown to

exhibit 4-hydroxybutyryl-CoA transferase activity when implemented as part of a pathway to produce 4-hydroxybutyate and 1,4-butanediol (Burk, et al., WO/2008/115840 (2008)). An additional candidate enzyme, encoded by *abfT-1*, from *P. gingivalis* can be inferred by sequence homology. Another 4-hydroxybutyryl-CoA transferase is encoded by the gene product of *abfT* from *Clostridium aminobutyricum* (Gerhardt et al., Arch.Microbiol 174:189-199 (2000)).

Table 22.

Protein	GenBank ID	Organism
cat2	YP_001396397	*Clostridium kluyveri*
abfT-2	NP_906037	*Porphyromonas gingivalis*
abfT-1	NP_904965.1	*Porphyromonas gingivalis*
abfT	CAB60036	*Clostridium aminobutyricum*

Exemplary phosphate transferring acyltransferases include phosphotransacetylase, encoded by *pta*, and phosphotransbutyrylase, encoded by *ptb*. The *pta* gene from *E. coli* encodes an enzyme that can convert acetyl-CoA into acetyl-phosphate, and vice versa (Suzuki, T. 1969 Biochim.Biophys.Acta 191:559-569 (1969)). This enzyme can also utilize propionyl-CoA instead of acetyl-CoA forming propionate in the process (Hesslinger et al., Mol.Microbiol 27:477-492 (1998)). Similarly, the *ptb* gene from *C. acetobutylicum* encodes an enzyme that can convert butyryl-CoA into butyryl-phosphate (Huang et al., J.Mol.Microbiol Biotechnol 2:33-38 (2000); (Walter et al., Gene 134:107-111 (1993)). This same enzyme was shown to have activity on 4-hydroxybutyryl-CoA when implemented as part of a pathway to produce 1,4-butanediol WO/2008/115840 (2008). Additional *ptb* genes can be found in butyrate-producing bacterium L2-50 (Ljungdahl and Andreesen, Methods Enzymol. 53:360-372 (1978) and *Bacillus megaterium* (Vazquez et al., Curr.Microbiol 42:345-349 (2001)).

Table 23.

Protein	GenBank ID	Organism
pta	NP_416800.1	*Escherichia coli*
ptb	NP_349676	*Clostridium acetobutylicum*
ptb	AAR19757.1	butyrate-producing bacterium L2-50
ptb	CAC07932.1	*Bacillus megaterium*

Exemplary kinases include the *E. coli* acetate kinase, encoded by *ackA* (Skarstedt and Silverstein, J.Biol.Chem.251:6775-6783 (1976)) , the *C.* acetobutylicum butyrate kinases, encoded by *buk1* and *buk2* (Huang et al., 2000 J.Mol.Microbiol Biotechnol 2:33-38 (2000); Walter et al., Gene 134:107-111 (1993)), and the *E. coli* gamma-glutamyl kinase, encoded by *proB* (Smith et al., J.Bacteriol. 157:545-551 (1984)). These enzymes phosphorylate acetate,

butyrate, and glutamate, respectively. The a*ckA* gene product from *E. coli* also phosphorylates propionate (Hesslinger et al., Mol.Microbiol 27:477-492 (1998)). The gene product of *buk1* from *C. acetobutylicum* was shown in Burk et al., WO/2008/115840 (2008) to have activity on 4-hydroxybutyryl-CoA when implemented as part of a pathway to produce 1,4-butanediol.

Table 24.

Protein	GenBank ID	Organism
ackA	NP_416799.1	*Escherichia coli*
buk1	NP_349675	*Clostridium acetobutylicum*
buk2	Q97II1	*Clostridium acetobutylicum*
proB	NP_414777.1	*Escherichia coli*

Alcohol-forming 4-hydroxybutyryl-CoA reductase enzymes catalyze the 2 reduction steps required to form 1,4-butanediol from 4-hydroxybutyryl-CoA. Exemplary 2-step oxidoreductases that convert an acyl-CoA to alcohol include those that transform substrates such as acetyl-CoA to ethanol (e.g., *adhE* from *E. coli* (Kessler et al., FEBS.Lett. 281:59-63 (1991)) and butyryl-CoA to butanol (e.g. *adhE2* from *C. acetobutylicum* (Fontaine et al., J.Bacteriol. 184:821-830 (2002)). The *adhE2* enzyme from *C. acetobutylicum* was specifically shown in ref. Burk et al., WO/2008/115840 (2008). to produce BDO from 4-hydroxybutyryl-CoA. In addition to reducing acetyl-CoA to ethanol, the enzyme encoded by *adhE* in *Leuconostoc mesenteroides* has been shown to oxide the branched chain compound isobutyraldehyde to isobutyryl-CoA (Kazahaya et al., J.Gen.Appl.Microbiol. 18:43-55 (1972); Koo et al., Biotechnol Lett. 27:505-510 (2005)).

Table 25.

Protein	GenBank ID	Organism
adhE	NP_415757.1	*Escherichia coli*
adhE2	AAK09379.1	*Clostridium acetobutylicum*
adhE	AAV66076.1	*Leuconostoc mesenteroides*

Another exemplary enzyme can convert malonyl-CoA to 3-HP. An NADPH-dependent enzyme with this activity has characterized in *Chloroflexus aurantiacus* where it participates in the 3-hydroxypropionate cycle (Hugler et al., J.Bacteriol. 184:2404-2410 (2000); Strauss and Fuchs, Eur.J.Biochem. 215:633-643 (1993)). This enzyme, with a mass of 300 kDa, is highly substrate-specific and shows little sequence similarity to other known oxidoreductases (Hugler et al., J.Bacteriol. 184:2404-2410 (2002)). No enzymes in other organisms have been shown to catalyze this specific reaction; however there is bioinformatic evidence that other organisms may have similar pathways (Klatt et al., Environ.Microbiol. 9:2067-2078 (2007)). Enzyme candidates

in other organisms including *Roseiflexus castenholzii*, *Erythrobacter sp. NAP1* and marine gamma proteobacterium HTCC2080 can be inferred by sequence similarity.

Table 26.

Protein	GenBank ID	Organism
mcr	AAS20429.1	*Chloroflexus aurantiacus*
Rcas_2929	YP_001433009.1	*Roseiflexus castenholzii*
NAP1_02720	ZP_01039179.1	*Erythrobacter sp. NAP1*
MGP2080_00535	ZP_01626393.1	marine gamma proteobacterium HTCC2080

An alternative route to BDO from 4-hydroxybutyryl-CoA involves first reducing this compound to 4-hydroxybutanal. Several acyl-CoA dehydrogenases are capable of reducing an acyl-CoA to its corresponding aldehyde. Exemplary genes that encode such enzymes include the *Acinetobacter calcoaceticus acr1* encoding a fatty acyl-CoA reductase (Reiser and Somerville, Journal of Bacteriology 179:2969-2975 (1997)), the *Acinetobacter sp. M-1* fatty acyl-CoA reductase (Ishige et al., Appl.Environ.Microbiol. 68:1192-1195 (2002)), and a CoA- and NADP-dependent succinate semialdehyde dehydrogenase encoded by the *sucD* gene in *Clostridium kluyveri* (Sohling and Gottschalk, J Bacteriol. 178:871-880 (1996)). *SucD* of *P. gingivalis* is another succinate semialdehyde dehydrogenase (Takahashi et al., J.Bacteriol. 182:4704-4710 (2000)). These succinate semialdehyde dehydrogenases were specifically shown in ref. Burk et al., WO/2008/115840 (2008) to convert 4-hydroxybutyryl-CoA to 4-hydroxybutanal as part of a pathway to produce 1,4-butanediol. The enzyme acylating acetaldehyde dehydrogenase in *Pseudomonas sp*, encoded by *bphG*, is yet another capable enzyme as it has been demonstrated to oxidize and acylate acetaldehyde, propionaldehyde, butyraldehyde, isobutyraldehyde and formaldehyde (Powlowski et al., J Bacteriol. 175:377-385 (1993)).

Table 27.

Protein	GenBank ID	Organism
acr1	YP_047869.1	*Acinetobacter calcoaceticus*
acr1	AAC45217	*Acinetobacter baylyi*
acr1	BAB85476.1	*Acinetobacter* sp. Strain M-1
sucD	P38947.1	*Clostridium kluyveri*
sucD	NP_904963.1	*Porphyromonas gingivalis*
bphG	BAA03892.1	*Pseudomonas sp*

An additional enzyme type that converts an acyl-CoA to its corresponding aldehyde is malonyl-CoA reductase which transforms malonyl-CoA to malonic semialdehyde. Malonyl-CoA reductase is a key enzyme in autotrophic carbon fixation via the 3-hydroxypropionate cycle in

thermoacidophilic archael bacteria (Berg et al., Science 318:1782-1786 (2007); Thauer, R. K. Science 318:1732-1733 (2007)). The enzyme utilizes NADPH as a cofactor and has been characterized in *Metallosphaera* and *Sulfolobus spp* (Alber et al., J.Bacteriol.188:8551-8559 (2006); Hugler et al., J.Bacteriol. 184:2404-2410 (2002)). The enzyme is encoded by *Msed_0709* in *Metallosphaera sedula* (Alber et al., J.Bacteriol. 188:8551-8559 (2006); Berg et al., Science 318:1782-1786 (2007)). A gene encoding a malonyl-CoA reductase from *Sulfolobus tokodaii* was cloned and heterologously expressed in *E. coli* (Alber et al., J.Bacteriol. 188:8551-8559 (2006)). Although the aldehyde dehydrogenase functionality of these enzymes is similar to the bifunctional dehydrogenase from *Chloroflexus aurantiacus*, there is little sequence similarity. Both malonyl-CoA reductase enzyme candidates have high sequence similarity to aspartate-semialdehyde dehydrogenase, an enzyme catalyzing the reduction and concurrent dephosphorylation of aspartyl-4-phosphate to aspartate semialdehyde. Additional gene candidates can be found by sequence homology to proteins in other organisms including *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius*.

Table 28.

Protein	GenBank ID	Organism
Msed_0709	YP_001190808.1	*Metallosphaera sedula*
mcr	NP_378167.1	*Sulfolobus tokodaii*
asd-2	NP_343563.1	*Sulfolobus solfataricus*
Saci_2370	YP_256941.1	*Sulfolobus acidocaldarius*

Enzymes exhibiting 1,4-butanediol dehydrogenase activity are capable of forming 1,4-butanediol from 4-hydroxybutanal. Exemplary genes encoding enzymes that catalyze the conversion of an aldehyde to alcohol (i.e., alcohol dehydrogenase or equivalently aldehyde reductase) include *alrA* encoding a medium-chain alcohol dehydrogenase for C2-C14 (Tani et al., Appl.Environ.Microbiol. 66:5231-5235 (2000)), ADH2 from *Saccharomyces cerevisiae* Atsumi et al. Nature 451:86-89 (2008)), *yqhD* from *E. coli* which has preference for molecules longer than C(3) (Sulzenbacher et al., Journal of Molecular Biology 342:489-502 (2004)), and *bdh* I and *bdh* II from *C. acetobutylicum* which converts butyryaldehyde into butanol (Walter et al., Journal of Bacteriology 174:7149-7158 (1992)).

Table 29.

Protein	GenBank ID	Organism
alrA	BAB12273.1	*Acinetobacter* sp. Strain M-1
ADH2	NP_014032.1	*Saccharymyces cerevisiae*
yqhD	NP_417484.1	*Escherichia coli*
bdh I	NP_349892.1	*Clostridium acetobutylicum*
bdh II	NP_349891.1	*Clostridium acetobutylicum*

Enzymes exhibiting 4-hydroxybutyrate dehydrogenase activity (EC 1.1.1.61) also fall into this category. Such enzymes have been characterized in *Ralstonia eutropha* (Bravo et al., J.Forensic Sci. 49:379-387 (2004)), *Clostridium kluyveri* (Wolff and Kenealy, Protein Expr.Purif. 6:206-212 (1995)) and *Arabidopsis thaliana* (Breitkreuz et al., J.Biol.Chem. 278:41552-41556 (2003)).

Table 30.

Protein	GenBank ID	Organism
4hbd	YP_726053.1	*Ralstonia eutropha* H16
4hbd	L21902.1	*Clostridium kluyveri* DSM 555
4hbd	Q94B07	*Arabidopsis thaliana*

The first step in the cloning and expression process is to express in *E. coli* the minimal set of genes (e.g., *MtaA*, *MtaB*, and *MtaC*) necessary to produce Me-THF from methanol. These methyltransferase activities use Coenzyme B_{12} (cobalamin) as a cofactor. In *Moorella thermoacetica*, a cascade of methyltransferase proteins mediate incorporation of methanol derived methyl groups into the acetyl-CoA synthase pathway. Recent work (Das et al., Proteins 67:167-176 (2007)) indicates that MtaABC are encoded by Moth_1208-09 and Moth_2346. These genes are cloned via proof-reading PCR and linked together for expression in a high-copy number vector such as pZE22-S under control of the repressible PA1-lacO1 promoter (Lutz and Bujard, Nucleic Acids Res 25:1203-1210 (1997)). Cloned genes are verified by PCR and or restriction enzyme mapping to demonstrate construction and insertion of the 3-gene set into the expression vector. DNA sequencing of the presumptive clones is carried out to confirm the expected sequences of each gene. Once confirmed, the final construct is expressed in *E. coli* K-12 (MG1655) cells by addition of IPTG inducer between 0.05 and 1 mM final concentration. Expression of the cloned genes is monitored using SDS-PAGE of whole cell extracts. To optimize levels of soluble vs. pellet (potentially inclusion body origin) protein, the affect of titration of the promoter on these levels can be examined. If no acceptable expression is obtained, higher or lower copy number vectors or variants in promoter strength are tested.

To determine if expression of the MtaABC proteins from *M. thermoacetica* confers upon *E. coli* the ability to transfer methyl groups from methanol to tetrahydrofolate (THF) the recombinant strain is fed methanol at various concentrations. Activity of the methyltransferase system is assayed anaerobically as described for vanillate as a methyl source in *M. thermoacetica* (Naidu and Ragsdale, J Bacteriol. 183:3276-3281 (2001)) or for *Methanosarcina barkeri* methanol methyltransferase (Sauer et al., Eur.J Biochem. 243:670-677 (1997); Tallant

and Krzycki, J Bacteriol. 178:1295-1301 (1996); Tallant and Krzycki, J Bacteriol. 179:6902-6911 (1997); Tallant et al., J Biol Chem. 276:4485-4493 (2001)). For a positive control, *M. thermoacetica* cells are cultured in parallel and assayed anaerobically to confirm endogenous methyltransferase activity. Demonstration of dependence on exogenously added coenzyme B_{12} confirms methanol:corrinoid methyltransferase activity in *E. coli*.

Once methyltransferase expression is achieved, further work is performed towards optimizing the expression. Titrating the promoter in the expression vector enables the testing of a range of expression levels. This is then used as a guide towards the expression required in single-copy, or enables the determination of whether or not a single-copy of these genes allows sufficient expression. If so, the methyltransferase genes are integrated into the chromosome as a single, synthetic operon. This entails targeted integration using RecET-based 'recombineering' (Angrand et al., Nucleic Acids Res 27:e16 (1999); Muyrers et al., Nucleic Acids Res 27:1555-1557 (1999); Zhang et al., 1998 Nat.Genet. 20:123-128 (1998)). A potential issue with RecET-based integration of a cassette and removal of a FRT or loxP-bounded selectable marker by FLP or Cre is the production of a recombination scar at each integration site. While problems caused by this can be minimized by a number of methods, other means that do not leave genomic scars are available. The standard alternative, is to introduce the desired genes using integrative 'suicide' plasmids coupled to counter-selection such as that allowed by the *Bacillus sacB* gene (Link et al., J Bacteriol. 179:6228-6237 (1997)); in this way, markerless and scar less insertions at any location in the *E. coli* chromosome can be generated. The final goal is a strain of *E. coli* K-12 expressing methanol:corrinoid methyltransferase activity under an inducible promoter and in single copy (chromosomally integrated).

Using standard PCR methods, entire ACS/CODH operons are assembled into low or medium copy number vectors such as pZA33-S (P15A-based) or pZS13-S (pSC101-based). As described for the methyltransferase genes, the structure and sequence of the cloned genes are confirmed. Expression is monitored via protein gel electrophoresis of whole-cell lysates grown under strictly anaerobic conditions with the requisite metals (Ni, Zn, Fe) and coenzyme B_{12} provided. As necessary, the gene cluster is modified for *E. coli* expression by identification and removal of any apparent terminators and introduction of consensus ribosomal binding sites chosen from sites known to be effective in *E. coli* (Barrick et al., Nucleic Acids Res 22:1287-1295 (1994); Ringquist et al., Mol.Microbiol 6:1219-1229 (1992)). However, each gene cluster is cloned and expressed in a manner parallel to its native structure and expression. This helps ensure the desired stoichiometry between the various gene products—most of which interact with each other. Once satisfactory expression of the CODH/ACS gene cluster under anaerobic

conditions is achieved, the ability of cells expressing these genes to fix CO and/or CO_2 into cellular carbon is assayed. Initial conditions employ strictly anaerobically grown cells provided with exogenous glucose as a carbon and energy source via substrate-level phosphorylation or anaerobic respiration with nitrate as an electron acceptor. Additionally, exogenously provided CH_3-THF is added to the medium.

The ACS/CODH genes are cloned and expressed in cells also expressing the methanol-methyltransferase system. This is achieved by introduction of compatible plasmids expressing ACS/CODH into MTR-expressing cells. For added long-term stability, the ACS/CODH and MTR genes can also be integrated into the chromosome. After strains of *E. coli* capable of utilizing methanol to produce Me-THF and of expressing active CODH/ACS gene are made, they are assayed for the ability to utilize both methanol and syngas for incorporation into acetyl-CoA, acetate, and cell mass. Initial conditions employ strictly anaerobically grown cells provided with exogenous glucose as a carbon and energy source. Alternatively, or in addition to glucose, nitrate will be added to the fermentation broth to serve as an electron acceptor and initiator of growth. Anaerobic growth of *E. coli* on fatty acids, which are ultimately metabolized to acetyl-CoA, has been demonstrated in the presence of nitrate (Campbell et al., Molecular Microbiology 47:793-805 (2003)). Oxygen can also be provided as long as its intracellular levels are maintained below any inhibition threshold of the engineered enzymes. 'Synthetic syngas' of a composition suitable for these experiments is employed along with methanol. ^{13}C-labeled methanol or ^{13}C-labeled CO are provided to the cells and analytical mass spectrometry is employed to measure incorporation of the labeled carbon into acetate and cell mass (e.g., proteinogenic amino acids).

The pyruvate ferredoxin oxidoreductase genes from *M. thermoacetica*, *D. africanus*, and *E. coli* are cloned and expressed in strains exhibiting MTR and ACS/CODH activities. Conditions, promoters, etc., are described above. Given the large size of the PFOR genes and oxygen sensitivity of the corresponding enzymes, tests will be performed using low or single-copy plasmid vectors or single-copy chromosomal integrations. Activity assays described in ref. (Furdui and Ragsdale, J Biol Chem. 275:28494-28499 (2000)) will be applied to demonstrate activity. In addition, demonstration of growth on the gaseous carbon sources and methanol in the absence of an external electron acceptor will provide further evidence for PFOR activity *in vivo*.

The endogenous hydrogen-utilizing hydrogenase activity of the host organism is tested by growing the cells as described above in the presence and absence of hydrogen. If a dramatic shift towards the formation of more reduced products during fermentation is observed (e.g.,

increased ethanol as opposed to acetate), this indicates that endogenous hydrogenase activity is sufficiently active. In this case, no heterologous hydrogenases are cloned and expressed. If the native enzymes do not have sufficient activity or reduce the needed acceptor, the genes encoding an individual hydrogenase complex are cloned and expressed in strains exhibiting MTR, ACS/CODH, and PFOR activities. Conditions, promoters, etc., are described above.

The nonnative genes needed for isopropanol synthesis are cloned on expression plasmids as described previously. The host strain also expresses methanol methyltransferase activity, CODH/ACS activity, and possibly PFOR and hydrogenase activities. At this point, these (CODH/ACS, etc.) genes are integrated into the genome and expressed from promoters that can be used constitutively or with inducers (i.e., PA1-lacO1 is inducible in cells containing *lacI* or is otherwise constitutive). Once expression and yields of isopropanol are optimized, the base strain is further modified by integration of a single copy of these genes at a neutral locus. Given the relatively limited number of genes (at minimum, 3, and at most, 6), Applicants construct an artificial operon encoding the required genes. This operon is introduced using integrative plasmids and is coupled to counter-selection methods such as that allowed by the *Bacillus sacB* gene (Link et al., J Bacteriol. 179:6228-6237 (1997)). In this way, markerless and scar less insertions at any location in the *E. coli* chromosome can be generated. Optimization involves altering gene order as well as ribosomal binding sites and promoters.

To over express any native genes, for example, the native *atoB* (b2224) gene of *E. coli* which can serve as an alternative to the *C. acetobutylicum* acetyl-coenzyme A [CoA] acetyltransferase required for isopropanol production, RecET-based methods are applied to integrate a stronger upstream promoter. In the case of *atoB*, this gene is the last in an operon and the next gene downstream (*yfaP*) is both non-essential and in the opposite orientation. Therefore, polarity should not be an issue. A cassette containing a selectable marker such as spectinomycin resistance or chloramphenicol resistance flanked by FRT or loxP sites is used to select for introduction of a strong constitutive promoter (e.g., pL). Once the correct clone is obtained and validated, using qRT-PCR, FLP or Cre expression is used to select for removal of the FRT- or loxP-bounded marker.

The nonnative genes needed for 4-hydroxybutyrate synthesis are cloned on expression plasmids as described previously. The host strain also expresses methanol methyltransferase activity, CODH/ACS activity, and possibly PFOR and hydrogenase activities. At this point, these (CODH/ACS, etc.) genes are integrated into the genome and expressed from promoters that can be used constitutively or with inducers (i.e., PA1-lacO1 is inducible in cells containing

lacI or is otherwise constitutive). Once expression and yields of 4-hydroxybutyrate are optimized, the base strain is further modified by integration of a single copy of these genes at a neutral locus. Given the relatively limited number of genes (at minimum, 5, and at most, 6), an artificial operon encoding the required genes can be constructed. This operon is introduc ed using integrative plasmids and is coupled to counter-selection methods such as that allowed by the *Bacillus sacB* gene (Link et al., J Bacteriol. 179:6228-6237 (1997)). In this way, markerless and scar less insertions at any location in the *E. coli* chromosome can be generated. Optimization involves altering gene order as well as ribosomal binding sites and promoters.

The nonnative genes needed for 1,4-butanediol synthesis are cloned on expression plasmids as described previously. The host strain also expresses methanol methyltransferase activity, CODH/ACS activity, and possibly PFOR and hydrogenase activities. At this point, these (CODH/ACS, etc.) genes are integrated into the genome and expressed from promoters that can be used constitutively or with inducers (i.e., PA1-lacO1 is inducible in cells containing *lacI* or is otherwise constitutive). Once expression and yields of 1,4-butanediol are optimized, the base strain is further modified by integration of a single copy of these genes at a neutral locus. Given the relatively limited number of genes (at minimum, 5, and at most, 6), an artificial operon encoding the required genes can be constructed. This operon is introduced using integrative plasmids and is coupled to counter-selection methods such as that allowed by the *Bacillus sacB* gene (Link et al., J Bacteriol. 179:6228-6237 (1997)). In this way, markerless and scar less insertions at any location in the *E. coli* chromosome can be generated. Optimization involves altering gene order as well as ribosomal binding sites and promoters.

Engineering the capability to convert synthesis gas into acetyl-CoA, the central metabolite from which all cell mass components and many valuable products can be derived, into a foreign host such as *E. coli* can be accomplished following the expression of exogenous genes that encode various proteins of the Wood-Ljungdahl pathway. This pathway is highly active in acetogenic organisms such as *Moorella thermoacetica (formerly, Clostridium thermoaceticum)*, which has been the model organism for elucidating the Wood-Ljungdahl pathway since its isolation in 1942 (Fontaine et al., J Bacteriol. 43:701-715 (1942)). The Wood-Ljungdahl pathway comprises of two branches: the Eastern (or methyl) branch that enables the conversion of CO_2 to methyltetrahydrofolate (Me-THF) and the Western (or carbonyl) branch that enables the conversion of methyl-THF, CO, and Coenzyme-A into acetyl-CoA (Figure 3). In some embodiments, the present invention provides a non-naturally occurring microorganism expressing genes encoding enzymes that catalyze the methyl and carbonyl branches of the

Wood-Ljungdahl pathway. Such an organism is capable of converting CO, CO_2, and/or H_2 into acetyl-CoA, cell mass, and products.

Another organism of the present invention contains three capabilities which are depicted in Figure 3A: 1) a functional methyl branch of the Wood-Ljungdahl pathway which enables the conversion of THF and CO_2 to 5-methyl-tetrahydrofolate, 2) the ability to combine CO, Coenzyme A, and the methyl group of Me-THF to form acetyl-CoA, and 3) the ability to synthesize isopropanol from acetyl-CoA. The fifth organism described in this invention, depicted in Figure 3B, contains a functional methyl branch of the Wood-Ljungdahl pathway, the ability to synthesize acetyl-CoA, and the ability to synthesize 4-hydroxybutyrate from acetyl-CoA. The sixth organism described in this invention, depicted in Figure 3C, contains a functional methyl branch of the Wood-Ljungdahl pathway, the ability to synthesize acetyl-CoA, and the ability to synthesize 1,4-butanediol from acetyl-CoA.

These three organisms are able to 'fix' carbon from exogenous CO and/or CO_2 to synthesize acetyl-CoA, cell mass, and products. A host organism engineered with these capabilities that also naturally possesses the capability for anapleurosis (e.g., *E. coli*) can grow on the syngas-generated acetyl-CoA in the presence of a suitable external electron acceptor such as nitrate. This electron acceptor is required to accept electrons from the reduced quinone formed via succinate dehydrogenase. A further advantage of adding an external electron acceptor is that additional energy for cell growth, maintenance, and product formation can be generated from respiration of acetyl-CoA. An alternative strategy involves engineering a pyruvate ferredoxin oxidoreductase (PFOR) enzyme into the strain to enable synthesis of biomass precursors in the absence of an external electron acceptor. A further characteristic of the engineered organism is the capability for extracting reducing equivalents from molecular hydrogen. This enables a high yield of reduced products such as ethanol, butanol, isobutanol, isopropanol, 1,4-butanediol, succinic acid, fumaric acid, malic acid, 4-hydroxybutyric acid, 3-hydroxypropionic acid, lactic acid, adipic acid, 2-hydroxyisobutyric acid, 3-hydroxyisobutyric acid, methacrylic acid, and acrylic acid.

The organisms provided herein can produce acetyl-CoA, cell mass, and targeted chemicals, more specifically isopropanol, 4-hydroxybutyrate, and 1,4-butanediol, from: 1) CO, 2) CO_2 and H_2, 3) CO, CO_2, and H_2, 4) synthesis gas comprising CO and H_2, and 5) synthesis gas comprising CO, CO_2, and H_2.

Successfully engineering any of these pathways into an organism involves identifying an appropriate set of enzymes, cloning their corresponding genes into a production host, optimizing

the stability and expression of these genes, optimizing fermentation conditions, and assaying for product formation following fermentation. Below are described enzymes that catalyze steps 1 through 5 of the pathways depicted in Figures 3A through 3C. These enzymes are required to enable the conversion of synthesis gas to acetyl-CoA. Enzymes for steps 6 through 17 in Figure 3A and steps 6 through 20 in Figures 3B and 3C were described above. To engineer a production host for the utilization of syngas, one or more exogenous DNA sequence(s) encoding the requisite enzymes can be expressed in the microorganism.

Formate dehydrogenase is a two subunit selenocysteine-containing protein that catalyzes the incorporation of CO_2 into formate in *Moorella thermoacetica* (Andreesen and Ljungdahl, J.Bacteriol. 116:867-873 (1973); Li et al., J.Bacteriol. 92:405-412 (1966); Yamamoto et al., J.Biol.Chem. 258:1826-1832 (1983). The loci, Moth_2312 and Moth_2313 are actually one gene that is responsible for encoding the alpha subunit of formate dehydrogenase while the beta subunit is encoded by Moth_2314 (Pierce et al., Environ.Microbiol. (2008)). Another set of genes encoding formate dehydrogenase activity with a propensity for CO_2 reduction is encoded by Sfum_2703 through Sfum_2706 in *Syntrophobacter fumaroxidans* (de Bok et al., Eur.J.Biochem. 270:2476-2485 (2003)); Reda et al., Proc.Natl.Acad.Sci.U S.A. 105:10654-10658 (2008)). Similar to their *M. thermoacetica* counterparts, Sfum_2705 and Sfum_2706 are actually one gene. A similar set of genes presumed to carry out the same function are encoded by CHY_0731, CHY_0732, and CHY_0733 in *C. hydrogenoformans* (Wu et al., PLoS Genet. 1:e65 (2005)).

Table 31.

Protein	GenBank ID	Organism
Moth_2312	YP_431142	*Moorella thermoacetica*
Moth_2313	YP_431143	*Moorella thermoacetica*
Moth_2314	YP_431144	*Moorella thermoacetica*
Sfum_2703	YP_846816.1	*Syntrophobacter fumaroxidans*
Sfum_2704	YP_846817.1	*Syntrophobacter fumaroxidans*
Sfum_2705	YP_846818.1	*Syntrophobacter fumaroxidans*
Sfum_2706	YP_846819.1	*Syntrophobacter fumaroxidans*
CHY_0731	YP_359585.1	*Carboxydothermus hydrogenoformans*
CHY_0732	YP_359586.1	*Carboxydothermus hydrogenoformans*
CHY_0733	YP_359587.1	*Carboxydothermus hydrogenoformans*

Formyltetrahydrofolate synthetase ligates formate to tetrahydrofolate at the expense of one ATP. This reaction is catalyzed by the gene product of Moth_0109 in *M. thermoacetica* (Lovell et al., Arch.Microbiol 149:280-285 (1988); Lovell et al., Biochemistry 29:5687-5694 (1990); O'brien et al., Experientia.Suppl. 26:249-262 (1976), *FHS* in *Clostridium aciduric*

(Whitehead and Rabinowitz, J.Bacteriol. 167:205-209(1986); Whitehead and Rabinowitz, J.Bacteriol. 170:3255-3261 (1988)), and CHY_2385 in *C. hydrogenoformans* (Wu et al., PLoS Genet. 1:e65 (2005)).

Table 32.

Protein	GenBank ID	Organism
Moth_0109	YP_428991.1	*Moorella thermoacetica*
CHY_2385	YP_361182.1	*Carboxydothermus hydrogenoformans*
FHS	P13419.1	*Clostridium acidurici*

In *M. thermoacetica*, *E. coli*, and *C. hydrogenoformans*, methenyltetrahydrofolate cyclohydrolase and methylenetetrahydrofolate dehydrogenase are carried out by the bi-functional gene products of Moth_1516, *folD*, and CHY_1878, respectively (D'Ari and Rabinowitz, J.Biol.Chem. 266:23953-23958 (1991); Pierce et al., Environ.Microbiol (2008); Wu et al., PLoS Genet. 1:e65 (2005)).

Table 33.

Protein	GenBank ID	Organism
Moth_1516	YP_430368.1	*Moorella thermoacetica*
folD	NP_415062.1	*Escherichia coli*
CHY_1878	YP_360698.1	*Carboxydothermus hydrogenoformans*

The final step of the methyl branch of the Wood-Ljungdahl pathway is catalyzed by methylenetetrahydrofolate reductase. In *M. thermoacetica*, this enzyme is oxygen-sensitive and contains an iron-sulfur cluster (Clark and Ljungdahl, J Biol Chem. 259:10845-10849 (1984)). This enzyme is encoded by *metF* in *E. coli* (Sheppard et al., J.Bacteriol. 181:718-725 (1999)) and CHY_1233 in *C. hydrogenoformans* (Wu et al., PLoS Genet. 1:e65 (2005)). The *M. thermoacetica* genes, and its *C. hydrogenoformans* counterpart, are located near the CODH/ACS gene cluster, separated by putative hydrogenase and heterodisulfide reductase genes.

Table 34.

Protein	GenBank ID	Organism
metF	NP_418376.1	*Escherichia coli*
CHY_1233	YP_360071.1	*Carboxydothermus hydrogenoformans*

While *E. coli* naturally possesses the capability for some of the required transformations (*i.e.*, methenyltetrahydrofolate cyclohydrolase, methylenetetrahydrofolate dehydrogenase, methylenetetrahydrofolate reductase), it is thought that the methyl branch enzymes from acetogens may have significantly higher (50 – 100X) specific activities than those from non-acetogens (Morton et al., Genetics and molecular biology of anaerobic bacteria, p. 389-406,

Springer Verlag, New York (1992.)). The formate dehydrogenase also appears to be specialized for anaerobic conditions (Ljungdahl and Andreesen, FEBS Lett. 54:279-282 (1975)). Therefore, various non-native versions of each of these are expressed in the strain of *E. coli* capable of methanol and syngas utilization. Specifically, these genes are cloned and combined into an expression vector designed to express them as a set. Initially, a high or medium copy number vector is chosen (using ColE1 or P15A replicons). One promoter that can be used is a strongly constitutive promoter such as lambda pL or an IPTG-inducible version of this, pL-lacO (Lutz and Bujard, Nucleic Acids Res 25:1203-1210 (1997)). To make an artificial operon, one 5' terminal promoter is placed upstream of the set of genes and each gene receives a consensus rbs element. The order of genes is based on the natural order whenever possible. Ultimately, the genes are integrated into the *E. coli* chromosome. Enzyme assays are performed as described in (Clark and Ljungdahl, J Biol Chem. 259:10845-10849 (1984); Clark and Ljungdahl, Methods Enzymol. 122:392-399 (1986); D'Ari and Rabinowitz, J.Biol.Chem. 266:23953-23958 (1991); de Mata and Rabinowitz, J.Biol.Chem. 255:2569-2577 (1980); Ljungdahl and Andreesen, Methods Enzymol. 53:360-372 (1978); Lovell et al., 1988 Arch.Microbiol 149:280-285 (1988); Yamamoto et al., J.Biol.Chem. 258:1826-1832 (1983)).

After strains of *E. coli* expressing both the carbonyl and methyl branches of the Wood-Ljungdahl pathway are constructed, they are assayed for the ability to utilize syngas consisting of CO, CO_2, and H_2, for incorporation into acetyl-CoA, cell mass, and isopropanol or 1,4-butanediol. Initial conditions employ strictly anaerobically grown cells provided with exogenous glucose as a carbon and energy source. Alternatively, or in addition to glucose, nitrate will be added to the fermentation broth to serve as an electron acceptor and initiator of growth. Anaerobic growth of *E. coli* on fatty acids, which are ultimately metabolized to acetyl-CoA, has been demonstrated in the presence of nitrate (Campbell et al., Molecular Microbiology 47:793-805 (2003)). Oxygen can also be provided as long as its intracellular levels are maintained below any inhibition threshold of the engineered enzymes. 'Synthetic syngas' of a composition suitable for these experiments is employed. ^{13}C-labeled CO and/or CO_2 are provided to the cells and analytical mass spectrometry is employed to measure incorporation of the labeled carbon into acetate, isopropanol, 4-hydroxybutyrate, 1,4-butanediol, and cell mass (e.g., proteinogenic amino acids).

The invention is described herein with general reference to the metabolic reaction, reactant or product thereof, or with specific reference to one or more nucleic acids or genes encoding an enzyme associated with or catalyzing, or a protein associated with, the referenced metabolic reaction, reactant or product. Unless otherwise expressly stated herein, those skilled

in the art will understand that reference to a reaction also constitutes reference to the reactants and products of the reaction. Similarly, unless otherwise expressly stated herein, reference to a reactant or product also references the reaction, and reference to any of these metabolic constituents also references the gene or genes encoding the enzymes that catalyze or proteins involved in the referenced reaction, reactant or product. Likewise, given the well known fields of metabolic biochemistry, enzymology and genomics, reference herein to a gene or encoding nucleic acid also constitutes a reference to the corresponding encoded enzyme and the reaction it catalyzes or a protein associated with the reaction as well as the reactants and products of the reaction.

The non-naturally occurring microbial organisms of the invention can be produced by introducing expressible nucleic acids encoding one or more of the enzymes or proteins participating in one or more isopropanol, 4-hydroxybutyrate, or 1,4-butanediol biosynthetic pathways. Depending on the host microbial organism chosen for biosynthesis, nucleic acids for some or all of a particular isopropanol, 4-hydroxybutyrate, or 1,4-butanediol biosynthetic pathway can be expressed. For example, if a chosen host is deficient in one or more enzymes or proteins for a desired biosynthetic pathway, then expressible nucleic acids for the deficient enzyme(s) or protein(s) are introduced into the host for subsequent exogenous expression. Alternatively, if the chosen host exhibits endogenous expression of some pathway genes, but is deficient in others, then an encoding nucleic acid is needed for the deficient enzyme(s) or protein(s) to achieve isopropanol, 4-hydroxybutyrate, or 1,4-butanediol biosynthesis. Thus, a non-naturally occurring microbial organism of the invention can be produced by introducing exogenous enzyme or protein activities to obtain a desired biosynthetic pathway or a desired biosynthetic pathway can be obtained by introducing one or more exogenous enzyme or protein activities that, together with one or more endogenous enzymes or proteins, produces a desired product such as isopropanol, 4-hydroxybutyrate, or 1,4-butanediol.

Depending on the isopropanol, 4-hydroxybutyrate, or 1,4-butanediol biosynthetic pathway constituents of a selected host microbial organism, the non-naturally occurring microbial organisms of the invention will include at least one exogenously expressed isopropanol, 4-hydroxybutyrate, or 1,4-butanediol pathway-encoding nucleic acid and up to all encoding nucleic acids for one or more isopropanol, 4-hydroxybutyrate, or 1,4-butanediol biosynthetic pathways. For example, isopropanol, 4-hydroxybutyrate, or 1,4-butanediol biosynthesis can be established in a host deficient in a pathway enzyme or protein through exogenous expression of the corresponding encoding nucleic acid. In a host deficient in all enzymes or proteins of an isopropanol, 4-hydroxybutyrate, or 1,4-butanediol pathway,

exogenous expression of all enzyme or proteins in the pathway can be included, although it is understood that all enzymes or proteins of a pathway can be expressed even if the host contains at least one of the pathway enzymes or proteins. For example, exogenous expression of all enzymes or proteins in a pathway for production of isopropanol, 4-hydroxybutyrate, or 1,4-butanediol can be included.

Given the teachings and guidance provided herein, those skilled in the art will understand that the number of encoding nucleic acids to introduce in an expressible form will, at least, parallel the isopropanol, 4-hydroxybutyrate, or 1,4-butanediol pathway deficiencies of the selected host microbial organism. Therefore, a non-naturally occurring microbial organism of the invention can have one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, up to all nucleic acids encoding the enzymes or proteins constituting an isopropanol, 4-hydroxybutyrate, or 1,4-butanediol biosynthetic pathway disclosed herein. In some embodiments, the non-naturally occurring microbial organisms also can include other genetic modifications that facilitate or optimize isopropanol, 4-hydroxybutyrate, or 1,4-butanediol biosynthesis or that confer other useful functions onto the host microbial organism. One such other functionality can include, for example, augmentation of the synthesis of one or more of the isopropanol, 4-hydroxybutyrate, or 1,4-butanediol pathway precursors such as acetyl-CoA.

Generally, a host microbial organism is selected such that it produces the precursor of an isopropanol, 4-hydroxybutyrate, or 1,4-butanediol pathway, either as a naturally produced molecule or as an engineered product that either provides *de novo* production of a desired precursor or increased production of a precursor naturally produced by the host microbial organism. For example, acetyl-CoA is produced naturally in a host organism such as *E. coli*. A host organism can be engineered to increase production of a precursor, as disclosed herein. In addition, a microbial organism that has been engineered to produce a desired precursor can be used as a host organism and further engineered to express enzymes or proteins of an isopropanol, 4-hydroxybutyrate, or 1,4-butanediol pathway.

In some embodiments, a non-naturally occurring microbial organism of the invention is generated from a host that contains the enzymatic capability to synthesize isopropanol, 4-hydroxybutyrate, or 1,4-butanediol. In this specific embodiment it can be useful to increase the synthesis or accumulation of an isopropanol, 4-hydroxybutyrate, or 1,4-butanediol pathway product to, for example, drive isopropanol, 4-hydroxybutyrate, or 1,4-butanediol pathway reactions toward isopropanol, 4-hydroxybutyrate, or 1,4-butanediol production. Increased

synthesis or accumulation can be accomplished by, for example, overexpression of nucleic acids encoding one or more of the above-described isopropanol, 4-hydroxybutyrate, or 1,4-butanediol pathway enzymes or proteins. Over expression the enzyme or enzymes and/or protein or proteins of the isopropanol, 4-hydroxybutyrate, or 1,4-butanediol pathway can occur, for example, through exogenous expression of the endogenous gene or genes, or through exogenous expression of the heterologous gene or genes. Therefore, naturally occurring organisms can be readily generated to be non-naturally occurring microbial organisms of the invention, for example, producing isopropanol, 4-hydroxybutyrate, or 1,4-butanediol, through overexpression of one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, that is, up to all nucleic acids encoding isopropanol, 4-hydroxybutyrate, or 1,4-butanediol biosynthetic pathway enzymes or proteins. In addition, a non-naturally occurring organism can be generated by mutagenesis of an endogenous gene that results in an increase in activity of an enzyme in the isopropanol, 4-hydroxybutyrate, or 1,4-butanediol biosynthetic pathway.

In particularly useful embodiments, exogenous expression of the encoding nucleic acids is employed. Exogenous expression confers the ability to custom tailor the expression and/or regulatory elements to the host and application to achieve a desired expression level that is controlled by the user. However, endogenous expression also can be utilized in other embodiments such as by removing a negative regulatory effector or induction of the gene's promoter when linked to an inducible promoter or other regulatory element. Thus, an endogenous gene having a naturally occurring inducible promoter can be up-regulated by providing the appropriate inducing agent, or the regulatory region of an endogenous gene can be engineered to incorporate an inducible regulatory element, thereby allowing the regulation of increased expression of an endogenous gene at a desired time. Similarly, an inducible promoter can be included as a regulatory element for an exogenous gene introduced into a non-naturally occurring microbial organism.

It is understood that, in methods of the invention, any of the one or more exogenous nucleic acids can be introduced into a microbial organism to produce a non-naturally occurring microbial organism of the invention. The nucleic acids can be introduced so as to confer, for example, an isopropanol, 4-hydroxybutyrate, or 1,4-butanediol biosynthetic pathway onto the microbial organism. Alternatively, encoding nucleic acids can be introduced to produce an intermediate microbial organism having the biosynthetic capability to catalyze some of the required reactions to confer isopropanol, 4-hydroxybutyrate, or 1,4-butanediol biosynthetic capability. For example, a non-naturally occurring microbial organism having an isopropanol, 4-

hydroxybutyrate, or 1,4-butanediol biosynthetic pathway can comprise at least two exogenous nucleic acids encoding desired enzymes or proteins. Thus, it is understood that any combination of two or more enzymes or proteins of a biosynthetic pathway can be included in a non-naturally occurring microbial organism of the invention. Similarly, it is understood that any combination of three or more enzymes or proteins of a biosynthetic pathway can be included in a non-naturally occurring microbial organism of the invention and so forth, as desired, so long as the combination of enzymes and/or proteins of the desired biosynthetic pathway results in production of the corresponding desired product. Similarly, any combination of four, or more enzymes or proteins of a biosynthetic pathway as disclosed herein can be included in a non-naturally occurring microbial organism of the invention, as desired, so long as the combination of enzymes and/or proteins of the desired biosynthetic pathway results in production of the corresponding desired product.

In addition to the biosynthesis of isopropanol, 4-hydroxybutyrate, or 1,4-butanediol as described herein, the non-naturally occurring microbial organisms and methods of the invention also can be utilized in various combinations with each other and with other microbial organisms and methods well known in the art to achieve product biosynthesis by other routes. For example, one alternative to produce isopropanol, 4-hydroxybutyrate, or 1,4-butanediol other than use of the isopropanol, 4-hydroxybutyrate, or 1,4-butanediol producers is through addition of another microbial organism capable of converting an isopropanol, 4-hydroxybutyrate, or 1,4-butanediol pathway intermediate to isopropanol, 4-hydroxybutyrate, or 1,4-butanediol. One such procedure includes, for example, the fermentation of a microbial organism that produces an isopropanol, 4-hydroxybutyrate, or 1,4-butanediol pathway intermediate. The isopropanol, 4-hydroxybutyrate, or 1,4-butanediol pathway intermediate can then be used as a substrate for a second microbial organism that converts the isopropanol, 4-hydroxybutyrate, or 1,4-butanediol pathway intermediate to isopropanol, 4-hydroxybutyrate, or 1,4-butanediol. The isopropanol, 4-hydroxybutyrate, or 1,4-butanediol pathway intermediate can be added directly to another culture of the second organism or the original culture of the isopropanol, 4-hydroxybutyrate, or 1,4-butanediol pathway intermediate producers can be depleted of these microbial organisms by, for example, cell separation, and then subsequent addition of the second organism to the fermentation broth can be utilized to produce the final product without intermediate purification steps.

In other embodiments, the non-naturally occurring microbial organisms and methods of the invention can be assembled in a wide variety of subpathways to achieve biosynthesis of, for example, isopropanol, 4-hydroxybutyrate, or 1,4-butanediol. In these embodiments, biosynthetic

pathways for a desired product of the invention can be segregated into different microbial organisms, and the different microbial organisms can be co-cultured to produce the final product. In such a biosynthetic scheme, the product of one microbial organism is the substrate for a second microbial organism until the final product is synthesized. For example, the biosynthesis of isopropanol, 4-hydroxybutyrate, or 1,4-butanediol can be accomplished by constructing a microbial organism that contains biosynthetic pathways for conversion of one pathway intermediate to another pathway intermediate or the product. Alternatively, isopropanol, 4-hydroxybutyrate, or 1,4-butanediol also can be biosynthetically produced from microbial organisms through co-culture or co-fermentation using two organisms in the same vessel, where the first microbial organism produces an isopropanol, 4-hydroxybutyrate, or 1,4-butanediol intermediate and the second microbial organism converts the intermediate to isopropanol, 4-hydroxybutyrate, or 1,4-butanediol.

Given the teachings and guidance provided herein, those skilled in the art will understand that a wide variety of combinations and permutations exist for the non-naturally occurring microbial organisms and methods of the invention together with other microbial organisms, with the co-culture of other non-naturally occurring microbial organisms having subpathways and with combinations of other chemical and/or biochemical procedures well known in the art to produce isopropanol, 4-hydroxybutyrate, or 1,4-butanediol.

Sources of encoding nucleic acids for an isopropanol, 4-hydroxybutyrate, or 1,4-butanediol pathway enzyme or protein can include, for example, any species where the encoded gene product is capable of catalyzing the referenced reaction. Such species include both prokaryotic and eukaryotic organisms including, but not limited to, bacteria, including archaea and eubacteria, and eukaryotes, including yeast, plant, insect, animal, and mammal, including human. Exemplary species for such sources include, for example, *Escherichia coli*, as well as other exemplary species disclosed herein or available as source organisms for corresponding genes. However, with the complete genome sequence available for now more than 550 species (with more than half of these available on public databases such as the NCBI), including 395 microorganism genomes and a variety of yeast, fungi, plant, and mammalian genomes, the identification of genes encoding the requisite isopropanol, 4-hydroxybutyrate, or 1,4-butanediol biosynthetic activity for one or more genes in related or distant species, including for example, homologues, orthologs, paralogs and nonorthologous gene displacements of known genes, and the interchange of genetic alterations between organisms is routine and well known in the art. Accordingly, the metabolic alterations enabling biosynthesis of isopropanol, 4-hydroxybutyrate, or 1,4-butanediol described herein with reference to a particular organism such as *E. coli* can be

readily applied to other microorganisms, including prokaryotic and eukaryotic organisms alike. Given the teachings and guidance provided herein, those skilled in the art will know that a metabolic alteration exemplified in one organism can be applied equally to other organisms.

In some instances, such as when an alternative isopropanol, 4-hydroxybutyrate, or 1,4-butanediol biosynthetic pathway exists in an unrelated species, isopropanol, 4-hydroxybutyrate, or 1,4-butanediol biosynthesis can be conferred onto the host species by, for example, exogenous expression of a paralog or paralogs from the unrelated species that catalyzes a similar, yet non-identical metabolic reaction to replace the referenced reaction. Because certain differences among metabolic networks exist between different organisms, those skilled in the art will understand that the actual gene usage between different organisms may differ. However, given the teachings and guidance provided herein, those skilled in the art also will understand that the teachings and methods of the invention can be applied to all microbial organisms using the cognate metabolic alterations to those exemplified herein to construct a microbial organism in a species of interest that will synthesize isopropanol, 4-hydroxybutyrate, or 1,4-butanediol.

Host microbial organisms can be selected from, and the non-naturally occurring microbial organisms generated in, for example, bacteria, yeast, fungus or any of a variety of other microorganisms applicable to fermentation processes. Exemplary bacteria include species selected from *Escherichia coli, Klebsiella oxytoca, Anaerobiospirillum succiniciproducens, Actinobacillus succinogenes, Mannheimia succiniciproducens, Rhizobium etli, Bacillus subtilis, Corynebacterium glutamicum, Gluconobacter oxydans, Zymomonas mobilis, Lactococcus lactis, Lactobacillus plantarum, Streptomyces coelicolor, Clostridium acetobutylicum, Pseudomonas fluorescens*, and *Pseudomonas putida*. Exemplary yeasts or fungi include species selected from *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces lactis, Kluyveromyces marxianus, Aspergillus terreus, Aspergillus niger* and *Pichia pastoris*. *E. coli* is a particularly useful host organism since it is a well characterized microbial organism suitable for genetic engineering. Other particularly useful host organisms include yeast such as *Saccharomyces cerevisiae*.

Methods for constructing and testing the expression levels of a non-naturally occurring isopropanol, 4-hydroxybutyrate, or 1,4-butanediol-producing host can be performed, for example, by recombinant and detection methods well known in the art. Such methods can be found described in, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Third Ed., Cold Spring Harbor Laboratory, New York (2001); and Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, MD (1999).

Exogenous nucleic acid sequences involved in a pathway for production of isopropanol, 4-hydroxybutyrate, or 1,4-butanediol can be introduced stably or transiently into a host cell using techniques well known in the art including, but not limited to, conjugation, electroporation, chemical transformation, transduction, transfection, and ultrasound transformation. For exogenous expression in *E. coli* or other prokaryotic cells, some nucleic acid sequences in the genes or cDNAs of eukaryotic nucleic acids can encode targeting signals such as an N-terminal mitochondrial or other targeting signal, which can be removed before transformation into prokaryotic host cells, if desired. For example, removal of a mitochondrial leader sequence led to increased expression in *E. coli* (Hoffmeister et al., *J. Biol. Chem.* 280:4329-4338 (2005)). For exogenous expression in yeast or other eukaryotic cells, genes can be expressed in the cytosol without the addition of leader sequence, or can be targeted to mitochondrion or other organelles, or targeted for secretion, by the addition of a suitable targeting sequence such as a mitochondrial targeting or secretion signal suitable for the host cells. Thus, it is understood that appropriate modifications to a nucleic acid sequence to remove or include a targeting sequence can be incorporated into an exogenous nucleic acid sequence to impart desirable properties. Furthermore, genes can be subjected to codon optimization with techniques well known in the art to achieve optimized expression of the proteins.

An expression vector or vectors can be constructed to include one or more isopropanol, 4-hydroxybutyrate, or 1,4-butanediol biosynthetic pathway encoding nucleic acids as exemplified herein operably linked to expression control sequences functional in the host organism. Expression vectors applicable for use in the microbial host organisms of the invention include, for example, plasmids, phage vectors, viral vectors, episomes and artificial chromosomes, including vectors and selection sequences or markers operable for stable integration into a host chromosome. Additionally, the expression vectors can include one or more selectable marker genes and appropriate expression control sequences. Selectable marker genes also can be included that, for example, provide resistance to antibiotics or toxins, complement auxotrophic deficiencies, or supply critical nutrients not in the culture media. Expression control sequences can include constitutive and inducible promoters, transcription enhancers, transcription terminators, and the like which are well known in the art. When two or more exogenous encoding nucleic acids are to be co-expressed, both nucleic acids can be inserted, for example, into a single expression vector or in separate expression vectors. For single vector expression, the encoding nucleic acids can be operationally linked to one common expression control sequence or linked to different expression control sequences, such as one inducible promoter and one constitutive promoter. The transformation of exogenous nucleic acid sequences involved in a metabolic or synthetic pathway can be confirmed using methods

well known in the art. Such methods include, for example, nucleic acid analysis such as Northern blots or polymerase chain reaction (PCR) amplification of mRNA, or immunoblotting for expression of gene products, or other suitable analytical methods to test the expression of an introduced nucleic acid sequence or its corresponding gene product. It is understood by those skilled in the art that the exogenous nucleic acid is expressed in a sufficient amount to produce the desired product, and it is further understood that expression levels can be optimized to obtain sufficient expression using methods well known in the art and as disclosed herein.

The invention provides a method for producing isopropanol that include s culturing a non-naturally occurring microbial organism having an isopropanol pathway. The pathway includes at least one exogenous nucleic acid encoding an isopropanol pathway enzyme expressed in a sufficient amount to produce isopropanol under conditions and for a sufficient period of time to produce isopropanol. The isopropanol pathway comprising an acetoacetyl-CoA thiolase, an acetoacetyl-CoA:acetate:CoA transferase, an acetoacetate decarboxylase, and an isopropanol dehydrogenase. An alternative isopropanol pathway comprises succinyl-CoA:3-ketoacid CoA transferase, acetoacetate decarboxylase, and an isopropanol dehydrogenase.

In embodiments where an organism has a methanol methyltransferase, culturing can be carried out utilizing a feedstock such as 1) methanol and CO, 2) methanol, CO_2, and H_2, 3) methanol, CO, CO_2, and H_2, 4) methanol and synthesis gas comprising CO and H_2, and 5) methanol and synthesis gas comprising CO, CO_2, and H_2.

In embodiments where an organism has a formate dehydrogenase, a formyltetrahydrofolate synthetase, a methenyltetrahydrofolate cyclohydrolase, a methylenetetrahydrofolate dehydrogenase, and a methylenetetrahydrofolate reductase, the organism can utilize a feedstock such as 1) CO, 2) CO_2 and H_2, 3) CO and CO_2, 4) synthesis gas comprising CO and H_2, and 5) synthesis gas comprising CO, CO_2, and H_2.

Similarly, 4-hydroxybutyrate or 1,4-butanediol can also be produced by culturing the appropriate organisms as described herein above.

Suitable purification and/or assays to test for the production of isopropanol, 4-hydroxybutyrate, or 1,4-butanediol can be performed using well known methods. Suitable replicates such as triplicate cultures can be grown for each engineered strain to be tested. For example, product and byproduct formation in the engineered production host can be monitored. The final product and intermediates, and other organic compounds, can be analyzed by methods such as HPLC (High Performance Liquid Chromatography), GC-MS (Gas Chromatography-

Mass Spectroscopy) and LC-MS (Liquid Chromatography-Mass Spectroscopy) or other suitable analytical methods using routine procedures well known in the art. The release of product in the fermentation broth can also be tested with the culture supernatant. Byproducts and residual glucose can be quantified by HPLC using, for example, a refractive index detector for glucose and alcohols, and a UV detector for organic acids (Lin et al., *Biotechnol. Bioeng.* 90:775-779 (2005)), or other suitable assay and detection methods well known in the art. The individual enzyme or protein activities from the exogenous DNA sequences can also be assayed using methods well known in the art (see, for example, WO/2008/115840 and Hanai et al., Appl Environ Microbiol 73:7814-7818 (2007)).

The isopropanol, 4-hydroxybutyrate, or 1,4-butanediol can be separated from other components in the culture using a variety of methods well known in the art. Such separation methods include, for example, extraction procedures as well as methods that include continuous liquid-liquid extraction, pervaporation, membrane filtration, membrane separation, reverse osmosis, electrodialysis, distillation, crystallization, centrifugation, extractive filtration, ion exchange chromatography, size exclusion chromatography, adsorption chromatography, and ultrafiltration. All of the above methods are well known in the art.

Any of the non-naturally occurring microbial organisms described herein can be cultured to produce and/or secrete the biosynthetic products of the invention. For example, the isopropanol, 4-hydroxybutyrate, or 1,4-butanediol producers can be cultured for the biosynthetic production of isopropanol, 4-hydroxybutyrate, or 1,4-butanediol.

For the production of isopropanol, 4-hydroxybutyrate, or 1,4-butanediol, the recombinant strains are cultured in a medium with carbon source and other essential nutrients. It is highly desirable to maintain anaerobic conditions in the fermenter to reduce the cost of the overall process. Such conditions can be obtained, for example, by first sparging the medium with nitrogen and then sealing the flasks with a septum and crimp-cap. For strains where growth is not observed anaerobically, microaerobic conditions can be applied by perforating the septum with a small hole for limited aeration. Exemplary anaerobic conditions have been described previously and are well-known in the art. Exemplary aerobic and anaerobic conditions are described, for example, in United State Patent application serial No. 11/891,602, filed August 10, 2007. Fermentations can be performed in a batch, fed-batch or continuous manner, as disclosed herein.

If desired, the pH of the medium can be maintained at a desired pH, in particular neutral pH, such as a pH of around 7 by addition of a base, such as NaOH or other bases, or acid, as

needed to maintain the culture medium at a desirable pH. The growth rate can be determined by measuring optical density using a spectrophotometer (600 nm), and the glucose uptake rate by monitoring carbon source depletion over time.

In addition to renewable feedstocks such as those exemplified above, the isopropanol, 4-hydroxybutyrate, or 1,4-butanediol microbial organisms of the invention also can be modified for growth on syngas as its source of carbon. In this specific embodiment, one or more proteins or enzymes are expressed in the isopropanol, 4-hydroxybutyrate, or 1,4-butanediol producing organisms to provide a metabolic pathway for utilization of syngas or other gaseous carbon source.

Although organisms of the present invention are designed to utilize syngas and/or methanol as a growth source, they may also utilize, for example, any carbohydrate source which can supply a source of carbon to the non-naturally occurring microorganism. Such sources include, for example, sugars such as glucose, xylose, arabinose, galactose, mannose, fructose and starch. Other sources of carbohydrate include, for example, renewable feedstocks and biomass. Exemplary types of biomasses that can be used as feedstocks in the methods of the invention include cellulosic biomass, hemicellulosic biomass and lignin feedstocks or portions of feedstocks. Such biomass feedstocks contain, for example, carbohydrate substrates useful as carbon sources such as glucose, xylose, arabinose, galactose, mannose, fructose and starch. Given the teachings and guidance provided herein, those skilled in the art will understand that renewable feedstocks and biomass other than those exemplified above also can be used for culturing the microbial organisms of the invention for the production of isopropanol, 4-hydroxybutyrate, or 1,4-butanediol.

Accordingly, given the teachings and guidance provided herein, those skilled in the art will understand that a non-naturally occurring microbial organism can be produced that secretes the biosynthesized compounds of the invention when grown on a carbon source such as CO and/or CO2. Such compounds include, for example, isopropanol, 4-hydroxybutyrate, or 1,4-butanediol and any of the intermediate metabolites in the isopropanol, 4-hydroxybutyrate, or 1,4-butanediol pathway. All that is required is to engineer in one or more of the required enzyme or protein activities to achieve biosynthesis of the desired compound or intermediate including, for example, inclusion of some or all of the isopropanol, 4-hydroxybutyrate, or 1,4-butanediol biosynthetic pathways. Accordingly, the invention provides a non-naturally occurring microbial organism that produces and/or secretes isopropanol, 4-hydroxybutyrate, or 1,4-butanediol when grown on a carbohydrate or other carbon source and produces and/or secretes any of the

intermediate metabolites shown in the isopropanol, 4-hydroxybutyrate, or 1,4-butanediol pathway when grown on a carbohydrate or other carbon source. The isopropanol, 4-hydroxybutyrate, or 1,4-butanediol producing microbial organisms of the invention can initiate synthesis from an intermediate, for example, acetyl-CoA.

The non-naturally occurring microbial organisms of the invention are constructed using methods well known in the art as exemplified herein to exogenously express at least one nucleic acid encoding an isopropanol, 4-hydroxybutyrate, or 1,4-butanediol pathway enzyme or protein in sufficient amounts to produce isopropanol, 4-hydroxybutyrate, or 1,4-butanediol. It is understood that the microbial organisms of the invention are cultured under conditions sufficient to produce isopropanol, 4-hydroxybutyrate, or 1,4-butanediol. Following the teachings and guidance provided herein, the non-naturally occurring microbial organisms of the invention can achieve biosynthesis of isopropanol, 4-hydroxybutyrate, or 1,4-butanediol resulting in intracellular concentrations between about 0.1-2000 mM or more. Generally, the intracellular concentration of isopropanol, 4-hydroxybutyrate, or 1,4-butanediol is between about 3-1800 mM, particularly between about 5-1700 mM and more particularly between about 8-1600 mM, including about 100 mM, 200 mM, 500 mM, 800 mM, or more. Intracellular concentrations between and above each of these exemplary ranges also can be achieved from the non-naturally occurring microbial organisms of the invention.

In some embodiments, culture conditions include anaerobic or substantially anaerobic growth or maintenance conditions. Exemplary anaerobic conditions have been described previously and are well known in the art. Exemplary anaerobic conditions for fermentation processes are described herein and are described, for example, in U.S. patent application serial No. 11/891,602, filed August 10, 2007. Any of these conditions can be employed with the non-naturally occurring microbial organisms as well as other anaerobic conditions well known in the art. Under such anaerobic conditions, the isopropanol, 4-hydroxybutyrate, or 1,4-butanediol producers can synthesize isopropanol, 4-hydroxybutyrate, or 1,4-butanediol at intracellular concentrations of 5-10 mM or more as well as all other concentrations exemplified herein. It is understood that, even though the above description refers to intracellular concentrations, isopropanol, 4-hydroxybutyrate, or 1,4-butanediol producing microbial organisms can produce isopropanol, 4-hydroxybutyrate, or 1,4-butanediol intracellularly and/or secrete the product into the culture medium.

The culture conditions can include, for example, liquid culture procedures as well as fermentation and other large scale culture procedures. As described herein, particularly useful

yields of the biosynthetic products of the invention can be obtained under anaerobic or substantially anaerobic culture conditions.

As described herein, one exemplary growth condition for achieving biosynthesis of isopropanol, 4-hydroxybutyrate, or 1,4-butanediol includes anaerobic culture or fermentation conditions. In certain embodiments, the non-naturally occurring microbial organisms of the invention can be sustained, cultured or fermented under anaerobic or substantially anaerobic conditions. Briefly, anaerobic conditions refers to an environment devoid of oxygen. Substantially anaerobic conditions include, for example, a culture, batch fermentation or continuous fermentation such that the dissolved oxygen concentration in the medium remains between 0 and 10% of saturation. Substantially anaerobic conditions also includes growing or resting cells in liquid medium or on solid agar inside a sealed chamber maintained with an atmosphere of less than 1% oxygen. The percent of oxygen can be maintained by, for example, sparging the culture with an N_2/CO_2 mixture or other suitable non-oxygen gas or gases.

The culture conditions described herein can be scaled up and grown continuously for manufacturing of isopropanol, 4-hydroxybutyrate, or 1,4-butanediol. Exemplary growth procedures include, for example, fed-batch fermentation and batch separation; fed-batch fermentation and continuous separation, or continuous fermentation and continuous separation. All of these processes are well known in the art. Fermentation procedures are particularly useful for the biosynthetic production of commercial quantities of isopropanol, 4-hydroxybutyrate, or 1,4-butanediol. Generally, and as with non-continuous culture procedures, the continuous and/or near-continuous production of isopropanol, 4-hydroxybutyrate, or 1,4-butanediol will include culturing a non-naturally occurring isopropanol, 4-hydroxybutyrate, or 1,4-butanediol producing organism of the invention in sufficient nutrients and medium to sustain and/or nearly sustain growth in an exponential phase. Continuous culture under such conditions can be include, for example, 1 day, 2, 3, 4, 5, 6 or 7 days or more. Additionally, continuous culture can include 1 week, 2, 3, 4 or 5 or more weeks and up to several months. Alternatively, organisms of the invention can be cultured for hours, if suitable for a particular application. It is to be understood that the continuous and/or near-continuous culture conditions also can include all time intervals in between these exemplary periods. It is further understood that the time of culturing the microbial organism of the invention is for a sufficient period of time to produce a sufficient amount of product for a desired purpose.

Fermentation procedures are well known in the art. Briefly, fermentation for the biosynthetic production of isopropanol, 4-hydroxybutyrate, or 1,4-butanediol can be utilized in,

for example, fed-batch fermentation and batch separation; fed-batch fermentation and continuous separation, or continuous fermentation and continuous separation. Examples of batch and continuous fermentation procedures are well known in the art.

In addition to the above fermentation procedures using the isopropanol, 4-hydroxybutyrate, or 1,4-butanediol producers of the invention for continuous production of substantial quantities of isopropanol, 4-hydroxybutyrate, or 1,4-butanediol, the isopropanol, 4-hydroxybutyrate, or 1,4-butanediol producers also can be, for example, simultaneously subjected to chemical synthesis procedures to convert the product to other compounds or the product can be separated from the fermentation culture and sequentially subjected to chemical conversion to convert the product to other compounds, if desired.

Important process considerations for a syngas fermentation are high biomass concentration and good gas-liquid mass transfer (Bredwell et al., Biotechnol Prog. 15:834-844 (1999). The solubility of CO in water is somewhat less than that of oxygen. Continuously gas-sparged fermentations can be performed in controlled fermenters with constant off-gas analysis by mass spectrometry and periodic liquid sampling and analysis by GC and HPLC. The liquid phase can function in batch mode. Fermentation products such as alcohols, organic acids, and residual glucose along with residual methanol are quantified by HPLC (Shimadzu, Columbia MD), for example, using an Aminex® series of HPLC columns (for example, HPX-87 series) (BioRad, Hercules CA), using a refractive index detector for glucose and alcohols, and a UV detector for organic acids. The growth rate is determined by measuring optical density using a spectrophotometer (600 nm). All piping in these systems is glass or metal to maintain anaerobic conditions. The gas sparging is performed with glass frits to decrease bubble size and improve mass transfer. Various sparging rates are tested, ranging from about 0.1 to 1 vvm (vapor volumes per minute). To obtain accurate measurements of gas uptake rates, periodic challenges are performed in which the gas flow is temporarily stopped, and the gas phase composition is monitored as a function of time.

In order to achieve the overall target productivity, methods of cell retention or recycle are employed. One method to increase the microbial concentration is to recycle cells via a tangential flow membrane from a sidestream. Repeated batch culture can also be used, as previously described for production of acetate by *Moorella* (Sakai et al., J Biosci.Bioeng 99:252-258 (2005)). Various other methods can also be used (Bredwell et al., Biotechnol Prog. 15:834-844 (1999); Datar et al., Biotechnol Bioeng 86:587-594 (2004)). Additional optimization can be

tested such as overpressure at 1.5 atm to improve mass transfer (Najafpour and Younesi, Enzyme and Microbial Technology 38[1-2], 223-228 (2006)).

Once satisfactory performance is achieved using pure H_2/CO as the feed, synthetic gas mixtures are generated containing inhibitors likely to be present in commercial syngas. For example, a typical impurity profile is 4.5% CH_4, 0.1% C_2H_2, 0.35% C_2H_6, 1.4% C_2H_4, and 150 ppm nitric oxide (Datar et al., Biotechnol Bioeng 86:587-594 (2004)). Tars, represented by compounds such as benzene, toluene, ethylbenzene, p-xylene, o-xylene, and naphthalene, are added at ppm levels to test for any effect on production. For example, it has been shown that 40 ppm NO is inhibitory to *C. carboxidivorans* (Ahmed and Lewis, Biotechnol Bioeng 97:1080-1086 (2007)). Cultures are tested in shake-flask cultures before moving to a fermentor. Also, different levels of these potential inhibitory compounds are tested to quantify the effect they have on cell growth. This knowledge is used to develop specifications for syngas purity, which is utilized for scale up studies and production. If any particular component is found to be difficult to decrease or remove from syngas used for scale up, an adaptive evolution procedure is utilized to adapt cells to tolerate one or more impurities.

To generate better producers, metabolic modeling can be utilized to optimize growth conditions. Modeling can also be used to design gene knockouts that additionally optimize utilization of the pathway (see, for example, U.S. patent publications US 2002/0012939, US 2003/0224363, US 2004/0029149, US 2004/0072 723, US 2003/0059792, US 2002/0168654 and US 2004/0009466, and U.S. Patent No. 7,127,379). Modeling analysis allows reliable predictions of the effects on cell growth of shifting the metabolism towards more efficient production of isopropanol, 4-hydroxybutyrate, or 1,4-butanediol.

One computational method for identifying and designing metabolic alterations favoring biosynthesis of a desired product is the OptKnock computational framework (Burgard et al., *Biotechnol. Bioeng.* 84:647-657 (2003)). OptKnock is a metabolic modeling and simulation program that suggests gene deletion strategies that result in genetically stable microorganisms which overproduce the target product. Specifically, the framework examines the complete metabolic and/or biochemical network of a microorganism in order to suggest genetic manipulations that force the desired biochemical to become an obligatory byproduct of cell growth. By coupling biochemical production with cell growth through strategically placed gene deletions or other functional gene disruption, the growth selection pressures imposed on the engineered strains after long periods of time in a bioreactor lead to improvements in performance as a result of the compulsory growth-coupled biochemical production. Lastly, when gene

deletions are constructed there is a negligible possibility of the designed strains reverting to their wild-type states because the genes selected by OptKnock are to be completely removed from the genome. Therefore, this computational methodology can be used to either identify alternative pathways that lead to biosynthesis of a desired product or used in connection with the non-naturally occurring microbial organisms for further optimization of biosynthesis of a desired product.

Briefly, OptKnock is a term used herein to refer to a computational method and system for modeling cellular metabolism. The OptKnock program relates to a framework of models and methods that incorporate particular constraints into flux balance analysis (FBA) models. These constraints include, for example, qualitative kinetic information, qualitative regulatory information, and/or DNA microarray experimental data. OptKnock also computes solutions to various metabolic problems by, for example, tightening the flux boundaries derived through flux balance models and subsequently probing the performance limits of metabolic networks in the presence of gene additions or deletions. OptKnock computational framework allows the construction of model formulations that enable an effective query of the performance limits of metabolic networks and provides methods for solving the resulting mixed-integer linear programming problems. The metabolic modeling and simulation methods referred to herein as OptKnock are described in, for example, U.S. publication 2002/0168654, filed January 10, 2002, in International Patent No. PCT/US02/00660, filed January 10, 2002, and U.S. patent application serial No. 11/891,602, filed August 10, 2007.

Another computational method for identifying and designing metabolic alterations favoring biosynthetic production of a product is a metabolic modeling and simulation system termed SimPheny®. This computational method and system is described in, for example, U.S. publication 2003/0233218, filed June 14, 2002, and in International Patent Application No. PCT/US03/18838, filed June 13, 2003. SimPheny® is a computational system that can be used to produce a network model *in silico* and to simulate the flux of mass, energy or charge through the chemical reactions of a biological system to define a solution space that contains any and all possible functionalities of the chemical reactions in the system, thereby determining a range of allowed activities for the biological system. This approach is referred to as constraints-based modeling because the solution space is defined by constraints such as the known stoichiometry of the included reactions as well as reaction thermodynamic and capacity constraints associated with maximum fluxes through reactions. The space defined by these constraints can be interrogated to determine the phenotypic capabilities and behavior of the biological system or of its biochemical components.

These computational approaches are consistent with biological realities because biological systems are flexible and can reach the same result in many different ways. Biological systems are designed through evolutionary mechanisms that have been restricted by fundamental constraints that all living systems must face. Therefore, constraints-based modeling strategy embraces these general realities. Further, the ability to continuously impose further restrictions on a network model via the tightening of constraints results in a reduction in the size of the solution space, thereby enhancing the precision with which physiological performance or phenotype can be predicted.

Given the teachings and guidance provided herein, those skilled in the art will be able to apply various computational frameworks for metabolic modeling and simulation to design and implement biosynthesis of a desired compound in host microbial organisms. Such metabolic modeling and simulation methods include, for example, the computational systems exemplified above as SimPheny® and OptKnock. For illustration of the invention, some methods are described herein with reference to the OptKnock computation framework for modeling and simulation. Those skilled in the art will know how to apply the identification, design and implementation of the metabolic alterations using OptKnock to any of such other metabolic modeling and simulation computational frameworks and methods well known in the art.

The methods described above will provide one set of metabolic reactions to disrupt. Elimination of each reaction within the set or metabolic modification can result in a desired product as an obligatory product during the growth phase of the organism. Because the reactions are known, a solution to the bilevel OptKnock problem also will provide the associated gene or genes encoding one or more enzymes that catalyze each reaction within the set of reactions. Identification of a set of reactions and their corresponding genes encoding the enzymes participating in each reaction is generally an automated process, accomplished through correlation of the reactions with a reaction database having a relationship between enzymes and encoding genes.

Once identified, the set of reactions that are to be disrupted in order to achieve production of a desired product are implemented in the target cell or organism by functional disruption of at least one gene encoding each metabolic reaction within the set. One particularly useful means to achieve functional disruption of the reaction set is by deletion of each encoding gene. However, in some instances, it can be beneficial to disrupt the reaction by other genetic aberrations including, for example, mutation, deletion of regulatory regions such as promoters or cis binding sites for regulatory factors, or by truncation of the coding sequence at any of a number of

locations. These latter aberrations, resulting in less than total deletion of the gene set can be useful, for example, when rapid assessments of the coupling of a product are desired or when genetic reversion is less likely to occur.

To identify additional productive solutions to the above described bilevel OptKnock problem which lead to further sets of reactions to disrupt or metabolic modifications that can result in the biosynthesis, including growth-coupled biosynthesis of a desired product, an optimization method, termed integer cuts, can be implemented. This method proceeds by iteratively solving the OptKnock problem exemplified above with the incorporation of an additional constraint referred to as an integer cut at each iteration. Integer cut constraints effectively prevent the solution procedure from choosing the exact same set of reactions identified in any previous iteration that obligatorily couples product biosynthesis to growth. For example, if a previously identified growth-coupled metabolic modification specifies reactions 1, 2, and 3 for disruption, then the following constraint prevents the same reactions from being simultaneously considered in subsequent solutions. The integer cut method is well known in the art and can be found described in, for example, Burgard et al., *Biotechnol. Prog.* 17:791-797 (2001). As with all methods described herein with reference to their use in combination with the OptKnock computational framework for metabolic modeling and simulation, the integer cut method of reducing redundancy in iterative computational analysis also can be applied with other computational frameworks well known in the art including, for example, SimPheny®.

The methods exemplified herein allow the construction of cells and organisms that biosynthetically produce a desired product, including the obligatory coupling of production of a target biochemical product to growth of the cell or organism engineered to harbor the identified genetic alterations. Therefore, the computational methods described herein allow the identification and implementation of metabolic modifications that are identified by an *in silico* method selected from OptKnock or SimPheny®. The set of metabolic modifications can include, for example, addition of one or more biosynthetic pathway enzymes and/or functional disruption of one or more metabolic reactions including, for example, disruption by gene deletion.

As discussed above, the OptKnock methodology was developed on the premise that mutant microbial networks can be evolved towards their computationally predicted maximum-growth phenotypes when subjected to long periods of growth selection. In other words, the approach leverages an organism's ability to self-optimize under selective pressures. The OptKnock framework allows for the exhaustive enumeration of gene deletion combinations that

force a coupling between biochemical production and cell growth based on network stoichiometry. The identification of optimal gene/reaction knockouts requires the solution of a bilevel optimization problem that chooses the set of active reactions such that an optimal growth solution for the resulting network overproduces the biochemical of interest (Burgard et al., *Biotechnol. Bioeng.* 84:647-657 (2003)).

An *in silico* stoichiometric model of *E. coli* metabolism can be employed to identify essential genes for metabolic pathways as exemplified previously and described in, for example, U.S. patent publications US 2002/0012939, US 2003/0224363, US 2004/0029149, US 2004/0072723, US 2003/0059792, US 2002/0168 654 and US 2004/0009466, and in U.S. Patent No. 7,127,379. As disclosed herein, the OptKnock mathematical framework can be applied to pinpoint gene deletions leading to the growth-coupled production of a desired product. Further, the solution of the bilevel OptKnock problem provides only one set of deletions. To enumerate all meaningful solutions, that is, all sets of knockouts leading to growth-coupled production formation, an optimization technique, termed integer cuts, can be implemented. This entails iteratively solving the OptKnock problem with the incorporation of an additional constraint referred to as an integer cut at each iteration, as discussed above.

It is understood that modifications which do not substantially affect the activity of the various embodiments of this invention are also included within the definition of the invention provided herein. Accordingly, the following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

ACS/CODH GENE INSERTIONS IN E. COLI

This example describes the creation of *E. coli* plasmids that express the *M. thermoacetica* ACS/CODH operon genes including those required for CODH, ACS, methyltransferase, and the corrinoid iron-sulfur protein. This example further describes the expression these in *E. coli* resulting in observable CO oxidation activity, methyltransferase activity, and corrinoid iron-sulfur protein activity. Finally, this example demonstrates that *E. coli* tolerates high CO concentrations, and may even consume CO when the CO-utilizing gene products from *M. thermoacetica* are expressed.

Expression vectors were chosen from the set described by Lutz and Bujard (Lutz and Bujard, Nucleic Acids Res 25:1203-1210 (1997)); these come with compatible replicons that cover a range of copy numbers. Additionally, each contains prA1-laco1; this T7 early gene

promoter is inducible by IPTG and can lead to very high levels of transcription in the presence of IPTG and represses in other conditions. The ACS/CODH-encoding operon was cloned from Moth_1204 (*cooC*) to Moth_1197; a second version containing only Moth_1203 to Moth_1197 was also constructed. Both of these fragments (10 – 11 kbp) were confirmed by DNA sequence analysis. These were constructed in both p15A and ColE1-based vectors for medium to high copy numbers.

To estimate the final concentrations recombinant proteins, SDS-PAGE followed by Western blot analyses were performed on the same cell extracts used in the CO oxidation, ACS, methyltransferase, and corrinoid Fe-S assays. The antisera used were polyclonal to purified *M. thermoacetica* ACS/CODH and Mtr proteins and were visualized using an alkaline phosphatase-linked goat-anti-rabbit secondary antibody. The Westerns Blots are shown in Figure 4A and 4B. Amounts of CODH in ACS90 and ACS91 were estimated at 50 ng by comparison to the control lanes.

A carbon monoxide oxidation assay (Seravalli et al., Biochemistry 43:3944-3955 (2004)) was used to test whether or not functional expression of the CODH-encoding genes from *M. thermoacetica* was achieved. Cultures of *E. coli* MG1655 containing either an empty vector, or the vectors expressing "Acs90" or "Acs91" were grown in Terrific Broth under anaerobic conditions (with supplements of cyanocobalamin, ferrous iron, and reducing agents) until reaching medium to high density at which point, IPTG was added to a final concentration of 0.2 mM to induce the promoter. After 3.5 hrs of growth at 37 C, the cells were harvested and spun down prior to lysis with lysozyme and mild detergents. There is a benchmark figure of *M. thermoacetica* CODH specific activity, 500 U at 55C or ~60U at 25C. This assay employed reduction of methyl viologen in the presence of CO. This is measured at 578 nm in stoppered, anaerobic, glass cuvettes. Reactions positive for CO oxidation by CODH turned a deep violet color (see Figure 5). About 0.5% of the cellular protein was CODH as estimated by Western blotting; therefore, the data in Table 35 are approximately 50X less than the 500 U/mg activity of pure *M. thermoacetica* CODH. Nevertheless, this experiment did clearly demonstrate CO oxidation activity in recombinant *E. coli* with a much smaller amount in the negative controls. The small amount of CO oxidation (CH_3 viologen reduction) seen in the negative controls indicates that *E. coli* may have a limited ability to reduce CH_3 viologen.

Table 35.

ACS90	7.7 mg/ml	ACS91	11.8 mg/ml	
Mta98	9.8 mg/ml	Mta99	11.2 mg/ml	
Extract	**Vol**	**OD/**	**U/ml**	**U/mg**
ACS90	10 microliters	0.073	0.376	0.049
ACS91	10 microliters	0.096	0.494	0.042
Mta99	10 microliters	0.0031	0.016	0.0014
ACS90	10 microliters	0.099	0.51	0.066
Mta99	25 microliters	0.012	0.025	0.0022
ACS91	25 microliters	0.215	0.443	0.037
Mta98	25 microliters	0.019	0.039	0.004
ACS91	10 microliters	0.129	0.66	0.056
Averages				
ACS90	0.057 U/mg			
ACS91	0.045 U/mg			
Mta99	0.0018 U/mg			

This assay is an *in vitro* reaction that synthesizes acetyl-CoA from methyl-tetrahydrofolate, CO, and CoA using ACS/CODH, methyltransferase, and CFeSP (Raybuck et al., Biochemistry 27:7698-7702 (1988)). By adding or leaving out each of the enzymes involved, this assay can be used for a wide range of experiments, from testing one or more purified enzymes or cell extracts for activity, to determining the kinetics of the reaction under various conditions or with limiting amounts of substrate or enzyme. Samples of the reaction taken at various time points are quenched with 1M HCl, which liberates acetate from the acetyl-CoA end product. After purification with Dowex columns, the acetate can be analyzed by chromatography, mass spectrometry, or by measuring radioactivity. The exact method will be determined by the specific substrates used in the reaction.

This assay was run in order to determine if the ACS/CODH operon expressed in *E. coli* expresses the Fe-S corrinoid protein activity. Therefore, 14C-labeled methyl-THF was used as a labeled substrate to measure acetate synthesis by radioactivity incorporation into isolated acetate samples. Six different conditions were tested:

Purified ACS/CODH, MeTr, and CFeSP as a positive control
Purified ACS/CODH with ACS90 cell extract
Purified ACS/CODH with ACS91 cell extract
Purified ACS/CODH, MeTr with ACS90 cell extract
Purified ACS/CODH, MeTr with ACS91 cell extract
Purified ACS/CODH, MeTr with as much ACS91 cell extract as possible (excluding the MES buffer)

The reaction was assembled in the anaerobic chamber in assay vials filled with CO. The total reaction volume was small compared to the vial volume, reagents were added prior to filling with CO, a gas-tight Hamilton syringe was used and the reagents were kept anaerobic. The reaction (~60ul total) consisted of the cell extract (except #1), CoA, Ti(III)citrate, MES (except #6), purified ACS/CODH, 14C-methyl-tetrahydrofolate, methyl-viologen, and ferredoxin. Additionally, purified MeTr was added to #1, #4-6 and purified CFeSP was added to #1.

The reaction was carried out in the anaerobic chamber in a sand bath at 55°. The final reagent added was the 14C-methyl-tetrahydrofolate, which started the reaction (t = 0s). An initial sample was taken immediately, followed by samples at 30 minutes, 1 hour, and 2 hours. These time points are not exact, as the 6 conditions were run concurrently (since this experiment was primarily a qualitative one). The 15ul samples were added to 15ul of 1M HCl in scintillation vials. After counting the reaction mixtures, it was determined that the corrinoid Fe-S protein in ACS90 extracts was active with total activity approaching approximately 1/5 of the positive control.

Within the ACS/CODH operon is encoded an essential methyltransferase activity that catalyzes the transfer of CH_3 from methyl-tetrahydrofolate to the ACS complex as part of the synthesis of acetyl-CoA (i.e. this is the step that the methyl and carbonyl paths join together). Within the operon in *M. thermoacetica*, the Mtr-encoding gene is Moth_1197 and comes after the main CODH and ACS subunits. Therefore, Mtr activity would constitute indirect evidence that the more proximal genes can be expressed.

Mtr activity was assayed by spectroscopy. Specifically, methylated CFeSP, with Co(III), has a small absorption peak at ~450nm, while non-methylated CFeSP, with Co(I), has a large peak at ~390nm. This spectrum is due to both the cobalt and iron-sulfur cluster chromophores. Additionally, it should be noted that the CFeSP can spontaneously oxidize to Co(II), which creates a broad absorption peak at ~470nm (Seravalli et al., Biochemistry 38:5728-5735 (1999)). See Figure 6 for the results from *E. coli* cells containing ACS90.

To test whether or not *E. coli* can grow anaerobically in the presence of saturating amounts of CO, 120 ml serum bottles with 50 ml of Terrific Broth medium (plus $NiCl_2$, $Fe(II)NH_4SO_4$, and cyanocobalamin) were made in anaerobic conditions. One half of these bottles were equilibrated with nitrogen gas for 30 min. and one half was equilibrated with CO gas for 30 min. An empty vector (pZA33) was used as a control and that and both ACS90 and ACS91 were tested with both N_2 and CO. All were grown for 36 hrs with shaking (250 rpm) at 37C. At the end of the 36 period, examination of the flasks showed high amounts of growth in

all (Figure 7). The bulk of the observed growth occurred overnight with a long lag of some (low but visible) density. Inocula sizes were ~0.5 ml from *E. coli* stocks.

The final CO concentrations are measured using an assay of the spectral shift of myoglobin upon exposure to CO. Myoglobin reduced with sodium dithionite has an absorbance peak at 435 nm; this peak is shifted to 423 nm with CO. Due to the low wavelength (and need to record a whole spectrum from 300 nm on upwards) quartz cuvettes must be used. CO concentration is measured against a standard curve and depends upon the Henry's Law constant for CO of maximum water solubility = 970 micromolar at 20C and 1 atm.

The results shown in Table 36 are very encouraging. Growth reached similar levels (by visual inspection) whether or not a strain was cultured in the presence of CO or not. Furthermore, the negative control had a final CO concentration of 930 micromolar vs. 688 and 728 micromolar for the ACS/CODH operon expressing strains. Clearly, the error in these measurements is high given the large standard deviations. Nevertheless, this test does allow two tentative conclusions: **1)** *E. coli* can tolerate exposure to CO under anaerobic conditions, and **2)** *E. coli* cells expressing the ACS/CODH operon might be metabolizing some of the CO. The second conclusion is significantly less certain than the first.

Table 36.

Stain and Growth Conditions pZA33-CO	Final CO concentration (micromolar)
ACS90-CO	638
	494
	734
	883
ave	687
SD	164
ACS91-CO	728
	812
	760
	611
ave.	728
SD	85

Throughout this application various publications have been referenced within parentheses. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

Although the invention has been described with reference to the disclosed embodiments, those skilled in the art will readily appreciate that the specific examples and studies detailed above are only illustrative of the invention. It should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

What is claimed is:

1. A non-naturally occurring microbial organism, comprising a microbial organism having an isopropanol pathway comprising at least one exogenous nucleic acid encoding an isopropanol pathway enzyme expressed in a sufficient amount to produce isopropanol, said isopropanol pathway enzyme comprising a succinyl-CoA:3-ketoacid-CoA transferase.

2. The organism of claim 1, wherein said succinyl-CoA:3-ketoacid-CoA transferase is encoded by one or more of the genes selected from the group consisting of *HPAG1_0676, HPAG1_0677, ScoA, ScoB, OXCT1, and OXCT2.*

3. The organism of claim 1, further comprising an acetoacetyl-CoA thiolase, an acetoacetate decarboxylase, and an isopropanol dehydrogenase.

4. The organism of claim 3, wherein said acetoacetyl-CoA thiolase is encoded by a gene selected from the group consisting of *atoB*, *thlA*, *thlB*, and *erg10.*

5. The organism of claim 3,wherein said acetoacetate decarboxylase is encoded by the gene *adc.*

6. The organism of claim 3, wherein said isopropanol dehydrogenase is encoded by a gene selected from the group consisting of *adh* and *ipdh.*

7. The organism of claim 1, further comprising at least one enzyme or polypeptide selected from the group consisting of a corrinoid protein, a methyltetrahydrofolate:corrinoid protein methyltransferase, a corrinoid iron-sulfur protein, a nickel-protein assembly protein, a ferredoxin, an acetyl-CoA synthase, a carbon monoxide dehydrogenase, a pyruvate ferredoxin oxidoreductase,and a hydrogenase.

8. The organism of claim 7, wherein said corrinoid protein is encoded by a gene selected from the group consisting of *mtaC*, *mtaC1*, *mtaC2*, and *mtaC3.*

9. The organism of claim 7, wherein said methyltetrahydrofolate:corrinoid protein methyltransferase is encoded by a gene selected from the group consisting of *mtaA*, and *mtaA1*, *mtaA2.*

10. The organism of claim 7, wherein said methyltetrahydrofolate:corrinoid protein methyltransferase is encoded by the gene *acsE*.

11. The organism of claim 7, wherein said corrinoid iron-sulfur protein is encoded by the gene acsD.

12. The organism of claim 7, wherein said nickel-protein assembly protein is encoded by at least one gene selected from the group consisting of *acsF* and *cooC*.

13. The organism of claim 7, wherein said ferredoxin is encoded by the gene *orf7*.

14. The organism of claim 7, wherein said acetyl-CoA synthase is encoded by at least one gene selected from the group consisting of *acsB* and *acsC*.

15. The organism of claim 7, wherein said carbon monoxide dehydrogenase is encoded by the gene *acsA*.

16. The organism of claim 7, wherein said pyruvate ferredoxin oxidoreductase is encoded by a gene selected from the group consisting of *por* and *ydbK*.

17. The organism of claim 7, wherein said hydrogenase is encoded by at least one gene selected from the group consisting of *hypA*, *hypB*, *hypC*, *hypD*, *hypE*, *hypF*, *moth_2175*, *moth_2176*, *moth_2177*, *moth_2178*, *moth_2179*, *moth_2180*, *moth_2181*, *hycA*, *hycB*, *hycC*, *hycD*, *hycE*, *hycF*, *hycG*, *hycH*, *hycI*, *hyfA*, *hyfB*, *hyfC*, *hyfD*, *hyfE*, *hyfF*, *hyfG*, *hyfH*, *hyfI*, *hyfJ*, *hyfR*, *moth_2182*, *moth_2183*, *moth_2184*, *moth_2185*, *moth_2186*, *moth_2187*, *moth_2188*, *moth_2189*, *moth_2190*, *moth_2191*, *moth_2192*, *moth_0439*, *moth_0440*, *moth_0441*, *moth_0442*, *moth_0809*, *moth_0810*, *moth_0811*, *moth_0812*, *moth_0813*, *moth_0814*, *moth_0815*, *moth_0816*, *moth_1193*, *moth_1194*, *moth_1195*, *moth_1196*, *moth_1717*, *moth_1718*, *moth_1719*, *moth_1883*, *moth_1884*, *moth_1885*, *moth_1886*, *moth_1887*, *moth_1888*, *moth_1452*, *moth_1453*, and *moth_1454*.

18. The organism of claim 7, further comprising the polypetide AcsEps encoded by the gene *acsEps*.

19. The organism of claim 7, further comprising at least one enzyme or polypeptide encoded by a gene selected from the group consisting of *codh*, *codh-I*, *cooF*, *hypA*, *cooH*, *cooU*, *cooX*, *cooL*, *cooK*, *cooM*, *cooT*, *cooJ*, and *codh-II*.

20. The organism of claim 7, further comprising a methanol methyltransferase.

21. The organism of claim 20, wherein said methanol methyltransferase is encoded by a gene selected from the group consisting of *mtaB*, *mtaB1*, *mtaB2*, and *mtaB3*.

22. The organism of claim 20, wherein said organism utilizes a feedstock selected from the group consisting of: 1) methanol and CO, 2) methanol, CO_2, and H_2, 3) methanol, CO, CO_2, and H_2, 4) methanol and synthesis gas comprising CO and H_2, and 5) methanol and synthesis gas comprising CO, CO_2, and H_2.

23. The organism of claim 7, further comprising a formate dehydrogenase, a formyltetrahydrofolate synthetase, a methenyltetrahydrofolate cyclohydrolase, a methylenetetrahydrofolate dehydrogenase, and a methylenetetrahydrofolate reductase.

24. The organism of claim 23, wherein said formate dehydrogenase is encoded by a gene selected from the group consisting of *moth_2312*, *moth _2313*, *moth_2314*, *sfum_2703*, *sfum_2704*, *sfum_2705*, *sfum_2706*, *chy_0731*, *chy_0732*, and *chy_0733*.

25. The organism of claim 23, wherein said formyltetrahydrofolate synthetase is encoded by a gene selected from the group consisting of *moth_0109*, *chy_2385*, and *fhs*.

26. The organism of claim 23, wherein said methenyltetrahydrofolate cyclohydrolase is encoded by a gene selected from the group consisting of *moth_1516*, *folD*, and *chy_1878*.

27. The organism of claim 23, wherein said methylenetetrahydrofolate dehydrogenase is encoded by a gene selected from the group consisting of *moth_1516*, *folD*, and *chy_1878*.

28. The organism of claim 23, wherein said methylenetetrahydrofolate reductase is encoded by a gene selected from the group consisting of *moth_1191*, *metF*, and *chy_1233*.

29. The organism of claim 23, wherein said organism utilizes a feedstock selected from the group consisting of: 1) CO, 2) CO_2 and H_2, 3) CO and CO_2, 4) synthesis gas comprising CO and H_2, and 5) synthesis gas comprising CO, CO_2, and H_2.

30. A non-naturally occurring microbial organism, comprising a microbial organism having a 4-hydroxybutryate pathway comprising at least one exogenous nucleic acid encoding an 4-hydroxybutryate pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutryate, said 4-hydroxybutryate pathway enzyme comprising an acetoacetyl-CoA thiolase, a 3-hydroxybutyryl-CoA dehydrogenase, a crotonase, a crotonyl-CoA hydratase, a 4-hydroxybutyryl-CoA transferase, a phosphotrans-4-hydroxybutyrylase, and a 4-hydroxybutyrate kinase.

31. The organism of claim 30, wherein said Acetoacetyl-CoA thiolase is encoded by a gene selected from the group consisting of *atoB*, *thlA*, *thlB*, and *erg10*.

32. The organism of claim 30, wherein said 3-Hydroxybutyryl-CoA dehydrogenase is encoded by a gene selected from the group consisting of *hbd*, *msed_1423*, *msed_0399*, *msed_0389*, and *msed_1933*.

33. The organism of claim 30, wherein said Crotonase is encoded by a gene selected from the group consisting of *crt*, *paaA*, *paaB*, *phaA*, *phaB*, *maoC*, *paaF*, and *paaG*.

34. The organism of claim 30, wherein said crotonyl-CoA hydratase is encoded by a gene selected from the group consisting of *abfD*, *msed_1321*, and *msed_1220*.

35. The organism of claim 30, wherein said 4-hydroxybutyryl-CoA transferase is encoded by a gene selected from the group consisting of *cat2*, *abfT-2*, *abfT-1*, and *abfT*.

36. The organism of claim 30, wherein said phosphotrans-4-hydroxybutyrylase is encoded by a gene selected from the group consisting of *pta* and *ptb*.

37. The organism of claim 30, wherein said 4-hydroxybutyrate kinase is encoded by a gene selected from the group consisting of *ackA*, *buk1*, *buk2*, and *proB*.

38. The organism of claim 30, further comprising at least one enzyme or polypeptide selected from the group consisting of, a corrinoid protein, a methyltetrahydrofolate:corrinoid protein methyltransferase, a corrinoid iron-sulfur protein, a nickel-protein assembly protein, a ferredoxin, an acetyl-CoA synthase, a carbon monoxide dehydrogenase, a pyruvate ferredoxin oxidoreductase,and a hydrogenase.

39. The organism of claim 38, wherein said corrinoid protein is encoded by a gene selected from the group consisting of *mtaC*, *mtaC1*, *mtaC2*, and *mtaC3*.

40. The organism of claim 38, wherein said methyltetrahydrofolate:corrinoid protein methyltransferase is encoded by a gene selected from the group consisting of *mtaA*, and *mtaA1*, *mtaA2*.

41. The organism of claim 38, wherein said methyltetrahydrofolate:corrinoid protein methyltransferase is encoded by the gene *acsE*.

42. The organism of claim 38, wherein said corrinoid iron-sulfur protein is encoded by the gene acsD.

43. The organism of claim 38, wherein said nickel-protein assembly protein is encoded by at least one gene selected from the group consisting of *acsF* and *cooC*.

44. The organism of claim 38, wherein said ferredoxin is encoded by the gene *orf7*.

45. The organism of claim 38, wherein said acetyl-CoA synthase is encoded by at least one gene selected from the group consisting of *acsB* and *acsC*.

46. The organism of claim 38, wherein said carbon monoxide dehydrogenase is encoded by the gene *acsA*.

47. The organism of claim 38, wherein said pyruvate ferredoxin oxidoreductase is encoded by a gene selected from the group consisting of *por* and *ydbK*.

48. The organism of claim 38, wherein said hydrogenase is encoded by at least one gene selected from the group consisting of *hypA*, *hypB*, *hypC*, *hypD*, *hypE*, *hypF*, *moth_2175*, *moth_2176*, *moth_2177*, *moth_2178*, *moth_2179*, *moth_2180*, *moth_2181*, *hycA*, *hycB*, *hycC*, *hycD*, *hycE*, *hycF*, *hycG*, *hycH*, *hycI*, *hyfA*, *hyfB*, *hyfC*, *hyfD*, *hyfE*, *hyfF*, *hyfG*, *hyfH*, *hyfI*, *hyfJ*, *hyfR*, *moth_2182*, *moth_2183*, *moth_2184*, *moth_2185*, *moth_2186*, *moth_2187*, *moth_2188*, *moth_2189*, *moth_2190*, *moth_2191*, *moth_2192*, *moth_0439*, *moth_0440*, *moth_0441*, *moth_0442*, *moth_0809*, *moth_0810*, *moth_0811*, *moth_0812*, *moth_0813*, *moth_0814*, *moth_0815*, *moth_0816*, *moth_1193*, *moth_1194*, *moth_1195*, *moth_1196*, *moth_1717*, *moth_1718*, *moth_1719*, *moth_1883*, *moth_1884*, *moth_1885*, *moth_1886*, *moth_1887*, *moth_1888*, *moth_1452*, *moth_1453*, and *moth_1454*.

49. The organism of claim 38, further comprising the polypetide AcsEps encoded by the gene *acsEps*.

50. The organism of claim 38, further comprising at least one enzyme or polypeptide encoded by a gene selected from the group consisting of *codh*, *codh-I*, *cooF*, *hypA*, *cooH*, *cooU*, *cooX*, *cooL*, *cooK*, *cooM*, *cooT*, *cooJ*, and *codh-II*.

51. The organism of claim 38, further comprising a methanol methyltransferase.

52. The organism of claim 51, wherein said methanol methyltransferase is encoded by a gene selected from the group consisting of *mtaB*, *mtaB1*, *mtaB2*, and *mtaB3*.

53. The organism of claim 51, wherein said organism utilizes a feedstock selected from the group consisting of: 1) methanol and CO, 2) methanol, CO_2, and H_2, 3) methanol, CO, CO_2, and H_2, 4) methanol and synthesis gas comprising CO and H_2, and 5) methanol and synthesis gas comprising CO, CO_2, and H_2.

54. The organism of claim 38, further comprising a formate dehydrogenase, a formyltetrahydrofolate synthetase, a methenyltetrahydrofolate cyclohydrolase, a methylenetetrahydrofolate dehydrogenase, and a methylenetetrahydrofolate reductase.

55. The organism of claim 54, wherein said formate dehydrogenase is encoded by a gene selected from the group consisting of *moth_2312*, *moth _2313*, *moth_2314*, *sfum_2703*, *sfum_2704*, *sfum_2705*, *sfum_2706*, *chy_0731*, *chy_0732*, and *chy_0733*.

56. The organism of claim 54, wherein said formyltetrahydrofolate synthetase is encoded by a gene selected from the group consisting of *moth_0109*, *chy_2385*, and *fhs*.

57. The organism of claim 54, wherein said methenyltetrahydrofolate cyclohydrolase is encoded by a gene selected from the group consisting of *moth_1516*, *folD*, and *chy_1878*.

58. The organism of claim 54, wherein said methylenetetrahydrofolate dehydrogenase is encoded by a gene selected from the group consisting of *moth_1516*, *folD*, and *chy_1878*.

59. The organism of claim 54, wherein said methylenetetrahydrofolate reductase is encoded by a gene selected from the group consisting of *moth_1191*, *metF*, and *chy_1233*.

60. The organism of claim 54, wherein said organism utilizes a feedstock selected from the group consisting of: 1) CO, 2) CO_2 and H_2, 3) CO and CO_2, 4) synthesis gas comprising CO and H_2, and 5) synthesis gas comprising CO, CO_2, and H_2.

61. A non-naturally occurring microbial organism comprising a microbial organism having a 1,4-butanediol pathway comprising at least one exogenous nucleic acid encoding a 1,4-butanediol pathway enzyme expressed in a sufficient amount to produce 1,4-butanediol, said 1,4-butanediol pathway enzyme comprising an acetoacetyl-CoA thiolase, a 3-Hydroxybutyryl-CoA dehydrogenase, a crotonase, a crotonyl-CoA hydratase, a 4-hydroxybutyryl-CoA reductase (alcohol forming), a 4-hydroxybutyryl-CoA reductase (aldehyde forming), and a 1,4-butanediol dehydrogenase; said organism further comprising an acetyl-CoA pathway comprising at least one exogenous nucleic acid encoding an acetyl-CoA pathway enzyme expressed in a sufficient amount to produce acetyl-CoA, said acetyl-CoA pathway enzyme comprising a corrinoid protein, a methyltetrahydrofolate:corrinoid protein methyltransferase, a corrinoid iron-sulfur protein, a nickel-protein assembly protein, a ferredoxin, an acetyl-CoA synthase, a carbon monoxide dehydrogenase, a pyruvate ferredoxin oxidoreductase,and a hydrogenase.

62. The organism of claim 61, wherein said acetoacetyl-CoA thiolase is encoded by a gene selected from the group consisting of *atoB*, *thlA*, *thlB*, and *erg10*.

63. The organism of claim 61, wherein said 3-Hydroxybutyryl-CoA dehydrogenase is encoded by a gene selected from the group consisting of *hbd*, *msed_1423*, *msed_0399*, *msed_0389*, and *msed_1933*.

64. The organism of claim 61, wherein said crotonase is encoded by a gene selected from the group consisting of *crt*, *paaA*, *paaB*, *phaA*, *phaB*, *maoC*, *paaF*, and *paaG*.

65. The organism of claim 61, wherein said crotonyl-CoA hydratase is encoded by a gene selected from the group consisting of *abfD*, *msed_1321*, and *msed_1220*.

66. The organism of claim 61, wherein said 4-hydroxybutyryl-CoA reductase (alcohol forming) is encoded by a gene selected from the group consisting of *adhE*, *adhE2*, *mcr*, *rcas_2929*, *nap1_02720*, and *mgp2080_00535*.

67. The organism of claim 61, wherein said 4-hydroxybutyryl-CoA reductase (aldehyde forming) is encoded by a gene selected from the group consisting of *acr1*, *sucD*, *bphG*, *msed_0709*, *mcr*, *asd-2*, and *saci_2370*.

68. The organism of claim 61, wherein said 1,4-butanediol dehydrogenase is encoded by a gene selected from the group consisting of *alrA*, *adh2*, *yqhD*, *bdh I*, *bdh II*, and *4hbd*.

69. The organism of claim 61, wherein said corrinoid protein is encoded by a gene selected from the group consisting of *mtaC*, *mtaC1*, *mtaC2*, and *mtaC3*.

70. The organism of claim 61, wherein said methyltetrahydrofolate:corrinoid protein methyltransferase is encoded by a gene selected from the group consisting of *mtaA*, and *mtaA1*, *mtaA2*.

71. The organism of claim 61, wherein said methyltetrahydrofolate:corrinoid protein methyltransferase is encoded by the gene *acsE*.

72. The organism of claim 61, wherein said corrinoid iron-sulfur protein is encoded by the gene acsD.

73. The organism of claim 61, wherein said nickel-protein assembly protein is encoded by at least one gene selected from the group consisting of *acsF* and *cooC*.

74. The organism of claim 61, wherein said ferredoxin is encoded by the gene *orf7*.

75. The organism of claim 61, wherein said acetyl-CoA synthase is encoded by at least one gene selected from the group consisting of *acsB* and *acsC*.

76. The organism of claim 61, wherein said carbon monoxide dehydrogenase is encoded by the gene *acsA*.

77. The organism of claim 61, wherein said pyruvate ferredoxin oxidoreductase is encoded by a gene selected from the group consisting of *por* and *ydbK*.

78. The organism of claim 61, wherein said hydrogenase is encoded by at least one gene selected from the group consisting of *hypA*, *hypB*, *hypC*, *hypD*, *hypE*, *hypF*, *moth_2175*, *moth_2176*, *moth_2177*, *moth_2178*, *moth_2179*, *moth_2180*, *moth_2181*, *hycA*, *hycB*, *hycC*, *hycD*, *hycE*, *hycF*, *hycG*, *hycH*, *hycI*, *hyfA*, *hyfB*, *hyfC*, *hyfD*, *hyfE*, *hyfF*, *hyfG*, *hyfH*, *hyfI*, *hyfJ*, *hyfR*, *moth_2182*, *moth_2183*, *moth_2184*, *moth_2185*, *moth_2186*, *moth_2187*, *moth_2188*, *moth_2189*, *moth_2190*, *moth_2191*, *moth_2192*, *moth_0439*, *moth_0440*, *moth_0441*, *moth_0442*, *moth_0809*, *moth_0810*, *moth_0811*, *moth_0812*, *moth_0813*, *moth_0814*, *moth_0815*, *moth_0816*, *moth_1193*, *moth_1194*, *moth_1195*, *moth_1196*, *moth_1717*, *moth_1718*, *moth_1719*, *moth_1883*, *moth_1884*, *moth_1885*, *moth_1886*, *moth_1887*, *moth_1888*, *moth_1452*, *moth_1453*, and *moth_1454*.

79. The organism of claim 61, further comprising the polypetide AcsEps encoded by the gene *acsEps*.

80. The organism of claim 61, further comprising at least one enzyme or polypeptide encoded by a gene selected from the group consisting of *codh*, *codh-I*, *cooF*, *hypA*, *cooH*, *cooU*, *cooX*, *cooL*, *cooK*, *cooM*, *cooT*, *cooJ*, and *codh-II*.

81. The organism of claim 61, further comprising a methanol methyltransferase.

82. The organism of claim 81, wherein said methanol methyltransferase is encoded by a gene selected from the group consisting of *mtaB*, *mtaB1*, *mtaB2*, and *mtaB3*.

83. The organism of claim 81, wherein said organism utilizes a feedstock selected from the group consisting of: 1) methanol and CO, 2) methanol, CO_2, and H_2, 3) methanol, CO, CO_2, and H_2, 4) methanol and synthesis gas comprising CO and H_2, and 5) methanol and synthesis gas comprising CO, CO_2, and H_2.

84. A non-naturally occurring microbial organism comprising a microbial organism having a 1,4-butanediol pathway comprising at least one exogenous nucleic acid encoding a 1,4-butanediol pathway enzyme expressed in a sufficient amount to produce 1,4-butanediol, said 1,4-butanediol pathway enzyme comprising an acetoacetyl-CoA thiolase, a 3-Hydroxybutyryl-CoA dehydrogenase, a crotonase, a crotonyl-CoA hydratase, a 4-hydroxybutyryl-CoA reductase (alcohol forming), a 4-hydroxybutyryl-CoA reductase (aldehyde forming), and a 1,4-butanediol dehydrogenase; said organism further comprising an acetyl-CoA pathway comprising at least one exogenous nucleic acid encoding an acetyl-CoA pathway enzyme expressed in a sufficient amount to produce acetyl-CoA, said acetyl-CoA pathway enzyme comprising an acetyl-CoA synthase, a formate dehydrogenase, a formyltetrahydrofolate synthetase, a methenyltetrahydrofolate cyclohydrolase, a methylenetetrahydrofolate dehydrogenase, and a methylenetetrahydrofolate reductase.

85. The organism of claim 84, wherein said formate dehydrogenase is encoded by a gene selected from the group consisting of *moth_2312*, *moth _2313*, *moth_2314*, *sfum_2703*, *sfum_2704*, *sfum_2705*, *sfum_2706*, *chy_0731*, *chy_0732*, and *chy_0733*.

86. The organism of claim 84, wherein said formyltetrahydrofolate synthetase is encoded by a gene selected from the group consisting of *moth_0109*, *chy_2385*, and *fhs*.

87. The organism of claim 84, wherein said methenyltetrahydrofolate cyclohydrolase is encoded by a gene selected from the group consisting of *moth_1516*, *folD*, and *chy_1878*.

88. The organism of claim 84, wherein said methylenetetrahydrofolate dehydrogenase is encoded by a gene selected from the group consisting of *moth_1516*, *folD*, and *chy_1878*.

89. The organism of claim 84, wherein said methylenetetrahydrofolate reductase is encoded by a gene selected from the group consisting of *moth_1191*, *metF*, and *chy_1233*.

90. The organism of claim 84, wherein said organism utilizes a feedstock selected from the group consisting of: 1) CO, 2) CO_2 and H_2, 3) CO and CO_2, 4) synthesis gas comprising CO and H_2, and 5) synthesis gas comprising CO, CO_2, and H_2.

91. A non-naturally occurring microbial organism comprising a microbial organism having an isopropanol pathway comprising at least one exogenous nucleic acid encoding an isopropanol pathway enzyme expressed in a sufficient amount to produce isopropanol, said isopropanol pathway enzyme comprising an acetoacetyl-CoA thiolase, an acetoacetyl-CoA:acetate:CoA transferase, an acetoacetate decarboxylase, and an isopropanol dehydrogenase; said organism further comprising at least one exogenous nucleic acid encoding an acetyl-CoA enzyme expressed in a sufficient amount to produce acetyl-CoA, said acetyl-CoA pathway enzyme comprising a methanol methyl transferase, a corrinoid protein, a methyltetrahydro-folate:corrinoid protein methyltransferase, a corrinoid iron-sulfur protein, a nickel-protein assembly protein, a ferredoxin, an acetyl-CoA synthase, a carbon monoxide dehydrogenase, a pyruvate ferredoxin oxidoreductase,and a hydrogenase.

92. The organism of claim 91, wherein said acetoacetyl-CoA:acetate:CoA transferase is encoded by a gene selected from the group consisting of *atoA*, *atoD*, *ctfA*, an *ctfB*.

93. A non-naturally occurring microbial organism, comprising a microbial organism having an isopropanol pathway comprising at least one exogenous nucleic acid encoding an isopropanol pathway enzyme expressed in a sufficient amount to produce isopropanol, said isopropanol pathway enzyme comprising an acetoacetyl-CoA thiolase, an acetoacetyl-CoA:acetate:CoA transferase, an acetoacetate decarboxylase, and an isopropanol dehydrogenase; said organism further comprising at least one exogenous nucleic acid encoding an acetyl-CoA enzyme expressed in a sufficient amount to produce acetyl-CoA, said acetyl-CoA pathway enzyme comprising an acetyl-CoA synthase, a formate dehydrogenase, a formyltetrahydrofolate synthetase, a methenyltetrahydrofolate cyclohydrolase, a methylenetetrahydrofolate dehydrogenase, and a methylenetetrahydrofolate reductase.

94. The organism of claim 93, wherein said acetoacetyl-CoA:acetate:CoA transferase is encoded by a gene selected from the group consisting of *atoA*, *atoD*, *ctfA*, an *ctfB*.

95. A method for producing isopropanol, comprising culturing a non-naturally occurring microbial organism having an isopropanol pathway, said pathway comprising at least one exogenous nucleic acid encoding an isopropanol pathway enzyme expressed in a sufficient amount to produce isopropanol under conditions and for a sufficient period of time to produce isopropanol, said isopropanol pathway comprising a succinyl-CoA:3-ketoacid-CoA transferase.

96. The method of claim 95, wherein said succinyl-CoA:3-ketoacid-CoA transferase is encoded by one or more of the genes selected from the group consisting of *HPAG1_0676, HPAG1_0677, ScoA, ScoB, OXCT1, and OXCT2.*

97. The method of claim 95, further comprising an acetoacetyl-CoA thiolase, an acetoacetate decarboxylase, and an isopropanol dehydrogenase.

98. The method of claim 95, wherein said acetoacetyl-CoA thiolase is encoded by a gene selected from the group consisting of *atoB*, *thlA*, *thlB*, and *erg10.*

99. The method of claim 95,wherein said acetoacetate decarboxylase is encoded by the gene *adc.*

100. The method of claim 95, wherein said isopropanol dehydrogenase is encoded by a gene selected from the group consisting of *adh* and *ipdh.*

101. The method of claim 95, wherein said organism further comprises at least one enzyme or polypeptide selected from the group consisting of, a corrinoid protein, a methyltetrahydrofolate:corrinoid protein methyltransferase, a corrinoid iron-sulfur protein, a nickel-protein assembly protein, a ferredoxin, an acetyl-CoA synthase, a carbon monoxide dehydrogenase, a pyruvate ferredoxin oxidoreductase,and a hydrogenase.

102. The method of claim 101, wherein said corrinoid protein is encoded by a gene selected from the group consisting of *mtaC*, *mtaC1*, *mtaC2*, and *mtaC3.*

103. The method of claim 101, wherein said methyltetrahydrofolate:corrinoid protein methyltransferase is encoded by a gene selected from the group consisting of *mtaA*, and *mtaA1*, *mtaA2.*

104. The method of claim 101, wherein said methyltetrahydrofolate:corrinoid protein methyltransferase is encoded by the gene *acsE*.

105. The method of claim 101, wherein said corrinoid iron-sulfur protein is encoded by the gene acsD.

106. The method of claim 101, wherein said nickel-protein assembly protein is encoded by at least one gene selected from the group consisting of *acsF* and *cooC*.

107. The method of claim 101, wherein said ferredoxin is encoded by the gene *orf7*.

108. The method of claim 101, wherein said acetyl-CoA synthase is encoded by at least one gene selected from the group consisting of *acsB* and *acsC*.

109. The method of claim 101, wherein said carbon monoxide dehydrogenase is encoded by the gene *acsA*.

110. The method of claim 101, wherein said pyruvate ferredoxin oxidoreductase is encoded by a gene selected from the group consisting of *por* and *ydbK*.

111. The method of claim 101, wherein said hydrogenase is encoded by at least one gene selected from the group consisting of *hypA*, *hypB*, *hypC*, *hypD*, *hypE*, *hypF*, *moth_2175*, *moth_2176*, *moth_2177*, *moth_2178*, *moth_2179*, *moth_2180*, *moth_2181*, *hycA*, *hycB*, *hycC*, *hycD*, *hycE*, *hycF*, *hycG*, *hycH*, *hycI*, *hyfA*, *hyfB*, *hyfC*, *hyfD*, *hyfE*, *hyfF*, *hyfG*, *hyfH*, *hyfI*, *hyfJ*, *hyfR*, *moth_2182*, *moth_2183*, *moth_2184*, *moth_2185*, *moth_2186*, *moth_2187*, *moth_2188*, *moth_2189*, *moth_2190*, *moth_2191*, *moth_2192*, *moth_0439*, *moth_0440*, *moth_0441*, *moth_0442*, *moth_0809*, *moth_0810*, *moth_0811*, *moth_0812*, *moth_0813*, *moth_0814*, *moth_0815*, *moth_0816*, *moth_1193*, *moth_1194*, *moth_1195*, *moth_1196*, *moth_1717*, *moth_1718*, *moth_1719*, *moth_1883*, *moth_1884*, *moth_1885*, *moth_1886*, *moth_1887*, *moth_1888*, *moth_1452*, *moth_1453*, and *moth_1454*.

112. The method of claim 101, further comprising the polypetide AcsEps encoded by the gene *acsEps*.

113. The method of claim 101, further comprising at least one enzyme or polypeptide encoded by a gene selected from the group consisting of *codh*, *codh-I*, *cooF*, *hypA*, *cooH*, *cooU*, *cooX*, *cooL*, *cooK*, *cooM*, *cooT*, *cooJ*, and *codh-II*.

114. The method of claim 101, further comprising a methanol methyltransferase.

115. The method of claim 114, wherein said methanol methyltransferase is encoded by a gene selected from the group consisting of *mtaB*, *mtaB1*, *mtaB2*, and *mtaB3*.

116. The method of claim 114, wherein said organism utilizes a feedstock selected from the group consisting of: 1) methanol and CO, 2) methanol, CO_2, and H_2, 3) methanol, CO, CO_2, and H_2, 4) methanol and synthesis gas comprising CO and H_2, and 5) methanol and synthesis gas comprising CO, CO_2, and H_2.

117. The method of claim 101, wherein said organism further comprises a formate dehydrogenase, a formyltetrahydrofolate synthetase, a methenyltetrahydrofolate cyclohydrolase, a methylenetetrahydrofolate dehydrogenase, and a methylenetetrahydrofolate reductase.

118. The method of claim 117, wherein said formate dehydrogenase is encoded by a gene selected from the group consisting of *moth_2312*, *moth _2313*, *moth_2314*, *sfum_2703*, *sfum_2704*, *sfum_2705*, *sfum_2706*, *chy_0731*, *chy_0732*, and *chy_0733*.

119. The method of claim 117, wherein said formyltetrahydrofolate synthetase is encoded by a gene selected from the group consisting of *moth_0109*, *chy_2385*, and *fhs*.

120. The method of claim 117, wherein said methenyltetrahydrofolate cyclohydrolase is encoded by a gene selected from the group consisting of *moth_1516*, *folD*, and *chy_1878*.

121. The method of claim 117, wherein said methylenetetrahydrofolate dehydrogenase is encoded by a gene selected from the group consisting of *moth_1516*, *folD*, and *chy_1878*.

122. The method of claim 117, wherein said methylenetetrahydrofolate reductase is encoded by a gene selected from the group consisting of *moth_1191*, *metF*, and *chy_1233*.

123. The method of claim 117, wherein said organism utilizes a feedstock selected from the group consisting of: 1) CO, 2) CO_2 and H_2, 3) CO and CO_2, 4) synthesis gas comprising CO and H_2, and 5) synthesis gas comprising CO, CO_2, and H_2.

124. A method for producing 4-hydroxybutyrate, comprising culturing a non-naturally occurring microbial organism having an 4-hydroxybutyrate pathway, said pathway comprising at least one exogenous nucleic acid encoding an 4-hydroxybutyrate pathway enzyme expressed in a sufficient amount to produce 4-hydroxybutyrate under conditions and for a sufficient period of time to produce 4-hydroxybutyrate, said 4-hydroxybutyrate pathway comprising an acetoacetyl-CoA thiolase, a 3-hydroxybutyryl-CoA dehydrogenase, a crotonase, a crotonyl-CoA hydratase, a 4-hydroxybutyryl-CoA transferase, a phosphotrans-4-hydroxybutyrylase, and a 4-hydroxybutyrate kinase.

125. The method of claim 124, wherein said Acetoacetyl-CoA thiolase is encoded by a gene selected from the group consisting of *atoB*, *thlA*, *thlB*, and *erg10*.

126. The method of claim 124, wherein said 3-Hydroxybutyryl-CoA dehydrogenase is encoded by a gene selected from the group consisting of *hbd*, *msed_1423*, *msed_0399*, *msed_0389*, and *msed_1933*.

127. The method of claim 124, wherein said Crotonase is encoded by a gene selected from the group consisting of *crt*, *paaA*, *paaB*, *phaA*, *phaB*, *maoC*, *paaF*, and *paaG*.

128. The method of claim 124, wherein said Crotonyl-CoA hydratase is encoded by a gene selected from the group consisting of *abfD*, *msed_1321*, and *msed_1220*.

129. The method of claim 124, wherein said 4-Hydroxybutyryl-CoA transferase is encoded by a gene selected from the group consisting of *cat2*, *abfT-2*, *abfT-1*, and *abfT*.

130. The method of claim 124, wherein said Phosphotrans-4-hydroxybutyrylase is encoded by a gene selected from the group consisting of *pta* and *ptb*.

131. The method of claim 124, wherein said 4-Hydroxybutyrate kinase is encoded by a gene selected from the group consisting of *ackA*, *buk1*, *buk2*, and *proB*.

132. The method of claim 124, wherein said organism further comprises at least one enzyme or polypeptide selected from the group consisting of, a corrinoid protein, a methyltetrahydrofolate:corrinoid protein methyltransferase, a corrinoid iron-sulfur protein, a nickel-protein assembly protein, a ferredoxin, an acetyl-CoA synthase, a carbon monoxide dehydrogenase, a pyruvate ferredoxin oxidoreductase,and a hydrogenase.

133. The method of claim 132, wherein said corrinoid protein is encoded by a gene selected from the group consisting of *mtaC*, *mtaC1*, *mtaC2*, and *mtaC3*.

134. The method of claim 132, wherein said methyltetrahydrofolate:corrinoid protein methyltransferase is encoded by a gene selected from the group consisting of *mtaA*, and *mtaA1*, *mtaA2*.

135. The method of claim 132, wherein said methyltetrahydrofolate:corrinoid protein methyltransferase is encoded by the gene *acsE*.

136. The method of claim 132, wherein said corrinoid iron-sulfur protein is encoded by the gene acsD.

137. The method of claim 132, wherein said nickel-protein assembly protein is encoded by at least one gene selected from the group consisting of *acsF* and *cooC*.

138. The method of claim 132, wherein said ferredoxin is encoded by the gene *orf7*.

139. The method of claim 132, wherein said acetyl-CoA synthase is encoded by at least one gene selected from the group consisting of *acsB* and *acsC*.

140. The method of claim 132, wherein said carbon monoxide dehydrogenase is encoded by the gene *acsA*.

141. The method of claim 132, wherein said pyruvate ferredoxin oxidoreductase is encoded by a gene selected from the group consisting of *por* and *ydbK*.

142. The method of claim 132, wherein said hydrogenase is encoded by at least one gene selected from the group consisting of *hypA*, *hypB*, *hypC*, *hypD*, *hypE*, *hypF*, *moth_2175*, *moth_2176*, *moth_2177*, *moth_2178*, *moth_2179*, *moth_2180*, *moth_2181*, *hycA*, *hycB*, *hycC*, *hycD*, *hycE*, *hycF*, *hycG*, *hycH*, *hycI*, *hyfA*, *hyfB*, *hyfC*, *hyfD*, *hyfE*, *hyfF*, *hyfG*, *hyfH*, *hyfI*, *hyfJ*, *hyfR*, *moth_2182*, *moth_2183*, *moth_2184*, *moth_2185*, *moth_2186*, *moth_2187*, *moth_2188*, *moth_2189*, *moth_2190*, *moth_2191*, *moth_2192*, *moth_0439*, *moth_0440*, *moth_0441*, *moth_0442*, *moth_0809*, *moth_0810*, *moth_0811*, *moth_0812*, *moth_0813*, *moth_0814*, *moth_0815*, *moth_0816*, *moth_1193*, *moth_1194*, *moth_1195*, *moth_1196*, *moth_1717*, *moth_1718*, *moth_1719*, *moth_1883*, *moth_1884*, *moth_1885*, *moth_1886*, *moth_1887*, *moth_1888*, *moth_1452*, *moth_1453*, and *moth_1454*.

143. The method of claim 132, further comprising the polypetide AcsEps encoded by the gene *acsEps*.

144. The method of claim 132, further comprising at least one enzyme or polypeptide encoded by a gene selected from the group consisting of *codh*, *codh-I*, *cooF*, *hypA*, *cooH*, *cooU*, *cooX*, *cooL*, *cooK*, *cooM*, *cooT*, *cooJ*, and *codh-II*.

145. The method of claim 132, further comprising a methanol methyltransferase.

146. The method of claim 145, wherein said methanol methyltransferase is encoded by a gene selected from the group consisting of *mtaB*, *mtaB1*, *mtaB2*, and *mtaB3*.

147. The method of claim 145, wherein said organism utilizes a feedstock selected from the group consisting of: 1) methanol and CO, 2) methanol, CO_2, and H_2, 3) methanol, CO, CO_2, and H_2, 4) methanol and synthesis gas comprising CO and H_2, and 5) methanol and synthesis gas comprising CO, CO_2, and H_2.

148. The method of claim 132, wherein said organism further comprises a formate dehydrogenase, a formyltetrahydrofolate synthetase, a methenyltetrahydrofolate cyclohydrolase, a methylenetetrahydrofolate dehydrogenase, and a methylenetetrahydrofolate reductase.

149. The method of claim 148, wherein said formate dehydrogenase is encoded by a gene selected from the group consisting of *moth_2312*, *moth _2313*, *moth_2314*, *sfum_2703*, *sfum_2704*, *sfum_2705*, *sfum_2706*, *chy_0731*, *chy_0732*, and *chy_0733*.

150. The method of claim 148, wherein said formyltetrahydrofolate synthetase is encoded by a gene selected from the group consisting of *moth_0109*, *chy_2385*, and *fhs*.

151. The method of claim 148, wherein said methenyltetrahydrofolate cyclohydrolase is encoded by a gene selected from the group consisting of *moth_1516*, *folD*, and *chy_1878*.

152. The method of claim 148, wherein said methylenetetrahydrofolate dehydrogenase is encoded by a gene selected from the group consisting of *moth_1516*, *folD*, and *chy_1878*.

153. The method of claim 148, wherein said methylenetetrahydrofolate reductase is encoded by a gene selected from the group consisting of *moth_1191*, *metF*, and *chy_1233*.

154. The method of claim 148, wherein said organism utilizes a feedstock selected from the group consisting of: 1) CO, 2) CO_2 and H_2, 3) CO and CO_2, 4) synthesis gas comprising CO and H_2, and 5) synthesis gas comprising CO, CO_2, and H_2.

155. A method for producing 1,4-butanediol, comprising culturing a non-naturally occurring microbial organism having an 1,4-butanediol pathway, said pathway comprising at least one exogenous nucleic acid encoding an 1,4-butanediol pathway enzyme expressed in a sufficient amount to produce 1,4-butanediol under conditions and for a sufficient period of time to produce 1,4-butanediol, said 1,4-butanediol pathway comprising an acetoacetyl-CoA thiolase, a 3-Hydroxybutyryl-CoA dehydrogenase, a crotonase, a crotonyl-CoA hydratase, a 4-hydroxybutyryl-CoA reductase (alcohol forming), a 4-hydroxybutyryl-CoA reductase (aldehyde forming), a 1,4-butanediol dehydrogenase; said organism further comprising an acetyl-CoA pathway comprising at least one exogenous nucleic acid encoding an acetyl-CoA pathway enzyme expressed in a sufficient amount to produce acetyl-CoA, said acetyl-CoA pathway enzyme comprising a corrinoid protein, a methyltetrahydrofolate:corrinoid protein methyltransferase, a corrinoid iron-sulfur protein, a nickel-protein assembly protein, a ferredoxin, an acetyl-CoA synthase, a carbon monoxide dehydrogenase, a pyruvate ferredoxin oxidoreductase,and a hydrogenase.

156. The method of claim 155, wherein said Acetoacetyl-CoA thiolase is encoded by a gene selected from the group consisting of *atoB*, *thlA*, *thlB*, and *erg10*.

157. The method of claim 155, wherein said 3-Hydroxybutyryl-CoA dehydrogenase is encoded by a gene selected from the group consisting of *hbd*, *msed_1423*, *msed_0399*, *msed_0389*, and *msed_1933*.

158. The method of claim 155, wherein said Crotonase is encoded by a gene selected from the group consisting of *crt*, *paaA*, *paaB*, *phaA*, *phaB*, *maoC*, *paaF*, and *paaG*.

159. The method of claim 155, wherein said Crotonyl-CoA hydratase is encoded by a gene selected from the group consisting of *abfD*, *msed_1321*, and *msed_1220*.

160. The method of claim 155, wherein said 4-hydroxybutyryl-CoA reductase (alcohol forming) is encoded by a gene selected from the group consisting of *adhE*, *adhE2*, *mcr*, *rcas_2929*, *nap1_02720*, and *mgp2080_00535*.

161. The method of claim 155, wherein said 4-hydroxybutyryl-CoA reductase (aldehyde forming) is encoded by a gene selected from the group consisting of *acr1*, *sucD*, *bphG*, *msed_0709*, *mcr*, *asd-2*, and *saci_2370*.

162. The method of claim 155, wherein said 1,4-butanediol dehydrogenase is encoded by a gene selected from the group consisting of *alrA*, *adh2*, *yqhD*, *bdh I*, *bdh II*, and *4hbd*.

163. The method of claim 155, wherein said corrinoid protein is encoded by a gene selected from the group consisting of *mtaC*, *mtaC1*, *mtaC2*, and *mtaC3*.

164. The method of claim 155, wherein said methyltetrahydrofolate:corrinoid protein methyltransferase is encoded by a gene selected from the group consisting of *mtaA*, and *mtaA1*, *mtaA2*.

165. The method of claim 155, wherein said methyltetrahydrofolate:corrinoid protein methyltransferase is encoded by the gene *acsE*.

166. The method of claim 155, wherein said corrinoid iron-sulfur protein is encoded by the gene acsD.

167. The method of claim 155, wherein said nickel-protein assembly protein is encoded by at least one gene selected from the group consisting of *acsF* and *cooC*.

168. The method of claim 155, wherein said ferredoxin is encoded by the gene *orf7*.

169. The method of claim 155, wherein said acetyl-CoA synthase is encoded by at least one gene selected from the group consisting of *acsB* and *acsC*.

170. The method of claim 155, wherein said carbon monoxide dehydrogenase is encoded by the gene *acsA*.

171. The method of claim 155, wherein said pyruvate ferredoxin oxidoreductase is encoded by a gene selected from the group consisting of *por* and *ydbK*.

172. The method of claim 155, wherein said hydrogenase is encoded by at least one gene selected from the group consisting of *hypA*, *hypB*, *hypC*, *hypD*, *hypE*, *hypF*, *moth_2175*, *moth_2176*, *moth_2177*, *moth_2178*, *moth_2179*, *moth_2180*, *moth_2181*, *hycA*, *hycB*, *hycC*, *hycD*, *hycE*, *hycF*, *hycG*, *hycH*, *hycI*, *hyfA*, *hyfB*, *hyfC*, *hyfD*, *hyfE*, *hyfF*, *hyfG*, *hyfH*, *hyfI*, *hyfJ*, *hyfR*, *moth_2182*, *moth_2183*, *moth_2184*, *moth_2185*, *moth_2186*, *moth_2187*, *moth_2188*, *moth_2189*, *moth_2190*, *moth_2191*, *moth_2192*, *moth_0439*, *moth_0440*, *moth_0441*, *moth_0442*, *moth_0809*, *moth_0810*, *moth_0811*, *moth_0812*, *moth_0813*, *moth_0814*, *moth_0815*, *moth_0816*, *moth_1193*, *moth_1194*, *moth_1195*, *moth_1196*, *moth_1717*, *moth_1718*, *moth_1719*, *moth_1883*, *moth_1884*, *moth_1885*, *moth_1886*, *moth_1887*, *moth_1888*, *moth_1452*, *moth_1453*, and *moth_1454*.

173. The method of claim 155, wherein said organism further comprises the polypetide AcsEps encoded by the gene *acsEps*.

174. The method of claim 155, further comprising at least one enzyme or polypeptide encoded by a gene selected from the group consisting of *codh*, *codh-I*, *cooF*, *hypA*, *cooH*, *cooU*, *cooX*, *cooL*, *cooK*, *cooM*, *cooT*, *cooJ*, and *codh-II*.

175. The method of claim 155, further comprising a methanol methyltransferase.

176. The method of claim 175, wherein said methanol methyltransferase is encoded by a gene selected from the group consisting of *mtaB*, *mtaB1*, *mtaB2*, and *mtaB3*.

177. The method of claim 175, wherein said organism utilizes a feedstock selected from the group consisting of: 1) methanol and CO, 2) methanol, CO_2, and H_2, 3) methanol, CO, CO_2, and H_2, 4) methanol and synthesis gas comprising CO and H_2, and 5) methanol and synthesis gas comprising CO, CO_2, and H_2.

178. A method for producing 1,4-butanediol, comprising culturing a non-naturally occurring microbial organism having an 1,4-butanediol pathway, said pathway comprising at least one exogenous nucleic acid encoding an 1,4-butanediol pathway enzyme expressed in a sufficient amount to produce 1,4-butanediol under conditions and for a sufficient period of time to produce 1,4-butanediol, said 1,4-butanediol pathway comprising an acetoacetyl-CoA thiolase, a 3-hydroxybutyryl-CoA dehydrogenase, a crotonase, a crotonyl-CoA hydratase, a 4-hydroxybutyryl-CoA reductase (alcohol forming), a 4-hydroxybutyryl-CoA reductase (aldehyde forming), a 1,4-butanediol dehydrogenase; said organism further comprising an acetyl-CoA pathway comprising at least one exogenous nucleic acid encoding an acetyl-CoA pathway enzyme expressed in a sufficient amount to produce acetyl-CoA, said acetyl-CoA pathway enzyme comprising an acetyl-CoA synthase, a formate dehydrogenase, a formyltetrahydrofolate synthetase, a methenyltetrahydrofolate cyclohydrolase, a methylenetetrahydrofolate dehydrogenase, and a methylenetetrahydrofolate reductase.

179. The method of claim 178, wherein said formate dehydrogenase is encoded by a gene selected from the group consisting of *moth_2312*, *moth _2313*, *moth_2314*, *sfum_2703*, *sfum_2704*, *sfum_2705*, *sfum_2706*, *chy_0731*, *chy_0732*, and *chy_0733*.

180. The method of claim 178, wherein said formyltetrahydrofolate synthetase is encoded by a gene selected from the group consisting of *moth_0109*, *chy_2385*, and *fhs*.

181. The method of claim 178, wherein said methenyltetrahydrofolate cyclohydrolase is encoded by a gene selected from the group consisting of *moth_1516*, *folD*, and *chy_1878*.

182. The method of claim 178, wherein said methylenetetrahydrofolate dehydrogenase is encoded by a gene selected from the group consisting of *moth_1516*, *folD*, and *chy_1878*.

183. The method of claim 178, wherein said methylenetetrahydrofolate reductase is encoded by a gene selected from the group consisting of *moth_1191*, *metF*, and *chy_1233*.

184. The method of claim 178, wherein said organism utilizes a feedstock selected from the group consisting of: 1) CO, 2) CO_2 and H_2, 3) CO and CO_2, 4) synthesis gas comprising CO and H_2, and 5) synthesis gas comprising CO, CO_2, and H_2.

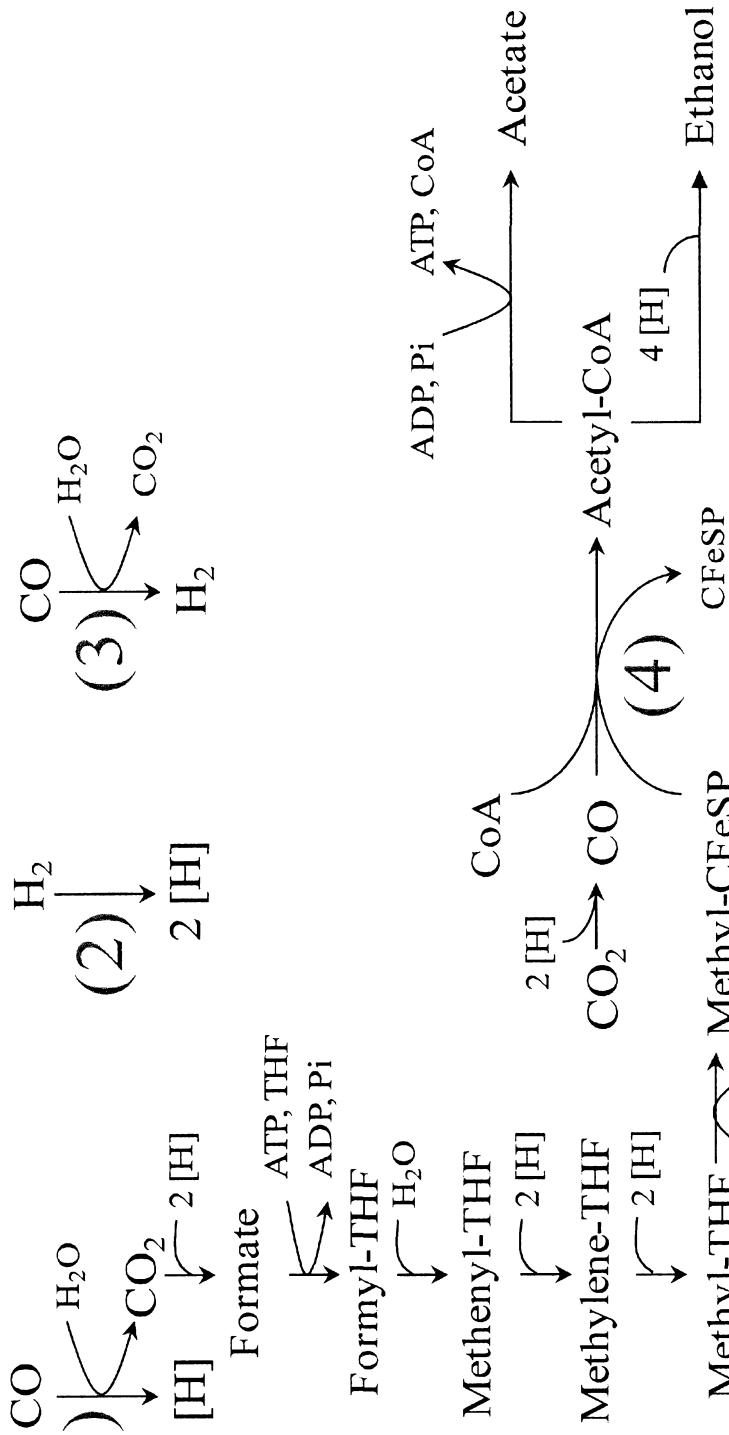
CO
(1)
H2O
CO2
2 [H]
2 [H]
Formate
ATP, THF
ADP, Pi
Formyl-THF
H2O
Methenyl-THF
2 [H]
Methylene-THF
2 [H]
Methyl-THF
CFeSP
THF
H2
(2)
2 [H]
CO
(3)
H2O
CO2
H2
CoA
2 [H]
CO2
CO
Acetyl-CoA
(4)
CFeSP
Methyl-CFeSP
ADP, Pi
ATP, CoA
Acetate
4 [H]
Ethanol

Figure 1

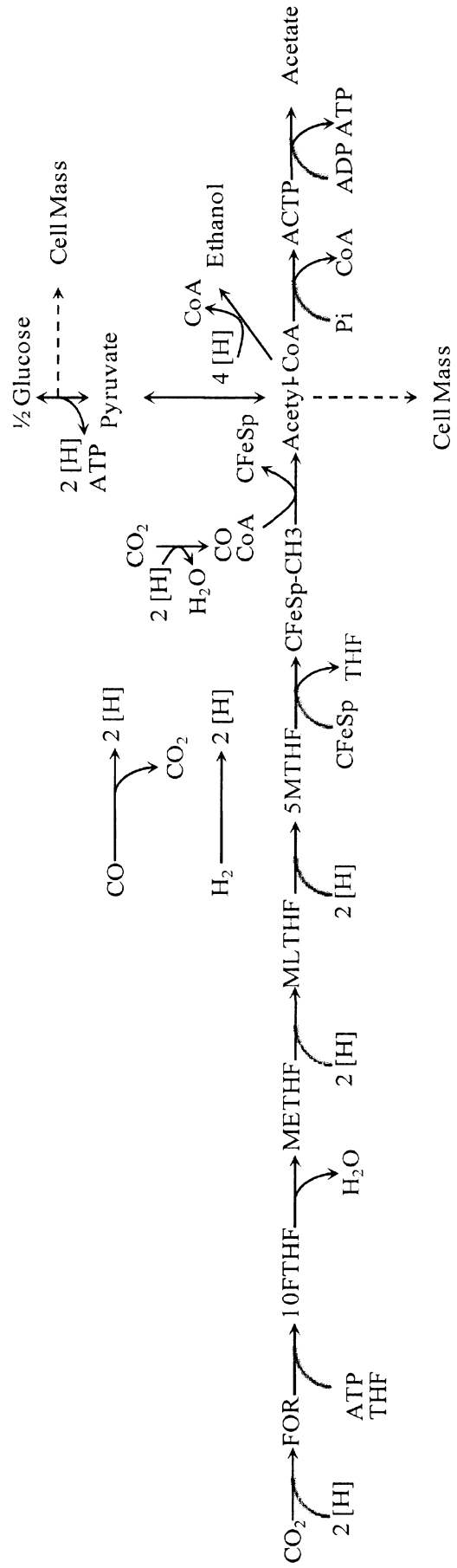
½ Glucose
Cell Mass
2 [H]
ATP
Pyruvate
CO
2 [H]
CO2
H2
2 [H]
CO2
2 [H]
H2O
CO
CoA
CFeSp
4 [H]
CoA
Ethanol
Acetyl-CoA
Cell Mass
ACTP
Pi
CoA
ADP
ATP
Acetate
CFeSp-CH3
CFeSp
THF
5MTHF
2 [H]
ML THF
2 [H]
METHF
2 [H]
H2O
10FTHF
ATP
THF
FOR
2 [H]
CO2

Figure 2A

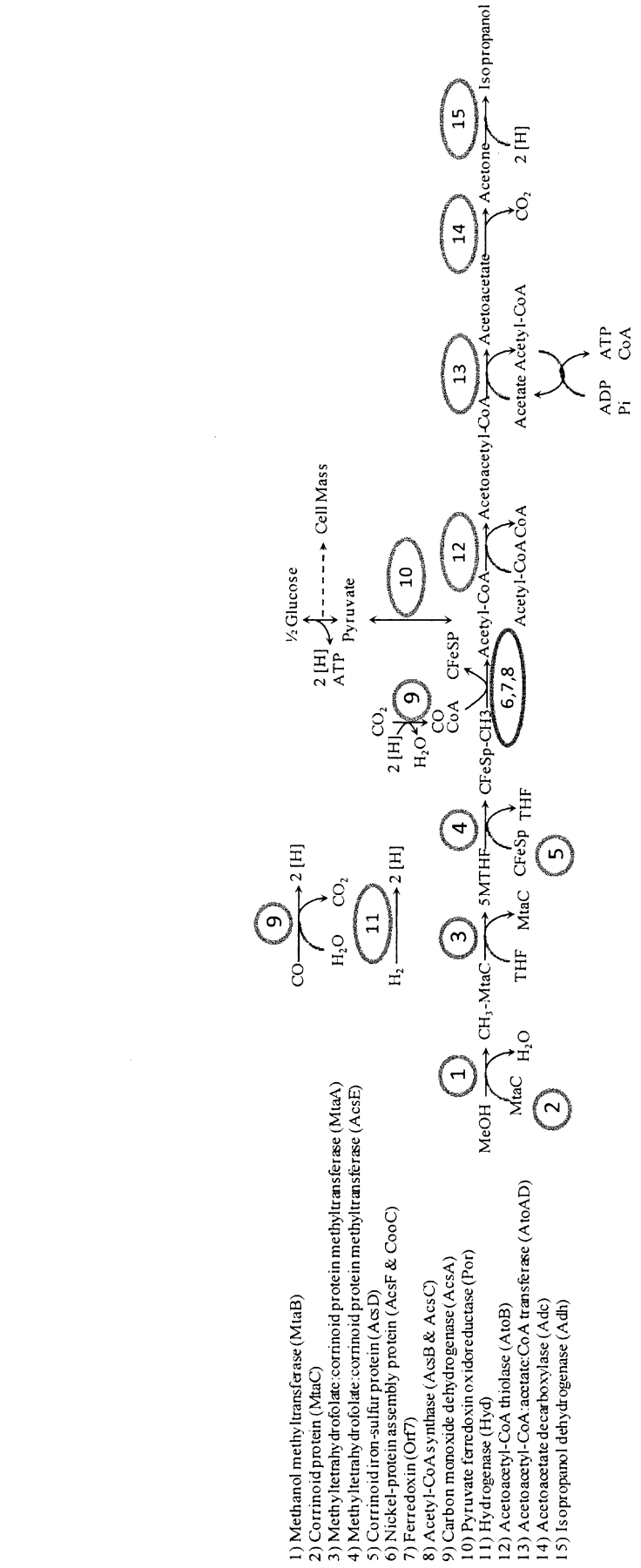
1) Methanol methyltransferase (MtaB)
2) Corrinoid protein (MtaC)
3) Methyltetrahydrofolate:corrinoid protein methyltransferase (MtaA)
4) Methyltetrahydrofolate:corrinoid protein methyltransferase (AcsE)
5) Corrinoid iron-sulfur protein (AcsD)
6) Nickel-protein assembly protein (AcsF & CooC)
7) Ferredoxin (Orf7)
8) Acetyl-CoA synthase (AcsB & AcsC)
9) Carbon monoxide dehydrogenase (AcsA)
10) Pyruvate ferredoxin oxidoreductase (Por)
11) Hydrogenase (Hyd)
12) Acetoacetyl-CoA thiolase (AtoB)
13) Acetoacetyl-CoA:acetate:CoA transferase (AtoAD)
14) Acetoacetate decarboxylase (Adc)
15) Isopropanol dehydrogenase (Adh)
½ Glucose
2 [H]
ATP
Pyruvate
Cell Mass
CO
H_2O
CO_2
2 [H]
H_2
2 [H]
CO_2
2 [H]
H_2O
CO
CoA
CFeSP
MeOH
MtaC
H_2O
CH_3-MtaC
THF
MtaC
5MTHF
CFeSp
THF
CFeSp-CH_3
Acetyl-CoA
Acetyl-CoA
CoA
Acetoacetyl-CoA
Acetate
Acetyl-CoA
Acetoacetate
CO_2
Acetone
2 [H]
Isopropanol
ADP
Pi
ATP
CoA

Figure 2B

1) Methanol methyltransferase (MtaB)
2) Corrinoid protein (MtaC)
3) Methyltetrahydrofolate:corrinoid protein methyltransferase (MtaA)
4) Methyltetrahydrofolate:corrinoid protein methyltransferase (AcsE)
5) Corrinoid iron-sulfur protein (AcsD)
6) Nickel-protein assembly protein (AcsF & CooC)
7) Ferredoxin (Orf7)
8) Acetyl-CoA synthase (AcsB & AcsC)
9) Carbon monoxide dehydrogenase (AcsA)
10) Pyruvate ferredoxin oxidoreductase (Por)
11) Hydrogenase (Hyd)
12) Acetoacetyl-CoA thiolase (AtoB)
13) 3-Hydroxybutyryl-CoA dehydrogenase (Hbd)
14) Crotonase (Crt)
15) Crotonyl-CoA hydratase (4-Budh)
16) 4-Hydroxybutyryl-CoA transferase
17) Phosphotrans-4-hydroxybutytylase
18) 4-Hydroxybutyrate kinase
½ Glucose
Cell Mass
2 [H]
ATP
Pyruvate
CO
H_2O
CO_2
2 [H]
H_2
2 [H]
CO_2
2 [H]
H_2O
CO
CoA
CFeSP
MeOH
MtaC
H_2O
CH_3-MtaC
THF
MtaC
5MTHF
CFeSp
THF
CFeSp-CH3
Acetyl-CoA
Acetyl-CoA
CoA
Acetoacetyl-CoA
2 [H]
3-Hydroxybutyryl-CoA
H_2O
H_2O
4-Hydroxybutyryl-CoA
Pi
CoA
4-Hydroxybutyryl-Pi
ADP
ATP
4-Hydroxybutyrate
ADP, Pi
ATP, CoA
Acetate
Acetyl-CoA
6,7,8
9
11
10
12
13
14
15
16
17
18
1
2
3
4
5

Figure 2C

1) Methanol methyltransferase (MtaB)
2) Corrinoid protein (MtaC)
3) Methyltetrahydrofolate:corrinoid protein methyltransferase (MtaA)
4) Methyltetrahydrofolate:corrinoid protein methyltransferase (AcsE)
5) Corrinoid iron-sulfur protein (AcsD)
6) Nickel-protein assembly protein (AcsF & CooC)
7) Ferredoxin (Orf7)
8) Acetyl-CoA synthase (AcsB & AcsC)
9) Carbon monoxide dehydrogenase (AcsA)
10) Pyruvate ferredoxin oxidoreductase (Por)
11) Hydrogenase (Hyd)
12) Acetoacetyl-CoA thiolase (AtoB)
13) 3-Hydroxybutyryl-CoA dehydrogenase (Hbd)
14) Crotonase (Crt)
15) Crotonyl-CoA hydratase (4-Budh)
16) 4-hydroxybutyryl-CoA reductase (alcohol forming)
17) 4-hydroxybutyryl-CoA reductase (aldehyde forming)
18) 1,4-butanediol dehydrogenase
½ Glucose
2 [H]
ATP
Pyruvate
Cell Mass
CO
H_2O
CO_2
H_2
2 [H]
CO_2
2 [H]
H_2O
CO
CoA
CFeSP
MeOH
CH_3-MtaC
MtaC
H_2O
THF
5MTHF
MtaC
CFeSp
THF
CFeSp-CH_3
Acetyl-CoA
Acetyl-CoA
CoA
Acetoacetyl-CoA
2 [H]
3-Hydroxybutyryl-CoA
H_2O
Crotonoyl-CoA
H_2O
4-Hydroxybutyryl-CoA
2 [H]
CoA
4-Hydroxybutanal
2 [H]
1,4-Butanediol
4 [H]
CoA
1
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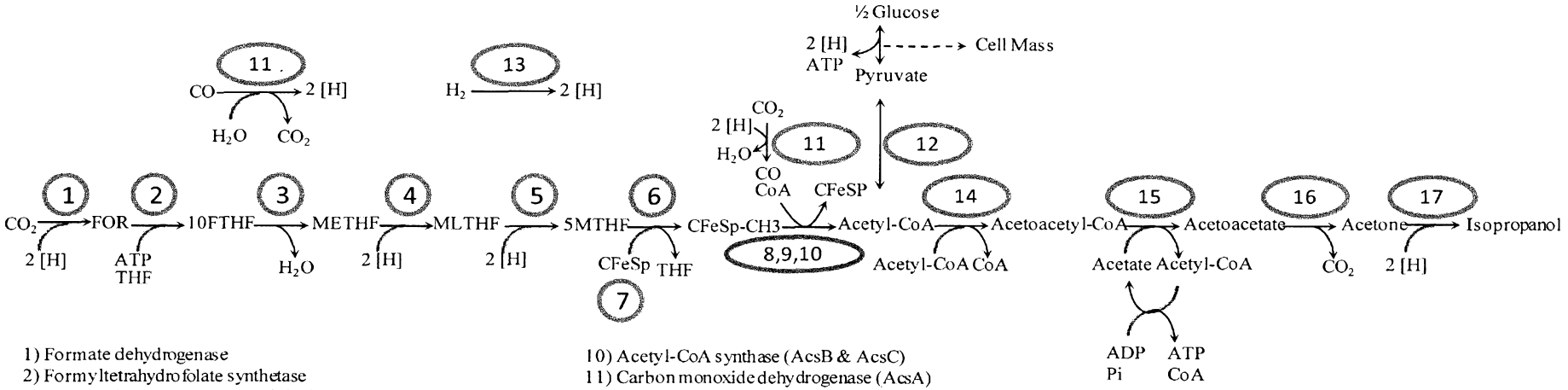
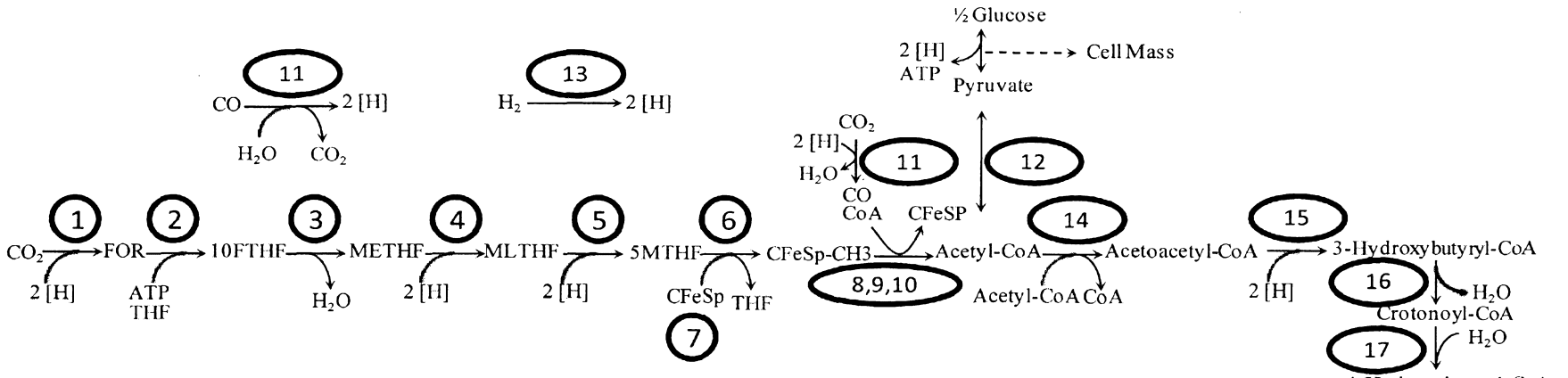
4
5
6,7,8
9
10
11
12
13
14
15
16
17
18

Figure 2D



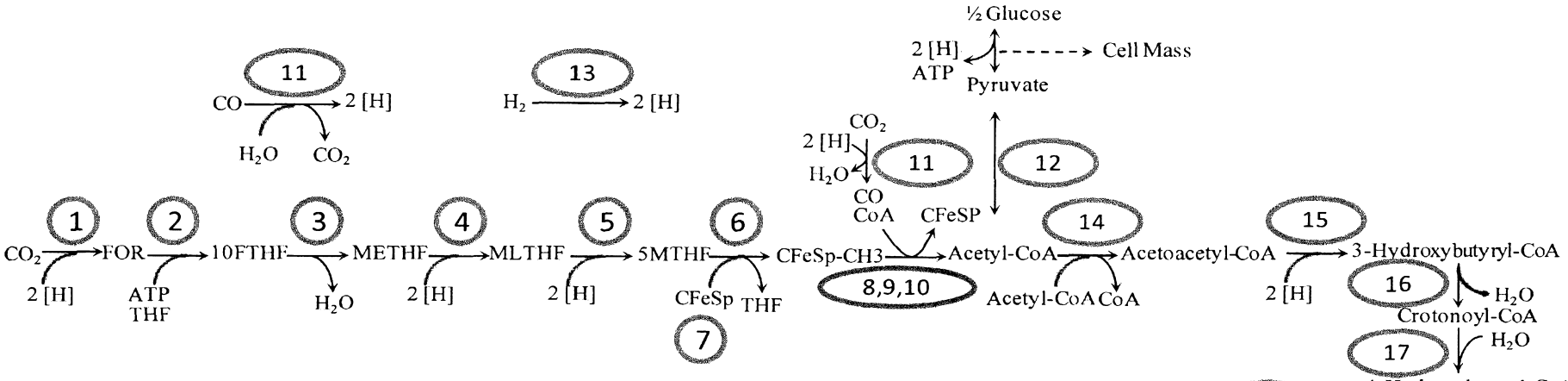
1) Formate dehydrogenase
2) Formyltetrahydrofolate synthetase
3) Methenyltetrahydrofolate cyclohydrolase
4) Methylenetetrahydrofolate dehydrogenase
5) Methylenetetrahydrofolate reductase
6) Methyltetrahydrofolate:corrinoid protein methyltransferase (AcsE)
7) Corrinoid iron-sulfur protein (AcsD)
8) Nickel-protein assembly protein (AcsF & CooC)
9) Ferredoxin (Orf7)
10) Acetyl-CoA synthase (AcsB & AcsC)
11) Carbon monoxide dehydrogenase (AcsA)
12) Pyruvate ferredoxin oxidoreductase (Por)
13) Hydrogenase (Hyd)
14) Acetoacetyl-CoA thiolase (AtoB)
15) Acetoacetyl-CoA:acetate:CoA transferase (AtoAD)
16) Acetoacetate decarboxylase (Adc)
17) Isopropanol dehydrogenase (Adh)

Figure 3A



1) Formate dehydrogenase
2) Formyltetrahydrofolate synthetase
3) Methenyltetrahydrofolate cyclohydrolase
4) Methylenetetrahydrofolate dehydrogenase
5) Methylenetetrahydrofolate reductase
6) Methyltetrahydrofolate:corrinoid protein methyltransferase (AcsE)
7) Corrinoid iron-sulfur protein (AcsD)
8) Nickel-protein assembly protein (AcsF & CooC)
9) Ferredoxin (Orf7)
10) Acetyl-CoA synthase (AcsB & AcsC)
11) Carbon monoxide dehydrogenase (AcsA)
12) Pyruvate ferredoxin oxidoreductase (Por)
13) Hydrogenase (Hyd)
14) Acetoacetyl-CoA thiolase (AtoB)
15) 3-Hydroxybutyryl-CoA dehydrogenase (Hbd)
16) Crotonase (Crt)
17) Crotonyl-CoA hydratase (4-Budh)
18) 4-Hydroxybutyryl-CoA transferase
19) Phosphotrans-4-hydroxybutyrylase
20) 4-Hydroxybutyrate kinase

Figure 3B



1) Formate dehydrogenase
2) Formyltetrahydrofolate synthetase
3) Methenyltetrahydrofolate cyclohydrolase
4) Methylenetetrahydrofolate dehydrogenase
5) Methylenetetrahydrofolate reductase
6) Methyltetrahydrofolate:corrinoid protein methyltransferase (AcsE)
7) Corrinoid iron-sulfur protein (AcsD)
8) Nickel-protein assembly protein (AcsF & CooC)
9) Ferredoxin (Orf7)
10) Acetyl-CoA synthase (AcsB & AcsC)
11) Carbon monoxide dehydrogenase (AcsA)
12) Pyruvate ferredoxin oxidoreductase (Por)
13) Hydrogenase (Hyd)
14) Acetoacetyl-CoA thiolase (AtoB)
15) 3-Hydroxybutyryl-CoA dehydrogenase (Hbd)
16) Crotonase (Crt)
17) Crotonyl-CoA hydratase (4-Budh)
18) 4-Hydroxybutyryl-CoA reductase (alcohol forming)
19) 4-Hydroxybutyryl-CoA reductase (aldehyde forming)
20) 1,4-Butanediol dehydrogenase

Figure 3C

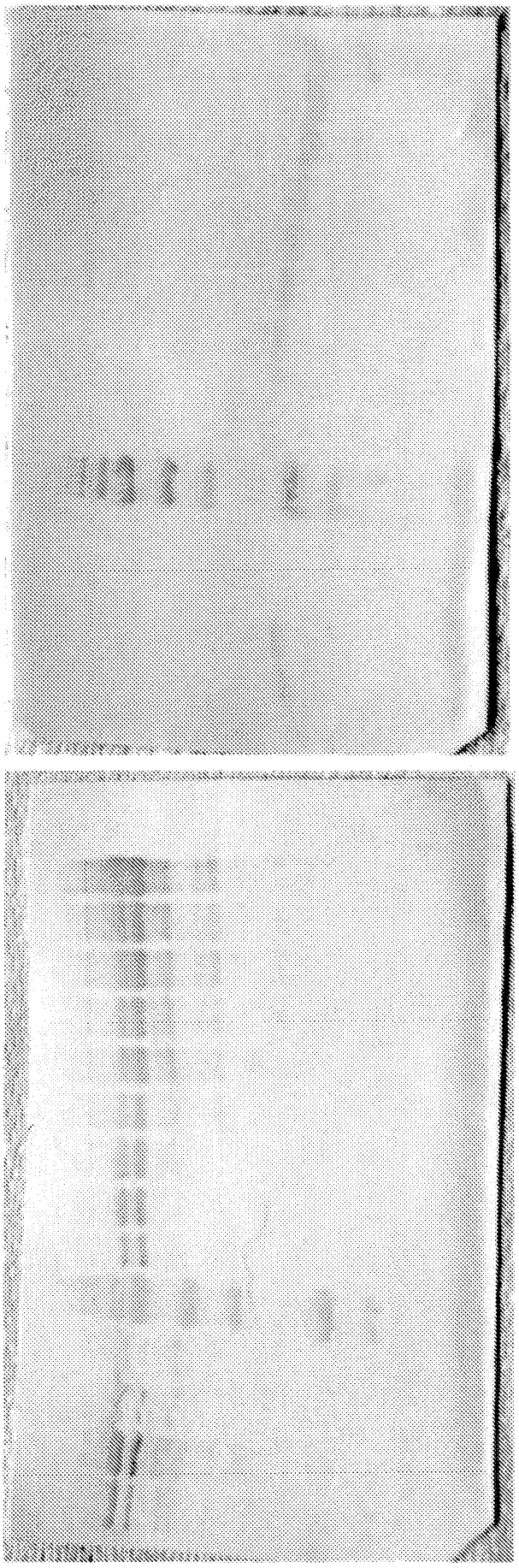


Figure 4

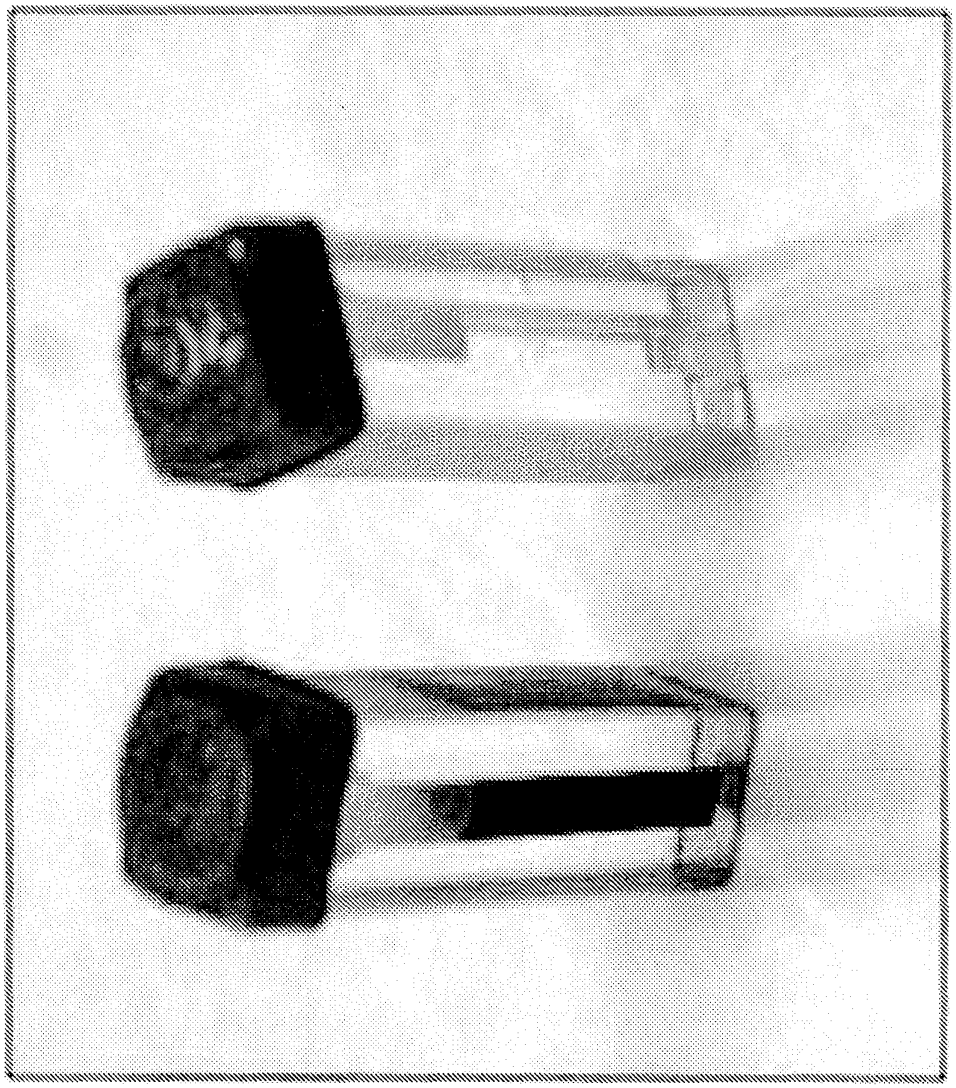


Figure 5

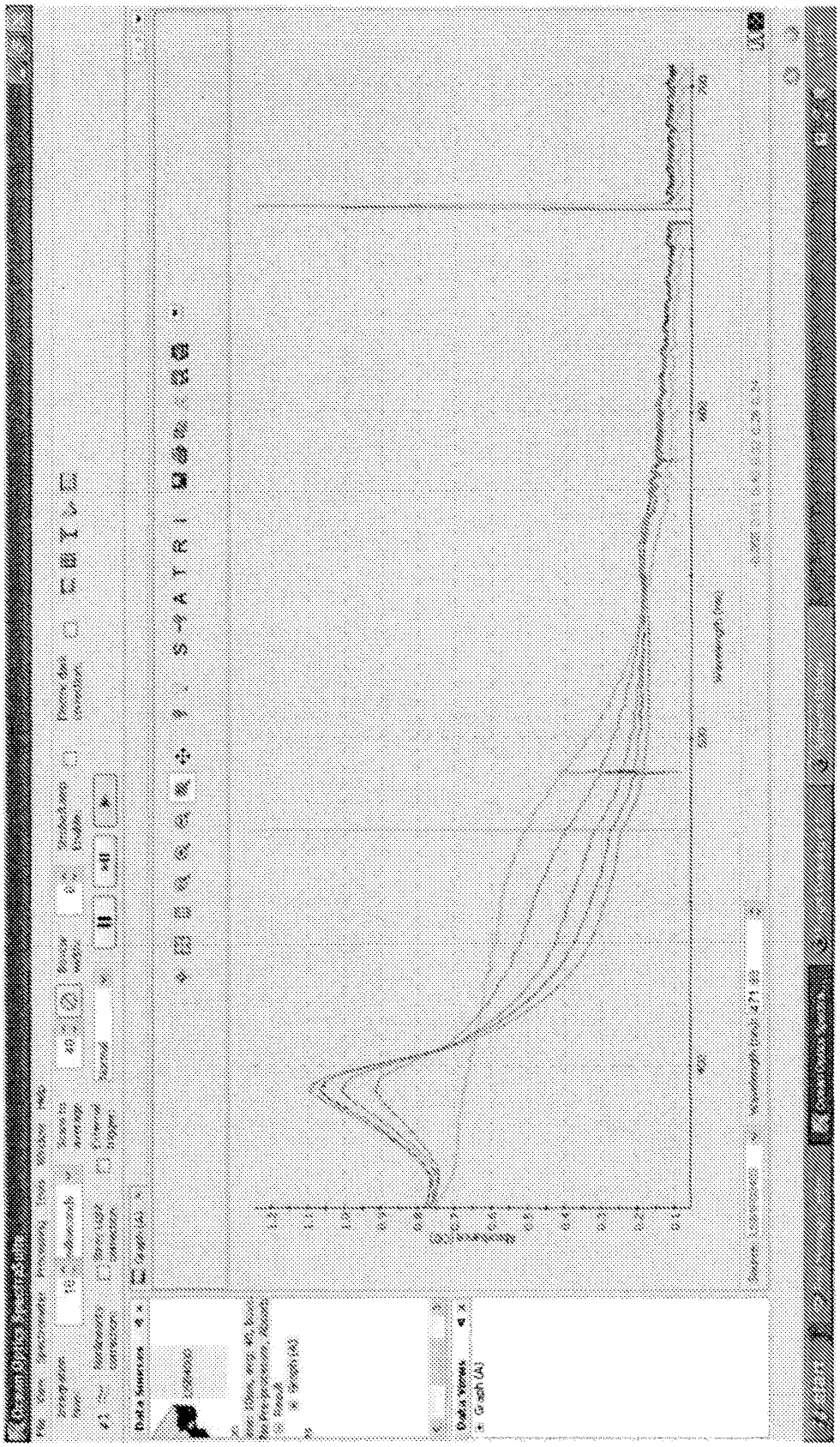


Figure 6

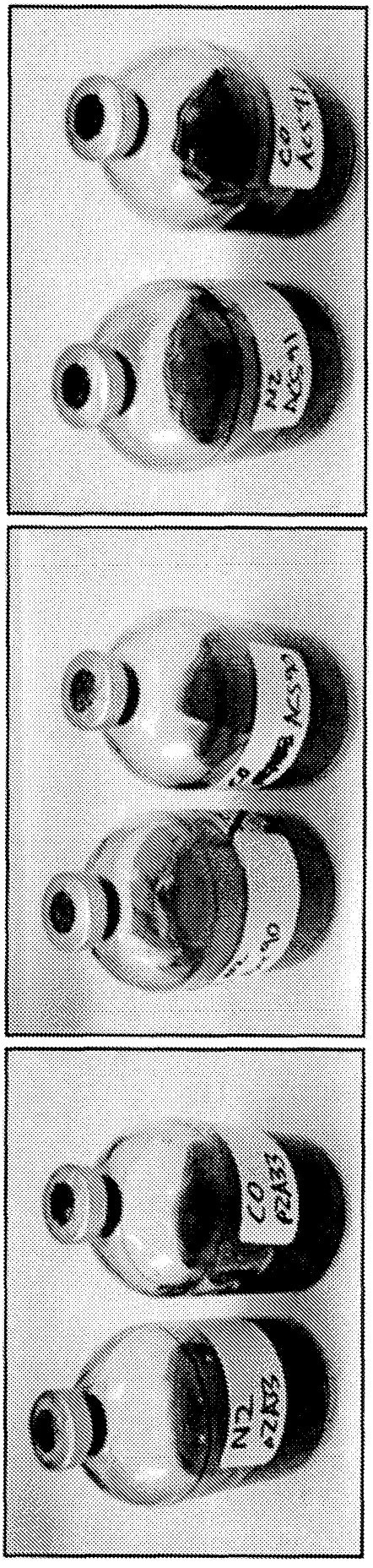


Figure 7