



(86) **Date de dépôt PCT/PCT Filing Date:** 2013/05/30
(87) **Date publication PCT/PCT Publication Date:** 2013/12/05
(85) **Entrée phase nationale/National Entry:** 2014/11/26
(86) **N° demande PCT/PCT Application No.:** NZ 2013/000092
(87) **N° publication PCT/PCT Publication No.:** 2013/180581
(30) **Priorité/Priority:** 2012/05/30 (US61/653,348)

(51) **Cl.Int./Int.Cl. C12P 7/62** (2006.01),
C12N 1/21 (2006.01), **C12N 15/53** (2006.01),
C12N 15/63 (2006.01), **C12N 15/74** (2006.01)

(71) **Demandeur/Applicant:**
LANZATECH NEW ZEALAND LIMITED, NZ

(72) **Inventeurs/Inventors:**
CHEN, WENDY, NZ;
KOEPEKE, MICHAEL, NZ

(74) **Agent:** BERESKIN & PARR LLP/S.E.N.C.R.L.,S.R.L.

(54) **Titre : MICRO-ORGANISMES RECOMBINANTS ET LEURS UTILISATIONS**
(54) **Title: RECOMBINANT MICROORGANISMS AND USES THEREFOR**

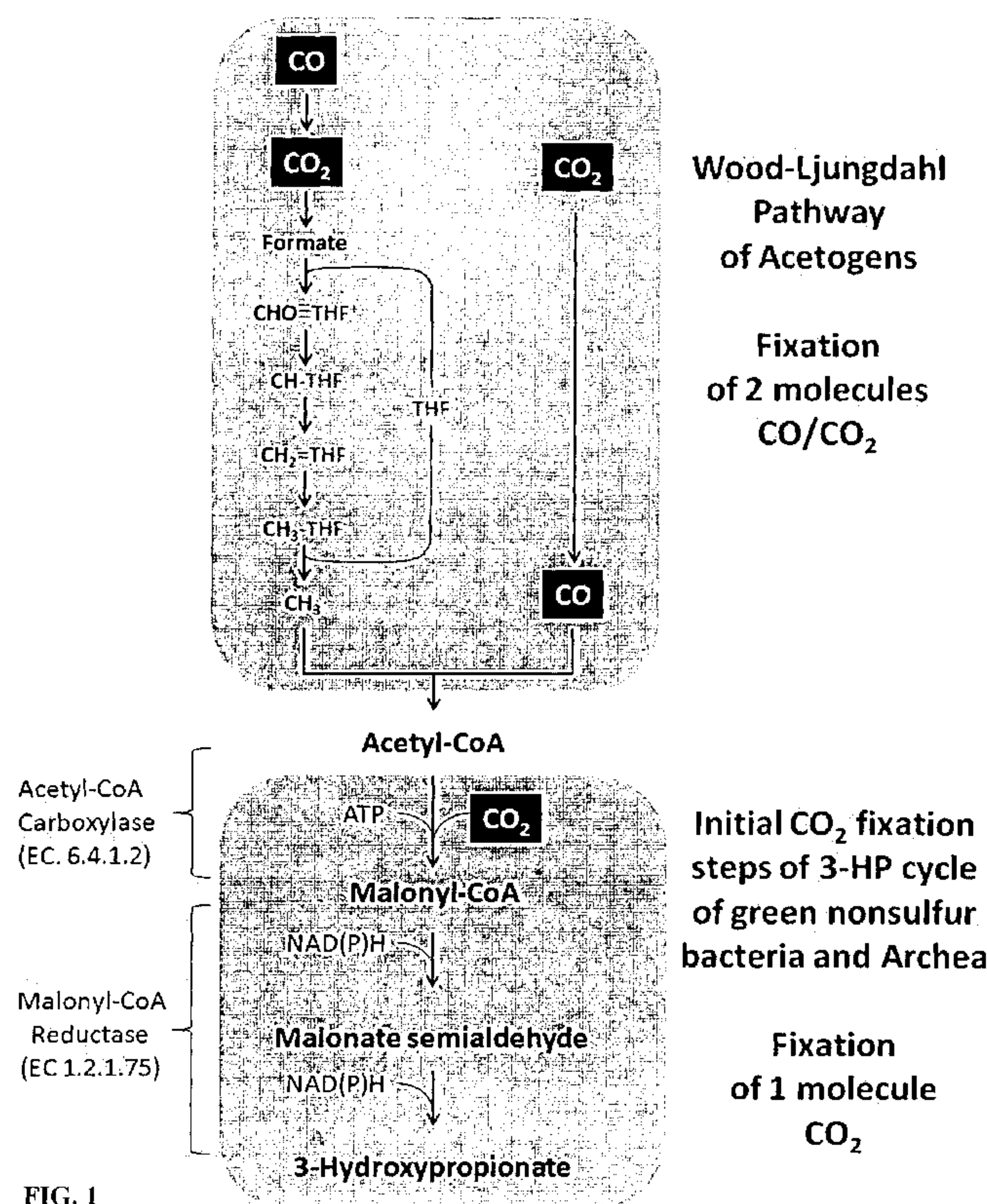
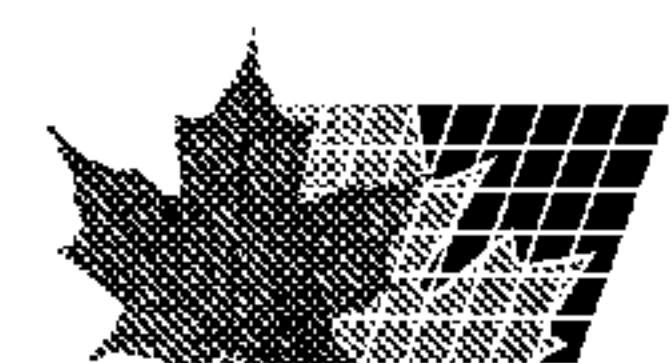


FIG. 1

(57) **Abrégé/Abstract:**

Bacteria are genetically engineered to produce 3-hydroxypropionate (3-HP). The bacteria are carboxydutrophic acetogens. The bacteria produce acetyl-coA using the Wood-Ljungdahl pathway for fixing CO/CO₂. A malonyl-coA reductase from a bacterium that contains such an enzyme is introduced. Additionally, an acetyl-coA carboxylase may also be introduced. The production of 3-HP can be improved by overproduction of acetyl-CoA carboxylase or by overproduction of biotin. This can be effected by improved promoters or higher copy number or enzymes that are catalytically more efficient.



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau

WIPO | PCT



(10) International Publication Number

WO 2013/180581 A1

(43) International Publication Date
5 December 2013 (05.12.2013)

(51) International Patent Classification:

C12P 7/62 (2006.01) C12N 15/74 (2006.01)
C12N 15/63 (2006.01) C12N 1/21 (2006.01)
C12N 15/53 (2006.01)

(21) International Application Number:

PCT/NZ2013/000092

(22) International Filing Date:

30 May 2013 (30.05.2013)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

61/653,348 30 May 2012 (30.05.2012) US

(71) Applicant: LANZATECH NEW ZEALAND LIMITED
[NZ/NZ]; 24 Balfour Road, Parnell, Auckland 1052 (NZ).(72) Inventors: CHEN, Wendy; 24 Balfour Road, Parnell,
Auckland 1052 (NZ). KOEPKE, Michael; 24 Balfour
Road, Parnell, Auckland 1052 (NZ).(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,

DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME,
MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ,
OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC,
SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ,
UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,
TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,
EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: RECOMBINANT MICROORGANISMS AND USES THEREFOR

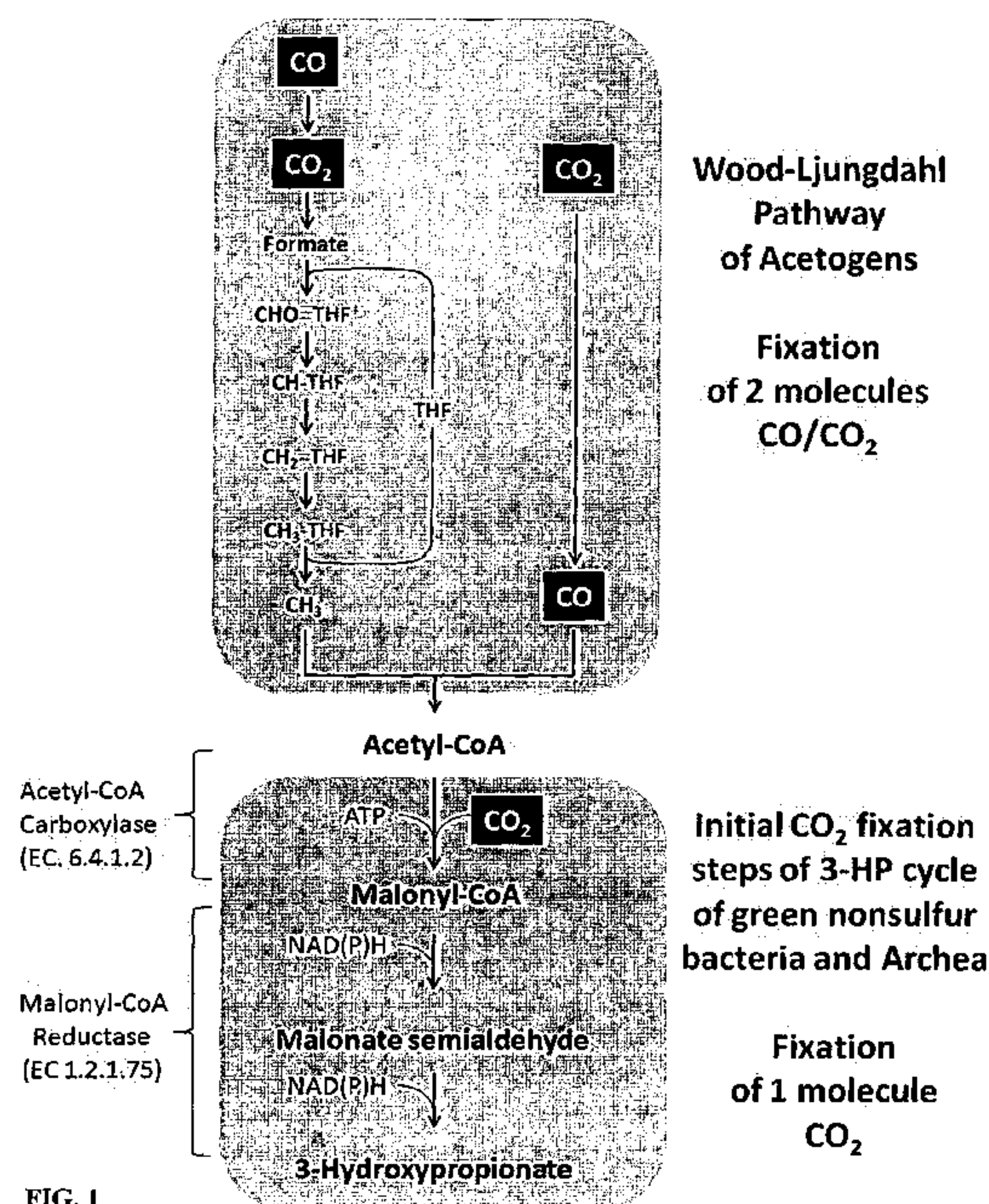


FIG. 1

(57) **Abstract:** Bacteria are genetically engineered to produce 3-hydroxypropionate (3-HP). The bacteria are carboxydophilic acetogens. The bacteria produce acetyl-coA using the Wood-Ljungdahl pathway for fixing CO/CO₂. A malonyl-coA reductase from a bacterium that contains such an enzyme is introduced. Additionally, an acetyl-coA carboxylase may also be introduced. The production of 3-HP can be improved by overproduction of acetyl-CoA carboxylase or by overproduction of biotin. This can be effected by improved promoters or higher copy number or enzymes that are catalytically more efficient.

WO 2013/180581 A1

RECOMBINANT MICROORGANISMS AND USES THEREFOR

TECHNICAL FIELD OF THE INVENTION

[001] The present invention relates to recombinant microorganisms and methods for the production of 3-Hydroxypropionate [3-HP] by microbial fermentation of a substrate comprising CO and/or CO₂.

BACKGROUND OF THE INVENTION

[002] 3-Hydroxypropionate [3-HP] is a platform chemical, acting as precursor for production of polymer materials and as chemical feedstock. Poly(3-hydroxypropionicacid) [P(3-HP)] is a biodegradable polymer with promising characteristics such as unusual high heat stability.

[003] 3-HP can be used to derive a number of valuable industrial chemicals including: acrylic acid which is used in the manufacture of paint, paper, adhesives, textiles, speciality coatings, ink, and superabsorbent polymer polyacrylates; 1,3-propanediol which is of use as a solvent, adhesive, cosmetic, or to make polytrimethylene terephthalate used in carpet and textiles; 3-hydroxypropionaldehyde which is used in the preparation of foods, as a feed additive, and as a preservative in the nutritional industry.

[004] 3-HP is listed as third most important renewable chemical by the US department of energy and a global market opening for 3-HP has been estimated to be 3.63 million tons per year (Paster et al, 2003, US DOE report: 48–49).

[005] It is an object of the invention to provide recombinant microorganisms and a method for the production of 3-HP by microbial fermentation which may provide one or more advantages over known methods, or to at least to provide the public with a useful choice.

SUMMARY OF INVENTION

[006] The invention generally provides, *inter alia*, methods for the production of 3-HP by microbial fermentation of a substrate comprising CO and/or CO₂, and recombinant

microorganisms of use in such methods. It combines two different CO₂ fixation pathways to produce a single metabolic product.

- [007] In a first aspect, the invention provides an anaerobic acetogenic recombinant microorganism capable of producing 3-HP and optionally one or more other products by fermentation of a substrate comprising CO and/or CO₂.
- [008] In one particular embodiment, the microorganism is adapted to express one or more enzymes (or one or more subunits thereof) in the 3-HP biosynthesis pathway which enzymes are not naturally present in a parental microorganism from which the recombinant microorganism is derived. In another embodiment, the microorganism is adapted to over-express one or more enzymes (or one or more subunits thereof) in the 3-HP biosynthesis pathway, which enzymes are naturally present in a parental microorganism from which the recombinant microorganism is derived. In one embodiment, the microorganism is adapted to express one or more enzymes (or one or more subunits thereof) in the 3-HP-biosynthesis pathway which are not naturally present in a parental microorganism and over-express one or more enzymes (or one or more subunits thereof) in the 3-HP biosynthesis pathway which are naturally present in a parental microorganism.
- [009] In one embodiment, the one or more enzymes are chosen from the group consisting of: Malonyl-CoenzymeA reductase (EC 1.2.1.75); Acetyl-CoA Carboxylase(ACC) (EC 6.4.1.2); and a functionally equivalent variant of any one thereof.
- [010] In one embodiment, the parental microorganism is capable of fermenting a substrate comprising CO and/or CO₂ to produce Acetyl-CoA but not of converting Acetyl-CoA to 3-HP and the recombinant microorganism is adapted to express one or more enzymes (or one or more subunits thereof) involved in the conversion of Acetyl-CoA to 3-HP.
- [011] In one embodiment, the microorganism comprises one or more exogenous nucleic acids adapted to increase expression of one or more nucleic acids native to the parental microorganism and which one or more nucleic acids encode one or more of the enzymes (or one or more subunits thereof) referred to herein before.

- [012] In one embodiment, the one or more exogenous nucleic acid adapted to increase expression is a regulatory element. In one embodiment, the regulatory element is a promoter.
- [013] In one embodiment, the promoter is a constitutive promoter. In one embodiment, the promoter is selected from the group comprising Wood-Ljungdahl gene cluster or Phosphotransacetylase/Acetate kinase operon promoters.
- [014] In one embodiment, the microorganism comprises one or more exogenous nucleic acids encoding and adapted to express one or more of the enzymes (or one or more subunits thereof) referred to herein before. In one embodiment, the microorganisms comprise one or more exogenous nucleic acid encoding and adapted to express at least two of the enzymes (or one or more subunits thereof).
- [015] In one embodiment, the one or more exogenous nucleic acid is a nucleic acid construct or vector, in one particular embodiment a plasmid, encoding one or more of the enzymes referred to hereinbefore in any combination.
- [016] In one embodiment, the exogenous nucleic acid is an expression plasmid.
- [017] In one embodiment, the parental microorganism is selected from the group of anaerobic acetogens.
- [018] In one particular embodiment, the parental microorganism is selected from the group of carboxydotrophic acetogenic bacteria, in one embodiment from the group comprising *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, *Clostridium ragsdalei*, *Clostridium carboxidivorans*, *Clostridium drakei*, *Clostridium scatologenes*, *Clostridium aceticum*, *Clostridium formicoaceticum*, *Clostridium magnum*, *Butyribacterium methylotrophicum*, *Acetobacterium woodii*, *Alkalibaculum bacchii*, *Blautia producta*, *Eubacterium limosum*, *Moorella thermoacetica*, *Moorella thermautotrophica*, *Sporomusa ovata*, *Sporomusa silvacetica*, *Sporomusa sphaeroides*, *Oxobacter pfennigii*, and *Thermoanaerobacter kiuvi*.

- [019] In one embodiment the parental microorganism is *Clostridium autoethanogenum* or *Clostridium ljungdahlii*. In one particular embodiment, the microorganism is *Clostridium autoethanogenum* DSM23693. In another particular embodiment, the microorganism is *Clostridium ljungdahlii* DSM13528 (or ATCC55383).
- [020] In one embodiment, the parental microorganism lacks one or more genes encoding Malonyl-CoenzymeA reductase and/or Acetyl-CoA carboxylase, or one or more subunits thereof.
- [021] In a second aspect, the invention provides a nucleic acid encoding one or more enzymes (or one or more subunits thereof) which when expressed in a microorganism allows the microorganism to produce 3-HP by fermentation of substrate comprising CO and/or CO₂.
- [022] In one embodiment, the nucleic acid encodes two or more enzymes (or one or more subunits thereof) which when expressed in a microorganism allows the microorganism to produce 3-HP by fermentation of substrate comprising CO.
- [023] In one embodiment, the enzymes are chosen from Malonyl-CoenzymeA reductase and Acetyl CoA carboxylase, and a functionally equivalent variant of any one or more thereof.
- [024] In one embodiment, the nucleic acid comprises nucleic acid sequences encoding Malonyl-CoenzymeA reductase, Acetyl CoA carboxylase, or a functionally equivalent variant of any one or more thereof, in any order.
- [025] In one embodiment, the nucleic acid encoding Malonyl-CoenzymeA reductase has the sequence of SEQ ID NO: 1 or GI:163848165, Caur 2614, or is a functionally equivalent variant thereof.
- [026] In one embodiment, the nucleic acid encoding Acetyl CoA carboxylase comprises the sequences SEQ ID NO: 18, 20, 22, and 24 (or CLJU_c42100-40, GI: 9447826-31, and GI:163847210 -11, Caur_1647 -48, GI:163849262, Caur_3739, GI:163848951, Caur_3421, GI:163846951, Caur_1378), or a functionally equivalent variant of any

one or more thereof. Acetyl CoA carboxylase may be comprised of a number of subunits. These may be encoded on one or more nucleic acids, if desired.

- [027] In one embodiment, the nucleic acids of the invention further comprise a promoter. In one embodiment, the promoter allows for constitutive expression of the genes under its control. In a particular embodiment a Wood-Ljungdahl cluster promoter is used. In another particular embodiment, a Phosphotransacetylase/Acetate kinase operon promoter is used. In one particular embodiment, the promoter is from *C. autoethanogenum*.
- [028] In a third aspect, the invention provides a nucleic acid construct or vector comprising one or more nucleic acid of the second aspect.
- [029] In one particular embodiment, the nucleic acid construct or vector is an expression construct or vector. In one particular embodiment, the expression construct or vector is a plasmid.
- [030] In a fourth aspect, the invention provides host organisms comprising any one or more of the nucleic acids of the seventh aspect or vectors or constructs of the third aspect.
- [031] In a fifth aspect, the invention provides a composition comprising an expression construct or vector as referred to in the third aspect of the invention and a methylation construct or vector.
- [032] Preferably, the composition is able to produce a recombinant microorganism according to the first aspect of the invention.
- [033] In one particular embodiment, the expression construct/vector and/or the methylation construct/vector is a plasmid.
- [034] In a sixth aspect, the invention provides a method for the production of 3-HP, and optionally one or more other products, by microbial fermentation comprising fermenting a substrate comprising CO and/or CO₂ using a recombinant microorganism of the first aspect of the invention.

[035] In one embodiment the method comprises the steps of:

- (a) providing a substrate comprising CO and/or CO₂ to a bioreactor containing a culture of one or more microorganism of the first aspect of the invention; and
- (b) anaerobically fermenting the culture in the bioreactor to produce 3-HP.

[036] In one embodiment the method comprises the steps of:

- (a) capturing CO- and/or CO₂-containing gas produced as a result of the industrial process, before the gas is released into the atmosphere;
- (b) the anaerobic fermentation of the CO- and/or CO₂-containing gas to produce at least 3-HP by a culture containing one or more microorganism of the first aspect of the invention.

[037] In particular embodiments of the method aspects, the microorganism is maintained in an aqueous culture medium.

[038] In particular embodiments of the method aspects, the fermentation of the substrate takes place in a bioreactor.

[039] Preferably, the substrate comprising CO and/or CO₂ is a gaseous substrate comprising CO and/or CO₂. In one embodiment, the substrate comprises an industrial waste gas. In certain embodiments, the gas is steel mill waste gas or syngas.

[040] In a particular embodiment, the substrate is a substrate comprising CO.

[041] In embodiments of the invention where the substrate comprises CO₂, but no CO, the substrate preferably also comprises H₂.

[042] In one embodiment, the substrate comprises CO and CO₂. In one embodiment, the substrate comprises CO₂ and H₂. In another embodiment, the substrate comprises CO, CO₂, and H₂.

[043] In one embodiment, the substrate will typically contain a major proportion of CO, such as at least about 20% to about 100% CO by volume, from 20% to 70% CO by volume, from 30% to 60% CO by volume, and from 40% to 55% CO by volume. In

particular embodiments, the substrate comprises about 25%, or about 30%, or about 35%, or about 40%, or about 45%, or about 50% CO, or about 55% CO, or about 60% CO by volume.

- [044] In certain embodiments the methods further comprise the step of recovering 3-HP and optionally one or more other products from the fermentation broth.
- [045] In a seventh aspect, the invention provides 3-HP when produced by the method of the sixth aspect.
- [046] In another aspect, the invention provides a method for the production of a microorganism of the first aspect of the invention comprising transforming a parental microorganism with one or more exogenous nucleic acid such that the microorganism is capable of producing 3-HP, and optionally one or more other products, by fermentation of a substrate comprising CO and/or CO₂, wherein the parental microorganism is not capable of producing 3-HP by fermentation of a substrate comprising CO and/or CO₂.
- [047] In one particular embodiment, a parental microorganism is transformed with one or more exogenous nucleic acid adapted to express one or more enzymes in the 3-HP biosynthesis pathway which are not naturally present in the parental microorganism. In another embodiment, a parental microorganism is transformed with one or more nucleic acid adapted to over-express one or more enzymes in the 3-HP biosynthesis pathway which are naturally present in the parental microorganism. In another embodiment, a parental microorganism is transformed with one or more exogenous nucleic acid adapted to express one or more enzymes in the 3-HP biosynthesis pathway which are not naturally present in the parental microorganism and over-express one or more enzymes in the 3-HP biosynthesis pathway which are naturally present in the parental microorganism.
- [048] In certain embodiments, the one or more enzymes are as herein before described.
- [049] In certain embodiment, the parental microorganism is as herein before described.

- [050] According to one embodiment a process is provided for converting CO or CO₂ into 3-hydroxypropionate (3-HP). A gaseous CO-containing and/or CO₂-containing substrate is passed to a bioreactor containing a culture of carboxydutrophic, acetogenic bacteria in a culture medium such that the bacteria convert the CO and/or CO₂ to 3-HP. The carboxydutrophic acetogenic bacteria are genetically engineered to express a malonyl-Coenzyme A reductase. They also express an acetyl-CoA carboxylase, whether native or exogenous. The 3-HP is recovered from the bioreactor.
- [051] According to another embodiment an isolated, genetically engineered, carboxydutrophic, acetogenic bacterium is provided that comprises a nucleic acid encoding a malonyl-Coenzyme A reductase. The nucleic acid is exogenous to the host bacteria. The bacteria express the malonyl-Coenzyme A reductase and the bacteria acquire the ability to fix three molecules of CO or CO₂ into one molecule of 3-hydroxypropionate (3-HP). The malonyl-Coenzyme A reductase is typically at least 85 % identical to the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 1.
- [052] The bacteria may further comprise an exogenous nucleic acid encoding acetyl-Coenzyme A carboxylase. The acetyl-CoA carboxylase is typically at least 85 % identical to the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 18-21. The nucleic acid may be operably linked to a promoter. The nucleic acid may have been codon optimized. The nucleic acid or the encoded carboxylase may be from a nonsulfur, photosynthetic bacterium. The bacteria may be selected from the group consisting of *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, *Clostridium ragsdalei*, *Clostridium carboxidivorans*, *Clostridium drakei*, *Clostridium scatologenes*, *Clostridium aceticum*, *Clostridium formicoaceticum*, *Clostridium magnum*, *Butyribacterium methylotrophicum*, *Acetobacterium woodii*, *Alkalibaculum bacchii*, *Blautia producta*, *Eubacterium limosum*, *Moorella thermoacetica*, *Moorella thermautotrophica*, *Sporomusa ovata*, *Sporomusa silvacetica*, *Sporomusa sphaeroides*, *Oxobacter pfennigii*, and *Thermoanaerobacter kiuvi*. The donor bacterium of the exogenous nucleic acid may be a nonsulfur, photosynthetic bacterium such as, *Chloroflexus auranticus*, *Metallosphaera*, and *Sulfolobus* spp.

[053] The genetically engineered bacteria may be cultured by growing in a medium comprising a gaseous carbon source. The carbon source may comprise CO and/or CO₂, which may be used as either or both of an energy source or a carbon source. The bacteria may optionally be grown under strictly anaerobic conditions. The carbon source may comprise an industrial waste product or off-gas.

[054] The invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively, in any or all combinations of two or more of said parts, elements or features, and where specific integers are mentioned herein which have known equivalents in the art to which the invention relates, such known equivalents are deemed to be incorporated herein as if individually set forth.

BRIEF DESCRIPTION OF THE DRAWINGS

[055] These and other aspects of the present invention, which should be considered in all its novel aspects, will become apparent from the following description, which is given by way of example only, with reference to the accompanying figures, in which:

[056] Figure 1: Combination of two CO₂ fixing pathways for sustainable production of 1 molecule 3-HP from 3 molecules of CO or CO₂.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[057] The following description of preferred embodiments is given in general terms. The invention is further elucidated from the disclosure given under the heading "Examples" herein below, which provides experimental data supporting the invention, specific examples of various aspects of the invention, and means of performing the invention.

[058] The inventors have surprisingly been able to engineer a carboxydutrophic acetogenic microorganism to produce 3-Hydroxypropionate (3-HP) by fermentation of a substrate comprising CO and/or CO₂. This offers an alternative means for the production of 3-HP which may have benefits over the current methods for the production of 3-HP. In addition, it offers a means of using carbon monoxide from

industrial processes which would otherwise be released into the atmosphere and pollute the environment.

- [059] In engineering the microorganisms of the invention, the inventors have surprisingly been able to combine two separate CO₂ fixation pathways, as illustrated in Figure 1. This provides for sustainable fermentation to produce 3-HP using a substrate comprising CO and/or a substrate comprising CO₂. Two pathways fixing CO₂ are thus linked to produce a desired product.
- [060] In one embodiment, the invention describes fixation of three molecules of CO₂ into one molecule of 3-HP by combining two separate CO₂ fixation pathways (Fig. 1), the Wood-Ljungdahl pathways of acetogens that allows fixation of two molecules of CO₂, and the initial carbon fixation steps of the 3-HP cycle allows fixation of another molecule of CO₂. CO₂ could also be replaced with carbon monoxide (CO), as the key enzyme of the Wood-Ljungdahl pathway, a CO dehydrogenase (CODH) is able to convert CO into CO₂ and energy in a biological water gas shift reaction ($\text{CO} + \text{H}_2\text{O} \rightleftharpoons \text{CO}_2 + \text{H}_2$). Any mixture of CO and CO₂ can be used. When CO₂ alone is used, energy in form of Hydrogen or electricity may need to be supplied, while CO can serve as both carbon and energy source.
- [061] While the inventors have demonstrated the efficacy of the invention in *Clostridium autoethanogenum*, the invention is applicable to the wider group of anaerobic acetogenic microorganisms and fermentation on substrates comprising CO and/or CO₂, as discussed above and further herein.
- [062] As referred to herein, a “fermentation broth” is a culture medium comprising at least a nutrient media and bacterial cells.
- [063] As referred to herein, a “shuttle microorganism” is a microorganism in which a methyltransferase enzyme is expressed and is distinct from the destination microorganism.

- [064] As referred to herein, a “destination microorganism” is a microorganism in which the genes included on an expression construct/vector are expressed and is distinct from the shuttle microorganism. This is also called a host microorganism.
- [065] The term “main fermentation product” is intended to mean the one fermentation product which is produced in the highest concentration and/or yield. There may be one or more fermentation products. The most prevalent may or may not be the most commercially valuable.
- [066] The terms “increasing the efficiency,” “increased efficiency,” and the like, when used in relation to a fermentation process, include, but are not limited to, increasing one or more of the rate of growth of microorganisms catalysing the fermentation, the growth and/or product production rate at elevated product concentrations, the volume of desired product produced per volume of substrate consumed, the rate of production or level of production of the desired product, and the relative proportion of the desired product produced compared with other by-products of the fermentation.
- [067] The phrase “substrate comprising carbon monoxide” and like terms should be understood to include any substrate in which carbon monoxide is available to one or more strains of bacteria for growth and/or fermentation, for example.
- [068] The phrase “gaseous substrate comprising carbon monoxide” and like phrases and terms includes any gas which contains a level of carbon monoxide. In certain embodiments the substrate contains at least about 20% to about 100% CO by volume, from 20% to 70% CO by volume, from 30% to 60% CO by volume, and from 40% to 55% CO by volume. In particular embodiments, the substrate comprises about 25%, or about 30%, or about 35%, or about 40%, or about 45%, or about 50% CO, or about 55% CO, or about 60% CO by volume.
- [069] While it is not necessary for a substrate comprising CO to contain any hydrogen, the presence of H₂ should not be detrimental to product formation in accordance with methods of the invention. In particular embodiments, the presence of hydrogen results in an improved overall efficiency of alcohol production. For example, in particular embodiments, the substrate may comprise an approx 2:1, or 1:1, or 1:2 ratio

of H₂: CO. In one embodiment the substrate comprises about 30% or less H₂ by volume, 20% or less H₂ by volume, about 15% or less H₂ by volume or about 10% or less H₂ by volume. In other embodiments, the substrate stream comprises low concentrations of H₂, for example, less than 5%, or less than 4%, or less than 3%, or less than 2%, or less than 1%, or is substantially hydrogen free. The substrate may also contain some CO₂ for example, such as about 1% to about 80% CO₂ by volume, or 1% to about 30% CO₂ by volume. In one embodiment the substrate comprises less than or equal to about 20% CO₂ by volume. In particular embodiments the substrate comprises less than or equal to about 15% CO₂ by volume, less than or equal to about 10% CO₂ by volume, less than or equal to about 5% CO₂ by volume or substantially no CO₂.

[070] The phrase “substrate comprising carbon dioxide” and like terms should be understood to include any substrate in which carbon dioxide is available to one or more strains of bacteria for growth and/or fermentation, for example. Substrates comprising carbon dioxide may further comprise hydrogen and/or carbon monoxide.

[071] The phrase “gaseous substrate comprising carbon dioxide” and like phrases and terms includes any gas which contains a level of carbon dioxide. In certain embodiments the substrate contains at least about 10% to about 60% CO₂ by volume, from 20% to 50% CO₂ by volume, from 30% to 60% CO₂ by volume, and from 40% to 55% CO₂ by volume. In particular embodiments, the substrate comprises about 20%, or about 25%, or about 30%, or about 35%, or about 40%, or about 45%, or about 50% CO, or about 55% CO, or about 60% CO₂ by volume.

[072] Preferably, a substrate comprising CO₂ will also contain a level of CO or H₂. In particular embodiments, the substrate comprises a CO₂:H₂ ratio of at least about 1:1, or at least about 1:2, or at least about 1:3, or at least about 1:4, or at least about 1:5.

[073] In the description which follows, embodiments of the invention are described in terms of delivering and fermenting a “gaseous substrate containing CO and/or CO₂.” However, it should be appreciated that the gaseous substrate may be provided in alternative forms. For example, the gaseous substrate containing CO and/or CO₂ may be provided dissolved in a liquid. Essentially, a liquid is saturated with a carbon

monoxide containing gas and then that liquid is added to the bioreactor. This may be achieved using standard methodology. By way of example, a microbubble dispersion generator (Hensirisak et. al. Scale-up of microbubble dispersion generator for aerobic fermentation; Applied Biochemistry and Biotechnology Volume 101, Number 3 / October, 2002) could be used. By way of further example, the gaseous substrate containing CO may be adsorbed onto a solid support. Such alternative methods are encompassed by use of the term "substrate containing CO and/or CO₂" and the like.

- [074] In particular embodiments of the invention, the CO-containing gaseous substrate (or a gaseous substrate comprising CO₂, or CO and CO₂, or CO₂ and H₂ and CO) is an industrial off or waste gas. "Industrial waste or off gases" should be taken broadly to include any gases comprising CO and/or CO₂ produced by an industrial process and include gases produced as a result of ferrous metal products manufacturing, non-ferrous products manufacturing, petroleum refining processes, gasification of coal, gasification of biomass, electric power production, carbon black production, and coke manufacturing. Further examples may be provided elsewhere herein.
- [075] Unless the context requires otherwise, the phrases "fermenting", "fermentation process" or "fermentation reaction" and the like, as used herein, are intended to encompass both the growth phase and product biosynthesis phase of the process. As will be described further herein, in some embodiments the bioreactor may comprise a first growth reactor and a second fermentation reactor. As such, the addition of metals or compositions to a fermentation reaction should be understood to include addition to either or both of these reactors.
- [076] The term "bioreactor" includes a fermentation device consisting of one or more vessels and/or towers or piping arrangement, which includes the Continuous Stirred Tank Reactor (CSTR), Immobilized Cell Reactor (ICR), Trickle Bed Reactor (TBR), Bubble Column, Gas Lift Fermenter, Static Mixer, or other vessel or other device suitable for gas-liquid contact. In some embodiments the bioreactor may comprise a first growth reactor and a second fermentation reactor. As such, when referring to the addition of substrate to the bioreactor or fermentation reaction it should be understood to include addition to either or both of these reactors where appropriate.

[077] “Exogenous nucleic acids” are nucleic acids which originate outside of the microorganism to which they are introduced. Exogenous nucleic acids may be derived from any appropriate source, including, but not limited to, the microorganism to which they are to be introduced, strains or species of microorganisms which differ from the organism to which they are to be introduced, or they may be artificially or recombinantly created. When an organism is genetically engineered or recombinant, it contains sequences that are adjacent to different sequences than in the naturally occurring microorganism. In one embodiment, the exogenous nucleic acids represent nucleic acid sequences naturally present within the microorganism to which they are to be introduced, and they are introduced to increase expression of or over-express a particular gene (for example, by increasing the copy number of the sequence (for example a gene), or introducing a strong or constitutive promoter to increase expression). In another embodiment, the exogenous nucleic acids represent nucleic acid sequences not naturally present within the microorganism to which they are to be introduced and allow for the expression of a product not naturally present within the microorganism or increased expression of a gene native to the microorganism (for example in the case of introduction of a regulatory element such as a promoter). The exogenous nucleic acid may be adapted to integrate into the genome of the microorganism to which it is to be introduced or to remain in an extra-chromosomal state. The exogenous sequence may come from a heterologous source, for example, another species, genus, family, or kingdom. In any event, the bacterium so produced is non-naturally occurring, having a genetic complement which is different from the naturally occurring, either by sequence differences or by copy number differences, for example.

[078] It should be appreciated that the invention may be practised using nucleic acids whose sequence varies from the sequences specifically exemplified herein provided they perform substantially the same function. For nucleic acid sequences that encode a protein or peptide this means that the encoded protein or peptide has substantially the same function. For nucleic acid sequences that represent promoter sequences, the variant sequence will have the ability to promote expression of one or more genes. Such nucleic acids may be referred to herein as “functionally equivalent variants.” By way of example, functionally equivalent variants of a nucleic acid include allelic variants, fragments of a gene, genes which include mutations (deletion, insertion,

nucleotide substitutions and the like) and/or polymorphisms and the like. Homologous genes from other microorganisms may also be considered as examples of functionally equivalent variants of the sequences specifically exemplified herein.

[079] These include homologous genes in species such as *Clostridium ljungdahlii*, *Chloroflexus auranticus*, *Metallosphaera* or *Sulfolobus spp*, details of which are publicly available on websites such as Genbank or NCBI. The phrase “functionally equivalent variants” should also be taken to include nucleic acids whose sequence varies as a result of codon optimisation for a particular organism. “Functionally equivalent variants” of a nucleic acid herein will preferably have at least approximately 70%, preferably approximately 80%, more preferably approximately 85%, preferably approximately 90%, preferably approximately 95% or greater nucleic acid sequence identity with the nucleic acid identified.

[080] It should also be appreciated that the invention may be practised using polypeptides whose sequence varies from the amino acid sequences specifically exemplified herein. These variants may be referred to herein as “functionally equivalent variants.” A functionally equivalent variant of a protein or a peptide includes those proteins or peptides that share at least 40%, preferably 50%, preferably 60%, preferably 70%, preferably 75%, preferably 80%, preferably 85%, preferably 90%, preferably 95% or greater amino acid identity with the protein or peptide identified and has substantially the same function as the peptide or protein of interest. Such variants include within their scope fragments of a protein or peptide wherein the fragment comprises a truncated form of the polypeptide wherein deletions may be from 1 to 5, to 10, to 15, to 20, to 25 amino acids, and may extend from residue 1 through 25 at either terminus of the polypeptide, and wherein deletions may be of any length within the region; or may be at an internal location. Functionally equivalent variants of the specific polypeptides herein should also be taken to include polypeptides expressed by homologous genes in other species of bacteria, for example as exemplified in the previous paragraph.

[081] “Substantially the same function” as used herein is intended to mean that the nucleic acid or polypeptide is able to perform the function of the nucleic acid or polypeptide of which it is a variant. For example, a variant of an enzyme of the invention will be

able to catalyse the same reaction as that enzyme. However, it should not be taken to mean that the variant has the same level of activity as the polypeptide or nucleic acid of which it is a variant.

- [082] One may assess whether a functionally equivalent variant has substantially the same function as the nucleic acid or polypeptide of which it is a variant using any number of known methods. However, by way of example, the methods outlined by Hügler et al (2002, *J. Bacteriol.* 184: 2404–2410 or Kroeger et al (2011, *Anal. Biochem.* 411: 100-5) may be used to measure the activity of Malonyl-coenzyme A reductase and Acetyl Co-A carboxylase, respectively.
- [083] “Over-express,” “over expression,” and like terms and phrases when used in relation to the invention should be taken broadly to include any increase in expression of one or more protein as compared to the expression level of the protein of a parental microorganism under the same conditions. It should not be taken to mean that the protein is expressed at any particular level.
- [084] A “parental microorganism” is a microorganism used to generate a recombinant microorganism of the invention. The parental microorganism may be one that occurs in nature (*i.e.*, a wild type microorganism) or one that has been previously modified but which does not express or over-express one or more of the enzymes the subject of the present invention. Accordingly, the recombinant microorganisms of the invention have been modified to express or over-express one or more enzymes that were not expressed or over-expressed in the parental microorganism.
- [085] The terms nucleic acid “constructs” or “vectors” and like terms should be taken broadly to include any nucleic acid (including DNA and RNA) suitable for use as a vehicle to transfer genetic material into a cell. The terms should be taken to include plasmids, viruses (including bacteriophage), cosmids and artificial chromosomes. Constructs or vectors may include one or more regulatory elements, an origin of replication, a multicloning site and/or a selectable marker. In one particular embodiment, the constructs or vectors are adapted to allow expression of one or more genes encoded by the construct or vector. Nucleic acid constructs or vectors include naked nucleic acids as well as nucleic acids formulated with one or more agents to

facilitate delivery to a cell (for example, liposome-conjugated nucleic acid, an organism in which the nucleic acid is contained).

- [086] The “3-HP biosynthesis pathway” is the enzymatic pathway allowing for the conversion of Acetyl-CoA to Malonyl-CoA to Malonate semialdehyde to 3-HP. Unless the context clearly requires otherwise, reference to an enzyme in the 3-HP biosynthesis pathway should be taken to include reference to any one or more subunits of the enzyme. By way of example only, Acetyl CoA carboxylase may comprise four subunits.

Microorganisms

- [087] As discussed herein before, the invention provides a recombinant microorganism capable of producing 3-HP, and optionally one or more other products, by fermentation of a substrate comprising CO and/or CO₂.
- [088] In one particular embodiment, the microorganism is adapted to express one or more enzymes (or one or more subunits thereof) in the 3-HP biosynthesis pathway which are not naturally present in a parental microorganism from which it is derived. In another embodiment, the microorganism is adapted to over-express one or more enzymes (or one or more subunits thereof) in the 3-HP biosynthesis pathway which are naturally present in the parental microorganism.
- [089] In one embodiment, the parental microorganism is capable of fermenting a substrate comprising CO to produce Acetyl-CoA but not of converting Acetyl-CoA to 3-HP and the recombinant microorganism is adapted to express one or more enzymes (or one or more subunits thereof) involved in the conversion of Acetyl-CoA to 3-HP. In one embodiment, the parental microorganism is capable of converting Acetyl CoA to Malonyl CoA, but not of converting Malonyl CoA to 3-HP. In another embodiment, the parental microorganism is capable of converting Malonyl CoA to 3-HP but not of converting Acetyl CoA to Malonyl CoA.
- [090] In one embodiment the one or more enzymes in the 3-HP biosynthesis pathway are chosen from the group consisting: Malonyl-coenzyme A reductase; Acetyl CoA carboxylase; and a functionally equivalent variant of any one or more thereof.

- [091] The microorganism may be adapted to express or over-express the one or more enzymes (or one or more subunits thereof) by any number of recombinant methods including, for example, increasing expression of native genes within the microorganism (for example, by introducing a stronger or constitutive promoter to drive expression of a gene), increasing the copy number of a gene encoding a particular enzyme by introducing exogenous nucleic acids encoding and adapted to express the enzyme, introducing an exogenous nucleic acid encoding and adapted to express an enzyme not naturally present within the parental microorganism.
- [092] In certain embodiments, the parental microorganism may be transformed to provide a combination of increased or over-expression of one or more genes native to the parental microorganism and introduction of one or more genes not native to the parental microorganism. For example, one or more genes encoding one or more enzyme in the 3-HP biosynthesis pathway may be native to the parental microorganism but it may not include one or more other genes encoding one or more other enzyme in the pathway. The microorganism could for example be engineered to over-express native Acetyl CoA carboxylase and to introduce a Malonyl CoA reductase gene encoding an enzyme for the conversion of Malonyl-CoA to 3-HP (*e.g.*, Malonyl CoA reductase). Alternatively, the microorganism could be engineered to over-express native Malonyl CoA reductase and to introduce a gene encoding Acetyl CoA carboxylase. Skilled persons will appreciate various other combinations of use in the invention.
- [093] By way of example only, exemplary sequence information for Malonyl CoA reductase is provided in the form of SEQ ID NO: 1 herein, and also on public databases with the accession numbers YP_001636209.1/ Caur_2614, GI:163848165. By way of additional example, exemplary sequence information for Acetyl CoA carboxylase is provided in the form of SEQ ID NO: 18-21 herein, and also on public databases with the accession numbers NC_014328.1-33.1/CLJU_c42100-40, GI: 9447826-31, and GI:163847210-11, Caur_1647-48, YP_001635254.1-55.1; GI:163849262, Caur_3739, YP_001637306.1; GI:163848951, Caur_3421, YP_001636995.1; GI:163846951, Caur_1378, YP_001634995.1. Naturally occurring or synthetic enzymes may be used. Typically the enzymes will have at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least

92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, identity with the sequence encoded by a nucleic acid according to SEQ ID NO:1 or 18-21.

[094] The enzymes (and any corresponding genes encoding them) of use in the microorganisms of the invention may be derived from any appropriate source, including different genera and species of bacteria, or other organisms. However, in one embodiment, the Malonyl-coenzymeA reductase is that derived from *Chloroflexus auranticus*, *Clostridium ljungdahlii*, *Metallosphaera* or *Sulfolobus spp.* In one embodiment, the Malonyl-coenzyme A reductase has the amino acid sequence exemplified above, or it is a functionally equivalent variant thereof. In one embodiment, the Acetyl CoA carboxylase is that derived from *Clostridium ljungdahlii*, *Chloroflexus auranticus*, *Metallosphaera* or *Sulfolobus spp.* In one embodiment, the Acetyl CoA carboxylase has the amino acid sequence exemplified herein before, or it is a functionally equivalent variant thereof.

[095] Malonyl-CoA reductase (EC 1.2.1.75) belongs to the group of short-chain reductases (SDRs) and can be obtained from bacteria as green non-sulfur phototrophic bacteria (Chloroflexi) *Chloroflexus aurantiacus* (YP_001636209.1; AAS20429.1), *Chloroflexus aggregans* (YP_002462600.1), *Oscillochloris trichoides* (WP_006561105.1), *Roseiflexus castenholzii* (YP_001433009.1) or *Roseiflexus sp.* (YP_001277512.1), and in alpha-proteobacteria as *Erythrobacter sp.* (WP_007163680), and as gamma proteobacteria (WP_009019528.1, WP_007234918.1, WP_009021869.1, WP_009470571.1), and can be obtained from thermoacidophilic archaea as *Crenarchaeotes Sulfolobus tokodaii* (NP_378167.1), *Acidianus hospitalis* (YP_004459517.1), *Metallosphaera cuprina* (YP_004410014.1) *Metallosphaera sedula* (YP_001190808.1), *Sulfolobus solfataricus* (NP_343563.1), *Metallosphaera yellowstonensis* (WP_009071519.1), *Sulfolobus islandicus* (YP_002844727.1; YP_002833533.1; YP_002830795.1), *Sulfolobus acidocaldarius* (YP_256941.1; YP_256733.1) and as *Archaeoglobus profundus* (YP_003401535.1), and as *Candidatus Chloracidobacterium thermophilum* (YP_004863680.1) or *Caldiarchaeum subterraneum* (BAJ47902.1).

- [096] Acetyl CoA carboxylase (EC 1.2.1.75) belongs to the group of biotin dependent carboxylases and can be obtained from bacteria as green non-sulfur phototrophic bacteria (Chloroflexi) as *Chloroflexus aurantiacus* (YP_001635254.1-55.1; YP_001637306.1; YP_001636995.1; YP_001634995.1), or carboxydophilic acetogens as *C. ljungdahlii* (NC_014328.1-33.1).
- [097] In one embodiment, the microorganism comprises one or more exogenous nucleic acids adapted to increase expression of one or more nucleic acids native to the parental microorganism and which one or more nucleic acids encode one or more of the enzymes (or one or more subunits thereof) referred to herein before. In one embodiment, the one or more exogenous nucleic acid adapted to increase expression is a regulatory element. In one embodiment, the regulatory element is a promoter. In one embodiment, the promoter is a constitutive promoter that is preferably highly active under appropriate fermentation conditions. Inducible promoters could also be used. In preferred embodiments, the promoter is selected from the group comprising Wood-Ljungdahl gene cluster or Phosphotransacetylase/Acetate kinase operon promoters. It will be appreciated by those of skill in the art that other promoters which can direct expression, preferably a high level of expression under appropriate fermentation conditions, would be effective as alternatives to the exemplified embodiments. When a promoter is in a position such that it drives expression of a downstream coding sequence it is referred to as operably linked.
- [098] In one embodiment, the microorganism comprises one or more exogenous nucleic acids encoding and adapted to express one or more of the enzymes (or one or more subunits thereof) referred to herein before. In one embodiment, the microorganisms comprise one or more exogenous nucleic acid encoding and adapted to express at least two of the enzymes (or one or more subunits thereof).
- [099] In one particular embodiment, the microorganism comprises one or more exogenous nucleic acids encoding Malonyl-CoenzymeA reductase or a functionally equivalent variant thereof. In one particular embodiment, the microorganism comprises one or more exogenous nucleic acids encoding Acetyl CoA carboxylase or a functionally equivalent variant thereof. Acetyl CoA carboxylase may be comprised of 4 subunits, with each subunit encoded by a different gene. These genes may be combined in a

single nucleic acid or two or more nucleic acids which together encode the whole enzyme. In addition, a particular parental microorganism may contain genes for only one, two, or three of these subunits. Accordingly, the invention encompasses engineering the microorganism using one or more exogenous nucleic to express one, two or three of the subunits only. Similarly, it encompasses engineering the microorganism to over-express one or more of the subunits if the genes are native to the microorganism. Combinations of over-expression of native subunit genes and introduction of any missing subunit genes is also envisaged.

[0100] In one embodiment, the Malonyl-CoenzymeA reductase is encoded by a nucleic acid comprising SEQ ID NO: 1, or a functionally equivalent variant thereof. In one embodiment, the Acetyl CoA carboxylase is encoded by one or more nucleic acid comprising SEQ ID NO: 18, 19, 20 and 21, or a functionally equivalent variant of any one or more thereof. Alternatively, the enzymes may be encoded by a nucleic acid sequence as described in a publicly available database, for example, as listed herein before.

[0101] The microorganism may comprise one or more exogenous nucleic acids. Where it is desirable to transform the parental microorganism with two or more genetic elements (such as genes or regulatory elements (for example a promoter)) they may be contained on one or more exogenous nucleic acids.

[0102] In one embodiment, the one or more exogenous nucleic acid is a nucleic acid construct or vector, in one particular embodiment a plasmid, encoding one or more of the enzymes referred to hereinbefore in any combination.

[0103] The exogenous nucleic acids may remain extra-chromosomal upon transformation of the parental microorganism or may integrate into the genome of the parental microorganism. Accordingly, they may include additional nucleotide sequences adapted to assist integration (for example, a region which allows for homologous recombination and targeted integration into the host genome) or expression and replication of an extrachromosomal construct (for example, origin of replication, promoter and other regulatory elements or sequences).

[0104] In one embodiment, the exogenous nucleic acids encoding one or enzymes (or one or more subunits thereof) as mentioned herein before will further comprise a promoter adapted to promote expression of the one or more enzymes encoded by the exogenous nucleic acids. In one embodiment, the promoter is a constitutive promoter that is preferably highly active under appropriate fermentation conditions. Inducible promoters could also be used. In preferred embodiments, the promoter is selected from the group comprising Wood-Ljungdahl gene cluster and Phosphotransacetylase/Acetate kinase promoters. It will be appreciated by those of skill in the art that other promoters which can direct expression, preferably a high level of expression under appropriate fermentation conditions, would be effective as alternatives to the exemplified embodiments.

[0105] In one embodiment, the exogenous nucleic acid is an expression plasmid.

[0106] In one embodiment, the parental microorganism is selected from the group of anaerobic acetogens.

[0107] In one particular embodiment, the parental microorganism is selected from the group of carboxydophilic acetogenic bacteria. In certain embodiments the microorganism is selected from the group comprising *Clostridium autoethanogenum*, *Clostridium ljungdahlii*, *Clostridium ragsdalei*, *Clostridium carboxidivorans*, *Clostridium drakei*, *Clostridium scatologenes*, *Clostridium aceticum*, *Clostridium formicoaceticum*, *Clostridium magnum*, *Butyribacterium methylotrophicum*, *Acetobacterium woodii*, *Alkalibaculum bacchii*, *Blautia producta*, *Eubacterium limosum*, *Moorella thermoacetica*, *Moorella thermautotrophica*, *Sporomusa ovata*, *Sporomusa silvacetica*, *Sporomusa sphaeroides*, *Oxobacter pfennigii*, and *Thermoanaerobacter kiuvi*.

[0108] In one particular embodiment, the parental microorganism is selected from the cluster of ethanologenic, acetogenic Clostridia comprising the species *C. autoethanogenum*, *C. ljungdahlii*, and *C. ragsdalei* and related isolates. These include but are not limited to strains *C. autoethanogenum* JAI-1^T (DSM10061) [Abrini J, Naveau H, Nyns E-J: *Clostridium autoethanogenum*, sp. nov., an anaerobic bacterium that produces ethanol from carbon monoxide. Arch Microbiol 1994, 4: 345-351], *C. autoethanogenum*

LBS1560 (DSM19630) [Simpson SD, Forster RL, Tran PT, Rowe MJ, Warner IL: Novel bacteria and methods thereof. International patent 2009, WO/2009/064200], *C. autoethanogenum* LBS1561 (DSM23693), *C. ljungdahlii* PETC^T (DSM13528 = ATCC 55383) [Tanner RS, Miller LM, Yang D: *Clostridium ljungdahlii* sp. nov., an Acetogenic Species in Clostridial rRNA Homology Group I. Int J Syst Bacteriol 1993, 43: 232-236], *C. ljungdahlii* ERI-2 (ATCC 55380) [Gaddy JL: *Clostridium* stain which produces acetic acid from waste gases. US patent 1997, 5,593,886], *C. ljungdahlii* C-01 (ATCC 55988) [Gaddy JL, Clausen EC, Ko C-W: Microbial process for the preparation of acetic acid as well as solvent for its extraction from the fermentation broth. US patent, 2002, 6,368,819], *C. ljungdahlii* O-52 (ATCC 55989) [Gaddy JL, Clausen EC, Ko C-W: Microbial process for the preparation of acetic acid as well as solvent for its extraction from the fermentation broth. US patent, 2002, 6,368,819], *C. ragsdalei* P11^T (ATCC BAA-622) [Huhnke RL, Lewis RS, Tanner RS: Isolation and Characterization of novel Clostridial Species. International patent 2008, WO 2008/028055], related isolates such as “*C. coskatii*” [Zahn *et al* - Novel ethanogenic species *Clostridium coskatii* (US Patent Application number US20110229947)] and “*Clostridium* sp.” (Tyurin *et al.*, 2012, *J. Biotech Res.* 4: 1-12), or mutated strains such as *C. ljungdahlii* OTA-1 (Tirado-Acevedo O. Production of Bioethanol from Synthesis Gas Using *Clostridium ljungdahlii*. PhD thesis, North Carolina State University, 2010). These strains form a subcluster within the Clostridial rRNA cluster I, and their 16S rRNA gene is more than 99% identical with a similar low GC content of around 30%. However, DNA-DNA reassociation and DNA fingerprinting experiments showed that these strains belong to distinct species [Huhnke RL, Lewis RS, Tanner RS: Isolation and Characterization of novel Clostridial Species. International patent 2008, WO 2008/028055].

- [0109] All species of this cluster have a similar morphology and size (logarithmic growing cells are between 0.5-0.7 x 3-5 µm), are mesophilic (optimal growth temperature between 30-37 °C) and strictly anaerobe [Tanner RS, Miller LM, Yang D: *Clostridium ljungdahlii* sp. nov., an Acetogenic Species in Clostridial rRNA Homology Group I. Int J Syst Bacteriol 1993, 43: 232-236; Abrini J, Naveau H, Nyns E-J: *Clostridium autoethanogenum*, sp. nov., an anaerobic bacterium that produces ethanol from carbon monoxide. Arch Microbiol 1994, 4: 345-351; Huhnke RL, Lewis RS, Tanner RS: Isolation and Characterization of novel Clostridial Species.

International patent 2008, WO 2008/028055]. Moreover, they all share the same major phylogenetic traits, such as same pH range (pH 4-7.5, with an optimal initial pH of 5.5-6), strong autotrophic growth on CO containing gases with similar growth rates, and a similar metabolic profile with ethanol and acetic acid as main fermentation end product, and small amounts of 2,3-butanediol and lactic acid formed under certain conditions. [Tanner RS, Miller LM, Yang D: *Clostridium ljungdahlii* sp. nov., an Acetogenic Species in Clostridial rRNA Homology Group I. Int J Syst Bacteriol 1993, 43: 232-236; Abrini J, Naveau H, Nyns E-J: *Clostridium autoethanogenum*, sp. nov., an anaerobic bacterium that produces ethanol from carbon monoxide. Arch Microbiol 1994, 4: 345-351; Huhnke RL, Lewis RS, Tanner RS: Isolation and Characterization of novel Clostridial Species. International patent 2008, WO 2008/028055]. Indole production was observed with all three species as well. However, the species differentiate in substrate utilization of various sugars (*e.g.*, rhamnose, arabinose), acids (*e.g.*, gluconate, citrate), amino acids (*e.g.*, arginine, histidine), or other substrates (*e.g.*, betaine, butanol). Moreover some of the species were found to be auxotrophic for certain vitamins (*e.g.*, thiamine, biotin) while others were not.

[0110] In one embodiment, the parental strain uses CO as its sole carbon and energy source.

[0111] In one embodiment the parental microorganism is *Clostridium autoethanogenum* or *Clostridium ljungdahlii*. In one particular embodiment, the microorganism is *Clostridium autoethanogenum* DSM23693. In another particular embodiment, the microorganism is *Clostridium ljungdahlii* DSM13528 (or ATCC55383).

[0112] In one embodiment, the parental microorganism lacks one or more genes encoding Malonyl-coenzymeA reductase or Acetyl CoA carboxylase (or one or more subunit thereof).

Nucleic acids

[0113] The invention also provides nucleic acids and nucleic acid constructs of use in generating a recombinant microorganism of the invention.

- [0114] In one embodiment, the nucleic acids comprise one or more sequences encoding one or more of the enzymes (or one or more subunits thereof) in the 3-HP biosynthesis pathway which when expressed in a microorganism allows the microorganism to produce 3-HP by fermentation of substrate comprising CO and/or CO₂. In one particular embodiment, the invention provides a nucleic acid encoding two or more enzymes (or one or more subunits thereof) which when expressed in a microorganism allows the microorganism to produce 3-HP by fermentation of substrate comprising CO and/or CO₂.
- [0115] In one particular embodiment, the enzymes are chosen from Malonyl CoA reductase, Acetyl CoA carboxylase and a functionally equivalent variant of any one or more thereof.
- [0116] In one embodiment, a nucleic acid of the invention comprises one or more nucleic acid sequences encoding Malonyl-CoenzymeA reductase, Acetyl CoA carboxylase or a functionally equivalent variant of any one or more thereof, in any order.
- [0117] In one embodiment, a nucleic acid of the invention comprises one or more nucleic acid sequences encoding one or more subunit of Acetyl CoA carboxylase or a functionally equivalent variant of any one or more thereof, in any order.
- [0118] Exemplary amino acid sequences and nucleic acid sequence encoding each of the above enzymes are provided herein or can be obtained from GenBank as mentioned hereinbefore. However, skilled persons will readily appreciate alternative nucleic acids sequences encoding the enzymes or functionally equivalent variants thereof, having regard to the information contained herein, in GenBank and other databases, and the genetic code.
- [0119] In one embodiment, Malonyl-CoenzymeA reductase has a sequence as herein before described or is a functionally equivalent variant thereof.
- [0120] In one embodiment, the nucleic acid sequence encoding Acetyl CoA carboxylase has a sequence as herein before described or is a functionally equivalent variant thereof.

[0121] In one embodiment, the nucleic acids of the invention will further comprise a promoter. In one embodiment, the promoter allows for constitutive expression of the genes under its control. However, inducible promoters may also be employed. Persons of skill in the art will readily appreciate promoters of use in the invention. Preferably, the promoter can direct a high level of expression under appropriate fermentation conditions. In a particular embodiment a Wood-Ljungdahl cluster promoter is used. In another embodiment, a Phosphotransacetylase/Acetate kinase promoter is used. In another embodiment a pyruvate: ferredoxin oxidoreductase promoter, an Rnf complex operon promoter or an ATP synthase operon promoter. In one particular embodiment, the promoter is from *C. autoethanogenum*.

[0122] The nucleic acids of the invention may remain extra-chromosomal upon transformation of a parental microorganism or may be adapted for integration into the genome of the microorganism. Accordingly, nucleic acids of the invention may include additional nucleotide sequences adapted to assist integration (for example, a region which allows for homologous recombination and targeted integration into the host genome) or stable expression and replication of an extrachromosomal construct (for example, origin of replication, promoter and other regulatory sequences).

[0123] In one embodiment, the nucleic acid is nucleic acid construct or vector. In one particular embodiment, the nucleic acid construct or vector is an expression construct or vector, however other constructs and vectors, such as those used for cloning are encompassed by the invention. In one particular embodiment, the expression construct or vector is a plasmid.

[0124] It will be appreciated that an expression construct/vector of the present invention may contain any number of regulatory elements in addition to the promoter as well as additional genes suitable for expression of further proteins if desired. In one embodiment the expression construct/vector includes one promoter. In another embodiment, the expression construct/vector includes two or more promoters. In one particular embodiment, the expression construct/vector includes one promoter for each gene to be expressed. In one embodiment, the expression construct/vector includes one or more ribosomal binding sites, preferably a ribosomal binding site for each gene to be expressed.

[0125] It will be appreciated by those of skill in the art that the nucleic acid sequences and construct/vector sequences described herein may contain standard linker nucleotides such as those required for ribosome binding sites and/or restriction sites. Such linker sequences should not be interpreted as being required and do not provide a limitation on the sequences defined.

[0126] Nucleic acids and nucleic acid constructs, including expression constructs/vectors of the invention may be constructed using any number of techniques known in the art. For example, chemical synthesis or recombinant techniques may be used. Such techniques are described, for example, in Sambrook et al (Molecular Cloning: A laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989). Further exemplary techniques are described in the Examples section herein after. Essentially, the individual genes and regulatory elements will be operably linked to one another such that the genes can be expressed to form the desired proteins. Suitable vectors for use in the invention will be appreciated by those of ordinary skill in the art. However, by way of example, the following vectors may be suitable: pMTL80000 vectors, pIMP1, pJIR750, and the plasmids exemplified in the Examples section herein after.

[0127] It should be appreciated that nucleic acids of the invention may be in any appropriate form, including RNA, DNA, or cDNA.

[0128] The invention also provides host organisms, particularly microorganisms, and including viruses, bacteria, and yeast, comprising any one or more of the nucleic acids described herein.

[0129] The one or more exogenous nucleic acids may be delivered to a parental microorganism as naked nucleic acids or may be formulated with one or more agents to facilitate the transformation process (for example, liposome-conjugated nucleic acid, an organism in which the nucleic acid is contained). The one or more nucleic acids may be DNA, RNA, or combinations thereof, as is appropriate. Restriction inhibitors may be used in certain embodiments; see, for example Murray, N.E. *et al.* (2000) *Microbial. Molec. Biol. Rev.* 64, 412.)

[0130] The microorganisms of the invention may be prepared from a parental microorganism and one or more exogenous nucleic acids using any number of techniques known in the art for producing recombinant microorganisms. By way of example only, transformation (including transduction or transfection) may be achieved by electroporation, ultrasonication, polyethylene glycol-mediated transformation, chemical or natural competence, or conjugation. Suitable transformation techniques are described for example in, Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A laboratory Manual, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, 1989.

[0131] In certain embodiments, due to the restriction systems which are active in the microorganism to be transformed, it is necessary to methylate the nucleic acid to be introduced into the microorganism. This can be done using a variety of techniques, including those described below, and further exemplified in the Examples section herein after.

[0132] By way of example, in one embodiment, a recombinant microorganism of the invention is produced by a method comprises the following steps: introduction into a shuttle microorganism of (i) of an expression construct/vector as described herein and (ii) a methylation construct/vector comprising a methyltransferase gene; expression of the methyltransferase gene; isolation of one or more constructs/vectors from the shuttle microorganism; and, introduction of the one or more construct/vector into a destination microorganism.

[0133] In one embodiment, the methyltransferase gene of step B is expressed constitutively. In another embodiment, expression of the methyltransferase gene of step B is induced.

[0134] The shuttle microorganism is a microorganism, preferably a restriction negative microorganism that facilitates the methylation of the nucleic acid sequences that make up the expression construct/vector. In a particular embodiment, the shuttle microorganism is a restriction negative *E. coli*, *Bacillus subtilis*, or *Lactococcus lactis*.

[0135] The methylation construct/vector comprises a nucleic acid sequence encoding a methyltransferase.

[0136] Once the expression construct/vector and the methylation construct/vector are introduced into the shuttle microorganism, the methyltransferase gene present on the methylation construct/vector is induced. Induction may be by any suitable promoter system although in one particular embodiment of the invention, the methylation construct/vector comprises an inducible lac promoter and is induced by addition of lactose or an analogue thereof, more preferably isopropyl- β -D-thio-galactoside (IPTG). Other suitable promoters include the ara, tet, or T7 system. In a further embodiment of the invention, the methylation construct/vector promoter is a constitutive promoter.

[0137] In a particular embodiment, the methylation construct/vector has an origin of replication specific to the identity of the shuttle microorganism so that any genes present on the methylation construct/vector are expressed in the shuttle microorganism. Preferably, the expression construct/vector has an origin of replication specific to the identity of the destination microorganism so that any genes present on the expression construct/vector are expressed in the destination microorganism.

[0138] Expression of the methyltransferase enzyme results in methylation of the genes present on the expression construct/vector. The expression construct/vector may then be isolated from the shuttle microorganism according to any one of a number of known methods. By way of example only, the methodology described in the Examples section described hereinafter may be used to isolate the expression construct/vector.

[0139] In one particular embodiment, both construct/vector are concurrently isolated.

[0140] The expression construct/vector may be introduced into the destination microorganism using any number of known methods. However, by way of example, the methodology described in the Examples section hereinafter may be used. Since the expression construct/vector is methylated, the nucleic acid sequences present on

the expression construct/vector are able to be incorporated into the destination microorganism and successfully expressed.

[0141] It is envisaged that a methyltransferase gene may be introduced into a shuttle microorganism and over-expressed. Thus, in one embodiment, the resulting methyltransferase enzyme may be collected using known methods and used *in vitro* to methylate an expression plasmid. The expression construct/vector may then be introduced into the destination microorganism for expression. In another embodiment, the methyltransferase gene is introduced into the genome of the shuttle microorganism followed by introduction of the expression construct/vector into the shuttle microorganism, isolation of one or more constructs/vectors from the shuttle microorganism and then introduction of the expression construct/vector into the destination microorganism.

[0142] It is envisaged that the expression construct/vector and the methylation construct/vector as defined above may be combined to provide a composition of matter. Such a composition has particular utility in circumventing restriction barrier mechanisms to produce the recombinant microorganisms of the invention.

[0143] In one particular embodiment, the expression construct/vector and/or the methylation construct/vector are plasmids.

[0144] Persons of ordinary skill in the art will appreciate a number of suitable methyltransferases of use in producing the microorganisms of the invention. However, by way of example the *Bacillus subtilis* phage Φ T1 methyltransferase and the methyltransferase described in the Examples herein after may be used. In one embodiment, the methyltransferase has the amino acid sequence of SEQ ID NO: 6, or is a functionally equivalent variant thereof. Nucleic acids encoding suitable methyltransferases will be readily appreciated having regard to the sequence of the desired methyltransferase and the genetic code. In one embodiment, the nucleic acid encoding a methyltransferase is as described in the Examples herein after (for example the nucleic acid of SEQ ID NO: 26, or it is a functionally equivalent variant thereof).

[0145] Any number of constructs/vectors adapted to allow expression of a methyltransferase gene may be used to generate the methylation construct/vector. However, by way of example, the plasmid described in the Examples section hereinafter may be used (for example, SEQ ID NO: 7).

Methods of production

[0146] The invention provides a method for the production of 3-HP and optionally one or more other products by microbial fermentation comprising fermenting a substrate comprising CO and/or CO₂ using a recombinant microorganism of the invention. Preferably, 3-HP is the main fermentation product. The methods of the invention may be used to reduce the total atmospheric carbon emissions from an industrial process.

[0147] Preferably, the fermentation comprises the steps of anaerobically fermenting a substrate in a bioreactor to produce at least 3-HP using a recombinant microorganism of the invention.

[0148] In one embodiment the method comprises the steps of:

- (a) providing a substrate comprising CO and/or CO₂ to a bioreactor containing a culture of one or more microorganism of the invention; and
- (b) anaerobically fermenting the culture in the bioreactor to produce at least 3-HP.

[0149] In one embodiment the method comprises the steps of:

- (a) capturing CO- and/or CO₂-containing gas produced as a result of the industrial process, before the gas is released into the atmosphere;
- (b) the anaerobic fermentation of the CO- and/or CO₂-containing gas to produce the at least 3-HP by a culture containing one or more microorganism of the invention.

[0150] In one embodiment, the substrate comprises CO. In one embodiment, the substrate comprises CO₂ and CO. In another embodiment, the substrate comprises CO₂ and H₂. In another embodiment, the substrate comprises CO₂ and CO and H₂.

[0151] In one particular embodiment of the invention, the gaseous substrate fermented by the microorganism is a gaseous substrate containing CO. The gaseous substrate may be a CO-containing waste gas obtained as a by-product of an industrial process, or from some other source such as from automobile exhaust fumes. In certain embodiments, the industrial process is selected from the group consisting of ferrous metal products manufacturing, such as a steel mill, non-ferrous products manufacturing, petroleum refining processes, gasification of coal, electric power production, carbon black production, ammonia production, methanol production and coke manufacturing. In these embodiments, the CO-containing gas may be captured from the industrial process before it is emitted into the atmosphere, using any convenient method. The CO may be a component of syngas (gas comprising carbon monoxide and hydrogen). The CO produced from industrial processes is normally flared off to produce CO₂ and therefore the invention has particular utility in reducing CO₂ greenhouse gas emissions and producing butanol for use as a biofuel. Depending on the composition of the gaseous CO-containing substrate, it may also be desirable to treat it to remove any undesired impurities, such as dust particles before introducing it to the fermentation. For example, the gaseous substrate may be filtered or scrubbed using known methods.

[0152] In particular embodiments of the invention, the gaseous substrate fermented by the microorganism is a gaseous substrate comprising CO₂ and H₂. The CO₂/H₂ containing substrate may be a waste gas obtained as a by-product of an industrial process. In certain embodiments the industrial process is selected from the group consisting of hydrogen production. In certain embodiments the gaseous substrate comprising CO₂ and H₂ may be a blended gas stream, wherein at least a portion of the gas stream is derived from one or more industrial process is blended with at least a portion of CO₂ or H₂ to optimise the CO₂:H₂ ratio of the gaseous substrate. This may be particularly beneficial for industrial gas streams rich in either CO₂ or H₂. Examples of industrial process which produce by-product gas streams which can be used as a source for a CO₂ and H₂ substrate, or a CO₂ and H₂ blended substrate include coke manufacturing, refinery processes, ammonia production processes, methanol production processes, acetic acid production, natural gas refineries and power plants.

[0153] It will be appreciated that for growth of the bacteria and conversion of gas to products comprising 3-HP to occur, a suitable liquid nutrient medium in addition to the CO- and/or CO₂ -containing substrate gas will need to be fed to the bioreactor. The substrate and media may be fed to the bioreactor in a continuous, batch or batch fed fashion. A nutrient medium will contain vitamins and minerals sufficient to permit growth of the micro-organism used. Anaerobic media suitable for fermentation to produce one or more products using CO and/or CO₂ are known in the art. For example, suitable media are described Biebel (2001). In one embodiment of the invention the media is as described in the Examples section herein after.

[0154] The fermentation should desirably be carried out under appropriate conditions for the fermentation supporting the conversion of the gas to products comprising 3-HP to occur. Reaction conditions that should be considered include pressure, temperature, gas flow rate, liquid flow rate, media pH, media redox potential, agitation rate (if using a continuous stirred tank reactor), inoculum level, maximum gas substrate concentrations to ensure that CO and/or CO₂ in the liquid phase does not become limiting, and maximum product concentrations to avoid product inhibition.

[0155] In addition, it is often desirable to increase the CO and/or CO₂ concentration of a substrate stream (or CO and/or CO₂ partial pressure in a gaseous substrate) and thus increase the efficiency of fermentation reactions where CO and/or CO₂ is a substrate. Operating at increased pressures allows a significant increase in the rate of CO and/or CO₂ transfer from the gas phase to the liquid phase where it can be taken up by the micro-organism as a carbon source to make products comprising 3-HP. This in turn means that the retention time (defined as the liquid volume in the bioreactor divided by the input gas flow rate) can be reduced when bioreactors are maintained at elevated pressure rather than atmospheric pressure. The optimum reaction conditions will depend partly on the particular micro-organism of the invention used. However, in general, it is preferred that the fermentation be performed at pressure higher than ambient pressure. Also, since a given CO- and/or CO₂ -to-at least 3-HP conversion rate is in part a function of the substrate retention time, and achieving a desired retention time in turn dictates the required volume of a bioreactor, the use of pressurized systems can greatly reduce the volume of the bioreactor required, and consequently the capital cost of the fermentation equipment. According to examples

given in U.S. Patent No. 5,593,886, reactor volume can be reduced in linear proportion to increases in reactor operating pressure, i.e. bioreactors operated at 10 atmospheres of pressure need only be one tenth the volume of those operated at 1 atmosphere of pressure.

[0156] By way of example, the benefits of conducting a gas-to-ethanol fermentation at elevated pressures has been described. For example, WO 02/08438 describes gas-to-ethanol fermentations performed under pressures of 30 psig and 75 psig, giving ethanol productivities of 150 g/l/day and 369 g/l/day respectively. However, example fermentations performed using similar media and input gas compositions at atmospheric pressure were found to produce between 10 and 20 times less ethanol per litre per day.

[0157] It is also desirable that the rate of introduction of the CO and/or CO₂ -containing gaseous substrate is such as to ensure that the concentration of CO and/or CO₂ in the liquid phase does not become limiting. This is because a consequence of CO- and/or CO₂ -limited conditions may be that one or more product is consumed by the culture.

[0158] The composition of gas streams used to feed a fermentation reaction can have a significant impact on the efficiency and/or costs of that reaction. For example, O₂ may reduce the efficiency of an anaerobic fermentation process. Processing of unwanted or unnecessary gases in stages of a fermentation process before or after fermentation can increase the burden on such stages (to products comprising where the gas stream is compressed before entering a bioreactor, unnecessary energy may be used to compress gases that are not needed in the fermentation). Accordingly, it may be desirable to treat substrate streams, particularly substrate streams derived from industrial sources, to remove unwanted components and increase the concentration of desirable components.

[0159] In certain embodiments a culture of a bacterium of the invention is maintained in an aqueous culture medium. Preferably the aqueous culture medium is a minimal anaerobic microbial growth medium. Suitable media are known in the art and described for example in U.S. Patent Nos. 5,173,429 and 5,593,886 and WO 02/08438, and as described in the Examples section herein after.

[0160] 3-HP, or a mixed stream containing 3-HP and/or one or more other products, may be recovered from the fermentation broth by methods known in the art, such as fractional distillation or evaporation, pervaporation, gas stripping and extractive fermentation, including for example, liquid-liquid extraction.

[0161] In certain preferred embodiments of the invention, 3-HP and one or more products are recovered from the fermentation broth by continuously removing a portion of the broth from the bioreactor, separating microbial cells from the broth (conveniently by filtration), and recovering one or more products from the broth. Alcohols may conveniently be recovered for example by distillation. Acetone may be recovered for example by distillation. Any acids produced may be recovered for example by adsorption on activated charcoal. The separated microbial cells are preferably returned to the fermentation bioreactor. The cell free permeate remaining after any alcohol(s) and acid(s) have been removed is also preferably returned to the fermentation bioreactor. Additional nutrients (such as B vitamins) may be added to the cell free permeate to replenish the nutrient medium before it is returned to the bioreactor.

[0162] Also, if the pH of the broth was adjusted as described above to enhance adsorption of acetic acid to the activated charcoal, the pH should be re-adjusted to a similar pH to that of the broth in the fermentation bioreactor, before being returned to the bioreactor.

[0163] 3-HP may be recovered following fermentation using any appropriate methodology including but not limited to pervaporation, reverse osmosis, and liquid liquid extraction techniques.

EXAMPLES

[0164] The invention will now be described in more detail with reference to the following non-limiting examples.

Example 1

[0165] Two CO₂ fixing pathways, the linear Wood-Ljungdahl pathway of acetogens and the 3-HP cycle found in Green nonsulfur bacteria and Archea (Thauer, 2007, Science,

318: 1732-33) were combined to present a sustainable route towards the platform chemical 3-Hydroxypropionate (3-HP). This route allows fixation of 3 molecules of CO or CO₂ into one molecule of 3-HP. A carboxydutrophic acetogenic organism, *Clostridium autoethanogenum*, was chosen and metabolically engineered with genes performing the initial CO₂ fixation step from the nonsulfur photosynthetic bacterium *Chloroflexus auranticus* (Fig. 1).

[0166] Carboxydutrophic acetogens such as *Clostridium autoethanogenum* or *Clostridium ljungdahlii* are able to grow autotrophically by fixing two molecules of CO or CO₂ and fusing them to form acetyl-CoA. Nonsulfur photosynthetic bacterium such as *Chloroflexus auranticus* are able to fix CO₂ in a cyclic process. They use acetyl-CoA as starting point and fuse it in an ATP dependent step catalyzed by an acetyl-CoA carboxylase (EC. 6.4.1.2) to form malonyl-CoA, which can then be reduced to 3-HP, the central intermediate of this cycle by action of a Malonyl-Coenzyme A reductase (EC 1.2.1.75) (Huegler et al, 2002, *J. Bacteriol.* 184: 2404-10). A Malonyl-Coenzyme A reductase gene, enzyme (GI:163848165, Caur_2614; YP_001636209.1), was introduced into the carboxydutrophic organism to form a new metabolic route that fixes three molecules of CO or CO₂ into 3-HP. An acetyl-CoA carboxylase was identified to be already present in the host organisms as part of fatty acid biosynthesis (*C. autoethanogenum*: SEQ ID NO: 18-21; *C. ljungdahlii*: CLJU_c42100-40, GI: 9447826-31, NC_014328.1-33.1) but the *Chloroflexus auranticus* acetyl-CoA carboxylase (GI:163847210 -11, Caur_1647 -48, YP_001635254.1-55.1; GI:163849262, Caur_3739, YP_001637306.1; GI:163848951, Caur_3421, YP_001636995.1; GI:163846951, Caur_1378, YP_001634995.1) can be introduced in addition to the essential Malonyl-Coenzyme A reductase.

Materials and Methods

Microorganisms and Growth Conditions

[0167] *C. autoethanogenum* DSM23693 is a derivative of *C. autoethanogenum* DSM10061 sourced from DSMZ (The German Collection of Microorganisms and Cell Cultures, Inhoffenstraße 7 B, 38124 Braunschweig, Germany).

[0168] *E. coli* XL1-Blue MRF' Kan was purchased from Stratagene (Santa Clara, CA 95051-7201, USA).

[0169] *E. coli* was cultivated under aerobic conditions, while all other strains were grown strictly anaerobically in a volume of 50 ml liquid media in serum bottles with fructose (heterotrophic growth) or 30 psi CO-containing steel mill gas (collected from New Zealand Steel site in Glenbrook, NZ; composition: 44% CO, 32% N₂, 22% CO₂, 2% H₂) in the headspace (autotrophic growth).

[0170] Media were prepared using standard anaerobic techniques (Hungate RE: A roll tube method for cultivation of strict anaerobes, in Norris JR and Ribbons DW (eds.), *Methods in Microbiology*, vol. 3B. Academic Press, New York, 1969: 117-132; Wolfe RS: Microbial formation of methane. *Adv Microb Physiol* 1971, 6: 107-146) according to formulations are given in Tab. 1-3. For solid media, 1.2 % Bacto agar (BD, Frankton Lakes, NJ 07417, USA) was added.

[0171] All strains were grown at 37 °C except as otherwise stated.

Table 1: PETC-MES medium (*C. autoethanogenum* pH5.6)

Media component	Concentration per 1.0L of media
NH ₄ Cl	1 g
KCl	0.1 g
MgSO ₄ .7H ₂ O	0.2 g
NaCl	0.8 g
KH ₂ PO ₄	0.2 g
CaCl ₂	0.02 g
Trace metal solution (see below)	10 ml
Wolfe's vitamin solution (see below)	10 ml
Yeast Extract	2 g
Resazurin (2 g/L stock)	0.5 ml
2-(N-morpholino)ethanesulfonic acid (MES)	20 g
Sodium acetate	0.25 g
Reducing agent	0.006-0.008 % (v/v)
Fructose (for heterotrophic growth)	5 g
Trace metal solution	per L of stock

Nitrilotriacetic Acid	2 g
MnSO ₄ .H ₂ O	1 g
Fe (SO ₄) ₂ (NH ₄) ₂ .6H ₂ O	0.8 g
CoCl ₂ .6H ₂ O	0.2 g
ZnSO ₄ .7H ₂ O	0.2 mg
CuCl ₂ .2H ₂ O	0.02 g
NaMoO ₄ .2H ₂ O	0.02 g
Na ₂ SeO ₃	0.02 g
NiCl ₂ .6H ₂ O	0.02 g
Na ₂ WO ₄ .2H ₂ O	0.02 g
Reducing agent stock	per 100 mL of stock
NaOH	0.9 g
Cystein.HCl	4 g
Na ₂ S	4 g

Table 2: Luria Bertani medium LB (*E. coli*)

Media component	Concentration per 1.0L of media
Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g

Table 3: M9 minimal media (*E. coli*)

Media component	Concentration per 1.0L of media
Na ₂ HPO ₄	6 g
KH ₂ PO ₄	3 g
NaCl	0.5 g
NH ₄ Cl	1 g
100 mM MgSO ₄	10ml
Glucose	20%
CaCl ₂	10 mM
Thiamine-HCl	100 mM

Construction of expression plasmid with Malonyl-Coenzyme A reductase from *C. aurantiacus*

[0172] Standard recombinant DNA and molecular cloning techniques were used (Sambrook, J., and Russell, D., Molecular cloning: A Laboratory Manual 3rd Ed., Cold Spring Harbour Lab Press, Cold Spring Harbour, NY, 2001). The DNA sequence of Malonyl-Coenzyme A reductase from *C.aurantiacus* was obtained from NCBI GenBank (GI:163848165, Caur_2614; YP_001636209.1).

[0173] The Malonyl-Coenzyme A reductase from *Chloroflexus aurantiacus* was codon-optimised (SEQ ID NO: 1) and synthesized by ATG:Biosynthetics GmbH (Merzhausen, Germany), flanked by *Nde*I and *Eco*I restriction sites for further sub-cloning. The Phosphotransacetylase/Acetate kinase operon promoter ($P_{pta-ack}$) of *C. autoethanogenum* was used for expression of Malonyl-Coenzyme A reductase. All DNA sequences used are given in Table 4.

Table 4: Sequences used for expression plasmid with Malonyl-Coenzyme A reductase from *C. aurantiacus*

Description	Source	SEQ ID NO:
Malonyl-Coenzyme A reductase	Codon-optimised	1
Phosphotransacetylase/Acetate kinase operon promoter region	<i>Clostridium autoethanogenum</i> DSM10061	2

[0174] The promoter region of the phosphotransacetylase-acetate kinase operon ($P_{pta-ack}$) (SEQ ID NO: 17) was amplified using primers Ppta-ack-NotI-F (SEQ ID NO: 8: GAGCGGCCGCAATATGATATTTATGTCC) and Ppta-ack-NdeI-R (SEQ ID NO: 9: TTCCATATGTTTCATGTTTCATTTCCTCC) and cloned into the *E. coli*-*Clostridium* shuttle vector pMTL 85141 (FJ797651.1; Nigel Minton, University of Nottingham, UK) [Heap JT, Pennington OJ, Cartman ST, Minton NP. A modular system for *Clostridium* shuttle plasmids. J Microbiol Methods. 2009, 78: 79-85] using *Not*I and *Nde*I restriction sites and strain XL1-Blue MRF' Kan.

[0175] The antibiotic resistance gene in the created plasmid pMTL 85145 was subsequently replaced with an erythromycin resistance gene from pMTL 82254 (FJ797646.1; Nigel Minton, University of Nottingham, UK) [Heap JT, Pennington OJ, Cartman ST, Minton NP. A modular system for Clostridium shuttle plasmids. J Microbiol Methods. 2009, 78: 79-85] using *FseI* and *PmeI* restriction sites and strain XL1-Blue MRF' Kan. The created plasmid pMTL 85245 (SEQ ID NO: 3) and a 1625 bp fragment of the *repL* gene from pMTL83151 (FJ797647.1; Nigel Minton, University of Nottingham, UK) [Heap JT, Pennington OJ, Cartman ST, Minton NP. A modular system for Clostridium shuttle plasmids. J Microbiol Methods. 2009, 78: 79-85] were both cut with *FseI* and *AscI*. A ligation was performed resulting in plasmid pMTL83245.

[0176] The created plasmid pMTL 83245 (SEQ ID NO: 4) and the 3660 bp codon-optimised product of the Malonyl-Coenzyme A reductase gene were both cut with *NdeI* and *EcoRI*. A ligation was performed and ligation products were subsequently transformed into *E. coli* XL1-Blue MRF' Kan resulting in plasmid pMTL83245-SDR (SEQ ID NO: 5). DNA sequencing using oligonucleotides (given in Table 5) confirmed successful cloning of the Malonyl-Coenzyme A reductase gene without mutations.

Table 5: Primers used for confirmation of successful SDR cloning

Oligonucleotide Name	DNA Sequence (5' to 3')	SEQ ID NO:
M13R	CAGGAAACAGCTATGAC	10
SDR_seqR1	AGCAGCTTCTATCTGATCACCTGC	11
SDR_seqR2	TGCTCTAATGCTGCTACGTCATTG	12
SDR_seqR3	TGCAAGTTCACTCTGAATCATTGC	13
SDR_seqR4	ACATGGTGCTGGTTCATGACTAG	14
SDR_seqR5	TCTAGCACCAAGTTCTCTTGCTG	15
M13 (-21)	TGTAAAACGACGGCCAG	16

Determination of enzyme activities

[0177] The recombinant strain containing the plasmid pMTL 83245-SDR was grown up in LB medium containing the appropriate antibiotics under aerobic conditions overnight. The cells were inoculated into fresh LB medium with an initial OD₆₀₀ of 0.1. The cells were harvested at logarithmic phase (OD₆₀₀ ~0.6), and centrifuged at 13,000×g and 4 °C for 10 mins. The cell pellet was washed twice with 100mM Tris-HCl (pH 7.8) and resuspended in the same wash buffer containing protease inhibitor and mixed with 1.44 g of 100 µm glass beads. Tubes were chilled on ice for 5 min prior to disruption in a Mini Bead Beater (Biospec Products) through 5 cycles of 1 min beating at 5,000 rpm followed by 1 min on ice between cycles. After lysis, the sample was centrifuged (13,000 × g, 4 °C for 10 mins) and the supernatant aliquoted and stored at -80 °C until analysis. Protein content of the extracts was determined using a commercial kit (Pierce® Microplate BCA Protein Assay Kit-Reducing Agent Compatible, Thermo Scientific).

[0178] Malonyl-CoA reductase activity was determined at 45°C using the method reported by Hugler et al. (Hugler, M., Menendez, C., Schagger, H., Fuchs, G., 2002. Malonyl-coenzyme A reductase from *Chloroflexus aurantiacus*, a key enzyme of the 3-hydroxypropionate cycle for autotrophic CO₂ fixation. J. Bacteriol. 184 (9), 2404–2410). For routine assays, the enzyme lysate was pre-incubated at 45 °C in 100 mM Tris-HCl buffer (pH 7.8), containing 3 mM 1,4-dithioerythritol, 2 mM MgCl₂, and 0.3 mM NADPH for 10 mins. The reaction was initiated by the addition of 0.3 mM malonyl-CoA. The amount of NADPH consumed was determined using a molar extinction coefficient ($\Delta\epsilon_{365}$) of 3400M⁻¹ cm⁻¹. One unit of SDR activity was defined as the amount of enzyme required to oxidize 2 µmol of NADPH to NADP⁺ per min. To study the effect of temperature on the activity of SDR, the assay was also performed at 37 °C.

Methylation of expression plasmid with Malonyl-Coenzyme A reductase from *C. aurantiacus*

[0179] Methylation of the 3-HP expression plasmid pMTL83245-SDR was performed *in vivo* in *E. coli* using a synthesized hybrid Type II methyltransferase gene (SEQ ID NO: 6) designed from methyltransferase genes from *C. autoethanogenum*, *C. ragsdalei* and *C. ljungdahlii*. The methyltransferase (SEQ ID NO: 6) was synthesised and fused with

an inducible *lac* promoter in vector pGS20 (ATG:biosynthetics GmbH, Merzhausen, Germany) (SEQ ID NO: 7).

[0180] Both expression plasmid and methylation plasmid were transformed into the same cells of restriction negative *E. coli* XL1-Blue MRF' Kan, which is possible due to their compatible Gram-(-) origins of replication (high copy ColE1 in expression plasmid and low copy p15A in methylation plasmid). *In vivo* methylation was induced by addition of 1 mM IPTG, and methylated plasmids were isolated using QIAGEN Plasmid Midi Kit (QIAGEN GmbH, Hilden, Germany). The resulting mixture was used for transformation experiments with *C. autoethanogenum* DSM23693, but only the abundant (high-copy) expression plasmid which has a Gram-(+) replication origin (*repH*) is able to replicate in *Clostridia*.

Transformation of methylated 3-HP expression plasmid in *C. autoethanogenum*

[0181] To make competent cells of *C. autoethanogenum* DSM23693, a 50 ml culture (PETC media (Table 1) with steel mill gas and fructose as carbon source; 37 °C) was subcultured to fresh media for 3 consecutive days. These cells were used to inoculate 50 ml PETC media containing 40 mM DL-threonine at an OD_{600nm} of 0.05. When the culture reached an OD_{600nm} of 0.45, the cells were transferred into an anaerobic chamber and harvested at 4,700 x g and 4 °C. The culture was twice washed with ice-cold electroporation buffer (270 mM sucrose, 1 mM MgCl₂, 7 mM sodium phosphate, pH 7.4) and finally suspended in a volume of 600 µl fresh electroporation buffer. This mixture was transferred into a pre-cooled electroporation cuvette with a 0.4 cm electrode gap containing ~10 µg of the methylated plasmid mix. Since an additional Type I restriction system was identified in the genome of *C. ljungdahlii* compared to *C. autoethanogenum*, 1 µl of a Type I restriction inhibitor (EPICENTRE Biotechnologies, Madison, WI 53713, USA) was added to the plasmid mix. The cells were mixed with plasmid and restriction inhibitor and immediately pulsed using a Gene pulser Xcell electroporation system (Bio-Rad Laboratories, Hercules, CA 94547, USA) with the following settings: 2.5 kV, 600 Ω, and 25 µF. Time constants were between 3.7-5.1 ms. For regeneration, the culture was transferred in 5 ml PETC-MES media (Table 1), which increased recovery of the cells. The culture was monitored at a wavelength of 600 nm using a Spectronic Helios Epsilon Spectrophotometer (Thermo Fisher Scientific Inc., Waltham MA 02454, USA) equipped with a tube

holder. Once growth was observed (one doubling), the culture was scaled up to 10 ml and later 50 ml PETC-MES media containing each 5 µg/ml clarithromycin and 30 psi steel mill gas in the headspace as sole carbon source.

Analysis of metabolites

[0182] HPLC analysis of 3-hydroxypropionate (3-HP) and other metabolites was performed using an Agilent 1100 Series HPLC system equipped with a RID (Refractive Index Detector) operated at 35 °C and an Aminex HPX-87H column (300 x 7.8 mm, particle size 5 µm) kept at 35 °C. Slightly acidified water was used (0.005 M H₂SO₄) as mobile phase with a flow rate of 0.6 ml/min. To remove proteins and other cell residues, 400 µl samples were mixed with 100 µl of a 1 % (w/v) 5-Sulfosalicylic acid in 1M sulphuric acid and centrifuged at 14,000 rpm for 3 min to separate precipitated residues. 10 µl of the supernatant were then injected into the HPLC for analyses.

Results

Production of 3-HP in transformed cells

3-HP production from CO and CO₂/H₂ with C. aurantiacus pathway genes in C. autoethanogenum:

[0183] Growth experiments were carried out with transformed *C. autoethanogenum* DSM23693 carrying plasmid pMTL83245-SDR in 50 ml PETC-MES media (Table 1; without fructose) in serum with rubber stoppers and 30 psi steel mill gas (collected from New Zealand Steel site in Glenbrook, NZ; composition: 44% CO, 32% N₂, 22% CO₂, 2% H₂) in the headspace as sole energy and carbon source. 3-HP production was confirmed using HPLC analysis.

Example 2

Improving 3-HP production via increasing biotin biosynthesis

[0184] Fixation of CO₂ onto acetyl-CoA by acetyl-CoA carboxylase (ACC) is mediated by biotin (vitamin B7, vitamin H). Biotin is required as co-factor for this carboxylation reaction.

[0185] Acetyl-CoA carboxylase is a complex consisting of four different subunits, AccA, AccB, AccC, and AccD, where biotin is covalently bound to subunit AccB. Carboxylation of biotin is catalyzed by subunit AccC with the expense of ATP. The subsequent transfer of CO₂ from carboxylated biotin to acetyl-CoA by AccA and AccD results in formation of malonyl-CoA. In the first step of the activation of the acetyl-CoA carboxylase complex, the binding of biotin to AccB is catalyzed by biotin-[acetyl-CoA carboxylase] ligase (holoenzyme synthetase).

[0186] To improve CO₂ fixation and 3-HP production via acetyl-CoA carboxylase in carboxydophilic acetogens, the pool of biotin co-factor can be increased by over-expression of genes involved in the biosynthesis pathway of this co-factor. Biotin biosynthesis includes enzymes 6-carboxyhexanoate--CoA ligase [EC:6.2.1.14], 8-amino-7-oxononanoate synthase [EC:2.3.1.47], adenosylmethionine-8-amino-7-oxononanoate aminotransferase [EC:2.6.1.62], biotin synthetase [EC:2.8.1.6], biotin-[acetyl-CoA-carboxylase] ligase [EC:6.3.4.15], biotinidase [EC:3.5.1.12], biotin--protein ligase [EC:6.3.4.15; EC:6.3.4.11; EC:6.3.4.10; EC:6.3.4.9], ethiobiotin synthetase [EC:6.3.3.3], type III pantothenate kinase [EC:2.7.1.33].

Example 3

Improving 3-HP production via limiting fatty acid biosynthesis

[0187] Malonyl-CoA is also a precursor for fatty acid biosynthesis. To increase 3-HP production, the rate of fatty acid biosynthesis can be limited in favour for 3-HP production. A malonyl-CoA:acyl carrier protein transacylase (FabD) [EC:2.3.1.39] initiates the elongation of fatty acid chains. The gene encoding this enzyme can be downregulated or activity of this enzyme decreased to prevent malonyl-CoA from being used for the native fatty acid biosynthesis.

[0188] The invention has been described herein, with reference to certain preferred embodiments, in order to enable the reader to practice the invention without undue experimentation. However, a person having ordinary skill in the art will readily recognise that many of the components and parameters may be varied or modified to a certain extent or substituted for known equivalents without departing from the scope of the invention. It should be appreciated that such modifications and equivalents are herein incorporated as if individually set forth. Titles, headings, or the like are

provided to enhance the reader's comprehension of this document, and should not be read as limiting the scope of the present invention.

[0189] The entire disclosures of all applications, patents and publications, cited above and below, if any, are hereby incorporated by reference. However, the reference to any applications, patents and publications in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that they constitute valid prior art or form part of the common general knowledge in any country in the world.

[0190] Throughout this specification and any claims which follow, unless the context requires otherwise, the words "comprise," "comprising" and the like, are to be construed in an inclusive sense as opposed to an exclusive sense, that is to say, in the sense of "including, but not limited to."

We claim:

1. A process for converting CO and/or CO₂ into 3-hydroxypropionate (3-HP), the process comprising:

passing a gaseous CO-containing and/or CO₂-containing substrate to a bioreactor containing a culture of carboxydophilic, acetogenic bacteria in a culture medium such that the bacteria convert the CO and/or CO₂ to 3-HP, and

recovering the 3-HP from the bioreactor,

wherein the carboxydophilic acetogenic bacteria are genetically engineered to express a malonyl-Coenzyme A reductase.
2. Isolated, genetically engineered, carboxydophilic, acetogenic bacteria which comprise a nucleic acid encoding a malonyl-Coenzyme A reductase, whereby the bacteria express the malonyl-Coenzyme A reductase and the bacteria are able to fix three molecules of CO or CO₂ into one molecule of 3-hydroxypropionate (3-HP).
3. The isolated, genetically engineered, carboxydophilic, acetogenic bacteria of claim 2 further comprising a nucleic acid encoding acetyl-Coenzyme A carboxylase from a nonsulfur, photosynthetic bacterium, wherein the nucleic acid is operably linked to a promoter.
4. The isolated, genetically engineered, carboxydophilic, acetogenic bacteria of claim 2 which are selected from the group consisting of *Clostridium autoethanogenum*, *Clostridium ljundahlii*, *Clostridium ragsdalei*, *Clostridium carboxidivorans*, *Clostridium drakei*, *Clostridium scatologenes*, *Clostridium aceticum*, *Clostridium formicoaceticum*, *Clostridium magnum*, *Butyribacterium methylotrophicum*, *Acetobacterium woodii*, *Alkalibaculum bacchii*, *Blautia producta*, *Eubacterium limosum*, *Moorella thermoacetica*, *Moorella thermautotrophica*, *Sporomusa ovata*, *Sporomusa silvacetica*, *Sporomusa sphaeroides*, *Oxobacter pfennigii*, and *Thermoanaerobacter kiuvi*.
5. The isolated, genetically engineered, carboxydophilic, acetogenic bacteria of claim 4 which are a *Clostridium* species selected from the group consisting of *C. ljundahlii*, and *C. autoethanogenum*.

6. The isolated, genetically engineered, carboxydophilic, acetogenic bacteria of claim 3 wherein the nonsulfur, photosynthetic bacterium is selected from the group consisting of *Clostridium ljungdahlii*, *Metallosphaera* and *Sulfolobus* spp.
7. The isolated, genetically engineered, carboxydophilic, acetogenic bacteria of claim 3 wherein the nonsulfur, photosynthetic bacterium is *Chloroflexus auranticus*.
8. The isolated, genetically engineered, carboxydophilic, acetogenic bacteria of claim 2 wherein the nucleic acid encoding malonyl-Coenzyme A reductase has been codon optimized.
9. A method of culturing the isolated, genetically engineered, carboxydophilic, acetogenic bacteria of claim 2 comprising, growing the bacteria in a medium comprising a gaseous carbon source, wherein the carbon source comprises CO and/or CO₂.
10. A method of culturing the bacteria of isolated, genetically engineered, carboxydophilic, acetogenic claim 2 comprising, growing the bacteria in a medium comprising an energy source, wherein the energy source comprises CO and/or CO₂.
11. The method of claim 9 or 10 wherein the culturing is strictly anaerobic.
12. The method of claim 9 or 10 wherein the carbon source comprises an industrial waste product or off-gas.
13. The method of claim 9 or 10 wherein the bacteria further comprise an exogenous nucleic acid encoding acetyl-Coenzyme A carboxylase from a nonsulfur, photosynthetic bacterium, wherein the nucleic acid is operably linked to a promoter.
14. The process of claim 1 wherein the malonyl-Coenzyme A reductase is from a non-sulfur, photosynthetic bacterium.
15. The process of claim 14 wherein the malonyl-Coenzyme A reductase is from *Chloroflexus auranticus*.

16. The isolated, genetically engineered, carboxydophilic, acetogenic bacteria of claim 2 wherein the malonyl-Coenzyme A reductase is at least 85 % identical to the amino acid sequence encoded by the nucleotide sequence of SEQ ID NO: 1.

1/1

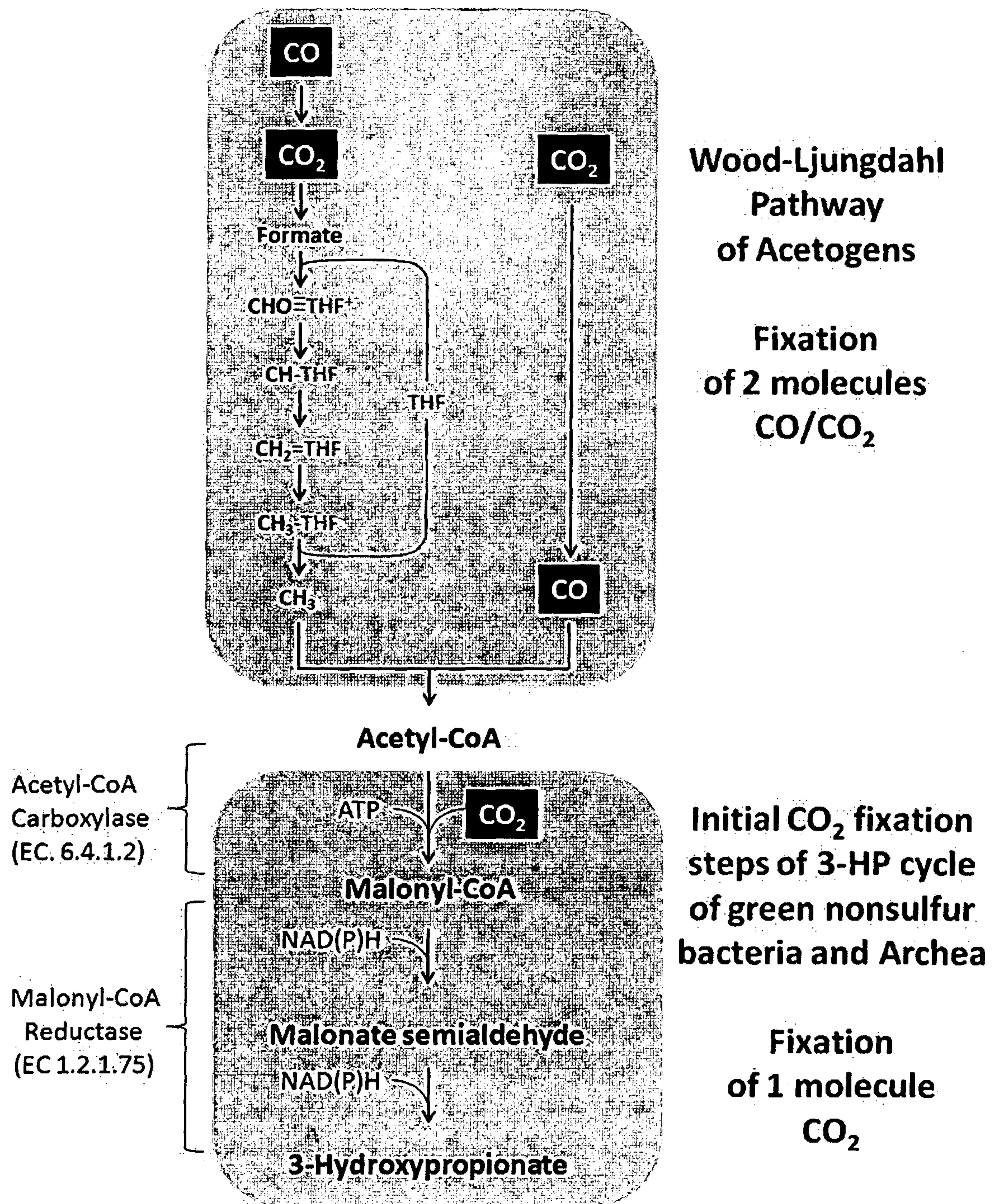


FIG. 1

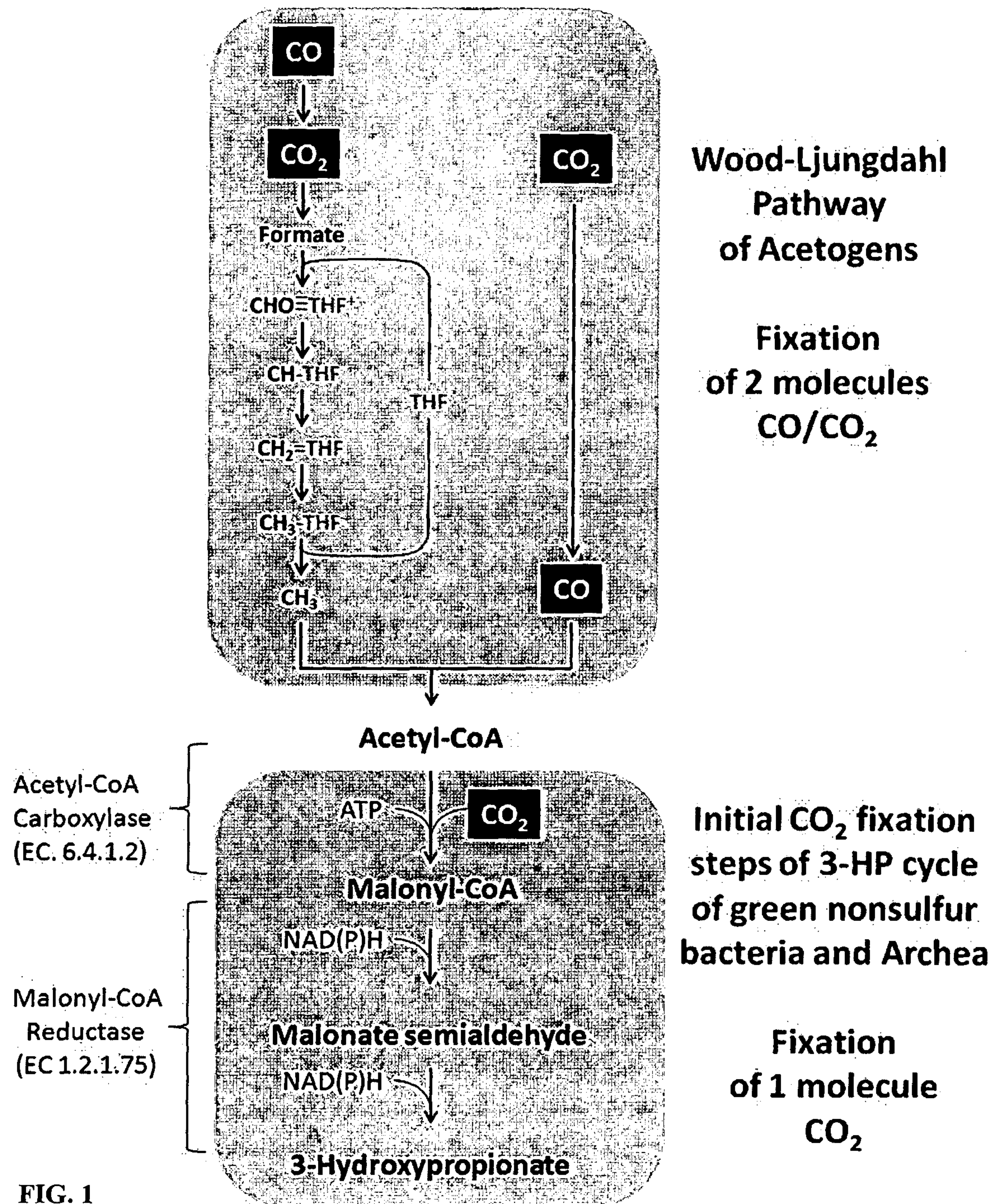


FIG. 1