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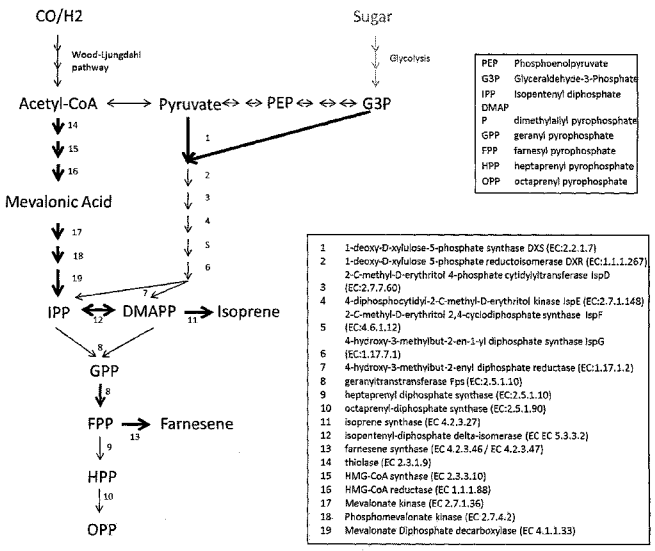
(54) Title: RECOMBINANT MICROORGANISMS AND USES THEREFOR



(57) Abstract: Terpenes are valuable commercial products used in a diverse number of industries. Terpenes may be produced from petrochemical sources and from terpene feed-stocks, such as turpentine. However, these production methods are expensive, unsustainable and often cause environmental problems including contributing to climate change. Microbial fermentation provides an alternative option for the production of terpenes. One or more terpenes and/or precursors can be produced by microbial fermentation of a substrate comprising CO. Recombinant microorganisms may be used in such methods. Carboxydotrophic, acetogenic, recombinant microorganisms can be used in such methods. The recombinant microorgnsims may contain exogenous mevalonate (MVA) pathway enzymes and/or DXS pathway enzymes, for example.

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RECOMBINANT METABOLIC MICROORGANISMS AND USES THEREFOR

FIELD OF THE INVENTION

[0001] The present invention relates to recombinant microorganisms and methods for the production of terpenes and/or precursors thereof by microbial fermentation of a substrate comprising CO.

BACKGROUND OF THE INVENTION

[0002] Terpenes are a diverse class of naturally occurring chemicals composed of five-carbon isoprene units. Terpene derivatives include terpenoids (also known as isoprenoids) which may be formed by oxidation or rearrangement of the carbon backbone or a number of functional group additions or rearrangments.

[0003] Examples of terpenes include: isoprene (C5 hemiterpene), farnesene (C15 Sesquiterpenes), artemisinin (C15 Sesquiterpenes), citral (C10 Monoterpenes), carotenoids (C40 Tetraterpenes), menthol (C10 Monoterpenes), Camphor (C10 Monoterpenes), and cannabinoids.

[0004] Terpenes are valuable commercial products used in a diverse number of industries. The highest tonnage uses of terpenes are as resins, solvents, fragrances and vitamins. For example, isoprene is used in the production of synthetic rubber (cis-1,4-polyisoprene) for example in the tyre industry; farnesene is used as an energy dense drop-in fuel used for transportation or as jet-fuel; artemisinin is used as a malaria drug; and citral, carotenoids, menthol, camphor, and cannabinoids are used in the manufacture of pharmaceuticals, butadiene, and as aromatic ingredients.

[0005] Terpenes may be produced from petrochemical sources and from terpene feed-stocks, such as turpentine. For example, isoprene is produced petrochemically as a by-product of naphtha or oil cracking in the production of ethylene. Many terpenes are also extracted in relatively small quantities from natural sources. However, these production methods are expensive, unsustainable and often cause environmental problems including contributing to climate change.

[0006] Due to the extremely flammable nature of isoprene, known methods of production require extensive safeguards to limit potential for fire and explosions.

[0007] It is an object of the invention to overcome one or more of the disadvantages of the prior art, or at least to provide the public with an alternative means for producing terpenes and other related products.

SUMMARY OF INVENTION

[0008] Microbial fermentation provides an alternative option for the production of terpenes. Terpenes are ubiquitous in nature, for example they are involved in bacterial cell wall biosynthesis, and they are produced by some trees (for example poplar) to protect leaves from UV light exposure. However, not all bacteria comprise the necessary cellular machinery to produce terpenes and/or their precursors as metabolic products. For example, carboxydotrophic acetogens, such as *C. autoethanogenum* or *C. ljungdahlii*, which are able to ferment substrates comprising carbon monoxide to produce products such as ethanol, are not known to produce and emit any terpenes and/or their precursors as metabolic products. In addition, most bacteria are not known to produce any terpenes which are of commercial value.

[0009] The invention generally provides, *inter alia*, methods for the production of one or more terpenes and/or precursors thereof by microbial fermentation of a substrate comprising CO, and recombinant microorganisms of use in such methods.

[0010] In a first aspect, the invention provides a carboxydotrophic acetogenic recombinant microorganism capable of producing one or more terpenes and/or precursors thereof and optionally one or more other products by fermentation of a substrate comprising CO.

[0011] In one particular embodiment, the microorganism is adapted to express one or more enzymes in the mevalonate (MVA) pathway not present in a parental microorganism from which the recombinant microorganism is derived (may be referred to herein as an exogenous enzyme). In another embodiment, the microorganism is adapted to over-express one or more enzymes in the mevalonate (MVA) pathway which are present in a parental microorganism

from which the recombinant microorganism is derived (may be referred to herein as an endogenous enzyme).

[0012] In a further embodiment, the microorganism is adapted to:

a) express one or more exogenous enzymes in the mevalonate (MVA) pathway and/or overexpress one or more endogenous enzyme in the mevalonate (MVA) pathway; and

b) express one or more exogenous enzymes in the DXS pathway and/or overexpress one or more endogenous enzymes in the DXS pathway.

[0013] In one embodiment, the one or more enzymes from the mevalonate (MVA) pathway is selected from the group consisting of:

a) thiolase (EC 2.3.1.9),

b) HMG-CoA synthase (EC 2.3.3.10),

c) HMG-CoA reductase (EC 1.1.1.88),

d) Mevalonate kinase (EC 2.7.1.36),

e) Phosphomevalonate kinase (EC 2.7.4.2),

f) Mevalonate Diphosphate decarboxylase (EC 4.1.1.33), and

g) a functionally equivalent variant of any one thereof.

[0014] In a further embodiment, the one or more enzymes from the DXS pathway is selected from the group consisting of:

a) 1-deoxy-D-xylulose-5-phosphate synthase DXS (EC:2.2.1.7),

b) 1-deoxy-D-xylulose 5-phosphate reductoisomerase DXR (EC:1.1.1.267),

c) 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase IspD (EC:2.7.7.60),

d) 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase IspE (EC:2.7.1.148),

e) 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase IspF (EC:4.6.1.12),

f) 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase IspG (EC:1.17.7.1),

g) 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (EC:1.17.1.2), and

h) a functionally equivalent variant of any one thereof.

[0015] In a further embodiment, one or more further exogenous or endogenous enzymes are expressed or over-expressed to result in the production of a terpene compound or a precursor

thereof wherein the exogenous enzyme that is expressed, or the endogenous enzyme that is overexpressed, is selected from the group consisting of:

a) geranyltranstransferase Fps (EC:2.5.1.10),
b) heptaprenyl diphosphate synthase (EC:2.5.1.10),
c) octaprenyl-diphosphate synthase (EC:2.5.1.90),
d) isoprene synthase (EC 4.2.3.27),
e) isopentenyl-diphosphate delta-isomerase (EC 5.3.3.2),
f) farnesene synthase (EC 4.2.3.46 / EC 4.2.3.47), and
g) a functionally equivalent variant of any one thereof.

[0016] 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 mevalonic acid or isopentenyl pyrophosphate (IPP) and the recombinant microorganism is adapted to express one or more enzymes involved in the mevalonate pathway.

[0017] In one embodiment, the one or more terpene and/or precursor thereof is chosen from mevalonic acid, IPP, dimethylallyl pyrophosphate (DMAPP), isoprene, geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP) and farnesene.

[0018] In one embodiment, the microorganism comprises one or more exogenous nucleic acids adapted to increase expression of one or more endogenous nucleic acids and which one or more endogenous nucleic acids encode one or more of the enzymes referred to herein before.

[0019] In one embodiment, the one or more exogenous nucleic acids 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. In one embodiment, the promoter is selected from the group comprising Wood-Ljungdahl gene cluster or Phosphotransacetylase/Acetate kinase operon promoters.

[0020] In one embodiment, the microorganism comprises one or more exogenous nucleic acids encoding and adapted to express one or more of the enzymes referred to hereinbefore. In one embodiment, the microorganisms comprise one or more exogenous nucleic acids

encoding and adapted to express at least two of the enzymes. In other embodiments, the microorganism comprises one or more exogenous nucleic acids encoding and adapted to express at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine or more of the enzymes.

[0021] 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.

[0022] In one embodiment, the exogenous nucleic acid is an expression plasmid.

[0023] In one particular embodiment, the parental microorganism is selected from the group of carboxydotrophic 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.*

[0024] 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).

[0025] In one embodiment, the parental microorganism lacks one or more genes in the DXS pathway and/or the mevalonate (MVA) pathway. In one embodiment, the parental microorganism lacks one or more genes encoding an enzyme selected from the group consisting of:

a) thiolase (EC 2.3.1.9),

b) HMG-CoA synthase (EC 2.3.3.10),

c) HMG-CoA reductase (EC 1.1.1.88),

d) Mevalonate kinase (EC 2.7.1.36),
e) Phosphomevalonate kinase (EC 2.7.4.2),
f) Mevalonate Diphosphate decarboxylase (EC 4.1.1.33),
g) 1-deoxy-D-xylulose-5-phosphate synthase DXS (EC:2.2.1.7),
h) 1-deoxy-D-xylulose 5-phosphate reductoisomerase DXR (EC:1.1.1.267),
i) 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase IspD (EC:2.7.7.60),
j) 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase IspE (EC:2.7.1.148),
k) 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase IspF (EC:4.6.1.12),
l) 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase IspG (EC:1.17.7.1),
m) 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (EC:1.17.1.2), and
n) a functionally equivalent variant of any one thereof.

[0026] In a second aspect, the invention provides a nucleic acid encoding one or more enzymes which when expressed in a microorganism allows the microorganism to produce one or more terpenes and/or precursors thereof by fermentation of a substrate comprising CO.

[0027] In one embodiment, the nucleic acid encodes two or more enzymes which when expressed in a microorganism allows the microorganism to produce one or more terpenes and/or precursors thereof by fermentation of a substrate comprising CO. In one embodiment, a nucleic acid of the invention encodes at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine or more of such enzymes.

[0028] In one embodiment, the nucleic acid encodes one or more enzymes in the mevalonate (MVA) pathway. In one embodiment, the one or more enzymes is chosen from the group consisting of:

a) thiolase (EC 2.3.1.9),
b) HMG-CoA synthase (EC 2.3.3.10),
c) HMG-CoA reductase (EC 1.1.1.88),
d) Mevalonate kinase (EC 2.7.1.36),
e) Phosphomevalonate kinase (EC 2.7.4.2),
f) Mevalonate Diphosphate decarboxylase (EC 4.1.1.33), and
g) a functionally equivalent variant of any one thereof.

[0029] In a particular embodiment, the nucleic acid encodes thiolase (which may be an acetyl CoA c-acetyltransferase), HMG-CoA synthase and HMG-CoA reductase,

[0030] In a further embodiment, the nucleic acid encodes one or more enzymes in the mevalonate (MVA) pathway and one or more further nucleic acids in the DXS pathway pathway. In one embodiment, the one or more enzymes from the DXS pathway is selected from the group consisting of:

a) 1-deoxy-D-xylulose-5-phosphate synthase DXS (EC:2.2.1.7),
b) 1-deoxy-D-xylulose 5-phosphate reductoisomerase DXR (EC:1.1.1.267),
c) 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase IspD (EC:2.7.7.60),
d) 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase IspE (EC:2.7.1.148),
e) 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase IspF (EC:4.6.1.12),
f) 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase IspG (EC:1.17.7.1),
g) 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (EC:1.17.1.2), and
h) a functionally equivalent variant of any one thereof.

[0031] In a further embodiment, the nucleic acid encodes one or more further exogenous or endogenous enzymes are expressed or over-expressed to result in the production of a terpene compound or a precursor thereof wherein the exogenous nucleic acid that is expressed, or the endogenous enzyme that is overexpressed, encodes and enzyme selected from the group consisting of:

a) geranyltranstransferase Fps (EC:2.5.1.10),
b) heptaprenyl diphosphate synthase (EC:2.5.1.10),
c) octaprenyl-diphosphate synthase (EC:2.5.1.90),
d) isoprene synthase (EC 4.2.3.27),
e) isopentenyl-diphosphate delta-isomerase (EC 5.3.3.2),
f) farnesene synthase (EC 4.2.3.46 / EC 4.2.3.47), and
g) a functionally equivalent variant of any one thereof.

[0032] In one embodiment, the nucleic acid encoding thiolase (EC 2.3.1.9) has the sequence SEQ ID NO: 40 or is a functionally equivalent variant thereof.

[0033] In one embodiment, the nucleic acid encoding thiolase (EC 2.3.1.9) is acetyl CoA c-acetyl transferase that has the sequence SEQ ID NO: 41 or is a functionally equivalent variant thereof.

[0034] In one embodiment, the nucleic acid encoding HMG-CoA synthase (EC 2.3.3.10) has the sequence SEQ ID NO: 42 or is a functionally equivalent variant thereof.

[0035] In one embodiment, the nucleic acid encoding HMG-CoA reductase (EC 1.1.1.88) has the sequence SEQ ID NO: 43 or is a functionally equivalent variant thereof.

[0036] In one embodiment, the nucleic acid encoding Mevalonate kinase (EC 2.7.1.36) has the sequence SEQ ID NO: 51 or is a functionally equivalent variant thereof.

[0037] In one embodiment, the nucleic acid encoding Phosphomevalonate kinase (EC 2.7.4.2) has the sequence SEQ ID NO: 52 or is a functionally equivalent variant thereof.

[0038] In one embodiment, the nucleic acid encoding Mevalonate Diphosphate decarboxylase (EC 4.1.1.33) has the sequence SEQ ID NO: 53 or is a functionally equivalent variant thereof.

[0039] In one embodiment, the nucleic acid encoding 1-deoxy-D-xylulose-5-phosphate synthase DXS (EC:2.2.1.7) has the sequence SEQ ID NO: 1 or is a functionally equivalent variant thereof.

[0040] In one embodiment, the nucleic acid encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase DXR (EC:1.1.1.267) has the sequence SEQ ID NO: 3 or is a functionally equivalent variant thereof.

[0041] In one embodiment, the nucleic acid encoding 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase IspD (EC:2.7.7.60) has the sequence SEQ ID NO: 5 or is a functionally equivalent variant thereof.

[0042] In one embodiment, the nucleic acid encoding 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase IspE (EC:2.7.1.148) has the sequence SEQ ID NO: 7 or is a functionally equivalent variant thereof.

[0043] In one embodiment, the nucleic acid encoding 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase IspF (EC:4.6.1.12) has the sequence SEQ ID NO: 9 or is a functionally equivalent variant thereof.

[0044] In one embodiment, the nucleic acid encoding 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase IspG (EC:1.17.7.1) has the sequence SEQ ID NO: 11 or is a functionally equivalent variant thereof.

[0045] In one embodiment, the nucleic acid encoding 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (EC:1.17.1.2) has the sequence SEQ ID NO: 13 or is a functionally equivalent variant thereof.

[0046] In one embodiment, the nucleic acid encoding geranyltranstransferase Fps has the sequence SEQ ID NO: 15, or it is a functionally equivalent variant thereof.

[0047] In one embodiment, the nucleic acid encoding heptaprenyl diphosphate synthase has the sequence SEQ ID NO: 17, or it is a functionally equivalent variant thereof.

[0048] In one embodiment, the nucleic acid encoding octaprenyl-diphosphate synthase (EC:2.5.1.90) wherein the octaprenyl-diphosphate synthase is polyprenyl synthetase is encoded by sequence SEQ ID NO: 19, or it is a functionally equivalent variant thereof.

[0049] In one embodiment, the nucleic acid encoding isoprene synthase (ispS) has the sequence SEQ ID NO: 21, or it is a functionally equivalent variant thereof.

[0050] In one embodiment, the nucleic acid encoding Isopentenyl-diphosphate delta-isomerase (idi) has the sequence SEQ ID NO: 54, or it is a functionally equivalent variant thereof.

[0051] In one embodiment, the nucleic acid encoding farnesene synthase has the sequence SEQ ID NO: 57, or it is a functionally equivalent variant thereof.

[0052] In one embodiment, the nucleic acid encodes the following enzymes:

a) isoprene synthase;

b) Isopentenyl-diphosphate delta-isomerase (idi); and

c) 1-deoxy-D-xylulose-5-phosphate synthase DXS;

or functionally equivalent variants thereof.

[0053] In one embodiment, the nucleic acid encodes the following enzymes:

a) Thiolase;

b) HMG-CoA synthase;

c) HMG-CoA reductase;

d) Mevalonate kinase;

e) Phosphomevalonate kinase;

f) Mevalonate Diphosphate decarboxylase;

g) Isopentenyl-diphosphate delta-isomerase (idi); and

h) isoprene synthase;

or functionally equivalent variants thereof.

[0054] In one embodiment, the nucleic acid encodes the following enzymes:

a) geranyltranstransferase Fps; and

b) farnesene synthase

or functionally equivalent variants thereof.

[0055] 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.*

[0056] In a third aspect, the invention provides a nucleic acid construct or vector comprising one or more nucleic acid of the second aspect.

[0057] 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.

[0058] In a fourth aspect, the invention provides host organisms comprising any one or more of the nucleic acids of the second aspect or vectors or constructs of the third aspect.

[0059] 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.

[0060] Preferably, the composition is able to produce a recombinant microorganism according to the first aspect of the invention.

[0061] In one particular embodiment, the expression construct/vector and/or the methylation construct/vector is a plasmid.

[0062] In a sixth aspect, the invention provides a method for the production of one or more terpenes and/or precursors thereof and optionally one or more other products by microbial fermentation comprising fermenting a substrate comprising CO using a recombinant microorganism of the first aspect of the invention.

[0063] In one embodiment the method comprises the steps of:

(a) providing a substrate comprising CO to a bioreactor containing a culture of one or more microorganisms of the first aspect of the invention; and

(b) anaerobically fermenting the culture in the bioreactor to produce at least one terpene and/or precursor thereof.

[0064] In one embodiment the method comprises the steps of:

(a) capturing CO-containing gas produced as a result of the industrial process;

(b) anaerobic fermentation of the CO-containing gas to produce at least one terpene and/or precursor thereof by a culture containing one or more microorganism of the first aspect of the invention.

[0065] In particular embodiments of the method aspects, the microorganism is maintained in an aqueous culture medium.

[0066] In particular embodiments of the method aspects, the fermentation of the substrate takes place in a bioreactor.

[0067] In one embodiment, the one or more terpene and/or precursor thereof is chosen from mevalonic acid, IPP, dimethylallyl pyrophosphate (DMAPP), isoprene, geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP) and farnesene.

[0068] Preferably, the substrate comprising CO is a gaseous substrate comprising CO. In one embodiment, the substrate comprises an industrial waste gas. In certain embodiments, the gas is steel mill waste gas or syngas.

[0069] 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.

[0070] In certain embodiments the methods further comprise the step of recovering a terpene and/or precursor thereof and optionally one or more other products from the fermentation broth.

[0071] In a seventh aspect, the invention provides one or more terpene and/or precursor thereof when produced by the method of the sixth aspect. In one embodiment, the one or more terpene and/or precursor thereof is chosen from the group consisting of mevalonic acid, IPP, dimethylallyl pyrophosphate (DMAPP), isoprene, geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP) and farnesene.

[0072] In another aspect, the invention provides a method for the production of a microorganism of the first aspect of the invention comprising transforming a carboxydotrophic acetogenic parental microorganism by introduction of one or more nucleic

acids such that the microorganism is capable of producing, or increasing the production of, one or more terpenes and/or precursors thereof and optionally one or more other products by fermentation of a substrate comprising CO, wherein the parental microorganism is not capable of producing, or produces at a lower level, the one or more terpene and/or precursor thereof by fermentation of a substrate comprising CO.

[0073] In one particular embodiment, a parental microorganism is transformed by introducing one or more exogenous nucleic acids adapted to express one or more enzymes in the mevalonate (MVA) pathway and optionally the DXS pathway. In another embodiment, a parental microorganism is transformed with one or more nucleic acids adapted to over-express one or more enzymes in the mevalonate (MVA) pathway and optionally the DXS pathway which are naturally present in the parental microorganism.

[0074] In certain embodiments, the one or more enzymes are as herein before described.

[0075] In one embodiment an isolated, genetically engineered, carboxydotrophic, acetogenic bacteria are provided which comprise an exogenous nucleic acid encoding an enzyme in a mevalonate pathway or in a DXS pathway or in a terpene biosynthesis pathway, whereby the bacteria express the enzyme. The enzyme is selected from the group consisting of:

a) thiolase (EC 2.3.1.9);
b) HMG-CoA synthase (EC 2.3.3.10);
c) HMG-CoA reductase (EC 1.1.1.88);
d) Mevalonate kinase (EC 2.7.1.36);
e) Phosphomevalonate kinase (EC 2.7.4.2);
f) Mevalonate Diphosphate decarboxylase (EC 4.1.1.33); 1-deoxy-D-xylulose-5-phosphate synthase DXS (EC:2.2.1.7);
g) 1-deoxy-D-xylulose 5-phosphate reductoisomerase DXR (EC:1.1.1.267);
h) 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase IspD (EC:2.7.7.60);
i) 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase IspE (EC:2.7.1.148);
j) 2-C-methyl-D-erythritol 2;4-cyclodiphosphate synthase IspF (EC:4.6.1.12);
k) 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase IspG (EC:1.17.7.1);
l) 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (EC:1.17.1.2); geranyltranstransferase Fps (EC:2.5.1.10);

m) heptaprenyl diphosphate synthase (EC:2.5.1.10);
n) octaprenyl-diphosphate synthase (EC:2.5.1.90);
o) isoprene synthase (EC 4.2.3.27);
p) isopentenyl-diphosphate delta-isomerase (EC 5.3.3.2); and
q) farnesene synthase (EC 4.2.3.46 / EC 4.2.3.47).

[0076] In some aspects the bacteria do not express the enzyme in the absence of said nucleic acid. In some aspects the bacteria which express the enzyme under anaerobic conditions.

[0077] One embodiment provides a plasmid which can replicate in a carboxydotrophic, acetogenic bacteria. The plasmid comprises a nucleic acid encoding an enzyme in a mevalonate pathway or in a DXS pathway or in a terpene biosynthesis pathway, whereby when the plasmid is in the bacteria, the enzyme is expressed by said bacteria. The enzyme is selected from the group consisting of:

a) thiolase (EC 2.3.1.9);
b) HMG-CoA synthase (EC 2.3.3.10);
c) HMG-CoA reductase (EC 1.1.1.88);
d) Mevalonate kinase (EC 2.7.1.36);
e) Phosphomevalonate kinase (EC 2.7.4.2);
f) Mevalonate Diphosphate decarboxylase (EC 4.1.1.33); 1-deoxy-D-xylulose-5-phosphate synthase DXS (EC:2.2.1.7);
g) 1-deoxy-D-xylulose 5-phosphate reductoisomerase DXR (EC:1.1.1.267);
h) 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase IspD (EC:2.7.7.60);
i) 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase IspE (EC:2.7.1.148);
j) 2-C-methyl-D-erythritol 2;4-cyclodiphosphate synthase IspF (EC:4.6.1.12);
k) 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase IspG (EC:1.17.7.1);
l) 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (EC:1.17.1.2); geranyltranstransferase Fps (EC:2.5.1.10);
m) heptaprenyl diphosphate synthase (EC:2.5.1.10);
n) octaprenyl-diphosphate synthase (EC:2.5.1.90);
o) isoprene synthase (EC 4.2.3.27);
p) isopentenyl-diphosphate delta-isomerase (EC 5.3.3.2); and
q) farnesene synthase (EC 4.2.3.46 / EC 4.2.3.47).

[0078] A process is provided in another embodiment for converting CO and/or CO_2 into isoprene. The process comprises: passing a gaseous CO-containing and/or CO_2-containing substrate to a bioreactor containing a culture of carboxydotrophic, acetogenic bacteria in a culture medium such that the bacteria convert the CO and/or CO_2 to isoprene, and recovering the isoprene from the bioreactor. The carboxydotrophic acetogenic bacteria are genetically engineered to express a isoprene synthase.

[0079] Another embodiment provides an isolated, genetically engineered, carboxydotrophic, acetogenic bacteria which comprise a nucleic acid encoding an isoprene synthase. The bacteria express the isoprene synthase and the bacteria are able to convert imethylallyldiphosphate to isoprene. In one aspect the isoprene synthase is a *Populus tremuloides* enzyme. In another aspect the nucleic acid is codon optimized. In still another aspect, expression of the isoprene synthase is under the transcriptional control of a promoter for a pyruvate: ferredoxin oxidoreductase gene from *Clostridium autoethanogenum.*

[0080] Another embodiment provides a process for converting CO and/or CO_2 into isopentyldiphosphate (IPP). The process comprises: passing a gaseous CO-containing and/or CO_2-containing substrate to a bioreactor containing a culture of carboxydotrophic, acetogenic bacteria in a culture medium such that the bacteria convert the CO and/or CO_2 to isopentyldiphosphate (IPP), and recovering the IPP from the bioreactor. The carboxydotrophic acetogenic bacteria are genetically engineered to express a isopentyldiphosphate delta isomerase.

[0081] Still another embodiment provides isolated, genetically engineered, carboxydotrophic, acetogenic bacteria which comprise a nucleic acid encoding an isopentyldiphosphate delta isomerase. The bacteria express the isopentyldiphosphate delta isomerase and the bacteria are able to convert dimethylallyldiphosphate to isopentyldiphosphate. In some aspects the nucleic acid encodes a *Clostridium beijerinckii* isopentyldiphosphate delta isomerase. In other aspects, the nucleic acid is under the transcriptional control of a promoter for a pyruvate: ferredoxin oxidoreductase gene from *Clostridium autoethanogenum.* In still other aspects, the nucleic acid is under the transcriptional control of a promoter for a pyruvate: ferredoxin oxidoreductase gene from *Clostridium autoethanogenum* and downstream of a second nucleic acid encoding an isoprene synthase.

[0082] Still another embodiment provides a process for converting CO and/or CO_2 into isopentyldiphosphate (IPP) and/or isoprene. The process comprises: passing a gaseous CO-containing and/or CO_2-containing substrate to a bioreactor containing a culture of carboxydotrophic, acetogenic bacteria in a culture medium such that the bacteria convert the CO and/or CO_2 to isopentyldiphosphate (IPP) and/or isoprene, and recovering the IPP and/or isoprene from the bioreactor. The carboxydotrophic acetogenic bacteria are genetically engineered to have an increased copy number of a nucleic acid encoding a deoxyxylulose 5-phosphate synthase (DXS) enzyme, wherein the increased copy number is greater than 1 per genome.

[0083] Yet another embodiment provides isolated, genetically engineered, carboxydotrophic, acetogenic bacteria which comprise a copy number of greater than 1 per genome of a nucleic acid encoding a deoxyxylulose 5-phosphate synthase (DXS) enzyme. In some aspects, the isolated, genetically engineered, carboxydotrophic, acetogenic bacteria may further comprise a nucleic acid encoding an isoprene synthase. In other aspects, the isolated, genetically engineered, carboxydotrophic, acetogenic bacteria of may further comprise a nucleic acid encoding an isopentyldiphosphate delta isomerase. In still other aspects the isolated, genetically engineered, carboxydotrophic, acetogenic bacteria may further comprise a nucleic acid encoding an isopentyldiphosphate delta isomerase and a nucleic acid encoding an isoprene synthase.

[0084] Another embodiment provides isolated, genetically engineered, carboxydotrophic, acetogenic bacteria which comprise a nucleic acid encoding a phosphomevalonate kinase (PMK). The bacteria express the encoded enzyme and the enzyme is not native to the bacteria. In some aspects the enzymes are *Staphylococcus aureus* enzymes. In some aspects the enzyme is expressed under the control of one or more *C. autoethanogenum* promoters. In some apects the bacteria further comprise a nucleic acid encoding thiolase (thlA/vraB), a nucleic acid encoding a HMG-CoA synthase (HMGS), and a nucleic acid encoding an HMG-CoA reductase (HMGR). In some aspects the thiolase is Clostridium acetobutylicum thiolase. In some aspects the bacteria further comprise a nucleic acid encoding a mevalonate disphosphate decarboxylase (PMD).

[0085] Still another embodiment provides isolated, genetically engineered, carboxydotrophic, acetogenic bacteria which comprise an exogenous nucleic acid encoding alpha-farnesene synthase. In some aspects the nucleic acid is codon optimized for expression in *C. autoethanogenum.* In some aspects the alpha-farnesene synthase is a *Malus* x *domestica* alpha-farnesene synthase. In some aspects the bacteria further comprise a nucleic acid segment encoding geranyltranstransferase. In some aspects
the gernayltranstransferase is an *E. coli* geranyltranstransferase.

[0086] Suitable isolated, genetically engineered, carboxydotrophic, acetogenic bacteria for any of the aspects or embodiments of the invention 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.*

[0087] 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 FIGURES

[0088] 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.

[0089] Figure 1: Pathway diagram for production of terpenes, gene targets described in this application are highlighted with bold arrows.

[0090] Figure 2: Genetic map of plasmid pMTL 85146-ispS

[0091] Figure 3: Genetic map of plasmid pMTL 85246-ispS-idi

[0092] Figure 4: Genetic map of plasmid pMTL 85246-ispS-idi-dxs

[0093] Figure 5: Sequencing results for plasmid pMTL 85246-ispS-idi-dxs

[0094] Figure 6: Comparison of energetics for production of terpenes from CO via DXS and mevalonate pathway

[0095] Figure 7: Mevalonate pathway

[0096] Figure 8: Agarose gel electrophoresis image confirming presence of isoprene expression plasmid pMTL 85246-ispS-idi in C. autoethanogenum transformants. Lanes 1, and 20 show 100 bp Plus DNA Ladder. Lane 3-6, 9-12, 15-18 show PCR with isolated plasmids from 4 different clones as template, each in the following order: colE1, ermB, and idi. Lanes 2, 8, and 14 show PCR without template as negative control, each in the following order: colE1, ermB, and idi. Lanes 7, 13, and 19 show PCR with pMTL 85246-ispS-idi from E. coli as positive control, each in the following order: colE1, ermB, and idi.

[0097] Figure 9 - Mevalonate expression plasmid pMTL8215-Pptaack-thlA-HMGS-Patp-HMGR

[0098] Figure 10 - Isoprene expression plasmid pMTL 8314-Pptaack-thlA-HMGS-Patp-HMGR-Prnf-MK-PMK-PMD-Pfor-idi-ispS

[0099] Figure 11 - Farnesene expression plasmid pMTL8314-Pptaack-thlA-HMGS-Patp-HMGR-Prnf-MK-PMK-PMD-Pfor-idi-ispA-FS

[0100] Figure 12 - Genetic map of plasmid pMTL 85246-ispS-idi-dxs

[0101] Figure 13 – Amplification chart for gene expression experiment with C. autoethanogenum carrying plasmid pMTL 85146-ispS

[0102] Figure 14 – Amplification chart for gene expression experiment with C. autoethanogenum carrying plasmid pMTL 85246-ispS-idi

[0103] Figure 15 - Amplification chart for gene expression experiment with C. autoethanogenum carrying plasmid pMTL 85246-ispS-idi-dxs

[0104] Figure 16 - PCR check for the presence of the plasmid pMTL8314Prnf-MK-PMK-PMD-Pfor-idi-ispA-FS. Expected band size 1584 bp. The DNA marker Fermentas 1kb DNA ladder.

[0105] Figure 17 - Growth curve for transformed C. autoethanogemun carrying plasmid pMTL8314Prnf-MK-PMK-PMD-Pfor-idi-ispA-FS

[0106] Figure 18 - RT-PRC data showing the expression of the genes Mevalonate kinase (MK SEQ ID NO: 51), Phosphomevalonate Kinase (PMK SEQ ID NO: 52), Mevalonate Diphosphate Decarboxylase (PMD SEQ ID NO: 53), Isopentyl-diphosphate Delta-isomerase (idi SEQ ID NO: 54), Geranyltranstransferase (ispA SEQ ID NO: 56) and Farnesene synthase (FS SEQ ID NO: 57).

[0107] Figure 19 - GC-MS detection and conformation of the presence of farnesene in 1mM mevalonate spiked cultures carrying pMTL8314Prnf-MK-PMK-PMD-Pfor-idi-ispA-FS. GC-MS chromatogram scanned for peaks containing ions with a mass of 93. Chromatogram 1 and 2 are transformed C. autoethanogenum , 3 is beta-farnesene standard run at the same time as the C. autoethanogenum samples. 4 is E. coli carrying the plasmids pMTL8314Prnf-MK-PMK-PMD-Pfor-idi-ispA-FS grown on M9 Glucose showing alpha-farnesene production and 5 is beta-farnesene standard run at the time of the E. coli samples. The difference in retention time between the E. coli and the C. autoethanogenum samples are due to minor changes to the instrument. However the difference in retention time between the beta-farnesene standard and the produced alpha-farnesene are the exact same in both cases, which together with the match in mass spectra's confirm the production of alpha-farnesene in C. autoethanogenum.

[0108] Figure 20 - MS spectrums for peaks labeled 1A and 2A in figure 19. The MS spectra's matches up with the NIST database spectra (figure 21) confirming the peak is alpha-farnesene.

[0109] Figure 21 - MS spectrum for alpha-farnesen from the NIST Mass Spectral Database.

DETAILED DESCRIPTION OF THE INVENTION

[0110] The following is a description of the present invention, including preferred embodiments thereof, 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.

[0111] The inventors have surprisingly been able to engineer a carboxydotrophic acetogenic microorganism to produce terpene and precursors thereof including isoprene and farnesene by fermentation of a substrate comprising CO. This offers an alternative means for the production of these products which may have benefits over the current methods for their production. 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.

[0112] As referred to herein, a "fermentation broth" is a culture medium comprising at least a nutrient media and bacterial cells.

[0113] As referred to herein, a "shuttle microorganism" is a microorganism in which a methyltransferase enzyme is expressed and is distinct from the destination microorganism.

[0114] 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.

[0115] The term "main fermentation product" is intended to mean the one fermentation product which is produced in the highest concentration and/or yield.

[0116] 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.

[0117] 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.

[0118] 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.

[0119] While it is not necessary for the substrate to contain any hydrogen, the presence of H_2 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 H_2:CO. In one embodiment the substrate comprises about 30% or less H_2 by volume, 20% or less H_2 by volume, about 15% or less H_2 by volume or about 10% or less H_2 by volume. In other embodiments, the substrate stream comprises low concentrations of H_2, 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 CO_2 for example, such as about 1% to about 80% CO_2 by volume, or 1% to about 30% CO_2 by volume. In one embodiment the substrate comprises less than or equal to about 20% CO_2 by volume. In particular embodiments the substrate comprises less than or equal to about 15% CO_2 by volume, less than or equal to about 10% CO_2 by volume, less than or equal to about 5% CO_2 by volume or substantially no CO_2.

[0120] In the description which follows, embodiments of the invention are described in terms of delivering and fermenting a "gaseous substrate containing CO". However, it should be appreciated that the gaseous substrate may be provided in alternative forms. For example, the gaseous substrate containing CO 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 the like.

[0121] In particular embodiments of the invention, the CO-containing gaseous substrate is an industrial off or waste gas. "Industrial waste or off gases" should be taken broadly to include any gases comprising CO 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.

[0122] 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.

[0123] 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.

[0124] "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 (for example in a parental microorganism from which the recombinant microorganism is derived), 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. 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.

[0125] "Exogenous" may also be used to refer to proteins. This refers to a protein that is not present in the parental microorganism from which the recombinant microorganism is derived.

[0126] The term "endogenous" as used herein in relation to a recombinant microorganism and a nucleic acid or protein refers to any nucleic acid or protein that is present in a parental microorganism from which the recombinant microorganism is derived.

[0127] 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. These include homologous genes in species such as *Clostridium acetobutylicum, Clostridium beijerinckii, C. saccharobutylicum and C. saccharoperbutylacetonicum,* 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.

[0128] 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.

[0129] "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.

[0130] 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 described by Silver et al. (1991, *Plant Physiol.* 97: 1588-1591) or Zhao et al. (2011, *Appl Microbiol Biotechnol*, 90:1915–1922) for the isoprene synthase enzyme, by Green et al. (2007, *Phytochemistry*; 68:176–188) for the farnesene synthase enzyme, by Kuzuyama et al. (2000, *J. Bacteriol.* 182, 891-897) for the 1-deoxy-D-xylulose 5-phosphate synthase Dxs, by Berndt and Schlegel (1975, *Arch. Microbiol.* 103, 21-30) or by Stim-Herndon et al. (1995, *Gene* 154: 81-85) for the thiolase, by Cabano et al. (1997, *Insect Biochem. Mol. Biol.* 27: 499-505) for the HMG-CoA synthase, by Ma et al. (2011, *Metab. Engin.*, 13:588–597) for the HMG-CoA reductase and mevalonate kinase enzyme, by Herdendorf and Miziorko (2007, *Biochemistry*, 46: 11780-8) for the phosphomevalonate kinase, and by Krepkiy et al. (2004, *Protein Sci.* 13: 1875-1881) for the mevalonate diphosphate decarboxylase. It is also possible to identify genes of DXS and mevalonate pathway using inhibitors like fosmidomycin or mevinoline as described by Trutko et al. (2005, *Microbiology* 74: 153-158).

[0131] "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 proteins (including expression of one or more nucleic acids encoding same) as compared to the expression level of the protein (including nucleic acids) of a parental microorganism under the same conditions. It should not be taken to mean that the protein (or nucleic acid) is expressed at any particular level.

[0132] 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 (ie 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 that are the subject of the

present invention. Accordingly, the recombinant microorganisms of the invention may have been modified to express or over-express one or more enzymes that were not expressed or over-expressed in the parental microorganism.

[0133] 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).

[0134] A "terpene" as referred to herein should be taken broadly to include any compound made up of C_5 isoprene units joined together including simple and complex terpenes and oxygen-containing terpene compounds such as alcohols, aldehydes and ketones. Simple terpenes are found in the essential oils and resins of plants such as conifers. More complex terpenes include the terpenoids and vitamin A, carotenoid pigments (such as lycopene), squalene, and rubber. Examples of monoterpenes include, but are not limited to isoprene, pinene, nerol, citral, camphor, menthol, limonene. Examples of *sesquiterpenes* include but are not limited to nerolidol, farnesol. Examples of *diterpenes* include but are not limited to phytol, vitamin A_1. Squalene is an example of a *triterpene*, and carotene (provitamin A_1) is a *tetraterpene*.

[0135] A "terpene precursor" is a compound or intermediate produced during the reaction to form a terpene starting from Acetyl CoA and optionally pyruvate. The term refers to a precursor compound or intermediate found in the mevalonate (MVA) pathway and optionally the DXS pathway as well as downstream precursors of longer chain terpenes, such as FPP and GPP. In particular embodiments, it includes but is not limited to mevalonic acid, IPP, dimethylallyl pyrophosphate (DMAPP), geranyl pyrophosphate (GPP) and farnesyl pyrophosphate (FPP).

[0136] The "DXS pathway" is the enzymatic pathway from pyruvate and D-glyceraldehyde-3-phosphate to DMAPP or IPP. It is also known as the deoxyxylulose 5-phosphate (DXP/DXPS/DOXP or DXS) / methylerythritol phosphate (MEP) pathway.

[0137] The "mevalonate (MVA) pathway" is the enzymatic pathway from acetyl-CoA to IPP.

Microorganisms

[0138] Two pathways for production of terpenes are known, the deoxyxylulose 5-phosphate (DXP/DXPS/DOXP or DXS) / methylerythritol phosphate (MEP) pathway (Hunter et al., 2007, *J. Biol. chem.* 282: 21573-77) starting from pyruvate and D-glyceraldehyde-3-phosphate (G3P), the two key intermediates in the glycolysis, and the mevalonate (MVA) pathway (Miziorko, 2011, *Arch Biochem Biophys*, 505: 131-143) starting from acetyl-CoA. Many different classes of microorganisms have been investigated for presence of either of these pathways (Lange et al., 2000, *PNAS*, 97: 13172-77; Trutko et al., 2005, *Microbiology*, 74: 153-158; Julsing et al., 2007, *Appl Microbiol Biotechnol*, 75: 1377-84), but not carboxydotrophic acetogens. The DXS pathway for example was found to be present in *E. coli*, *Bacillus*, or *Mycobacterium*, while the mevalonate pathway is present in yeast *Saccharomyces*, *Cloroflexus*, or *Myxococcus*.

[0139] Genomes of carboxydotrophic acetogens *C. autoethanogenum*, *C. ljungdahlii* were analysed by the inventors for presence of either of the two pathways. All genes of the DXS pathway were identified in *C. autoethanogenum* and *C. ljungdahlii* (Table 1), while the mevalonate pathway is absent. Additionally, carboxydotrophic acetogens such as *C. autoethanogenum* or *C. ljungdahlii* are not known to produce any terpenes as metabolic end products.

Table 1: Terpene biosynthesis genes of the DXS pathway identified in *C. autoethanogenum* and *C. ljungdahlii*:

Gene/Enzyme	*C. autoethanogenum*	*C. ljungdahlii*
1-deoxy-D-xylulose-5-phosphate synthase DXS	SEQ ID NO: 1-2	YP_003779286.1; GI: 300854302, CLJU_c11160

(EC:2.2.1.7)		
1-deoxy-D-xylulose 5-phosphate reductoisomerase DXR (EC:1.1.1.267)	SEQ ID NO: 3-4	YP_003779478.1; GI: 300854494, CLJU_c13080
2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase IspD (EC:2.7.7.60)	SEQ ID NO: 5-6	YP_003782252.1 GI: 300857268, CLJU_c41280
4-diphosphocytidyl-2-C-methyl-D-erythritol kinase IspE (EC:2.7.1.148)	SEQ ID NO: 7-8	YP_003778403.1; GI: 300853419, CLJU_c02110
2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase IspF (EC:4.6.1.12)	SEQ ID NO: 9-10	YP_003778349.1; GI: 300853365, CLJU_c01570
4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase IspG (EC:1.17.7.1)	SEQ ID NO: 11-12	YP_003779480.1; GI: 300854496, CLJU_c13100
4-hydroxy-3-methylbut-2-enyl diphosphate reductase (EC:1.17.1.2)	SEQ ID NO: 13-14	YP_003780294.1; GI: 300855310, CLJU_c21320

[0140] Genes for downstream synthesis of terpenes from isoprene units were also identified in both organisms (Table 2).

Gene/Enzyme	*C. autoethanogenum*	*C. ljungdahlii*
geranyltranstransferase Fps (EC:2.5.1.10)	SEQ ID NO: 15-16	YP_003779285.1; GI: 300854301, CLJU_c11150
heptaprenyl diphosphate synthase (EC:2.5.1.10)	SEQ ID NO: 17-18	YP_003779312.1; GI: 300854328, CLJU_c11420

octaprenyl-diphosphate synthase [EC:2.5.1.90]	SEQ ID NO: 19-20	YP_003782157.1; GI: 300857173, CLJU_c40310

[0141] Terpenes are energy dense compounds, and their synthesis requires the cell to invest energy in the form of nucleoside triposphates such as ATP. Using sugar as a substrate requires sufficient energy to be supplied from glycolysis to yield several molecules of ATP. The production of terpenes and/or their precursors via the DXS pathway using sugar as a substrate proceeds in a relatively straightforward manner due to the availability of pyruvate and D-glyceraldehyde-3-phosphate (G3P), G3P being derived from C5 pentose and C6 hexose sugars. These C5 and C6 molecules are thus relatively easily converted into C5 isoprene units from which terpenes are composed.

[0142] For anaerobic acetogens using a C1 substrate like CO or CO2, it is more difficult to synthesise long molecules such as hemiterpenoids from C1 units. This is especially true for longer chain terpenes like C10 monoterpenes, C15 sesquiterpenes, or C40 tetraterpenes. To date the product with most carbon atoms reported in acetogens (both native and recombinant organisms) are C4 compounds butanol (Köpke et al., 2011, *Curr. Opin. Biotechnol.* 22: 320-325; Schiel-Bengelsdorf and Dürre, 2012, *FEBS Letters*: 10.1016/j.febslet.2012.04.043; Köpke et al., 2011, *Proc. Nat. Sci. U.S.A.* 107: 13087-92; US patent 2011/0236941) and 2,3-butanediol (Köpke et al., 2011, Appl. Environ. Microbiol. 77:5467-75). The inventors have shown that it is surprisingly possible to anaerobically produce these longer chain terpene molecules using the C1 feedstock CO via the acetyl CoA intermediate.

[0143] Energetics of the Wood-Ljungdahl pathway of anaerobic acetogens are just emerging, but unlike under aerobic growth conditions or glycolysis of sugar fermenting organisms no ATP is gained in the Wood-Ljungdahl pathway by substrate level phosphorylation, in fact activation of CO_2 to formate actually requires one molecule of ATP and a membrane gradient is required. The inventors note that it is important that a pathway for product formation is energy efficient. The inventors note that in acetogens the substrate CO or CO_2 is channeled directly into acetyl-CoA, which represents the most direct route to terpenes and/or their precursors, especially when compared to sugar based systems, with only six reactions

required (Fig. 1). Though less ATP is available in carboxydotrophic acetogens, the inventors believe that this more direct pathway may sustain a higher metabolic flux (owing to higher chemical motive force of intermediate reactions). A highly effective metabolic flux is important as several intermediates in the terpene biosynthesis pathway, such as key intermediates Mevalonate and FPP, are toxic to most bacteria when not turned over efficiently.
Despite having a higher ATP availability, this problem of intermediate toxicity can be a a bottleneck in production of terpenes from sugar.

[0144] When comparing the energetics of terpene precursor IPP and DMAPP production from CO (Fig. 6) via the mevalonate pathway versus the DXS pathway, the inventors noted that the mevalonate pathway requires less nucleoside triposphates as ATP, less reducing equivalents, and is also more direct when compared to the DXS pathway with only six necessary reaction steps from acetyl-CoA. This provides advantages in the speed of the reactions and metabolic fluxes and increases overall energy efficiency. Additionally, the lower number of enzymes required simplifies the recombination method required to produce a recombinant microorganism.

[0145] No acetogens with a mevalonate pathway have been identified, but the inventors have shown that it is possible to introduce the mevalonate pathway and optionally the DXS pathway into a carboxydotrophic acetogen such as *Clostridium autoethanogenum* or *C. ljungdahlii* to efficiently produce terpenes and/or precursors thereof from the C1 carbon substrate CO. They contemplate that this is applicable to all carboxydotrophic acetogenic microorganisms.

[0146] Additionally, the production of terpenes and/or precursors thereof has never been shown to be possible using recombinant microorganisms under anaerobic conditions. Anaerobic production of isoprene has the advantage of providing a safer operating environment because isoprene is extremely flammable in the presence of oxygen and has a lower flammable limit (LFL) of 1.5-2.0 % and an upper flammable (UFL) limit of 2.0-12 % at room temperature and atmospheric pressure. As flames cannot occur in the absence of oxygen, the inventors believe that an anaerobic fermentation process is desirable as it would be safer across all product concentrations, gas compositions, temperature and pressure ranges.

[0147] As discussed hereinbefore, the invention provides a recombinant microorganism capable of producing one or more terpenes and/or precursors thereof, and optionally one or more other products, by fermentation of a substrate comprising CO.

[0148] In a further embodiment, the microorganism is adapted to:
express one or more exogenous enzymes from the mevalonate (MVA) pathway and/or overexpress one or more endogenous enzyme from the mevalonate (MVA) pathway; and
a) express one or more exogenous enzymes from the DXS pathway and/or overexpress one or more endogenous enzymes from the DXS pathway.

[0149] In one embodiment, the parental microorganism from which the recombinant microorganism is derived is capable of fermenting a substrate comprising CO to produce Acetyl CoA, but not of converting Acetyl CoA to mevalonic acid or isopentenyl pyrophosphate (IPP) and the recombinant microorganism is adapted to express one or more enzymes involved in the mevalonate pathway.

[0150] The microorganism may be adapted to express or over-express the one or more enzymes 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.

[0151] In one embodiment, the one or more enzymes are from the mevalonate (MVA) pathway and are selected from the group consisting of:
a) thiolase (EC 2.3.1.9),
b) HMG-CoA synthase (EC 2.3.3.10),
c) HMG-CoA reductase (EC 1.1.1.88),
d) Mevalonate kinase (EC 2.7.1.36),
e) Phosphomevalonate kinase (EC 2.7.4.2),
f) Mevalonate Diphosphate decarboxylase (EC 4.1.1.33), and

g) a functionally equivalent variant of any one thereof.

[0152] In a further embodiment, the optional one or more enzymes are from the DXS pathway is selected from the group consisting of:

a) 1-deoxy-D-xylulose-5-phosphate synthase DXS (EC:2.2.1.7),
b) 1-deoxy-D-xylulose 5-phosphate reductoisomerase DXR (EC:1.1.1.267),
c) 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase IspD (EC:2.7.7.60),
d) 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase IspE (EC:2.7.1.148),
e) 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase IspF (EC:4.6.1.12),
f) 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase IspG (EC:1.17.7.1),
g) 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (EC:1.17.1.2), and
h) a functionally equivalent variant of any one thereof.

[0153] In a further embodiment, one or more exogenous or endogenous further enzymes are expressed or over-expressed to result in the production of a terpene compound and/or precursor thereof wherein the exogenous enzyme that is expressed, or the endogenous enzyme that is overexpressed is selected from the group consisting of:

a) geranyltranstransferase Fps (EC:2.5.1.10),
b) heptaprenyl diphosphate synthase (EC:2.5.1.10),
c) octaprenyl-diphosphate synthase (EC:2.5.1.90),
d) isoprene synthase (EC 4.2.3.27),
e) isopentenyl-diphosphate delta-isomerase (EC EC 5.3.3.2),
f) farnesene synthase (EC 4.2.3.46 / EC 4.2.3.47), and
g) a functionally equivalent variant of any one thereof.

[0154] By way of example only, sequence information for each of the enzymes is listed in the figures herein.

[0155] The enzymes 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 enzymes are derived from *Staphylococcus aureus.*

[0156] In one embodiment, the enzyme isoprene synthase (ispS) is derived from *Poplar tremuloides*. In a further embodiment, it has the nucleic acid sequence exemplified in SEQ ID NO: 21 hereinafter, or it is a functionally equivalent variant thereof.

[0157] In one embodiment, the enzyme deoxyxylulose 5-phosphate synthase is derived from *C.* autoethanogenum, encoded by the nucleic acid sequence exemplified in SEQ ID NO: 1 and/or with the amino acid sequence exemplified in SEQ ID NO: 2 hereinafter, or it is a functionally equivalent variant thereof.

[0158] In one embodiment, the enzyme 1-deoxy-D-xylulose 5-phosphate reductoisomerase DXR is derived from *C. autoethanogenum* and is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 3 or is a functionally equivalent variant thereof.

[0159] In one embodiment, the enzyme 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase IspD is derived from *C. autoethanogenum* and is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 5 or is a functionally equivalent variant thereof.

[0160] In one embodiment, the enzyme 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase IspE is derived from *C. autoethanogenum* and is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 7 or is a functionally equivalent variant thereof.

[0161] In one embodiment, the enzyme 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase IspF is derived from *C. autoethanogenum* and is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 9 or is a functionally equivalent variant thereof.

[0162] In one embodiment, the enzyme 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase IspG is derived from *C. autoethanogenum* and is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 11 or is a functionally equivalent variant thereof.

[0163] In one embodiment, the enzyme 4-hydroxy-3-methylbut-2-enyl diphosphate reductase is derived from *C. autoethanogenum* and is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 13 or is a functionally equivalent variant thereof.

[0164] In one embodiment, the enzyme mevalonate kinase (MK) is derived from *Staphylococcus* aureus subsp. aureus Mu50 and is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 51 hereinafter, or it is a functionally equivalent variant thereof.

[0165] In one embodiment, the enzyme phosphomevalonate kinase (PMK) is derived from *Staphylococcus aureus* subsp. aureus Mu50 and is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 52 hereinafter, or it is a functionally equivalent variant thereof.

[0166] In one embodiment, the enzyme mevalonate diphosphate decarboxylase (PMD) is derived from *Staphylococcus aureus* subsp. aureus Mu50 and is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 53 hereinafter, or it is a functionally equivalent variant thereof.

[0167] In one embodiment, the enzyme Isopentenyl-diphosphate delta-isomerase (idi) is derived from *Clostridium beijerinckii* and is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 54 hereinafter, or it is a functionally equivalent variant thereof.

[0168] In one embodiment, the enzyme thiolase (thIA) is derived from *Clostridium acetobutylicum* ATCC824 and is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 40 hereinafter, or it is a functionally equivalent variant thereof.

[0169] In one embodiment, the enzyme is a thiolase enzyme, and is an acetyl-CoA c-acetyltransferase (vraB) derived from *Staphylococcus aureus* subsp. aureus Mu50 and is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 41 hereinafter, or it is a functionally equivalent variant thereof.

[0170] In one embodiment, the enzyme 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) is derived from Staphylococcus aureus subsp. aureus Mu50 and is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 42 hereinafter, or it is a functionally equivalent variant thereof.

[0171] In one embodiment, the enzyme Hydroxymethylglutaryl-CoA reductase (HMGR) is derived from Staphylococcus aureus subsp. aureus Mu50 and is encoded by the nucleic acid

sequence exemplified in SEQ ID NO: 43 hereinafter, or it is a functionally equivalent variant thereof.

[0172] In one embodiment, the enzyme Geranyltranstransferase (ispA) is derived from *Escherichia coli str.* K-12 *substr.* MG1655 is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 56 hereinafter, or it is a functionally equivalent variant thereof.

[0173] In one embodiment, the enzyme heptaprenyl diphosphate synthase is derived from *C. autoethanogenum* and is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 17 or is a functionally equivalent variant thereof.

[0174] In one embodiment, the enzyme polyprenyl synthetase is derived from *C. autoethanogenum* and is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 19 or is a functionally equivalent variant thereof.

[0175] In one embodiment, the enzyme Alpha-farnesene synthase (FS) is derived from *Malus* x *domestica* and is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 57 hereinafter, or it is a functionally equivalent variant thereof.

[0176] The enzymes and functional variants of use in the microorganisms may be identified by assays known to one of skill in the art. In particular embodiments, the enzyme isoprene synthase may be identified by the method outlined Silver et al. (1991, *Plant Physiol.* 97: 1588-1591) or Zhao et al. (2011, *Appl Microbiol Biotechnol*, 90:1915–1922). In a further particular embodiment, the enzyme farnesene synthase may be identified by the method outlined in Green et al., 2007, Phytochemistry; 68:176–188. In further particular embodiments, enzymes from the mevalonate pathway may be identified by the method outlined in Cabano et al. (1997, *Insect Biochem. Mol. Biol.* 27: 499-505) for the HMG-CoA synthase, Ma et al. (2011, *Metab. Engin.*, 13:588–597) for the HMG-CoA reductase and mevalonate kinase enzyme, Herdendorf and Miziorko (2007, *Biochemistry*, 46: 11780-8) for the phosphomevalonate kinase, and Krepkiy et al. (2004, *Protein Sci.* 13: 1875-1881) for the mevalonate diphosphate decarboxylase. Ma et al., 2011, *Metab. Engin.*, 13:588–597. The 1-deoxy-D-xylulose 5-phosphate synthase of the DXS pathway can be assayed using the method outlined in Kuzuyama et al. (2000, *J. Bacteriol.* 182, 891-897). It is also possible to

identify genes of DXS and mevalonate pathway using inhibitors like fosmidomycin or mevinoline as described by Trutko et al. (2005, *Microbiology* 74: 153-158).

[0177] In one embodiment, the microorganism comprises one or more exogenous nucleic acids adapted to increase expression of one or more endogenous nucleic acids and which one or more endogenous nucleic acids encode one or more of the enzymes 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.

[0178] In one embodiment, the microorganism comprises one or more exogenous nucleic acids encoding and adapted to express one or more of the enzymes referred to herein before. In one embodiment, the microorganisms comprise one or more exogenous nucleic acid encoding and adapted to express at least two, at least of the enzymes. In other embodiments, the microorganism comprises one or more exogenous nucleic acid encoding and adapted to express at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine or more of the enzymes.

[0179] In one particular embodiment, the microorganism comprises one or more exogenous nucleic acid encoding an enzyme of the invention or a functionally equivalent variant thereof.

[0180] 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.

[0181] 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.

[0182] The exogenous nucleic acids may remain extra-chromosomal upon transformation of the parental microorganism or may intergrate 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).

[0183] In one embodiment, the exogenous nucleic acids encoding one or enzymes 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.

[0184] In one embodiment, the exogenous nucleic acid is an expression plasmid.

[0185] In one particular embodiment, the parental microorganism is selected from the group of carboxydotrophic 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.*

[0186] 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-1T (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 ethanologenic 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].

[0187] 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 auxotroph to certain vitamins (e.g. thiamine, biotin) while others were not.

[0188] In one embodiment, the parental carboxydotrophic acetogenic microorganism is selected from the group consisting of *Clostridium autoethanogenum, Clostridium ljungdahlii, Clostridium ragsdalei, Clostridium carboxidivorans, Clostridium drakei, Clostridium* scatologenes, *Butyribacterium limosum, Butyribacterium methylotrophicum, Acetobacterium woodii, Alkalibaculum bacchii, Blautia producta, Eubacterium limosum, Moorella thermoacetica, Moorella thermautotrophica, Oxobacter pfennigii*, and *Thermoanaerobacter kiuvi.*

[0189] In one particular embodiment of the first or second aspects, the parental microorganism is selected from the group of carboxydotrophic Clostridia comprising *Clostridium autoethanogenum, Clostridium ljungdahlii, Clostridium ragsdalei, Clostridium carboxidivorans, Clostridium drakei, Clostridium scatologenes, Clostridium aceticum, Clostridium formicoaceticum, Clostridium magnum.*

[0190] In a one embodiment, the microorganism is selected from a cluster of carboxydotrophic 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, Naveau, & Nyns, 1994), *C. autoethanogenum* LBS1560 (DSM19630) (WO/2009/064200), *C. autoethanogenum* LBS1561 (DSM23693), *C. ljungdahlii* PETC^T (DSM13528 = ATCC 55383) (Tanner, Miller, & Yang, 1993), *C. ljungdahlii* ERI-2 (ATCC 55380) (US patent 5,593,886), *C. ljungdahlii* C-01 (ATCC 55988) (US patent 6,368,819), *C. ljungdahlii* O-52 (ATCC 55989) (US patent 6,368,819), or "*C. ragsdalei* P11^T" (ATCC BAA-622) (WO 2008/028055), and related isolates such as "*C. coskatii*" (US patent 2011/0229947), "*Clostridium sp.* MT351" (Michael Tyurin & Kiriukhin, 2012) and mutant strains thereof 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).

[0191] These strains form a subcluster within the Clostridial rRNA cluster I (Collins et al., 1994), having at least 99% identity on 16S rRNA gene level, although being distinct species as determined by DNA-DNA reassociation and DNA fingerprinting experiments (WO 2008/028055, US patent 2011/0229947).

[0192] The strains of this cluster are defined by common characteristics, having both a similar genotype and phenotype, and they all share the same mode of energy conservation and fermentative metabolism. The strains of this cluster lack cytochromes and conserve energy via an Rnf complex.

[0193] All strains of this cluster have a genome size of around 4.2 MBp (Köpke et al., 2010) and a GC composition of around 32 %mol (Abrini et al., 1994; Köpke et al., 2010; Tanner et al., 1993) (WO 2008/028055; US patent 2011/0229947), and conserved essential key gene operons encoding for enzymes of Wood-Ljungdahl pathway (Carbon monoxide dehydrogenase, Formyl-tetrahydrofolate synthetase, Methylene-tetrahydrofolate dehydrogenase, Formyl-tetrahydrofolate cyclohydrolase, Methylene-tetrahydrofolate reductase, and Carbon monoxide dehydrogenase/Acetyl-CoA synthase), hydrogenase, formate dehydrogenase, Rnf complex (*rnfCDGEAB*), pyruvate:ferredoxin oxidoreductase, aldehyde:ferredoxin oxidoreductase (Köpke et al., 2010, 2011). The organization and number

of Wood-Ljungdahl pathway genes, responsible for gas uptake, has been found to be the same in all species, despite differences in nucleic and amino acid sequences (Köpke et al., 2011).

[0194] The strains all 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 (Abrini et al., 1994; Tanner et al., 1993)(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 metabolic profile with ethanol and acetic acid as main fermentation end product, with small amounts of 2,3-butanediol and lactic acid formed under certain conditions (Abrini et al., 1994; Köpke et al., 2011; Tanner et al., 1993) 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). Some of the species were found to be auxotroph to certain vitamins (e.g. thiamine, biotin) while others were not. Reduction of carboxylic acids into their corresponding alcohols has been shown in a range of these organisms (Perez, Richter, Loftus, & Angenent, 2012).

[0195] The traits described are therefore not specific to one organism like *C. autoethanogenum* or *C. ljungdahlii*, but rather general traits for carboxydotrophic, ethanol-synthesizing Clostridia. Thus, the invention can be anticipated to work across these strains, although there may be differences in performance.

[0196] The recombinant carboxydotrophic acetogenic microorganisms of the invention may be prepared from a parental carboxydotrophic acetogenic 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, electrofusion, ultrasonication, polyethylene glycol-mediated transformation, conjugation, or chemical and natural competence. Suitable transformation techniques are described for example in Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A laboratory Manual, Cold Spring Harbour Labrotary Press, Cold Spring Harbour, 1989.

[0197] Electroporation has been described for several carboxydotrophic acetogens as *C. ljungdahlii* (Köpke et al., 2010; Leang, Ueki, Nevin, & Lovley, 2012) (PCT/NZ2011/000203; WO2012/053905), *C. autoethanogenum* (PCT/NZ2011/000203; WO2012/053905), *Acetobacterium woodii* (Strätz, Sauer, Kuhn, & Dürre, 1994) or *Moorella thermoacetica* (Kita et al., 2012) and is a standard method used in many *Clostridia* such as *C. acetobutylicum* (Mermelstein, Welker, Bennett, & Papoutsakis, 1992), *C. cellulolyticum* (Jennert, Tardif, Young, & Young, 2000) or *C. thermocellum* (MV Tyurin, Desai, & Lynd, 2004).

[0198] Electrofusion has been described for acetogenic *Clostridium sp.* MT351 (Tyurin and Kiriukhin, 2012).

[0199] Prophage induction has been described for carboxydotrophic acetogen as well in case of *C. scatologenes* (Prasanna Tamarapu Parthasarathy, 2010, Development of a Genetic Modification System in *Clostridium scatologenes* ATCC 25775 for Generation of Mutants, Masters Project Western Kentucky University).

[0200] Conjugation has been described as method of choice for acetogen *Clostridium difficile* (Herbert, O'Keeffe, Purdy, Elmore, & Minton, 2003) and many other Clostridia including *C. acetobuylicum* (Williams, Young, & Young, 1990).

[0201] In one embodiment, the parental strain uses CO as its sole carbon and energy source.

[0202] 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).

Nucleic acids

[0203] The invention also provides one or more nucleic acids or nucleic acid constructs of use in generating a recombinant microorganism of the invention.

[0204] In one embodiment, the nucleic acid comprises sequences encoding one or more of the enzymes in the mevalonate (MVA) pathway and optionally the DXS pathway which when expressed in a microorganism allows the microorganism to produce one or more terpenes and/or precursors thereof by fermentation of a substrate comprising CO. In one particular embodiment, the invention provides a nucleic acid encoding two or more enzymes which when expressed in a microorganism allows the microorganism to produce one or more terpene and/or precursor thereof by fermentation of substrate comprising CO. In one embodiment, a nucleic acid of the invention encodes three, four, five or more of such enzymes.

[0205] In one embodiment, the one or more enzymes encoded by the nucleic acid are from the mevalonate (MVA) pathway and are selected from the group consisting of:

a) thiolase (EC 2.3.1.9),
b) HMG-CoA synthase (EC 2.3.3.10),
c) HMG-CoA reductase (EC 1.1.1.88),
d) Mevalonate kinase (EC 2.7.1.36),
e) Phosphomevalonate kinase (EC 2.7.4.2),
f) Mevalonate Diphosphate decarboxylase (EC 4.1.1.33), and
g) a functionally equivalent variant of any one thereof.

[0206] In a further embodiment, the one or more optional enzymes encoded by the nucleic acid are from the DXS pathway are selected from the group consisting of:

a) 1-deoxy-D-xylulose-5-phosphate synthase DXS (EC:2.2.1.7),
b) 1-deoxy-D-xylulose 5-phosphate reductoisomerase DXR (EC:1.1.1.267),
c) 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase IspD (EC:2.7.7.60),
d) 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase IspE (EC:2.7.1.148),
e) 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase IspF (EC:4.6.1.12),
f) 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase IspG (EC:1.17.7.1),
g) 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (EC:1.17.1.2), and
h) a functionally equivalent variant of any one thereof.

[0207] In a further embodiment, the nucleic acid encodes one or more further enzymes that are expressed or over-expressed to result in the production of a terpene compound and/or

precursor thereof wherein the exogenous enzyme that is expressed, or the endogenous enzyme that is overexpressed is selected from the group consisting of:

a) geranyltranstransferase Fps (EC:2.5.1.10),
b) heptaprenyl diphosphate synthase (EC:2.5.1.10),
c) octaprenyl-diphosphate synthase (EC:2.5.1.90),
d) isoprene synthase (EC 4.2.3.27),
e) isopentenyl-diphosphate delta-isomerase (EC EC 5.3.3.2),
f) farnesene synthase (EC 4.2.3.46 / EC 4.2.3.47), and
g) a functionally equivalent variant of any one thereof.

[0208] Exemplary amino acid sequences and nucleic acid sequences 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 acid 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.

[0209] In a further embodiment, the nucleic acid encoding thiolase (thIA) derived from *Clostridium* acetobutylicum ATCC824 is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 40 hereinafter, or it is a functionally equivalent variant thereof.

[0210] In a further embodiment, the nucleic acid encoding thiolase wherein the thiolase is acetyl-CoA c-acetyltransferase (vraB) derived from *Staphylococcus aureus* subsp. aureus Mu50 is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 41 hereinafter, or it is a functionally equivalent variant thereof.

[0211] In a further embodiment, the nucleic acid encoding 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) derived from *Staphylococcus aureus* subsp. aureus Mu50 is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 42 hereinafter, or it is a functionally equivalent variant thereof.

[0212] In a further embodiment, the nucleic acid encoding Hydroxymethylglutaryl-CoA reductase (HMGR) derived from *Staphylococcus aureus* subsp. aureus Mu50 is encoded by

the nucleic acid sequence exemplified in SEQ ID NO: 43 hereinafter, or it is a functionally equivalent variant thereof.

[0213] In a further embodiment, the nucleic acid encoding mevalonate kinase (MK) derived from *Staphylococcus aureus* subsp. aureus Mu50 is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 51 hereinafter, or it is a functionally equivalent variant thereof.

[0214] In a further embodiment, the nucleic acid encoding phosphomevalonate kinase (PMK) derived from *Staphylococcus aureus* subsp. aureus Mu50 is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 52 hereinafter, or it is a functionally equivalent variant thereof.

[0215] In a further embodiment, the nucleic acid encoding mevalonate diphosphate decarboxylase (PMD) derived from *Staphylococcus aureus* subsp. aureus Mu50 is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 53 hereinafter, or it is a functionally equivalent variant thereof.

[0216] In a further embodiment, the nucleic acid encoding deoxyxylulose 5-phosphate synthase derived from *C. autoethanogenum,* is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 1 and/or with the amino acid sequence exemplified in SEQ ID NO: 2 hereinafter, or it is a functionally equivalent variant thereof.

[0217] In one embodiment, the nucleic acid encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase DXR (EC:1.1.1.267) has the sequence SEQ ID NO: 3 or is a functionally equivalent variant thereof.

[0218] In one embodiment, the nucleic acid encoding 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase IspD (EC:2.7.7.60) has the sequence SEQ ID NO: 5 or is a functionally equivalent variant thereof.

[0219] In one embodiment, the nucleic acid encoding 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase IspE (EC:2.7.1.148) has the sequence SEQ ID NO: 7 or is a functionally equivalent variant thereof.

[0220] In one embodiment, the nucleic acid encoding 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase IspF (EC:4.6.1.12) has the sequence SEQ ID NO: 9 or is a functionally equivalent variant thereof.

[0221] In one embodiment, the nucleic acid encoding 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase IspG (EC:1.17.7.1) has the sequence SEQ ID NO: 11 or is a functionally equivalent variant thereof.

[0222] In one embodiment, the nucleic acid encoding 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (EC:1.17.1.2) has the sequence SEQ ID NO: 13 or is a functionally equivalent variant thereof.

[0223] In a further embodiment, the nucleic acid encoding Geranyltranstransferase (ispA) derived from *Escherichia coli str.* K-12 *substr.* MG1655 is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 56 hereinafter, or it is a functionally equivalent variant thereof.

[0224] In one embodiment, the nucleic acid encoding heptaprenyl diphosphate synthase has the sequence SEQ ID NO: 17, or it is a functionally equivalent variant thereof.

[0225] In one embodiment, the nucleic acid encoding octaprenyl-diphosphate synthase (EC:2.5.1.90) wherein the octaprenyl-diphosphate synthase is polyprenyl synthetase is encoded by sequence SEQ ID NO: 19, or it is a functionally equivalent variant thereof.

[0226] In one embodiment, the nucleic acid encoding isoprene synthase (ispS) derived from Poplar *tremuloides* is exemplified in SEQ ID NO: 21 hereinafter, or it is a functionally equivalent variant thereof.

[0227] In a further embodiment, the nucleic acid encoding Isopentenyl-diphosphate delta-isomerase (idi) derived from *Clostridium beijerinckii* is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 54 hereinafter, or it is a functionally equivalent variant thereof.

[0228] In a further embodiment, the nucleic acid encoding Alpha-farnesene synthase (FS) derived from *Malus* x *domestica* is encoded by the nucleic acid sequence exemplified in SEQ ID NO: 57 hereinafter, or it is a functionally equivalent variant thereof.

[0229] 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 kindase 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.*

[0230] The nucleic acids of the invention may remain extra-chromosomal upon transformation of a parental microorganism or may be adapted for intergration 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).

[0231] 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.

[0232] 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.

[0233] 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.

[0234] Nucleic acids and nucleic acid constructs, including expression constructs/vectors of the invention may be constructed using any number of techniques standard 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.

[0235] It should be appreciated that nucleic acids of the invention may be in any appropriate form, including RNA, DNA, or cDNA.

[0236] 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.

Methods of producing organisms

[0237] 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 tranformation 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.)

[0238] 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 Labrotary Press, Cold Spring Harbour, 1989.

[0239] 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.

[0240] By way of example, in one embodiment, a recombinant microorganism of the invention is produced by a method comprises the following steps:

b) 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;
c) expression of the methyltransferase gene;
d) isolation of one or more constructs/vectors from the shuttle microorganism; and,
e) introduction of the one or more construct/vector into a destination microorganism.

[0241] 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.

[0242] 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 subtillis, or Lactococcus lactis.*

[0243] The methylation construct/vector comprises a nucleic acid sequence encoding a methyltransferase.

[0244] 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.

[0245] 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.

[0246] 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.

[0247] In one particular embodiment, both construct/vector are concurrently isolated.

[0248] 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.

[0249] 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.

[0250] 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.

[0251] In one particular embodiment, the expression construct/vector and/or the methylation construct/vector are plasmids.

[0252] 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 ΦT1 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: 60 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: 63, or it is a functionally equivalent variant thereof).

[0253] 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.

Methods of production

[0254] The invention provides a method for the production of one or more terpenes and/or precursors thereof, and optionally one or more other products, by microbial fermentation comprising fermenting a substrate comprising CO using a recombinant microorganism of the invention. Preferably, the one or more terpene and/or precursor thereof is the main fermentation product. The methods of the invention may be used to reduce the total atmospheric carbon emissions from an industrial process.

[0255] Preferably, the fermentation comprises the steps of anaerobically fermenting a substrate in a bioreactor to produce at least one or more terpenes and/or a precursor thereof using a recombinant microorganism of the invention.

[0256] In one embodiment, the one or more terpene and/or precursor thereof is chosen from mevalonic acid, IPP, dimethylallyl pyrophosphate (DMAPP), isoprene, geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP) and farnesene.

[0257] Instead of producing isoprene directly from terpenoid key intermediates IPP and DMAPP then using this to synthesise longer chain terpenes, it is also possible to synthesise longer chain terpenes, such as C10 Monoterpenoids or C15 Sesquiterpenoids, directly via a geranyltransferase (see Table 6). From C15 Sesquiterpenoid building block farnesyl-PP it is possible to produce farnesene, which, similarly to ethanol, can be used as a transportation fuel.

[0258] In one embodiment the method comprises the steps of:

(a) providing a substrate comprising CO 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 one or more terpene and/or precursor thereof.

[0259] In one embodiment the method comprises the steps of:

a) capturing CO-containing gas produced as a result of the industrial process;
b) anaerobic fermentation of the CO-containing gas to produce the at least one or more terpene and/or precursor thereof by a culture containing one or more microorganism of the invention.

[0260] In an 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 CO_2 and therefore the invention has particular utility in reducing CO_2 greenhouse gas emissions and producing a terpene 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.

[0261] It will be appreciated that for growth of the bacteria and CO-to-at least one or more terpene and/or precursor thereof to occur, in addition to the CO-containing substrate gas, a suitable liquid nutrient medium 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 a terpene and/or a prescursor thereof

using CO 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.

[0262] The fermentation should desirably be carried out under appropriate conditions for the CO-to-the at least one or more terpene and/or precursor thereof fermentation 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 in the liquid phase does not become limiting, and maximum product concentrations to avoid product inhibition.

[0263] In addition, it is often desirable to increase the CO concentration of a substrate stream (or CO partial pressure in a gaseous substrate) and thus increase the efficiency of fermentation reactions where CO is a substrate. Operating at increased pressures allows a significant increase in the rate of CO transfer from the gas phase to the liquid phase where it can be taken up by the micro-organism as a carbon source for the production of at least one or more terpene and/or precursor thereof. 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-to-at least one or more terpene and/or precursor thereof 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 US 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.

[0264] 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.

[0265] It is also desirable that the rate of introduction of the CO-containing gaseous substrate is such as to ensure that the concentration of CO in the liquid phase does not become limiting. This is because a consequence of CO-limited conditions may be that one or more product is consumed by the culture.

[0266] 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 (e.g. 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.

[0267] 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.

[0268] Terpenes and/or precursors thereof, or a mixed stream containing one or more terpenes, precursors thereof 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.

[0269] In certain preferred embodiments of the invention, the one or more terpene and/or precursor thereof 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.

[0270] 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.

EXAMPLES

[0271] The invention will now be described in more detail with reference to the following non-limiting examples.

Example 1 - Expression of isoprene synthase in *C. autoethanogenum* for production of isoprene from CO

[0272] The inventors have identified terpene biosynthesis genes in carboxydotrophic acetogens such as *C. autoethanogenum* and *C. ljungdahlii*. A recombinant organism was engineered to produce isoprene. Isoprene is naturally emitted by some plant such as poplar to protect its leave from UV radiation. Isoprene synthase (EC 4.2.3.27) gene of Poplar was codon optimized and introduced into a carboxydotrophic acetogen *C. autoethanogenum* to produce isoprene from CO. The enzyme takes key intermediate DMAPP (Dimethylallyl diphosphate) of terpenoid biosynthesis to isoprene in an irreversible reaction (Fig. 1).

Strains and growth conditions:

[0273] All subcloning steps were performed in *E. coli* using standard strains and growth conditions as described earlier (Sambrook et al, Molecular Cloning: A laboratory Manual,

Cold Spring Harbour Labrotary Press, Cold Spring Harbour, 1989; Ausubel et al, Current protocols in molecular biology, John Wiley & Sons, Ltd., Hoboken, 1987).

[0274] *C. autoethanogenum* DSM10061 and DSM23693 (a derivative of DSM10061) were obtained from DSMZ (The German Collection of Microorganisms and Cell Cultures, Inhoffenstraße 7 B, 38124 Braunschweig, Germany). Growth was carried out at 37° C using strictly anaerobic conditions and techniques (Hungate, 1969, Methods in Microbiology, vol. 3B. Academic Press, New York: 117-132; Wolfe, 1971, *Adv. Microb. Physiol.*, 6: 107-146). Chemically defined PETC media without yeast extract (Table 1) and 30 psi carbon monoxide containing steel mill waste gas (collected from New Zealand Steel site in Glenbrook, NZ; composition: 44% CO, 32% N_2, 22% CO_2, 2% H_2) as sole carbon and energy source was used.

Table 1

Media component	**Concentration per 1.0L of media**
NH_4Cl	1 g
KCl	0.1 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.8 g
KH_2PO_4	0.1 g
$CaCl_2$	0.02 g
Trace metal solution	10 ml
Wolfe's vitamin solution	10 ml
Resazurin (2 g/L stock)	0.5 ml
$NaHCO_3$	2 g
Reducing agent	0.006-0.008 % (v/v)
Distilled water	Up to 1 L, pH 5.5 (adjusted with HCl)

Wolfe's vitamin solution	**per L of Stock**

Biotin	2 mg
Folic acid	2 mg
Pyridoxine hydrochloride	10 mg
Riboflavin	5 mg
Nicotinic acid	5 mg
Calcium D-(+)-pantothenate	5 mg
Vitamin B_{12}	0.1 mg
p-Aminobenzoic acid	5 mg
Lipoic acid	5 mg
Thiamine	5 mg
Distilled water	To 1 L

Trace metal solution	**per L of stock**
Nitrilotriacetic Acid	2 g
$MnSO_4.H_2O$	1 g
$Fe(SO_4)_2(NH_4)_2.6H_2O$	0.8 g
$CoCl_2.6H_2O$	0.2 g
$ZnSO_4.7H_2O$	0.2 mg
$CuCl_2.2H_2O$	0.02 g
$NaMoO_4.2H_2O$	0.02 g
Na_2SeO_3	0.02 g
$NiCl_2.6H_2O$	0.02 g
$Na_2WO_4.2H_2O$	0.02 g
Distilled water	To 1 L

Reducing agent stock	**per 100 mL of stock**
NaOH	0.9 g

Cystein.HCl	4 g
Na_2S	4 g
Distilled water	To 100 mL

Construction of expression plasmid:

[0275] Standard Recombinant DNA and molecular cloning techniques were used in this invention (Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A laboratory Manual, Cold Spring Harbour Laboratory Press, Cold Spring Harbour, 1989; Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K: Current protocols in molecular biology. John Wiley & Sons, Ltd., Hoboken, 1987). The isoprene synthase of *Poplar tremuloides* (AAQ16588.1; GI:33358229) was codon-optimized (SEQ ID NO: 21) and synthesized. A promoter region of the Pyruvate:ferredoxin oxidoreductase of *C. autoethanogenum* (SEQ ID NO: 22) was used to express the gene.

[0276] Genomic DNA from *Clostridium autoethanogenum* DSM23693 was isolated using a modified method by Bertram and Dürre (1989). A 100-ml overnight culture was harvested (6,000 x g, 15 min, 4 °C), washed with potassium phosphate buffer (10 mM, pH 7.5) and suspended in 1.9 ml STE buffer (50 mM Tris-HCl, 1 mM EDTA, 200 mM sucrose; pH 8.0). 300 µl lysozyme (~100,000 U) was added and the mixture was incubated at 37 °C for 30 min, followed by addition of 280 µl of a 10 % (w/v) SDS solution and another incubation for 10 min. RNA was digested at room temperature by addition of 240 µl of an EDTA solution (0.5 M, pH 8), 20 µl Tris-HCl (1 M, pH 7.5), and 10 µl RNase A (Fermentas Life Sciences). Then, 100 µl Proteinase K (0.5 U) was added and proteolysis took place for 1-3 h at 37 °C. Finally, 600 µl of sodium perchlorate (5 M) was added, followed by a phenol-chloroform extraction and an isopropanol precipitation. DNA quantity and quality was inspected spectrophotometrically. The Pyruvate:ferredoxin oxidoreductase promoter sequence was amplified by PCR using oligonucleotides Ppfor-NotI-F (SEQ ID NO: 23: AAGCGGCCGCAAAATAGTTGATAATAATGC) and Ppfor-NdeI-R (SEQ ID NO: 24: TACGCATATGAATTCCTCTCCTTTTCAAGC) using iProof High Fidelity DNA Polymerase (Bio-Rad Laboratories) and the following program: initial denaturation at 98 °C for 30 seconds, followed by 32 cycles of denaturation (98 °C for 10 seconds), annealing (50-

62 °C for 30-120 seconds) and elongation (72 °C for 30-90 seconds), before a final extension step (72 °C for 10 minutes).

Construction of isoprene synthase expression plasmid:

[0277] Construction of an expression plasmid was performed in *E. coli* DH5α-T1^R (Invitrogen) and XL1-Blue MRF' Kan (Stratagene). In a first step, the amplified P_{pfor} promoter region was cloned into the *E. coli-Clostridium* shuttle vector pMTL85141 (FJ797651.1; Nigel Minton, University of Nottingham; Heap et al., 2009) using *Not*I and *Nde*I restriction sites, generating plasmid pMTL85146. As a second step, *ispS* was cloned into pMTL85146 using restriction sites *NdeI* and *EcoRI*, resulting in plasmid pMTL 85146-ispS (Fig. 2, SEQ ID NO: 25).

Transformation and expression in C. autoethanogenum

[0278] Prior to transformation, DNA was methylated *in vivo* in *E. coli* using a synthesized hybrid Type II methyltransferase (SEQ ID NO: 63) co-expressed on a methylation plasmid (SEQ ID NO: 64) designed from methyltransferase genes from *C. autoethanogenum*, *C. ragsdalei* and *C. ljungdahlii* as described in US patent 2011/0236941.

[0279] Both expression plasmid and methylation plasmid were transformed into same cells of restriction negative E. coli XL1-Blue MRF' Kan (Stratagene), 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). The resulting mixture was used for transformation experiments with *C. autoethanogenum* DSM23693, but only the abundant (high-copy) expression plasmid has a Gram-(+) replication origin (*repL*) allowing it to replicate in Clostridia.

Transformation into C. autoethanogenum:

[0280] During the complete transformation experiment, *C. autoethanogenum* DSM23693 was grown in PETC media (Table 1) supplemented with 1 g/L yeast extract and 10 g/l fructose as well as 30 psi steel mill waste gas (collected from New Zealand Steel site in Glenbrook, NZ; composition: 44% CO, 32% N_2, 22% CO_2, 2% H_2) as carbon source.

[0281] To make competent cells, a 50 ml culture of *C. autoethanogenum* DSM23693 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 0.4, 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 1 µg of the methylated plasmid mixture and immediately pulsed using the Gene pulser Xcell electroporation system (Bio-Rad) with the following settings: 2.5 kV, 600 Ω, and 25 µF. Time constants of 3.7-4.0 ms were achieved. The culture was transferred into 5 ml fresh media. Regeneration of the cells was monitored at a wavelength of 600 nm using a Spectronic Helios Epsilon Spectrophotometer (Thermo) equipped with a tube holder. After an initial drop in biomass, the cells started growing again. Once the biomass has doubled from that point, the cells were harvested, suspended in 200 µl fresh media and plated on selective PETC plates (containing 1.2 % Bacto™ Agar (BD)) with appropriate antibiotics 4 µg/ml Clarithromycin or 15 µg/ml thiamphenicol. After 4-5 days of inoculation with 30 psi steel mill gas at 37 °C, colonies were visible.

[0282] The colonies were used to inoculate 2 ml PETC media with antibiotics. When growth occurred, the culture was scaled up into a volume of 5 ml and later 50 ml with 30 psi steel mill gas as sole carbon source.

Confirmation of the successful transformation:

[0283] To verify the DNA transfer, a plasmid mini prep was performed from 10 ml culture volume using Zyppy plasmid miniprep kit (Zymo). Since the quality of the isolated plasmid was not sufficient for a restriction digest due to Clostridial exonuclease activity [Burchhardt and Dürre, 1990], a PCR was performed with the isolated plasmid with oligonucleotide pairs colE1-F (SEQ ID NO: 65: CGTCAGACCCCGTAGAAA) plus colE1-R (SEQ ID NO: 66: CTCTCCTGTTCCGACCCT). PCR was carried out using iNtRON Maximise Premix PCR kit (Intron Bio Technologies) with the following conditions: initial denaturation at 94 °C for 2 minutes, followed by 35 cycles of denaturation (94 °C for 20 seconds), annealing (55 °C for

20 seconds) and elongation (72 °C for 60 seconds), before a final extension step (72 °C for 5 minutes).

[0284] To confirm the identity of the clones, genomic DNA was isolated (see above) from 50 ml cultures of *C. autoethanogenum* DSM23693. A PCR was performed against the 16s rRNA gene using oligonucleotides fD1 (SEQ ID NO: 67: CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG) and rP2 (SEQ ID NO: 68: CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT) [Weisberg et al., 1991] and iNtRON Maximise Premix PCR kit (Intron Bio Technologies) with the following conditions: initial denaturation at 94 °C for 2 minutes, followed by 35 cycles of denaturation (94 °C for 20 seconds), annealing (55 °C for 20 seconds) and elongation (72 °C for 60 seconds), before a final extension step (72 °C for 5 minutes). Sequencing results were at least 99.9 % identity against the 16s rRNA gene (*rrsA*) of *C. autoethanogenum* (Y18178, GI:7271109).

Expression of isoprene synthase gene

[0285] qRT-PCR experiments were performed to confirm successful expression of introduced isoprene synthase gene in *C. autoethanogenum*.

[0286] A culture harboring isoprene synthase plasmid pMTL 85146-ispS and a control culture without plasmid was grown in 50 mL serum bottles and PETC media (Table 1) with 30 psi steel mill waste gas (collected from New Zealand Steel site in Glenbrook, NZ; composition: 44% CO, 32% N_2, 22% CO_2, 2% H_2) as sole energy and carbon source. 0.8 mL samples were taken during logarithmic growth phase at an OD_{600nm} of around 0.5 and mixed with 1.6 mL RNA protect reagent (Qiagen). The mixture was centrifuged (6,000 x g, 5 min, 4 °C), and the cell sediment snap frozen in liquid nitrogen and stored at -80 °C until RNA extraction. Total RNA was isolated using RNeasy Mini Kit (Qiagen) according to protocol 5 of the manual. Disruption of the cells was carried out by passing the mixture through a syringe 10 times, and eluted in 50 µL of RNase/DNase-free water. After DNase I treatment using DNA-free™ Kit (Ambion), the reverse transcription step was then carried out using SuperScript III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). RNA was checked using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and by gel electrophoresis. A non-RT control was performed for every oligonucleotide pair. All qRT-PCR reactions were

performed in duplicate using a MyiQ™ Single Colour Detection System (Bio-Rad Labratories, Carlsbad, CA, USA) in a total reaction volume of 15 µL with 25 ng of cDNA template, 67 nM of each oligonucleotide (Table 2), and 1x iQ™ SYBR® Green Supermix (Bio-Rad Labratories, Carlsbad, CA, USA). The reaction conditions were 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s and 72 °C for 30 s. For detection of oligonucleotide dimerisation or other artifacts of amplification, a melting-curve analysis was performed immediately after completion of the qPCR (38 cycles of 58 °C to 95 °C at 1 °C/s). Two housekeeping genes (guanylate kinase and formate tetrahydrofolate ligase) were included for each cDNA sample for normalization. Determination of relative gene expression was conducted using Relative Expression Software Tool (REST©) 2008 V2.0.7 (38). Dilution series of cDNA spanning 4 log units were used to generate standard curves and the resulting amplification efficiencies to calculate concentration of mRNA.

Table 2: Oligonucleotides for qRT-PCR

Target	**Oligonucleotide Name**	**DNA Sequence (5` to 3`)**	**SEQ ID NO:**
Guanylate kinase (*gnk*)	GnK-F	TCAGGACCTTCTGGAACTGG	108
	GnK-R	ACCTCCCCTTTTCTTGGAGA	109
Formate tetrahydrofolate ligase (FoT4L)	FoT4L-F	CAGGTTTCGGTGCTGACCTA	110
	FoT4L-F	AACTCCGCCGTTGTATTTCA	111
Isoprene Synthase	ispS-F	AGG CTG AAT TTC TTA CAC TTC TTG A	69
	ispS-R	GTA ACT CCA TCA AAT CCT CCA CTA C	70

[0287] While no amplification was observed with the wild-type strain using oligonucleotide pair ispS, a signal with the ispS oligonucleotide pair was measured for the strain carrying plasmid pMTL 85146-ispS, confirming successful expression of the ispS gene.

Example 2 - Expression of Isopentenyl-diphosphate delta-isomerase to convert between key terpene precursors DMAPP (Dimethylallyl diphosphate) and IPP (Isopentenyl diphosphate)

[0288] Availability and balance of precursors DMAPP (Dimethylallyl diphosphate) and IPP (Isopentenyl diphosphate) is crucial for production of terpenes. While the DXS pathway synthesizes both IPP and DMAPP equally, in the mevalonate pathway the only product is IPP. Production of isoprene requires only the precursor DMAPP to be present in conjunction with an isoprene synthase, while for production of higher terpenes and terpenoids, it is required to have equal amounts of IPP and DMAPP available to produce Geranyl-PP by a geranyltransferase.

Construction of isopentenyl-diphosphate delta-isomerase expression plasmid:

[0289] An Isopentenyl-diphosphate delta-isomerase gene *idi* from *C. beijerinckii* (Gene ID:5294264), encoding an Isopentenyl-diphosphate delta-isomerase (YP_001310174.1), was cloned downstream of *ispS*. The gene was amplified using oligonucleotide Idi-Cbei-SacI-F (SEQ ID NO: 26: GTGAGCTCGAAAGGGGAAATTAAATG) and Idi-Cbei-KpnI-R (SEQ ID NO: 27: ATGGTACCCCAAATCTTTATTTAGACG) from genomic DNA of *C. beijerinckii* NCIMB8052, obtained using the same method as described above for *C. autoethanogenum*. The PCR product was cloned into vector pMTL 85146-ispS using *SacI* and *KpnI* restriction sites to yield plasmid pMTL85146-ispS-idi (SEQ ID NO: 28). The antibiotic resistance marker was exchanged from *catP* to *ermB* (released from vector pMTL82254 (FJ797646.1; Nigel Minton, University of Nottingham; Heap et al., 2009) using restriction enzymes *Pme*I and *Fse*I to form plasmid pMTL85246-ispS-idi (Fig. 3).

[0290] Transformation and expression in *C. autoethanogenum* was carried out as decribed for plasmid pMTL 85146-ispS. After successful transformation, growth experiment was carried out in 50 mL 50 mL serum bottles and PETC media (Table 1) with 30 psi steel mill waste gas (collected from New Zealand Steel site in Glenbrook, NZ; composition: 44% CO, 32% N_2, 22% CO_2, 2% H_2) as sole energy and carbon source. To confirm that the plasmid has been successfully introduced, plasmid mini prep DNA was carried out from transformants as described previously. PCR against the isolated plasmid using oligonucleotide pairs that target colE1 (colE1-F: SEQ ID NO: 65: CGTCAGACCCCGTAGAAA and colE1-R: SEQ ID NO: 66: CTCTCCTGTTCCGACCCT), ermB (ermB-F: SEQ ID NO: 106:

TTTGTAATTAAGAAGGAG and ermB-R: SEQ ID NO: 107: GTAGAATCCTTCTTCAAC) and idi (Idi-Cbei-SacI-F: SEQ ID NO: 26: GTGAGCTCGAAAGGGGAAATTAAATG and Idi-Cbei-KpnI-R: SEQ ID NO: 27: ATGGTACCCCAAATCTTTATTTAGACG) confirmed transformation success (Figure 8). Similarly, genomic DNA from these transformants were extracted, and the resulting 16s rRNA amplicon using oligonucleotides fD1 and rP2 (see above) confirmed 99.9 % identity against the 16S rRNA gene of *C. autoethanogenum* (Y18178, GI:7271109).

[0291] Successful confirmation of gene expression was carried out as described above using a oligonucleotide pair against Isopentenyl-diphosphate delta-isomerase gene *idi* (idi-F, SEQ ID NO: 71: ATA CGT GCT GTA GTC ATC CAA GAT A and idiR, SEQ ID NO: 72: TCT TCA AGT TCA CAT GTA AAA CCC A) and a sample from a serum bottle growth experiment with C. autoethanogenum carrying plasmid pMTL 85146-ispS-idi. A signal for the isoprene synthase gene ispS was also observed (Fig. 14).

Example 3 - Overexpression of DXS pathway

[0292] To improve flow through the DXS pathway, genes of the pathway were overexpressed. The initial step of the pathway, converting pyruvate and D-glyceraldehyde-3-phosphate (G3P) into deoxyxylulose 5-phosphate (DXP/DXPS/DOXP), is catalyzed by an deoxyxylulose 5-phosphate synthase (DXS).

Construction of DXS overexpression expression plasmid:

[0293] The *dxs* gene of *C. autoethanogenum* was amplified from genomic DNA with oligonucleotides Dxs-SalI-F (SEQ ID NO: 29: GCAGTCGACTTTATTAAAGGGATAGATAA) and Dxs-XhoI-R (SEQ ID NO: 30: TGCTCGAGTTAAAATATATGACTTACCTCTG) as described for other genes above. The amplified gene was then cloned into plasmid pMTL85246-ispS-idi with *SalI* and *XhoI* to produce plasmid pMTL85246-ispS-idi-dxs (SEQ ID NO: 31 and Fig. 4). DNA sequencing using oligonucleotides given in Table 3 confirmed successful cloning of *ispS*, *idi*, and *dxs* without mutations (Fig. 5). The *ispS* and *idi* genes are as described in example 1 and 2 respectively.

Table 3: Oligonucleotides for sequencing

Oligonucleotide Name	DNA Sequence (5' to 3')	SEQ ID NO:
M13R	CAGGAAACAGCTATGAC	32
Isoprene-seq1	GTTATTCAAGCTACACCTTT	33
Isoprene-seq2	GATTGGTAAAGAATTAGCTG	34
Isoprene-seq3	TCAAGAAGCTAAGTGGCT	35
Isoprene-seq4	CTCACCGTAAAGGAACA	36
Isoprene-seq5	GCTAGCTAGAGAAATTAGAA	37
Isoprene-seq6	GGAATGGCAAAATATCTTGA	38
Isoprene-seq7	GAAACACATCAGGGAATATT	39

Transformation and expression in C. autoethanogenum

[0294] Transformation and expression in *C. autoethanogenum* was carried out as decribed for plasmid pMTL 85146-ispS. After successful transformation, a growth experiment was carried out in 50 mL 50 mL serum bottles and PETC media (Table 1) with 30 psi steel mill waste gas (collected from New Zealand Steel site in Glenbrook, NZ; composition: 44% CO, 32% N_2, 22% CO_2, 2% H_2) as sole energy and carbon source. Confirmation of gene expression was carried out as described above from a sample collected at OD_{600nm} = 0.75. Oligonucleotide pair dxs-F (SEQ ID NO: 73: ACAAAGTATCTAAGACAGGAGGTCA) and dxs-R (SEQ ID NO: 74: GATGTCCCACATCCCATATAAGTTT) was used to measure expression of gene *dxs* in both wild-type strain and strain carrying plasmid pMTL 85146-ispS-idi-dxs. mRNA levels in the strain carrying the plasmid were found to be over 3 times increased compared to the wild-type (Fig. 15). Biomass was normalized before RNA extraction.

Example 4 - Introduction and Expression of Mevalonate pathway

[0295] The first step of the mevalonate pathway (Fig. 7) is catalyzed by a thiolase that converts two molecules of acetyl-CoA into acetoacetyl-CoA (and HS-CoA). This enzyme has been successfully expressed in carboxydotrophic acetogens *Clostridium autoethanogenum* and *C. ljungdahlii* by the same inventors (US patent 2011/0236941). Constructs for the remaining genes of the mevalonate pathway have been designed.

Construction of mevalonate expression plasmid:

[0296] 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 three genes required for mevalonate synthesis via the upper part of the mavalonate pathway, i.e., thiolase (thlA/vraB), HMG-CoA synthase (HMGS) and HMG-CoA reductase (HMGR), were codon-optimised as an operon (P_{ptaack}-thlA/vraB-HMGS-P_{atp}-HMGR).

[0297] The Phosphotransacetylase/Acetate kinase operon promoter ($P_{pta\text{-}ack}$) of *C. autoethanogenum* (SEQ ID NO: 61) was used for expression of the thiolase and HMG-CoA synthase while a promoter region of the ATP synthase (P_{atp}) of *C. autoethanogenum* was used for expression of the HMG-CoA reductase. Two variants of thiolase, *thlA* from *Clostridium acetobutylicum* and *vraB* from *Staphylococcus aureus*, were synthesised and flanked by *Nde*I and *EcoR*I restriction sites for further sub-cloning. Both HMG-CoA synthase (HMGS) and HMG-CoA reductase (HMGR) were synthesised from *Staphylococcus aureus* and flanked by *EcoRI-SacI* and *KpnI-XbaI* restriction sites respectively for further sub-cloning. All optimized DNA sequences used are given in Table 4.

Table 4: Sequences of mevalonate expression plasmid

Description	**Source**	**SEQ ID NO:**
Thiolase (*thlA*)	*Clostridium acetobutylicum* ATCC 824; NC_003030.1; GI: 1119056	40
Acetyl-CoA c-acetyltransferase (vraB)	*Staphylococcus aureus* subsp. *aureus* Mu50; NC_002758.2; region: 652965..654104; including GI: 15923566	41
3-hydroxy-3-methylglutaryl-CoA synthase (HMGS)	*Staphylococcus aureus* subsp. *aureus* Mu50; NC_002758.2; region: 2689180..2690346; including GI: 15925536	42
Hydroxymethylglutaryl-CoA	*Staphylococcus aureus* subsp. *aureus*	43

reductase (HMGR)	Mu50; NC_002758.2; region: complement(2687648..2688925); including GI: 15925535	
Phosphotransacetylase-acetate kinase operon ($P_{pta\text{-}ack}$)	*Clostridium autoethanogenum* DSM10061	44
ATP synthase promoter (P_{atp})	*Clostridium autoethanogenum* DSM10061	45

[0298] The ATP synthase promoter (P_{atp}) together with the hydroxymethylglutaryl-CoA reductase (HMGR) was amplified using oligonucleotides pUC57-F (SEQ ID NO: 46: AGCAGATTGTACTGAGAGTGC) and pUC57-R (SEQ ID NO: 47: ACAGCTATGACCATGATTACG) and pUC57- Patp-HMGR as a template. The 2033 bp amplified fragment was digested with SacI and XbaI and ligated into the *E.* coli-*Clostridium* shuttle vector pMTL 82151 (FJ7976; Nigel Minton, University of Nottingham, UK; Heap et al., 2009, *J Microbiol Methods.* 78: 79-85) resulting in plasmid pMTL 82151-Patp-HMGR (SEQ ID NO: 76).

[0299] 3-hydroxy-3-methylglutaryl-CoA synthase (HMGS) was amplified from the codon-synthesised plasmid pGH-seq3.2 using oligonucleotides EcoRI-HMGS_F (SEQ ID NO: 77: AGCCGTGAATTCGAGGCTTTTACTAAAAACA) and EcoRI-HMGS_R (SEQ ID NO: 78: AGGCGTCTAGATGTTCGTCTCTACAAATAATT). The 1391 bp amplified fragment was digested with SacI and EcoRI and ligated into the previously created plasmid pMTL 82151-Patp-HMGR to give pMTL 82151-HMGS-Patp-HMGR (SEQ ID NO: 79). The created plasmid pMTL 82151-HMGS-Patp-HMGR (SEQ ID NO: 79) and the 1768 bp codon-optimised operon of P_{ptaack}-thlA/vraB were both cut with *Not*I and *Eco*RI. A ligation was performed and subsequently transformed into *E. coli* XL1-Blue MRF' Kan resulting in plasmid pMTL8215- P_{ptaack}-thlA/vraB-HMGS-P_{atp}-HMGR (SEQ ID NO: 50).

[0300] The five genes required for synthesis of terpenoid key intermediates from mevalonate via the bottom part of the mevalonate pathway, i.e., mevalonate kinase (MK), phosphomevalonate kinase (PMK), mevalonate diphosphate decarboxylase (PMD), isopentenyl-diphosphate delta-isomerase (idi) and isoprene synthase (ispS) were codon-optimised by ATG:Biosynthetics GmbH (Merzhausen, Germany). Mevalonate kinase (MK),

phosphomevalonate kinase (PMK) and mevalonate diphosphate decarboxylase (PMD) were obtained from *Staphylococcus aureus.*

[0301] The promoter region of the RNF Complex (P_{rnf}) of *C. autoethanogenum* (SEQ ID NO: 62) was used for expression of mevalonate kinase (MK), phosphomevalonate kinase (PMK) and mevalonate diphosphate decarboxylase (PMD), while the promoter region of the Pyruvate:ferredoxin oxidoreductase (P_{for}) of *C. autoethanogenum* (SEQ ID NO: 22) was used for expression of isopentenyl-diphosphate delta-isomerase (idi) and isoprene synthase (ispS). All DNA sequences used are given in Table 5. The codon-optimised Prnf-MK was amplified from the synthesised plasmid pGH- Prnf-MK-PMK-PMD with oligonucleotides NotI-XbaI-Prnf-MK_F (SEQ ID NO: 80: ATGCGCGGCCGCTAGGTCTAGAATATCGATACAGATAAAAAAATATATAATACA G) and SalI-Prnf-MK_R (SEQ ID NO: 81: TGGTTCTGTAACAGCGTATTCACCTGC). The amplified gene was then cloned into plasmid pMTL83145 (SEQ ID NO: 49) with NotI and SalI to produce plasmid pMTL8314-Prnf-MK (SEQ ID NO: 82). This resulting plasmid and the 2165bp codon optimised fragment PMK-PMD was subsequently digested with SalI and HindIII. A ligation was performed resulting in plasmid pMTL 8314-Prnf-MK-PMK-PMD (SEQ ID NO: 83).

[0302] The isoprene expression plasmid without the mevalonate pathway was created by ligating the isoprene synthase (ispS) flanked by restriction sites AgeI and NheI to the previously created farnesene plasmid, pMTL 8314-Prnf-MK-PMK-PMD-Pfor-idi-ispA-FS (SEQ ID NO:91) to result in plasmid pMTL8314-Prnf-MK-PMK-PMD-Pfor-idi-ispS (SEQ ID NO:84). The final isoprene expression plasmid, pMTL 8314-Pptaack-thlA-HMGS-Patp-HMGR-Prnf-MK-PMK-PMD-Pfor-idi-ispS (SEQ ID NO:. 58, figure 10) is created by ligating the 4630 bp fragment of Pptaack-thlA-HMGS-Patp-HMGR from pMTL8215-Pptaack-thlA-HMGS-Patp-HMGR (SEQ ID NO: 50) with pMTL 8314-Prnf-MK-PMK-PMD-Pfor-idi-ispS (SEQ ID NO: 84) using restriction sites NotI and XbaI.

Table 5: Sequences of isoprene expression plasmid from mevalonate pathway

Description	**Source**	**SEQ ID NO:**

Mevalonate kinase (MK)	*Staphylococcus aureus* subsp. *aureus* Mu50; NC_002758.2; region: 665080..665919; including GI:15923580	51
Phosphomevalonate kinase (PMK)	*Staphylococcus aureus* subsp. *aureus* Mu50; NC_002758.2; region: 666920..667996; including GI:15923582	52
Mevalonate diphosphate decarboxylase (PMD)	*Staphylococcus aureus* subsp. *aureus* Mu50; NC_002758.2; region: 665924..666907; including GI:15923581	53
Isoprene synthase (isIS)	isoprene synthase of *Poplar tremuloides* (AAQ16588.1; GI:33358229)	21
Isopentenyl-diphosphate delta-isomerase (idi)	*Clostridium beijerinckii* NCIMB 8052; YP_001310174.1; region: complement(3597793..3598308); including GI:150017920	54
RNF Complex promoter (P_{rnf})	*Clostridium autoethanogenum* DSM10061	55

Example 5 - Introduction of farnesene synthase in *C. autoethanogenum* for production of farnesene from CO via the mevalonate pathway

[0303] Instead of producing isoprene directly from terpenoid key intermediates IPP and DMAPP then using this to synthesise longer chain terpenes, it is also possible to synthesise longer chain terpenes, such as C10 Monoterpenoids or C15 Sesquiterpenoids, directly via a geranyltransferase (see Table 6). From C15 Sesquiterpenoid building block farnesyl-PP it is possible to produce farnesene, which, similarly to ethanol, can be used as a transportation fuel.

Construction of farnesene expression plasmid

[0304] The two genes required for farnesene synthesis from IPP and DMAPP via the mevalonate pathway, i.e., geranyltranstransferase (ispA) and alpha-farnesene synthase (FS)

were codon-optimised. Geranyltranstransferase (ispA) was obtained from *Escherichia coli str.* K-12 *substr.* MG1655 and alpha-farnesene synthase (FS) was obtained from *Malus* x *domestica.* All DNA sequences used are given in Table 6. The codon-optimised idi was amplified from the synthesised plasmid pMTL83245-Pfor-FS-idi (SEQ ID NO: 85) with via the mevalonate pathways idi_F (SEQ ID NO: 86: AGGCACTCGAGATGGCAGAGTATATAATAGCAGTAG) and idi_R2 (SEQ ID NO:87: AGGCGCAAGCTTGGCGCACCGGTTTATTTAAATATCTTATTTTCAGC). The amplified gene was then cloned into plasmid pMTL83245-Pfor with XhoI and HindIII to produce plasmid pMTL83245-Pfor-idi (SEQ ID NO: 88). This resulting plasmid and the 1754bp codon optimised fragment of farnesene synthase (FS) was subsequently digested with HindIII and NheI . A ligation was performed resulting in plasmid pMTL83245-Pfor-idi-FS (SEQ ID NO: 89). The 946bp fragment of ispA and pMTL83245-Pfor-idi-FS was subsequently digested with AgeI and HindIII and ligated to create the resulting plasmid pMTL83245-Pfor-idi-ispA-FS (SEQ ID NO: 90). The farnesene expression plasmid without the upper mevalonate pathway was created by ligating the 2516bp fragment of Pfor-idi-ispA-FS from pMTL83245-Pfor-idi-ispA-FS to pMTL 8314-Prnf-MK-PMK-PMD to result in plasmid pMTL 8314-Prnf-MK-PMK-PMD-Pfor-idi-ispA-FS (SEQ ID NO: 91). The final farnesene expression plasmid pMTL83145-thlA-HMGS-Patp-HMGR-Prnf-MK-PMK-PMD-Pfor-idi-ispA-FS (SEQ ID NO: 59 and figure 18) is created by ligating the 4630 bp fragment of Pptaack-thlA-HMGS-Patp-HMGR from pMTL8215- Pptaack-thlA-HMGS-Patp-HMGR (SEQ ID NO: 50) with pMTL 8314-Prnf-MK-PMK-PMD-Pfor-idi-ispA-FS (SEQ ID NO: 91) using restriction sites NotI and XbaI.

Table 6: Sequences of farnesene expression plasmid from mevalonate pathway

Description	**Source**	**SEQ ID NO:**
Geranyltranstransferase (ispA)	*Escherichia coli str.* K-12 *substr.* MG1655; NC_000913.2; region: complement(439426..440325); including GI:16128406	56
Alpha-farnesene synthase	*Malus* x *domestica*;	57

(FS)	AY787633.1; GI:60418690	

Transformation into C. autoethanogenum

[0305] Transformation and expression in *C. autoethanogenum* was carried out as described in example 1.

Confirmation of successful transformation

[0306] The presence of pMTL8314-Prnf-MK-PMK-PMD-Pfor-idi-ispA-FS (SEQ ID NO: 59) was confirmed by colony PCR using oligonucleotides repHF (SEQ ID NO: 92:AAGAAGGGCGTATATGAAAACTTGT) andcatR (SEQ ID NO: 93: TTCGTTTACAAAACGGCAAATGTGA)which selectively amplifies a portion of the garm +ve perplicon and most of the *cat* gene on the pMTL831xxx series palsmids. Yielding a band of 1584 bp (figure 16).

Expression of lower mevalonate pathway in C. autoethanogenum

[0307] Confirmation of expression of the lower mevalonate pathway genes Mevalonate kinase (MK SEQ ID NO: 51), Phosphomevalonate Kinase (PMK SEQ ID NO: 52), Mevalonate Diphosphate Decarboxylase (PMD SEQ ID NO: 53), Isopentyl-diphosphate Delta-isomerase (idi; SEQ ID NO: 54), Geranyltranstransferase (ispA ; SEQ ID NO: 56) and Farnesene synthase (FS SEQ ID NO: 57) was done as described above in example 1. Using oligonucleotides listed in table 7.

Table 7: List of oligonucleotides used for the detection of expression of the genes in the lower mevalonate pathway carried on plasmid pMTL8314Prnf-MK-PMK-PMD-Pfor-idi-ispA-FS (SEQ ID NO: 91)

Target	Oligonucleotide Name	DNA Sequence (5' to 3')	SEQ ID NO: NO.

Mevalonate kinase	MK-RTPCR-F	GTGCTGGTAGAGGTGGTTCA	94
	MK-RTPCR-R	CCAAGTATGTGCTGCACCAG	95
Phosphomevalonate Kinase	PMK-RTPCR-F	ATATCAGACCCACACGCAGC	96
	PMK-RTPCR-R	AATGCTTCATTGCTATGTCACATG	97
Mevalonate Diphosphate Decarboxylase	PMD-RTPCR-F	GCAGAAGCAAAGGCAGCAAT	98
	PMD-RTPCR-R	TTGATCCAAGATTTGTAGCATGC	99
Isopentyl-diphosphate Delta-isomerase	idi-RTPCR-F	GGACAAACACTTGTTGTAGTCACC	100
	idi-RTPCR-R	TCAAGTTCGCAAGTAAATCCCA	101
Geranyltranstransferase	ispA-RTPCR-F	ACCAGCAATGGATGACGATG	102
	ispA-RTPCR-R	AGTTTGTAAAGCGTCACCTGC	103
Farnesene synthase	FS-RTPCR-F	AAGCTAGTAGATGGTGGGCT	104
	FS-RTPCR-R	AATGCTACACCTACTGCGCA	105

[0308] Rt-PCR data confirming expression of all genes in the lower mevalonate pathway is shown in figure 18, this data is also summarised in Table 8.

Table 8: Avarage CT values for the genes genes Mevalonate kinase (MK SEQ ID NO: 51), Phosphomevalonate Kinase (PMK SEQ ID NO: 52), Mevalonate Diphosphate Decarboxylase (PMD SEQ ID NO: 53), Isopentyl-diphosphate Delta-isomerase (idi SEQ ID NO: 54), Geranyltranstransferase (ispA SEQ ID NO: 56) and Farnesene synthase (FS SEQ ID NO: 57). for two independent samples taken from the two starter cultures for the mevalonate feeding experiment (see below).

Gene	Sample 1 (Ct Mean)	Sample 2 (Ct Mean)
MK	21.9	20.82
PMK	23.64	22.81

PMD	24	22.83
Idi	24.23	27.54
ispA	23.92	23.22
FS	21.28 (single Ct)	21.95 (single Ct)
HK (rho)	31.5	28.88

Production of alpha-farnesene from mevalonate

[0309] After conformation of successfully transformed of the plasmid pMTL8314-Prnf-MK-PMK-PMD-Pfor-idi-ispA-FS, a growth experiment was carried out in 50ml PETC media (Table 1) in 250ml serum bottles with 30psi Real Mill Gas (collected from New Zealand Steel site in Glenbrook, NZ; composition: 44% CO, 32% N_2, 22% CO_2, 2% H_2) as sole energy and carbon source. All cultures were incubated at 37°C on an orbital shaker adapted to hold serum bottles. Transformants were first grown up to an OD600 of ~0.4 before being subcultured into fresh media supplemented with 1mM mevalonic acid. Controls without mevalonic acid were set up at the same time from the same culture. Samples for GC-MS (Gas Chromatography – Mass Spectroscopy) were taken at each time point. Figure 17 shows a representative growth curve for 2 control cultures and two cultures fed 1mM mevalonate. Farnesene was detected in the samples taken at 66 h and 90 h after start of experiment (figure 19-21).

Detection of alpha-farnesene by Gas Chromatography – Mass Spetctroscopy

[0310] For GC-MS detection of alpha-farnesene hexane extraction was performed on 5 ml of culture by adding 2 ml hexane and shaking vigorously to mix in a sealed glass balch tube. The tubes were then incubated in a sonicating water bath for 5 min to encourage phase separation. 400µl hexane extract were transferred to a GC vail and loaded on to the auto loader. The samples was analysed on a VARIAN GC3800 MS4000 iontrap GC/MS (Varian Inc, CA, USA. Now Agilent Technologies) with a EC-1000 column 0.25 µm film thickness (Grace Davidson, OR, USA) Varian MS workstation (Varian Inc, Ca. Now Agilent Technologies, CA, USA) and and NIST MS Search 2.0 (Agilent Technologies, CA, USA). Injection volume of 1µl with Helium carrier gas flow rate of 1 ml per min.

[0311] 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.

[0312] 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.

[0313] 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. Isolated, genetically engineered, carboxydotrophic, acetogenic bacteria which comprise an exogenous nucleic acid encoding an enzyme in a mevalonate pathway or in a DXS pathway or in a terpene biosynthesis pathway, whereby the bacteria express the enzyme, said enzyme selected from the group consisting of:
    a) thiolase (EC 2.3.1.9);
    b) HMG-CoA synthase (EC 2.3.3.10);
    c) HMG-CoA reductase (EC 1.1.1.88);
    d) Mevalonate kinase (EC 2.7.1.36);
    e) Phosphomevalonate kinase (EC 2.7.4.2);
    f) Mevalonate Diphosphate decarboxylase (EC 4.1.1.33); 1-deoxy-D-xylulose-5-phosphate synthase DXS (EC:2.2.1.7);
    g) 1-deoxy-D-xylulose 5-phosphate reductoisomerase DXR (EC:1.1.1.267);
    h) 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase IspD (EC:2.7.7.60);
    i) 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase IspE (EC:2.7.1.148);
    j) 2-C-methyl-D-erythritol 2;4-cyclodiphosphate synthase IspF (EC:4.6.1.12);
    k) 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase IspG (EC:1.17.7.1);
    l) 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (EC:1.17.1.2); geranyltranstransferase Fps (EC:2.5.1.10);
    m) heptaprenyl diphosphate synthase (EC:2.5.1.10);
    n) octaprenyl-diphosphate synthase (EC:2.5.1.90);
    o) isoprene synthase (EC 4.2.3.27);
    p) isopentenyl-diphosphate delta-isomerase (EC 5.3.3.2); and
    q) farnesene synthase (EC 4.2.3.46 / EC 4.2.3.47).
2. The bacteria of claim 1 wherein in the absence of said nucleic acid, the bacteria do not express the enzyme.
3. The bacteria of claim 1 which express the enzyme under anaerobic conditions.
4. A plasmid which can replicate in a carboxydotrophic, acetogenic bacteria which comprises a nucleic acid encoding an enzyme in a mevalonate pathway or in a DXS pathway or in a terpene biosynthesis pathway, whereby when the plasmid is in said bacteria the enzyme is expressed by said bacteria, said enzyme selected from the group consisting of:

thiolase (EC 2.3.1.9);

a) HMG-CoA synthase (EC 2.3.3.10);
b) HMG-CoA reductase (EC 1.1.1.88);
c) Mevalonate kinase (EC 2.7.1.36);
d) Phosphomevalonate kinase (EC 2.7.4.2);
e) Mevalonate Diphosphate decarboxylase (EC 4.1.1.33); 1-deoxy-D-xylulose-5-phosphate synthase DXS (EC:2.2.1.7);
f) 1-deoxy-D-xylulose 5-phosphate reductoisomerase DXR (EC:1.1.1.267);
g) 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase IspD (EC:2.7.7.60);
h) 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase IspE (EC:2.7.1.148);
i) 2-C-methyl-D-erythritol 2;4-cyclodiphosphate synthase IspF (EC:4.6.1.12);
j) 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase IspG (EC:1.17.7.1);
k) 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (EC:1.17.1.2); geranyltranstransferase Fps (EC:2.5.1.10);
l) heptaprenyl diphosphate synthase (EC:2.5.1.10);
m) octaprenyl-diphosphate synthase (EC:2.5.1.90);
n) isoprene synthase (EC 4.2.3.27);
o) isopentenyl-diphosphate delta-isomerase (EC 5.3.3.2); and
p) farnesene synthase (EC 4.2.3.46 / EC 4.2.3.47).

5. A process for converting CO and/or CO_2 into isoprene, the process comprising:
passing a gaseous CO-containing and/or CO_2-containing substrate to a bioreactor containing a culture of carboxydotrophic, acetogenic bacteria in a culture medium such that the bacteria convert the CO and/or CO_2 to isoprene, and
recovering the isoprene from the bioreactor,
wherein the carboxydotrophic acetogenic bacteria are genetically engineered to express a isoprene synthase.
6. Isolated, genetically engineered, carboxydotrophic, acetogenic bacteria which comprise a nucleic acid encoding an isoprene synthase, whereby the bacteria express the isoprene synthase and the bacteria are able to convert dimethylallyldiphosphate to isoprene.
7. The isolated, genetically engineered, carboxydotrophic, acetogenic bacteria of claim 6 wherein the isoprene synthase is a *Populus tremuloides* enzyme.
8. The isolated, genetically engineered, carboxydotrophic, acetogenic bacteria of claim 6 wherein the nucleic acid is codon optimized.

9. The isolated, genetically engineered, carboxydrotrophic, acetogenic bacteria of claim 6 wherein expression of the isoprene synthase is under the transcriptional control of a promoter for a pyruvate: ferredoxin oxidoreductase gene from *Clostridium autoethanogenum.*
10. A process for converting CO and/or CO_2 into isopentyldiphosphate (IPP), the process comprising:

passing a gaseous CO-containing and/or CO_2-containing substrate to a bioreactor containing a culture of carboxydotrophic, acetogenic bacteria in a culture medium such that the bacteria convert the CO and/or CO_2 to isopentyldiphosphate (IPP), and

recovering the IPP from the bioreactor,

wherein the carboxydotrophic acetogenic bacteria are genetically engineered to express a isopentyldiphosphate delta isomerase.

11. Isolated, genetically engineered, carboxydotrophic, acetogenic bacteria which comprise a nucleic acid encoding an isopentyldiphosphate delta isomerase, whereby the bacteria express the isopentyldiphosphate delta isomerase and the bacteria are able to convert dimethylallyldiphosphate to isopentyldiphosphate.
12. The isolated, genetically engineered, carboxydotrophic, acetogenic bacteria of claim 11 wherein the nucleic acid encodes a *Clostridium beijerinckii* isopentyldiphosphate delta isomerase.
13. The isolated, genetically engineered, carboxydotrophic, acetogenic bacteria of claim 11 wherein the nucleic acid is under the transcriptional control of a promoter for a pyruvate: ferredoxin oxidoreductase gene from *Clostridium autoethanogenum.*
14. The isolated, genetically engineered, carboxydotrophic, acetogenic bacteria of claim 11 wherein the nucleic acid is under the transcriptional control of a promoter for a pyruvate: ferredoxin oxidoreductase gene from *Clostridium autoethanogenum* and downstream of a second nucleic acid encoding an isoprene synthase .
15. A process for converting CO and/or CO_2 into isopentyldiphosphate (IPP) and/or isoprene, the process comprising:

passing a gaseous CO-containing and/or CO_2-containing substrate to a bioreactor containing a culture of carboxydotrophic, acetogenic bacteria in a culture medium such that the bacteria convert the CO and/or CO_2 to isopentyldiphosphate (IPP) and/or isoprene, and

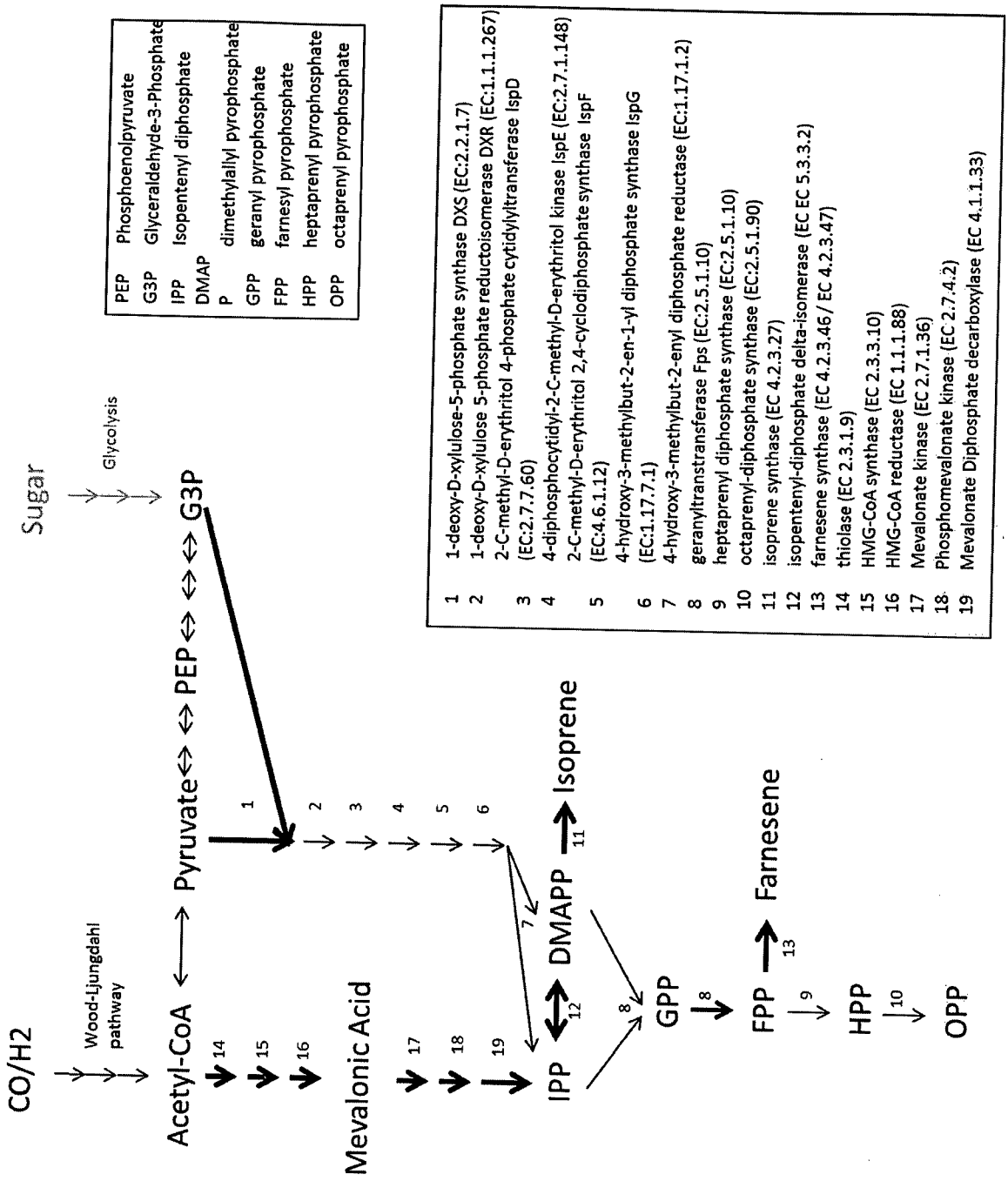
recovering the IPP and/or isoprene from the bioreactor,

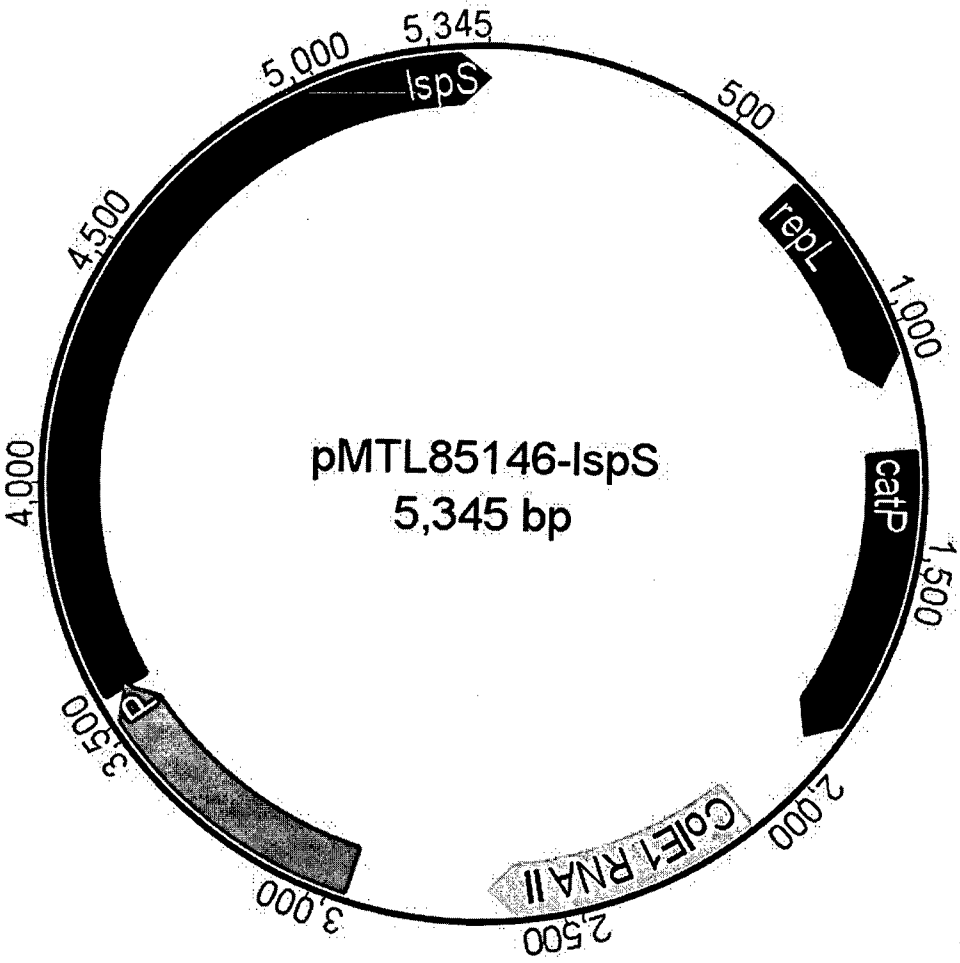
wherein the carboxydotrophic acetogenic bacteria are genetically engineered to have an increased copy number of a nucleic acid encoding a deoxyxylulose 5-phosphate synthase (DXS) enzyme, wherein the increased copy number is greater than 1 per genome.

16. Isolated, genetically engineered, carboxydotrophic, acetogenic bacteria which comprise a copy number of greater than 1 per genome of a nucleic acid encoding a deoxyxylulose 5-phosphate synthase (DXS) enzyme.
17. The isolated, genetically engineered, carboxydotrophic, acetogenic bacteria of claim 16 further comprising a nucleic acid encoding an isoprene synthase.
18. The isolated, genetically engineered, carboxydotrophic, acetogenic bacteria of claim 16 further comprising a nucleic acid encoding an isopentyldiphosphate delta isomerase.
19. The isolated, genetically engineered, carboxydotrophic, acetogenic bacteria of claim 16 further comprising a nucleic acid encoding an isopentyldiphosphate delta isomerase and a nucleic acid encoding an isoprene synthase.
20. Isolated, genetically engineered, carboxydotrophic, acetogenic bacteria which comprise a nucleic acid encoding a phosphomevalonate kinase (PMK), whereby the bacteria express the encoded enzyme, wherein the enzyme is not native to the bacteria.
21. The isolated, genetically engineered, carboxydotrophic, acetogenic bacteria of claim 20 wherein the enzymes are *Staphylococcus aureus* enzymes.
22. The isolated, genetically engineered, carboxydotrophic, acetogenic bacteria of claim 20 wherein the enzyme is expressed under the control of one or more *C. autoethanogenum* promoters.
23. The isolated, genetically engineered, carboxydotrophic, acetogenic bacteria of claim 20 further comprising a nucleic acid encoding thiolase (thlA/vraB), a nucleic acid encoding a HMG-CoA synthase (HMGS), and a nucleic acid encoding an HMG-CoA reductase (HMGR).
24. The isolated, genetically engineered, carboxydotrophic, acetogenic bacteria of claim 23 wherein the thiolase is Clostridium acetobutylicum thiolase.
25. The isolated, genetically engineered, carboxydotrophic, acetogenic bacteria of claim 20 further comprising a nucleic acid encoding a mevalonate disphosphate decarboxylase (PMD).
26. Isolated, genetically engineered, carboxydotrophic, acetogenic bacteria which comprise an exogenous nucleic acid encoding alpha-farnesene synthase.

27. The isolated, genetically engineered, carboxydotrophic, acetogenic bacteria of claim 26 wherein the nucleic acid is codon optimized for expression in *C. autoethanogenum*..
28. The bacteria of claim 26 wherein the alpha-farnesene synthase is a *Malus* x *domestica* alpha-farnesene synthase.
29. The isolated, genetically engineered, carboxydotrophic, acetogenic bacteria of claim 26 further comprising a nucleic acid segment encoding geranyltranstransferase.
30. The isolated, genetically engineered, carboxydotrophic, acetogenic bacteria of claim 29 wherein the gernayltranstransferase is an *E. coli* geranyltranstransferase.

The isolated, genetically engineered, carboxydotrophic, acetogenic bacteria of claim 1, 6, 11, 20, or 26 which is 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*


CO/H2
Wood-Ljungdahl pathway
Sugar
Glycolysis
Acetyl-CoA
Pyruvate
PEP
G3P
Mevalonic Acid
IPP
DMAPP
Isoprene
GPP
FPP
Farnesene
HPP
OPP
PEP Phosphoenolpyruvate
G3P Glyceraldehyde-3-Phosphate
IPP Isopentenyl diphosphate
DMAP dimethylallyl pyrophosphate
GPP geranyl pyrophosphate
FPP farnesyl pyrophosphate
HPP heptaprenyl pyrophosphate
OPP octaprenyl pyrophosphate
1 1-deoxy-D-xylulose-5-phosphate synthase DXS (EC:2.2.1.7)
2 1-deoxy-D-xylulose 5-phosphate reductoisomerase DXR (EC:1.1.1.267)
2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase IspD
3 (EC:2.7.7.60)
4 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase IspE (EC:2.7.1.148)
2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase IspF
5 (EC:4.6.1.12)
4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase IspG
6 (EC:1.17.7.1)
7 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (EC:1.17.1.2)
8 geranyltranstransferase Fps (EC:2.5.1.10)
9 heptaprenyl diphosphate synthase (EC:2.5.1.10)
10 octaprenyl-diphosphate synthase (EC:2.5.1.90)
11 isoprene synthase (EC 4.2.3.27)
12 isopentenyl-diphosphate delta-isomerase (EC EC 5.3.3.2)
13 farnesene synthase (EC 4.2.3.46/ EC 4.2.3.47)
14 thiolase (EC 2.3.1.9)
15 HMG-CoA synthase (EC 2.3.3.10)
16 HMG-CoA reductase (EC 1.1.1.88)
17 Mevalonate kinase (EC 2.7.1.36)
18 Phosphomevalonate kinase (EC 2.7.4.2)
19 Mevalonate Diphosphate decarboxylase (EC 4.1.1.33)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lspS
repL
catP
ColE1 RNA II
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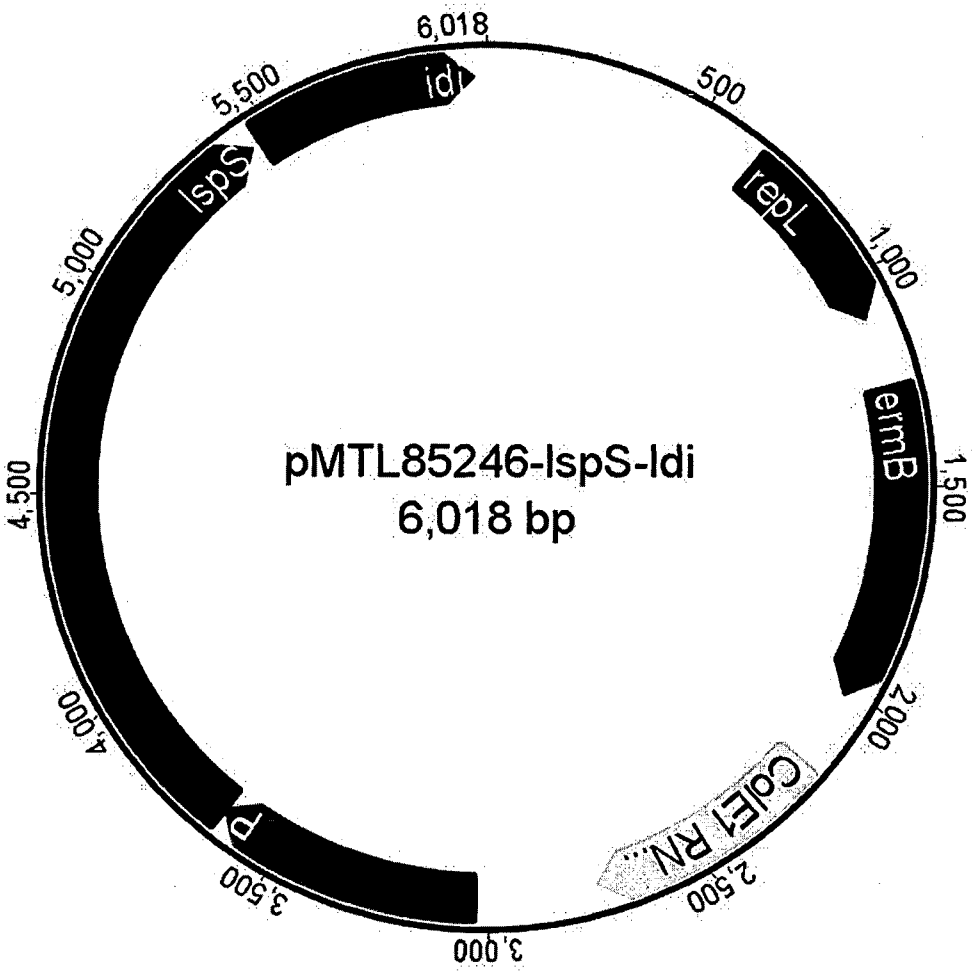
4,000
4,500
5,000

FIG. 2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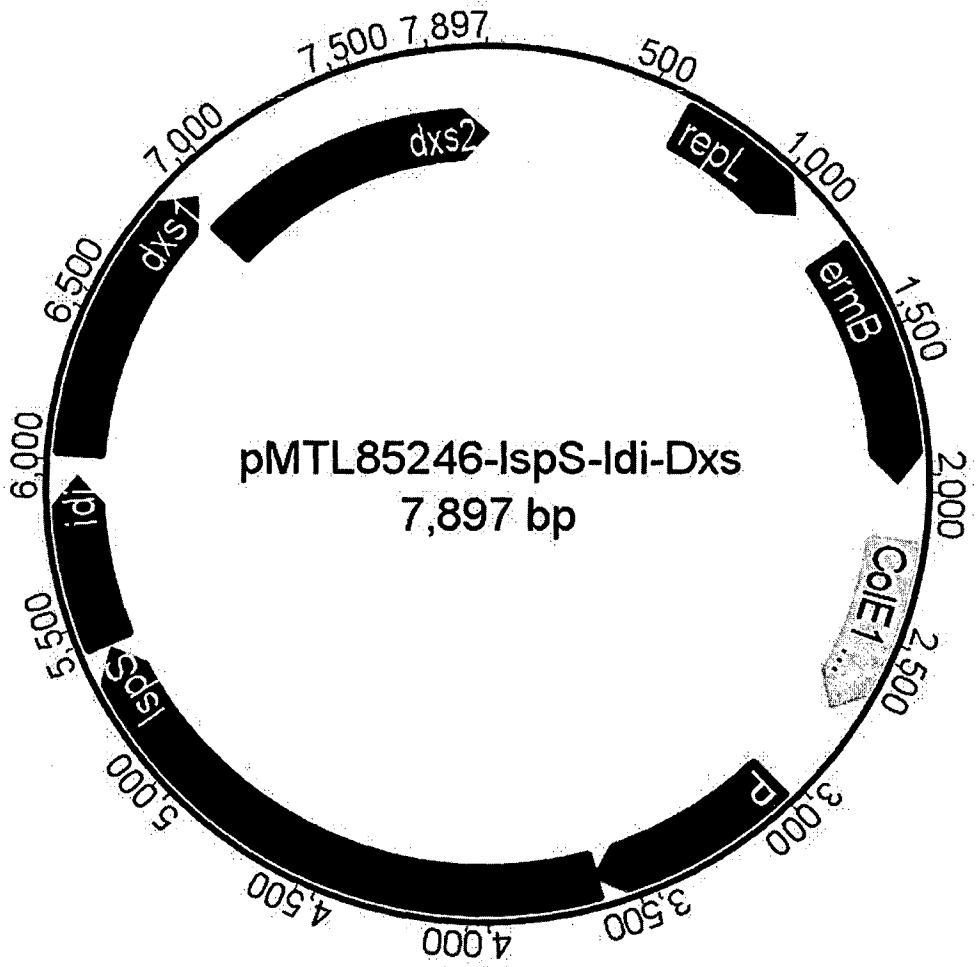
ermB
ColE1 RN...
P
lspS

FIG. 3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dxs2
repL
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ColE1
P
IspS
idi
dxs1
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2,000
2,500
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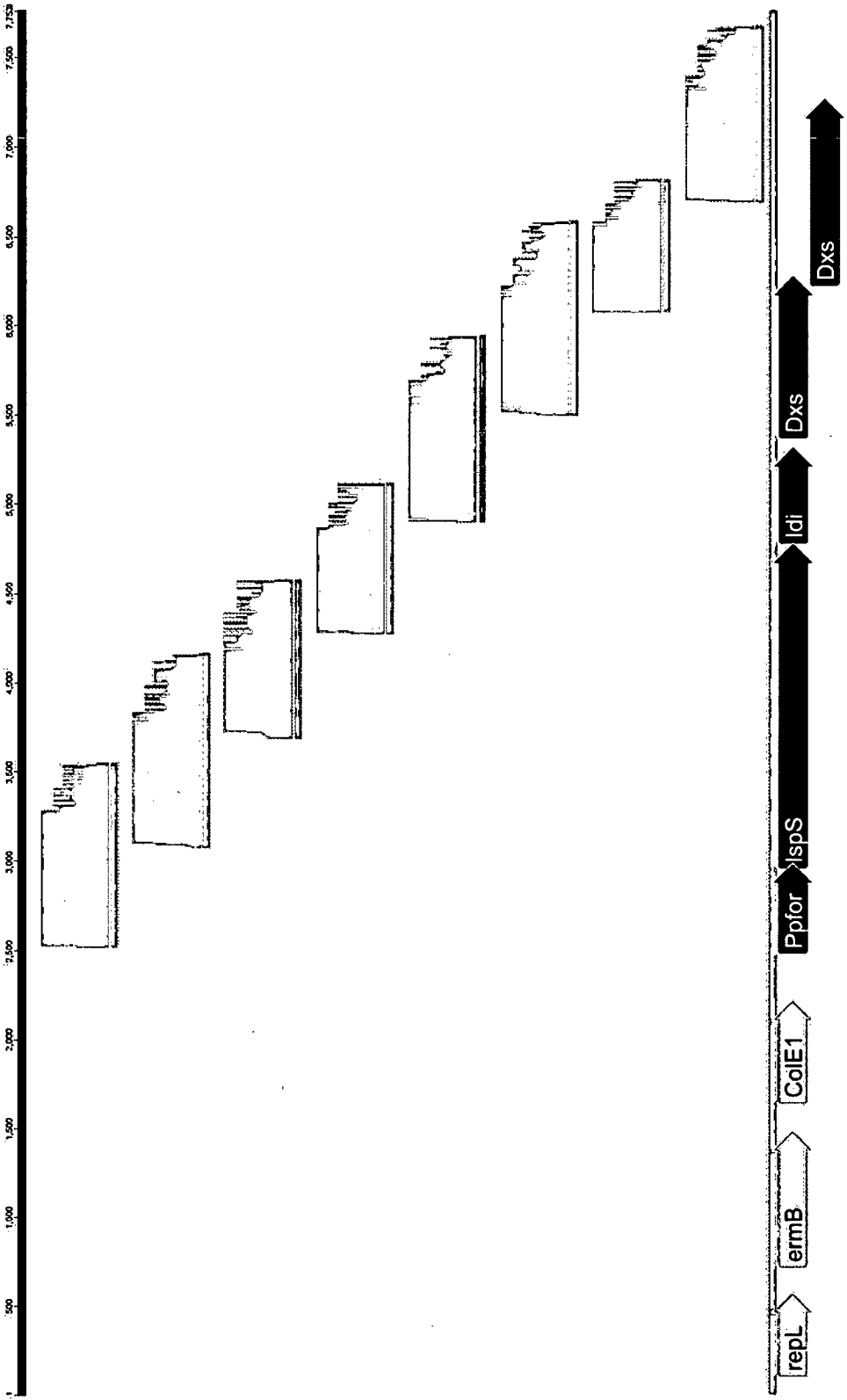
6,500
7,000
7,500
7,897

FIG. 4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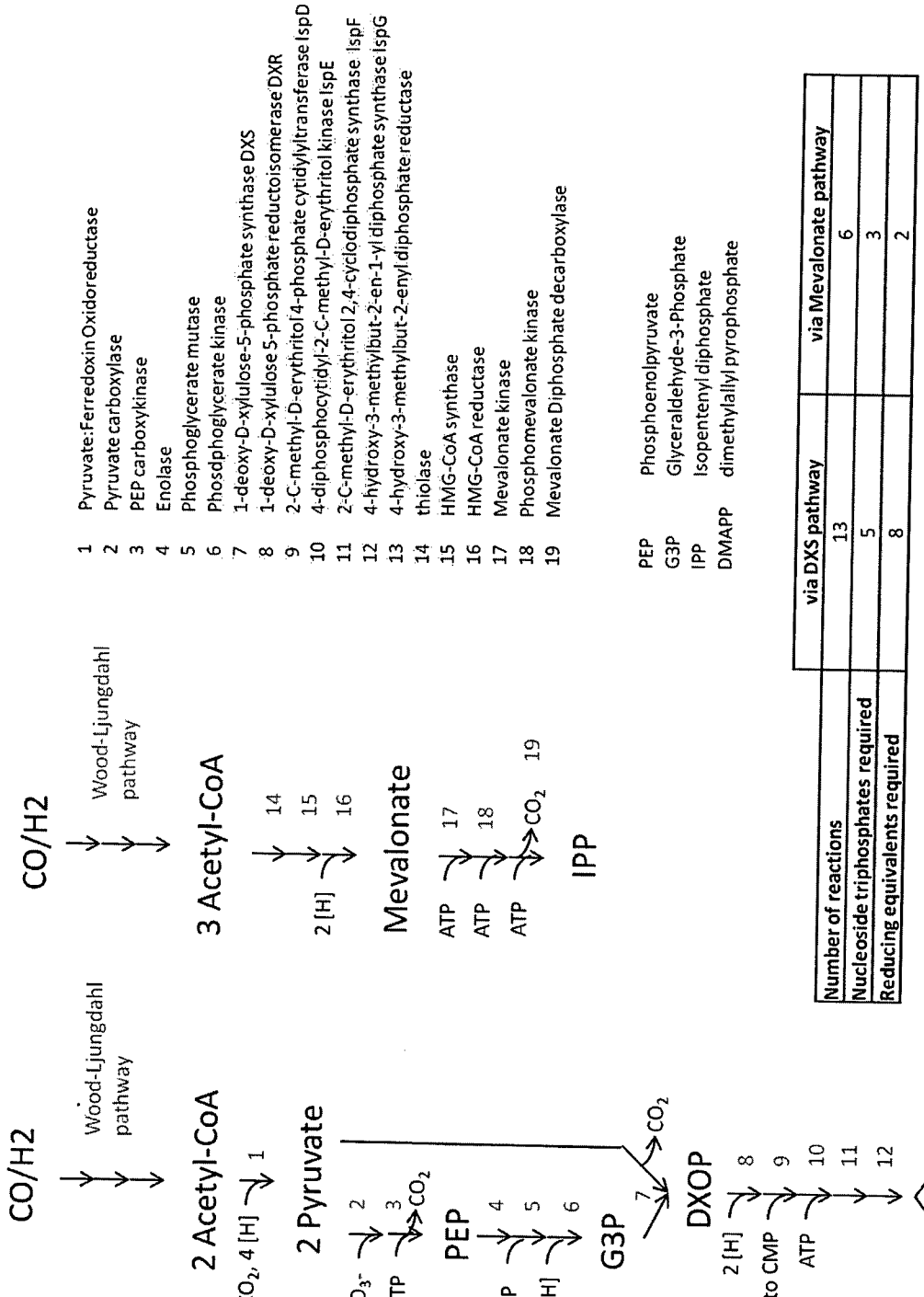
ermB
ColE1
Ppfor
IspS
Idi
Dxs
Dxs



	via DXS pathway	via Mevalonate pathway
Number of reactions	13	6
Nucleoside triphosphates required	5	3
Reducing equivalents required	8	2

FIG. 6

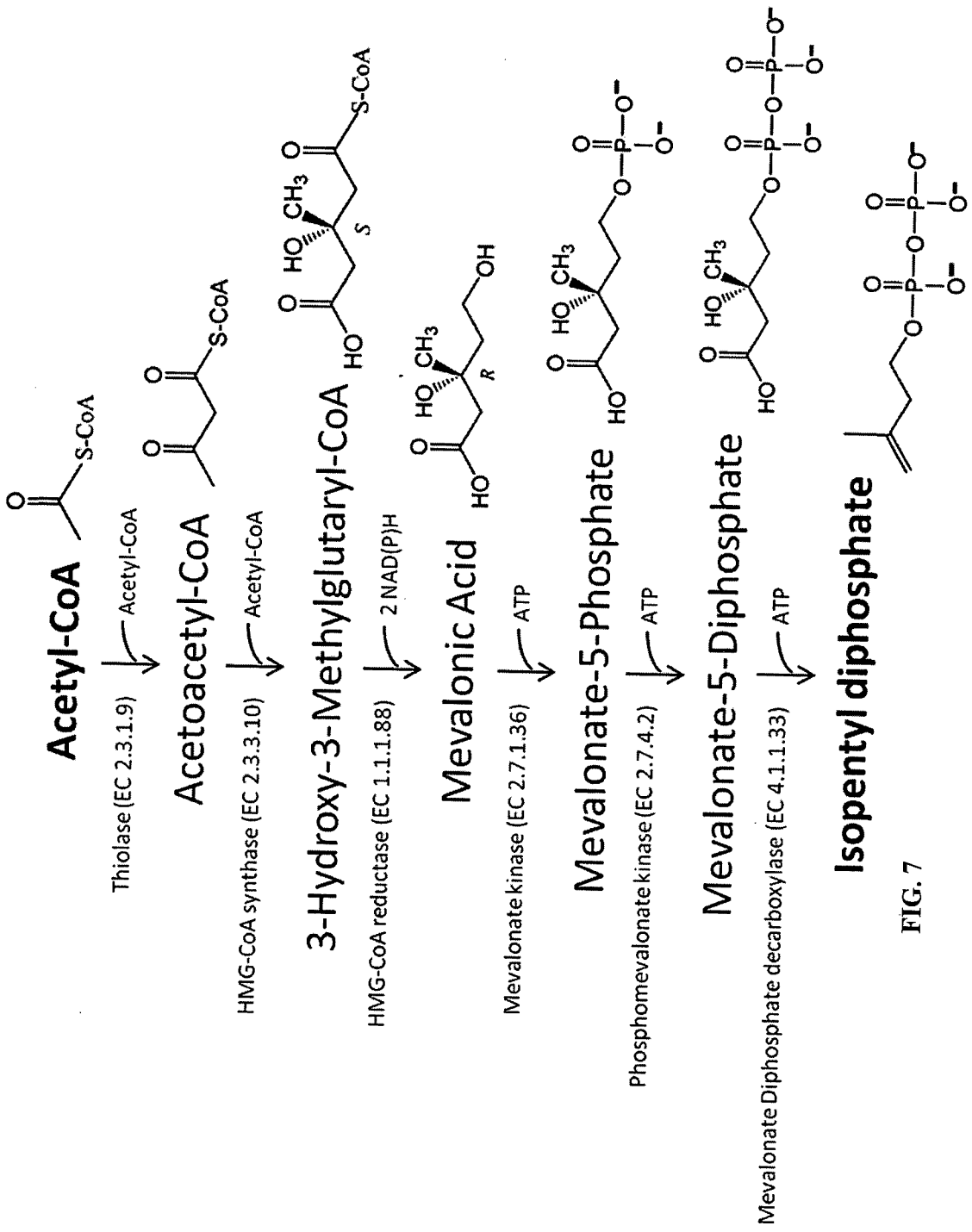

Acetyl-CoA
Thiolase (EC 2.3.1.9)
Acetyl-CoA
Acetoacetyl-CoA
HMG-CoA synthase (EC 2.3.3.10)
Acetyl-CoA
3-Hydroxy-3-Methylglutaryl-CoA
HMG-CoA reductase (EC 1.1.1.88)
2 NAD(P)H
Mevalonic Acid
Mevalonate kinase (EC 2.7.1.36)
ATP
Mevalonate-5-Phosphate
Phosphomevalonate kinase (EC 2.7.4.2)
ATP
Mevalonate-5-Diphosphate
Mevalonate Diphosphate decarboxylase (EC 4.1.1.33)
ATP
Isopentyl diphosphate

FIG. 7

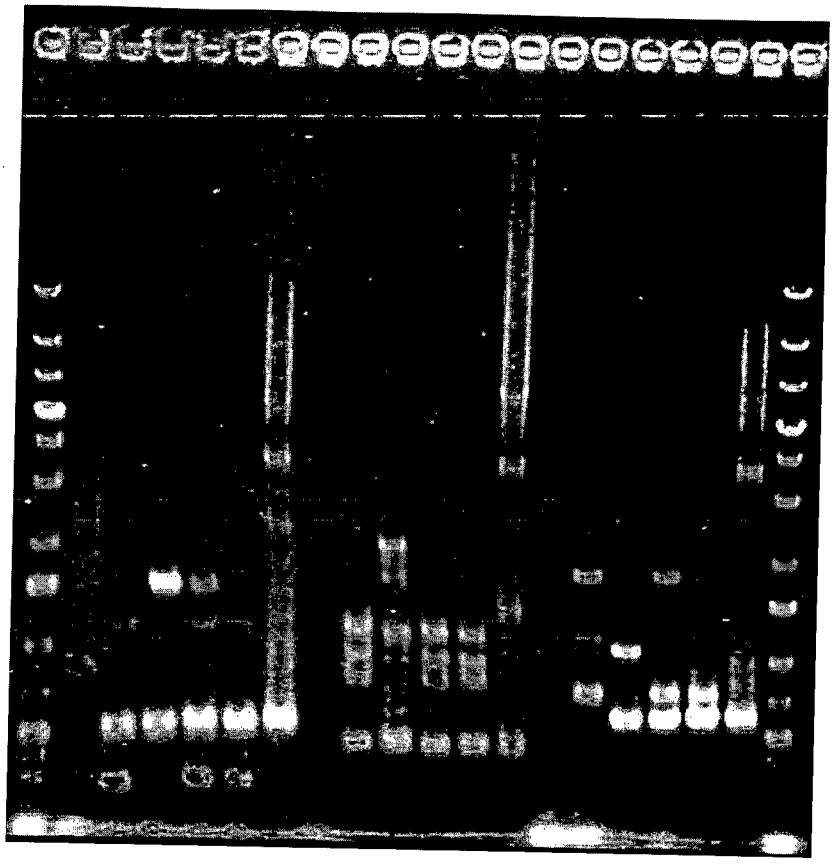


FIG. 8

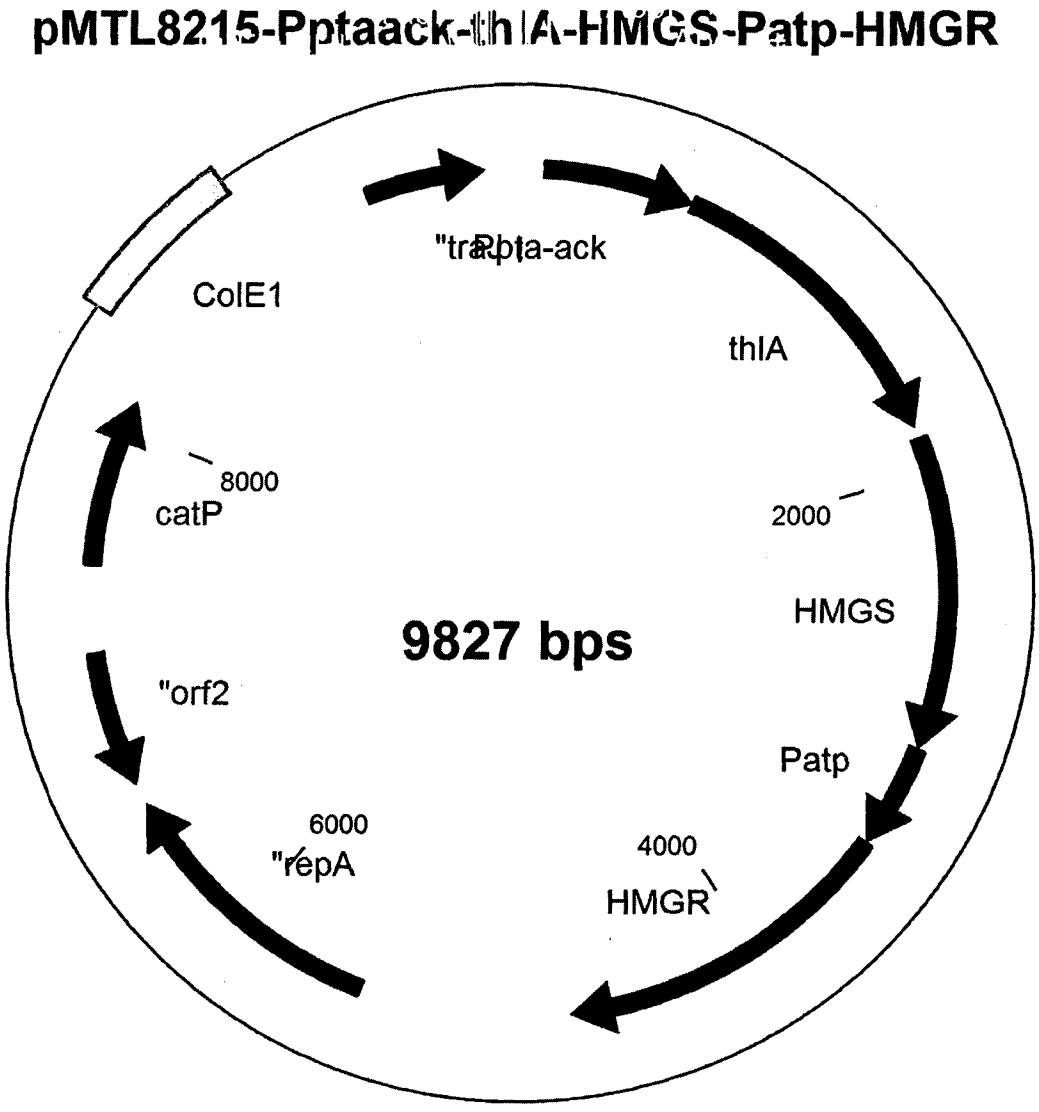

pMTL8215-Pptaack-thlA-HMGS-Patp-HMGR
"traRpta-ack
ColE1
thlA
8000
catP
2000
HMGS
9827 bps
"orf2
Patp
6000
"repA
4000
HMGR

FIG. 9

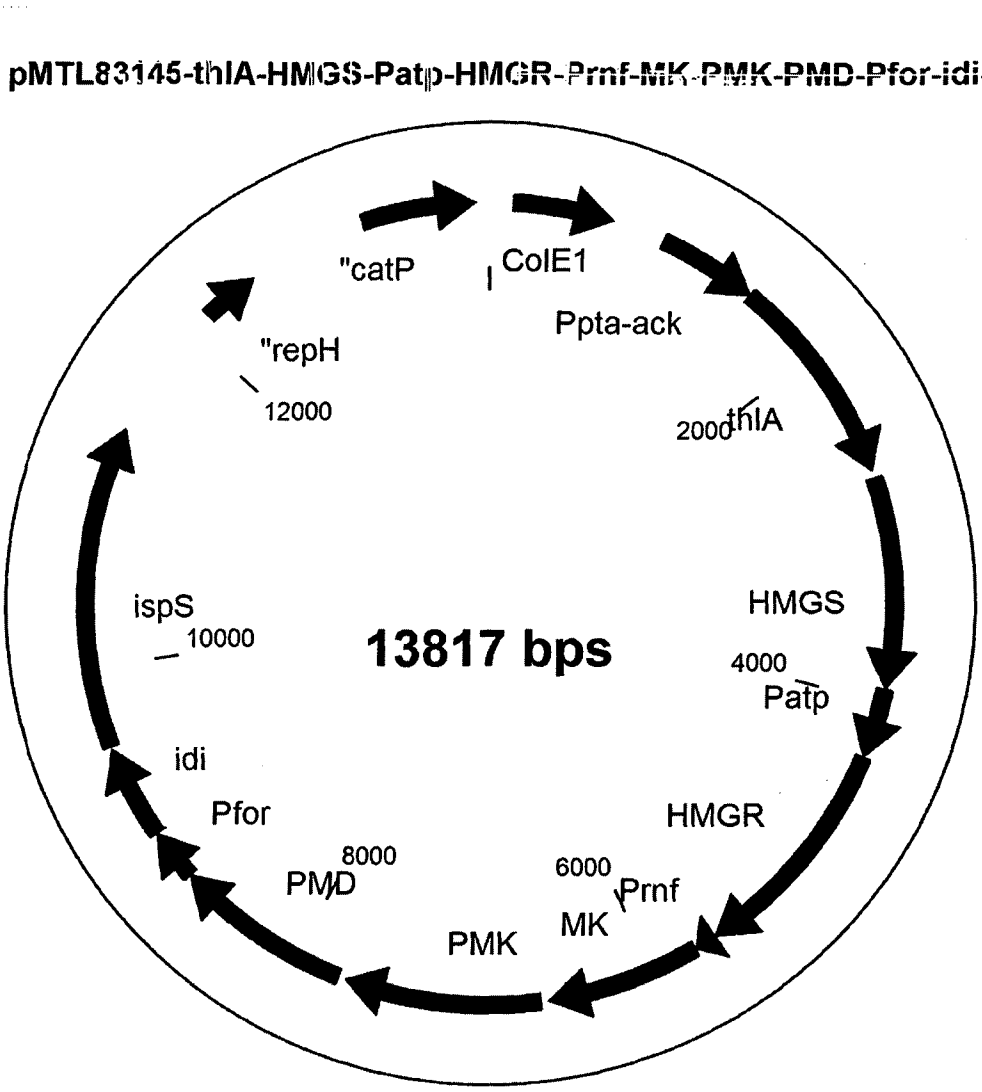

pMTL83145-thlA-HMGS-Patp-HMGR-Prnf-MK-PMK-PMD-Pfor-idi-ispS
"catP
ColE1
Ppta-ack
"repH
12000
2000
thlA
HMGS
ispS
10000
13817 bps
4000
Patp
idi
Pfor
HMGR
8000
PMD
6000
Prnf
MK
PMK

FIG. 10

pMTL83145-thlA-HMGS-Patp-HMGR-Prnf-MK-PMK-PMD-Pfor-idi-ispA-FS

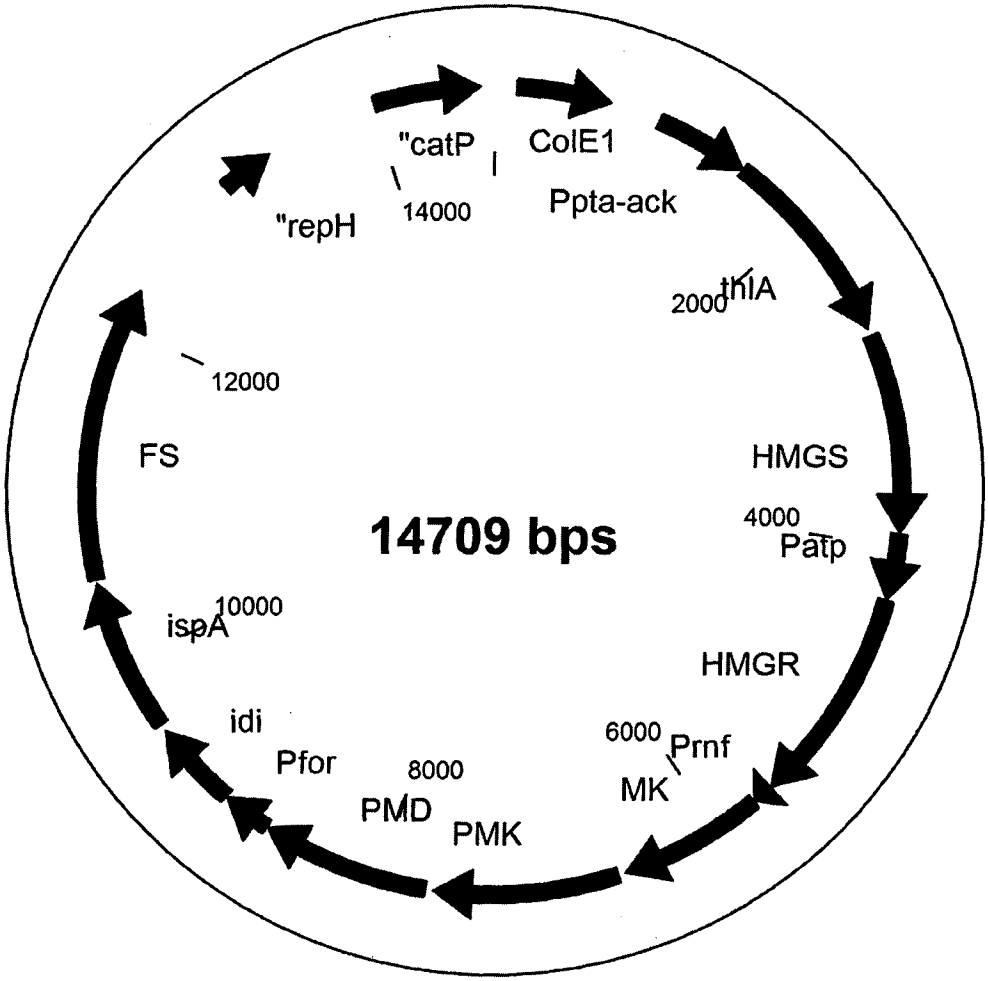


FIG. 11

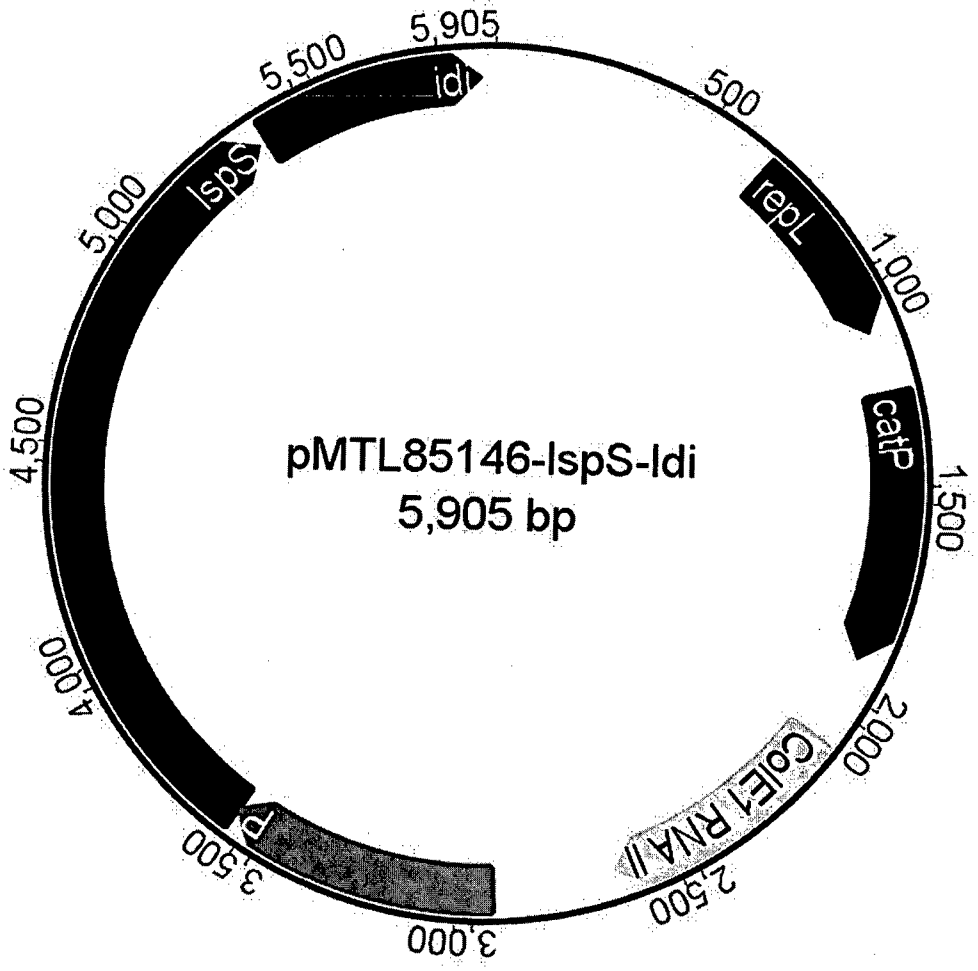
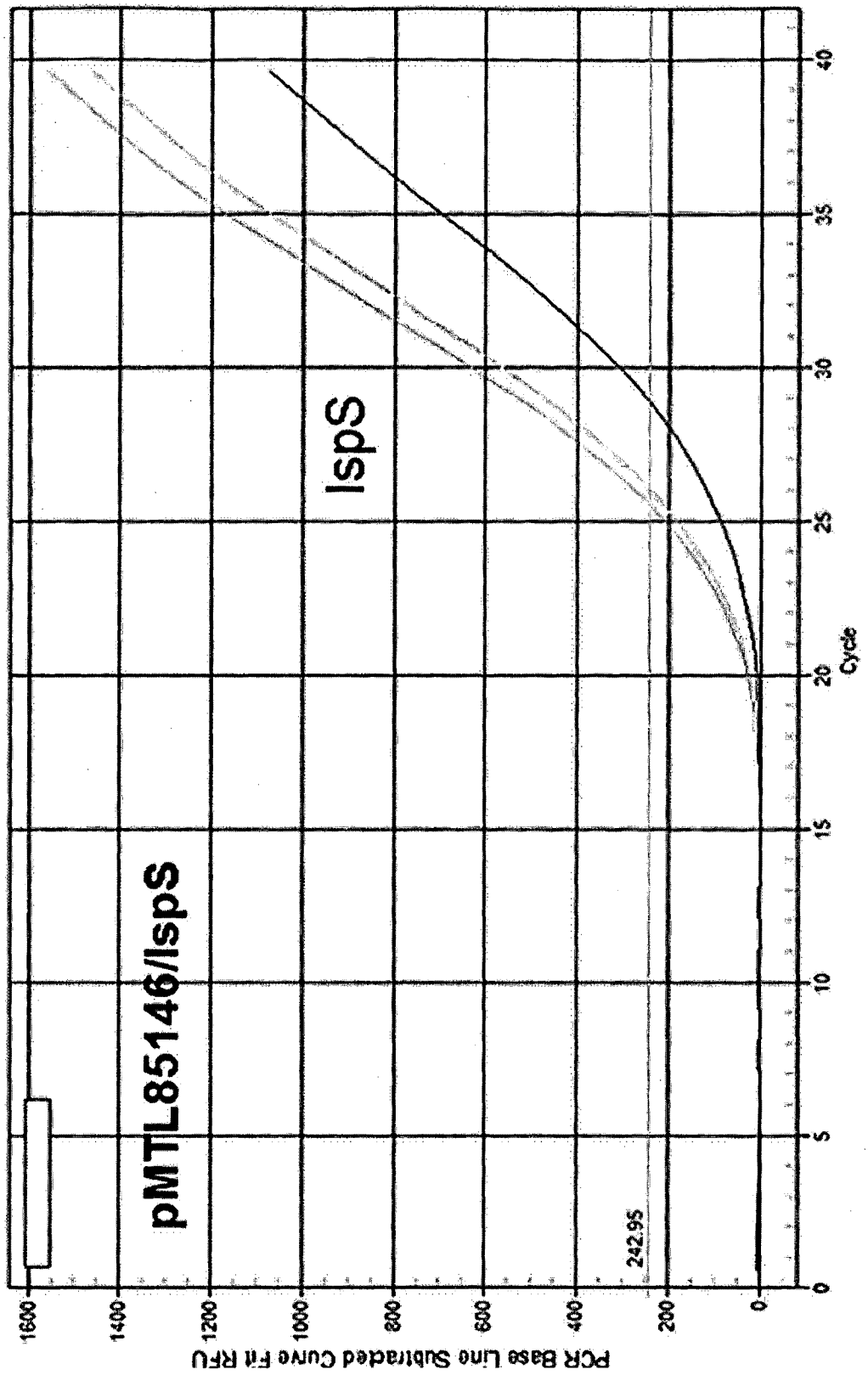
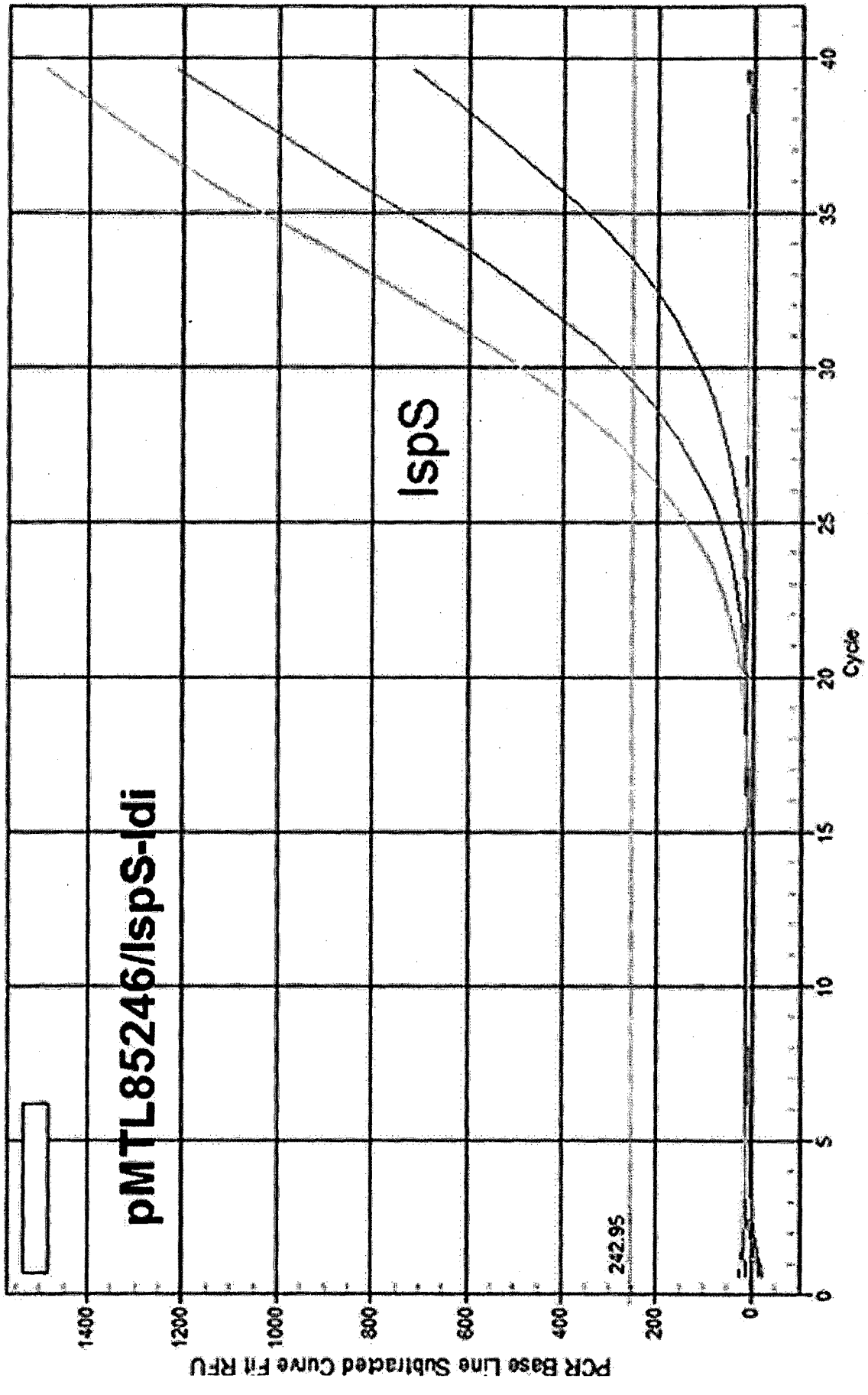

pMTL85146-lspS-Idi
5,905 bp
5,905
500
1,000
1,500
2,000
2,500
3,000
3,500
4,000
4,500
5,000
5,500
idi
repL
catP
ColE1 RNA II
lspS

FIG. 12


pMTL85146/lspS
lspS
242.95
PCR Base Line Subtracted Curve Fit RFU
Cycle
0
200
400
600
800
1000
1200
1400
1600
0
5
10
15
20
25
30
35
40


pMTL85246/lspS-ldi
lspS
242.95
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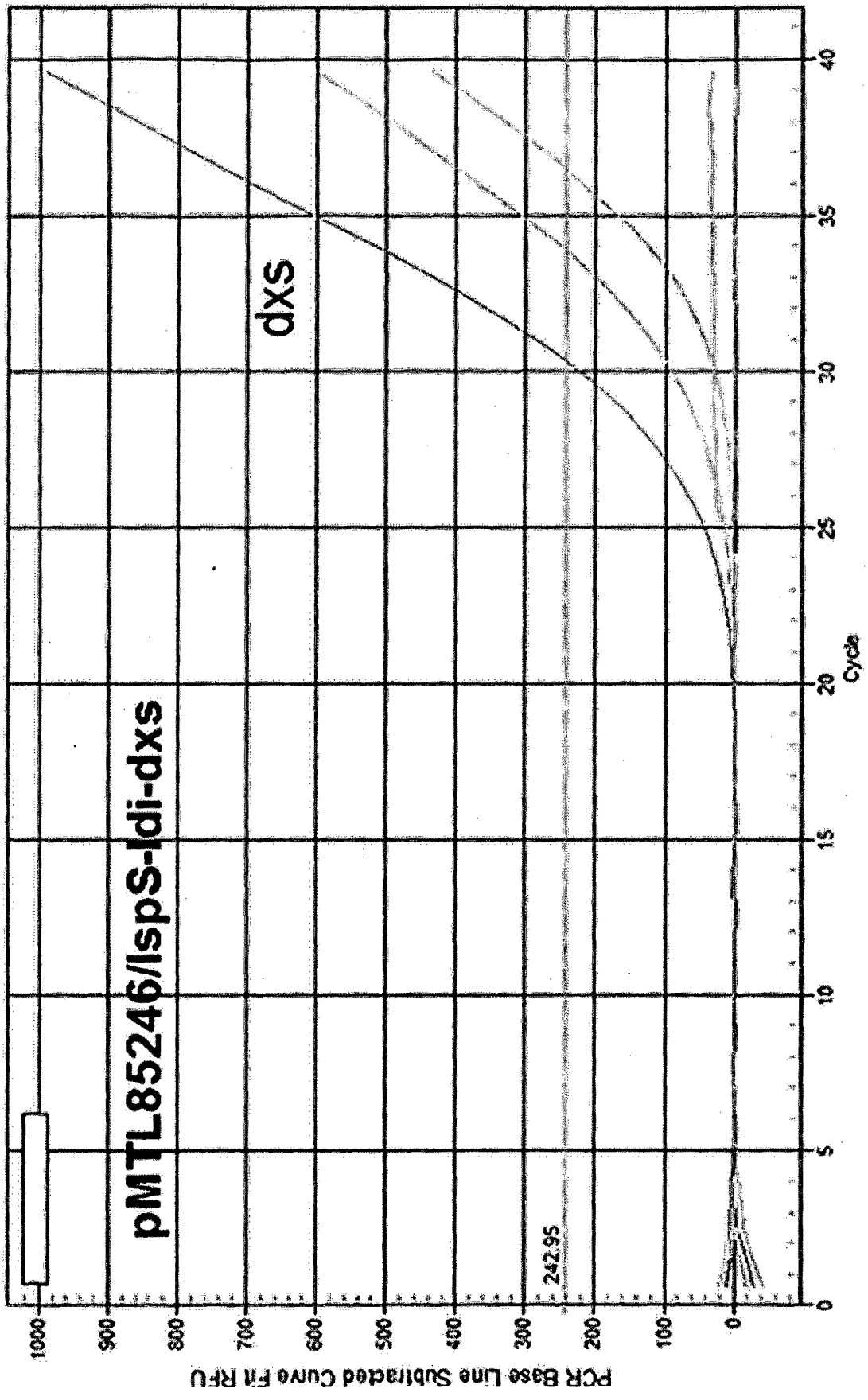
1000
1200
1400
5
10
15
20
25
30
35
40


pMTL85246/IspS-Idi-dxs
dxs
242.95
PCR Base Line Subtracted Curve Fit RFU
Cycle
0
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300
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700
800
900
1000
5
10
15
20
25
30
35
40

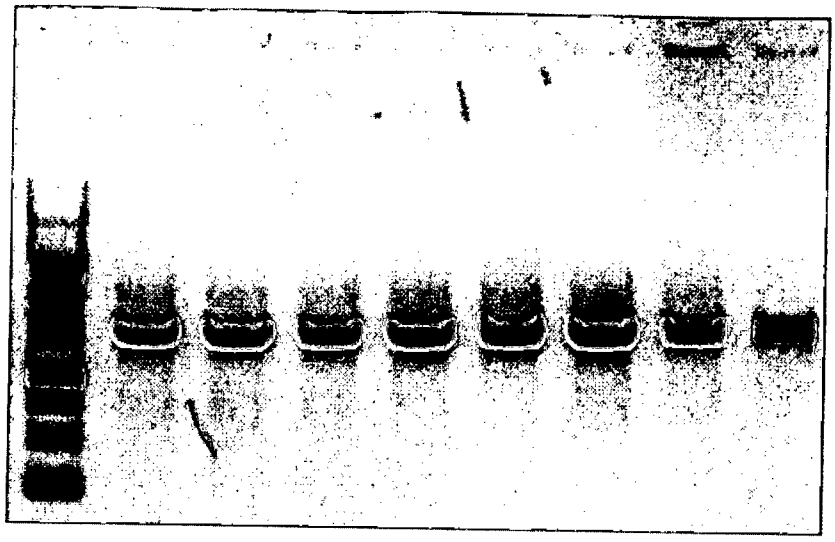

FIG. 16

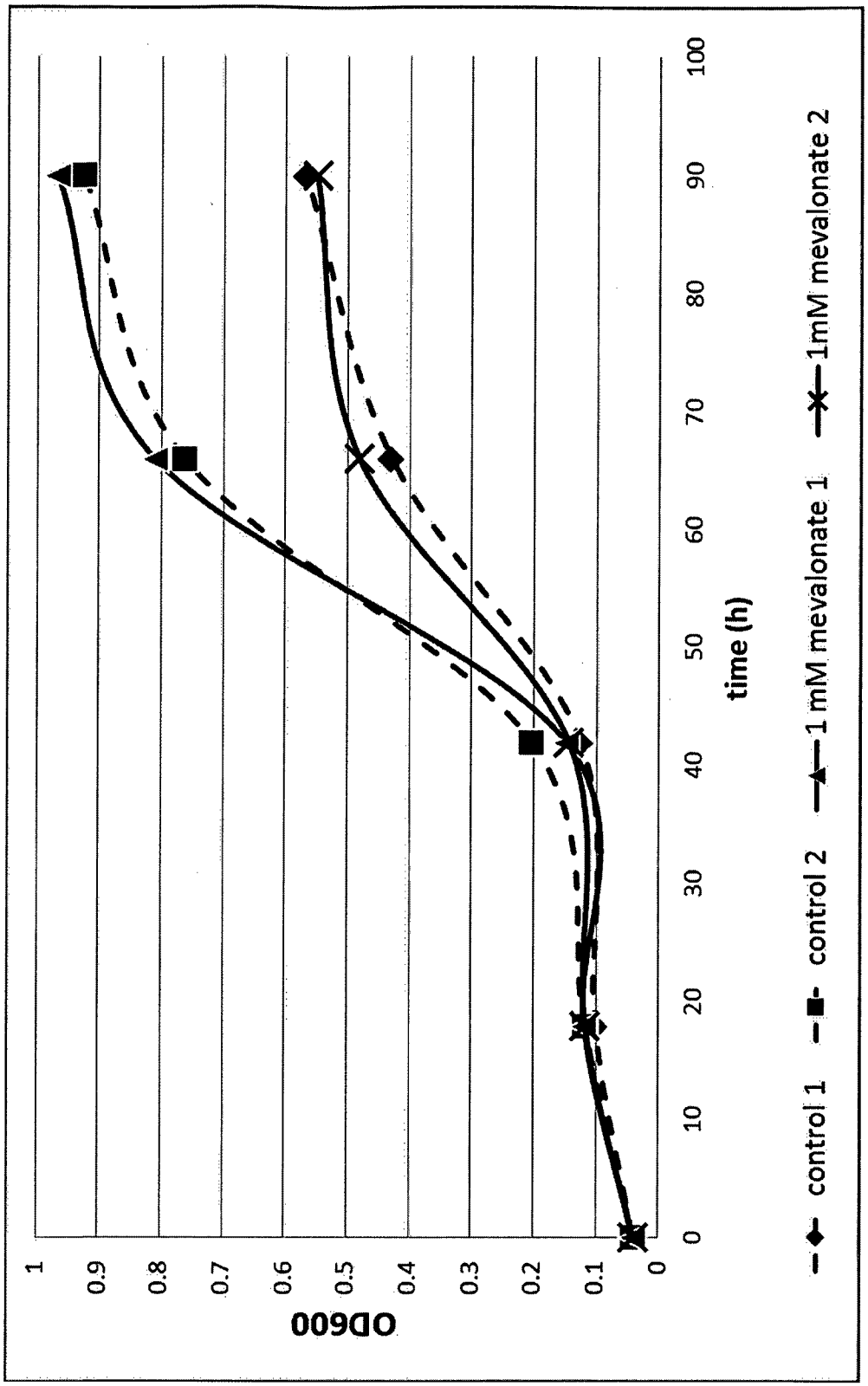
OD600
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
80
90
100
time (h)
control 1
control 2
1 mM mevalonate 1
1mM mevalonate 2

FIG. 17

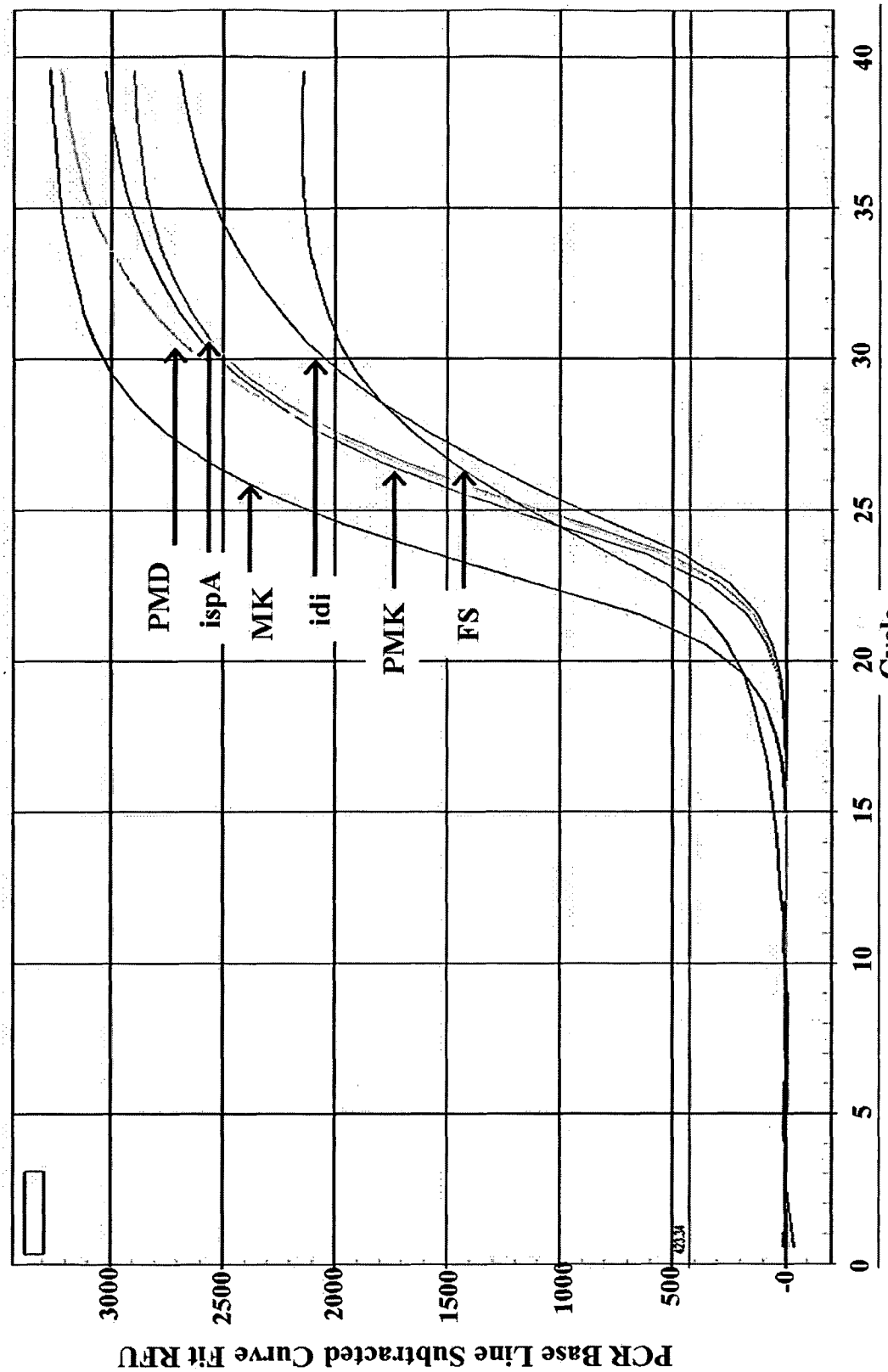
PCR Base Line Subtracted Curve Fit RFU
3000
2500
2000
1500
1000
500
-0
0
5
10
15
20
25
30
35
40
Cycle
PMD
ispA
MK
idi
PMK
FS

FIG. 18

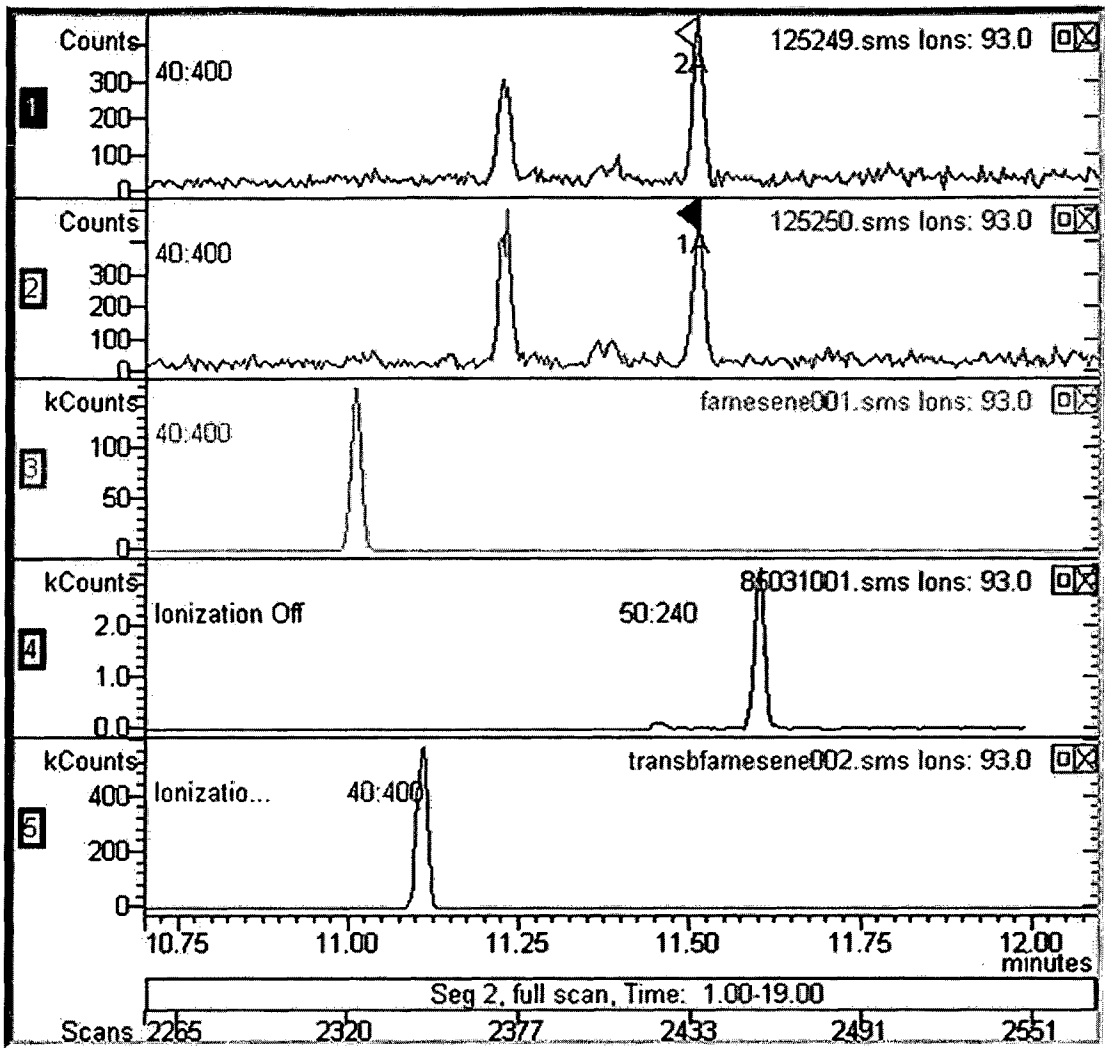
Counts
40:400
125249.sms Ions: 93.0
2A
125250.sms Ions: 93.0
1A
kCounts
farnesene001.sms Ions: 93.0
Ionization Off
50:240
86031001.sms Ions: 93.0
Ionizatio...
transbfarnesene002.sms Ions: 93.0
10.75
11.00
11.25
11.50
11.75
12.00
minutes
Seg 2, full scan, Time: 1.00-19.00
Scans 2265
2320
2377
2433
2491
2551

FIG. 19

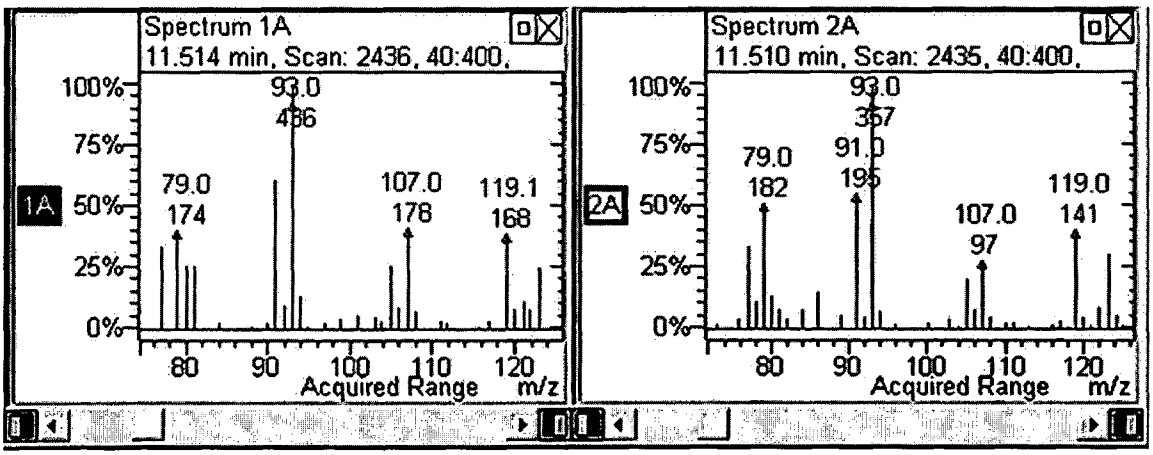
Spectrum 1A
11.514 min, Scan: 2436, 40:400,
93.0
486
79.0
174
107.0
176
119.1
168
Acquired Range
m/z
Spectrum 2A
11.510 min, Scan: 2435, 40:400,
93.0
357
79.0
182
91.0
195
107.0
97
119.0
141
Acquired Range
m/z

FIG. 20

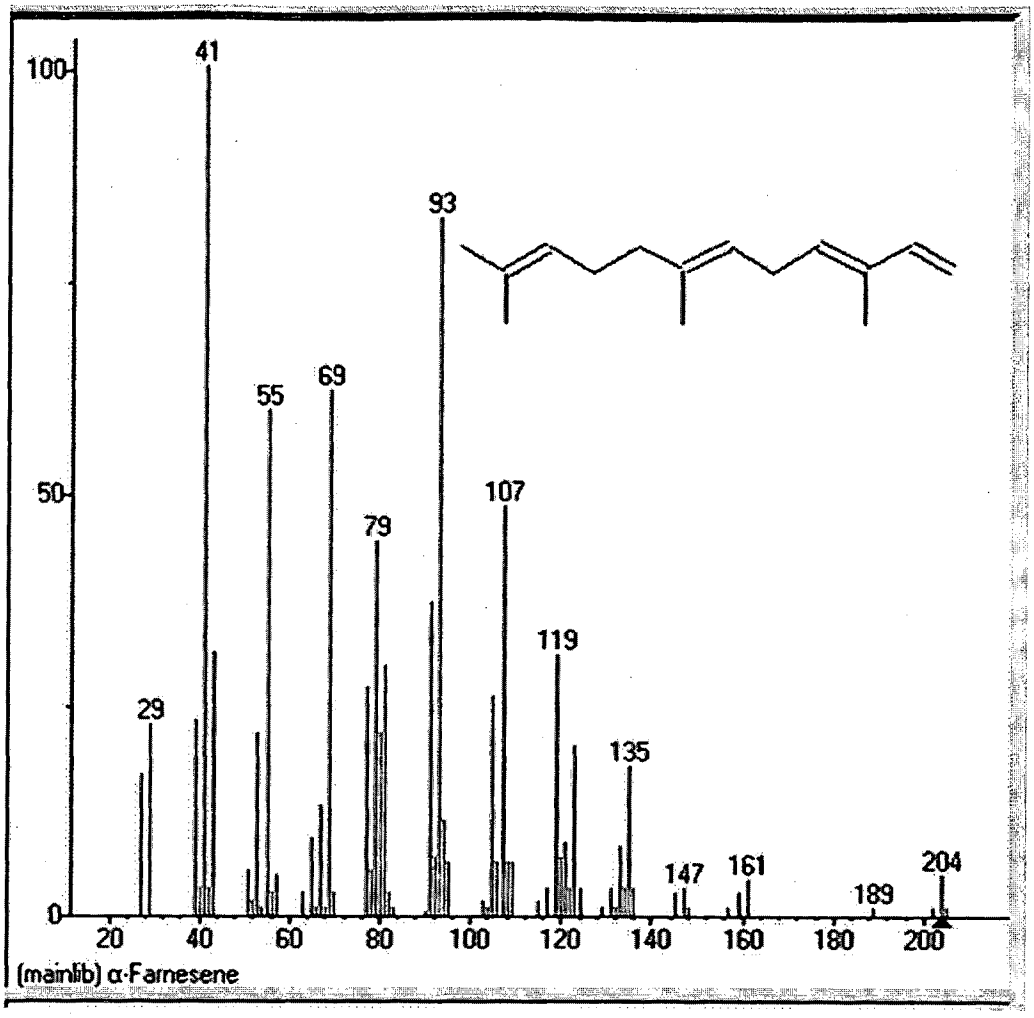
100
50
0
41
93
55
69
79
107
119
29
135
147
161
189
204
20
40
60
80
100
120
140
160
180
200
(mainlib) α-Farnesene

FIG. 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ2013/000095

A. CLASSIFICATION OF SUBJECT MATTER

C12N 1/21(2006.01)i, C12N 15/52(2006.01)i, C12N 15/63(2006.01)i, C12N 15/74(2006.01)i, C12P 5/02(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N 1/21; C12M 1/00; C12P 5/02; C12P 7/16; C12N 1/00; C12M 3/00; C12N 15/52; C12N 15/63; C12N 15/74

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & keywords: acetogeic bacteria, mevalonate, DXS, terpene, pathway, CO, CO2, isoprene synthase, isopentyldiphosphate delta isomerase

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2009-111513 A1 (JOULE BIOTECHNOLOGIES, INC.) 11 September 2009 See abstract; paragraphs [00051], [00054], [00119], [00124], [00137], [00250] and [00539]; table 22; claims 1, 38-48 and 53-56.	1-4,15,16,20-24
Y		5-8,10-12,17-19,26-28
A		9,13,14,25,29,30
Y	US 2011-0014672 A1 (CHOTANI, G. K. et al.) 20 January 2011 See abstract; paragraphs [0035], [0044], [0188], [0192], [0244], [0228], [0320], [0580] and [0583]; claims 1-8.	5-8,10-12,17-19,26-28
A	WO 2008-137092 A2 (ACIDOPHIL, LLC) 13 November 2008 See abstract; claims 1-6, 17 and 20-22.	1-30
A	YANG, J. et al., `Enhancing production of bio-isoprene using hybrid MVA pathway and isoprene synthase in E. coli`, PLoS One, 27 April 2012, Vol. 7, Issue 4, e33509(pp. 1-7). See abstract; pages 2 and 5; figures 1-2.	1-30
A	US 2011-0236941 A1 (KOEPKE, M. et al.) 29 September 2011 See abstract; claims 66-70.	1-30

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 October 2013 (11.10.2013)	**11 October 2013 (11.10.2013)**

Name and mailing address of the ISA/KR
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/NZ2013/000095

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Information on patent family members

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PCT/NZ2013/000095

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		WO 2012-053905 A1	26/04/2012