METHODS OF PRODUCING ISOPRENE AND A CO-PRODUCT

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 61/141,652, filed December 30, 2008, and to U.S. Provisional Patent Application No. 61/187,934, filed June 17, 2009, the disclosures of which are incorporated herein by reference in their entireties.

BACKGROUND OF THE INVENTION

[0002] Isoprene (2-methyl-1,3-butadiene) is the critical starting material for a variety of synthetic polymers, most notably synthetic rubbers. Isoprene is naturally produced by a variety of microbial, plant, and animal species. In particular, two pathways have been identified for the biosynthesis of isoprene: the mevalonate (MVA) pathway and the non-mevalonate (DXP) pathway (Figures 19A and 19B). However, the yield of isoprene from naturally-occurring organisms is commercially unattractive. About 800,000 tons per year of *cis*-polyisoprene are produced from the polymerization of isoprene; most of this polyisoprene is used in the tire and rubber industry. Isoprene is also copolymerized for use as a synthetic elastomer in other products such as footwear, mechanical products, medical products, sporting goods, and latex.

[0003] Currently, the tire and rubber industry is based on the use of natural and synthetic rubber. Natural rubber is obtained from the milky juice of rubber trees or plants found in the rainforests of Africa. Synthetic rubber is based primarily on butadiene polymers. For these polymers, butadiene is obtained as a co-product from ethylene and propylene manufacture.

[0004] While isoprene can be obtained by fractionating petroleum, the purification of this material is expensive and time-consuming. Petroleum cracking of the C5 stream of hydrocarbons produces only about 15% isoprene. Thus, more economical methods for producing isoprene are needed. In particular, methods that produce isoprene at rates, titers, and purity that are sufficient to meet the demands of a robust commercial process are desirable. Also desired are systems for producing isoprene from inexpensive starting materials.

BRIEF SUMMARY OF THE INVENTION

[0005] The invention provides cells capable of co-producing isoprene and a co-product under oxygen-limited conditions, cells in oxygen-limited culture that co-produce isoprene and a co-product, methods of producing isoprene and a co-product, and compositions comprising isoprene and a co-product. In one aspect, provided herein are cells capable of co-producing isoprene and a co-product selected from the group consisting of ethanol, 1,3-propanediol, and hydrogen under oxygen-limited conditions, comprising a heterologous nucleic acid encoding an isoprene synthase polypeptide, wherein the cells (i) have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of the co-product greater than about 0.1 mg/L_{broth}/hr; or (ii) produce isoprene at a rate between about 400 nmole/g_{wcm}/hr to about 2.0×10^5 nmole/g_{wcm}/hr and produce the co-product at a rate between about 0.01 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr. In some embodiments, the cells are grown in oxygen limited culture. In some embodiments, the heterologous nucleic acid encoding an isoprene synthase polypeptide is operably linked to a promoter. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide. In some embodiments, the plant isoprene synthase polypeptide is from *Populus alba*. In some embodiments, the cells further comprise a heterologous nucleic acid encoding an MVA pathway polypeptide, a DXS polypeptide, or an IDI polypeptide.

[0006] In some embodiments, the MVA pathway polypeptide is an upper MVA pathway polypeptide. In some embodiments, the MVA pathway polypeptide is a lower MVA pathway polypeptide. In some embodiments, the upper MVA pathway polypeptide is selected from the group consisting of: (i) an acetoacetyl-Coenzyme A synthase (thiolase) polypeptide; (ii) a 3-hydroxy-3-methylglutaryl-Coenzyme A synthase polypeptide; and (iii) a 3-hydroxy-3-methylglutaryl-Coenzyme A reductase polypeptide. In some embodiments, the upper MVA pathway polypeptide is from the genus *Enterococcus*. In some embodiments, the upper MVA pathway polypeptide is from *Enterococcus faecalis*. In some embodiments, the lower MVA pathway polypeptide is selected from the group consisting of: (i) mevalonate kinase (MVK); (ii) phosphomevalonate kinase (PMK); (iii) diphosphomevalonate decarboxylase (MVD); and (iv) isopentenyl diphosphate isomerase (IDI). In some embodiments, the lower MVA pathway polypeptide is an MVK polypeptide. In some embodiments, the MVK polypeptide is

from the genus *Methanosarcina*. In some embodiments, the MVK polypeptide is from *Methanosarcina mazei*.

[0007] In some embodiments, the co-product is ethanol. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a polypeptide involved in ethanol fermentation. In some embodiments, the polypeptide involved in ethanol fermentation is an alcohol dehydrogenase B (adhB) polypeptide, an alcohol dehydrogenase E (adhE) polypeptide, or a pyruvate decarboxylase (pdc) polypeptide. In some embodiments, the co-product is 1,3-propanediol. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a polypeptide involved in the glycerol pathway or the 1,3-propanediol pathway. In some embodiments, the polypeptide involved in the glycerol pathway or the 1,3-propanediol pathway is dihydroxyacetone phosphate reductase (DAR1), glycerol-phosphate phosphatase (GPP2), glycerol dehydratase B1 (dhaB1), glycerol dehydratase B2 (dhaB2), glycerol dehydratase B3 (dhaB3), dhaX, orfX, orfY, 1,3-propanediol oxidoreductase (dhaT), glycerol dehydrogenase (dhaD), or dihydroxyacetone kinase (dhaK). In some embodiments, the polypeptide involved in the glycerol pathway or the 1,3-propanediol pathway is dihydroxyacetone phosphate reductase (DAR1), glycerol-phosphate phosphatase (GPP2), glycerol dehydratase B1 (dhaB1), glycerol dehydratase B2 (dhaB2), glycerol dehydratase B3 (dhaB3), dhaX, orfX, and orfY. In some embodiments, the co-product is hydrogen. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a hydrogenase polypeptide. In some embodiments, the hydrogenase polypeptide is a ferredoxin-dependent hydrogenase polypeptide, a NADPH-dependent hydrogenase polypeptide, or an oxygen-tolerant hydrogenase polypeptide.

[0008] In another aspect, provided herein are methods of co-producing isoprene and a co-product, the method comprising: (a) culturing cells capable of co-producing isoprene and a co-product selected from the group consisting of ethanol, 1,3-propanediol, and hydrogen under conditions suitable for the co-production of isoprene and the co-product, wherein the cells comprise a heterologous nucleic acid encoding an isoprene synthase polypeptide; and (b) co-producing isoprene and the co-product, wherein the cells (i) have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of the co-product greater than about 0.1 mg/L_{broth}/hr; or (ii) produce isoprene at a rate between about 400 nmole/g_{wcm}/hr to about 2.0×10^5 nmole/g_{wcm}/hr and produce the co-product at a rate between about 0.01 mmol/L_{broth}/hr and about 200

mmol/L_{broth}/hr. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the heterologous nucleic acid encoding an isoprene synthase polypeptide is operably linked to a promoter. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide. In some embodiments, the plant isoprene synthase polypeptide is from *Populus alba*. In some embodiments, the cells further comprise a heterologous nucleic acid encoding an MVA pathway polypeptide, a DXS polypeptide, or an IDI polypeptide.

[0009] In some embodiments, the MVA pathway polypeptide is an upper MVA pathway polypeptide. In some embodiments, the MVA pathway polypeptide is a lower MVA pathway polypeptide. In some embodiments, the upper MVA pathway polypeptide is selected from the group consisting of: (i) an acetoacetyl-Coenzyme A synthase (thiolase) polypeptide; (ii) a 3-hydroxy-3-methylglutaryl-Coenzyme A synthase polypeptide; and (iii) a 3-hydroxy-3-methylglutaryl-Coenzyme A reductase polypeptide. In some embodiments, the upper MVA pathway polypeptide is from the genus *Enterococcus*. In some embodiments, the upper MVA pathway polypeptide is from *Enterococcus faecalis*. In some embodiments, the lower MVA pathway polypeptide is selected from the group consisting of: (i) mevalonate kinase (MVK); (ii) phosphomevalonate kinase (PMK); (iii) diphosphomevalonate decarboxylase (MVD); and (iv) isopentenyl diphosphate isomerase (IDI). In some embodiments, the lower MVA pathway polypeptide is an MVK polypeptide. In some embodiments, the MVK polypeptide is from the genus *Methanosarcina*. In some embodiments, the MVK polypeptide is from *Methanosarcina mazei*.

[0010] In some embodiments, the co-product is ethanol. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a polypeptide involved in ethanol fermentation. In some embodiments, the polypeptide involved in ethanol fermentation is an alcohol dehydrogenase B (adhB) polypeptide, an alcohol dehydrogenase E (adhE) polypeptide, or a pyruvate decarboxylase (pdc) polypeptide. In some embodiments, the co-product is 1,3-propanediol. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a polypeptide involved in the glycerol pathway or the 1,3-propanediol pathway. In some embodiments, the polypeptide involved in the glycerol pathway or the 1,3-propanediol pathway is dihydroxyacetone phosphate reductase (DAR1), glycerol-phosphate phosphatase (GPP2), glycerol dehydratase B1 (dhaB1), glycerol dehydratase B2 (dhaB2), glycerol dehydratase B3 (dhaB3), dhaX, orfX, orfY, 1,3-propanediol oxidoreductase (dhaT),

glycerol dehydrogenase (dhaD), or dihydroxyacetone kinase (dhaK). In some embodiments, the polypeptide involved in the glycerol pathway or the 1,3-propanediol pathway is dihydroxyacetone phosphate reductase (DAR1), glycerol-phosphate phosphatase (GPP2), glycerol dehydratase B1 (dhaB1), glycerol dehydratase B2 (dhaB2), glycerol dehydratase B3 (dhaB3), dhaX, orfX, and orfY. In some embodiments, the co-product is hydrogen. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a hydrogenase polypeptide. In some embodiments, the hydrogenase polypeptide is a ferredoxin-dependent hydrogenase polypeptide, a NADPH-dependent hydrogenase polypeptide, or an oxygen-tolerant hydrogenase polypeptide.

[0011] In another aspect, provided herein are cells in oxygen-limited culture that co-produce isoprene and hydrogen. In some embodiments, the invention provides cells in oxygen-limited culture that produce isoprene at a rate greater than about 400 nmole of isoprene per gram of wet cell mass per hour (nmole/g_{wcm}/hr) and produce hydrogen at a rate greater than about 125 nmole of hydrogen per gram of wet cell mass per hour (nmole/g_{wcm}/hr). In some embodiments, the cells comprise a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells produce isoprene at a rate between about 400 nmole/g_{wcm}/hr and about 2.0×10^5 nmole/g_{wcm}/hr, and hydrogen at a rate between about 125 nmole/g_{wcm}/hr and about 1.25×10^4 nmole/g_{wcm}/hr. In some embodiments, the cells are capable of co-producing isoprene and hydrogen under oxygen-limited conditions. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a mevalonic acid (MVA) pathway polypeptide, a 1-deoxyxylulose-5-phosphate synthase (DXS) polypeptide, or an isopentenyl-diphosphate delta-isomerase (IDI) polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a deoxyxylulose-5-phosphate (DXP) pathway polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a hydrogenase polypeptide. In some embodiments, the hydrogenase polypeptide is a ferredoxin-dependent hydrogenase polypeptide, a NADPH-dependent hydrogenase polypeptide, or an oxygen-tolerant hydrogenase polypeptide. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source

(*e.g.*, a hydrolyzed biomass carbon source), polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells are cultured in the presence of oxygen under conditions where 0.5 moles of oxygen are taken up per mole of isoprene produced. In some embodiments, the cells are grown anaerobically, in the absence of oxygen.

[0012] In another aspect, provided herein are cells in oxygen-limited culture that co-produce isoprene and hydrogen, wherein the cells have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of hydrogen greater than about 0.005 mg/L_{broth}/hr. In some embodiments, the invention provides cells in oxygen-limited culture that have a peak volumetric productivity of isoprene greater than about 0.5 mg/L_{broth}/hr and a peak volumetric productivity of hydrogen greater than about 5 mg/L_{broth}/hr. In some embodiments, the cells are capable of co-producing isoprene and hydrogen under oxygen-limited conditions. In some embodiments, the cells comprise a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a mevalonic acid (MVA) pathway polypeptide, a DXS polypeptide, or an IDI polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a deoxyxylulose-5-phosphate (DXP) pathway polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a hydrogenase polypeptide. In some embodiments, the hydrogenase polypeptide is a ferredoxin-dependent hydrogenase polypeptide, a NADPH-dependent hydrogenase polypeptide, or an oxygen-tolerant hydrogenase polypeptide. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source (*e.g.*, a hydrolyzed biomass carbon source), polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells are cultured in the presence of oxygen under conditions where 0.5 moles of oxygen are taken up per mole of

isoprene produced. In some embodiments, the cells are grown anaerobically, in the absence of oxygen.

[0013] In another aspect, provided herein are cells in oxygen-limited culture that co-produce isoprene and hydrogen, wherein the cells convert more than about 0.002 molar percent of the carbon in a cell culture medium into isoprene, and produce hydrogen in an amount equivalent to more than about 0.024 molar percent of the carbon that the cells consume from a cell culture medium. In some embodiments, the cells are capable of co-producing isoprene and hydrogen under oxygen-limited conditions. In some embodiments, the cells comprise a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a mevalonic acid (MVA) pathway polypeptide, a DXS polypeptide, or an IDI polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a deoxyxylulose-5-phosphate (DXP) pathway polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a hydrogenase polypeptide. In some embodiments, the hydrogenase polypeptide is a ferredoxin-dependent hydrogenase polypeptide, a NADPH-dependent hydrogenase polypeptide, or an oxygen-tolerant hydrogenase polypeptide. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source (*e.g.*, a hydrolyzed biomass carbon source), polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells are cultured in the presence of oxygen under conditions where 0.5 moles of oxygen are taken up per mole of isoprene produced. In some embodiments, the cells are grown anaerobically, in the absence of oxygen.

[0014] In another aspect, provided herein are cells in oxygen-limited culture that co-produce isoprene and hydrogen, wherein the cells produce isoprene and hydrogen in a ratio ranging from at least one molar percent of isoprene for every three molar percent of hydrogen to at least one molar percent of isoprene for every four molar percent of hydrogen. In some

embodiments, the cells are capable of co-producing isoprene and hydrogen under oxygen-limited conditions. In some embodiments, the cells comprise a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a mevalonic acid (MVA) pathway polypeptide, a DXS polypeptide, or an IDI polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a deoxyxylulose-5-phosphate (DXP) pathway polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a hydrogenase polypeptide. In some embodiments, the hydrogenase polypeptide is a ferredoxin-dependent hydrogenase polypeptide, a NADPH-dependent hydrogenase polypeptide, or an oxygen-tolerant hydrogenase polypeptide. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source (*e.g.*, a hydrolyzed biomass carbon source), polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells are cultured in the presence of oxygen under conditions where 0.5 moles of oxygen are taken up per mole of isoprene produced. In some embodiments, the cells are grown anaerobically, in the absence of oxygen.

[0015] In another aspect, provided herein are cells in oxygen-limited culture that co-produce isoprene and hydrogen, wherein the cells produce isoprene at a volumetric pressure greater than about 3.6×10^{-6} atmospheres (equivalent to 10 $\mu g/L_{offgas}$) and produce hydrogen at a volumetric pressure greater than about 0.55×10^{-6} atmospheres. In some embodiments, the cells produce isoprene at a volumetric pressure between about 3.6×10^{-6} atmospheres and about 0.45 atmospheres. In some embodiments, the cells produce hydrogen at a volumetric pressure between about 0.55×10^{-6} atmospheres and about 1.0×10^{-2} atmospheres. In some embodiments, the cells are capable of co-producing isoprene and hydrogen under oxygen-limited conditions. In some embodiments, the cells comprise a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid

encoding a mevalonic acid (MVA) pathway polypeptide, a DXS polypeptide, or an IDI polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a deoxyxylulose-5-phosphate (DXP) pathway polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a hydrogenase polypeptide. In some embodiments, the hydrogenase polypeptide is a ferredoxin-dependent hydrogenase polypeptide, a NADPH-dependent hydrogenase polypeptide, or an oxygen-tolerant hydrogenase polypeptide. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source (*e.g.*, a hydrolyzed biomass carbon source), polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells are cultured in the presence of oxygen under conditions where 0.5 moles of oxygen are taken up per mole of isoprene produced. In some embodiments, the cells are grown anaerobically, in the absence of oxygen.

[0016] In another aspect, provided herein are cells in oxygen-limited culture that coproduce isoprene and hydrogen, comprising a heterologous nucleic acid encoding an isoprene synthase polypeptide, wherein the cells: (i) produce isoprene at a rate greater than about 400 nmole/g_{wcm}/hr and produce hydrogen at a rate greater than about 125 nmole/g_{wcm}/hr; (ii) have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of hydrogen greater than about 0.05 mg/L_{broth}/hr; or (iii) convert more than about 0.002 molar percent of the carbon that the cells consume from a cell culture medium into isoprene, and produce hydrogen equivalent to more than about 0.024 molar percent of the carbon that the cells consume from a cell culture medium. In some embodiments, the cells are capable of co-producing isoprene and hydrogen under oxygen-limited conditions. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a mevalonic acid (MVA) pathway polypeptide, a DXS polypeptide, or an IDI polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a deoxyxylulose-5-phosphate (DXP) pathway polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a hydrogenase polypeptide. In some embodiments, the hydrogenase polypeptide is

a ferredoxin-dependent hydrogenase polypeptide, a NADPH-dependent hydrogenase polypeptide, or an oxygen-tolerant hydrogenase polypeptide. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source (*e.g.*, a hydrolyzed biomass carbon source), polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells are cultured in the presence of oxygen under conditions where 0.5 moles of oxygen are taken up per mole of isoprene produced. In some embodiments, the cells are grown anaerobically, in the absence of oxygen.

[0017] In another aspect, provided herein are cells in oxygen-limited culture that co-produce isoprene and hydrogen, comprising a heterologous nucleic acid encoding an isoprene synthase polypeptide, wherein the heterologous nucleic acid is operably linked to a promoter, wherein the cells produce isoprene and hydrogen in a ratio ranging from at least one molar percent of isoprene for every three molar percent of hydrogen to at least one molar percent of isoprene for every four molar percent of hydrogen. In some embodiments, the cells are capable of co-producing isoprene and hydrogen under oxygen-limited conditions. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a mevalonic acid (MVA) pathway polypeptide, a DXS polypeptide, or an IDI polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a deoxyxylulose-5-phosphate (DXP) pathway polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a hydrogenase polypeptide. In some embodiments, the hydrogenase polypeptide is a ferredoxin-dependent hydrogenase polypeptide, a NADPH-dependent hydrogenase polypeptide, or an oxygen-tolerant hydrogenase polypeptide. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source (*e.g.*, a hydrolyzed biomass carbon source), polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a

component from a yeast extract. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells are cultured in the presence of oxygen under conditions where 0.5 moles of oxygen are taken up per mole of isoprene produced. In some embodiments, the cells are grown anaerobically, in the absence of oxygen.

[0018] In another aspect, provided herein are cells in oxygen-limited culture that co-produce isoprene and a 2- (C2) or 3-carbon (C3) alcohol or diol. In some embodiments, the C2- or C3-alcohol or diol is ethanol. Thus in one aspect, provided herein are cells in oxygen-limited culture that co-produce isoprene and ethanol, wherein the cells have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of ethanol greater than about 0.1 mg/L_{broth}/hr. In some embodiments, the invention provides cells in oxygen-limited culture that have a peak volumetric productivity of isoprene greater than about 0.5 mg/L_{broth}/hr and a peak volumetric productivity of ethanol greater than about 0.1 mg/L_{broth}/hr. In some embodiments, the cells are capable of co-producing isoprene and a 2- (C2) or 3-carbon (C3) alcohol or diol under oxygen-limited conditions. In some embodiments, the C2- or C3-alcohol or diol is ethanol. In some embodiments, the cells comprise a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a mevalonic acid (MVA) pathway polypeptide, a DXS polypeptide, or an IDI polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a deoxyxylulose-5-phosphate (DXP) pathway polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding an ethanol fermentation-related polypeptide. In some embodiments, the ethanol fermentation-related polypeptide is an alcohol dehydrogenase polypeptide. In some embodiments, the ethanol fermentation-related polypeptide is a pyruvate decarboxylase polypeptide. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source (*e.g.*, a hydrolyzed biomass carbon source), polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells are cultured in the

presence of oxygen under conditions where 0.5 moles of oxygen are taken up per mole of isoprene produced. In some embodiments, the cells are grown anaerobically, in the absence of oxygen.

[0019] In some embodiments, the C2- or C3-alcohol or diol is 1,2-propanediol. Thus in another aspect, provided herein are cells in oxygen-limited culture that co-produce isoprene and 1,2-propanediol, wherein the cells have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of 1,2-propanediol greater than about 0.1 mg/L_{broth}/hr. In some embodiments, the invention provides cells in oxygen-limited culture that have a peak volumetric productivity of isoprene greater than about 0.5 mg/L_{broth}/hr and a peak volumetric productivity of 1,2-propanediol greater than about 0.1 mg/L_{broth}/hr. In some embodiments, the cells are capable of co-producing isoprene and a 2- (C2) or 3-carbon (C3) alcohol or diol under oxygen-limited conditions. In some embodiments, the C2- or C3-alcohol or diol is 1,2-propanediol. In some embodiments, the cells comprise a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a mevalonic acid (MVA) pathway polypeptide, a DXS polypeptide, or an IDI polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a deoxyxylulose-5-phosphate (DXP) pathway polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding one or more polypeptides involved in the glycerol pathway or the 1,3-propanediol pathway. In some embodiments, the polypeptide involved in the glycerol pathway or the 1,3-propanediol pathway is dihydroxyacetone phosphate reductase (DAR1), glycerol-phosphate phosphatase (GPP2), glycerol dehydratase B1 (dhaB1), glycerol dehydratase B2 (dhaB2), glycerol dehydratase B3 (dhaB3), dhaX, orfX, orfY, 1,3-propanediol oxidoreductase (dhaT), glycerol dehydrogenase (dhaD), or dihydroxyacetone kinase (dhaK). In some embodiments, the polypeptide involved in the glycerol pathway or the 1,3-propanediol pathway is dihydroxyacetone phosphate reductase (DAR1), glycerol-phosphate phosphatase (GPP2), glycerol dehydratase B1 (dhaB1), glycerol dehydratase B2 (dhaB2), glycerol dehydratase B3 (dhaB3), dhaX, orfX, and orfY. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid,

phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source (*e.g.*, a hydrolyzed biomass carbon source), polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells are cultured in the presence of oxygen under conditions where 0.5 moles of oxygen are taken up per mole of isoprene produced. In some embodiments, the cells are grown anaerobically, in the absence of oxygen.

[0020] In some embodiments, the C2- or C3-alcohol or diol is 1,3-propanediol. Thus in another aspect, provided herein are cells in oxygen-limited culture that co-produce isoprene and 1,3-propanediol, wherein the cells have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of 1,3-propanediol greater than about 0.1 mg/L_{broth}/hr. In some embodiments, the invention provides cells in oxygen-limited culture that have a peak volumetric productivity of isoprene greater than about 0.5 mg/L_{broth}/hr and a peak volumetric productivity of 1,3-propanediol greater than about 0.1 mg/L_{broth}/hr. In some embodiments, the cells are capable of co-producing isoprene and a 2- (C2) or 3-carbon (C3) alcohol or diol under oxygen-limited conditions. In some embodiments, the C2- or C3-alcohol or diol is 1,3-propanediol. In some embodiments, the cells comprise a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a mevalonic acid (MVA) pathway polypeptide, a DXS polypeptide, or an IDI polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a deoxyxylulose-5-phosphate (DXP) pathway polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding one or more polypeptides involved in the glycerol pathway or the 1,3-propanediol pathway. In some embodiments, the polypeptide involved in the glycerol pathway or the 1,3-propanediol pathway is dihydroxyacetone phosphate reductase (DAR1), glycerol-phosphate phosphatase (GPP2), glycerol dehydratase B1 (dhaB1), glycerol dehydratase B2 (dhaB2), glycerol dehydratase B3 (dhaB3), dhaX, orfX, orfY, 1,3-propanediol oxidoreductase (dhaT), glycerol dehydrogenase (dhaD), or dihydroxyacetone kinase (dhaK). In some embodiments, the polypeptide involved in the glycerol pathway or the 1,3-propanediol pathway is dihydroxyacetone phosphate reductase (DAR1), glycerol-phosphate phosphatase (GPP2), glycerol dehydratase B1 (dhaB1), glycerol dehydratase B2

(dhaB2), glycerol dehydratase B3 (dhaB3), dhaX, orfX, and orfY. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source (*e.g.*, a hydrolyzed biomass carbon source), polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells are cultured in the presence of oxygen under conditions where 0.5 moles of oxygen are taken up per mole of isoprene produced. In some embodiments, the cells are grown anaerobically, in the absence of oxygen.

[0021] In another aspect, provided herein are methods of co-producing isoprene and hydrogen, the methods comprising (a) culturing cells under conditions suitable for the co-production of isoprene and hydrogen; and (b) co-producing isoprene and hydrogen, wherein the cells produce isoprene at a rate greater than about 400 nmole/g_{wcm}/hr and produce hydrogen at a rate greater than about 125 nmole/g_{wcm}/hr. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells comprise a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells produce isoprene at a rate between about 400 nmole/g_{wcm}/hr and about 2.0×10^5 nmole/g_{wcm}/hr, and hydrogen at a rate between about 125 nmole/g_{wcm}/hr and about 1.25×10^4 nmole/g_{wcm}/hr. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a mevalonic acid (MVA) pathway polypeptide, a DXS polypeptide, or an IDI polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a deoxyxylulose-5-phosphate (DXP) pathway polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a hydrogenase polypeptide. In some embodiments, the hydrogenase polypeptide is a ferredoxin-dependent hydrogenase polypeptide, a NADPH-dependent hydrogenase polypeptide, or an oxygen-tolerant hydrogenase polypeptide. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable

carbon source (*e.g.*, a hydrolyzed biomass carbon source), polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells are cultured in the presence of oxygen under conditions where 0.5 moles of oxygen are taken up per mole of isoprene produced. In some embodiments, the cells are grown anaerobically, in the absence of oxygen. In some embodiments, the method also includes recovering isoprene and hydrogen produced by the cells. In some embodiments, the method includes purifying isoprene produced by the cells. In some embodiments, the method includes purifying hydrogen produced by the cells. In some embodiments, the method includes polymerizing the isoprene.

[0022] In another aspect, provided herein are methods of co-producing isoprene and hydrogen, the methods comprising (a) culturing cells under conditions suitable for the co-production of isoprene and hydrogen; and (b) co-producing isoprene and hydrogen, wherein the cells have an average volumetric productivity of isoprene greater than about 0.1 $mg/L_{broth}/hr$ and an average volumetric productivity of hydrogen greater than about 0.05 $mg/L_{broth}/hr$. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells comprise a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells produce isoprene at a peak volumetric productivity of isoprene greater than about 0.5 $mg/L_{broth}/hr$, and hydrogen at a peak volumetric productivity of isoprene greater than about 5 $mg/L_{broth}/hr$. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a mevalonic acid (MVA) pathway polypeptide, a DXS polypeptide, or an IDI polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a deoxyxylulose-5-phosphate (DXP) pathway polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a hydrogenase polypeptide. In some embodiments, the hydrogenase polypeptide is a ferredoxin-dependent hydrogenase polypeptide, a NADPH-dependent hydrogenase polypeptide, or an oxygen-tolerant hydrogenase polypeptide. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source (*e.g.*, a hydrolyzed biomass carbon

source), polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells are cultured in the presence of oxygen under conditions where 0.5 moles of oxygen are taken up per mole of isoprene produced. In some embodiments, the cells are grown anaerobically, in the absence of oxygen. In some embodiments, the method also includes recovering isoprene and hydrogen produced by the cells. In some embodiments, the method includes purifying isoprene produced by the cells. In some embodiments, the method includes purifying hydrogen produced by the cells. In some embodiments, the method includes polymerizing the isoprene.

[0023] In another aspect, provided herein are methods of co-producing isoprene and hydrogen, the methods comprising (a) culturing cells under conditions suitable for the co-production of isoprene and hydrogen; and (b) co-producing isoprene and hydrogen, wherein the cells convert more than about 0.002 molar percent of the carbon that the cells consume from a cell culture medium into isoprene, and produce hydrogen equivalent to more than about 0.024 molar percent of the carbon that the cells consume from the culture medium. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells comprise a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells produce isoprene at a rate between about 400 nmole/g_{wcm}/hr and about 2.0×10^5 nmole/g_{wcm}/hr, and hydrogen at a rate between about 125 nmole/g_{wcm}/hr and about 1.25×10^4 nmole/g_{wcm}/hr. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a mevalonic acid (MVA) pathway polypeptide, a DXS polypeptide, or an IDI polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a hydrogenase polypeptide. In some embodiments, the hydrogenase polypeptide is a ferredoxin-dependent hydrogenase polypeptide, a NADPH-dependent hydrogenase polypeptide, or an oxygen-tolerant hydrogenase polypeptide. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source (*e.g.*, a hydrolyzed biomass carbon source), polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells

are grown in oxygen-limited culture. In some embodiments, the cells are cultured in the presence of oxygen under conditions where 0.5 moles of oxygen are taken up per mole of isoprene produced. In some embodiments, the cells are grown anaerobically, in the absence of oxygen. In some embodiments, the method also includes recovering isoprene and hydrogen produced by the cells. In some embodiments, the method includes purifying isoprene produced by the cells. In some embodiments, the method includes purifying hydrogen produced by the cells. In some embodiments, the method includes polymerizing the isoprene.

[0024] In another aspect, provided herein are methods of co-producing isoprene and a 2-(C2) or 3-carbon (C3) alcohol or diol. In some embodiments, the C2- or C3-alcohol or diol is ethanol. Thus in one aspect, provided herein are methods of co-producing isoprene and ethanol, the methods comprising (a) culturing cells under conditions suitable for the co-production of isoprene and ethanol; and (b) co-producing isoprene and ethanol, wherein the cells have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of ethanol greater than about 0.1 mg/L_{broth}/hr. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells comprise a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells produce isoprene at a peak volumetric productivity of isoprene greater than about 0.5 mg/L_{broth}/hr, and ethanol at a peak volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a mevalonic acid (MVA) pathway polypeptide, a DXS polypeptide, or an IDI polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a deoxyxylulose-5-phosphate (DXP) pathway polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding an ethanol fermentation-related polypeptide. In some embodiments, the ethanol fermentation-related polypeptide is an alcohol dehydrogenase polypeptide. In some embodiments, the ethanol fermentation-related polypeptide is a pyruvate decarboxylase polypeptide. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source

(*e.g.*, a hydrolyzed biomass carbon source), polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells are cultured in the presence of oxygen under conditions where 0.5 moles of oxygen are taken up per mole of isoprene produced. In some embodiments, the cells are grown anaerobically, in the absence of oxygen. In some embodiments, the method also includes recovering isoprene and ethanol produced by the cells. In some embodiments, the method includes purifying isoprene produced by the cells. In some embodiments, the method includes purifying ethanol produced by the cells. In some embodiments, the method includes polymerizing the isoprene.

[0025] In some embodiments, the C2- or C3-alcohol or diol is 1,2-propanediol. Thus in one aspect, provided herein are methods of co-producing isoprene and 1,2-propanediol, the methods comprising (a) culturing cells under conditions suitable for the co-production of isoprene and 1,2-propanediol; and (b) co-producing isoprene and 1,2-propanediol, wherein the cells have an average volumetric productivity of isoprene greater than about 0.1 $mg/L_{broth}/hr$ and an average volumetric productivity of 1,2-propanediol greater than about 0.1 $mg/L_{broth}/hr$. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells comprise a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a mevalonic acid (MVA) pathway polypeptide, a DXS polypeptide, or an IDI polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a deoxyxylulose-5-phosphate (DXP) pathway polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding one or more polypeptides involved in the glycerol pathway or the 1,3-propanediol pathway. In some embodiments, the polypeptide involved in the glycerol pathway or the 1,3-propanediol pathway is dihydroxyacetone phosphate reductase (DAR1), glycerol-phosphate phosphatase (GPP2), glycerol dehydratase B1 (dhaB1), glycerol dehydratase B2 (dhaB2), glycerol dehydratase B3 (dhaB3), dhaX, orfX, orfY, 1,3-propanediol oxidoreductase (dhaT), glycerol dehydrogenase (dhaD), or dihydroxyacetone kinase (dhaK). In some embodiments, the polypeptide involved in the glycerol pathway or the 1,3-propanediol pathway is dihydroxyacetone phosphate reductase (DAR1), glycerol-phosphate phosphatase (GPP2), glycerol dehydratase B1 (dhaB1), glycerol dehydratase B2 (dhaB2), glycerol dehydratase B3 (dhaB3), dhaX, orfX, and orfY. In some

embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source (*e.g.*, a hydrolyzed biomass carbon source), polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells are cultured in the presence of oxygen under conditions where 0.5 moles of oxygen are taken up per mole of isoprene produced. In some embodiments, the cells are grown anaerobically, in the absence of oxygen.

[0026] In some embodiments, the C2- or C3-alcohol or diol is 1,3-propanediol. Thus in one aspect, provided herein are methods of co-producing isoprene and 1,3-propanediol, the methods comprising (a) culturing cells under conditions suitable for the co-production of isoprene and 1,3-propanediol; and (b) co-producing isoprene and 1,3-propanediol, wherein the cells have an average volumetric productivity of isoprene greater than about 0.1 $mg/L_{broth}/hr$ and an average volumetric productivity of 1,3-propanediol greater than about 0.1 $mg/L_{broth}/hr$. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells comprise a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a mevalonic acid (MVA) pathway polypeptide, a DXS polypeptide, or an IDI polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding a deoxyxylulose-5-phosphate (DXP) pathway polypeptide. In some embodiments, the cells further comprise a heterologous nucleic acid encoding one or more polypeptides involved in the glycerol pathway or the 1,3-propanediol pathway. In some embodiments, the polypeptide involved in the glycerol pathway or the 1,3-propanediol pathway is dihydroxyacetone phosphate reductase (DAR1), glycerol-phosphate phosphatase (GPP2), glycerol dehydratase B1 (dhaB1), glycerol dehydratase B2 (dhaB2), glycerol dehydratase B3 (dhaB3), dhaX, orfX, orfY, 1,3-propanediol oxidoreductase (dhaT), glycerol dehydrogenase (dhaD), or dihydroxyacetone kinase (dhaK). In some embodiments, the polypeptide involved in the glycerol pathway or the 1,3-propanediol pathway is dihydroxyacetone phosphate reductase (DAR1), glycerol-phosphate phosphatase (GPP2), glycerol dehydratase B1 (dhaB1), glycerol

dehydratase B2 (dhaB2), glycerol dehydratase B3 (dhaB3), dhaX, orfX, and orfY. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate (*e.g.*, xylose or glucose), acetate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source (*e.g.*, a hydrolyzed biomass carbon source), polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells are cultured in the presence of oxygen under conditions where 0.5 moles of oxygen are taken up per mole of isoprene produced. In some embodiments, the cells are grown anaerobically, in the absence of oxygen.

[0027] In some embodiments of any of the various aspects described herein, the MVA pathway polypeptide is an upper MVA pathway polypeptide. In some embodiments, the MVA pathway polypeptide is a lower MVA pathway polypeptide. In some embodiments, the upper MVA pathway polypeptide is selected from the group consisting of: (i) an acetoacetyl-Coenzyme A synthase (thiolase) polypeptide; (ii) a 3-hydroxy-3-methylglutaryl-Coenzyme A synthase polypeptide; and (iii) a 3-hydroxy-3-methylglutaryl-Coenzyme A reductase polypeptide. In some embodiments, the upper MVA pathway polypeptide is from the genus *Enterococcus*. In some embodiments, the upper MVA pathway polypeptide is from *Enterococcus faecalis*. In some embodiments, the lower MVA pathway polypeptide is selected from the group consisting of: (i) mevalonate kinase (MVK); (ii) phosphomevalonate kinase (PMK); (iii) diphosphomevalonate decarboxylase (MVD); and (iv) isopentenyl diphosphate isomerase (IDI). In some embodiments, the lower MVA pathway polypeptide is an MVK polypeptide. In some embodiments, the MVK polypeptide is from the genus *Methanosarcina*. In some embodiments, the MVK polypeptide is from *Methanosarcina mazei*.

[0028] In another aspect, provided herein are compositions comprising isoprene and hydrogen. In some embodiments, the compositions comprise isoprene and hydrogen in ratios ranging from at least one molar percent of isoprene for every three molar percent of hydrogen to at least one molar percent of isoprene for every four molar percent of hydrogen. In some embodiments, the composition further comprises from 1 to 11 molar percent isoprene and from 4 to 44 molar percent hydrogen. In some embodiments, the composition further

comprises oxygen, carbon dioxide, or nitrogen. In some embodiments, the composition further comprises from 0 to 21 molar percent oxygen, from 18 to 44 molar percent carbon dioxide, and from 0 to 78 molar percent nitrogen. In some embodiments, the composition further comprises 1.0×10^{-4} molar percent or less of non-methane volatile impurities. In some embodiments, the non-methane volatile impurities comprise one or more of the following: 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, acetaldehyde, methanethiol, methyl acetate, 1-propanol, diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene, 2,3-cycloheptenolpyridine, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol) and citronellol (3,7-dimethyl-6-octen-1-ol) or a linear isoprene polymer (such as a linear isoprene dimer or a linear isoprene trimer derived from the polymerization of multiple isoprene units). In some embodiments, the non-methane volatile impurities comprise one or more of the following: the isoprene composition includes one or more of the following: an alcohol, an aldehyde, an ester or a ketone (such as any of the alcohols, aldehyes, esters or ketones described herein). In some embodiments, the isoprene composition includes (i) an alcohol and an aldehyde, (ii) an alcohol and a ketone, (iii) an aldehyde and a ketone, or (iv) an alcohol, an aldehyde, and a ketone. In some embodiments, the non-methane volatile impurities comprise one or more of the following: methanol, acetaldehyde, ethanol, methanethiol, 1-butanol, 3-methyl-1-propanol, acetone, acetic acid, 2-butanone, 2-methyl-1-butanol, or indole.

[0029] It is to be understood that one, some, or all of the properties of the various embodiments described herein may be combined to form other embodiments of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0030] Figure 1 is the nucleotide sequence of a kudzu isoprene synthase gene codon-optimized for expression in *E. coli* (SEQ ID NO:1). The atg start codon is in italics, the stop codon is in bold and the added *Pst*I site is underlined.

[0031] Figure 2 is a map of pTrcKudzu.

[0032] Figures 3A-C are the nucleotide sequence of pTrcKudzu (SEQ ID NO:2). The RBS is underlined, the kudzu isoprene synthase start codon is in bold capitol letters and the stop codon is in bold, capital letters. The vector backbone is pTrcHis2B.

[0033] Figure 4 is a map of pETNHisKudzu.

[0034] Figures 5A-C are the nucleotide sequence of pETNHisKudzu (SEQ ID NO:3).

[0035] Figure 6 is a map of pCL-lac-Kudzu.

[0036] Figures 7A-C are the nucleotide sequence of pCL-lac-Kudzu (SEQ ID NO:4).

[0037] Figure 8A is a graph showing the production of isoprene in *E. coli* BL21 cells with no vector.

[0038] Figure 8B is a graph showing the production of isoprene in *E. coli* BL21 cells with pCL-lac-Kudzu

[0039] Figure 8C is a graph showing the production of isoprene in *E. coli* BL21 cells with pTrcKudzu.

[0040] Figure 8D is a graph showing the production of isoprene in *E. coli* BL21 cells with pETN-HisKudzu.

[0041] Figure 9A is a graph showing OD over time of fermentation of *E. coli* BL21/pTrcKudzu in a 14 liter fed batch fermentation.

[0042] Figure 9B is a graph showing isoprene production over time of fermentation of *E. coli* BL21/pTrcKudzu in a 14 liter fed batch fermentation.

[0043] Figure 10A is a graph showing the production of isoprene in *Panteoa citrea.* Control cells without recombinant kudzu isoprene synthase. Grey diamonds represent isoprene synthesis, black squares represent OD_{600}.

[0044] Figure 10B is a graph showing the production of isoprene in *Panteoa citrea* expressing pCL-lac Kudzu. Grey diamonds represent isoprene synthesis, black squares represent OD_{600}.

[0045] Figure 10C is a graph showing the production of isoprene in *Panteoa citrea* expressing pTrcKudzu. Grey diamonds represent isoprene synthesis, black squares represent OD_{600}.

[0046] Figure 11 is a graph showing the production of isoprene in *Bacillus subtilis* expressing recombinant isoprene synthase. BG3594comK is a *B. subtilis* strain without plasmid (native isoprene production). CF443 is *B. subtilis* strain BG3594comK with pBSKudzu (recombinant isoprene production). IS on the y-axis indicates isoprene.

[0047] Figures 12A-C are the nucleotide sequence of pBS Kudzu #2 (SEQ ID NO:5).

[0048] Figure 13 is the nucleotide sequence of kudzu isoprene synthase codon-optimized for expression in *Yarrowia* (SEQ ID NO:6).

[0049] Figure 14 is a map of pTrex3g comprising a kudzu isoprene synthase gene codon-optimized for expression in *Yarrowia*.

[0050] Figures 15A-C are the nucleotide sequence of vector pSPZ1(MAP29Spb) (SEQ ID NO:7).

[0051] Figure 16 is the nucleotide sequence of the synthetic kudzu (*Pueraria montana*) isoprene gene codon-optimized for expression in *Yarrowia* (SEQ ID NO:8).

[0052] Figure 17 is the nucleotide sequence of the synthetic hybrid poplar (*Populus alba x Populus tremula*) isoprene synthase gene (SEQ ID NO:9). The ATG start codon is in bold and the stop codon is underlined.

[0053] Figure 18A shows a schematic outlining construction of vectors pYLA 1, pYL1 and pYL2.

[0054] Figure 18B shows a schematic outlining construction of the vector pYLA(POP1).

[0055] Figure 18C shows a schematic outlining construction of the vector pYLA(KZ1)

[0056] Figure 18D shows a schematic outlining construction of the vector pYLI(KZ1)

[0057] Figure 18E shows a schematic outlining construction of the vector pYLI(MAP29)

[0058] Figure 18F shows a schematic outlining construction of the vector pYLA(MAP29)

[0059] Figure 19A shows the MVA and DXP metabolic pathways for isoprene (based on F. Bouvier *et al.*, Progress in Lipid Res. 44: 357-429, 2005). The following description includes alternative names for each polypeptide in the pathways and a reference that discloses an assay for measuring the activity of the indicated polypeptide (each of these references are each hereby incorporated by reference in their entireties, particularly with respect to assays for polypeptide activity for polypeptides in the MVA and DXP pathways). **Mevalonate Pathway: AACT**; Acetyl-CoA acetyltransferase, MvaE, EC 2.3.1.9. Assay: J. Bacteriol., 184: 2116–2122, 2002; **HMGS**; Hydroxymethylglutaryl-CoA synthase, MvaS, EC 2.3.3.10. Assay: J. Bacteriol., 184: 4065–4070, 2002; **HMGR**; 3-Hydroxy-3-methylglutaryl-CoA reductase, MvaE, EC 1.1.1.34. Assay: J. Bacteriol., 184: 2116–2122, 2002; **MVK**; Mevalonate kinase, ERG12, EC 2.7.1.36. Assay: Curr Genet 19:9-14, 1991. **PMK**; Phosphomevalonate kinase, ERG8, EC 2.7.4.2, Assay: Mol Cell Biol., 11:620–631, 1991; **DPMDC**; Diphosphomevalonate decarboxylase, MVD1, EC 4.1.1.33. Assay: Biochemistry, 33:13355-13362, 1994; **IDI**; Isopentenyl-diphosphate delta-isomerase, IDI1, EC 5.3.3.2. Assay: J. Biol. Chem. 264:19169-19175, 1989. **DXP Pathway: DXS**; 1-Deoxyxylulose-5-phosphate synthase, dxs, EC 2.2.1.7. Assay: PNAS, 94:12857-62, 1997; **DXR**; 1-Deoxy-D-xylulose 5-phosphate reductoisomerase, dxr, EC 2.2.1.7. Assay: Eur. J. Biochem. 269:4446–4457, 2002; **MCT**; 4-Diphosphocytidyl-2C-methyl-D-erythritol synthase, IspD, EC 2.7.7.60. Assay: PNAS, 97: 6451–6456, 2000; **CMK**; 4-Diphosphocytidyl-2-C-methyl-D-erythritol kinase, IspE, EC 2.7.1.148. Assay: PNAS, 97:1062-1067, 2000; **MCS**; 2C-Methyl-D-erythritol 2,4-cyclodiphosphate synthase, IspF, EC 4.6.1.12. Assay: PNAS, 96:11758-11763, 1999; **HDS**; 1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase, ispG, EC 1.17.4.3. Assay: J. Org. Chem., 70:9168 -9174, 2005; **HDR**; 1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase, IspH, EC 1.17.1.2. Assay: JACS, 126:12847-12855, 2004.

[0060] Figure 19B illustrates the classical and modified MVA pathways. 1, acetyl-CoA acetyltransferase (**AACT**); 2, HMG-CoA synthase (**HMGS**); 3, HMG-CoA reductase (**HMGR**); 4, mevalonate kinase (**MVK**); 5, phosphomevalonate kinase (**PMK**); 6, diphosphomevalonate decarboxylase (**MVD or DPMDC**); 7, isopentenyl diphosphate isomerase (**IDI**); 8, phosphomevalonate decarboxylase (**PMDC**); 9, isopentenyl phosphate kinase (**IPK**). The classical MVA pathway proceeds from reaction 1 through reaction 7 via reactions 5 and 6, while a modified MVA pathway goes through reactions 8 and 9. P and PP in the structural formula are phosphate and pyrophosphate, respectively. This figure was taken from Koga and Morii, *Microbiology and Mol. Biology Reviews*, 71:97-120, 2007,

which is incorporated by reference in its entirety, particular with respect to nucleic acids and polypetides of the modified MVA pathway. The modified MVA pathway is present, for example, in some Archaeal organisms, such as *Methanosarcina mazei.*

[0061] Figure 20 shows graphs representing results of the GC-MS analysis of isoprene production by recombinant *Y. lipolytica* strains without (left) or with (right) a kudzu isoprene synthase gene. The arrows indicate the elution time of the authentic isoprene standard.

[0062] Figure 21 is a map of pTrcKudzu yIDI DXS Kan.

[0063] Figures 22A-D are the nucleotide sequence of pTrcKudzu yIDI DXS Kan (SEQ ID NO:10).

[0064] Figure 23A is a graph showing production of isoprene from glucose in BL21/pTrcKudzukan. Time 0 is the time of induction with IPTG (400 μmol). The x-axis is time after induction; the y-axis is OD_{600} and the y2-axis is total productivity of isoprene (μg/L headspace or specific productivity (μg/L headspace/OD). Diamonds represent OD_{600}, circles represent total isoprene productivity (μg/L) and squares represent specific productivity of isoprene (μg/L/OD).

[0065] Figure 23B is a graph showing production of isoprene from glucose in BL21/pTrcKudzu yIDI kan. Time 0 is the time of induction with IPTG (400 μmol). The x-axis is time after induction; the y-axis is OD_{600} and the y2-axis is total productivity of isoprene (μg/L headspace or specific productivity (μg/L headspace/OD). Diamonds represent OD_{600}, circles represent total isoprene productivity (μg/L) and squares represent specific productivity of isoprene (μg/L/OD).

[0066] Figure 23C is a graph showing production of isoprene from glucose in BL21/pTrcKudzu DXS kan. Time 0 is the time of induction with IPTG (400 μmol). The x-axis is time after induction; the y-axis is OD_{600} and the y2-axis is total productivity of isoprene (μg/L headspace or specific productivity (μg/L headspace/OD). Diamonds represent OD_{600}, circles represent total isoprene productivity (μg/L) and squares represent specific productivity of isoprene (μg/L/OD).

[0067] Figure 23D is a graph showing production of isoprene from glucose in BL21/pTrcKudzu yIDI DXS kan. Time 0 is the time of induction with IPTG (400 μmol). The

x-axis is time after induction; the y-axis is OD_{600} and the y2-axis is total productivity of isoprene (μg/L headspace or specific productivity (μg/L headspace/OD). Diamonds represent OD_{600}, circles represent total isoprene productivity (μg/L) and squares represent specific productivity of isoprene (μg/L/OD).

[0068] Figure 23E is a graph showing production of isoprene from glucose in BL21/pCL PtrcKudzu. Time 0 is the time of induction with IPTG (400 μmol). The x-axis is time after induction; the y-axis is OD_{600} and the y2-axis is total productivity of isoprene (μg/L headspace or specific productivity (μg/L headspace/OD). Diamonds represent OD_{600}, circles represent total isoprene productivity (μg/L) and squares represent specific productivity of isoprene (μg/L/OD).

[0069] Figure 23F is a graph showing production of isoprene from glucose in BL21/pCL PtrcKudzu yIDI. Time 0 is the time of induction with IPTG (400 μmol). The x-axis is time after induction; the y-axis is OD_{600} and the y2-axis is total productivity of isoprene (μg/L headspace or specific productivity (μg/L headspace/OD). Diamonds represent OD_{600}, circles represent total isoprene productivity (μg/L) and squares represent specific productivity of isoprene (μg/L/OD).

[0070] Figure 23G is a graph showing production of isoprene from glucose in BL21/pCL PtrcKudzu DXS. Time 0 is the time of induction with IPTG (400 μmol). The x-axis is time after induction; the y-axis is OD_{600} and the y2-axis is total productivity of isoprene (μg/L headspace or specific productivity (μg/L headspace/OD). Diamonds represent OD_{600}, circles represent total isoprene productivity (μg/L) and squares represent specific productivity of isoprene (μg/L/OD).

[0071] Figure 23H is a graph showing production of isoprene from glucose in BL21/pTrcKudzuIDIDXSkan. The arrow indicates the time of induction with IPTG (400 μmol). The x-axis is time after induction; the y-axis is OD_{600} and the y2-axis is total productivity of isoprene (μg/L headspace or specific productivity (μg/L headspace/OD). Black diamonds represent OD_{600}, black triangles represent isoprene productivity (μg/L) and white squares represent specific productivity of isoprene (μg/L/OD).

[0072] Figure 24 is a map of pTrcKKDyIkIS kan.

[0073] Figures 25A-D are a nucleotide sequence of pTrcKKDyIkIS kan (SEQ ID NO:11).

[0074] Figure 26 is a map of pCL PtrcUpperPathway.

[0075] Figures 27A-27D is a nucleotide sequence of pCL PtrcUpperPathway (SEQ ID NO:12).

[0076] Figure 28 shows a map of the cassette containing the lower MVA pathway and yeast idi for integration into the *B. subtilis* chromosome at the nprE locus. nprE upstream/downstream indicates 1 kb each of sequence from the nprE locus for integration. aprE promoter (alkaline serine protease promoter) indicates the promoter (-35, -10, +1 transcription start site, RBS) of the *aprE* gene. MVK1 indicates the yeast mevalonate kinase gene. RBS-PMK indicates the yeast phosphomevalonte kinase gene with a *Bacillus* RBS upstream of the start site. RBS-MPD indicates the yeast diphosphomevalonate decarboxylase gene with a *Bacillus* RBS upstream of the start site. RBS-IDI indicates the yeast idi gene with a *Bacillus* RBS upstream of the start site. Terminator indicates the terminator alkaline serine protease transcription terminator from *B. amyliquefaciens*. SpecR indicates the spectinomycin resistance marker. "nprE upstream repeat for amp." indicates a direct repeat of the upstream region used for amplification.

[0077] Figures 29A-D are a nucleotide sequence of cassette containing the lower MVA pathway and yeast idi for integration into the *B. subtilis* chromosome at the *nprE* locus (SEQ ID NO:13).

[0078] Figure 30 is a map of p9796-poplar.

[0079] Figures 31A-B are a nucleotide sequence of p9796-poplar (SEQ ID NO:14).

[0080] Figure 32 is a map of pTrcPoplar.

[0081] Figures 33A-C are a nucleotide sequence of pTrcPoplar (SEQ ID NO:15).

[0082] Figure 34 is a map of pTrcKudzu yIDI Kan.

[0083] Figures 35A-C are a nucleotide sequence of pTrcKudzu yIDI Kan (SEQ ID NO:16).

[0084] Figure 36 is a map of pTrcKudzuDXS Kan.

[0085] Figures 37A-C are a nucleotide sequence of pTrcKudzuDXS Kan (SEQ ID NO:17).

[0086] Figure 38 is a map of pCL PtrcKudzu.

[0087] Figures 39A-C are a nucleotide sequence of pCL PtrcKudzu (SEQ ID NO:18).

[0088] Figure 40 is a map of pCL PtrcKudzu A3.

[0089] Figures 41A-C are a nucleotide sequence of pCL PtrcKudzu A3 (SEQ ID NO:19).

[0090] Figure 42 is a map of pCL PtrcKudzu yIDI.

[0091] Figures 43A-C are a nucleotide sequence of pCL PtrcKudzu yIDI (SEQ ID NO:20).

[0092] Figure 44 is a map of pCL PtrcKudzu DXS.

[0093] Figures 45A-D are a nucleotide sequence of pCL PtrcKudzu DXS (SEQ ID NO:21).

[0094] Figures 46A-E show graphs representing isoprene production from biomass feedstocks. Panel A shows isoprene production from corn stover, Panel B shows isoprene production from bagasse, Panel C shows isoprene production from softwood pulp, Panel D shows isoprene production from glucose, and Panel E shows isoprene production from cells with no additional feedstock. Grey squares represent OD_{600} measurements of the cultures at the indicated times post-inoculation and black triangles represent isoprene production at the indicated times post-inoculation.

[0095] Figure 47A shows a graph representing isoprene production by BL21 (λDE3) pTrcKudzu yIDI DXS (kan) in a culture with no glucose added. Squares represent OD_{600}, and triangles represent isoprene produced (μg/ml).

[0096] Figure 47B shows a graph representing isoprene production from 1% glucose feedstock invert sugar by BL21 (λDE3) pTrcKudzu yIDI DXS (kan). Squares represent OD_{600}, and triangles represent isoprene produced (μg/ml).

[0097] Figure 47C shows a graph representing isoprene production from 1% invert sugar feedstock by BL21 (λDE3) pTrcKudzu yIDI DXS (kan). Squares represent OD_{600}, and triangles represent isoprene produced (μg/ml).

[0098] Figure 47D shows a graph representing isoprene production from 1% AFEX corn stover feedstock by BL21 (λDE3) pTrcKudzu yIDI DXS (kan). Squares represent OD_{600}, and triangles represent isoprene produced (μg/ml).

[0099] Figures 48A-C show graphs demonstrating the effect of yeast extract of isoprene production. Panel A shows the time course of optical density within fermentors fed with varying amounts of yeast extract. Panel B shows the time course of isoprene titer within fermentors fed with varying amounts of yeast extract. The titer is defined as the amount of isoprene produced per liter of fermentation broth. Panel C shows the effect of yeast extract on isoprene production in *E. coli* grown in fed-batch culture.

[0100] Figures 49A-C show graphs demonstrating isoprene production from a 500 L bioreactor with *E. coli* cells containing the pTrcKudzu + yIDI + DXS plasmid. Panel A shows the time course of optical density within the 500-L bioreactor fed with glucose and yeast extract. Panel B shows the time course of isoprene titer within the 500-L bioreactor fed with glucose and yeast extract. The titer is defined as the amount of isoprene produced per liter of fermentation broth. Panel C shows the time course of total isoprene produced from the 500-L bioreactor fed with glucose and yeast extract.

[0101] Figure 50 is a map of pJMupperpathway2.

[0102] Figures 51A-C are the nucleotide sequence of pJMupperpathway2 (SEQ ID NO:22).

[0103] Figure 52 is a map of pBS Kudzu #2.

[0104] Figure 53A is a graph showing growth during fermentation time of *Bacillus* expressing recombinant kudzu isoprene synthase in 14 liter fed batch fermentation. Black diamonds represent a control strain (BG3594comK) without recombinant isoprene synthase (native isoprene production) and grey triangles represent CF443, *Bacillus* strain BG3594comK with pBSKudzu (recombinant isoprene production).

[0105] Figure 53B is a graph showing isoprene production during fermentation time of *Bacillus* expressing recombinant kudzu isoprene synthase in 14 liter fed batch fermentation. Black diamonds represent a control strain (BG3594comK) without recombinant isoprene

synthase (native isoprene production) and grey triangles represent CF443, *Bacillus* strain BG3594comK with pBSKudzu (recombinant isoprene production).

[0106] Figure 54 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0107] Figure 55 is a time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth.

[0108] Figure 56 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0109] Figure 57 is a time course of optical density within the 15-L bioreactor fed with glycerol.

[0110] Figure 58 is a time course of isoprene titer within the 15-L bioreactor fed with glycerol. The titer is defined as the amount of isoprene produced per liter of fermentation broth.

[0111] Figure 59 is a time course of total isoprene produced from the 15-L bioreactor fed with glycerol.

[0112] Figures 60A-60C are the time courses of optical density, mevalonic acid titer, and specific productivity within the 150-L bioreactor fed with glucose.

[0113] Figures 61A-61C are the time courses of optical density, mevalonic acid titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0114] Figures 62A-62C are the time courses of optical density, mevalonic acid titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0115] Figure 63A-63C are the time courses of optical density, isoprene titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0116] Figures 64A-64C are the time courses of optical density, isoprene titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0117] Figures 65A-65C are the time courses of optical density, isoprene titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0118] Figures 66A-66C are the time courses of optical density, isoprene titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0119] Figure 67A-67C are the time courses of optical density, isoprene titer, and specific productivity within the 15-L bioreactor fed with glucose.

[0120] Figure 68 is a graph of the calculated adiabatic flame temperatures for Series A as a function of fuel concentration for various oxygen levels. The figure legend lists the curves in the order in which they appear in the graph. For example, the first entry in the figure legend (isoprene in air at 40 °C) corresponds to the highest curve in the graph.

[0121] Figure 69 is a graph of the calculated adiabatic flame temperatures for Series B as a function of fuel concentration for various oxygen levels with 4% water. The figure legend lists the curves in the order in which they appear in the graph.

[0122] Figure 70 is a graph of the calculated adiabatic flame temperatures for Series C as a function of fuel concentration for various oxygen levels with 5% CO_2. The figure legend lists the curves in the order in which they appear in the graph.

[0123] Figure 71 is a graph of the calculated adiabatic flame temperatures for Series D as a function of fuel concentration for various oxygen levels with 10% CO_2. The figure legend lists the curves in the order in which they appear in the graph.

[0124] Figure 72 is a graph of the calculated adiabatic flame temperatures for Series E as a function of fuel concentration for various oxygen levels with 15% CO_2. The figure legend lists the curves in the order in which they appear in the graph.

[0125] Figure 73 is a graph of the calculated adiabatic flame temperatures for Series F as a function of fuel concentration for various oxygen levels with 20% CO_2. The figure legend lists the curves in the order in which they appear in the graph.

[0126] Figure 74 is a graph of the calculated adiabatic flame temperatures for Series G as a function of fuel concentration for various oxygen levels with 30% CO_2. The figure legend lists the curves in the order in which they appear in the graph.

[0127] Figure 75A is a table of the conversion of the CAFT Model results from weight percent to volume percent for series A.

[0128] Figure 75B is a graph of the flammability results from the CAFT model for Series A in Figure 68 plotted as volume percent.

[0129] Figure 76A is a table of the conversion of the CAFT Model results from weight percent to volume percent for series B.

[0130] Figure 76B is a graph of the flammability results from the CAFT model for Series B in Figure 69 plotted as volume percent.

[0131] Figure 77 is a figure depicting the flammability test vessel.

[0132] Figure 78A is a graph of the flammability Curve for Test Series 1: 0% Steam, 0 psig, and 40°C.

[0133] Figure 78B is a table summarizing the explosion and non-explosion data points for Test Series 1.

[0134] Figure 78C is a graph of the flammability curve for Test Series 1 compared with the CAFT Model.

[0135] Figure 79A is a graph of the flammability curve for Test Series 2: 4% Steam, 0 psig, and 40°C.

[0136] Figure 79B is a table summarizing the explosion and non-explosion data points for Test Series 2.

[0137] Figure 79C is a graph of the flammability curve for Test Series 2 compared with the CAFT Model.

[0138] Figures 80A-B are a table of the detailed experimental conditions and results for Test Series 1.

[0139] Figure 81 is a table of the detailed experimental conditions and results for Test Series 2.

[0140] Figure 82 is a graph of the calculated adiabatic flame temperature plotted as a function of fuel concentration for various nitrogen/oxygen ratios at 3 atmospheres of pressure.

[0141] Figure 83 is a graph of the calculated adiabatic flame temperature plotted as a function of fuel concentration for various nitrogen/oxygen ratios at 1 atmosphere of pressure.

[0142] Figure 84 is a graph of the flammability envelope constructed using data from Figure 82 and following the methodology described in Example 13. The experimental data points (circles) are from tests described herein that were conducted at 1 atmosphere initial system pressure.

[0143] Figure 85 is a graph of the flammability envelope constructed using data from Figure 83 and following the methodology described in Example 13. The experimental data points (circles) are from tests described herein that were conducted at 1 atmosphere initial system pressure.

[0144] Figure 86A is a GC/MS chromatogram of fermentation off-gas.

[0145] Figure 86B is an expansion of Fig 86A to show minor volatiles present in fermentation off-gas.

[0146] Figure 87A is a GC/MS chromatogram of trace volatiles present in off-gas following cryo-trapping at -78°C.

[0147] Figure 87B is a GC/MS chromatogram of trace volatiles present in off-gas following cryo-trapping at -196°C.

[0148] Figure 87C is an expansion of Figure 87B.

[0149] Figure 87D is an expansion of Figure 87C.

[0150] Figures 88A-B are GC/MS chromatogram comparing C5 hydrocarbons from petroleum-derived isoprene (Figure 88A) and biologically produced isoprene (Figure 88B). The standard contains three C5 hydrocarbon impurities eluting around the main isoprene peak (Figure 88A). In contrast, biologically produced isoprene contains amounts of ethanol and acetone (run time of 3.41 minutes) (Figure 88A).

[0151] Figure 89 is a graph of the analysis of fermentation off-gas of an *E. coli* BL21 (DE3) pTrcIS strain expressing a Kudzu isoprene synthase and fed glucose with 3 g/L yeast extract.

[0152] Figure 90 shows the structures of several impurities that are structurally similar to isoprene and may also act as polymerization catalyst poisons.

[0153] Figure 91 is a map of pTrcHis2AUpperPathway (also called pTrcUpperMVA).

[0154] Figures 92A-92C are the nucleotide sequence of pTrcHis2AUpperPathway (also called pTrcUpperMVA) (SEQ ID NO:23).

[0155] Figure 93 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0156] Figure 94 is a time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth.

[0157] Figure 95 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0158] Figure 96 is a time course of optical density within the 15-L bioreactor fed with invert sugar.

[0159] Figure 97 is a time course of isoprene titer within the 15-L bioreactor fed with invert sugar. The titer is defined as the amount of isoprene produced per liter of fermentation broth.

[0160] Figure 98 is a time course of total isoprene produced from the 15-L bioreactor fed with invert sugar.

[0161] Figure 99 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0162] Figure 100 is a time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth.

[0163] Figure 101 is a time course of isoprene specific activity from the 15-L bioreactor fed with glucose.

[0164] Figure 102 is a map of pCLPtrcUpperPathwayHGS2.

[0165] Figures 103A-103C are the nucleotide sequence of pCLPtrcUpperPathwayHGS2 (SEQ ID NO:24).

[0166] Figure 104 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0167] Figure 105 is a time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth.

[0168] Figure 106 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0169] Figure 107 is a map of plasmid MCM330 (FRT-cm-FRT-gi1.2-KKDy at attTn7).

[0170] Figures 108A-108C are the nucleotide sequence of plasmid MCM330 (SEQ ID NO:25).

[0171] Figure 109 is a map of pET24D-Kudzu.

[0172] Figures 110A-B are the nucleotide sequence of pET24D-Kudzu (SEQ ID NO:26).

[0173] Figure 111A is a time course of optical density within the 15-L bioreactor fed with glucose.

[0174] Figure 111B is a time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth.

[0175] Figure 111C is a time course of specific productivity of isoprene in the 15-L bioreactor fed with glucose.

[0176] Figure 112A is a map of the *M. mazei* archeal Lower Pathway operon.

[0177] Figures 112B-C are the nucleotide sequence of the *M. mazei* archeal lower Pathway operon (SEQ ID NO:27).

[0178] Figure 113A is a map of MCM382 – pTrcKudzuMVK(mazei).

[0179] Figures 113B-C are the nucleotide sequence of MCM382 – pTrcKudzuMVK(mazei) (SEQ ID NO:28).

[0180] Figure 114A is a map of MCM376 - MVK from *M. mazei* archeal Lower in pET200D.

[0181] Figures 114B-C are the nucleotide sequence of MCM376 - MVK from *M. mazei* archeal Lowerin pET200D (SEQ ID NO:29).

[0182] Figures 115A-115D demonstrate that over-expression of MVK and isoprene synthase results in increased isoprene production. Accumulated isoprene and CO_2 from MCM401 and MCM343 during growth on glucose in 100 mL bioreactors with 100 and 200 uM IPTG induction of isoprene production was measured over a 22 hour time course. Figure 115A is a graph of the accumulated isoprene (%) from MCM343. Figure 115B is a graph of the accumulated isoprene (%) from MCM401. Figure 115C is a graph of the accumulated CO_2 (%) from MCM343. Figure 115D is a graph of the accumulated CO_2 (%) from MCM401.

[0183] Figure 116 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0184] Figure 117 is a time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth.

[0185] Figure 118 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0186] Figure 119 is a graph of the total carbon dioxide evolution rate (TCER), or metabolic activity profile, within the 15-L bioreactor fed with glucose.

[0187] Figure 120 is a graph of the cell viability during isoprene production within the 15-L bioreactor fed with glucose. TVC/OD is the total viable counts (colony forming units) in 1 mL of broth per optical density unit (OD_{550}).

[0188] Figure 121 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0189] Figure 122 is a time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth.

[0190] Figure 123 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0191] Figure 124 is a time course of volumetric productivity within the 15-L bioreactor fed with glucose. The volumetric productivity is defined as the amount of isoprene produced per liter of broth per hour.

[0192] Figure 125 is a time course of instantaneous yield within the 15-L bioreactor fed with glucose. The instantaneous yield is defined as the amount of isoprene (gram) produced per amount of glucose (gram) fed to the bioreactor (w/w) during the time interval between the data points.

[0193] Figure 126 is a graph of the total carbon dioxide evolution rate (TCER), or metabolic activity profile, within the 15-L bioreactor fed with glucose.

[0194] Figure 127 is cell viability during isoprene production within the 15-L bioreactor fed with glucose. TVC/OD is the total viable counts (colony forming units) in 1 mL of broth per optical density unit (OD_{550}).

[0195] Figure 128 is a time course of optical density within the 15-L bioreactor fed with glucose.

[0196] Figure 129 is a time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth.

[0197] Figure 130 is a time course of total isoprene produced from the 15-L bioreactor fed with glucose.

[0198] Figure 131 is a graph of total carbon dioxide evolution rate (TCER), or metabolic activity profile, within the 15-L bioreactor fed with glucose.

[0199] Figure 132 is a graph showing that a transient decrease in the airflow to the bioreactor caused a spike in the concentration of isoprene in the offgas that did not cause a dramatic decrease in metabolic activity (TCER). TCER, or metabolic activity, is the total carbon dioxide evolution rate.

[0200] Figure 133 is a graph of the cell viability during isoprene production within the 15-L bioreactor fed with glucose. TVC/OD is the total viable counts (colony forming units) in 1 mL of broth per optical density unit (OD_{550}).

[0201] Figure 134 is a time course of optical density within the 15-L bioreactor fed with glucose. Dotted vertical lines denote the time interval when isoprene was introduced into the bioreactor at a rate of 1 g/L/hr.

[0202] Figure 135 is total carbon dioxide evolution rate (TCER), or metabolic activity profile, within the 15-L bioreactor fed with glucose. Dotted vertical lines denote the time interval when isoprene was introduced into the bioreactor at a rate of 1 g/L/hr.

[0203] Figure 136 is cell viability during isoprene production within the 15-L bioreactor fed with glucose. TVC/OD is the total viable counts (colony forming units) in 1 mL of broth per optical density unit (OD_{550}). Dotted vertical lines denote the time interval when isoprene was introduced into the bioreactor at a rate of 1 g/L/hr.

[0204] Figures 137A-B are the sequence of *Populus alba* pET24a: isoprene synthase gene highlighted in bold letters (SEQ ID NO:30).

[0205] Figures 137C-D are the sequence of *Populus nigra* pET24a: isoprene synthase gene highlighted in bold letters (SEQ ID NO:31).

[0206] Figures 137E-F are the sequence of *Populus tremuloides* pET24a (SEQ ID NO:32).

[0207] Figure 137G is the amino acid sequence of *Populus tremuloides* isoprene synthase gene (SEQ ID NO:33).

[0208] Figures 137H-I are the sequence of *Populus trichocarpa* pET24a: isoprene synthase gene highlighted in bold letters (SEQ ID NO:34).

[0209] Figures 137J-K are the sequence of *Populus tremula* × *Populus alba* pET24a: isoprene synthase gene highlighted in bold letters (SEQ ID NO:35).

[0210] Figure 137L is a map of MCM93 which contains the kudzu IspS coding sequence in a pCR2.1 backbone.

[0211] Figures 137M-N are the sequence of MCM93 (SEQ ID NO:36).

[0212] Figure 137O is a map of pET24D-Kudzu.

[0213] Figures 137P-Q are the sequence of pET24D-Kudzu (SEQ ID NO:37).

[0214] Figure 138 is isoprene synthase expression data for various poplar species as measured in the whole cell head space assay. Y-axis is ug/L/OD of isoprene produced by 0.2 mL of a culture induced with IPTG.

[0215] Figure 139 is relative activity of Poplar isoprene synthase enzymes as measured by DMAPP assay. Poplar enzymes have significantly higher activity than the isoprene synthase from Kudzu. Poplar [*alba x tremula*] only had traces (< 1%) of activity and is not shown in the plot.

[0216] Figure 140 is a map of pDONR221:19430 - hybrid_HGS.

[0217] Figure 141 is the nucleotide sequence of pDONR221:19430 - hybrid_HGS, the sequence of Kudzu isoprene synthase codon-optimized for yeast (SEQ ID NO:38).

[0218] Figure 142A is a map of pDW14.

[0219] Figures 142B-C are the complete nucleotide sequence of pDW14 (SEQ ID NO:39).

[0220] Figure 143 shows induced INVSc-1 strains harboring pDW14 or pYES-DEST52. Figure 143A. A 4-12% bis tris gel (Novex, Invitrogen) of lysates generated from INVSc-1 strains induced with galactose and stained with SimplyBlue SafeStain (Invitrogen). Figure 143B. Western blot analysis of the same strains using the WesternBreeze kit (Invitrogen). Lanes are as follows: 1, INVSc-1 + pYES-DEST52; 2, INVSc-1 + pDW14 (isolate 1); 3,

INVSc-1 + pDW14 (isolate 2). MW (in kDa) is indicated (using the SeeBlue Plus2 molecular weight standard).

[0221] Figure 144 shows induced INVSc-1 strains harboring pDW14 or pYES-DEST52. Figure 144A. OD_{600} of galactose-induced strains prior to lysis. The y-axis is OD_{600}. Figure 144B. DMAPP assay of isoprene synthase headspace in control and isoprene synthase-harboring strains. Specific activity was calculated as μg HG/L/OD. Samples are as follows: Control, INVSc-1 + pYES-DEST52; HGS-1, INVSc-1 + pDW14 (isolate 1); HGS-2, INVSc-1 + pDW14 (isolate 2).

[0222] Figure 145A is a map of codon optimized isoprene synthase fluo-opt2v2.

[0223] Figure 145B is the nucleotide sequence of codon optimized isoprene synthase fluo-opt2v2 (SEQ ID NO:40).

[0224] Figure 146A is a map of pBBR1MCS5.

[0225] Figures 146B-C are the nucleotide sequence of pBBR1MCS5 (SEQ ID NO:41).

[0226] Figure 147A is a map of pBBR5HGSOpt2_2.

[0227] Figures 147B-C are the nucleotide sequence of pBBR5HGSOpt2_2 (SEQ ID NO:42).

[0228] Figure 148 is a graph of CER versus fermentation time for strain MCM401, uninduced, induced with IPTG (4 x 50 μmol) or IPTG (2 x 100 μmol).

[0229] Figure 149 shows concentration of glucose in sugar cane solutions, pH adjusted or not, as a function of the number of autoclaving cycles (one cycle = 30 min).

[0230] Figure 150 shows growth curves (OD_{600} as a function of time) of *Pseudomonas putida* F1 and *Pseudomonas fluorescens* ATCC13525 on glucose, sugar cane, and inverted sugar cane.

[0231] Figure 151 shows growth curves (OD_{600} as a function of time) of *E. coli* BL21(DE3), MG1655, ATCC11303 and B REL 606 on glucose, sugar cane, and inverted sugar cane.

[0232] Figure 152 is a map of plasmid pET24 P.alba HGS.

[0233] Figure 153A-B are the nucleotide sequence of plasmid pET24 P.alba HGS (SEQ ID NO:43).

[0234] Figure 154 is a schematic diagram showing restriction sites used for endonuclease digestion to construct plasmid EWL230 and compatible cohesive ends between BspHI and NcoI sites.

[0235] Figure 155 is a map of plasmid EWL230.

[0236] Figures 156A-B are the nucleotide sequence of plasmid EWL230 (SEQ ID NO:44).

[0237] Figure 157 is a schematic diagram showing restriction sites used for endonuclease digestion to construct plasmid EWL244 and compatible cohesive ends between NsiI and PstI sites.

[0238] Figure 158 is a map of plasmid EWL244.

[0239] Figures 159A-B are the nucleotide sequence of plasmid EWL244 (SEQ ID NO:45).

[0240] Figure 160A is a map of the *M. mazei* Archaeal Lower Pathway operon.

[0241] Figures 160B-C are the nucleotide sequence of the *M. mazei* Archaeal Lower Pathway operon (SEQ ID NO:46).

[0242] Figure 161A is a map of MCM376-MVK from *M. mazei* Archaeal Lowerin pET200D.

[0243] Figures 161B-C are the nucleotide sequence of MCM376-MVK from *M. mazei* Archaeal Lowerin pET200D (SEQ ID NO:47).

[0244] Figure 162 is a map of plasmid pBBRCMPGI1.5-pgl.

[0245] Figures 163A-B are the nucleotide sequence of plasmid pBBRCMPGI1.5-pgl (SEQ ID NO:48).

[0246] Figures 164A-F are graphs of isoprene production by *E. coli* strain expressing *M. mazei* mevalonate kinase, *P. alba* isoprene synthase, and *pgl* (RHM111608-2), and grown in

fed-batch culture at the 15-L scale. Figure 164A shows the time course of optical density within the 15-L bioreactor fed with glucose. Figure 164B shows the time course of isoprene titer within the 15-L bioreactor fed with glucose. The titer is defined as the amount of isoprene produced per liter of fermentation broth. Method for calculating isoprene: cumulative isoprene produced in 59 hrs, g/Fermentor volume at 59 hrs, L [=] g/L broth. Figure 164C also shows the time course of isoprene titer within the 15-L bioreactor fed with glucose. Method for calculating isoprene: $\int$(Instantaneous isoprene production rate, g/L/hr)dt from t = 0 to 59 hours [=] g/L broth. Figure 164D shows the time course of total isoprene produced from the 15-L bioreactor fed with glucose. Figure 164E shows volumetric productivity within the 15-L bioreactor fed with glucose. Figure 164F shows carbon dioxide evolution rate (CER), or metabolic activity profile, within the 15-L bioreactor fed with glucose.

[0247] Figures 165A-B are graphs showing analysis of off-gas from fermentation in 15L bioreactors. Sample A is strain RM111608-2 sampled at 64.8 hours. Sample B is strain EWL256 was *E. coli* BL21 (DE3), pCL upper, cmR-gi1.2-yKKDyI, pTrcAlba-mMVK sampled at 34.5 hours. Hydrogen is detected above the baseline (0.95×10^{-8} torr) for both samples.

[0248] Figure 166 shows growth of a *S. cerevisiae* strain expressing codon-optimized Kudzu IspS (DW112) or a control strain expressing URA3 (DW114) measured by OD_{600} before and after growth in sealed 20-ml GC vials in SC minimal medium with different carbon sources. Strains were grown aerobically in 0.5% glucose (Entry OD), and then grown anaerobically for 48 hours with an additional 1% raffinose or 2% galactose (Post Anaerobic OD). A. Growth of DW112, which harbors the galactose-inducible IspS. B. Growth of DW114, which harbors the vector control.

[0249] Figure 167 is raw GC traces of headspace gas produced by *S. cerevisiae* strains. A. 112G – DW112 (IspS-expressing) grown and induced in 0.5 % glucose, 2 % galactose. B. 112R – DW112 grown in 0.5% glucose, 1% raffinose. C. 114G – DW114 (control) grown and induced in 0.5 % glucose, 2 % galactose. D. 114R – DW114 grown in 0.5% glucose, 1% raffinose. The only detectable peak for isoprene, in sample 112G, is circled.

[0250] Figure 168 shows raw HPLC traces of compounds produced by *S. cerevisiae* strains. A. 112G – DW112 (IspS-expressing) grown and induced in 0.5 % glucose, 2 %

galactose. B. 112R – DW112 grown in 0.5% glucose, 1% raffinose. C. 114G – DW114 (control) grown and induced in 0.5 % glucose, 2 % galactose. D. 114R – DW114 grown in 0.5% glucose, 1% raffinose. Ethanol peaks are circled.

[0251] Figure 169 shows a schematic of the DXP pathway and the pathway for ethanol fermentation with pyruvate decarboxylase.

[0252] Figure 170 shows a schematic of the reactions around pyruvate in *E. coli*. Enzymes endogenous to *E. coli* are shown in blue. Enzymes derived from *Zymomonas mobilis* are shown in red. Numbers listed on the arrows are the Michaelis-Menten constant (K_m)(mM) and the catalytic rate constant (K_{cat})(1/s), in that order. Where only one number is listed, is the K_m (mM).

[0253] Figure 171A is a map of plasmid pBBR5-Ptrcpdc; Figures 171B-C are the nucleotide sequence of plasmid pBBR5-Ptrcpdc (SEQ ID NO:148), encoding *Zymomonas mobilis* pyruvate decarboxylase under the control of the Trc promoter.

[0254] Figure 172A is a map of plasmid pDu-39. Figures 172B-D are the nucleotide sequence of plasmid pDu-39 (SEQ ID NO:151).

[0255] Figure 173 is a map of plasmid pMCM72.

[0256] Figure 174A is a map of plasmid pMCM596. Figures 174B-D are the nucleotide sequence of plasmid pMCM596 (SEQ ID NO:154).

[0257] Figure 175 is a growth curve of strain CMP182 and strain CMP183 in TM3 + 0.55 glucose + antibiotic, plus 0.1% (squares) or 1% (triangles) yeast extract.

[0258] Figure 176 shows ethanol concentration and isoprene specific productivity (in arbitrary units) in the flasks containing 0.1% (A) (5 hours after induction) and 1 % (B) (2 hours after induction) yeast extract. Both products are produced simultaneously.

[0259] Figure 177 shows fermentation products after 5 hours of induction in the 1 % yeast extract flasks. The strain expressing pdc shows a higher concentration in ethanol, confirming the fact that pdc was expressed and active. As expected from comparing K_ms for ldhA and pdc, pyruvate flux to lactate is interrupted once pdc is expressed. Also, in the strain

expressing pdc, more carbon is going towards acetaldehyde than towards acetyl-CoA, leading to a decrease of acetate.

[0260] Figure 178 is a map of plasmid pDU47-3-pET24a-P.alba (-3).

[0261] Figures 179A-B are the sequence of plasmid pDU47-3-pET24a-P.alba (-3) (SEQ ID NO:159).

[0262] Figure 180 is a map of plasmid pBBR-Ppdc-HGS1.

[0263] Figures 181A-B are the sequence of plasmid pBBR-Ppdc-HGS1 (SEQ ID NO:160).

[0264] Figure 182 shows production of isoprene by *Zymomonas mobilis* ZM4, pBBR1-MCS and *Zymomonas mobilis* ZM4, pBBR1-Ppdc-HGS1.

[0265] Figure 183A shows a map of plasmid pDW15 (SEQ ID NO:161), expressing the upper MVA pathway polypeptides *mvaE* and *mvaS* from *Enterobacter faecalis*. Figures 183B-D are the sequence of pDW15.

[0266] Figure 184A shows a map of plasmid pSYCO109. Figures 184B-F are the sequence of pSYCO109 (SEQ ID NO:162).

[0267] Figure 185A shows a map of plasmid pSYCO109F1.1. Figures 185B-F are the sequence of pSYCO109F1.1 (SEQ ID NO:163).

[0268] Figure 186 shows the chromosomal organization of *E. coli* K12 MG1655 around the pgl gene. Brackets ([]) indicate the region deleted in *E. coli* BL21 compared to *E. coli* K12 MG1655, and restored in *E. coli* strain CMP241. The circled gene is ybgS. The forward arrow (→) indicates the annealing site of the galMR primer (SEQ ID NO:187). The reverse arrow (←) indicates the annealing site of the galMF primer (SEQ ID NO:186).

[0269] Figure 187A shows the production of glycerol and/or 1,3-propanediol by *E. coli* strain CMP249 in the presence of 200 μM IPTG, plus or minus 125 mg/L vitamin B12. Closed symbols: glycerol, open symbols: 1,3-PDO. Grey: + B12, black: – B12. EFT : elapsed fermentation time. Figure 187B shows production of isoprene by *E. coli* strain CMP249 in the presence of 200 μM IPTG, plus or minus 125 mg/L vitamin B12. Grey: + B12, black: – B12. EFT : elapsed fermentation time. Figure 187C shows an OD profile and glucose

consumption by *E. coli* strain CMP249 in the presence of 200 μM IPTG, plus or minus 125 mg/L vitamin B12. Closed symbols: glucose, open symbols: OD. Grey: + B12, black: – B12. EFT : elapsed fermentation time. Figure 187D shows molar yield of 1,3-propanediol and glycerol in *E. coli* strain CMP249 grown in the presence of 200 μM IPTG, plus or minus 125 mg/L vitamin B12. Grey: + B12, black: – B12. EFT: elapsed fermentation time.

[0270] Figure 188 shows the time course of optical density in a 15-L bioreactor containing *E. coli* strain CMP239 fed with glucose.

[0271] Figure 189 shows the time course of isoprene titer in a 15-L bioreactor containing *E. coli* strain CMP239 fed with glucose. The isoprene titer is defined as the amount of isoprepe produced per liter of fermentation broth. Equation for calculating Isoprene Titer: ∫(Instantaneous isoprene production rate, g/L/hr)dt from t = 0 to t hrs [=] g/L broth.

[0272] Figure 190 shows the time course of total isoprene produced from the 15-L bioreactor containing *E. coli* strain CMP239 fed with glucose.

[0273] Figure 191 shows the specific productivity of isoprene in the 15-L bioreactor containing *E. coli* strain CMP239 fed with glucose. Equation for calculating Specific Productivity levels: (mg isoprene$_t$ – mg isoprene$_{to}$) / (OD550$_t$ * L broth$_t$ - OD550$_{to}$ * L broth$_{to}$) / (t – t$_0$) [=] mg isoprene/OD/L/hr.

[0274] Figure 192 shows the time course of 1,3-propanediol titer in the 15-L bioreactor containing *E. coli* strain CMP239 fed with glucose. The titer is defined as the amount of material produced per liter of fermentation broth. Equation for calculating 1,3-propanediol titer: Total material produced, g / volume fermentor broth, L [=] g/L broth.

[0275] Figure 193 shows the time course of total 1,3-propanediol produced from the 15-L bioreactor containing *E. coli* strain CMP239 fed with glucose.

[0276] Figure 194 shows the specific productivity of 1,3-PDO in the 15-L bioreactor containing *E. coli* strain CMP239 fed with glucose. Equation for calculating Specific Productivity levels: (mg 1,3-PDO$_t$ – mg 1,3-PDO $_{to}$) / (OD550$_t$ * L broth$_t$ - OD550$_{to}$ * L broth$_{to}$) / (t – t$_0$) [=] mg isoprene/OD/L/hr.

[0277] Figure 195 shows the time course of glycerol titer hin the 15-L bioreactor containing *E. coli* strain CMP239 fed with glucose. The glycerol titer is defined as the amount of material produced per liter of fermentation broth. Equation for calculating glycerol titer: Total material produced, g / volume fermentor broth, L [=] g/L broth.

[0278] Figure 196 shows the time course of total glycerol produced from the 15-L bioreactor containing *E. coli* strain CMP239 fed with glucose.

[0279] Figure 197 shows the specific productivity of glycerol in the 15-L bioreactor containing *E. coli* strain CMP239 fed with glucose. Equation for calculating Specific Productivity levels: (mg glycerol$_t$ – mg glycerol$_{to}$) / (OD550$_t$ * L broth$_t$ - OD550$_{to}$ * L broth$_{to}$) / (t – t$_0$) [=] mg isoprene/OD/L/hr.

DETAILED DESCRIPTION

[0280] The invention provides, *inter alia*, compositions and methods for the production of isoprene and a co-product. In one aspect, the co-product is hydrogen. In another aspect, the co-product is a C2- or C3-alcohol or diol. In some embodiments, the C2- or C3-alcohol or diol is ethanol. In some embodiments, the C2- or C3-alcohol or diol is 1,2-propanediol. In some embodiments, the C2- or C3-alcohol or diol is 1,3-propanediol.

[0281] Provided herein are cells in oxygen-limited culture for co-production of isoprene and hydrogen, methods of co-producing isoprene and hydrogen by culturing such cells under conditions suitable for the co-production of isoprene and hydrogen, and compositions comprising isoprene and hydrogen. In some embodiments, the compositions further comprise oxygen, carbon dioxide, or nitrogen, and 1.0×10^{-4} molar percent or less of non-methane volatile hydrocarbons. Both isoprene and hydrogen can be recovered and purified as necessary. Recovered isoprene can be polymerized to produce synthetic rubber. Recovered hydrogen can be used to power the fermentation process, thereby reducing the cost of isoprene production, reducing potential hazards associated with accumulation of high oxygen concentrations during conventional fermentation, and reducing the overall 'carbon footprint' of the process.

[0282] Existing aerobic systems for production of isoprene produce hydrogen gas via either the mevalonic acid ("MVA") pathway or the 1-deoxy-D-xylulose 5-phosphate ("DXP") pathway with molecular oxygen (O_2) as the primary electron acceptor. Both the

DXP and MVA pathways start with glucose, require oxygen input, and evolve small amounts of hydrogen gas. At current peak isoprene productivity (*e.g.*, ~6 g/L/hr), isoprene-producing aerobic cultures have an oxygen uptake rate ("OUR") of >200 mmol/L/hr. Conversion of glucose to hydrogen gas via the MVA pathway spills excess reducing equivalents that need to be disposed of, but releasing that excess to O_2 poses at least two problems: first, the combination of hydrogen gas and O_2 poses a safety hazard, and second, high OUR fermentations are capital and energy intensive. Because excess reducing equivalents represent potential energy, it would be useful to capture those excess reducing equivalents as H_2 instead of dumping them to O_2. Furthermore, the H_2 produced could be used to power the fermentation process, thereby directly reducing costs and indirectly reducing the overall 'carbon footprint' of the process. Hydrogen has been produced by both batch and continuous system fermentation using recombinant *E. coli* BL21. *See, e.g.*, G. Chittibabu et al., "Feasibility studies on the fermentative hydrogen production by recombinant Escherichia coli BL-21," *Process Biochem.* 41(3):682-688 (2006), which is incorporated herein by reference, particularly with reference to production of hydrogen by fermentation with recombinant *E. coli* BL21.

[0283] There are at least three routes for getting excess reducing equivalents to hydrogenase in a bacterial system such as *E. coli*. First, using endogenous bacterial enzymes, such as the *E. coli* pyruvate formate lyase/formate dehydrogenase/formate hydrogen lyase/hydrogenase-3 system. *See, e.g.*, Gerhard Gottschalk "Bacterial Metabolism," at pp. 194-196 (Springer Series in Microbiology, 1st ed. 1979). Second, by providing a heterologous electron capture system, such as glyceraldehyde-3-phosphate oxidoreductase ("GAPOR") and/or pyruvate oxidoreductase ("POR") with ferredoxin oxidoreductase, coupled with a heterologous hydrogenase activity, such as ferredoxin-dependent *Clostridium acetobutulicum* hydrogenase A (HydA). *See, e.g.*, King et al., (2006), which is incorporated herein by reference in its entirety, particularly with respect to production of hydrogen by HydA and three HydA-associated maturation enzymes (HydE, HydG, and HydF). Third, by providing a heterologous electron transfer system, such as NAD(P)H to NADPH ferredoxin oxidoreductase (NFOR)(*see, e.g.*, Viet et al., (2008)), which is incorporated herein by reference in its entirety, particularly with respect to production of hydrogen by NFOR; *see also* PCT Publication No. WO/2007/089901, which is incorporated herein by reference in its entirety, particularly with respect to optimization of *E. coli* strains for production of hydrogen) or *Clostridium kluyveri* NADH ferredoxin oxidoreductase (RnfCDGEAB)

(Henning Seedorf et al., "The genome of *Clostridium kluyveri*, a strict anaerobe with unique metabolic features," *Proc. Nat'l Acad. Sci. U.S.A.* 105(6):2128-2133 (2008), which is incorporated herein by reference in its entirety, particularly with reference to NADH ferredoxin oxidoreductase, and with reference to components of the anaerobic ethanol-acetate fermentation pathway), coupled with a heterologous hydrogenase activity, such as ferredoxin-dependent *Clostridium acetobutulicum* hydrogenase A (HydA). *See, e.g.*, King et al., (2006).

[0284] Thus, one strategy provided herein for capturing excess reducing equivalents as H_2 involves engineering a bacterial system to produce isoprene via anaerobic fermentation and to co-produce hydrogen by expression of an endogenous hydrogenase system. For example, isoprene-producing *E. coli* cells with functional H_2 flux can be engineered to express *E. coli* hydrogenase-3 (Hyd-3) polypeptides, *E. coli* pyruvate formate lyase ("PFL"), and the *E. coli* formate hydrogen lyase (FHL) complex, which produces hydrogen gas from formate and CO_2 under anaerobic conditions at acidic pH (*see, e.g.*, Akihito Yoshida et al., "Efficient induction of formate hydrogen lyase of aerobically grown *Escherichia coli* in a three-step biohydrogen production process," *Appl. Microbiol. Biotechnol.* 74:754-760 (2007), which is incorporated herein by reference in its entirety, particularly with respect to the induction of expression of formate hydrogen lyase in *E. coli*).

[0285] A second strategy provided herein for capturing excess reducing equivalents as H_2 involves engineering a hybrid system for the co-production of isoprene and hydrogen under oxygen-limited conditions. Such a system would co-produce isoprene and hydrogen while utilizing less oxygen than current aerobic culture conditions. Most hydrogenases are oxygen-sensitive to some degree, however, but bacterial strains can be engineered to express an oxygen-tolerant or oxygen-insensitive hydrogenase, such as, for example, *Rubrivivax gelatinosus* hydrogenase (*see, e.g.*, P.C. Maness et al., "Characterization of the oxygen tolerance of a hydrogenase linked to a carbon monoxide oxidation pathway in *Rubrivivax gelatinosus*," *Appl. Environ. Microbiol.* 68(6):2633-2636 (2002), which is incorporated herein by reference in its entirety, particularly with respect to *R. gelatinosus* hydrogenase), or *Ralstonia eutropha* hydrogenase (*see, e.g.*, Burgdorf et al., (2005), which is incorporated herein by reference in its entirety, particularly with respect to *R. eutropha* hydrogenase polypeptides). Alternatively, heterologous nucleic acids encoding conventional oxygen-sensitive hydrogenase polypeptides can be mutagenized and screened to identify O_2-tolerant or O_2-insensitive hydrogenase mutants using standard methods and assays (*see, e.g.*, L.E.

Nagy et al., "Application of gene-shuffling for the rapid generation of novel [FeFe]-hydrogenase libraries," *Biotechnol. Letts.* 29(3)421-430 (2007), which is incorporated herein by reference, particularly with respect to mutagenesis and screening for oxygen tolerant hydrogenase polypeptides).

[0286] A third strategy provided herein for capturing excess reducing equivalents as H_2 involves engineering an obligate anaerobic bacterium to co-produce isoprene and hydrogen. Such a system would co-produce isoprene and hydrogen in anaerobic culture. For example, an obligate anaerobe can be engineered, for example, to express glyceraldehyde-3-phosphate oxidoreductase ("GAPOR") and/or pyruvate oxidoreductase ("POR"), ferredoxin oxidoreductase, NADPH ferredoxin oxidoreductase (NFOR) or *Clostridium kluyveri* NADH ferredoxin oxidoreductase (RnfCDGEAB), coupled with a heterologous hydrogenase activity, such as ferredoxin-dependent *Clostridium acetobutulicum* hydrogenase A (HydA) (*see, e.g.*, King et al., (2006), which is incorporated herein by reference in its entirety, particularly with respect to production of hydrogen by HydA and three HydA-associated maturation enzymes (HydE, HydG, and HydF)) or NADPH-dependent *Pyrococcus furiosus* hydrogenase (*see, e.g.*, J. Woodward et al., "Enzymatic production of biohydrogen," *Nature* 405(6790):1015-15 (2000), which is incorporated herein by reference in its entirety, particularly with respect to production of hydrogen by NADPH-dependent *P. furiosus* hydrogenase).

[0287] In any of the strategies described herein, hydrogen yields can be maximized by also blocking non-productive metabolic pathways, including those that produce fermentation side products such as lactate, acetate, pyruvate, ethanol, succinate, and glycerol or those involved in hydrogen reuptake, and by expressing an appropriate set of hydrogenase and/or other metabolic regulatory proteins, such as, for example, hydrogenase maturation proteins or transcription factors. *See, e.g.*, Toshinori Maeda et al., "Enhanced hydrogen production from glucose by metabolically engineered *Escherichia coli*," *Appl. Microbiol. Biotechnol.* 77(4):879-890 (2007), which is incorporated by reference in its entirety, particularly with respect to production of *E. coli* strains with modified glucose metabolism.

[0288] In some embodiments, the C2- or C3-alcohol or diol is ethanol. Provided herein are cells in oxygen-limited culture for co-production of isoprene and ethanol, methods of co-producing isoprene and ethanol by culturing such cells under conditions suitable for the co-production of isoprene and ethanol, and compositions comprising isoprene, comprising ethanol or comprising isoprene and ethanol. In some embodiments, the compositions further

comprise oxygen, carbon dioxide, or nitrogen, and 1.0×10^{-4} molar percent or less of non-methane volatile hydrocarbons. Both isoprene and ethanol can be recovered and purified as necessary. Recovered isoprene can be polymerized to produce synthetic rubber. Recovered ethanol can be used to power the fermentation process, thereby reducing the cost of isoprene production, reducing potential hazards associated with accumulation of high ethanol concentrations during conventional fermentation, and reducing the overall 'carbon footprint' of the process.

[0289] Co-generation of isoprene and ethanol provides a way to increase the theoretical yield of isoprene from glucose by the DXP pathway, as the ATP generated in the production of ethanol can be utilized in the pathway to make isoprene. Moreover, the process would run anaerobically, decreasing capital investment for oxygen transfer. The process could even run in existing ethanol plants, in terms of tank stirring. Co-generation of isoprene and ethanol can be done in a variety of cell types, including yeast, such as *Saccharomyces cerevisiae*, and bacteria, such as *Escherichia coli* and *Zymomonas mobilis*. While *E. coli* can produce ethanol when it is grown anaerobically, using the enzyme adhE to go from acetyl-CoA to ethanol via acetaldehyde, ethanol production can be improved by expressing one or more enzymes associated with biochemical reactions around pyruvate in *E. coli* or other bacteria, such as *Zymomonas mobilis*. For example, ethanol production in *E. coli* can be greatly improved by co-expression of pyruvate decarboxylase (pdc) from *Zymomonas mobilis* (*see* Example 28).

[0290] In some embodiments, the C2- or C3-alcohol or diol is 1,2-propanediol. Provided herein are cells in oxygen-limited culture for co-production of isoprene and 1,2-propanediol, methods of co-producing isoprene and 1,2-propanediol by culturing such cells under conditions suitable for the co-production of isoprene and 1,2-propanediol, and compositions comprising isoprene, comprising 1,2-propanediol or comprising isoprene and 1,2-propanediol. In some embodiments, the compositions further comprise oxygen, carbon dioxide, or nitrogen, and 1.0×10^{-4} molar percent or less of non-methane volatile hydrocarbons. Both isoprene and 1,2-propanediol can be recovered and purified as necessary. Recovered isoprene can be polymerized to produce synthetic rubber. Recovered 1,2-propanediol can be used to power the fermentation process, thereby reducing the cost of isoprene production, reducing potential hazards associated with accumulation of high 1,2-

propanediol concentrations during conventional fermentation, and reducing the overall 'carbon footprint' of the process.

[0291] In some embodiments, the C2- or C3-alcohol or diol is 1,3-propanediol. Provided herein are cells in oxygen-limited culture for co-production of isoprene and 1,3-propanediol, methods of co-producing isoprene and 1,3-propanediol by culturing such cells under conditions suitable for the co-production of isoprene and 1,3-propanediol, and compositions comprising isoprene, comprising 1,3-propanediol or comprising isoprene and 1,3-propanediol. In some embodiments, the compositions further comprise oxygen, carbon dioxide, or nitrogen, and 1.0×10^{-4} molar percent or less of non-methane volatile hydrocarbons. Both isoprene and 1,3-propanediol can be recovered and purified as necessary. Recovered isoprene can be polymerized to produce synthetic rubber. Recovered 1,3-propanediol can be used to power the fermentation process, thereby reducing the cost of isoprene production, reducing potential hazards associated with accumulation of high 1,3-propanediol concentrations during conventional fermentation, and reducing the overall 'carbon footprint' of the process.

Definitions

[0292] Unless defined otherwise, the meanings of all technical and scientific terms used herein are those commonly understood by one of skill in the art to which this invention belongs. Singleton, *et al.*, Dictionary of Microbiology and Molecular Biology, 2nd ed., John Wiley and Sons, New York (1994), and Hale & Marham, The Harper Collins Dictionary of Biology, Harper Perennial, N.Y. (1991) provide one of skill with a general dictionary of many of the terms used in this invention. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary. One of skill in the art will also appreciate that any methods and materials similar or equivalent to those described herein can also be used to practice or test the invention.

[0293] The headings provided herein are not limitations of the various aspects or embodiments of the invention which can be had by reference to the specification as a whole.

[0294] For use herein, unless clearly indicated otherwise, use of the terms "a", "an," and the like refers to one or more.

[0295] Reference to "about" a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter per se. For example, description referring to "about X" includes description of "X." Numeric ranges are inclusive of the numbers defining the range.

[0296] It is understood that aspects and embodiments of the invention described herein include "comprising," "consisting," and "consisting essentially of" aspects and embodiments.

[0297] As used herein, the term "C2- or C3-alcohol or diol" includes, but is not limited to, ethanol (CAS No. 64-17-5), 1-propanol (CAS No. 71-23-8), 2-propanol (CAS No. 67-63-0), 1,2-propanediol (CAS No. 57-55-6), 1,3-propanediol (CAS No. 504-63-2), and glycerol (CAS No. 56-81-5). Unless otherwise indicated, the term "1,2-propanediol" refers to 1,2-(R)-propanediol, 1,2-(S)-propanediol, or a racemic mixture of 1,2-(R/S)-propanediol.

[0298] As used herein, the term "polypeptides" includes polypeptides, proteins, peptides, fragments of polypeptides, and fusion polypeptides.

[0299] As used herein, an "isolated polypeptide" is not part of a library of polypeptides, such as a library of 2, 5, 10, 20, 50 or more different polypeptides and is separated from at least one component with which it occurs in nature. An isolated polypeptide can be obtained, for example, by expression of a recombinant nucleic acid encoding the polypeptide.

[0300] By "heterologous polypeptide" is meant a polypeptide whose amino acid sequence is not identical to that of another polypeptide naturally expressed in the same host cell. In particular, a heterologous polypeptide is not identical to a wild-type polypeptide that is found in the same host cell in nature.

[0301] "Codon degeneracy" refers to divergence in the genetic code permitting variation of the nucleotide sequence without affecting the amino acid sequence of an encoded polypeptide. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a nucleic acid for improved expression in a host cell, it is desirable in some embodiments to design the nucleic acid such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

[0302] As used herein, a "nucleic acid" refers to two or more deoxyribonucleotides and/or ribonucleotides covalently joined together in either single or double-stranded form. It is to be understood that mutations, including single nucleotide mutations, can occur within a nucleic acid as defined herein.

[0303] By "recombinant nucleic acid" is meant a nucleic acid of interest that is free of one or more nucleic acids (*e.g.*, genes) which, in the genome occurring in nature of the organism from which the nucleic acid of interest is derived, flank the nucleic acid of interest. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (*e.g.*, a cDNA, a genomic DNA fragment, or a cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It is to be understood that mutations, including single nucleotide mutations, can occur within a nucleic acid as defined herein.

[0304] By "heterologous nucleic acid" is meant a nucleic acid whose nucleic acid sequence is not identical to that of another nucleic acid naturally found in the same host cell. In particular, a heterologous nucleic acid is not identical to a wild-type nucleic acid that is found in the same host cell in nature.

[0305] As used herein, a "vector" means a construct that is capable of delivering, and desirably expressing one or more nucleic acids of interest in a host cell. Examples of vectors include, but are not limited to, plasmids, viral vectors, DNA or RNA expression vectors, cosmids, and phage vectors.

[0306] As used herein, an "expression control sequence" means a nucleic acid sequence that directs transcription of a nucleic acid of interest. An expression control sequence can be a promoter, such as a constitutive or an inducible promoter, or an enhancer. An "inducible promoter" is a promoter that is active under environmental or developmental regulation. The expression control sequence is operably linked to the nucleic acid segment to be transcribed.

[0307] The term "selective marker" or "selectable marker" refers to a nucleic acid capable of expression in a host cell that allows for ease of selection of those host cells containing an introduced nucleic acid or vector. Examples of selectable markers include, but are not limited to, antibiotic resistance nucleic acids (*e.g.*, kanamycin, ampicillin, carbenicillin, gentamicin,

hygromycin, phleomycin, bleomycin, neomycin, or chloramphenicol) and/or nucleic acids that confer a metabolic advantage, such as a nutritional advantage on the host cell. Exemplary nutritional selective markers include those markers known in the art as *amd*S, *arg*B, and *pyr4*.

Isoprene

[0308] As used herein, the term "isoprene" or "2-methyl-1,3-butadiene" (CAS# 78-79-5) refers to the direct and final volatile C5 hydrocarbon product from the elimination of pyrophosphate from 3,3-dimethylallyl pyrophosphate (DMAPP), and does not involve the linking or polymerization of one or more isopentenyl diphosphate (IPP) molecules to one or more DMAPP molecules. The term "isoprene" is not generally intended to be limited to its method of production unless indicated otherwise herein.

[0309] The vast majority of isoprene is derived from petrochemical sources as an impure C5 hydrocarbon fraction which requires extensive purification before the material is suitable for polymerization. Several impurities are particularly problematic given their structural similarity to isoprene and the fact that they can act as polymerization catalyst poisons. Such compounds include 1,3-cyclopentadiene, *trans*-1,3-pentadiene, *cis*-1,3-pentadiene, 1,4-pentadiene, 1-pentyne, 2-pentyne, 3-methyl-l-butyne, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, and *cis*-pent-3-ene-1-yne (Figure 90). In some embodiments, the isoprene composition of the invention is substantially free of any contaminating unsaturated C5 hydrocarbons. As described further in Example 10, no detectable amount of unsaturated C5 hydrocarbons other than isoprene (such as 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 3-methyl-l-butyne, *trans*-piperylene, *cis*-piperylene, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne) was found in isoprene compositions produced using the methods described herein. Some isoprene compositions produced using the methods described herein contain ethanol, acetone, and C5 prenyl alcohols as determined by GC/MS analysis. All of these components are far more readily removed from the isoprene stream than the isomeric C5 hydrocarbon fractions that are present in isoprene compositions derived from petrochemical sources. Accordingly, in some embodiments, the isoprene compositions of the invention require minimal treatment in order to be of polymerization grade.

[0310] In one aspect, compositions and methods of the invention increase the rate of isoprene production and increase the total amount of isoprene that is produced. For example, cell culture systems that generate 4.8 x 10^4 nmole/g_{wcm}/hr of isoprene have been produced (Table 1). The efficiency of these systems is demonstrated by the conversion of about 2.2% of the carbon that the cells consume from a cell culture medium into isoprene. As shown in the Examples and Table 2, approximately 3 g of isoprene per liter of broth was generated. If desired, even greater amounts of isoprene can be obtained using other conditions, such as those described herein. In some embodiments, a renewable carbon source is used for the production of isoprene. In some embodiments, the production of isoprene is decoupled from the growth of the cells. In some embodiments, the concentrations of isoprene and any oxidants are within the nonflammable ranges to reduce or eliminate the risk that a fire may occur during production or recovery of isoprene. The compositions and methods of the present invention are desirable because they allow high isoprene yield per cell, high carbon yield, high isoprene purity, high productivity, low energy usage, low production cost and investment, and minimal side reactions. This efficient, large scale, biosynthetic process for isoprene production provides an isoprene source for synthetic isoprene-based rubber and provides a desirable, low-cost alternative to using natural rubber.

[0311] As discussed further below, the amount of isoprene produced by cells can be greatly increased by introducing a heterologous nucleic acid encoding an isoprene synthase polypeptide (*e.g.*, a plant isoprene synthase polypeptide) into the cells. Isoprene synthase polypeptides convert dimethylallyl diphosphate (DMAPP) into isoprene. As shown in the Examples, a heterologous *Pueraria Montana* (kudzu) isoprene synthase polypeptide was expressed in a variety of host cells, such as *Escherichia coli, Panteoa citrea, Bacillus subtilis, Yarrowia lipolytica,* and *Trichoderma reesei*. All of these cells produced more isoprene than the corresponding cells without the heterologous isoprene synthase polypeptide. As illustrated in Tables 1 and 2, large amounts of isoprene are produced using the methods described herein. For example, *B. subtilis* cells with a heterologous isoprene synthase nucleic acid produced approximately 10-fold more isoprene in a 14 liter fermentor than the corresponding control *B. subtilis* cells without the heterologous nucleic acid (Table 2). The production of 300 mg of isoprene per liter of broth (mg/L, wherein the volume of broth includes both the volume of the cell medium and the volume of the cells) by *E. coli* and 30 mg/L by *B. subtilis* in fermentors indicates that significant amounts of isoprene can be generated (Table 2). If desired, isoprene can be produced on an even larger scale or other

conditions described herein can be used to further increase the amount of isoprene. The vectors listed in Tables 1 and 2 and the experimental conditions are described in further detail below and in the Examples section.

Table 1: Exemplary yields of isoprene from a shake flask using the cell cultures and methods of the invention. The assay for measuring isoprene production is described in Example I, part II. For this assay, a sample was removed at one or more time points from the shake flask and cultured for 30 minutes. The amount of isoprene produced in this sample was then measured. The headspace concentration and specific rate of isoprene production are listed in Table 1 and described further herein.

Strain	Isoprene Production in a Headspace vial*	
	Headspace concentration $\mu g/L_{gas}$	Specific Rate $\mu g/L_{broth}/hr/OD$ ($nmol/g_{wcm}/hr$)
E. coli BL21/ pTrcKudzu IS	1.40	53.2 (781.2)
E. coli BL21/ pCL DXS yidi Kudzu IS	7.61	289.1 (4.25×10^3)
E. coli BL21/MCM127 with kudzu IS and entire MVA pathway	23.0	874.1 (12.8×10^3)
E. coli BL21/ pET N-HisKudzu IS	1.49	56.6 (831.1)
Pantoea citrea /pTrcKudzu IS	0.66	25.1 (368.6)
E. coli w/ Poplar IS [Miller (2001)]	-	5.6 (82.2)
Bacillis licheniformis Fall US 5849970	-	4.2 (61.4)
Yarrowia lipolytica with kudzu isoprene synthase	~0.05 μg/L	~2 (~30)

Strain	Isoprene Production in a Headspace vial*	
	Headspace concentration $\mu g/L_{gas}$	Specific Rate $\mu g/L_{broth}/hr/OD$ ($nmol/g_{wcm}/hr$)
Trichoderma reesei with kudzu isoprene synthase	~0.05 μg/L	~2 (~30)
E. coli BL21/ pTrcKKD$_y$I$_k$IS with kudzu IS and lower MVA pathway	85.9	3.2×10^3 (4.8×10^4)

*Normalized to 1 mL of 1 OD_{600}, cultured for 1 hour in a sealed headspace vial with a liquid to headspace volume ratio of 1:19.

Table 2: Exemplary yields of isoprene in a fermentor using the cell cultures and methods of the invention. The assay for measuring isoprene production is described in Example I, part II. For this assay, a sample of the off-gas of the fermentor was taken and analyzed for the amount of isoprene. The peak headspace concentration (which is the highest headspace concentration during the fermentation), titer (which is the cumulative, total amount of isoprene produced per liter of broth), and peak specific rate of isoprene production (which is the highest specific rate during the fermentation) are listed in Table 2 and described further herein.

Strain	Isoprene Production in Fermentors		
	Peak Headspace concentration** ($\mu g/L_{gas}$)	Titer (mg/L_{broth})	Peak Specific rate $\mu g/L_{broth}/hr/OD$ ($nmol/g_{wcm}/hr$)
E. coli BL21 /pTrcKudzu with Kudzu IS	52	41.2	37 (543.3)
E. coli FM5/pTrcKudzu IS	3	3.5	21.4 (308.1)
E. coli BL21/ triple strain (DXS, yidi, IS)	285	300	240 (3.52×10^3)
E. coli FM5/ triple strain (DXS, yidi, IS)	50.8	29	180.8 (2.65×10^3)
E. coli/MCM127 with Kudzu IS and entire MVA	3815	3044	992.5

Strain	Isoprene Production in Fermentors		
	Peak Headspace concentration** ($\mu g/L_{gas}$)	Titer (mg/L_{broth})	Peak Specific rate $\mu g/L_{broth}/hr/OD$ ($nmol/g_{wcm}/hr$)
pathway			(1.46×10^4)
E. coli BL21/pCLPtrc UpperPathway gi1.2 integrated lower pathway pTrcKudzu	2418	1640	1248 (1.83×10^4)
E. coli BL21/MCM401 with 4 x 50 μM IPTG	13991	23805	3733 (5.49×10^4)
E. coli BL21/MCM401 with 2 x 1000 μM IPTG	22375	19541	5839.5 (8.59×10^4)
E. coli BL21/pCLPtrc UpperPathwayHGS2 - pTrcKKDyIkIS	3500	3300	1088 (1.60×10^4)
Bacillus subtilis wild-type	1.5	2.5	0.8 (11.7)
Bacillus pBS Kudzu IS	16.6	~30 (over 100 hrs)	5 (73.4)
Bacillus Marburg 6051 [Wagner and Fall (1999)]	2.04	0.61	24.5 (359.8)
Bacillus Marburg 6051 Fall US 5849970	0.7	0.15	6.8 (100)

**Normalized to an off-gas flow rate of 1 vvm (1 volume off-gas per 1 L_{broth} per minute).

[0312] Additionally, isoprene production by cells that contain a heterologous isoprene synthase nucleic acid can be enhanced by increasing the amount of a 1-deoxy-D-xylulose-5-phosphate synthase (DXS) polypeptide and/or an isopentenyl diphosphate isomerase (IDI) polypeptide expressed by the cells. For example, a DXS nucleic acid and/or an IDI nucleic acid can be introduced into the cells. The DXS nucleic acid may be a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid. Similarly, the IDI nucleic acid may be a heterologous nucleic acid or a duplicate copy of an endogenous nucleic acid. In some embodiments, the amount of DXS and/or IDI polypeptide is increased by replacing the endogenous DXS and/or IDI promoters or regulatory regions with other promoters and/or regulatory regions that result in greater transcription of the DXS and/or IDI nucleic acids. In some embodiments, the cells contain both a heterologous nucleic acid encoding an isoprene

synthase polypeptide (*e.g.*, a plant isoprene synthase nucleic acid) and a duplicate copy of an endogenous nucleic acid encoding an isoprene synthase polypeptide.

[0313] The encoded DXS and IDI polypeptides are part of the DXP pathway for the biosynthesis of isoprene (Figure 19A). DXS polypeptides convert pyruvate and D-glyceraldehyde-3-phosphate into 1-deoxy-D-xylulose-5-phosphate. While not intending to be bound by any particular theory, it is believed that increasing the amount of DXS polypeptide increases the flow of carbon through the DXP pathway, leading to greater isoprene production. IDI polypeptides catalyze the interconversion of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). While not intending to be bound by any particular theory, it is believed that increasing the amount of IDI polypeptide in cells increases the amount (and conversion rate) of IPP that is converted into DMAPP, which in turn is converted into isoprene.

[0314] For example, fermentation of *E. coli* cells with a kudzu isoprene synthase, *S. cerevisia* IDI, and *E. coli* DXS nucleic acids was used to produce isoprene. The levels of isoprene varied from 50 to 300 μg/L over a time period of 15 hours (Example 7, part VII).

[0315] In some embodiments, the presence of heterologous or extra endogenous isoprene synthase, IDI, and DXS nucleic acids causes cells to grow more reproducibly or remain viable for longer compared to the corresponding cell with only one or two of these heterologous or extra endogenous nucleic acids. For example, cells containing heterologous isoprene synthase, IDI, and DXS nucleic acids grew better than cells with only heterologous isoprene synthase and DXS nucleic acids or with only a heterologous isoprene synthase nucleic acid. Also, heterologous isoprene synthase, IDI, and DXS nucleic acids were successfully operably linked to a strong promoter on a high copy plasmid that was maintained by *E. coli* cells, suggesting that large amounts of these polypeptides could be expressed in the cells without causing an excessive amount of toxicity to the cells. While not intending to be bound to a particular theory, it is believed that the presence of heterologous or extra endogenous isoprene synthase and IDI nucleic acids may reduce the amount of one or more potentially toxic intermediates that would otherwise accumulate if only a heterologous or extra endogenous DXS nucleic acid was present in the cells.

[0316] In some embodiments, the production of isoprene by cells that contain a heterologous isoprene synthase nucleic acid is augmented by increasing the amount of a

MVA polypeptide expressed by the cells (Figures 19A and 19B). Exemplary MVA pathways polypeptides include any of the following polypeptides: acetyl-CoA acetyltransferase (AA-CoA thiolase) polypeptides, 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA synthase) polypeptides, 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) polypeptides, mevalonate kinase (MVK) polypeptides, phosphomevalonate kinase (PMK) polypeptides, diphosphomevalonte decarboxylase (MVD) polypeptides, phosphomevalonate decarboxylase (PMDC) polypeptides, isopentenyl phosphate kinase (IPK) polypeptides, IDI polypeptides, and polypeptides (*e.g.*, fusion polypeptides) having an activity of two or more MVA pathway polypeptides. For example, one or more MVA pathway nucleic acids can be introduced into the cells. In some embodiments, the cells contain the upper MVA pathway, which includes AA-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase nucleic acids. In some embodiments, the cells contain the lower MVA pathway, which includes MVK, PMK, MVD, and IDI nucleic acids. In some embodiments, the cells contain an entire MVA pathway that includes AA-CoA thiolase, HMG-CoA synthase, HMG-CoA reductase, MVK, PMK, MVD, and IDI nucleic acids. In some embodiments, the cells contain an entire MVA pathway that includes AA-CoA thiolase, HMG-CoA synthase, HMG-CoA reductase, MVK, PMDC, IPK, and IDI nucleic acids. The MVA pathway nucleic acids may be heterologous nucleic acids or duplicate copies of endogenous nucleic acids. In some embodiments, the amount of one or more MVA pathway polypeptides is increased by replacing the endogenous promoters or regulatory regions for the MVA pathway nucleic acids with other promoters and/or regulatory regions that result in greater transcription of the MVA pathway nucleic acids. In some embodiments, the cells contain both a heterologous nucleic acid encoding an isoprene synthase polypeptide (*e.g.*, a plant isoprene synthase nucleic acid) and a duplicate copy of an endogenous nucleic acid encoding an isoprene synthase polypeptide.

[0317] For example, *E. coli* cells containing a nucleic acid encoding a kudzu isoprene synthase polypeptide and nucleic acids encoding *Saccharomyces cerevisiae* MVK, PMK, MVD, and IDI polypeptides generated isoprene at a rate of 6.67 x 10^{-4} mol/L_{broth}/OD_{600}/hr (*see* Example 8). Additionally, a 14 liter fermentation of *E. coli* cells with nucleic acids encoding *Enterococcus faecalis* AA-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase polypeptides produced 22 grams of mevalonic acid (an intermediate of the MVA pathway). A shake flask of these cells produced 2-4 grams of mevalonic acid per liter. These results indicate that heterologous MVA pathways nucleic acids are active in *E. coli*. *E. coli*

cells that contain nucleic acids for both the upper MVA pathway and the lower MVA pathway as well as a kudzu isoprene synthase (strain MCM 127) produced significantly more isoprene (874 ug/L) compared to *E. coli* cells with nucleic acids for only the lower MVA pathway and the kudzu isoprene synthase (strain MCM 131) (*see* Table 3 and Example 8, part VIII).

[0318] In some embodiments, at least a portion of the cells maintain the heterologous isoprene synthase, DXS, IDI, and/or MVA pathway nucleic acid for at least about 5, 10, 20, 50, 75, 100, 200, 300, or more cell divisions in a continuous culture (such as a continuous culture without dilution). In some embodiments of any of the aspects of the invention, the nucleic acid comprising the heterologous or duplicate copy of an endogenous isoprene synthase, DXS, IDI, and/or MVA pathway nucleic acid also comprises a selective marker, such as a kanamycin, ampicillin, carbenicillin, gentamicin, hygromycin, phleomycin, bleomycin, neomycin, or chloramphenicol antibiotic resistance nucleic acid.

[0319] As indicated in Example 7, part VI, the amount of isoprene produced can be further increased by adding yeast extract to the cell culture medium. In this example, the amount of isoprene produced was linearly proportional to the amount of yeast extract in the cell medium for the concentrations tested (Figure 48C). Additionally, approximately 0.11 grams of isoprene per liter of broth was produced from a cell medium with yeast extract and glucose (Example 7, part VIII). Both of these experiments used *E. coli* cells with kudzu isoprene synthase, *S. cerevisia* IDI, and *E. coli* DXS nucleic acids to produce isoprene. Increasing the amount of yeast extract in the presence of glucose resulted in more isoprene being produced than increasing the amount of glucose in the presence of yeast extract. Also, increasing the amount of yeast extract allowed the cells to produce a high level of isoprene for a longer length of time and improved the health of the cells.

[0320] Isoprene production was also demonstrated using three types of hydrolyzed biomass (bagasse, corn stover, and soft wood pulp) as the carbon source (Figures 46A-C). *E. coli* cells with kudzu isoprene synthase, *S. cerevisia* IDI, and *E. coli* DXS nucleic acids produced as much isoprene from these hydrolyzed biomass carbon sources as from the equivalent amount of glucose (*e.g.*, 1% glucose, w/v). If desired, any other biomass carbon source can be used in the compositions and methods of the invention. Biomass carbon sources are desirable because they are cheaper than many conventional cell mediums, thereby facilitating the economical production of isoprene.

[0321] Additionally, invert sugar was shown to function as a carbon source for the generation of isoprene (Figures 47C and 96-98). For example, 2.4 g/L of isoprene was produced from cells expressing MVA pathway polypeptides and a Kudzu isoprene synthase (Example 8, part XV). Glycerol was as also used as a carbon source for the generation of 2.2 mg/L of isoprene from cells expressing a Kudzu isoprene synthase (Example 8, part XIV). Expressing a DXS nucleic acid, an IDI nucleic acid, and/or one or more MVA pathway nucleic acids (such as nucleic acids encoding the entire MVA pathway) in addition to an isoprene synthase nucleic acid may increase the production of isoprene from glycerol.

[0322] In some embodiments, an oil is included in the cell medium. For example, *B. subtilis* cells containing a kudzu isoprene synthase nucleic acid produced isoprene when cultured in a cell medium containing an oil and a source of glucose (Example 4, part III). In some embodiments, more than one oil (such as 2, 3, 4, 5, or more oils) is included in the cell medium. While not intending to be bound to any particular theory, it is believed that (i) the oil may increase the amount of carbon in the cells that is available for conversion to isoprene, (ii) the oil may increase the amount of acetyl-CoA in the cells, thereby increasing the carbon flow through the MVA pathway, and/or (ii) the oil may provide extra nutrients to the cells, which is desirable since much of the carbon in the cells is converted to isoprene rather than other products. In some embodiments, cells that are cultured in a cell medium containing oil naturally use the MVA pathway to produce isoprene or are genetically modified to contain nucleic acids for the entire MVA pathway. In some embodiments, the oil is partially or completely hydrolyzed before being added to the cell culture medium to facilitate the use of the oil by the host cells.

[0323] One of the major hurdles to commercial production of small molecules such as isoprene in cells (*e.g.*, bacteria) is the decoupling of production of the molecule from growth of the cells. In some embodiments for the commercially viable production of isoprene, a signficiant amount of the carbon from the feedstock is converted to isoprene, rather than to the growth and maintenance of the cells ("carbon efficiency"). In various embodiments, the cells convert greater than or about 0.0015, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.12, 0.14, 0.16, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, or 8.0% of the carbon in the cell culture medium into isoprene. In particular embodiments, a significant portion of the carbon from the feedstock that is converted to downstream products is converted to isoprene. As described further in Example 11, *E. coli*

cells expressing MVA pathway and kudzu isoprene synthase nucleic acids exhibited decoupling of the production of isoprene or the intermediate mevalonic acid from growth, resulting in high carbon efficiency. In particular, mevalonic acid was formed from cells expressing the upper MVA pathway from *Enterococcus faecalis*. Isoprene was formed from cells expressing the upper MVA pathway from *Enterococcus faecalis*, the lower MVA pathway from *Saccharomyces cerevisiae*, and the isoprene synthase from *Pueraria montana* (Kudzu). This decoupling of isoprene or mevalonic acid production from growth was demonstrated in four different strains of *E. coli*: BL21(LDE3), BL21(LDE3) Tuner, FM5, and MG1655. The first two *E. coli* strains are B strains, and the latter two are K12 strains. Decoupling of production from growth was also demonstrated in a variant of MG1655 with *ack* and *pta* genes deleted. This variant also demonstrated less production of acetate.

Exemplary Polypeptides and Nucleic Acids

[0324] Various isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor polypeptides and nucleic acids can be used in the compositions and methods of the invention.

[0325] In some embodiments, the fusion polypeptide includes part or all of a first polypeptide (*e.g.*, an isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor polypeptide or catalytically active fragment thereof) and may optionally include part or all of a second polypeptide (*e.g.*, a peptide that facilitates purification or detection of the fusion polypeptide, such as a His-tag). In some embodiments, the fusion polypeptide has an activity of two or more MVA pathway polypeptides (such as AA-CoA thiolase and HMG-CoA reductase polypeptides). In some embodiments, the polypeptide is a naturally-occurring polypeptide (such as the polypeptide encoded by an *Enterococcus faecalis mvaE* nucleic acid) that has an activity of two or more MVA pathway polypeptides.

[0326] In various embodiments, a polypeptide has at least or about 50, 100, 150, 175, 200, 250, 300, 350, 400, or more amino acids. In some embodiments, the polypeptide fragment contains at least or about 25, 50, 75, 100, 150, 200, 300, or more contiguous amino acids from a full-length polypeptide and has at least or about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100% of an activity of a corresponding full-length polypeptide. In particular embodiments, the polypeptide includes a segment of or the entire

amino acid sequence of any naturally-occurring isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor polypeptide. In some embodiments, the polypeptide has one or more mutations compared to the sequence of a wild-type (*i.e.*, a sequence occurring in nature) isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor polypeptide.

[0327] In some embodiments, the polypeptide is an isolated polypeptide. In some embodiments, the polypeptide is a heterologous polypeptide.

[0328] In some embodiments, the nucleic acid is a recombinant nucleic acid. In some embodiments, an isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor nucleic acid is operably linked to another nucleic acid encoding all or a portion of another polypeptide such that the recombinant nucleic acid encodes a fusion polypeptide that includes an isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor polypeptide and all or part of another polypeptide (*e.g.*, a peptide that facilitates purification or detection of the fusion polypeptide, such as a His-tag). In some embodiments, part or all of a recombinant nucleic acid is chemically synthesized.

[0329] In some embodiments, the nucleic acid is a heterologous nucleic acid. In particular embodiments, the nucleic acid includes a segment of or the entire nucleic acid sequence of any naturally-occurring isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor nucleic acid. In some embodiments, the nucleic acid includes at least or about 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, or more contiguous nucleotides from a naturally-occurring isoprene synthase nucleic acid DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor nucleic acid. In some embodiments, the nucleic acid has one or more mutations compared to the sequence of a wild-type (*i.e.*, a sequence occurring in nature) isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor nucleic acid. In some embodiments, the nucleic acid has one or more mutations (*e.g.*, a silent mutation) that increase the transcription or translation of isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, or transcription factor nucleic acid. In some embodiments, the nucleic acid is a degenerate variant of any nucleic acid encoding an isoprene synthase, DXS, IDI, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor polypeptide.

[0330] The accession numbers of exemplary isoprene synthase, DXS, IDI, and/or MVA pathway polypeptides and nucleic acids are listed in Appendix 1 (the accession numbers of Appendix 1 and their corresponding sequences are herein incorporated by reference in their entireties, particularly with respect to the amino acid and nucleic acid sequences of isoprene synthase, DXS, IDI, and/or MVA pathway polypeptides and nucleic acids). The Kegg database also contains the amino acid and nucleic acid sequences of numerous exemplary isoprene synthase, DXS, IDI, and/or MVA pathway polypeptides and nucleic acids (*see*, for example, the world-wide web at "genome.jp/kegg/pathway/map/map00100.html" and the sequences therein, which are each hereby incorporated by reference in their entireties, particularly with respect to the amino acid and nucleic acid sequences of isoprene synthase, DXS, IDI, and/or MVA pathway polypeptides and nucleic acids). In some embodiments, one or more of the isoprene synthase, DXS, IDI, and/or MVA pathway polypeptides and/or nucleic acids have a sequence identical to a sequence publicly available on December 12, 2007 or September 14, 2008 such as any of the sequences that correspond to any of the accession numbers in Appendix 1 or any of the sequences present in the Kegg database. Additional exemplary isoprene synthase, DXS, IDI, and/or MVA pathway polypeptides and nucleic acids are described further below.

Exemplary Isoprene Synthase Polypeptides and Nucleic Acids

[0331] As noted above, isoprene synthase polypeptides convert dimethylallyl diphosphate (DMAPP) into isoprene. Exemplary isoprene synthase polypeptides include polypeptides, fragments of polypeptides, peptides, and fusions polypeptides that have at least one activity of an isoprene synthase polypeptide. Standard methods can be used to determine whether a polypeptide has isoprene synthase polypeptide activity by measuring the ability of the polypeptide to convert DMAPP into isoprene *in vitro*, in a cell extract, or *in vivo*. In an exemplary assay, cell extracts are prepared by growing a strain (*e.g.*, the *E. coli*/pTrcKudzu strain described herein) in the shake flask method as described in Example 1. After induction is complete, approximately 10 mL of cells are pelleted by centrifugation at 7000 x *g* for 10 minutes and resuspended in 5 ml of PEB without glycerol. The cells are lysed using a French Pressure cell using standard procedures. Alternatively the cells are treated with lysozyme (Ready-Lyse lysozyme solution; EpiCentre) after a freeze/thaw at -80C.

[0332] Isoprene synthase polypeptide activity in the cell extract can be measured, for example, as described in Silver *et al.*, J. Biol. Chem. 270:13010-13016, 1995 and references

therein, which are each hereby incorporated by reference in their entireties, particularly with respect to assays for isoprene synthase polypeptide activity. DMAPP (Sigma) is evaporated to dryness under a stream of nitrogen and rehydrated to a concentration of 100 mM in 100 mM potassium phosphate buffer pH 8.2 and stored at -20 °C. To perform the assay, a solution of 5 μL of 1M $MgCl_2$, 1 mM (250 μg/ml) DMAPP, 65 μL of Plant Extract Buffer (PEB) (50 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 5% glycerol, and 2 mM DTT) is added to 25 μL of cell extract in a 20 ml Headspace vial with a metal screw cap and teflon coated silicon septum (Agilent Technologies) and cultured at 37 °C for 15 minutes with shaking. The reaction is quenched by adding 200 μL of 250 mM EDTA and quantified by GC/MS as described in Example 1, part II.

[0333] Exemplary isoprene synthase nucleic acids include nucleic acids that encode a polypeptide, fragment of a polypeptide, peptide, or fusion polypeptide that has at least one activity of an isoprene synthase polypeptide. Exemplary isoprene synthase polypeptides and nucleic acids include naturally-occurring polypeptides and nucleic acids from any of the source organisms described herein as well as mutant polypeptides and nucleic acids derived from any of the source organisms described herein.

[0334] In some embodiments, the isoprene synthase polypeptide or nucleic acid is from the family Fabaceae, such as the Faboideae subfamily. In some embodiments, the isoprene synthase polypeptide or nucleic acid is a polypeptide or nucleic acid from *Pueraria montana* (kudzu) (Sharkey *et al.*, Plant Physiology 137: 700-712, 2005), *Pueraria lobata*, poplar (such as *Populus alba*, *Populus nigra*, *Populus trichocarpa*, or *Populus alba x tremula* (CAC35696) Miller *et al.*, Planta 213: 483-487, 2001) aspen (such as *Populus tremuloides*) Silver *et al.*, JBC 270(22): 13010-1316, 1995), or English Oak (*Quercus robur*) (Zimmer *et al.*, WO 98/02550), which are each hereby incorporated by reference in their entireties, particularly with respect to isoprene synthase nucleic acids and the expression of isoprene synthase polypeptides. Suitable isoprene synthases include, but are not limited to, those identified by Genbank Accession Nos. AY341431, AY316691, AY279379, AJ457070, and AY182241, which are each hereby incorporated by reference in their entireties, particularly with respect to sequences of isoprene synthase nucleic acids and polypeptides. In some embodiments, the isoprene synthase polypeptide or nucleic acid is not a naturally-occurring polypeptide or nucleic acid from *Quercus robur* (*i.e.*, the isoprene synthase polypeptide or nucleic acid is an isoprene synthase polypeptide or nucleic acid other than a naturally-

occurring polypeptide or nucleic acid from *Quercus robur*). In some embodiments, the isoprene synthase nucleic acid or polypeptide is a naturally-occurring polypeptide or nucleic acid from poplar. In some embodiments, the isoprene synthase nucleic acid or polypeptide is not a naturally-occurring polypeptide or nucleic acid from poplar.

Exemplary DXS Polypeptides and Nucleic Acids

[0335] As noted above, 1-deoxy-D-xylulose-5-phosphate synthase (DXS) polypeptides convert pyruvate and D-glyceraldehyde-3-phosphate into 1-deoxy-D-xylulose-5-phosphate. Exemplary DXS polypeptides include polypeptides, fragments of polypeptides, peptides, and fusions polypeptides that have at least one activity of a DXS polypeptide. Standard methods (such as those described herein) can be used to determine whether a polypeptide has DXS polypeptide activity by measuring the ability of the polypeptide to convert pyruvate and D-glyceraldehyde-3-phosphate into 1-deoxy-D-xylulose-5-phosphate *in vitro*, in a cell extract, or *in vivo*. Exemplary DXS nucleic acids include nucleic acids that encode a polypeptide, fragment of a polypeptide, peptide, or fusion polypeptide that has at least one activity of a DXS polypeptide. Exemplary DXS polypeptides and nucleic acids include naturally-occurring polypeptides and nucleic acids from any of the source organisms described herein as well as mutant polypeptides and nucleic acids derived from any of the source organisms described herein.

Exemplary IDI Polypeptides and Nucleic Acids

[0336] Isopentenyl diphosphate isomerase polypeptides (isopentenyl-diphosphate delta-isomerase or IDI) catalyses the interconversion of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (*e.g.*, converting IPP into DMAPP and/or converting DMAPP into IPP). Exemplary IDI polypeptides include polypeptides, fragments of polypeptides, peptides, and fusions polypeptides that have at least one activity of an IDI polypeptide. Standard methods (such as those described herein) can be used to determine whether a polypeptide has IDI polypeptide activity by measuring the ability of the polypeptide to interconvert IPP and DMAPP *in vitro*, in a cell extract, or *in vivo*. Exemplary IDI nucleic acids include nucleic acids that encode a polypeptide, fragment of a polypeptide, peptide, or fusion polypeptide that has at least one activity of an IDI polypeptide. Exemplary IDI polypeptides and nucleic acids include naturally-occurring polypeptides and nucleic acids

from any of the source organisms described herein as well as mutant polypeptides and nucleic acids derived from any of the source organisms described herein.

Exemplary MVA Pathway Polypeptides and Nucleic Acids

[0337] Exemplary MVA pathway polypeptides include acetyl-CoA acetyltransferase (AA-CoA thiolase) polypeptides, 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA synthase) polypeptides, 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) polypeptides, mevalonate kinase (MVK) polypeptides, phosphomevalonate kinase (PMK) polypeptides, diphosphomevalonte decarboxylase (MVD) polypeptides, phosphomevalonate decarboxylase (PMDC) polypeptides, isopentenyl phosphate kinase (IPK) polypeptides, IDI polypeptides, and polypeptides (*e.g.*, fusion polypeptides) having an activity of two or more MVA pathway polypeptides. In particular, MVA pathway polypeptides include polypeptides, fragments of polypeptides, peptides, and fusions polypeptides that have at least one activity of an MVA pathway polypeptide. Exemplary MVA pathway nucleic acids include nucleic acids that encode a polypeptide, fragment of a polypeptide, peptide, or fusion polypeptide that has at least one activity of an MVA pathway polypeptide. Exemplary MVA pathway polypeptides and nucleic acids include naturally-occurring polypeptides and nucleic acids from any of the source organisms described herein as well as mutant polypeptides and nucleic acids derived from any of the source organisms described herein.

[0338] In particular, acetyl-CoA acetyltransferase polypeptides (AA-CoA thiolase or AACT) convert two molecules of acetyl-CoA into acetoacetyl-CoA. Standard methods (such as those described herein) can be used to determine whether a polypeptide has AA-CoA thiolase polypeptide activity by measuring the ability of the polypeptide to convert two molecules of acetyl-CoA into acetoacetyl-CoA *in vitro*, in a cell extract, or *in vivo*.

[0339] 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA synthase or HMGS) polypeptides convert acetoacetyl-CoA into 3-hydroxy-3-methylglutaryl-CoA. Standard methods (such as those described herein) can be used to determine whether a polypeptide has HMG-CoA synthase polypeptide activity by measuring the ability of the polypeptide to convert acetoacetyl-CoA into 3-hydroxy-3-methylglutaryl-CoA *in vitro*, in a cell extract, or *in vivo*.

[0340] 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase or HMGR) polypeptides convert 3-hydroxy-3-methylglutaryl-CoA into mevalonate. Standard methods (such as those described herein) can be used to determine whether a polypeptide has HMG-CoA reductase polypeptide activity by measuring the ability of the polypeptide to convert 3-hydroxy-3-methylglutaryl-CoA into mevalonate *in vitro*, in a cell extract, or *in vivo*.

[0341] Mevalonate kinase (MVK) polypeptides phosphorylates mevalonate to form mevalonate-5-phosphate. Standard methods (such as those described herein) can be used to determine whether a polypeptide has MVK polypeptide activity by measuring the ability of the polypeptide to convert mevalonate into mevalonate-5-phosphate *in vitro*, in a cell extract, or *in vivo*.

[0342] Phosphomevalonate kinase (PMK) polypeptides phosphorylates mevalonate-5-phosphate to form mevalonate-5-diphosphate. Standard methods (such as those described herein) can be used to determine whether a polypeptide has PMK polypeptide activity by measuring the ability of the polypeptide to convert mevalonate-5-phosphate into mevalonate-5-diphosphate *in vitro*, in a cell extract, or *in vivo*.

[0343] Diphosphomevalonte decarboxylase (MVD or DPMDC) polypeptides convert mevalonate-5-diphosphate into isopentenyl diphosphate (IPP). Standard methods (such as those described herein) can be used to determine whether a polypeptide has MVD polypeptide activity by measuring the ability of the polypeptide to convert mevalonate-5-diphosphate into IPP *in vitro*, in a cell extract, or *in vivo*.

[0344] Phosphomevalonate decarboxylase (PMDC) polypeptides convert mevalonate-5-phosphate into isopentenyl phosphate (IP). Standard methods (such as those described herein) can be used to determine whether a polypeptide has PMDC polypeptide activity by measuring the ability of the polypeptide to convert mevalonate-5-phosphate into IP *in vitro*, in a cell extract, or *in vivo*.

[0345] Isopentenyl phosphate kinase (IPK) polypeptides phosphorylate isopentyl phosphate (IP) to form isopentenyl diphosphate (IPP). Standard methods (such as those described herein) can be used to determine whether a polypeptide has IPK polypeptide activity by measuring the ability of the polypeptide to convert IP into IPP *in vitro*, in a cell extract, or *in vivo*.

[0346] Exemplary IDI polypeptides and nucleic acids are described above.

Exemplary Hydrogenase Polypeptides and Nucleic Acids

[0347] Hydrogenase polypeptides catalyze the reaction: $2H^+ + 2e^- \leftrightarrow H_2$. *In vitro* that reaction is reversible, but certain hydrogenases may work in only one direction *in vivo*, either oxidizing H_2 or reducing H^+. Hydrogenase polypeptides can be oxygen-sensitive, contain complex metal cofactors as part of their catalytic center and sometimes consist of multiple subunits, with hydrogenase gene expression sometimes involving additional accessory polypeptides, such as 'maturation' factors or transcription regulatory factors (*i.e.*, activators or repressors). Hydrogenases are classified into at least three broad groups based upon the type of metal cofactor in their catalytic center: (1) nickel-iron ("NiFe") hydrogenases have a nickel/iron cofactor; (2) iron-iron hydrogenases ("FeFe") have an iron/iron cofactor; and (3) iron/sulfur-free ("Fe") hydrogenases, which lack the 4Fe4S clusters found in groups (1) and (2), have an iron cofactor and a methenyl-tetrahydromethanopterin electron carrier. *See, e.g.*, Chung-Jung Chou et al., "Hydrogenesis in hyperthermophilic microorganisms: implications for biofuels," *Metabol. Eng.* 10:394-404 (2008), and Gönül Vardar-Schara et al., "Metabolically engineered bacteria for producing hydrogen via fermentation," *Microbial Biotechnol.* 1(2):107-125 (2008), both of which are incorporated herein by reference in their entireties, particularly with respect to the various types and classes of hydrogenases. Although many organisms contain multiple hydrogenases, few contain genes for both NiFe and FeFe hydrogenases.

[0348] The catalytic center of NiFe hydrogenases consists of a nickel atom and an iron atom, each with two carbon monoxide (CO) and two cyanide (CN^-) ligands. The NiFe hydrogenases all comprise at least a second subunit containing multiple iron-sulfur (Fe-S) centers for the transfer of electrons to and from the catalytic center. The NiFe hydrogenases can be subdivided into four main classes: (1) respiratory enzymes, which are part of multienzyme systems that couple the oxidation of H_2 to reduction of terminal electron acceptors such as SO_4^{2-} or NO_3^- under anaerobic conditions, or to O_2 in aerobic microorganisms; (2) H_2 sensors, which activate expression of the metabolically active NiFe hyrogenases; (3) cytoplasmic hydrogenases, containing multiple subunits able to utilize $NADP^+$, which are readily reversible in *vitro*, but *in vivo* may only oxidize H_2; and (4) membrane-bound, energy-conserving multienzyme complexes also found in bacteria and

Archaea. Chung-Jung Chou et al., "Hydrogenesis in hyperthermophilic microorganisms: implications for biofuels," *Metabol. Eng.* 10:394-404 (2008).

[0349] The catalytic center of FeFe hydrogenases contains a catalytic "H cluster" which coordinates a binuclear (FeFe) site bridged to a [4Fe-4S] center by a single protein (cysteine) ligand. The two iron atoms of the binuclear center each have two carbon monoxide (CO) and two cyanide (CN^-) ligands, and are also bridged by two sulfur atoms which are part of a small organic molecule. Most FeFe hydrogenases are monomeric enzymes of about 50 kilodaltons (kDa), and appear to function *in vivo* primarily to dispose of excess reducing equivalents by reducing protons to hydrogen gas. Chung-Jung Chou et al., "Hydrogenesis in hyperthermophilic microorganisms: implications for biofuels," *Metabol. Eng.* 10:394-404 (2008).

[0350] The catalytic center of Fe hydrogenases was originally thought to have an active site based on an organic cofactor with no metals involved, but was later shown to contain a mononuclear Fe atom. Despite the phylogenetic differences between the three types of hydrogenase, in addition to at least one iron atom, all three groups of hydrogenases also contain at least one carbon monoxide (CO) ligand to the iron atom in their active sites, which facilitates the catalytic oxidation of H_2 and the reduction of protons. Chung-Jung Chou et al., "Hydrogenesis in hyperthermophilic microorganisms: implications for biofuels," *Metabol. Eng.* 10:394-404 (2008).

[0351] Exemplary hydrogenase polypeptides include, but are not limited to, the *E. coli* hydrogenase-1 (Hyd-1) polypeptides, *E. coli* hydrogenase-2 (Hyd-2) polypeptides, *E. coli* hydrogenase-3 (Hyd-3) polypeptides, *E. coli* hydrogenase-4 (Hyd-4) polypeptides, *E. coli* formate hydrogen lyase (FHL) complex, which produces hydrogen gas from formate and CO_2 under anaerobic conditions at acidic pH (*see, e.g.*, Akihito Yoshida et al., "Efficient induction of formate hydrogen lyase of aerobically grown *Escherichia coli* in a three-step biohydrogen production process," *Appl. Microbiol. Biotechnol.* 74:754-760 (2007), which is incorporated herein by reference in its entirety, particularly with respect to the induction of expression of formate hydrogen lyase in *E. coli*), *Ralstonia eutropha* H16 hydrogenase (*R. eutropha* HoxH) *Rhodococcus opacus MR11* hydrogenase (*R. opacus* HoxH) polypeptides, *Synechosystis* sp. PCC 6803 hydrogenase (*Syn.* PCC 6803 HoxH) polypeptides, *Desulfovibrio gigas* hydrogenase (*D. gigas*) polypeptides, and *Desulfovibrio desulfuricans* ATCC 7757 hydrogenase (*D. desulfuricans*) polypeptides (*see, e.g.*, Gönül Vardar-Schara et al.,

"Metabolically engineered bacteria for producing hydrogen via fermentation," *Microbial Biotechnol.* 1(2):107-125 (2008), which is incorporated herein by reference in its entirety, particularly with respect to the various types and classes of hydrogenases) and polypeptides (*e.g.*, fusion polypeptides) having an activity of two or more hydrogenase polypeptides. In particular, hydrogenase polypeptides include polypeptides, fragments of polypeptides, peptides, and fusion polypeptides that have at least one activity of a hydrogenase polypeptide. Exemplary hydrogenase nucleic acids include nucleic acids that encode a polypeptide, fragment of a polypeptide, peptide, or fusion polypeptide that has at least one activity of a hydrogenase polypeptide, or at least one activity necessary for expression, processing, or maturation of a hydrogenase polypeptide. Exemplary hydrogenase polypeptides and nucleic acids include naturally-occurring polypeptides and nucleic acids from any of the source organisms described herein as well as mutant polypeptides and nucleic acids derived from any of the source organisms described herein.

[0352] *E. coli* Hyd-3, which is part of the anaerobic formate hydrogen lyase (FHL) complex, is encoded by the *hyc* operon (comprising the *hycA*, *hycB*, *hycC*, *hycD*, *hycE*, *hycF*, *hycG*, *hycH*, and *hycI* genes). *E. coli* Hyd-4 is encoded by the *hyf* operon (comprising the *hyfA*, *hyfB*, *hyfC*, *hyfD*, *hyfE*, *hyfF*, *hyfG*, *hyfH*, *hyfI*, *hyfJ*, and *hyfR* genes). *E. coli* FHL is encoded by six genes from the *hyc* operon (*hycB*, *hycC*, *hycD*, *hycE*, *hycF* and *hycG*) and the *fdhF* gene (encoding formate dehydrogenase H (Fdh-H)). Expression of the FHL complex can further involve expression of pyruvate formate lyase (*pfl*), FhlA, a transcription factor that activates transcription of *fdhF* and the *hyc* operon, or deletion/inactivation of HycA, a transcription factor encoded by the *hycA* gene that negatively regulates transcription of FHL. Co-production of isoprene and hydrogen can be improved by expression or inactivation/deletion of additional proteins involved in the regulation of gene expression for hydrogenases and other enzymes, such as, for example, iron-sulfur complex transcriptional regulator (*iscR*) (Kalim-Akhtar et al., "Deletion of iscR stimulates recombinant Clostridial Fe/Fe hydrogenase activity and H_2-accumulation in *Escherichia coli* BL21(DE3)," *Appl. Microbiol. Biotechnol.* 78:853–862 (2008), which is incorporated herein by reference in its entirety, particularly with reference to stimulation of Clostridial Fe/Fe hydrogenase activity and hydrogen accumulation in *E. coli* by deleting the *iscR* gene).

[0353] Exemplary ferredoxin-dependent hydrogenase polypeptides include, but are not limited to, *Clostridium acetobutulicum* hydrogenase A (HydA) (*see, e.g.*, P.W. King et al.,

"Functional studies of [FeFe] hydrogenase maturation in an *Escherichia coli* biosynthetic system," *J. Bacteriol.* 188(6):163-172 (2006), which is incorporated herein by reference in its entirety, particularly with respect to production of hydrogen by HydA and three HydA-associated maturation enzymes (HydE, HydG, and HydF), which may be expressed alone or in in conjunction with one or more of: (1) *Bacillus subtilis* NADPH ferredoxin oxidoreductase (NFOR) (*see, e.g.*, Viet et al., (2008)), which is incorporated herein by reference in its entirety, particularly with respect to production of hydrogen by NFOR; *see also* PCT Publication No. WO/2007/089901, which is incorporated herein by reference in its entirety, particularly with respect to optimization of *E. coli* strains for production of hydrogen), *Clostridium kluyveri* NADH ferredoxin oxidoreductase (RnfCDGEAB) (Henning Seedorf et al., "The genome of *Clostridium kluyveri*, a strict anaerobe with unique metabolic features," *Proc. Nat'l Acad. Sci. U.S.A.* 105(6):2128-2133 (2008), which is incorporated herein by reference in its entirety, particular with reference to NADH ferredoxin oxidoreductase, and with reference to components of the anaerobic ethanol-acetate fermentation pathway), or *Clostridium pasteuranium* ferredoxin oxidoreductase (Fdx); (2) glyceraldehyde-6-phosphate ferredoxin oxidoreductase ("GAPOR"); or (3) pyruvate ferredoxin oxidoreductase ("POR"), and polypeptides (*e.g.*, fusion polypeptides) having an activity of two or more hydrogenase polypeptides or of one or more hydrogenase polypeptides and an activity of one or more ferredoxin-dependent oxidoreductases. In particular, ferredoxin-dependent hydrogenase polypeptides include polypeptides, fragments of polypeptides, peptides, and fusion polypeptides that have at least one activity of a ferredoxin-dependent hydrogenase polypeptide.

[0354] Exemplary NADPH-dependent hydrogenase polypeptides include, but are not limited to thermophilic hydrogenase polypeptides such as *Pyrococcus furiosus* hydrogenase (*see, e.g.*, J. Woodward et al., "Enzymatic production of biohydrogen," *Nature* 405(6790):1014-1015 (2000)), and polypeptides (*e.g.*, fusion polypeptides) having an activity of two or more NADPH-dependent hydrogenase polypeptides. In particular, NADPH-dependent hydrogenase polypeptides include polypeptides, fragments of polypeptides, peptides, and fusion polypeptides that have at least one activity of a NADPH-dependent hydrogenase polypeptide.

[0355] Exemplary oxygen-tolerant or oxygen-insensitive hydrogenases include, but are not limited to, *Rubrivivax gelatinosus* hydrogenase (*see, e.g.*, P.C. Maness et al.,

"Characterization of the oxygen tolerance of a hydrogenase linked to a carbon monoxide oxidation pathway in *Rubrivivax gelatinosus*," *Appl. Environ. Microbiol.* 68(6):2633-2636 (2002), which is incorporated herein by reference in its entirety, particularly with respect to *R. gelatinosus* hydrogenase), and *Ralstonia eutropha* hydrogenase polypeptides (*see, e.g.*, T. Burgdorf et al., "[NiFe]-hydrogenases of *Ralstonia eutropha* H16: modular enzymes for oxygen-tolerant biological hydrogen oxidation," *J. Mol. Microbiol. Biotechnol.* 10(2-4):181-196 (2005), which is incorporated herein by reference in its entirety, particularly with respect to *R. eutropha* hydrogenase polypeptides). Alternatively, heterologous nucleic acids encoding hydrogenase polypeptides can be mutagenized and screened for O_2-tolerance or O_2-insensitivity using standard methods and assays (*see, e.g.*, L.E. Nagy et al., "Application of gene-shuffling for the rapid generation of novel [FeFe]-hydrogenase libraries," *Biotechnol. Letts.* 29(3)421-430 (2007), which is incorporated herein by reference, particularly with respect to mutagenesis and screening for oxygen tolerant hydrogenase polypeptides).

[0356] Standard methods (such as those described herein) can be used to determine whether a polypeptide has hydrogenase activity by measuring the ability of the polypeptide to produce hydrogen gas *in vitro*, in a cell extract, or *in vivo*.

Exemplary Polypeptides and Nucleic Acids for Genes Related to Production of Fermentation Side Products

[0357] In addition to expressing or over-expressing heterologous or native hydrogenases in *E. coli*, co-production of isoprene and hydrogen can be improved by inactivation of anaerobic biosynthetic pathways, thereby blocking the carbon flow to a variety of metabolites (*i.e.*, fermentation side products) produced under oxygen-limited or anaerobic conditions, including, but not limited to, lactate, acetate, pyruvate, ethanol, succinate, and glycerol. Exemplary polypeptides involved in the production of fermentation side products include formate dehydrogenase N, alpha subunit (*fdnG*), formate dehydrogenase O, large subunit (*fdoG*), nitrate reductase (*narG*), formate transporter A (*focA*), formate transporter B (*focB*), pyruvate oxidase (*poxB*), pyruvate dehydrogenase E1 component ackA/pta (*aceE*), alcohol dehydrogenase (*adhE*), fumarate reductase membrane protein (*frdC*), and lactate dehydrogenase (*ldh*A). *See, e.g.*, Toshinori Maeda et al., "Enhanced hydrogen production from glucose by metabolically engineered *Escherichia coli*," *Appl. Microbiol. Biotechnol.* 77(4):879-890 (2007), which is incorporated by reference in its entirety, particularly with respect to production of *E. coli* strains with modified glucose metabolism. Exemplary

polypeptides involved in the regulation or expression of genes involved in the production of fermentation side products that may also be inactivated to improve co-production of isoprene and hydrogen include, but are not limited to, repressor of formate hydrogen lyase (*hycA*), fumarate reductase regulator (*fnr*), acetyl-coenzyme A synthetase (*acs*), and formate dehydrogenase regulatory protein (*hycA*), which regulates expression of the transcriptional regulator fhlA (formate hydrogen lyase transcriptional activator).

Exemplary Polypeptides and Nucleic Acids for Genes Related to Hydrogen Re-uptake

[0358] Exemplary polypeptides involved in hydrogen re-uptake that may also be inactivated to improve co-production of isoprene and hydrogen include, but are not limited to, *E. coli* hydrogenase-1 (Hyd-1) (*hya* operon) and *E. coli* hydrogenase-2 (Hyd-2) (*hyb* operon). *E. coli* Hyd-1 is encoded by the *hya* operon (comprising the *hyaA*, *hyaB*, *hyaC*, *hyaD*, *hyaE*, and *hyaF* genes). *E. coli* Hyd-2 is encoded by the *hyb* operon (comprising the *hybA*, *hybB*, *hybC*, *hybD*, *hybE*, *hybF*, *hybG*, and *hybO* genes).

Exemplary Polypeptides and Nucleic Acids for Genes Related to Ethanol Fermentation

[0359] Exemplary polypeptides involved in ethanol fermentation include, but are not limited to, alcohol dehydrogenase B (adhB), alcohol dehydrogenase E (adhE) and pyruvate decarboxylase (pdc).

[0360] Alcohol dehydrogenases (adh) facilitate the interconversion between alcohols and aldehydes or ketones with the reduction of NAD+ to NADH. In humans and many other animals, they break down alcohols which could otherwise be toxic; in yeast and many bacteria, some alcohol dehydrogenases catalyze the opposite reaction as part of fermentation. In humans, adh exists in multiple forms as a dimer and is encoded by at least seven different genes. There are five classes (I-V) of alcohol dehydrogenase, but the primary hepatic form used in humans is class I. Class 1 consists of A,B, and C subunits that are encoded by the genes ADH1A, ADH1B, and ADH1C. Class I ADH is found in the lining of the stomach and in the liver, and catalyzes the oxidation of ethanol to acetaldehyde: $CH_3CH_2OH + NAD^+ \rightarrow CH_3CHO + NADH + H^+$ This allows the consumption of alcoholic beverages, but its evolutionary purpose is probably the breakdown of alcohols naturally contained in foods or produced by bacteria in the digestive tract.

[0361] Unlike humans, yeast and bacteria do not ferment glucose to lactate. Instead, they ferment it to ethanol and CO_2. In yeast and many bacteria, alcohol dehydrogenase plays an important part in fermentation: pyruvate resulting from glycolysis is converted to acetaldehyde and carbon dioxide, and the acetaldehyde is then reduced to ethanol by an alcohol dehydrogenase called adhE. The purpose of this latter step is the regeneration of NAD+, so that energy-generating glycolysis can continue. Pyruvate decarboxylase is a homotetrameric enzyme that catalyzes the decarboxylation of pyruvate to acetaldehyde and carbon dioxide. Under anaerobic conditions, this enzyme is part of the fermentation process that occurs in yeast, especially of the *Saccharomyces* genus, to produce ethanol by fermentation. Pyruvate decarboxylase is present in many bacteria as well, including *Excherichia sp.*, such as *E. coli*, and *Zymomonas sp.*, such as *Z. mobilis*.

Exemplary Glycerol Pathway or 1,3-Propanediol Pathway Polypeptides and Nucleic Acids

[0362] Exemplary glycerol pathway polypeptides include, but are not limited to, DAR1 (dihydroxyacetone phosphate reductase), GPP2 (glycerol-phosphate phosphatase). Exemplary 1,3-propanediol pathway polypeptides include, but are not limited to dhaB1-3 (dhaB1, dhaB2, and dhaB3; glycerol dehydratase B1, B2, and B3), dhaX, orfX (protein X), and orfY (protein Y), as well as glycerol dehydratase variants with improved reaction kinetics, including variants of dhaB1, dhaB2, and dhaB3, such as those described in US Patent Publication No. 2008/0293119 A1, which is incorporated herein by reference in its entirety, particularly with respect to disclosure regarding variant glycerol dehydratase variants with improved reaction kinetics. The dha regulon enables organisms such as *Klebsiella pneumoniae* to grow anaerobically on glycerol and produce 1,3-propanediol (1,3-PD). *Escherichia coli* does not have a dha system, and thus is unable to grow anaerobically on glycerol without an exogenous electron acceptor and does not produce 1,3-propanediol. The dha regulon comprises at least four genes: glycerol dehydratase (dhaB), 1,3-propanediol oxidoreductase (dhaT), glycerol dehydrogenase (dhaD), and dihydroxyacetone kinase (dhaK). All four activities were inducible by the presence of glycerol.

Exemplary Methods for Isolating Nucleic Acids

[0363] Isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids can be isolated using standard methods. Methods of

obtaining desired nucleic acids from a source organism of interest (such as a bacterial genome) are common and well known in the art of molecular biology (*see*, for example, WO 2004/033646 and references cited therein, which are each hereby incorporated by reference in their entireties, particularly with respect to the isolation of nucleic acids of interest). For example, if the sequence of the nucleic acid is known (such as any of the known nucleic acids described herein), suitable genomic libraries may be created by restriction endonuclease digestion and may be screened with probes complementary to the desired nucleic acid sequence. Once the sequence is isolated, the DNA may be amplified using standard primer directed amplification methods such as polymerase chain reaction (PCR) (U.S. Patent No. 4,683,202, which is incorporated by reference in its entirety, particularly with respect to PCR methods) to obtain amounts of DNA suitable for transformation using appropriate vectors.

[0364] Alternatively, isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids (such as any isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids with a known nucleic acid sequence) can be chemically synthesized using standard methods.

[0365] Additional isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor polypeptides and nucleic acids which may be suitable for use in the compositions and methods described herein can be identified using standard methods. For example, cosmid libraries of the chromosomal DNA of organisms known to produce isoprene naturally can be constructed in organisms such as *E. coli*, and then screened for isoprene production. In particular, cosmid libraries may be created where large segments of genomic DNA (35-45 kb) are packaged into vectors and used to transform appropriate hosts. Cosmid vectors are unique in being able to accommodate large quantities of DNA. Generally cosmid vectors have at least one copy of the cos DNA sequence which is needed for packaging and subsequent circularization of the heterologous DNA. In addition to the cos sequence, these vectors also contain an origin of replication such as ColEI and drug resistance markers such as a nucleic acid resistant to ampicillin or neomycin. Methods of using cosmid vectors for the transformation of suitable bacterial hosts are well described in Sambrook *et al.*, Molecular

Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989, which is hereby incorporated by reference in its entirety, particularly with respect to transformation methods.

[0366] Typically to clone cosmids, heterologous DNA is isolated using the appropriate restriction endonucleases and ligated adjacent to the cos region of the cosmid vector using the appropriate ligases. Cosmid vectors containing the linearized heterologous DNA are then reacted with a DNA packaging vehicle such as bacteriophage. During the packaging process, the cos sites are cleaved and the heterologous DNA is packaged into the head portion of the bacterial viral particle. These particles are then used to transfect suitable host cells such as *E. coli.* Once injected into the cell, the heterologous DNA circularizes under the influence of the cos sticky ends. In this manner, large segments of heterologous DNA can be introduced and expressed in host cells.

[0367] Additional methods for obtaining isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids include screening a metagenomic library by assay (such as the headspace assay described herein) or by PCR using primers directed against nucleotides encoding for a length of conserved amino acids (for example, at least 3 conserved amino acids). Conserved amino acids can be identified by aligning amino acid sequences of known isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor polypeptides. Conserved amino acids for isoprene synthase polypeptides can be identified based on aligned sequences of known isoprene synthase polypeptides. An organism found to produce isoprene naturally can be subjected to standard protein purification methods (which are well known in the art) and the resulting purified polypeptide can be sequenced using standard methods. Other methods are found in the literature (*see*, for example, Julsing *et al.*, *Applied. Microbiol. Biotechnol.* 75: 1377-84, 2007; Withers *et al.*, *Appl Environ Microbiol.* 73(19):6277-83, 2007, which are each hereby incorporated by reference in their entireties, particularly with respect to identification of nucleic acids involved in the synthesis of isoprene).

[0368] Additionally, standard sequence alignment and/or structure prediction programs can be used to identify additional DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor polypeptides and nucleic acids based on the similarity of their primary

and/or predicted polypeptide secondary structure with that of known DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor polypeptides and nucleic acids. Standard databases such as the swissprot-trembl database (world-wide web at "expasy.org", Swiss Institute of Bioinformatics Swiss-Prot group CMU - 1 rue Michel Servet CH-1211 Geneva 4, Switzerland) can also be used to identify isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription regulatory polypeptides and nucleic acids. The secondary and/or tertiary structure of an isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor polypeptide can be predicted using the default settings of standard structure prediction programs, such as PredictProtein (630 West, 168 Street, BB217, New York, N.Y. 10032, USA). Alternatively, the actual secondary and/or tertiary structure of an isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor polypeptide can be determined using standard methods. Additional isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids can also be identified by hybridization to probes generated from known isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids.

Exemplary Promoters and Vectors

[0369] Any of the isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids described herein can be included in one or more vectors. Accordingly, the invention also features vectors with one more nucleic acids encoding any of the isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor polypeptides that are described herein. In some embodiments, the vector contains a nucleic acid under the control of an expression control sequence.

[0370] In some embodiments, the vector contains a selective marker or selectable marker. Markers useful in vector systems for transformation of *Trichoderma* are known in the art (*see*, *e.g.*, Finkelstein, Chapter 6 in Biotechnology of Filamentous Fungi, Finkelstein *et al.*, *Eds.* Butterworth-Heinemann, Boston, MA, Chap. 6., 1992; and Kinghorn *et al.*, Applied Molecular Genetics of Filamentous Fungi, Blackie Academic and Professional, Chapman and Hall, London, 1992, which are each hereby incorporated by reference in their entireties, particularly with respect to selective markers). In some embodiments, the selective marker is the *amd*S nucleic acid, which encodes the enzyme acetamidase, allowing transformed cells to grow on acetamide as a nitrogen source. The use of an *A. nidulans amd*S nucleic acid as a selective marker is described in Kelley *et al.*, *EMBO J.* 4:475 – 479, 1985 and Penttila *et al.*, *Gene* 61:155-164, 1987 (which are each hereby incorporated by reference in their entireties, particularly with respect to selective markers). In some embodiments, an isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation, or transcription regulatory nucleic acid integrates into a chromosome of the cells without a selective marker.

[0371] Suitable vectors are those which are compatible with the host cell employed. Suitable vectors can be derived, for example, from a bacterium, a virus (such as bacteriophage T7 or a M-13 derived phage), a cosmid, a yeast, or a plant. Protocols for obtaining and using such vectors are known to those in the art (*see*, for example, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989, which is hereby incorporated by reference in its entirety, particularly with respect to the use of vectors).

[0372] Promoters are well known in the art. Any promoter that functions in the host cell can be used for expression of an isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid in the host cell. Initiation control regions or promoters, which are useful to drive expression of isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids in various host cells are numerous and familiar to those skilled in the art (*see*, for example, WO 2004/033646 and references cited therein, which are each hereby incorporated by reference in their entireties, particularly with respect to vectors for the expression of nucleic acids of interest).

Virtually any promoter capable of driving these nucleic acids is suitable for the present invention including, but not limited to, CYC1, HIS3, GAL1, GAL10, ADH1, PGK, PHO5, GAPDH, ADCI, TRP1, URA3, LEU2, ENO, and TPI (useful for expression in *Saccharomyces*); AOX1 (useful for expression in *Pichia*); and lac, trp, λP_L, λP_R, T7, tac, and trc (useful for expression in *E. coli*).

[0373] In some embodiments, a glucose isomerase promoter is used (*see*, for example, U.S. Patent No. 7,132,527 and references cited therein, which are each hereby incorporated by reference in their entireties, particularly with respect promoters and plasmid systems for expressing polypeptides of interest). Reported glucose isomerase promoter mutants can be used to vary the level of expression of the polypeptide encoded by a nucleic acid operably linked to the glucose isomerase promoter (U.S. Patent No. 7,132,527). In various embodiments, the glucose isomerase promoter is contained in a low, medium, or high copy plasmid (U.S. Patent No. 7,132,527).

[0374] In various embodiments, an isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid is contained in a low copy plasmid (*e.g.*, a plasmid that is maintained at about 1 to about 4 copies per cell), medium copy plasmid (*e.g.*, a plasmid that is maintained at about 10 to about 15 copies per cell), or high copy plasmid (*e.g.*, a plasmid that is maintained at about 50 or more copies per cell). In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid is operably linked to a T7 promoter. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid operably linked to a T7 promoter is contained in a medium or high copy plasmid. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid is operably linked to a Trc promoter. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid operably linked

to a Trc promoter is contained in a medium or high copy plasmid. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid is operably linked to a Lac promoter. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid operably linked to a Lac promoter is contained in a low copy plasmid. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid is operably linked to an endogenous promoter, such as an endogenous *Escherichia, Panteoa, Bacillus, Yarrowia, Streptomyces,* or *Trichoderma* promoter or an endogenous alkaline serine protease, isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor promoter. In some embodiments, the heterologous or extra endogenous isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid operably linked to an endogenous promoter is contained in a high copy plasmid. In some embodiments, the vector is a replicating plasmid that does not integrate into a chromosome in the cells. In some embodiments, part or all of the vector integrates into a chromosome in the cells.

[0375] In some embodiments, the vector is any vector which when introduced into a fungal host cell is integrated into the host cell genome and is replicated. Reference is made to the Fungal Genetics Stock Center Catalogue of Strains (FGSC, the world-wide web at "fgsc.net" and the references cited therein, which are each hereby incorporated by reference in their entireties, particularly with respect to vectors) for a list of vectors. Additional examples of suitable expression and/or integration vectors are provided in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989, Current Protocols in Molecular Biology (F. M. Ausubel *et al.* (eds) 1987, Supplement 30, section 7.7.18); van den Hondel *et al.* in Bennett and Lasure (Eds.) More Gene Manipulations in Fungi, Academic Press pp. 396-428, 1991; and U.S. Patent No. 5,874,276, which are each hereby incorporated by reference in their entireties, particularly with respect to vectors. Particularly useful vectors include pFB6, pBR322, PUC18, pUC100, and pENTR/D.

[0376] In some embodiments, an isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid is operably linked to a suitable promoter that shows transcriptional activity in a fungal host cell. The promoter may be derived from one or more nucleic acids encoding a polypeptide that is either endogenous or heterologous to the host cell. In some embodiments, the promoter is useful in a *Trichoderma* host. Suitable non-limiting examples of promoters include *cbh*1, *cbh*2, *egl*1, *egl*2, *pepA*, *hfb*1, *hfb*2, *xyn*1, and *amy*. In some embodiments, the promoter is one that is native to the host cell. For example, in some embodiments when *T. reesei* is the host, the promoter is a native *T. reesei* promoter. In some embodiments, the promoter is *T. reesei cbh1*, which is an inducible promoter and has been deposited in GenBank under Accession No. D86235, which is incorporated by reference in its entirety, particularly with respect to promoters. In some embodiments, the promoter is one that is heterologous to the fungal host cell. Other examples of useful promoters include promoters from the genes of *A. awamori* and *A. niger* glucoamylase (glaA) (Nunberg *et al.*, *Mol. Cell Biol.* 4:2306-2315, 1984 and Boel *et al.*, *EMBO J.* 3:1581-1585, 1984, which are each hereby incorporated by reference in their entireties, particularly with respect to promoters); *Aspergillus niger* alpha amylases, *Aspergillus oryzae* TAKA amylase, *T. reesei xln*1, and the *T. reesei cellobiohydrolase* 1 (EP 137280, which is incorporated by reference in its entirety, particularly with respect to promoters).

[0377] In some embodiments, the expression vector also includes a termination sequence. Termination control regions may also be derived from various genes native to the host cell. In some embodiments, the termination sequence and the promoter sequence are derived from the same source. In another embodiment, the termination sequence is endogenous to the host cell. A particularly suitable terminator sequence is *cbh*1 derived from a *Trichoderma* strain (such as *T. reesei*). Other useful fungal terminators include the terminator from an *A. niger* or *A. awamori* glucoamylase nucleic acid (Nunberg *et al.*, *Mol. Cell Biol.* 4:2306-2315, 1984 and Boel *et al.*, *EMBO J.* 3:1581-1585, 1984; which are each hereby incorporated by reference in their entireties, particularly with respect to fungal terminators). Optionally, a termination site may be included. For effective expression of the polypeptides, DNA encoding the polypeptide are linked operably through initiation codons to selected expression control regions such that expression results in the formation of the appropriate messenger RNA.

[0378] In some embodiments, the promoter, coding, region, and terminator all originate from the isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid to be expressed. In some embodiments, the coding region for an isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid is inserted into a general-purpose expression vector such that it is under the transcriptional control of the expression construct promoter and terminator sequences. In some embodiments, genes or part thereof are inserted downstream of the strong *cbh*1 promoter.

[0379] An isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid can be incorporated into a vector, such as an expression vector, using standard techniques (Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1982, which is hereby incorporated by reference in its entirety, particularly with respect to the screening of appropriate DNA sequences and the construction of vectors). Methods used to ligate the DNA construct comprising a nucleic acid of interest (such as an isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid), a promoter, a terminator, and other sequences and to insert them into a suitable vector are well known in the art. For example, restriction enzymes can be used to cleave the isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid and the vector. Then, the compatible ends of the cleaved isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid and the cleaved vector can be ligated. Linking is generally accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide linkers are used in accordance with conventional practice (*see*, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989, and Bennett and Lasure, More Gene Manipulations in Fungi, Academic Press, San Diego, pp 70–76, 1991, which are each hereby incorporated by reference in their entireties, particularly with respect to

oligonucleotide linkers). Additionally, vectors can be constructed using known recombination techniques (*e.g.*, Invitrogen Life Technologies, Gateway Technology).

[0380] In some embodiments, it may be desirable to over-express isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids at levels far higher than currently found in naturally-occurring cells. This result may be accomplished by the selective cloning of the nucleic acids encoding those polypeptides into multicopy plasmids or placing those nucleic acids under a strong inducible or constitutive promoter. Methods for over-expressing desired polypeptides are common and well known in the art of molecular biology and examples may be found in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989, which is hereby incorporated by reference in its entirety, particularly with respect to cloning techniques.

[0381] In some embodiments, it may be desirable to under-express (*e.g.*, mutate, inactivate, or delete) isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation, or transcription factor polypeptide-encoding nucleic acids at levels far below that those currently found in naturally-occurring cells. This result may be accomplished by the mutation or inactivation of transcriptional regulatory proteins required for expression of isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids, by deletion of the isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids, or by placing those nucleic acids under the control of a strong repressible promoter. Methods for mutating, inactivating, or deleting desired polypeptides are common and well known in the art of molecular biology and examples may be found in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989, which is hereby incorporated by reference in its entirety, particularly with respect to cloning and mutagenesis techniques.

[0382] The following resources include descriptions of additional general methodology useful in accordance with the invention: Kreigler, Gene Transfer and Expression; A Laboratory Manual,1990 and Ausubel *et al.*, Eds. Current Protocols in Molecular Biology,

1994, which are each hereby incorporated by reference in their entireties, particularly with respect to molecular biology and cloning techniques.

Exemplary Source Organisms

[0383] Isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids (and their encoded polypeptides) can be obtained from any organism that naturally contains isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids. As noted above, isoprene is formed naturally by a variety of organisms, such as bacteria, yeast, plants, and animals. Organisms contain the MVA pathway, DXP pathway, or both the MVA and DXP pathways for producing isoprene (Figures 19A and 19B). Thus, DXS nucleic acids can be obtained, *e.g.*, from any organism that contains the DXP pathway or contains both the MVA and DXP pathways. IDI and isoprene synthase nucleic acids can be obtained, *e.g.*, from any organism that contains the MVA pathway, DXP pathway, or both the MVA and DXP pathways. MVA pathway nucleic acids can be obtained, *e.g.*, from any organism that contains the MVA pathway or contains both the MVA and DXP pathways. Ethanol fermentation-related nucleic acids can be obtained, *e.g.*, from any organism that naturally produces alcohol from glucose or other carbon source. Glycerol pathway and/or 1,3-propanediol pathway related nucleic acids can be obtained, *e.g.*, from any organism that naturally as the ability to grow on glycerol as primary carbon source. Hydrogenase nucleic acids can be obtained, *e.g.*, from any organism that oxidizes hydrogen or reduces hydrogen ions. Fermentation side product genes can be obtained or identified, *e.g.*, from any organism that undergoes oxygen-limited or anaerobic respiration, such as glycolysis.

[0384] In some embodiments, the nucleic acid sequence of the isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic is identical to the sequence of a nucleic acid that is produced by any of the following organisms in nature. In some embodiments, the amino acid sequence of the isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor polypeptide is identical to the sequence of a polypeptide that is produced by any of the following organisms in nature.

In some embodiments, the isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acid or polypeptide is a mutant nucleic acid or polypeptide derived from any of the organisms described herein. As used herein, "derived from" refers to the source of the nucleic acid or polypeptide into which one or more mutations is introduced. For example, a polypeptide that is "derived from a plant polypeptide" refers to polypeptide of interest that results from introducing one or more mutations into the sequence of a wild-type (*i.e.*, a sequence occurring in nature) plant polypeptide.

[0385] In some embodiments, the source organism is a fungus, examples of which are species of *Aspergillus* such as *A. oryzae* and *A. niger,* species of *Saccharomyces* such as *S. cerevisiae*, species of *Schizosaccharomyces* such as *S. pombe*, and species of *Trichoderma* such as *T. reesei.* In some embodiments, the source organism is a filamentous fungal cell. The term "filamentous fungi" refers to all filamentous forms of the subdivision Eumycotina (*see*, Alexopoulos, C. J. (1962), Introductory Mycology, Wiley, New York). These fungi are characterized by a vegetative mycelium with a cell wall composed of chitin, cellulose, and other complex polysaccharides. The filamentous fungi are morphologically, physiologically, and genetically distinct from yeasts. Vegetative growth by filamentous fungi is by hyphal elongation and carbon catabolism is obligatory aerobic. The filamentous fungal parent cell may be a cell of a species of, but not limited to, *Trichoderma,* (*e.g., Trichoderma reesei,* the asexual morph of *Hypocrea jecorina*, previously classified as *T. longibrachiatum, Trichoderma viride, Trichoderma koningii, Trichoderma harzianum*) (Sheir-Neirs *et al.*, Appl. Microbiol. Biotechnol 20: 46-53, 1984; ATCC No. 56765 and ATCC No. 26921)*; Penicillium sp., Humicola sp.* (*e.g.*, *H. insolens, H. lanuginose,* or *H. grisea*)*; Chrysosporium sp.* (*e.g.*, *C. lucknowense*), *Gliocladium sp., Aspergillus sp.* (*e.g.*, *A. oryzae*, *A. niger*, *A sojae, A. japonicus*, *A. nidulans*, or *A. awamori*) (Ward *et al.*, Appl. Microbiol. Biotechnol. 39: 7380743, 1993 and Goedegebuur *et al.*, Genet 41: 89-98, 2002), *Fusarium sp., (e.g., F. roseum, F. graminum F. cerealis, F. oxysporuim*, or *F. venenatum), Neurospora sp., (e.g., N. crassa), Hypocrea sp., Mucor sp., (e.g., M. miehei), Rhizopus sp. and Emericella sp.* (*see also*, Innis *et al.*, *Sci*. 228: 21-26, 1985). The term "*Trichoderma*" or "*Trichoderma sp.*" or "*Trichoderma spp.*" refer to any fungal genus previously or currently classified as *Trichoderma*.

[0386] In some embodiments, the fungus is *A. nidulans, A. awamori, A. oryzae, A. aculeatus, A. niger, A. japonicus, T. reesei, T. viride, F. oxysporum,* or *F. solani*. Aspergillus strains are disclosed in Ward et al., Appl. Microbiol. Biotechnol. 39:738-743, 1993 and Goedegebuur et al., Curr Gene 41:89-98, 2002, which are each hereby incorporated by reference in their entireties, particularly with respect to fungi. In particular embodiments, the fungus is a strain of Trichoderma, such as a strain of T. reesei. Strains of T. reesei are known and non-limiting examples include ATCC No. 13631, ATCC No. 26921, ATCC No. 56764, ATCC No. 56765, ATCC No. 56767, and NRRL 15709, which are each hereby incorporated by reference in their entireties, particularly with respect to strains of T. reesei. In some embodiments, the host strain is a derivative of RL-P37. RL-P37 is disclosed in Sheir-Neiss *et al.*, Appl. Microbiol. Biotechnology 20:46–53, 1984, which is hereby incorporated by reference in its entirety, particularly with respect to strains of T. reesei.

[0387] In some embodiments, the source organism is a yeast, such as *Saccharomyces sp.*, *Schizosaccharomyces sp.*, *Pichia sp.*, or *Candida sp*. In some embodiments, the *Saccharomyces sp*. is *Saccharomyces cerevisiae*.

[0388] In some embodiments, the source organism is a bacterium, such as strains of *Bacillus* such as *B. lichenformis* or *B. subtilis,* strains of *Pantoea* such as *P. citrea*, strains of *Pseudomonas* such as *P. alcaligenes, P. putida, or P. fluorescens,* strains of *Streptomyces* such as *S. lividans* or *S. rubiginosus,* strains of *Corynebacterium* sp. such as *Corynebacterium glutamicum*, strains of *Rhodopseudomonas sp.* such as *Rhodopseudomonas palustris*, or strains of *Escherichia* such as *E. coli*.

[0389] As used herein, "the genus *Bacillus*" includes all species within the genus "*Bacillus*," as known to those of skill in the art, including but not limited to *B. subtilis*, *B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens*, *B. clausii*, *B. halodurans*, *B. megaterium, B. coagulans, B. circulans, B. lautus*, and *B. thuringiensis*. It is recognized that the genus *Bacillus* continues to undergo taxonomical reorganization. Thus, it is intended that the genus include species that have been reclassified, including but not limited to such organisms as *B. stearothermophilus*, which is now named "*Geobacillus stearothermophilus*." The production of resistant endospores in the presence of oxygen is considered the defining feature of the genus *Bacillus*, although this characteristic also applies to the recently named *Alicyclobacillus*, *Amphibacillus*,

Aneurinibacillus, *Anoxybacillus*, *Brevibacillus*, *Filobacillus*, *Gracilibacillus*, *Halobacillus*, *Paenibacillus*, *Salibacillus*, *Thermobacillus*, *Ureibacillus*, and *Virgibacillus*.

[0390] In some embodiments, the source organism is a gram-positive bacterium. Non-limiting examples include strains of *Streptomyces* (*e.g.*, *S. lividans*, *S. coelicolor*, or *S. griseus*) and *Bacillus*. In some embodiments, the source organism is a gram–negative bacterium, such as *E. coli., Rhodopseudomonas sp.* such as *Rhodopseudomonas palustris,* or *Pseudomonas sp.*, such as *P. alcaligenes, P. putida, or P. fluorescens*, *Zymonomas sp.*, such as *Z. mobilis*.

[0391] In some embodiments, the source organism is a plant, such as a plant from the family Fabaceae, such as the Faboideae subfamily. In some embodiments, the source organism is kudzu, poplar (such as *Populus alba x tremula* CAC35696), aspen (such as *Populus tremuloides*), or *Quercus robur*.

[0392] In some embodiments, the source organism is an algae, such as a green algae, red algae, glaucophytes, chlorarachniophytes, euglenids, chromista, or dinoflagellates.

[0393] In some embodiments, the source organism is a cyanobacteria, such as cyanobacteria classified into any of the following groups based on morphology: *Chroococcales*, *Pleurocapsales*, *Oscillatoriales*, *Nostocales*, or *Stigonematales*.

Exemplary Host Cells

[0394] A variety of host cells can be used to express isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor polypeptides and to co-produce isoprene and hydrogen in the methods of the claimed invention. Exemplary host cells include cells from any of the organisms listed in the prior section under the heading "*Exemplary Source Organisms.*" The host cell may be a cell that naturally produces isoprene or a cell that does not naturally produce isoprene. In some embodiments, the host cell naturally produces isoprene using the DXP pathway, and an isoprene synthase, DXS, and/or IDI nucleic acid is added to enhance production of isoprene using this pathway. In some embodiments, the host cell naturally produces isoprene using the MVA pathway, and an isoprene synthase and/or one or more MVA pathway nucleic acids are added to enhance production of isoprene using this pathway. In some embodiments, the host cell naturally

produces isoprene using the DXP pathway and one or more MVA pathway nucleic acids are added to produce isoprene using part or all of the MVA pathway as well as the DXP pathway. In some embodiments, the host cell naturally produces isoprene using both the DXP and MVA pathways and one or more isoprene synthase, DXS, IDI, or MVA pathway nucleic acids are added to enhance production of isoprene by one or both of these pathways.

[0395] In some embodiments, the host cell naturally produces isoprene using both the DXP and MVA pathways, and one or more isoprene synthase, DXS, IDI, or MVA pathway nucleic acids are added to enhance production of isoprene by one or both of these pathways, one or more hydrogenase nucleic acids are added to enhance hydrogen production and one or more fermentation side product-producing genes are inactivated or deleted to limit production of fermentation side products. In some embodiments, the host cell naturally co-produces isoprene and hydrogen using both the DXP and MVA pathways and one or more isoprene synthase, DXS, IDI, or MVA pathway nucleic acids are added to enhance production of isoprene by one or both of these pathways, one or more hydrogenase nucleic acids are added to enhance hydrogen production, one or more fermentation side product-producing genes are inactivated or deleted to limit production of fermentation side products, and one or more hydrogen reuptake genes are inactivated or deleted to increase hydrogen production. In some embodiments, the host cell naturally co-produces isoprene and hydrogen using both the DXP and MVA pathways and a hydrogenase, and one or more isoprene synthase, DXS, IDI, or MVA pathway nucleic acids are added to enhance production of isoprene by one or both of these pathways, one or more hydrogenase nucleic acids are added to enhance hydrogen production, one or more hydrogenase maturation nucleic acids are added to enhance hydrogen production, one or more fermentation side product-producing genes are inactivated or deleted to limit production of fermentation side products, and one or more hydrogen reuptake genes are inactivated or deleted to increase hydrogen production. In some embodiments, the host cell naturally co-produces isoprene and hydrogen using both the DXP and MVA pathways and one or more isoprene synthase, DXS, IDI, or MVA pathway nucleic acids are added to enhance production of isoprene by one or both of these pathways, one or more hydrogenase nucleic acids are added to enhance hydrogen production, one or more hydrogenase maturation nucleic acids are added to enhance hydrogen production, one or more transcription factor nucleic acids are added or inactivated or deleted to enhance hydrogenase production, one or more fermentation side product-producing genes are

inactivated or deleted to limit production of fermentation side products, and one or more hydrogen reuptake genes are inactivated or deleted to increase hydrogen production.

Exemplary Transformation Methods

[0396] Isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor nucleic acids or vectors containing them can be inserted into a host cell (*e.g.*, a plant cell, a fungal cell, a yeast cell, or a bacterial cell described herein) using standard techniques for expression of the encoded isoprene synthase, DXS, IDI, MVA pathway, ethanol fermentation-related, glycerol pathway, 1,3-propanediol pathway, hydrogenase, hydrogenase maturation and/or transcription factor polypeptide. Introduction of a DNA construct or vector into a host cell can be performed using techniques such as transformation, electroporation, nuclear microinjection, transduction, transfection (*e.g.*, lipofection mediated or DEAE-Dextrin mediated transfection or transfection using a recombinant phage virus), incubation with calcium phosphate DNA precipitate, high velocity bombardment with DNA-coated microprojectiles, and protoplast fusion. General transformation techniques are known in the art (*see*, *e.g.*, Current Protocols in Molecular Biology (F. M. Ausubel *et al.* (eds) Chapter 9, 1987; Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor, 1989; and Campbell *et al.*, *Curr. Genet.* 16:53-56, 1989, which are each hereby incorporated by reference in their entireties, particularly with respect to transformation methods). The expression of heterologous polypeptide in *Trichoderma* is described in U.S. Patent No. 6,022,725; U.S. Patent No. 6,268,328; U.S. Patent No. 7,262,041;WO 2005/001036; Harkki *et al.*, *Enzyme Microb. Technol.* 13:227-233, 1991; Harkki *et al.*, *Bio Technol.* 7:596-603, 1989; EP 244,234; EP 215,594; and Nevalainen *et al.*, "*The Molecular Biology of Trichoderma and its Application to the Expression of Both Homologous and Heterologous Genes*," in Molecular Industrial Mycology, *Eds.* Leong and Berka, Marcel Dekker Inc., NY pp. 129 – 148, 1992, which are each hereby incorporated by reference in their entireties, particularly with respect to transformation and expression methods). Reference is also made to Cao *et al.*, (*Sci.* 9:991–1001, 2000; EP 238023; and Yelton *et al.*, *Proceedings. Natl. Acad. Sci.* USA 81:1470-1474, 1984 (which are each hereby incorporated by reference in their entireties, particularly with respect to transformation methods) for transformation of *Aspergillus* strains. The introduced

nucleic acids may be integrated into chromosomal DNA or maintained as extrachromosomal replicating sequences.

[0397] Any method known in the art may be used to select transformants. In one non-limiting example, stable transformants including an *amd*S marker are distinguished from unstable transformants by their faster growth rate and the formation of circular colonies with a smooth, rather than ragged outline on solid culture medium containing acetamide. Additionally, in some cases a further test of stability is conducted by growing the transformants on a solid non-selective medium (*e.g.,* a medium that lacks acetamide), harvesting spores from this culture medium, and determining the percentage of these spores which subsequently germinate and grow on selective medium containing acetamide.

[0398] In some embodiments, fungal cells are transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a known manner. In one specific embodiment, the preparation of *Trichoderma sp.* for transformation involves the preparation of protoplasts from fungal mycelia (*see*, Campbell *et al.*, *Curr. Genet.* 16:53-56, 1989, which is incorporated by reference in its entirety, particularly with respect to transformation methods). In some embodiments, the mycelia are obtained from germinated vegetative spores. The mycelia are treated with an enzyme that digests the cell wall resulting in protoplasts. The protoplasts are then protected by the presence of an osmotic stabilizer in the suspending medium. These stabilizers include sorbitol, mannitol, potassium chloride, magnesium sulfate, and the like. Usually the concentration of these stabilizers varies between 0.8 M and 1.2 M. It is desirable to use about a 1.2 M solution of sorbitol in the suspension medium.

[0399] Uptake of DNA into the host *Trichoderma sp.* strain is dependent upon the calcium ion concentration. Generally, between about 10 mM $CaCl_2$ and 50 mM $CaCl_2$ is used in an uptake solution. In addition to the calcium ion in the uptake solution, other compounds generally included are a buffering system such as TE buffer (10 Mm Tris, pH 7.4; 1 mM EDTA) or 10 mM MOPS, pH 6.0 buffer (morpholinepropanesulfonic acid) and polyethylene glycol (PEG). While not intending to be bound to any particular theory, it is believed that the polyethylene glycol acts to fuse the cell membranes, thus permitting the contents of the medium to be delivered into the cytoplasm of the *Trichoderma sp.* strain and the plasmid DNA to be transferred to the nucleus. This fusion frequently leaves multiple copies of the plasmid DNA integrated into the host chromosome.

[0400] Usually a suspension containing the *Trichoderma sp.* protoplasts or cells that have been subjected to a permeability treatment at a density of 10^5 to 10^7/mL (such as 2 x 10^6/mL) are used in the transformation. A volume of 100 μL of these protoplasts or cells in an appropriate solution (*e.g.*, 1.2 M sorbitol and 50 mM $CaCl_2$) are mixed with the desired DNA. Generally, a high concentration of PEG is added to the uptake solution. From 0.1 to 1 volume of 25% PEG 4000 can be added to the protoplast suspension. In some embodiments, about 0.25 volumes are added to the protoplast suspension. Additives such as dimethyl sulfoxide, heparin, spermidine, potassium chloride, and the like may also be added to the uptake solution and aid in transformation. Similar procedures are available for other fungal host cells (*see*, *e.g.*, U.S. Patent Nos. 6,022,725 and 6,268,328, which are each hereby incorporated by reference in their entireties, particularly with respect to transformation methods).

[0401] Generally, the mixture is then cultured at approximately 0°C for a period of between 10 to 30 minutes. Additional PEG is then added to the mixture to further enhance the uptake of the desired nucleic acid sequence. The 25% PEG 4000 is generally added in volumes of 5 to 15 times the volume of the transformation mixture; however, greater and lesser volumes may be suitable. The 25% PEG 4000 is desirably about 10 times the volume of the transformation mixture. After the PEG is added, the transformation mixture is then cultured either at room temperature or on ice before the addition of a sorbitol and $CaCl_2$ solution. The protoplast suspension is then further added to molten aliquots of a growth medium. When the growth medium includes a growth selection (*e.g.*, acetamide or an antibiotic) it permits the growth of transformants only.

[0402] The transformation of bacterial cells may be performed according to conventional methods, *e.g.*, as described in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1982, which is hereby incorporated by reference in its entirety, particularly with respect to transformation methods.

Exemplary Cell Culture Media

[0403] The invention also includes a cell or a population of cells in culture that co-produce isoprene and hydrogen. By "cells in culture" is meant two or more cells in a solution (*e.g.*, a cell growth medium) that allows the cells to undergo one or more cell divisions. "Cells in culture" do not include plant cells that are part of a living, multicellular plant containing cells

that have differentiated into plant tissues. In various embodiments, the cell culture includes at least or about 10, 20, 50, 100, 200, 500, 1,000, 5,000, 10,000 or more cells.

[0404] By "cells in oxygen-limited culture" is meant two or more cells in a solution (*e.g.*, a cell growth medium) that allows the cell to under go one or more cell divisions, wherein the solution contains a limiting amount of oxygen. The term "oxygen-limited culture" means that the culture is either anoxic or contains less than the required amount of oxygen to support respiration via the biological transfer of reducing equivalents to oxygen, and also encompasses anaerobic cultures. Under oxygen-limited culture conditions, some electrons derived from carbon metabolism cannot be accepted because oxygen concentrations are too low, causing cells to switch to hydrogen production if they comprise the appropriate metabolic pathways for doing so. Oxygen-limited culture conditions occur when the oxygen transfer rate ("OTR") is less than the oxygen uptake rate ("OUR") indicated by dissolved oxygen concentrations of close to zero in culture medium.

[0405] Any carbon source can be used to cultivate the host cells. The term "carbon source" refers to one or more carbon-containing compounds capable of being metabolized by a host cell or organism. For example, the cell medium used to cultivate the host cells may include any carbon source suitable for maintaining the viability or growing the host cells.

[0406] In some embodiments, the carbon source is a carbohydrate (such as monosaccharide, disaccharide, oligosaccharide, or polysaccharides), invert sugar (*e.g.*, enzymatically treated sucrose syrup), glycerol, glycerine (*e.g.*, a glycerine byproduct of a biodiesel or soap-making process), dihydroxyacetone, one-carbon source, oil (*e.g.*, a plant or vegetable oil such as corn, palm, or soybean oil), animal fat, animal oil, fatty acid (*e.g.*, a saturated fatty acid, unsaturated fatty acid, or polyunsaturated fatty acid), lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, polypeptide (*e.g.,* a microbial or plant protein or peptide), renewable carbon source (*e.g.*, a biomass carbon source such as a hydrolyzed biomass carbon source), yeast extract, component from a yeast extract, polymer, acid, alcohol, aldehyde, ketone, amino acid, succinate, lactate, acetate, ethanol, or any combination of two or more of the foregoing. In some embodiments, the carbon source is a product of photosynthesis, including, but not limited to, glucose.

[0407] Exemplary monosaccharides include glucose and fructose; exemplary oligosaccharides include lactose and sucrose, and exemplary polysaccharides include starch

and cellulose. Exemplary carbohydrates include C6 sugars (*e.g.*, fructose, mannose, galactose, or glucose) and C5 sugars (*e.g.*, xylose or arabinose). In some embodiments, the cell medium includes a carbohydrate as well as one or more carbon sources other than a carbohydrate (*e.g.*, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source, or a component from a yeast extract). In some embodiments, the cell medium includes a carbohydrate as well as a polypeptide (*e.g.*, a microbial or plant protein or peptide). In some embodiments, the microbial polypeptide is a polypeptide from yeast or bacteria. In some embodiments, the plant polypeptide is a polypeptide from soy, corn, canola, jatropha, palm, peanut, sunflower, coconut, mustard, rapeseed, cottonseed, palm kernel, olive, safflower, sesame, or linseed.

[0408] In some embodiments, the concentration of the carbohydrate is at least or about 5 grams per liter of broth (g/L, wherein the volume of broth includes both the volume of the cell medium and the volume of the cells), such as at least or about 10, 15, 20, 30, 40, 50, 60, 80, 100, 150, 200, 300, 400, or more g/L. In some embodiments, the concentration of the carbohydrate is between about 50 and about 400 g/L, such as between about 100 and about 360 g/L, between about 120 and about 360 g/L, or between about 200 and about 300 g/L. In some embodiments, this concentration of carbohydrate includes the total amount of carbohydrate that is added before and/or during the culturing of the host cells.

[0409] In some embodiments, the cells are cultured under limited glucose conditions. By "limited glucose conditions" is meant that the amount of glucose that is added is less than or about 105% (such as about 100%) of the amount of glucose that is consumed by the cells. In particular embodiments, the amount of glucose that is added to the culture medium is approximately the same as the amount of glucose that is consumed by the cells during a specific period of time. In some embodiments, the rate of cell growth is controlled by limiting the amount of added glucose such that the cells grow at the rate that can be supported by the amount of glucose in the cell medium. In some embodiments, glucose does not accumulate during the time the cells are cultured. In various embodiments, the cells are cultured under limited glucose conditions for greater than or about 1, 2, 3, 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, or 70 hours. In various embodiments, the cells are cultured under limited glucose conditions for greater than or about 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 95, or 100% of the total length of time the cells are cultured. While not intending to be bound by

any particular theory, it is believed that limited glucose conditions may allow more favorable regulation of the cells.

[0410] In some embodiments, the cells are cultured in the presence of an excess of glucose. In particular embodiments, the amount of glucose that is added is greater than about 105% (such as about or greater than 110, 120, 150, 175, 200, 250, 300, 400, or 500%) or more of the amount of glucose that is consumed by the cells during a specific period of time. In some embodiments, glucose accumulates during the time the cells are cultured.

[0411] Exemplary lipids are any substance containing one or more fatty acids that are C4 and above fatty acids that are saturated, unsaturated, or branched.

[0412] Exemplary oils are lipids that are liquid at room temperature. In some embodiments, the lipid contains one or more C4 or above fatty acids (*e.g.*, contains one or more saturated, unsaturated, or branched fatty acid with four or more carbons). In some embodiments, the oil is obtained from soy, corn, canola, jatropha, palm, peanut, sunflower, coconut, mustard, rapeseed, cottonseed, palm kernel, olive, safflower, sesame, linseed, oleagineous microbial cells, Chinese tallow, or any combination of two or more of the foregoing.

[0413] Exemplary fatty acids include compounds of the formula RCOOH, where "R" is a hydrocarbon. Exemplary unsaturated fatty acids include compounds where "R" includes at least one carbon-carbon double bond. Exemplary unsaturated fatty acids include, but are not limited to, oleic acid, vaccenic acid, linoleic acid, palmitoleic acid, and arachidonic acid. Exemplary polyunsaturated fatty acids include compounds where "R" includes a plurality of carbon-carbon double bonds. Exemplary saturated fatty acids include compounds where "R" is a saturated aliphatic group. In some embodiments, the carbon source includes one or more C_{12}-C_{22} fatty acids, such as a C_{12} saturated fatty acid, a C_{14} saturated fatty acid, a C_{16} saturated fatty acid, a C_{18} saturated fatty acid, a C_{20} saturated fatty acid, or a C_{22} saturated fatty acid. In an exemplary embodiment, the fatty acid is palmitic acid. In some embodiments, the carbon source is a salt of a fatty acid (*e.g.*, an unsaturated fatty acid), a derivative of a fatty acid (*e.g.*, an unsaturated fatty acid), or a salt of a derivative of fatty acid (*e.g.*, an unsaturated fatty acid). Suitable salts include, but are not limited to, lithium salts, potassium salts, sodium salts, and the like. Di- and triglycerides are fatty acid esters of glycerol.

[0414] In some embodiments, the concentration of the lipid, oil, fat, fatty acid, monoglyceride, diglyceride, or triglyceride is at least or about 1 gram per liter of broth (g/L, wherein the volume of broth includes both the volume of the cell medium and the volume of the cells), such as at least or about 5, 10, 15, 20, 30, 40, 50, 60, 80, 100, 150, 200, 300, 400, or more g/L. In some embodiments, the concentration of the lipid, oil, fat, fatty acid, monoglyceride, diglyceride, or triglyceride is between about 10 and about 400 g/L, such as between about 25 and about 300 g/L, between about 60 and about 180 g/L, or between about 75 and about 150 g/L. In some embodiments, the concentration includes the total amount of the lipid, oil, fat, fatty acid, monoglyceride, diglyceride, or triglyceride that is added before and/or during the culturing of the host cells. In some embodiments, the carbon source includes both (i) a lipid, oil, fat, fatty acid, monoglyceride, diglyceride, or triglyceride and (ii) a carbohydrate, such as glucose. In some embodiments, the ratio of the lipid, oil, fat, fatty acid, monoglyceride, diglyceride, or triglyceride to the carbohydrate is about 1:1 on a carbon basis (*i.e.*, one carbon in the lipid, oil, fat, fatty acid, monoglyceride, diglyceride, or triglyceride per carbohydrate carbon). In particular embodiments, the amount of the lipid, oil, fat, fatty acid, monoglyceride, diglyceride, or triglyceride is between about 60 and 180 g/L, and the amount of the carbohydrate is between about 120 and 360 g/L.

[0415] Exemplary microbial polypeptide carbon sources include one or more polypeptides from yeast or bacteria. Exemplary plant polypeptide carbon sources include one or more polypeptides from soy, corn, canola, jatropha, palm, peanut, sunflower, coconut, mustard, rapeseed, cottonseed, palm kernel, olive, safflower, sesame, or linseed.

[0416] Exemplary renewable carbon sources include cheese whey permeate, cornsteep liquor, sugar beet molasses, barley malt, and components from any of the foregoing. Exemplary renewable carbon sources also include glucose, hexose, pentose and xylose present in biomass, such as corn, switchgrass, sugar cane, cell waste of fermentation processes, and protein by-product from the milling of soy, corn, or wheat. In some embodiments, the biomass carbon source is a lignocellulosic, hemicellulosic, or cellulosic material such as, but are not limited to, a grass, wheat, wheat straw, bagasse, sugar cane bagasse, soft wood pulp, corn, corn cob or husk, corn kernel, fiber from corn kernels, corn stover, switch grass, rice hull product, or a by-product from wet or dry milling of grains (*e.g.*, corn, sorghum, rye, triticate, barley, wheat, and/or distillers grains). Exemplary cellulosic materials include wood, paper and pulp waste, herbaceous plants, and fruit pulp. In some

embodiments, the carbon source includes any plant part, such as stems, grains, roots, or tubers. In some embodiments, all or part of any of the following plants are used as a carbon source: corn, wheat, rye, sorghum, triticate, rice, millet, barley, cassava, legumes, such as beans and peas, potatoes, sweet potatoes, bananas, sugarcane, and/or tapioca. In some embodiments, the carbon source is a biomass hydrolysate, such as a biomass hydrolysate that includes both xylose and glucose or that includes both sucrose and glucose.

[0417] In some embodiments, the renewable carbon source (such as biomass) is pretreated before it is added to the cell culture medium. In some embodiments, the pretreatment includes enzymatic pretreatment, chemical pretreatment, or a combination of both enzymatic and chemical pretreatment (*see*, for example, Farzaneh *et al.*, *Bioresource Technology* 96 (18): 2014-2018, 2005; U.S. Patent No. 6,176,176; U.S. Patent No. 6,106,888; which are each hereby incorporated by reference in their entireties, particularly with respect to the pretreatment of renewable carbon sources). In some embodiments, the renewable carbon source is partially or completely hydrolyzed before it is added to the cell culture medium.

[0418] In some embodiments, the renewable carbon source (such as corn stover) undergoes ammonia fiber expansion (AFEX) pretreatment before it is added to the cell culture medium (*see*, for example, Farzaneh *et al.*, *Bioresource Technology* 96 (18): 2014-2018, 2005). During AFEX pretreatment, a renewable carbon source is treated with liquid anhydrous ammonia at moderate temperatures (such as about 60 to about 100 °C) and high pressure (such as about 250 to about 300 psi) for about 5 minutes. Then, the pressure is rapidly released. In this process, the combined chemical and physical effects of lignin solubilization, hemicellulose hydrolysis, cellulose decrystallization, and increased surface area enables near complete enzymatic conversion of cellulose and hemicellulose to fermentable sugars. AFEX pretreatment has the advantage that nearly all of the ammonia can be recovered and reused, while the remaining serves as nitrogen source for microbes in downstream processes. Also, a wash stream is not required for AFEX pretreatment. Thus, dry matter recovery following the AFEX treatment is essentially 100%. AFEX is basically a dry to dry process. The treated renewable carbon source is stable for long periods and can be fed at very high solid loadings in enzymatic hydrolysis or fermentation processes. Cellulose and hemicellulose are well preserved in the AFEX process, with little or no degradation. There is no need for neutralization prior to the enzymatic hydrolysis of a renewable carbon source that has undergone AFEX pretreatment. Enzymatic

hydrolysis of AFEX-treated carbon sources produces clean sugar streams for subsequent fermentation use.

[0419] In some embodiments, the concentration of the carbon source (*e.g.*, a renewable carbon source) is equivalent to at least or about 0.1, 0.5, 1, 1.5 2, 3, 4, 5, 10, 15, 20, 30, 40, or 50% glucose (w/v). The equivalent amount of glucose can be determined by using standard HPLC methods with glucose as a reference to measure the amount of glucose generated from the carbon source. In some embodiments, the concentration of the carbon source (*e.g.*, a renewable carbon source) is equivalent to between about 0.1 and about 20% glucose, such as between about 0.1 and about 10% glucose, between about 0.5 and about 10% glucose, between about 1 and about 10% glucose, between about 1 and about 5% glucose, or between about 1 and about 2% glucose.

[0420] In some embodiments, the carbon source includes yeast extract or one or more components of yeast extract. In some embodiments, the concentration of yeast extract is at least 1 gram of yeast extract per liter of broth (g/L, wherein the volume of broth includes both the volume of the cell medium and the volume of the cells), such at least or about 5, 10, 15, 20, 30, 40, 50, 60, 80, 100, 150, 200, 300, or more g/L. In some embodiments, the concentration of yeast extract is between about 1 and about 300 g/L, such as between about 1 and about 200 g/L, between about 5 and about 200 g/L, between about 5 and about 100 g/L, or between about 5 and about 60 g/L. In some embodiments, the concentration includes the total amount of yeast extract that is added before and/or during the culturing of the host cells. In some embodiments, the carbon source includes both yeast extract (or one or more components thereof) and another carbon source, such as glucose. In some embodiments, the ratio of yeast extract to the other carbon source is about 1:5, about 1:10, or about 1:20 (w/w).

[0421] Additionally the carbon source may also be one-carbon substrates such as carbon dioxide, or methanol. Glycerol production from single carbon sources (*e.g.*, methanol, formaldehyde, or formate) has been reported in methylotrophic yeasts (Yamada *et al.*, *Agric. Biol. Chem.,* 53(2) 541-543, 1989, which is hereby incorporated by reference in its entirety, particularly with respect to carbon sources) and in bacteria (Hunter *et. al.*, *Biochemistry,* 24, 4148-4155, 1985, which is hereby incorporated by reference in its entirety, particularly with respect to carbon sources). These organisms can assimilate single carbon compounds, ranging in oxidation state from methane to formate, and produce glycerol. The pathway of carbon assimilation can be through ribulose monophosphate, through serine, or through xylulose-

momophosphate (Gottschalk, Bacterial Metabolism, Second Edition, Springer-Verlag: New York, 1986, which is hereby incorporated by reference in its entirety, particularly with respect to carbon sources). The ribulose monophosphate pathway involves the condensation of formate with ribulose-5-phosphate to form a six carbon sugar that becomes fructose and eventually the three carbon product glyceraldehyde-3-phosphate. Likewise, the serine pathway assimilates the one-carbon compound into the glycolytic pathway via methylenetetrahydrofolate.

[0422] In addition to one and two carbon substrates, methylotrophic organisms are also known to utilize a number of other carbon containing compounds such as methylamine, glucosamine and a variety of amino acids for metabolic activity. For example, methylotrophic yeast are known to utilize the carbon from methylamine to form trehalose or glycerol (Bellion *et al.*, *Microb. Growth Cl Compd.,* [Int. Symp.], 7th ed., 415-32. Editors: Murrell *et al.*, Publisher: Intercept, Andover, UK, 1993, which is hereby incorporated by reference in its entirety, particularly with respect to carbon sources). Similarly, various species of *Candida* metabolize alanine or oleic acid (Sulter *et al.*, *Arch. Microbiol.* 153(5), 485-9, 1990, which is hereby incorporated by reference in its entirety, particularly with respect to carbon sources).

[0423] In some embodiments, cells are cultured in a standard medium containing physiological salts and nutrients (*see*, *e.g.*, Pourquie, J. *et al.*, Biochemistry and Genetics of Cellulose Degradation, *eds.* Aubert *et al.*, Academic Press, pp. 71-86, 1988 and Ilmen *et al.*, *Appl. Environ. Microbiol.* 63:1298-1306, 1997, which are each hereby incorporated by reference in their entireties, particularly with respect to cell medias). Exemplary growth media are common commercially prepared media such as Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth, or Yeast medium (YM) broth. Other defined or synthetic growth media may also be used, and the appropriate medium for growth of particular host cells are known by someone skilled in the art of microbiology or fermentation science.

[0424] In addition to an appropriate carbon source, the cell medium desirably contains suitable minerals, salts, cofactors, buffers, and other components known to those skilled in the art suitable for the growth of the cultures or the enhancement of isoprene production (*see*, for example, WO 2004/033646 and references cited therein and WO 96/35796 and references cited therein, which are each hereby incorporated by reference in their entireties, particularly with respect cell medias and cell culture conditions). In some embodiments where an isoprene synthase, DXS, IDI, and/or MVA pathway nucleic acid is under the control of an

inducible promoter, the inducing agent (*e.g.*, a sugar, metal salt or antimicrobial), is desirably added to the medium at a concentration effective to induce expression of an isoprene synthase, DXS, IDI, and/or MVA pathway polypeptide. In some embodiments, cell medium has an antibiotic (such as kanamycin) that corresponds to the antibiotic resistance nucleic acid (such as a kanamycin resistance nucleic acid) on a vector that has one or more DXS, IDI, or MVA pathway nucleic acids.

Exemplary Cell Culture Conditions

[0425] Materials and methods suitable for the maintenance and growth of bacterial cultures are well known in the art. Exemplary techniques may be found in Manual of Methods for General Bacteriology Gerhardt *et al.*, *eds*), American Society for Microbiology, Washington, D.C. (1994) or Brock in Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA, which are each hereby incorporated by reference in their entireties, particularly with respect to cell culture techniques. In some embodiments, the cells are cultured in a culture medium under conditions permitting the expression of one or more isoprene synthase, DXS, IDI, or MVA pathway polypeptides encoded by a nucleic acid inserted into the host cells.

[0426] Standard cell culture conditions can be used to culture the cells (*see*, for example, WO 2004/033646 and references cited therein, which are each hereby incorporated by reference in their entireties, particularly with respect to cell culture and fermentation conditions). Cells are grown and maintained at an appropriate temperature, gas mixture, and pH (such as at about 20°C to about 37°C, at about 6% to about 84% CO_2, and at a pH between about 5 to about 9). In some embodiments, cells are grown at 35°C in an appropriate cell medium. In some embodiments, *e.g.*, cultures are cultured at approximately 28°C in appropriate medium in shake cultures or fermentors until the desired amount of isoprene and hydrogen co-production is achieved. In some embodiments, the pH ranges for fermentation are between about pH 5.0 to about pH 9.0 (such as about pH 6.0 to about pH 8.0 or about 6.5 to about 7.0). Reactions may be performed under aerobic, anoxic, or anaerobic conditions based on the requirements of the host cells. In some embodiments, the cells are cultured under oxygen-limited conditions. In some embodiments, the cells are cultured in the presence of oxygen under conditions where 0.5 moles of oxygen are taken up per mole of isoprene produced. In some embodiments, the cells are cultured under anaerobic conditions. Exemplary culture conditions for a given filamentous fungus are known in the art and may be

found in the scientific literature and/or from the source of the fungi such as the American Type Culture Collection and Fungal Genetics Stock Center.

[0427] In various embodiments, the cells are grown using any known mode of fermentation, such as batch, fed-batch, or continuous processes. In some embodiments, a batch method of fermentation is used. Classical batch fermentation is a closed system where the composition of the media is set at the beginning of the fermentation and is not subject to artificial alterations during the fermentation. Thus, at the beginning of the fermentation the cell medium is inoculated with the desired host cells and fermentation is permitted to occur adding nothing to the system. Typically, however, "batch" fermentation is batch with respect to the addition of carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. In batch systems, the metabolite and biomass compositions of the system change constantly until the time the fermentation is stopped. Within batch cultures, cells moderate through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. In some embodiments, cells in log phase are responsible for the bulk of the isoprene production. In some embodiments, cells in stationary phase produce isoprene.

[0428] In some embodiments, a variation on the standard batch system is used, such as the Fed-Batch system. Fed-Batch fermentation processes comprise a typical batch system with the exception that the carbon source is added in increments as the fermentation progresses. Fed-Batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of carbon source in the cell medium. Fed-batch fermentations may be performed with the carbon source (*e.g.*, glucose) in a limited or excess amount. Measurement of the actual carbon source concentration in Fed-Batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen, and the partial pressure of waste gases such as CO_2. Batch and Fed-Batch fermentations are common and well known in the art and examples may be found in Brock, Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., which is hereby incorporated by reference in its entirety, particularly with respect to cell culture and fermentation conditions.

[0429] In some embodiments, continuous fermentation methods are used. Continuous fermentation is an open system where a defined fermentation medium is added continuously to a bioreactor and an equal amount of conditioned medium is removed simultaneously for

processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth.

[0430] Continuous fermentation allows for the modulation of one factor or any number of factors that affect cell growth or isoprene production. For example, one method maintains a limiting nutrient such as the carbon source or nitrogen level at a fixed rate and allows all other parameters to moderate. In other systems, a number of factors affecting growth can be altered continuously while the cell concentration (*e.g.*, the concentration measured by media turbidity) is kept constant. Continuous systems strive to maintain steady state growth conditions. Thus, the cell loss due to media being drawn off is balanced against the cell growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes as well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology and a variety of methods are detailed by Brock, Biotechnology: A Textbook of Industrial Microbiology, Second Edition (1989) Sinauer Associates, Inc., which is hereby incorporated by reference in its entirety, particularly with respect to cell culture and fermentation conditions.

[0431] In some embodiments, cells are immobilized on a substrate as whole cell catalysts and subjected to fermentation conditions for isoprene production.

[0432] In some embodiments, bottles of liquid culture are placed in shakers in order to introduce oxygen to the liquid and maintain the uniformity of the culture. In some embodiments, an incubator is used to control the temperature, humidity, shake speed, and/or other conditions in which a culture is grown. The simplest incubators are insulated boxes with an adjustable heater, typically going up to ~65 °C. More elaborate incubators can also include the ability to lower the temperature (via refrigeration), or the ability to control humidity or CO_2 levels. Most incubators include a timer; some can also be programmed to cycle through different temperatures, humidity levels, *etc.* Incubators can vary in size from tabletop to units the size of small rooms.

[0433] If desired, a portion or all of the cell medium can be changed to replenish nutrients and/or avoid the build up of potentially harmful metabolic byproducts and dead cells. In the case of suspension cultures, cells can be separated from the media by centrifuging or filtering the suspension culture and then resuspending the cells in fresh media. In the case of adherent cultures, the media can be removed directly by aspiration and replaced. In some

embodiments, the cell medium allows at least a portion of the cells to divide for at least or about 5, 10, 20, 40, 50, 60, 65, or more cell divisions in a continuous culture (such as a continuous culture without dilution).

[0434] In some embodiments, a constitutive or leaky promoter (such as a Trc promoter) is used and a compound (such as IPTG) is not added to induce expression of the isoprene synthase, DXS, IDI, or MVA pathway nucleic acid(s) operably linked to the promoter. In some embodiments, a compound (such as IPTG) is added to induce expression of the isoprene synthase, DXS, IDI, or MVA pathway nucleic acid(s) operably linked to the promoter.

Exemplary Methods for Decoupling Isoprene Production from Cell Growth

[0435] Desirably, carbon from the feedstock is converted to isoprene rather than to the growth and maintenance of the cells. In some embodiments, the cells are grown to a low to medium OD_{600}, then production of isoprene is started or increased. This strategy permits a large portion of the carbon to be converted to isoprene.

[0436] In some embodiments, cells reach an optical density such that they no longer divide or divide extremely slowly, but continue to make isoprene for several hours (such as about 2, 4, 6, 8, 10, 15, 20, 25, 30, or more hours). For example, Figs. 60A-67C illustrate that cells may continue to produce a substantial amount of mevalonic acid or isoprene after the cells reach an optical density such that they no longer divide or divide extremely slowly. In some cases, the optical density at 550 nm decreases over time (such as a decrease in the optical density after the cells are no longer in an exponential growth phase due to cell lysis), and the cells continue to produce a substantial amount of mevalonic acid or isoprene. In some embodiments, the optical density at 550 nm of the cells increases by less than or about 50% (such as by less than or about 40, 30, 20, 10, 5, or 0%) over a certain time period (such as greater than or about 5, 10, 15, 20, 25, 30, 40, 50 or 60 hours), and the cells produce isoprene at greater than or about 1, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, or more nmole of isoprene/gram of cells for the wet weight of the cells/hour (nmole/g_{wcm}/hr) during this time period. In some embodiments, the amount of isoprene is between about 2 to about 5,000 nmole/g_{wcm}/hr, such as between about 2 to about 100 nmole/g_{wcm}/hr, about 100 to about 500 nmole/g_{wcm}/hr, about 150 to about 500 nmole/g_{wcm} /hr, about 500 to about 1,000

nmole/g_{wcm}/hr, about 1,000 to about 2,000 nmole/g_{wcm}/hr, or about 2,000 to about 5,000 nmole/g_{wcm}/hr. In some embodiments, the amount of isoprene is between about 20 to about 5,000 nmole/g_{wcm}/hr, about 100 to about 5,000 nmole/g_{wcm}/hr, about 200 to about 2,000 nmole/g_{wcm}/hr, about 200 to about 1,000 nmole/g_{wcm}/hr, about 300 to about 1,000 nmole/g_{wcm}/hr, or about 400 to about 1,000 nmole/g_{wcm}/hr.

[0437] In some embodiments, the optical density at 550 nm of the cells increases by less than or about 50% (such as by less than or about 40, 30, 20, 10, 5, or 0%) over a certain time period (such as greater than or about 5, 10, 15, 20, 25, 30, 40, 50 or 60 hours), and the cells produce a cumulative titer (total amount) of isoprene at greater than or about 1, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, 10,000, 50,000, 100,000, or more mg of isoprene/L of broth (mg/L_{broth}, wherein the volume of broth includes the volume of the cells and the cell medium) during this time period. In some embodiments, the amount of isoprene is between about 2 to about 5,000 mg/L_{broth}, such as between about 2 to about 100 mg/L_{broth}, about 100 to about 500 mg/L_{broth}, about 500 to about 1,000 mg/L_{broth}, about 1,000 to about 2,000 mg/L_{broth}, or about 2,000 to about 5,000 mg/L_{broth}. In some embodiments, the amount of isoprene is between about 20 to about 5,000 mg/L_{broth}, about 100 to about 5,000 mg/L_{broth}, about 200 to about 2,000 mg/L_{broth}, about 200 to about 1,000 mg/L_{broth}, about 300 to about 1,000 mg/L_{broth}, or about 400 to about 1,000 mg/L_{broth}.

[0438] In some embodiments, the optical density at 550 nm of the cells increases by less than or about 50% (such as by less than or about 40, 30, 20, 10, 5, or 0%) over a certain time period (such as greater than or about 5, 10, 15, 20, 25, 30, 40, 50 or 60 hours), and the cells convert greater than or about 0.0015, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.12, 0.14, 0.16, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, or 8.0% of the carbon in the cell culture medium into isoprene during this time period. In some embodiments, the percent conversion of carbon into isoprene is between such as about 0.002 to about 4.0%, about 0.002 to about 3.0%, about 0.002 to about 2.0%, about 0.002 to about 1.6%, about 0.002 to about 0.005%, about 0.005 to about 0.01%, about 0.01 to about 0.05%, about 0.05 to about 0.15%, 0.15 to about 0.2%, about 0.2 to about 0.3%, about 0.3 to about 0.5%, about 0.5 to about 0.8%, about 0.8 to about 1.0%, or about 1.0 to about 1.6%. In some embodiments, the percent conversion of carbon into isoprene is between about 0.002 to about

0.4%, 0.002 to about 0.16%, 0.04 to about 0.16%, about 0.005 to about 0.3%, about 0.01 to about 0.3%, or about 0.05 to about 0.3%.

[0439] In some embodiments, isoprene is only produced in stationary phase. In some embodiments, isoprene is produced in both the growth phase and stationary phase. In various embodiments, the amount of isoprene produced (such as the total amount of isoprene produced or the amount of isoprene produced per liter of broth per hour per OD_{600}) during stationary phase is greater than or about 2, 3, 4, 5, 10, 20, 30, 40, 50, or more times the amount of isoprene produced during the growth phase for the same length of time. In various embodiments, greater than or about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, 99% or more of the total amount of isoprene that is produced (such as the production of isoprene during a fermentation for a certain amount of time, such as 20 hours) is produced while the cells are in stationary phase. In various embodiments, greater than or about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, 99% or more of the total amount of isoprene that is produced (such as the production of isoprene during a fermentation for a certain amount of time, such as 20 hours) is produced while the cells divide slowly or not at all such that the optical density at 550 nm of the cells increases by less than or about 50% (such as by less than or about 40, 30, 20, 10, 5, or 0%). In some embodiments, isoprene is only produced in the growth phase.

[0440] In some embodiments, one or more MVA pathway, IDI, DXP, or isoprene synthase nucleic acids are placed under the control of a promoter or factor that is more active in stationary phase than in the growth phase. For example, one or more MVA pathway, IDI, DXP, or isoprene synthase nucleic acids may be placed under control of a stationary phase sigma factor, such as RpoS. In some embodiments, one or more MVA pathway, IDI, DXP, or isoprene synthase nucleic acids are placed under control of a promoter inducible in stationary phase, such as a promoter inducible by a response regulator active in stationary phase.

Production of Isoprene within Safe Operating Ranges

[0441] The production of isoprene within safe operating levels according to its flammability characteristics simplifies the design and construction of commercial facilities, vastly improves the ability to operate safely, and limits the potential for fires to occur. In particular, the optimal ranges for the production of isoprene are within the safe zone, *i.e.*, the nonflammable range of isoprene concentrations. In one such aspect, the invention features a

method for the production of isoprene within the nonflammable range of isoprene concentrations (outside the flammability envelope of isoprene).

[0442] Thus, computer modeling and experimental testing were used to determine the flammability limits of isoprene (such as isoprene in the presence of O_2, N_2, CO_2, or any combination of two or more of the foregoing gases) in order to ensure process safety. The flammability envelope is characterized by the lower flammability limit (LFL), the upper flammability limit (UFL), the limiting oxygen concentration (LOC), and the limiting temperature. For a system to be flammable, a minimum amount of fuel (such as isoprene) must be in the presence of a minimum amount of oxidant, typically oxygen. The LFL is the minimum amount of isoprene that must be present to sustain burning, while the UFL is the maximum amount of isoprene that can be present. Above this limit, the mixture is fuel rich and the fraction of oxygen is too low to have a flammable mixture. The LOC indicates the minimum fraction of oxygen that must also be present to have a flammable mixture. The limiting temperature is based on the flash point of isoprene and is that lowest temperature at which combustion of isoprene can propagate. These limits are specific to the concentration of isoprene, type and concentration of oxidant, inerts present in the system, temperature, and pressure of the system. Compositions that fall within the limits of the flammability envelope propagate combustion and require additional safety precautions in both the design and operation of process equipment.

[0443] The following conditions were tested using computer simulation and mathematical analysis and experimental testing. If desired, other conditions (such as other temperature, pressure, and permanent gas compositions) may be tested using the methods described herein to determine the LFL, UFL, and LOC concentrations.

(1) Computer simulation and mathematical analysis

Test Suite 1:

isoprene: 0 wt% - 14 wt%

O_2: 6 wt% - 21 wt%

N_2: 79 wt% - 94 wt%

Test Suite 2:

isoprene: 0 wt% - 14 wt%

O_2: 6 wt% - 21 wt%

N_2: 79 wt% - 94 wt%

Saturated with H_2O

Test Suite 3:

isoprene: 0 wt% - 14 wt%

O_2: 6 wt% - 21 wt%

N_2: 79 wt% - 94 wt%

CO_2: 5 wt% - 30 wt%

(2) Experimental testing for final determination of flammability limits

Test Suite 1:

isoprene: 0 wt% - 14 wt%

O_2: 6 wt% - 21 wt%

N_2: 79 wt% - 94 wt%

Test Suite 2:

isoprene: 0 wt% - 14 wt%

O_2: 6 wt% - 21 wt%

N_2: 79 wt% - 94 wt%

Saturated with H_2O

[0444] Simulation software was used to give an estimate of the flammability characteristics of the system for several different testing conditions. CO_2 showed no significant affect on the system's flammability limits. Test suites 1 and 2 were confirmed by experimental testing. The modeling results were in-line with the experimental test results. Only slight variations were found with the addition of water.

[0445] The LOC was determined to be 9.5 vol% for an isoprene, O_2, N_2, and CO_2 mixture at 40°C and 1 atmosphere. The addition of up to 30% CO_2 did not significantly affect the flammability characteristics of an isoprene, O_2, and N_2 mixture. Only slight variations in flammability characteristics were shown between a dry and water saturated isoprene, O_2, and

N_2 system. The limiting temperature is about -54 °C. Temperatures below about -54 °C are too low to propagate combustion of isoprene.

[0446] In some embodiments, the LFL of isoprene ranges from about 1.5 vol.% to about 2.0 vol%, and the UFL of isoprene ranges from about 2.0 vol.% to about 12.0 vol.%, depending on the amount of oxygen in the system. In some embodiments, the LOC is about 9.5 vol% oxygen. In some embodiments, the LFL of isoprene is between about 1.5 vol.% to about 2.0 vol%, the UFL of isoprene is between about 2.0 vol.% to about 12.0 vol.%, and the LOC is about 9.5 vol% oxygen when the temperature is between about 25 °C to about 55 °C (such as about 40 °C) and the pressure is between about 1 atmosphere and 3 atmospheres.

[0447] In some embodiments, isoprene is produced in the presence of less than about 9.5 vol% oxygen (that is, below the LOC required to have a flammable mixture of isoprene). In some embodiments in which isoprene is produced in the presence of greater than or about 9.5 vol% oxygen, the isoprene concentration is below the LFL (such as below about 1.5 vol.%). For example, the amount of isoprene can be kept below the LFL by diluting the isoprene composition with an inert gas (*e.g.*, by continuously or periodically adding an inert gas such as nitrogen to keep the isoprene composition below the LFL). In some embodiments in which isoprene is produced in the presence of greater than or about 9.5 vol% oxygen, the isoprene concentration is above the UFL (such as above about 12 vol.%). For example, the amount of isoprene can be kept above the UFL by using a system (such as any of the cell culture systems described herein) that produces isoprene at a concentration above the UFL. If desired, a relatively low level of oxygen can be used so that the UFL is also relatively low. In this case, a lower isoprene concentration is needed to remain above the UFL.

[0448] In some embodiments in which isoprene is produced in the presence of greater than or about 9.5 vol% oxygen, the isoprene concentration is within the flammability envelope (such as between the LFL and the UFL). In some embodiments when the isoprene concentration may fall within the flammability envelope, one or more steps are performed to reduce the probability of a fire or explosion. For example, one or more sources of ignition (such as any materials that may generate a spark) can be avoided. In some embodiments, one or more steps are performed to reduce the amount of time that the concentration of isoprene remains within the flammability envelope. In some embodiments, a sensor is used to detect when the concentration of isoprene is close to or within the flammability envelope. If desired, the concentration of isoprene can be measured at one or more time points during the culturing

of cells, and the cell culture conditions and/or the amount of inert gas can be adjusted using standard methods if the concentration of isoprene is close to or within the flammability envelope. In particular embodiments, the cell culture conditions (such as fermentation conditions) are adjusted to either decrease the concentration of isoprene below the LFL or increase the concentration of isoprene above the UFL. In some embodiments, the amount of isoprene is kept below the LFL by diluting the isoprene composition with an inert gas (such as by continuously or periodically adding an inert gas to keep the isoprene composition below the LFL).

[0449] In some embodiments, the amount of flammable volatiles other than isoprene (such as one or more sugars) is at least about 2, 5, 10, 50, 75, or 100-fold less than the amount of isoprene produced. In some embodiments, the portion of the gas phase other than isoprene gas comprises between about 0% to about 100% (volume) oxygen, such as between about 0% to about 10%, about 10% to about 20%, about 20% to about 30%, about 30% to about 40%, about 40% to about 50%, about 50% to about 60%, about 60% to about 70%, about 70% to about 80%, about 90% to about 90%, or about 90% to about 100% (volume) oxygen. In some embodiments, the portion of the gas phase other than isoprene gas comprises between about 0% to about 99% (volume) nitrogen, such as between about 0% to about 10%, about 10% to about 20%, about 20% to about 30%, about 30% to about 40%, about 40% to about 50%, about 50% to about 60%, about 60% to about 70%, about 70% to about 80%, about 90% to about 90%, or about 90% to about 99% (volume) nitrogen.

[0450] In some embodiments, the portion of the gas phase other than isoprene gas comprises between about 1% to about 50% (volume) CO_2, such as between about 1% to about 10%, about 10% to about 20%, about 20% to about 30%, about 30% to about 40%, or about 40% to about 50% (volume) CO_2.

[0451] In some embodiments, an isoprene composition also contains ethanol. For example, ethanol may be used for extractive distillation of isoprene, resulting in compositions (such as intermediate product streams) that include both ethanol and isoprene. Desirably, the amount of ethanol is outside the flammability envelope for ethanol. The LOC of ethanol is about 8.7 vol%, and the LFL for ethanol is about 3.3 vol% at standard conditions, such as about 1 atmosphere and about 60 °F (NFPA 69 *Standard on Explosion Prevention Systems*, 2008 edition, which is hereby incorporated by reference in its entirety, particularly with respect to LOC, LFL, and UFL values). In some embodiments, compositions that include isoprene and

ethanol are produced in the presence of less than the LOC required to have a flammable mixture of ethanol (such as less than about 8.7% vol%). In some embodiments in which compositions that include isoprene and ethanol are produced in the presence of greater than or about the LOC required to have a flammable mixture of ethanol, the ethanol concentration is below the LFL (such as less than about 3.3 vol.%).

[0452] In various embodiments, the amount of oxidant (such as oxygen) is below the LOC of any fuel in the system (such as isoprene or ethanol). In various embodiments, the amount of oxidant (such as oxygen) is less than about 60, 40, 30, 20, 10, or 5% of the LOC of isoprene or ethanol. In various embodiments, the amount of oxidant (such as oxygen) is less than the LOC of isoprene or ethanol by at least 2, 4, 5, or more absolute percentage points (vol %). In particular embodiments, the amount of oxygen is at least 2 absolute percentage points (vol %) less than the LOC of isoprene or ethanol (such as an oxygen concentration of less than 7.5 vol% when the LOC of isoprene is 9.5 vol%). In various embodiments, the amount of fuel (such as isoprene or ethanol) is less than or about 25, 20, 15, 10, or 5% of the LFL for that fuel.

High Efficiency Production and Recovery of Isoprene, a Volatile Hydrocarbon, by Fermentation

[0453] Methods are provided herein of producing isoprene comprising a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein the liquid phase concentration of isoprene is less than about 200 mg/L. In some embodiments, the liquid phase concentration of isoprene in the culture is less than about any of 175 mg/L, 150 mg/L, 125 mg/L, 100 mg/L, 75 mg/L, 50 mg/L, 25 mg/L, 20 mg/L, 15 mg/L, 10 mg/L, 5 mg/L, or 2.5 mg/L. In some embodiments, the liquid phase concentration of isoprene in culture is between about any of 0.1 mg/L to 200 mg/L, 1 mg/L to 200 mg/L, 1 mg/L to 150 mg/L, 1 mg/L to 100 mg/L, 1 mg/L to 50 mg/L, 1 mg/L to 25 mg/L, 1 mg/L to 20 mg/L, or 10 mg/L to 20 mg/L. In some embodiments, the isoprene produced is any concentration or amount disclosed in the section entitled "Exemplary Production of Isoprene." In some embodiments, the liquid phase concentration is below the solubility limit of isoprene.

[0454] In some embodiments of the methods, the cells produce greater than about 400 nmole/gwcm/hour of isoprene. In some embodiments, the amount of isoprene is between about any of 400 nmole/g_{wcm}/hour to 1 mole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 1

mmole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 40 mmole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 4 mmole/g_{wcm}/hour, 1 mmole/g_{wcm}/hour to 1.5 mmole/g_{wcm}/hour, 1.5 mmole/g_{wcm}/hour to 3 mmole/g_{wcm}/hour, 3 mmole/g_{wcm}/hour to 5 mmole/g_{wcm}/hour, 5 mmole/g_{wcm}/hour to 25 mmole/g_{wcm}/hour, 25 mmole/g_{wcm}/hour to 100 mmole/g_{wcm}/hour, 100 mmole/g_{wcm}/hour to 500 mmole/g_{wcm}/hour, or 500 mmole/g_{wcm}/hour to 1000 mmole/g_{wcm}/hour. In some embodiments, the amount of isoprene is about any of 1 mmole/g_{wcm}/hour, 1.5 mmole/g_{wcm}/hour, 2 mmole/g_{wcm}/hour, 3 mmole/g_{wcm}/hour, 4 mmole/g_{wcm}/hour, or 5 mmole/g_{wcm}/hour.

[0455] The low value for Henry's coefficient means that isoprene can be recovered from fermentation broth by gas stripping at low sparging rates, for example 0.01 vvm to 2 vvm. In some embodiments, the gas sparging rate is between about any of 0.1 vvm to 1 vvm, 0.01 vvm to 0.5 vvm, 0.2 vvm to 1 vvm, or 0.5 vvm to 1 vvm. In some embodiments, the gas sparging rate is about any of 0.1 vvm, 0.25 vvm, 0.5 vvm, 0.75 vvm, 1 vvm, 1.25 vvm, 1.5 vvm, 1.75 vvm, or 2 vvm. In some embodiments, the low sparging rates are maintained for the entire course of the fermentation run, during growth phase, or during stationary phase. In some embodiments, the low sparging rates are maintained for between about any of 1 hour to 5 hours, 5 hours to 10 hours, 10 hours to 20 hours, 20 hours to 30 hours, 30 hours to 40 hours, 40 hours to 50 hours, or 50 hours to 60 hours. The lower desirable gas sparge limit is defined by the point at which the aqueous phase becomes saturated with isoprene and a liquid organic phase forms. This can only occur below the boiling point of isoprene (34.1°C at 1 atm), above which a liquid isoprene phase will never form. At temperatures below the boiling point of isoprene, the formation of a liquid phase is determined by the aqueous solubility of isoprene, which is approximately 650 mg/L at 25°C. While it is highly desirable to avoid the formation of a liquid isoprene phase, it is not absolutely required provided that the cells can tolerate the presence of liquid isoprene without toxic effects.

[0456] In some embodiments, the oxygen, CO_2, and isoprene are any of the amounts or concentrations discussed in the section entitled "Production of Isoprene with Safe Operating Ranges." In some embodiments, all the oxygen is consumed by the cells while maintaining fully aerobic metabolism. In some embodiments, an excess of oxygen is used in order to satisfy the oxygen demands of the cells. Desirable ranges of oxygen in the off-gas are less than 20%, or less than 15% or less than 10% (v/v). Levels of oxygen below the limiting oxygen concentration required for combustion of isoprene (9.5% v/v at 1 atm) are particularly

desirable. In some embodiments, oxygen-enriched air is utilized with the purpose of allowing minimal gas sweep rates while satisfying the cellular oxygen demand. In some embodiments, the portion of the gas phase of the gas sweep comprises between about 0.1% to about 10%, about 10% to about 20%, or about 20% to about 30% (volume) oxygen. In some embodiments, isoprene fermentations are performed under high pressure in order minimize the amount of excess oxygen required to maintain the required dissolved oxygen levels in the liquid phase.

[0457] In some embodiments, the reduction of the gas sweep rate through the fermentor is advantageous for an integrated isoprene production process in that such conditions enrich the off-gas isoprene levels up to about 30,000 ug/L (about 1% v/v) without adversely affecting the physiology of the cells.

[0458] In some embodiments, reduced gas-sparge rates do not significantly adversely affect the physiology of the cells. In some embodiments, the carbon dioxide evolution rate of cells in culture with reduced gas-sparge rates is between about any of 1 x 10^{-18} mmol/L/hour to about 1 mol/L/hour, 1 mmol/L/hour to 1 mol/L/hour, 25 mmol/L/hour to 750 mmol/L/hour, 25 mmol/L/hour to 75 mmol/L/hour, 250 mmol/L/hour to 750 mmol/L/hour, or 450 mmol/L/hour to 550 mmol/L/hour. In some embodiments, the carbon dioxide evolution rate is about any of 50 mmol/L/hour, 100 mmol/L/hour, 150 mmol/L/hour, 200 mmol/L/hour, 250 mmol/L/hour, 300 mmol/L/hour, 350 mmol/L/hour, 400 mmol/L/hour, 450 mmol/L/hour, or 500 mmol/L/hour. In some embodiments, cell viability with reduced gas-sparge rates is reduced by less than about any of 1.75-fold, 1.5-fold, 1.25-fold, 1-fold, 0.75-fold, 0.5-fold, or 0.25-fold. In some embodiments, cell viability with reduced gas-sparge rates is reduced by about 2-fold. In some embodiments, cell viability with reduced gas-sparge rates of a cell expressing a MVA pathway and/or DXP pathway RNA and/or protein from one or more of a heterologous and/or duplicate copy of a MVA pathway and/or DXP pathway nucleic acid is compared to a control cell lacking one or more of a heterologous and/or duplicate copy of a MVA pathway and/or DXP pathway nucleic acid with reduced gas-sparge rates. In some embodiments, cell viability with reduced gas-sparge rates of a cell expressing a MVA pathway and/or DXP pathway RNA and/or protein from one or more of a heterologous and/or duplicate copy of a MVA pathway and/or DXP pathway nucleic acid under the control of an inducible promoter, wherein the promotor is induced, is compared to a control cell containing one or more of a heterologous and/or duplicate copy of a MVA pathway and/or

DXP pathway nucleic acid under the control of an inducible promoter, wherein the promotor is not induced (uninduced) with reduced gas-sparge rates. In some embodiments, the inducible promoter is a beta-galactosidase promotor.

[0459] In some embodiments, the fermentation of a genetically modified host organism that converts at least 5% of the total carbon consumed by the organism into a volatile, unsaturated hydrocarbon. In some embodiments, the production of an unsaturated hydrocarbon at such a rate as to be present in the fermentation off-gas at a level of at least about any of 100 ug/L, 500 ug/L, 1000 ug/L, 2, 500 ug/L, 5,000 ug/L, 7,500 ug/L, or 10,000 ug/L.

[0460] In some embodiments, the unsaturated hydrocarbon is recovered from the off-gas stream in a manner that is suited to high-rates of production, which correspond to concentrations in the offgas of at least about any of 100 ug/L, 500 ug/L, 1000 ug/L, 2,500 ug/L, 5,000 ug/L, 7,500 ug/L, or 10,000 ug/L. In some embodiments, the continuous extraction and recovery of an unsaturated hydrocarbon from the fermentation off-gas in particular at low gas sweep rates such that the resulting off-gas is enriched in the volatile component of interest. In some embodiments, recovery of the volatile hydrocarbon by methods that depend on elevated concentrations of the volatile. For example, efficient capture of isoprene in fermentation off-gas through the use of compression/condensation or extractive distillation technologies. Also contemplated is the use of activated carbon cartridges in addition to silica gel adsorbants, desorption and concentration of isoprene from carbon cartidges, and/or construction and fermentation of host organisms such as *E. coli strains* that can convert about 5% or more of the glucose substrate to isoprene and result in off-gas concentrations of greater than about 15,000 ug/L isoprene. Recovery methods include any of the methods described herein.

[0461] Also provided herein are methods of producing a compound, wherein the compound has one or more characteristics selected from the group consisting of (a) a Henry's law coefficient of less than about 250 M/atm and (b) a solubility in water of less than about 100 g/L. In some embodiments, the method comprises: a) culturing cells under suitable conditions for production of the compound, wherein gas is added (such as the addition of gas to a system such as a fermentation system) at a gas sparging rate between about 0.01 vvm to about 2 vvm; and b) producing the compound.

[0462] In some embodiments, the amount of the compound that partitions into the cell mass is not included in the liquid phase solubility values. In some embodiments, the liquid phase concentration is below the solubility limit of compound.

[0463] In some embodiments, the compounds can be continuously recovered from fermentation broth by gas stripping at moderate to low gas sparging rates, in particular those compounds with Henry's law coefficients of about any of less than 250 M/atm, 200 M/atm, 150 M/atm, 100 M/atm, 75 M/atm, 50 M/atm, 25 M/atm, 10 M/atm, 5 M/atm, or 1 M/atm. Examples include aldehydes such as acetaldehyde (15 M/atm), ketones such as acetone (30 M/atm) or 2-butanone (20 M/atm), or alcohols including methanol (220 M/atm), ethanol (200 M/atm), 1-butanol (120 m/atm) or C5 alcohols including 3-methyl-3-buten-1-ol, and 3-methyl-2-buten-1-ol (50- 100 M/atm). Esters of alcohols generally have lower Henry's constants than the respective alcohols, for example ethyl acetate (6-9 M/atm) or the acetyl esters of C5 alcohols (<5 M/atm).Compounds with Henry's law coefficients of less than 1M/atm are particularly desirable. Examples include hemiterpenes, monoterpenes, or sesquiterpenes, in addition to other hydrocarbons such as C1 to C5 hydrocarbons (*e.g.*, methane, ethane, ethylene, or propylene). In some embodiments, the hydrocarbons such as C1 to C5 hydrocarbons are saturated, unsaturated, or branched.

[0464] In general, there is a correlation between Henry's law coefficient and water solubility in that compounds with very low coefficients are sparingly soluble in water (substantially water insoluble). Although volatiles with infinite solubilities in water (*e.g.* acetone or ethanol) can be removed by gas stripping, desirable solubility limits are less than about any of 100 g/L, 75 g/L, 50 g/L, 25 g/L, 10 g/L, 5 g/L, or 1g/L.

[0465] In some embodiments of any of the methods of producing any of the compounds described above, the gas sparging rate is between about any of 0.1 vvm to 1 vvm, 0.2 vvm to 1 vvm, or 0.5 vvm to 1 vvm. In some embodiments, the gas sparging rate is about any of 0.1 vvm, 0.25 vvm, 0.5 vvm, 0.75 vvm, 1 vvm, 1.25 vvm, 1.5 vvm, 1.75 vvm, or 2 vvm. In some embodiments, the low sparging rates are maintained for the entire course of the fermentation run, during growth phase, or during stationary phase. In some embodiments, the low sparging rates are maintained for between about any of 1 hour to 5 hours, 5 hours to 10 hours, 10 hours to 20 hours, 20 hours to 30 hours, 30 hours to 40 hours, 40 hours to 50 hours, or 50 hours to 60 hours.

[0466] Any of the systems described herein can be used in the methods of producing a compound described above. Standard methods would be used to purify such as those described in the section entitled "Exemplary Purification Methods." Separation can be performed post-recovery for example, by distillation or selective adsorption techniques.

Exemplary Production of Isoprene

[0467] In some embodiments, the cells are cultured in a culture medium under conditions permitting the production of isoprene by the cells.

[0468] By "peak absolute productivity" is meant the maximum absolute amount of isoprene in the off-gas during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run). By "peak absolute productivity time point" is meant the time point during a fermentation run when the absolute amount of isoprene in the off-gas is at a maximum during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run). In some embodiments, the isoprene amount is measured at the peak absolute productivity time point. In some embodiments, the peak absolute productivity for the cells is about any of the isoprene amounts disclosed herein.

[0469] By "peak specific productivity" is meant the maximum amount of isoprene produced per cell during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run). By "peak specific productivity time point" is meant the time point during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run) when the amount of isoprene produced per cell is at a maximum. The peak specific productivity is determined by dividing the total productivity by the amount of cells, as determined by optical density at 600nm (OD_{600}). In some embodiments, the isoprene amount is measured at the peak specific productivity time point. In some embodiments, the peak specific productivity for the cells is about any of the isoprene amounts per cell disclosed herein.

[0470] By "peak volumetric productivity" is meant the maximum amount of isoprene produced per volume of broth (including the volume of the cells and the cell medium) during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run). By "peak specific volumetric productivity time point" is meant

the time point during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run) when the amount of isoprene produced per volume of broth is at a maximum. The peak specific volumetric productivity is determined by dividing the total productivity by the volume of broth and amount of time. In some embodiments, the isoprene amount is measured at the peak specific volumetric productivity time point. In some embodiments, the peak specific volumetric productivity for the cells is about any of the isoprene amounts per volume per time disclosed herein.

[0471] By "peak concentration" is meant the maximum amount of isoprene produced during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run). By "peak concentration time point" is meant the time point during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run) when the amount of isoprene produced per cell is at a maximum. In some embodiments, the isoprene amount is measured at the peak concentration time point. In some embodiments, the peak concentration for the cells is about any of the isoprene amounts disclosed herein.

[0472] By "average volumetric productivity" is meant the average amount of isoprene produced per volume of broth (including the volume of the cells and the cell medium) during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run). The average volumetric productivity is determined by dividing the total productivity by the volume of broth and amount of time. In some embodiments, the average specific volumetric productivity for the cells is about any of the isoprene amounts per volume per time disclosed herein.

[0473] By "cumulative total productivity" is meant the cumulative, total amount of isoprene produced during the culturing of cells for a particular period of time (*e.g.*, the culturing of cells during a particular fermentation run). In some embodiments, the cumulative, total amount of isoprene is measured. In some embodiments, the cumulative total productivity for the cells is about any of the isoprene amounts disclosed herein.

[0474] By "relative detector response" refers to the ratio between the detector response (such as the GC/MS area) for one compound (such as isoprene) to the detector response (such as the GC/MS area) of one or more compounds (such as all C5 hydrocarbons). The detector response may be measured as described herein, such as the GC/MS analysis performed with

an Agilent 6890 GC/MS system fitted with an Agilent HP-5MS GC/MS column (30 m x 250 μm; 0.25 μm film thickness). If desired, the relative detector response can be converted to a weight percentage using the response factors for each of the compounds. This response factor is a measure of how much signal is generated for a given amount of a particular compound (that is, how sensitive the detector is to a particular compound). This response factor can be used as a correction factor to convert the relative detector response to a weight percentage when the detector has different sensitivities to the compounds being compared. Alternatively, the weight percentage can be approximated by assuming that the response factors are the same for the compounds being compared. Thus, the weight percentage can be assumed to be approximately the same as the relative detector response.

[0475] In some embodiments, the cells in culture produce isoprene at greater than or about 1, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, or more nmole of isoprene/gram of cells for the wet weight of the cells/hour (nmole/g_{wcm}/hr). In some embodiments, the amount of isoprene is between about 2 to about 5,000 nmole/g_{wcm}/hr, such as between about 2 to about 100 nmole/g_{wcm}/hr, about 100 to about 500 nmole/g_{wcm}/hr, about 150 to about 500 nmole/g_{wcm} /hr, about 500 to about 1,000 nmole/g_{wcm}/hr, about 1,000 to about 2,000 nmole/g_{wcm}/hr, or about 2,000 to about 5,000 nmole/g_{wcm}/hr. In some embodiments, the amount of isoprene is between about 20 to about 5,000 nmole/g_{wcm}/hr, about 100 to about 5,000 nmole/g_{wcm}/hr, about 200 to about 2,000 nmole/g_{wcm}/hr, about 200 to about 1,000 nmole/g_{wcm}/hr, about 300 to about 1,000 nmole/g_{wcm}/hr, or about 400 to about 1,000 nmole/g_{wcm}/hr.

[0476] The amount of isoprene in units of nmole/g_{wcm}/hr can be measured as disclosed in U.S. Patent No. 5,849,970, which is hereby incorporated by reference in its entirety, particularly with respect to the measurement of isoprene production. For example, two mL of headspace (*e.g.*, headspace from a culture such as 2 mL of culture cultured in sealed vials at 32°C with shaking at 200 rpm for approximately 3 hours) are analyzed for isoprene using a standard gas chromatography system, such as a system operated isothermally (85°C) with an n-octane/porasil C column (Alltech Associates, Inc., Deerfield, Ill.) and coupled to a RGD2 mercuric oxide reduction gas detector (Trace Analytical, Menlo Park, CA) (*see*, for example, Greenberg et al, *Atmos. Environ.* 27A: 2689-2692, 1993; Silver *et al.*, *Plant Physiol.* 97:1588-1591, 1991, which are each hereby incorporated by reference in their entireties, particularly with respect to the measurement of isoprene production). The gas

chromatography area units are converted to nmol isoprene via a standard isoprene concentration calibration curve. In some embodiments, the value for the grams of cells for the wet weight of the cells is calculated by obtaining the A_{600} value for a sample of the cell culture, and then converting the A_{600} value to grams of cells based on a calibration curve of wet weights for cell cultures with a known A_{600} value. In some embodiments, the grams of the cells is estimated by assuming that one liter of broth (including cell medium and cells) with an A_{600} value of 1 has a wet cell weight of 1 gram. The value is also divided by the number of hours the culture has been incubating for, such as three hours.

[0477] In some embodiments, the cells in culture produce isoprene at greater than or about 1, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, 10,000, 100,000, or more ng of isoprene/gram of cells for the wet weight of the cells/hr (ng/g_{wcm}/h). In some embodiments, the amount of isoprene is between about 2 to about 5,000 ng/g_{wcm}/h, such as between about 2 to about 100 ng/g_{wcm}/h, about 100 to about 500 ng/g_{wcm}/h, about 500 to about 1,000 ng/g_{wcm}/h, about 1,000 to about 2,000 ng/g_{wcm}/h, or about 2,000 to about 5,000 ng/g_{wcm}/h. In some embodiments, the amount of isoprene is between about 20 to about 5,000 ng/g_{wcm}/h, about 100 to about 5,000 ng/g_{wcm}/h, about 200 to about 2,000 ng/g_{wcm}/h, about 200 to about 1,000 ng/g_{wcm}/h, about 300 to about 1,000 ng/g_{wcm}/h, or about 400 to about 1,000 ng/g_{wcm}/h. The amount of isoprene in ng/g_{wcm}/h can be calculated by multiplying the value for isoprene production in the units of nmole/g_{wcm}/hr discussed above by 68.1 (as described in Equation 5 below).

[0478] In some embodiments, the cells in culture produce a cumulative titer (total amount) of isoprene at greater than or about 1, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, 10,000, 50,000, 100,000, or more mg of isoprene/L of broth (mg/L_{broth}, wherein the volume of broth includes the volume of the cells and the cell medium). In some embodiments, the amount of isoprene is between about 2 to about 5,000 mg/L_{broth}, such as between about 2 to about 100 mg/L_{broth}, about 100 to about 500 mg/L_{broth}, about 500 to about 1,000 mg/L_{broth}, about 1,000 to about 2,000 mg/L_{broth}, or about 2,000 to about 5,000 mg/L_{broth}. In some embodiments, the amount of isoprene is between about 20 to about 5,000 mg/L_{broth}, about 100 to about 5,000 mg/L_{broth}, about 200 to about 2,000 mg/L_{broth}, about 200 to about 1,000 mg/L_{broth}, about 300 to about 1,000 mg/L_{broth}, or about 400 to about 1,000 mg/L_{broth}.

[0479] The specific productivity of isoprene in mg of isoprene/L of headspace from shake flask or similar cultures can be measured by taking a 1 ml sample from the cell culture at an OD_{600} value of approximately 1.0, putting it in a 20 mL vial, incubating for 30 minutes, and then measuring the amount of isoprene in the headspace (as described, for example, in Example I, part II). If the OD_{600} value is not 1.0, then the measurement can be normalized to an OD_{600} value of 1.0 by dividing by the OD_{600} value. The value of mg isoprene/L headspace can be converted to $mg/L_{broth}/hr/OD_{600}$ of culture broth by multiplying by a factor of 38. The value in units of $mg/L_{broth}/hr/OD_{600}$ can be multiplied by the number of hours and the OD_{600} value to obtain the cumulative titer in units of mg of isoprene/L of broth.

[0480] In some embodiments, the cells in culture have an average volumetric productivity of isoprene at greater than or about 0.1, 1.0, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1100, 1200, 1300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,100, 2,200, 2,300, 2,400, 2,500, 2,600, 2,700, 2,800, 2,900, 3,000, 3,100, 3,200, 3,300, 3,400, 3,500, or more mg of isoprene/L of broth/hr ($mg/L_{broth}/hr$, wherein the volume of broth includes the volume of the cells and the cell medium). In some embodiments, the average volumetric productivity of isoprene is between about 0.1 to about 3,500 $mg/L_{broth}/hr$, such as between about 0.1 to about 100 $mg/L_{broth}/hr$, about 100 to about 500 $mg/L_{broth}/hr$, about 500 to about 1,000 $mg/L_{broth}/hr$, about 1,000 to about 1,500 $mg/L_{broth}/hr$, about 1,500 to about 2,000 $mg/L_{broth}/hr$, about 2,000 to about 2,500 $mg/L_{broth}/hr$, about 2,500 to about 3,000 $mg/L_{broth}/hr$, or about 3,000 to about 3,500 $mg/L_{broth}/hr$. In some embodiments, the average volumetric productivity of isoprene is between about 10 to about 3,500 $mg/L_{broth}/hr$, about 100 to about 3,500 $mg/L_{broth}/hr$, about 200 to about 1,000 $mg/L_{broth}/hr$, about 200 to about 1,500 $mg/L_{broth}/hr$, about 1,000 to about 3,000 $mg/L_{broth}/hr$, or about 1,500 to about 3,000 $mg/L_{broth}/hr$.

[0481] In some embodiments, the cells in culture have a peak volumetric productivity of isoprene at greater than or about 0.5, 1.0, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1100, 1200, 1300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,100, 2,200, 2,300, 2,400, 2,500, 2,600, 2,700, 2,800, 2,900, 3,000, 3,100, 3,200, 3,300, 3,400, 3,500, 3,750, 4,000, 4,250, 4,500, 4,750, 5,000, 5,250, 5,500, 5,750, 6,000, 6,250, 6,500, 6,750, 7,000, 7,250, 7,500, 7,750, 8,000, 8,250, 8,500, 8,750, 9,000, 9,250, 9,500, 9,750, 10,000, 12,500, 15,000, or more mg of isoprene/L of broth/hr ($mg/L_{broth}/hr$, wherein the volume of broth includes the volume of the cells and the cell medium). In some

embodiments, the peak volumetric productivity of isoprene is between about 0.5 to about 15,000 mg/L_{broth}/hr, such as between about 0.5 to about 10 mg/L_{broth}/hr, about 1.0 to about 100 mg/L_{broth}/hr, about 100 to about 500 mg/L_{broth}/hr, about 500 to about 1,000 mg/L_{broth}/hr, about 1,000 to about 1,500 mg/L_{broth}/hr, about 1,500 to about 2,000 mg/L_{broth}/hr, about 2,000 to about 2,500 mg/L_{broth}/hr, about 2,500 to about 3,000 mg/L_{broth}/hr, about 3,000 to about 3,500 mg/L_{broth}/hr, about 3,500 to about 5,000 mg/L_{broth}/hr, about 5,000 to about 7,500 mg/L_{broth}/hr, about 7,500 to about 10,000 mg/L_{broth}/hr, about 10,000 to about 12,500 mg/L_{broth}/h, or about 12,500 to about 15,000 mg/L_{broth}/hr. In some embodiments, the peak volumetric productivity of isoprene is between about 10 to about 15,000 mg/L_{broth}/hr, about 100 to about 2,500 mg/L_{broth}/hr, about 1,000 to about 5,000 mg/L_{broth}/hr, about 2,500 to about 7,500 mg/L_{broth}/hr, about 5,000 to about 10,000 mg/L_{broth}/hr, about 7,500 to about 12,500 mg/L_{broth}/hr, or about 10,000 to about 15,000 mg/L_{broth}/hr.

[0482] The instantaneous isoprene production rate in mg/L_{broth}/hr in a fermentor can be measured by taking a sample of the fermentor off-gas, analyzing it for the amount of isoprene (in units such as mg of isoprene per L_{gas}) as described, for example, in Example I, part II and multiplying this value by the rate at which off-gas is passed though each liter of broth (*e.g.,* at 1 vvm (volume of air/volume of broth/minute) this is 60 L_{gas} per hour). Thus, an off-gas level of 1 mg/L_{gas} corresponds to an instantaneous production rate of 60 mg/L_{broth}/hr at air flow of 1 vvm. If desired, the value in the units mg/L_{broth}/hr can be divided by the OD_{600} value to obtain the specific rate in units of mg/L_{broth}/hr/OD. The average value of mg isoprene/L_{gas} can be converted to the total product productivity (grams of isoprene per liter of fermentation broth, mg/L_{broth}) by multiplying this average off-gas isoprene concentration by the total amount of off-gas sparged per liter of fermentation broth during the fermentation. Thus, an average off-gas isoprene concentration of 0.5 mg/L_{broth}/hr over 10 hours at 1 vvm corresponds to a total product concentration of 300 mg isoprene/L_{broth}.

[0483] In some embodiments, the cells in culture convert greater than or about 0.0015, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.12, 0.14, 0.16, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0, or 8.0% of the carbon in the cell culture medium into isoprene. In some embodiments, the percent conversion of carbon into isoprene is between such as about 0.002 to about 4.0%, about 0.002 to about 3.0%, about 0.002 to about 2.0%, about 0.002 to about 1.6%, about 0.002 to about 0.005%, about 0.005 to about 0.01%, about 0.01 to about 0.05%, about 0.05 to about 0.15%, 0.15 to about 0.2%, about 0.2

to about 0.3%, about 0.3 to about 0.5%, about 0.5 to about 0.8%, about 0.8 to about 1.0%, or about 1.0 to about 1.6%. In some embodiments, the percent conversion of carbon into isoprene is between about 0.002 to about 0.4%, 0.002 to about 0.16%, 0.04 to about 0.16%, about 0.005 to about 0.3%, about 0.01 to about 0.3%, or about 0.05 to about 0.3%.

[0484] The percent conversion of carbon into isoprene (also referred to as "% carbon yield") can be measured by dividing the moles carbon in the isoprene produced by the moles carbon in the carbon source (such as the moles of carbon in batched and fed glucose and yeast extract). This number is multiplied by 100% to give a percentage value (as indicated in Equation 1).

Equation 1

% Carbon Yield = (moles carbon in isoprene produced)/(moles carbon in carbon source) * 100

[0485] For this calculation, yeast extract can be assumed to contain 50% w/w carbon. As an example, for the 500 liter described in Example 7, part VIII, the percent conversion of carbon into isoprene can be calculated as shown in Equation 2.

Equation 2

% Carbon Yield = (39.1 g isoprene * 1/68.1mol/g * 5 C/mol)/[(181221 g glucose * 1/180 mol/g * 6 C/mol) + (17780 g yeast extract * 0.5 * 1/12 mol/g)] * 100 = 0.042%

[0486] For the two 500 liter fermentations described herein (Example 7, parts VII and VIII), the percent conversion of carbon into isoprene was between 0.04-0.06%. A 0.11-0.16% carbon yield has been achieved using 14 liter systems as described herein. Example 11, part V describes the 1.53% conversion of carbon to isoprene using the methods described herein.

[0487] One skilled in the art can readily convert the rates of isoprene production or amount of isoprene produced into any other units. Exemplary equations are listed below for interconverting between units.

Units for Rate of Isoprene production (total and specific)

Equation 3

1 g isoprene/L_{broth}/hr = 14.7 mmol isoprene/L_{broth}/hr (total volumetric rate)

Equation 4

1 nmol isoprene /g_{wcm}/hr = 1 nmol isoprene /L_{broth}/hr/OD_{600} (This conversion assumes that one liter of broth with an OD_{600} value of 1 has a wet cell weight of 1 gram.)

Equation 5

1 nmol isoprene/g_{wcm}/hr = 68.1 ng isoprene/g_{wcm}/hr (given the molecular weight of isoprene)

Equation 6

1 nmol isoprene/L_{gas} O_2/hr = 90 nmol isoprene/L_{broth}/hr (at an O_2 flow rate of 90 L/hr per L of culture broth)

Equation 7

1 ug isoprene/L_{gas} isoprene in off-gas = 60 ug isoprene/L_{broth}/hr at a flow rate of 60 L_{gas} per L_{broth} (1 vvm)

Units for Titer (total and specific)

Equation 8

1 nmol isoprene/mg cell protein = 150 nmol isoprene/L_{broth}/OD_{600} (This conversion assumes that one liter of broth with an OD_{600} value of 1 has a total cell protein of approximately 150 mg) (specific productivity)

Equation 9

1 g isoprene/L_{broth} = 14.7 mmol isoprene/L_{broth} (total titer)

[0488] If desired, Equation 10 can be used to convert any of the units that include the wet weight of the cells into the corresponding units that include the dry weight of the cells.

Equation 10

Dry weight of cells = (wet weight of cells)/3.3

[0489] If desired, Equation 11 can be used to convert between units of ppm and μg/L. In particular, "ppm" means parts per million defined in terms of μg/g (w/w). Concentrations of gases can also be expressed on a volumetric basis using "ppmv" (parts per million by volume), defined in terms of μL/L (vol/vol). Conversion of μg/L to ppm (*e.g.*, μg of analyte per g of gas) can be performed by determining the mass per L of off-gas (*i.e.*, the density of the gas). For example, a liter of air at standard temperature and pressure (STP; 101.3 kPa (1 bar) and 273.15K). has a density of approximately 1.29 g/L. Thus, a concentration of 1 ppm (μg/g) equals 1.29 μg/L at STP (Equation 11). The conversion of ppm (μg/g) to μg/L is a function of both pressure, temperature, and overall composition of the off-gas.

Equation 11

1 ppm (ug/g) equals 1.29 μg/L at standard temperature and pressure (STP; 101.3 kPa (1 bar) and 273.15K).

[0490] Conversion of ug/L to ppmv (*e.g.*, uL of analyte per L of gas) can be performed using the Universal Gas Law (equation 12). For example, an off-gas concentration of 1000 ug/L_{gas} corresponds to 14.7 $umol/L_{gas}$. The universal gas constant is 0.082057 L.atm $K^{-1}mol^{-1}$, so using equation 12, the volume occupied by 14.7 umol of HG at STP is equal to 0.329 mL. Therefore, the concentration of 1000 ug/L HG is equal to 329 ppmv or 0.0329% (v/v) at STP.

Equation 12

PV = nRT, where "P" is pressure, "V" is volume, "n" is moles of gas, "R" is the Universal gas constant, and "T" is temperature in Kelvin.

[0491] The amount of impurities in isoprene compositions are typically measured herein on a weight per volume (w/v) basis in units such as ug/L. If desired, measurements in units of ug/L can be converted to units of mg/m^3 using equation 13.

Equation 13

1 ug/L = 1 mg/m^3

[0492] In some embodiments encompassed by the invention, a cell comprising a heterologous nucleic acid encoding an isoprene synthase polypeptide produces an amount of isoprene that is at least or about 2-fold, 3-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold,

150-fold, 200-fold, 400-fold, or greater than the amount of isoprene produced from a corresponding cell grown under essentially the same conditions without the heterologous nucleic acid encoding the isoprene synthase polypeptide.

[0493] In some embodiments encompassed by the invention, a cell comprising a heterologous nucleic acid encoding an isoprene synthase polypeptide and one or more heterologous nucleic acids encoding a DXS, IDI, and/or MVA pathway polypeptide produces an amount of isoprene that is at least or about 2-fold, 3-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 150-fold, 200-fold, 400-fold, or greater than the amount of isoprene produced from a corresponding cell grown under essentially the same conditions without the heterologous nucleic acids.

[0494] In some embodiments, the isoprene composition comprises greater than or about 99.90, 99.92, 99.94, 99.96, 99.98, or 100% isoprene by weight compared to the total weight of all C5 hydrocarbons in the composition. In some embodiments, the composition has a relative detector response of greater than or about 99.90, 99.91, 99.92, 99.93, 99.94, 99.95, 99.96, 99.97, 99.98, 99.99, or 100% for isoprene compared to the detector response for all C5 hydrocarbons in the composition. In some embodiments, the isoprene composition comprises between about 99.90 to about 99.92, about 99.92 to about 99.94, about 99.94 to about 99.96, about 99.96 to about 99.98, about 99.98 to 100% isoprene by weight compared to the total weight of all C5 hydrocarbons in the composition.

[0495] In some embodiments, the isoprene composition comprises less than or about 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% C5 hydrocarbons other than isoprene (such 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 3-methyl-l-butyne, *trans*-piperylene, *cis*-piperylene, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne) by weight compared to the total weight of all C5 hydrocarbons in the composition. In some embodiments, the composition has a relative detector response of less than or about 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% for C5 hydrocarbons other than isoprene compared to the detector response for all C5 hydrocarbons in the composition. In some embodiments, the composition has a relative detector response of less than or about 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% for 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 3-methyl-l-butyne, *trans*-

piperylene, *cis*-piperylene, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne compared to the detector response for all C5 hydrocarbons in the composition. In some embodiments, the isoprene composition comprises between about 0.02 to about 0.04%, about 0.04 to about 0.06%, about 0.06 to 0.08%, about 0.08 to 0.10%, or about 0.10 to about 0.12% C5 hydrocarbons other than isoprene (such 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 3-methyl-l-butyne, *trans*-piperylene, *cis*-piperylene, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne) by weight compared to the total weight of all C5 hydrocarbons in the composition.

[0496] In some embodiments, the isoprene composition comprises less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ug/L of a compound that inhibits the polymerization of isoprene for any compound in the composition that inhibits the polymerization of isoprene. In some embodiments, the isoprene composition comprises between about 0.005 to about 50, such as about 0.01 to about 10, about 0.01 to about 5, about 0.01 to about 1, about 0.01 to about 0.5, or about 0.01 to about 0.005 ug/L of a compound that inhibits the polymerization of isoprene for any compound in the composition that inhibits the polymerization of isoprene. In some embodiments, the isoprene composition comprises less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ug/L of a hydrocarbon other than isoprene (such 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 3-methyl-l-butyne, *trans*-piperylene, *cis*-piperylene, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne). In some embodiments, the isoprene composition comprises between about 0.005 to about 50, such as about 0.01 to about 10, about 0.01 to about 5, about 0.01 to about 1, about 0.01 to about 0.5, or about 0.01 to about 0.005 ug/L of a hydrocarbon other than isoprene. In some embodiments, the isoprene composition comprises less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ug/L of a protein or fatty acid (such as a protein or fatty acid that is naturally associated with natural rubber).

[0497] In some embodiments, the isoprene composition comprises less than or about 10, 5, 1, 0.8, 0.5, 0.1, 0.05, 0.01, or 0.005 ppm of alpha acetylenes, piperylenes, acetonitrile, or 1,3-cyclopentadiene. In some embodiments, the isoprene composition comprises less than or about 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ppm of sulfur or allenes. In some embodiments, the isoprene composition comprises less than or about 30, 20, 15, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ppm of all acetylenes (such as 1-pentyne, 2-pentyne, 3-methyl-l-butyne, pent-4-ene-1-

yne, *trans*-pent-3-ene-1-yne, *cis*-pent-3-ene-1-yne, pentyne-1, butyne-2, 2MB1-3yne, and 1-pentyne-4yne). In some embodiments, the isoprene composition comprises less than or about 2000, 1000, 500, 200, 100, 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ppm of isoprene dimers, such as cyclic isoprene dimers (*e.g.*, cyclic C10 compounds derived from the dimerization of two isoprene units).

[0498] In some embodiments, the isoprene composition includes ethanol, acetone, a C5 prenyl alcohol (such as 3-methyl-3-buten-1-ol or 3-methyl-2-buten-1-ol), or any two or more of the foregoing. In particular embodiments, the isoprene composition comprises greater than or about 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 60, 80, 100, or 120 ug/L of ethanol, acetone, a C5 prenyl alcohol (such as 3-methyl-3-buten-1-ol or 3-methyl-2-buten-1-ol), or any two or more of the foregoing. In some embodiments, the isoprene composition comprises between about 0.005 to about 120, such as about 0.01 to about 80, about 0.01 to about 60, about 0.01 to about 40, about 0.01 to about 30, about 0.01 to about 20, about 0.01 to about 10, about 0.1 to about 80, about 0.1 to about 60, about 0.1 to about 40, about 5 to about 80, about 5 to about 60, or about 5 to about 40 ug/L of ethanol, acetone, a C5 prenyl alcohol, or any two or more of the foregoing.

[0499] In some embodiments, the isoprene composition includes one or more of the following components: 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, acetaldehyde, methanethiol, methyl acetate, 1-propanol, diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene, 2,3-cycloheptenolpyridine, or a linear isoprene polymer (such as a linear isoprene dimer or a linear isoprene trimer derived from the polymerization of multiple isoprene units). In various embodiments, the amount of one of these components relative to amount of isoprene in units of percentage by weight (*i.e.*, weight of the component divided by the weight of isoprene times 100) is greater than or about 0.01, 0.02, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, or 110% (w/w). In some embodiments, the relative detector response for the second compound compared to the detector response for isoprene is greater than or about 0.01, 0.02, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, or 110%. In various embodiments, the amount of one of these components relative to amount of

isoprene in units of percentage by weight (*i.e.*, weight of the component divided by the weight of isoprene times 100) is between about 0.01 to about 105 % (w/w), such as about 0.01 to about 90, about 0.01 to about 80, about 0.01 to about 50, about 0.01 to about 20, about 0.01 to about 10, about 0.02 to about 50, about 0.05 to about 50, about 0.1 to about 50, or 0.1 to about 20% (w/w).

[0500] In some embodiments, the isoprene composition includes one or more of the following: an alcohol, an aldehyde, an ester, or a ketone (such as any of the alcohols, aldehyes, esters, or ketones described herein). In some embodiments, the isoprene composition includes (i) an alcohol and an aldehyde, (ii) an alcohol and a ketone, (iii) an aldehyde and a ketone, or (iv) an alcohol, an aldehyde, and a ketone.

[0501] In some embodiments, the isoprene composition contains one or more of the following: methanol, acetaldehyde, ethanol, methanethiol, 1-butanol, 3-methyl-1-propanol, acetone, acetic acid, 2-butanone, 2-methyl-1-butanol, or indole. In some embodiments, the isoprene composition contains 1 ppm or more of one or more of the following: methanol, acetaldehyde, ethanol, methanethiol, 1-butanol, 3-methyl-1-propanol, acetone, acetic acid, 2-butanone, 2-methyl-1-butanol, or indole. In some embodiments, the concentration of more of one or more of the following: methanol, acetaldehyde, ethanol, methanethiol, 1-butanol, 3-methyl-1-propanol, acetone, acetic acid, 2-butanone, 2-methyl-1-butanol, or indole, is between about 1 to about 10,000 ppm in an isoprene composition (such as off-gas before it is purified). In some embodiments, the isoprene composition (such as off-gas after it has undergone one or more purification steps) includes one or more of the following: methanol, acetaldehyde, ethanol, methanethiol, 1-butanol, 3-methyl-1-propanol, acetone, acetic acid, 2-butanone, 2-methyl-1-butanol, or indole, at a concentration between about 1 to about 100 ppm, such as about 1 to about 10 ppm, about 10 to about 20 ppm, about 20 to about 30 ppm, about 30 to about 40 ppm, about 40 to about 50 ppm, about 50 to about 60 ppm, about 60 to about 70 ppm, about 70 to about 80 ppm, about 80 to about 90 ppm, or about 90 to about 100 ppm. Volatile organic compounds from cell cultures (such as volatile organic compounds in the headspace of cell cultures) can be analyzed using standard methods such as those described herein or other standard methods such as proton transfer reaction-mass spectrometry (*see*, for example, Bunge *et al.*, *Applied and Environmental Microbiology*, 74(7):2179-2186, 2008 which is hereby incorporated by reference in its entirety, particular with respect to the analysis of volatile organic compounds).

[0502] In some embodiments, the composition comprises greater than about 2 mg of isoprene, such as greater than or about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 mg of isoprene. In some embodiments, the composition comprises greater than or about 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 g of isoprene. In some embodiments, the amount of isoprene in the composition is between about 2 to about 5,000 mg, such as between about 2 to about 100 mg, about 100 to about 500 mg, about 500 to about 1,000 mg, about 1,000 to about 2,000 mg, or about 2,000 to about 5,000 mg. In some embodiments, the amount of isoprene in the composition is between about 20 to about 5,000 mg, about 100 to about 5,000 mg, about 200 to about 2,000 mg, about 200 to about 1,000 mg, about 300 to about 1,000 mg, or about 400 to about 1,000 mg. In some embodiments, greater than or about 20, 25, 30, 40, 50, 60, 70, 80, 90, or 95% by weight of the volatile organic fraction of the composition is isoprene.

[0503] In some embodiments, the composition includes ethanol. In some embodiments, the composition includes between about 75 to about 90% by weight of ethanol, such as between about 75 to about 80%, about 80 to about 85%, or about 85 to about 90% by weight of ethanol. In some embodiments in which the composition includes ethanol, the composition also includes between about 4 to about 15% by weight of isoprene, such as between about 4 to about 8%, about 8 to about 12%, or about 12 to about 15% by weight of isoprene.

[0504] In some embodiments encompassed by the invention, a cell comprising one or more heterologous nucleic acids encoding an isoprene synthase polypeptide, DXS polypeptide, IDI polypeptide, and/or MVA pathway polypeptide produces an amount of an isoprenoid compound (such as a compound with 10 or more carbon atoms that is formed from the reaction of one or more IPP molecules with one or more DMAPP molecules) that is greater than or about 2-fold, 3-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 150-fold, 200-fold, 400-fold, or greater than the amount of the isoprenoid compound produced from a corresponding cell grown under essentially the same conditions without the one or more heterologous nucleic acids. In some embodiments encompassed by the invention, a cell comprising one or more heterologous nucleic acids encoding an isoprene synthase polypeptide, DXS polypeptide, IDI polypeptide, and/or MVA pathway polypeptide produces an amount of a C5 prenyl alcohol (such as 3-methyl-3-buten-1-ol or 3-methyl-2-buten-1-ol) that is greater than or about 2-fold, 3-fold, 5-fold, 10-fold, 25-fold, 50-fold, 100-fold, 150-fold, 200-fold, 400-fold, or greater than the amount of the C5 prenyl alcohol produced from a

corresponding cell grown under essentially the same conditions without the one or more heterologous nucleic acids.

Exemplary Co-Production of Isoprene and Hydrogen

[0505] In some embodiments, any of the isoprene-producing cells described herein that comprise one or more heterologous nucleic acids encoding an isoprene synthase polypeptide, a DXS polypeptide, an IDI polypeptide, and/or an MVA pathway polypeptide operably linked to a promoter further comprise a heterologous nucleic acid also operably linked to a promoter encoding one or more hydrogenase polypeptides or one or more polypeptides involved in the regulation or expression of hydrogenase polypeptides (*e.g.*, hydrogenase maturation proteins or transcription factors). In some embodiments, any of the isoprene-producing cells described herein that comprise one or more heterologous nucleic acids encoding an isoprene synthase polypeptide, a DXS polypeptide, an IDI polypeptide, an MVA pathway polypeptide, one or more hydrogenase polypeptides or one or more polypeptides involved in the regulation or expression of hydrogenase polypeptides operably linked to a promoter further comprise a mutation or deletion inactivating one or more polypeptides involved in the production of fermentation side products, one or more polypeptides involved in the regulation or expression of genes for the production of fermentation side products, or one or more polypeptides involved in hydrogen reuptake. Such cells can co-produce isoprene and hydrogen.

[0506] In some embodiments of any of the aspects of the invention, the cells are bacterial cells, such as gram-positive bacterial cells (*e.g.*, *Bacillus* cells such as *Bacillus subtilis* cells or *Streptomyces* cells such as *Streptomyces lividans*, *Streptomyces coelicolor*, or *Streptomyces griseus* cells). In some embodiments of any of the aspects of the invention, the cells are gram-negative bacterial cells (*e.g.*, *Escherichia* cells such as *Escherichia coli* cells, *Rhodopseudomonas sp.* such as *Rhodopseudomonas palustris* cells, *Pseudomonas sp.* such as *Pseudomonas fluorescens* cells or *Pseudomonas putida* cells, or *Pantoea* cells such as *Pantoea citrea* cells). In some embodiments of any of the aspects of the invention, the cells are fungal, cells such as filamentous fungal cells (*e.g.*, *Trichoderma* cells such as *Trichoderma reesei* cells or *Aspergillus* cells such as *Aspergillus oryzae* and *Aspergillus niger*) or yeast cells (*e.g.*, *Yarrowia* cells such as *Yarrowia lipolytica* cells or *Sacchraomyces* cells such as *Saccaromyces cerevisiae*).

[0507] In some embodiments of any of the aspects of the invention, the isoprene synthase polypeptide is a polypeptide from a plant such as *Pueraria* (*e.g., Pueraria montana or Pueraria lobata*) or *Populus* (*e.g., Populus tremuloides*, *Populus alba*, *Populus nigra*, *Populus trichocarpa*, or the hybrid, *Populus alba x Populus tremula*).

[0508] In some embodiments of any of the aspects of the invention, the cells further comprise a heterologous nucleic acid encoding an IDI polypeptide. In some embodiments of any of the aspects of the invention, the cells further comprise an insertion of a copy of an endogenous nucleic acid encoding an IDI polypeptide. In some embodiments of any of the aspects of the invention, the cells further comprise a heterologous nucleic acid encoding a DXS polypeptide. In some embodiments of any of the aspects of the invention, the cells further comprise an insertion of a copy of an endogenous nucleic acid encoding a DXS polypeptide. In some embodiments of any of the aspects of the invention, the cells further comprise one or more nucleic acids encoding an IDI polypeptide and a DXS polypeptide. In some embodiments of any of the aspects of the invention, one nucleic acid encodes the isoprene synthase polypeptide, IDI polypeptide, and DXS polypeptide. In some embodiments of any of the aspects of the invention, one vector encodes the isoprene synthase polypeptide, IDI polypeptide, and DXS polypeptide. In some embodiments, the vector comprises a selective marker or a selectable marker, such as an antibiotic resistance nucleic acid.

[0509] In some embodiments of any of the aspects of the invention, the cells further comprise a heterologous nucleic acid encoding an MVA pathway polypeptide (such as an MVA pathway polypeptide from *Saccharomyces cerevisia* or *Enterococcus faecalis*). In some embodiments of any of the aspects of the invention, the cells further comprise an insertion of a copy of an endogenous nucleic acid encoding an MVA pathway polypeptide (such as an MVA pathway polypeptide from *Saccharomyces cerevisia* or *Enterococcus faecalis*). In some embodiments of any of the aspects of the invention, the cells comprise an isoprene synthase, DXS, and MVA pathway nucleic acid. In some embodiments of any of the aspects of the invention, the cells comprise an isoprene synthase nucleic acid, a DXS nucleic acid, an IDI nucleic acid, and a MVA pathway nucleic acid.

[0510] In some embodiments, the MVA pathway polypeptide is an upper MVA pathway polypeptide. In some embodiments, the MVA pathway polypeptide is a lower MVA pathway polypeptide. In some embodiments, the upper MVA pathway polypeptide is selected from the group consisting of: (i) an acetoacetyl-Coenzyme A synthase (thiolase) polypeptide; (ii) a 3-

hydroxy-3-methylglutaryl-Coenzyme A synthase polypeptide; and (iii) a 3-hydroxy-3-methylglutaryl-Coenzyme A reductase polypeptide. In some embodiments, the upper MVA pathway polypeptide is from the genus *Enterococcus*. In some embodiments, the upper MVA pathway polypeptide is from *Enterococcus faecalis*. In some embodiments, the lower MVA pathway polypeptide is selected from the group consisting of: (i) mevalonate kinase (MVK); (ii) phosphomevalonate kinase (PMK); (iii) diphosphomevalonate decarboxylase (MVD); and (iv) isopentenyl diphosphate isomerase (IDI). In some embodiments, the lower MVA pathway polypeptide is an MVK polypeptide. In some embodiments, the MVK polypeptide is from the genus *Methanosarcina*. In some embodiments, the MVK polypeptide is from *Methanosarcina mazei*.

[0511] In some embodiments, the isoprene-producing cells described herein further comprise a heterologous nucleic acid encoding a hydrogenase polypeptide operably linked to a promoter. In some embodiments, the hydrogenase polypeptide comprises *E. coli* hydrogenase-1 (Hyd-1), *E. coli* hydrogenase-2 (Hyd-2), *E. coli* hydrogenase-3 (Hyd-3), *E. coli* hydrogenase-4 (Hyd-4), *E. coli* formate hydrogen lyase (FHL) complex, which produces hydrogen gas from formate and CO_2 under anaerobic conditions at acidic pH, *Rhodococcus opacus MR11* hydrogenase (*R. opacus* HoxH), *Synechosystis* sp. PCC 6803 hydrogenase (*Syn*. PCC 6803 HoxH), *Desulfovibrio gigas* hydrogenase (*D. gigas*), and *Desulfovibrio desulfuricans ATCC 7757* hydrogenase (*D. desulfuricans*). In some embodiments, the isoprene-producing cells furthercomprising a heterologous nucleic acid encoding a hydrogenase polypeptide operably linked to a promoter further comprise *E. coli* hydrogenase-3 (Hyd-3), *E. coli* pyruvate formate lyase (*pfl*), and *E. coli* formate hydrogen lyase (FHL) complex.

[0512] In some embodiments, the hydrogenase polypeptide encodes a ferredoxin-dependent hydrogenase polypeptide. In some embodiments, the ferredoxin-dependent hydrogenase polypeptide comprises *Clostridium acetobutulicum* hydrogenase A (HydA), which can be expressed in conjunction with one or more of: (1) *Bacillus subtilis* NADPH ferredoxin oxidoreductase (NFOR) or *Clostridium kluyveri* NADH ferredoxin oxidoreductase (RnfCDGEAB), *Clostridium pasteuranium* ferredoxin oxidoreductase (Fdx); (2) glyceraldehyde-6-phosphate ferredoxin oxidoreductase (GAPOR); or (3) pyruvate ferredoxin oxidoreductase (POR). In some embodiments, the ferredoxin-dependent hydrogenase polypeptide *Clostridium acetobutulicum* hydrogenase A (HydA) is expressed with three

HydA-associated maturation enzymes (HydE, HydG, and HydF), and further in conjunction with one or more of: (1) *Bacillus subtilis* NADPH ferredoxin oxidoreductase (NFOR) or *Clostridium kluyveri* NADH ferredoxin oxidoreductase (RnfCDGEAB), *Clostridium pasteuranium* ferredoxin oxidoreductase (Fdx); (2) glyceraldehyde-6-phosphate ferredoxin oxidoreductase (GAPOR); or (3) pyruvate ferredoxin oxidoreductase (POR).

[0513] In some embodiments, the hydrogenase polypeptide encodes an NADPH-dependent hydrogenase polypeptide. In some embodiments, the NADPH-dependent hydrogenase polypeptide comprises *Pyrococcus furiosus* hydrogenase. In some embodiments, the hydrogenase polypeptide encodes an oxygen-tolerant hydrogenase. In some embodiments, the oxygen-tolerant hydrogenase comprises *Rubrivivax gelatinosus* hydrogenase, and *Ralstonia eutropha* hydrogenase.

[0514] In some embodiments, the isoprene-producing cells described herein further comprise a mutation or deletion inactivating a gene involved in regulation of hydrogenase activity, such as iron-sulfur complex transcriptional regulator (*iscR*) (Kalim-Akhtar et al., "Deletion of iscR stimulates recombinant Clostridial Fe/Fe hydrogenase activity and H_2-accumulation in *Escherichia coli* BL21(DE3)," *Appl. Microbiol. Biotechnol.* 78:853–862 (2008), which is incorporated herein by reference in its entirety, particularly with reference to stimulation of Clostridial Fe/Fe hydrogenase activity and hydrogen accumulation in *E. coli* by deleting the *iscR* gene).

[0515] In some embodiments, the isoprene-producing cells described herein further comprise a mutation or deletion inactivating a gene encoding one or more cellular polypeptides involved in production of fermentation side products, such as lactate, acetate, pyruvate, ethanol, succinate, and glycerol. In some embodiments, the inactivated polypeptides involved in production of fermentation side products comprise one or more polypeptides encoding formate dehydrogenase N, alpha subunit (*fdnG*), formate dehydrogenase O, large subunit (*fdoG*), nitrate reductase (*narG*), formate transporter A (*focA*), formate transporter B (*focB*), pyruvate oxidase (*poxB*), pyruvate dehydrogenase E1 component ackA/pta (*aceE*), alcohol dehydrogenase (*adhE*), fumarate reductase membrane protein (*frdC*), or lactate dehydrogenase (*ldh*A).

[0516] In some embodiments, the isoprene-producing cells described herein further comprise a mutation or deletion inactivating a gene encoding one or more cellular

polypeptides involved in the regulation or expression of genes involved in production of fermentation side products. In some embodiments, the inactivated polypeptides involved in the regulation or expression of genes involved in production of fermentation side products comprise repressor of formate hydrogen lyase (*hycA*), fumarate reductase regulator (*fnr*), acetyl-coenzyme A synthetase (*acs*), and formate dehydrogenase regulatory protein (*hycA*).

[0517] In some embodiments, the isoprene-producing cells described herein further comprise a mutation or deletion inactivating a gene encoding one or more cellular polypeptides involved in hydrogen re-uptake. In some embodiments, the inactivated polypeptides involved in hydrogen re-uptake comprise *E. coli* hydrogenase-1 (Hyd-1) (*hya* operon) and *E. coli* hydrogenase-2 (Hyd-2) (*hyb* operon).

[0518] In some embodiments of any of the aspects of the invention, the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor polypeptide or nucleic acid is operably linked to a T7 promoter, such as a T7 promoter contained in a medium or high copy plasmid. In some embodiments of any of the aspects of the invention, the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor nucleic acid is operably linked to a Trc promoter, such as a Trc promoter contained in a medium or high copy plasmid. In some embodiments of any of the aspects of the invention, the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor nucleic acid is operably linked to a Lac promoter, such as a Lac promoter contained in a low copy plasmid. In some embodiments of any of the aspects of the invention, the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor nucleic acid is operably linked to an endogenous promoter, such as an endogenous alkaline serine protease promoter. In some embodiments, the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor nucleic acid integrates into a chromosome of the cells without a selective marker or without a selectable marker.

[0519] In some embodiments, one or more MVA pathway, IDI, DXS, isoprene synthase, hydrogenase, hydrogenase maturation or transcription factor nucleic acids are placed under the control of a promoter or factor that is more active in stationary phase than in the growth phase. For example, one or more MVA pathway, IDI, DXS, isoprene synthase, hydrogenase,

hydrogenase maturation or transcription factor nucleic acids may be placed under control of a stationary phase sigma factor, such as RpoS. In some embodiments, one or more MVA pathway, IDI, DXS, isoprene synthase, hydrogenase, hydrogenase maturation or transcription factor nucleic acids are placed under control of a promoter inducible in stationary phase, such as a promoter inducible by a response regulator active in stationary phase.

[0520] In some embodiments of any of the aspects of the invention, at least a portion of the cells maintain the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor nucleic acid for at least or about 5, 10, 20, 40, 50, 60, 65, or more cell divisions in a continuous culture (such as a continuous culture without dilution). In some embodiments of any of the aspects of the invention, the nucleic acid comprising the isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, hydrogenase, hydrogenase maturation or transcription factor nucleic acid also comprises a selective marker or a selectable marker, such as an antibiotic resistance nucleic acid.

[0521] In some embodiments of any of the aspects of the invention, cells that co-produce isoprene and hydrogen are cultured in any of the culture media described herein, under oxygen-limited conditions to facilitate the co-production of isoprene and hydrogen by the cells. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells are grown in the presence of 0.5 moles of oxygen per mole of isoprene. In some embodiments, the cells are grown anaerobically, in the absence of oxygen.

[0522] In some embodiments, any of the cells described herein are grown in oxygen-limited culture and co-produce isoprene and hydrogen. In some embodiments, the cells in oxygen-limited culture produce isoprene at a rate greater than about 400 nmole/g_{wcm}/hr, and produce hydrogen at a rate greater than about 125 nmole/g_{wcm}/hr. In some embodiments, the cells in oxygen-limited culture produce isoprene at a rate between about 400 nmole/g_{wcm}/hr to about 2.0×10^5 nmole/g_{wcm}/hr and hydrogen at a rate between about 125 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr. In some embodiments, the cells in oxygen-limited culture produce isoprene at a rate between about 400 nmole/g_{wcm}/hr and about 2.0×10^5 nmole/g_{wcm}/hr, between about 500 nmole/g_{wcm}/hr and about 1.5×10^5 nmole/g_{wcm}/hr, between about 750 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 1000 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 2500 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 5000 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between

about 7500 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, and between about 1×10^4 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr. In some embodiments, the cells in oxygen-limited culture produce greater than about 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, or more nmole/g_{wcm}/hr isoprene. In some embodiments, the cells in oxygen-limited culture produce hydrogen at a rate between about 125 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 250 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 500 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 750 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 1000 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 1250 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 2500 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 5000 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 7500 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, and between about 1.00×10^4 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr. In some embodiments, the cells in oxygen-limited culture produce greater than about 125, 250, 500, 750, 1000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, 7,500, 10,000, or more nmole/g_{wcm}/hr hydrogen.

[0523] In some embodiments, any of the cells described herein are grown in oxygen-limited culture and co-produce isoprene and hydrogen. In some embodiments, the cells in oxygen-limited culture have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of hydrogen greater than about 0.005 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have a peak volumetric productivity of isoprene greater than about 1000 mg/L_{broth}/hr and a peak volumetric productivity of hydrogen greater than about 5 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have a peak volumetric productivity of isoprene greater than about 3000 mg/L_{broth}/hr and a peak volumetric productivity of hydrogen greater than about 5 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have a peak volumetric productivity of isoprene greater than about 5000 mg/L_{broth}/hr and a peak volumetric productivity of hydrogen greater than about 5 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have an average volumetric productivity of isoprene between about 0.1 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, and an average volumetric productivity of hydrogen between about 0.005 mg/L_{broth}/hr and about 5 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have an average volumetric productivity of isoprene between about 1 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 5

mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 10 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 25 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 50 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 100 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 250 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 500 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 1000 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, and between about 2500 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, and an average volumetric productivity of hydrogen between about 0.01 mg/L_{broth}/hr and about 5 mg/L_{broth}/hr, between about 0.025 mg/L_{broth}/hr and about 5 mg/L_{broth}/hr, between about 0.05 mg/L_{broth}/hr and about 5 mg/L_{broth}/hr, between about 0.1 mg/L_{broth}/hr and about 5 mg/L_{broth}/hr, between about 0.25 mg/L_{broth}/hr and about 5 mg/L_{broth}/hr, between about 0.5 mg/L_{broth}/hr and about 5 mg/L_{broth}/hr, between about 1 mg/L_{broth}/hr and about 5 mg/L_{broth}/hr, and between about 2.5 mg/L_{broth}/hr and about 5 mg/L_{broth}/hr.

[0524] In some embodiments, any of the cells described herein are grown in oxygen-limited culture and co-produce isoprene and hydrogen. In some embodiments, the cells in oxygen-limited culture convert more than about 0.002 molar percent of the carbon that the cells consume from a cell culture medium into isoprene, and produce hydrogen equivalent to more than about 0.024 molar percent of the carbon that the cells consume from a cell culture medium. In some embodiments, the cells in oxygen-limited culture convert more than about 0.002 molar percent of the carbon that the cells consume from a cell culture medium into isoprene, and produce hydrogen equivalent to more than about 400 molar percent of the carbon that the cells consumer from a cell culture medium.

[0525] In some embodiments, any of the cells described herein that co-produce isoprene and hydrogen are grown in oxygen-limited culture. In some embodiments, the cells in oxygen-limited culture co-produce isoprene and hydrogen in a ratio ranging from at least one molar percent of isoprene for every three molar percent of hydrogen to at least one molar percent of isoprene for every four molar percent of hydrogen. In some embodiments, the cells in oxygen-limited culture produce from 1 to 11 molar percent isoprene and from 3 to 33 molar percent hydrogen. In some embodiments, the cells produce from 1 to 11 molar percent isoprene and from 4 to 44 molar percent hydrogen. In some embodiments, the cells in oxygen-limited culture also produce oxygen, carbon dioxide, or nitrogen. In some embodiments, the cells in oxygen limited culture produce from 0 to 21 molar percent oxygen, from 18 to 44 molar percent carbon dioxide, and from 0 to 78 molar percent nitrogen.

[0526] In another aspect, provided herein are cells in oxygen-limited culture that co-produce isoprene and hydrogen, comprising a heterologous nucleic acid encoding an isoprene synthase polypeptide, wherein the cells: (i) produce isoprene at a rate greater than about 400 nmole/g_{wcm}/hr and produce hydrogen at a rate greater than about 125 nmole/g_{wcm}/hr; (ii) have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of hydrogen greater than about 0.005 mg/L_{broth}/hr; or (iii) convert more than about 0.002 molar percent of the carbon that the cells consume from a cell culture medium into isoprene, and produce hydrogen equivalent to more than about 0.024 molar percent of the carbon that the cells consume from a cell culture medium. In some embodiments, the cells are capable of co-producing isoprene and hydrogen under oxygen-limited conditions.

[0527] In some embodiments, the cells in oxygen-limited culture comprise a heterologous nucleic acid encoding an isoprene synthase polypeptide, wherein the heterologous nucleic acid is operably linked to a promoter, and wherein the cells produce greater than about 400 nmole/g_{wcm}/hr of isoprene and greater than about 125 nmole/g_{wcm}/hr of hydrogen. In some embodiments, the cells in oxygen-limited culture comprise a heterologous nucleic acid encoding an isoprene synthase polypeptide, wherein the heterologous nucleic acid is operably linked to a promoter, and wherein the cells have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of hydrogen greater than about 0.005 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture comprise a heterologous nucleic acid encoding an isoprene synthase polypeptide, wherein the heterologous nucleic acid is operably linked to a promoter, and wherein the cells convert more than about 0.002 molar percent of the carbon that the cells consume from a cell culture medium into isoprene, and more than about 0.024 molar percent of the carbon that the cells consume from a cell culture medium into hydrogen. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide.

[0528] In some embodiments, the cells in oxygen-limited culture comprising a heterologous nucleic acid encoding an isoprene synthase polypeptide produce isoprene at a rate between about 400 nmole/g_{wcm}/hr and about 2.0×10^5 nmole/g_{wcm}/hr, between about 500 nmole/g_{wcm}/hr and about 1.5×10^5 nmole/g_{wcm}/hr, between about 750 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 1000 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 2500 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between

about 5000 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 7500 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, and between about 1×10^4 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, and produce hydrogen at a rate between about 125 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 250 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 500 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 750 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 1000 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 1250 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 2500 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 5000 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 7500 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, and between about 1.00×10^4 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr.

[0529] In some embodiments, provided herein are methods of co-producing isoprene and hydrogen, the methods comprising: (a) culturing cells under conditions suitable for the co-production of isoprene and hydrogen; and (b) co-producing isoprene and hydrogen, wherein the cells produce greater than about 400 nmole/g_{wcm}/hour of isoprene, and wherein the cells produce greater than about 125 nmole/g_{wcm}/hr of hydrogen. In some embodiments, the cells are grown in oxygen-limited culture.

[0530] In some embodiments, the cells in oxygen-limited culture comprising a heterologous nucleic acid encoding an isoprene synthase polypeptide produce isoprene at a rate between about 400 nmole/g_{wcm}/hr and about 2.0×10^5 nmole/g_{wcm}/hr, between about 500 nmole/g_{wcm}/hr and about 1.5×10^5 nmole/g_{wcm}/hr, between about 750 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 1000 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 2500 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 5000 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 7500 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, and between about 1×10^4 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, and produce hydrogen at a rate between about 125 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 250 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 500 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 750 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 1000 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 1250 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 2500 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, between about 5000 nmole/g_{wcm}/hr to about 1.25×10^4

nmole/g_{wcm}/hr, between about 7500 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr, and between about 1.00×10^4 nmole/g_{wcm}/hr to about 1.25×10^4 nmole/g_{wcm}/hr.

[0531] In some embodiments, provided herein are methods of co-producing isoprene and hydrogen, the methods comprising: (a) culturing cells under conditions suitable for the co-production of isoprene and hydrogen; and (b) co-producing isoprene and hydrogen, wherein the cells have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of hydrogen greater than about 0.005 mg/L_{broth}/hr. In some embodiments, the cells are grown under oxygen-limited conditions.

[0532] In some embodiments, provided herein are methods of co-producing isoprene and hydrogen, the methods comprising: (a) culturing cells under conditions suitable for the co-production of isoprene and hydrogen; and (b) co-producing isoprene and hydrogen, wherein the cells convert more than about 0.002 molar percent of the carbon that the cells consume from a cell culture medium into isoprene, and produce hydrogen equivalent to more than about 0.024 molar percent of the carbon that the cells consume from a cell culture medium. In some embodiments, the cells are grown under oxygen-limited conditions.

[0533] In some embodiments, provided herein are compositions comprising isoprene and hydrogen in a ratio ranging from at least one molar percent of isoprene for every three molar percent of hydrogen to at least one molar percent of isoprene for every four molar percent of hydrogen, and 0.1 molar percent or less of volatile impurities. In some embodiments, the compositions further comprise from 1 to 11 molar percent isoprene and from 4 to 44 molar percent hydrogen. In some embodiments, the compositions further comprise oxygen, carbon dioxide, or nitrogen. In some embodiments, the compositions further comprise from 0 to 21 molar percent oxygen, from 18 to 44 molar percent carbon dioxide, and from 0 to 78 molar percent nitrogen. In some embodiments, the composition further comprises 1.0×10^{-4} molar percent or less of non-methane volatile impurities. In some embodiments, the non-methane volatile impurities comprise one or more of the following: 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, acetaldehyde, methanethiol, methyl acetate, 1-propanol, diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene, 2,3-cycloheptenolpyridine, 3-hexen-1-ol, 3-

hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol) and citronellol (3,7-dimethyl-6-octen-1-ol) or a linear isoprene polymer (such as a linear isoprene dimer or a linear isoprene trimer derived from the polymerization of multiple isoprene units). In some embodiments, the non-methane volatile impurities comprise one or more of the following: the isoprene composition includes one or more of the following: an alcohol, an aldehyde, an ester, or a ketone (such as any of the alcohols, aldehyes, esters or ketones described herein). In some embodiments, the isoprene composition includes (i) an alcohol and an aldehyde, (ii) an alcohol and a ketone, (iii) an aldehyde and a ketone, or (iv) an alcohol, an aldehyde, and a ketone. In some embodiments, the non-methane volatile impurities comprise one or more of the following: methanol, acetaldehyde, ethanol, methanethiol, 1-butanol, 3-methyl-1-propanol, acetone, acetic acid, 2-butanone, 2-methyl-1-butanol, or indole.

[0534] Also provided herein are methods of co-producing isoprene and hydrogen, the methods comprising: a) culturing cells under conditions suitable for the co-production of isoprene and hydrogen; and b) co-producing isoprene and hydrogen, wherein the peak concentration of the isoprene produced by the cells in oxygen-limited culture is greater than about 10 ng/L_{broth} and the hydrogen evolution rate of the cells is greater than about 0.0025 mmol/L_{broth}/hour. In some embodiments, the cells are grown under oxygen-limited conditions. In some embodiments of any of these methods, the hydrogen evolution rate is between about any of 0.0025 mmol/L_{broth}/hr and about 10 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 5 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 2.5 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 1 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 0.5 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 0.25 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 0.025 mmol/L_{broth}/hr, between about 0.025 mmol/L_{broth}/hr and about 0.5 mmol/L_{broth}/hr, between about 0.025 mmol/L_{broth}/hr and about 1 mmol/L_{broth}/hr, between about 0.025 mmol/L_{broth}/hr and about 2.5 mmol/L_{broth}/hr, between about 0.025 mmol/L_{broth}/hr and about 5 mmol/L_{broth}/hr, between about 0.025 mmol/L_{broth}/hr and about 10 mmol/L_{broth}/hr, between about 0.25 mmol/L_{broth}/hr and 1 mmol/L_{broth}/hr, between about 0.25 mmol/L_{broth}/hr and 2.5 mmol/L_{broth}/hr, between about 0.25 mmol/L_{broth}/hr and 2.5 mmol/L_{broth}/hr, between about 0.25 mmol/L_{broth}/hr and 10 mmol/L_{broth}/hr, between about 0.01 mmol/L_{broth}/hr and 10 mmol/L_{broth}/hr, between about 0.01 mmol/L_{broth}/hr and 50 mmol/L_{broth}/hr, between about 0.01 mmol/L_{broth}/hr and 100 mmol/L_{broth}/hr, and between about 0.01 mmol/L_{broth}/hr and 200 mmol/L_{broth}/hr.

[0535] Provided herein are also methods of co-producing isoprene and hydrogen comprising a) culturing cells under conditions suitable for the co-production of isoprene and hydrogen; and b) co-producing isoprene and hydrogen, wherein the liquid phase concentration of isoprene is less than about 200 mg/L, the cells produce greater than about 400 nmole/g_{wcm}/hour of isoprene, and the hydrogen evolution rate of the cells is greater than about 0.0025 mmol/L/hour. In some embodiments, the cells are grown under oxygen-limited conditions. In some embodiments, the liquid phase concentration of isoprene in the culture is less than about any of 175 mg/L, 150 mg/L, 125 mg/L, 100 mg/L, 75 mg/L, 50 mg/L, 25 mg/L, 20 mg/L, 15 mg/L, 10 mg/L, 5 mg/L, or 2.5 mg/L. In some embodiments, the liquid phase concentration of isoprene in culture is between about any of 0.1 mg/L to 200 mg/L, 1 mg/L to 200 mg/L, 1 mg/L to 150 mg/L, 1 mg/L to 100 mg/L, 1 mg/L to 50 mg/L, 1 mg/L to 25 mg/L, 1 mg/L to 20 mg/L, or 10 mg/L to 20 mg/L. In some embodiments of any of these methods, the hydrogen evolution rate is between about any of 0.0025 mmol/L_{broth}/hr and about 10 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 5 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 2.5 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 1 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 0.5 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 0.25 mmol/L_{broth}/hr, between about 0.0025 mmol/L_{broth}/hr and about 0.025 mmol/L_{broth}/hr, between about 0.025 mmol/L_{broth}/hr and about 0.5 mmol/L_{broth}/hr, between about 0.025 mmol/L_{broth}/hr and about 1 mmol/L_{broth}/hr, between about 0.025 mmol/L_{broth}/hr and about 2.5 mmol/L_{broth}/hr, between about 0.025 mmol/L_{broth}/hr and about 5 mmol/L_{broth}/hr, between about 0.025 mmol/L_{broth}/hr and about 10 mmol/L_{broth}/hr, between about 0.25 mmol/L_{broth}/hr and 1 mmol/L_{broth}/hr, between about 0.25 mmol/L_{broth}/hr and 2.5 mmol/L_{broth}/hr, between about 0.25 mmol/L_{broth}/hr and 2.5 mmol/L_{broth}/hr, and between about 0.25 mmol/L_{broth}/hr and 10 mmol/L_{broth}/hr.

[0536] In one aspect, provided herein are cells in oxygen-limited culture that co-produce isoprene and hydrogen. In some embodiments, the oxygen-limited culture is anaerobic. In some embodiments, the invention provides cells in oxygen-limited culture that produce greater than about 400 nmole/g_{wcm}/hr of isoprene and greater than about 125 nmole/g_{wcm}/hr of hydrogen. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid,

monoglyceride, diglyceride, triglyceride, renewable carbon source, polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are cultured under limited glucose conditions.

[0537] In some embodiments, provided herein are cells in oxygen-limited culture that convert more than about 0.002% of the carbon in a cell culture medium into isoprene and produce hydrogen equivalent to more than about 0.024 molar percent of the carbon in a cell culture medium. In some embodiments, the oxygen-limited culture is anaerobic. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source, polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are cultured under limited glucose conditions.

[0538] In some embodiments, provided herein are cells in oxygen-limited culture that comprise a heterologous nucleic acid encoding an isoprene synthase polypeptide. In some embodiments, the oxygen-limited culture is anaerobic. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source, polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are cultured under limited glucose conditions.

[0539] In one aspect, provided herein are methods of co-producing isoprene with another compound, such as methods of using any of the cells described herein to co-produce isoprene and hydrogen. In some embodiments, the method involves culturing cells under oxygen-limited conditions sufficient to produce greater than about 400 nmole/g_{wcm}/hr of isoprene and greater than about 125 nmole/g_{wcm}/hr of hydrogen. In some embodiments, the oxygen-limited culture is anaerobic. In some embodiments, the method also includes recovering the

isoprene and hydrogen produced by the cells. In some embodiments, the method further includes purifying the isoprene and the hydrogen produced by the cells. In some embodiments, the method includes polymerizing the isoprene. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source, polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are cultured under limited glucose conditions. In various embodiments, the amount of isoprene produced (such as the total amount of isoprene produced or the amount of isoprene produced per liter of broth per hour per OD_{600}) during stationary phase is greater than or about 2 or more times the amount of isoprene produced during the growth phase for the same length of time.

[0540] In some embodiments, the method includes culturing cells under oxygen-limited conditions sufficient to convert more than about 0.002% of the carbon (mol/mol) in a cell culture medium into isoprene and to produce hydrogen equivalent to more than about 0.024 molar percent of the carbon in a cell culture medium. In some embodiments, the oxygen-limited culture is anaerobic. In some embodiments, the method also includes recovering isoprene and hydrogen produced by the cells. In some embodiments, the method further includes purifying isoprene and hydrogen produced by the cells. In some embodiments, the method includes polymerizing the isoprene. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source, polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract.

[0541] In some embodiments of any of the aspects of the invention, the microbial polypeptide carbon source includes one or more polypeptides from yeast or bacteria. In some embodiments of any of the aspects of the invention, the plant polypeptide carbon source

includes one or more polypeptides from soy, corn, canola, jatropha, palm, peanut, sunflower, coconut, mustard, rapeseed, cottonseed, palm kernel, olive, safflower, sesame, or linseed.

[0542] In some embodiments, isoprene and hydrogen are only co-produced in stationary phase. In some embodiments, isoprene and hydrogen are co-produced in both the growth phase and stationary phase. In various embodiments, the amount of isoprene produced (such as the total amount of isoprene produced or the amount of isoprene produced per liter of broth per hour per OD_{600}) during stationary phase is greater than or about 2, 3, 4, 5, 10, 20, 30, 40, 50, or more times the amount of isoprene produced during the growth phase for the same length of time. In various embodiments, the amount of hydrogen produced (such as the total amount of hydrogen produced or the amount of hydrogen produced per liter of broth per hour per OD_{600}) during stationary phase is greater than or about 2, 3, 4, 5, 10, 20, 30, 40, 50, or more times the amount of hydrogen produced during the growth phase for the same length of time.

[0543] In some embodiments, the compositions provided herein comprise hydrogen and greater than or about 99.90, 99.92, 99.94, 99.96, 99.98, or 100% isoprene by weight compared to the total weight of all C5 hydrocarbons in the composition. In some embodiments, the composition comprises less than or about 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% C5 hydrocarbons other than isoprene (such 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 3-methyl-l-butyne, *trans*-piperylene, *cis*-piperylene, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne) by weight compared to the total weight of all C5 hydrocarbons in the composition. In some embodiments, the composition has less than or about 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% for 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 3-methyl-l-butyne, *trans*-piperylene, *cis*-piperylene, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne by weight compared to the total weight of all C5 hydrocarbons in the composition. In particular embodiments, the composition has greater than about 2 mg of isoprene and has greater than or about 99.90, 99.92, 99.94, 99.96, 99.98, or 100% isoprene by weight compared to the total weight of all C5 hydrocarbons in the composition. In some embodiments, the composition has less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 μg/L of a compound that inhibits the polymerization of isoprene for any

compound in the composition that inhibits the polymerization of isoprene. In particular embodiments, the composition also comprises greater than about 2 mg of isoprene and greater than about 0.48 mg of hydrogen.

[0544] In some embodiments, the volatile organic fraction of the gas phase has less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 μg/L of a compound that inhibits the polymerization of isoprene for any compound in the volatile organic fraction of the gas phase that inhibits the polymerization of isoprene. In some embodiments, the volatile organic fraction of the gas phase also has greater than about 2 mg of isoprene and greater than about 0.48 mg of hydrogen.

[0545] In some embodiments, the invention also features systems that include any of the cells and/or compositions described herein. In some embodiments, the system includes a reactor that chamber comprises cells in oxygen-limited culture that produce greater than about 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, or more nmole/g_{wcm}/hr isoprene and greater than about 125, 250, 500, 750, 1000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, 7,500, 10,000, or more nmole/g_{wcm}/hr hydrogen. In some embodiments, the system is not a closed system. In some embodiments, at least a portion of the isoprene is removed from the system. In some embodiments, the system includes a gas phase comprising isoprene and hydrogen. In various embodiments, the gas phase comprises any of the compositions described herein.

[0546] In one aspect, the invention provides a tire comprising polyisoprene. In some embodiments, the polyisoprene is produced by (i) polymerizing isoprene in any of the compositions described herein or (ii) polymerizing isoprene recovered from any of the compositions described herein. In some embodiments, the polyisoprene comprises *cis*-1,4-polyisoprene.

[0547] In one aspect, the invention features a product produced by any of the compositions or methods described herein.

Exemplary Co-Production of Isoprene and Ethanol

[0548] The invention also provides compositions and methods for co-production of isoprene and and a C2- or C3-alcohol or diol. In some embodiments, the C2-or C3-alcohol or diol is ethanol. In some embodiments, any of the isoprene-producing cells described herein

that comprise one or more heterologous nucleic acids encoding an isoprene synthase polypeptide, a DXS polypeptide, an IDI polypeptide, and/or an MVA pathway polypeptide operably linked to a promoter further comprise a heterologous nucleic acid also operably linked to a promoter encoding one or more polypeptides involved in ethanol fermentation or one or more polypeptides involved in the regulation or expression of one or more polypeptides involved in ethanol fermentation (*e.g.*, transcription factors and the like). In some embodiments, any of the isoprene-producing cells described herein that comprise one or more heterologous nucleic acids encoding an isoprene synthase polypeptide, a DXS polypeptide, an IDI polypeptide, an MVA pathway polypeptide, one or more polypeptides involved in ethanol fermentation or one or more polypeptides involved in the regulation or expression of one or more polypeptides involved in ethanol fermentation operably linked to a promoter further comprise a mutation or deletion inactivating one or more polypeptides involved in the production of fermentation side products, or one or more polypeptides involved in the regulation or expression of genes for the production of fermentation side products. Such cells can co-produce isoprene and ethanol.

[0549] In some embodiments of any of the aspects of the invention, the cells are bacterial cells, such as gram-positive bacterial cells (*e.g.*, *Bacillus* cells such as *Bacillus subtilis* cells or *Streptomyces* cells such as *Streptomyces lividans*, *Streptomyces coelicolor*, or *Streptomyces griseus* cells). In some embodiments of any of the aspects of the invention, the cells are gram-negative bacterial cells (*e.g.*, *Escherichia* cells such as *Escherichia coli* cells, *Rhodopseudomonas sp.* such as *Rhodopseudomonas palustris* cells, *Pseudomonas sp*. such as *Pseudomonas fluorescens* cells or *Pseudomonas putida* cells, *Pantoea* cells such as *Pantoea citrea* cells, or *Zymomonas* cells such as *Zymomonas mobilis* cells). In some embodiments of any of the aspects of the invention, the gram-negative bacterial cells are *E. coli*. In some embodiments of any of the aspects of the invention, the gram-negative bacterial cells are *Zymomonas mobilis*. In some embodiments of any of the aspects of the invention, the cells are fungal, cells such as filamentous fungal cells (*e.g.*, *Trichoderma* cells such as *Trichoderma reesei* cells or *Aspergillus* cells such as *Aspergillus oryzae* and *Aspergillus niger*) or yeast cells (*e.g.*, *Yarrowia* cells such as *Yarrowia lipolytica* cells or *Saccharomyces* cells such as *Saccharomyces cerevisiae*). In some embodiments of any of the aspects of the invention, the yeast cells are *S. cerevisiae*.

[0550] In some embodiments of any of the aspects of the invention, the isoprene synthase polypeptide is a polypeptide from a plant such as *Pueraria* (*e.g., Pueraria montana* or *Pueraria lobata*)(also known as "Kudzu") or *Populus* (*e.g.*, *Populus tremuloides*, *Populus alba*, *Populus nigra*, *Populus trichocarpa*, or the hybrid, *Populus alba x Populus tremula*).

[0551] In some embodiments of any of the aspects of the invention, the cells further comprise a heterologous nucleic acid encoding an IDI polypeptide. In some embodiments of any of the aspects of the invention, the cells further comprise an insertion of a copy of an endogenous nucleic acid encoding an IDI polypeptide. In some embodiments of any of the aspects of the invention, the cells further comprise a heterologous nucleic acid encoding a DXS polypeptide. In some embodiments of any of the aspects of the invention, the cells further comprise an insertion of a copy of an endogenous nucleic acid encoding a DXS polypeptide. In some embodiments of any of the aspects of the invention, the cells further comprise one or more nucleic acids encoding an IDI polypeptide and a DXS polypeptide. In some embodiments of any of the aspects of the invention, one nucleic acid encodes the isoprene synthase polypeptide, IDI polypeptide, and DXS polypeptide. In some embodiments of any of the aspects of the invention, one vector encodes the isoprene synthase polypeptide, IDI polypeptide, and DXS polypeptide. In some embodiments, the vector comprises a selective marker or a selectable marker, such as an antibiotic resistance nucleic acid.

[0552] In some embodiments of any of the aspects of the invention, the cells further comprise a heterologous nucleic acid encoding an MVA pathway polypeptide (such as an MVA pathway polypeptide from *Saccharomyces cerevisia* or *Enterococcus faecalis*). In some embodiments of any of the aspects of the invention, the cells further comprise an insertion of a copy of an endogenous nucleic acid encoding an MVA pathway polypeptide (such as an MVA pathway polypeptide from *Saccharomyces cerevisia* or *Enterococcus faecalis*). In some embodiments of any of the aspects of the invention, the cells comprise an isoprene synthase, DXS, and MVA pathway nucleic acid. In some embodiments of any of the aspects of the invention, the cells comprise an isoprene synthase nucleic acid, a DXS nucleic acid, an IDI nucleic acid, and a MVA pathway nucleic acid.

[0553] In some embodiments, the MVA pathway polypeptide is an upper MVA pathway polypeptide. In some embodiments, the MVA pathway polypeptide is a lower MVA pathway polypeptide. In some embodiments, the upper MVA pathway polypeptide is selected from the group consisting of: (i) an acetoacetyl-Coenzyme A synthase (thiolase) polypeptide; (ii) a 3-

hydroxy-3-methylglutaryl-Coenzyme A synthase polypeptide; and (iii) a 3-hydroxy-3-methylglutaryl-Coenzyme A reductase polypeptide. In some embodiments, the upper MVA pathway polypeptide is from the genus *Enterococcus*. In some embodiments, the upper MVA pathway polypeptide is from *Enterococcus faecalis*. In some embodiments, the lower MVA pathway polypeptide is selected from the group consisting of: (i) mevalonate kinase (MVK); (ii) phosphomevalonate kinase (PMK); (iii) diphosphomevalonate decarboxylase (MVD); and (iv) isopentenyl diphosphate isomerase (IDI). In some embodiments, the lower MVA pathway polypeptide is an MVK polypeptide. In some embodiments, the MVK polypeptide is from the genus *Methanosarcina*. In some embodiments, the MVK polypeptide is from *Methanosarcina mazei*.

[0554] In some embodiments of any of the aspects of the invention, the cells further comprise a heterologous nucleic acid encoding one or more polypeptides involved in ethanol fermentation or one or more polypeptides involved in the regulation or expression of one or more polypeptides involved in ethanol fermentation (*e.g.*, transcription factors and the like) operably linked to a promoter. In some embodiments of any of the aspects of the invention, the cells further comprise a heterologous nucleic acid encoding alcohol dehydrogenase B (adhB) from *Zymomonas mobilis* operably linked to a promoter. In some embodiments of any of the aspects of the invention, the cells further comprise a heterologous nucleic acid encoding alcohol dehydrogenase E (adhE) from *Zymomonas mobilis* operably linked to a promoter. In some embodiments of any of the aspects of the invention, the cells further comprise a heterologous nucleic acid encoding pyruvate decarboxylase (pdc) from *Zymomonas mobilis* operably linked to a promoter.

[0555] In some embodiments of any of the aspects of the invention, the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, ethanol fermentation-related and/or transcription factor polypeptide or nucleic acid is operably linked to a T7 promoter, such as a T7 promoter contained in a medium or high copy plasmid. In some embodiments of any of the aspects of the invention, the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, ethanol fermentation-related and/or transcription factor nucleic acid is operably linked to a Trc promoter, such as a Trc promoter contained in a medium or high copy plasmid. In some embodiments of any of the aspects of the invention, the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, ethanol fermentation-related and/or transcription factor nucleic acid is operably

linked to a Lac promoter, such as a Lac promoter contained in a low copy plasmid. In some embodiments of any of the aspects of the invention, the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, ethanol fermentation-related polypeptide or transcription factor nucleic acid is operably linked to an endogenous promoter, such as an endogenous alkaline serine protease promoter. In some embodiments, the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, ethanol fermentation-related and/or transcription factor nucleic acid integrates into a chromosome of the cells without a selective marker or without a selectable marker.

[0556] In some embodiments, one or more MVA pathway, IDI, DXS, isoprene synthase, ethanol fermentation-related and/or transcription factor nucleic acids are placed under the control of a promoter or factor that is more active in stationary phase than in the growth phase. For example, one or more MVA pathway, IDI, DXS, isoprene synthase, ethanol fermentation-related and/or transcription factor or transcription factor nucleic acids may be placed under control of a stationary phase sigma factor, such as RpoS. In some embodiments, one or more MVA pathway, IDI, DXS, isoprene synthase, ethanol fermentation-related and/or transcription factor or transcription factor nucleic acids are placed under control of a promoter inducible in stationary phase, such as a promoter inducible by a response regulator active in stationary phase.

[0557] In some embodiments of any of the aspects of the invention, at least a portion of the cells maintain the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, ethanol fermentation-related and/or transcription factor nucleic acid for at least or about 5, 10, 20, 40, 50, 60, 65, or more cell divisions in a continuous culture (such as a continuous culture without dilution). In some embodiments of any of the aspects of the invention, the nucleic acid comprising the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, ethanol fermentation-related and/or transcription factor nucleic acid also comprises a selective marker or a selectable marker, such as an antibiotic resistance nucleic acid.

[0558] In some embodiments of any of the aspects of the invention, cells that co-produce isoprene and ethanol are cultured in any of the culture media described herein, under oxygen-limited conditions to facilitate the co-production of isoprene and ethanol by the cells. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the

cells are grown in the presence of 0.5 moles of oxygen per mole of isoprene. In some embodiments, the cells are grown anaerobically, in the absence of oxygen.

[0559] In some embodiments, any of the cells described herein are grown in oxygen-limited culture and co-produce isoprene and ethanol. In some embodiments, the cells in oxygen-limited culture have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of ethanol greater than about 0.1 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have a peak volumetric productivity of isoprene greater than about 1000 mg/L_{broth}/hr and a peak volumetric productivity of ethanol greater than about 1500 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have a peak volumetric productivity of isoprene greater than about 3000 mg/L_{broth}/hr and a peak volumetric productivity of ethanol greater than about 4500 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have a peak volumetric productivity of isoprene greater than about 5000 mg/L_{broth}/hr and a peak volumetric productivity of ethanol greater than about 7500 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have an average volumetric productivity of isoprene between about 0.1 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, and an average volumetric productivity of ethanol between about 0.1 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have an average volumetric productivity of isoprene between about 1 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 5 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 10 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 25 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 50 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 100 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 250 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 500 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 1000 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, and between about 2500 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, and an average volumetric productivity of ethanol between about 0.1 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr, between about 1 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr, between about 10 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr, between about 100 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr, between about 500 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr, between about 1000 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr, between about 2500 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr, and between about 5000 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr.

[0560] In some embodiments, the cells in oxygen-limited culture comprise a heterologous nucleic acid encoding an isoprene synthase polypeptide, wherein the heterologous nucleic acid is operably linked to a promoter, and wherein the cells have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of ethanol greater than about 0.1 mg/L_{broth}/hr. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide.

[0561] In some embodiments, provided herein are methods of co-producing isoprene and ethanol, the methods comprising: (a) culturing cells under conditions suitable for the co-production of isoprene and ethanol; and (b) co-producing isoprene and ethanol, wherein the cells have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of ethanol greater than about 0.1 mg/L_{broth}/hr.

[0562] In some embodiments, provided herein are compositions comprising ethanol. In some embodiments, provided herein are compositions comprising isoprene. In some embodiments, the composition further comprises 1.0×10^{-4} molar percent or less of non-methane volatile impurities. In some embodiments, the non-methane volatile impurities comprise one or more of the following: 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, acetaldehyde, methanethiol, methyl acetate, 1-propanol, diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene, 2,3-cycloheptenolpyridine, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol) and citronellol (3,7-dimethyl-6-octen-1-ol) or a linear isoprene polymer (such as a linear isoprene dimer or a linear isoprene trimer derived from the polymerization of multiple isoprene units). In some embodiments, the non-methane volatile impurities comprise one or more of the following: the isoprene composition includes one or more of the following: an alcohol, an aldehyde, an ester or a ketone (such as any of the alcohols, aldehyes, esters or ketones described herein). In some embodiments, the isoprene composition includes (i) an alcohol and an aldehyde, (ii) an alcohol and a ketone, (iii) an aldehyde and a ketone, or (iv) an alcohol, an aldehyde, and a ketone. In some embodiments, the non-methane volatile impurities comprise one or more of

the following: methanol, acetaldehyde, ethanol, methanethiol, 1-butanol, 3-methyl-1-propanol, acetone, acetic acid, 2-butanone, 2-methyl-1-butanol, or indole.

[0563] Also provided herein are methods of co-producing isoprene and ethanol, the methods comprising: a) culturing cells under conditions suitable for the co-production of isoprene and ethanol; and b) co-producing isoprene and ethanol, wherein the peak concentration of the isoprene produced by the cells in oxygen-limited culture is greater than about 10 ng/L_{broth} and the ethanol production rate of the cells is greater than about 0.002 mmol/L_{broth}/hour. In some embodiments of any of these methods, the ethanol production rate is between about any of 0.002 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 0.01 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 0.05 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 0.1 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 0.5 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 1 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 5 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 10 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 25 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 50 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 75 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 100 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, and between about 150 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr.

[0564] Provided herein are also methods of co-producing isoprene and ethanol comprising a) culturing cells under conditions suitable for the co-production of isoprene and ethanol; and b) co-producing isoprene and ethanol, wherein the liquid phase concentration of isoprene is less than about 200 mg/L, the cells produce greater than about 400 nmole/g_{wcm}/hour of isoprene, and the ethanol production rate of the cells is greater than about 0.01 mmol/L_{broth}/hour. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the liquid phase concentration of isoprene in the culture is less than about any of 175 mg/L, 150 mg/L, 125 mg/L, 100 mg/L, 75 mg/L, 50 mg/L, 25 mg/L, 20 mg/L, 15 mg/L, 10 mg/L, 5 mg/L, or 2.5 mg/L. In some embodiments, the liquid phase concentration of isoprene in culture is between about any of 0.1 mg/L to 200 mg/L, 1 mg/L to 200 mg/L, 1 mg/L to 150 mg/L, 1 mg/L to 100 mg/L, 1 mg/L to 50 mg/L, 1 mg/L to 25 mg/L, 1 mg/L to 20 mg/L, or 10 mg/L to 20 mg/L. In some embodiments of any of these methods, the cells in oxygen-limited culture produce isoprene at a rate between about 400 nmole/g_{wcm}/hr and about 2.0×10^5 nmole/g_{wcm}/hr, between about 500 nmole/g_{wcm}/hr and

about 1.5×10^5 nmole/g_{wcm}/hr, between about 750 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 1000 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 2500 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 5000 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 7500 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, and between about 1×10^4 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, and produce ethanol at a rate between about any of 0.002 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 0.01 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 0.05 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 0.1 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 0.5 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 1 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 5 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 10 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 25 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 50 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 75 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 100 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, and between about 150 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr.

[0565] In one aspect, provided herein are cells in oxygen-limited culture that co-produce isoprene and ethanol. In some embodiments, the oxygen-limited culture is anaerobic. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source, polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are cultured under limited glucose conditions.

[0566] In some embodiments, provided herein are cells in oxygen-limited culture that comprise a heterologous nucleic acid encoding an isoprene synthase polypeptide. In some embodiments, the oxygen-limited culture is anaerobic. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty

acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source, polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are cultured under limited glucose conditions.

[0567] In one aspect, provided herein are methods of co-producing isoprene with another compound, such as methods of using any of the cells described herein to co-produce isoprene and ethanol. In some embodiments, the method involves culturing cells under oxygen-limited conditions. In some embodiments, the oxygen-limited culture is anaerobic. In some embodiments, the method also includes recovering the isoprene and ethanol produced by the cells. In some embodiments, the method further includes purifying the isoprene and the ethanol produced by the cells. In some embodiments, the method includes polymerizing the isoprene. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source, polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are cultured under limited glucose conditions. In various embodiments, the amount of isoprene produced (such as the total amount of isoprene produced or the amount of isoprene produced per liter of broth per hour per OD_{600}) during stationary phase is greater than or about 2 or more times the amount of isoprene produced during the growth phase for the same length of time.

[0568] In some embodiments of any of the aspects of the invention, the microbial polypeptide carbon source includes one or more polypeptides from yeast or bacteria. In some embodiments of any of the aspects of the invention, the plant polypeptide carbon source includes one or more polypeptides from soy, corn, canola, jatropha, palm, peanut, sunflower, coconut, mustard, rapeseed, cottonseed, palm kernel, olive, safflower, sesame, or linseed.

[0569] In some embodiments, isoprene and ethanol are only co-produced in stationary phase. In some embodiments, isoprene and ethanol are co-produced in both the growth phase and stationary phase. In various embodiments, the amount of isoprene produced (such as the total amount of isoprene produced or the amount of isoprene produced per liter of broth per

hour per OD_{600}) during stationary phase is greater than or about 2, 3, 4, 5, 10, 20, 30, 40, 50, or more times the amount of isoprene produced during the growth phase for the same length of time. In various embodiments, the amount of ethanol produced (such as the total amount of ethanol produced or the amount of ethanol produced per liter of broth per hour per OD_{600}) during stationary phase is greater than or about 2, 3, 4, 5, 10, 20, 30, 40, 50, or more times the amount of ethanol produced during the growth phase for the same length of time.

[0570] In some embodiments, the compositions provided herein comprise ethanol and greater than or about 99.90, 99.92, 99.94, 99.96, 99.98, or 100% isoprene by weight compared to the total weight of all C5 hydrocarbons in the composition. In some embodiments, the composition comprises less than or about 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% C5 hydrocarbons other than isoprene (such 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 3-methyl-l-butyne, *trans*-piperylene, *cis*-piperylene, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne) by weight compared to the total weight of all C5 hydrocarbons in the composition. In some embodiments, the composition has less than or about 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% for 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 3-methyl-l-butyne, *trans*-piperylene, *cis*-piperylene, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne by weight compared to the total weight of all C5 hydrocarbons in the composition. In particular embodiments, the composition has greater than about 2 mg of isoprene and has greater than or about 99.90, 99.92, 99.94, 99.96, 99.98, or 100% isoprene by weight compared to the total weight of all C5 hydrocarbons in the composition. In some embodiments, the composition has less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 μg/L of a compound that inhibits the polymerization of isoprene for any compound in the composition that inhibits the polymerization of isoprene. In particular embodiments, the composition also comprises greater than about 2 mg of isoprene and greater than about 0.48 mg of ethanol.

[0571] In some embodiments, the volatile organic fraction of the gas phase has less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 μg/L of a compound that inhibits the polymerization of isoprene for any compound in the volatile organic fraction of the gas phase that inhibits the polymerization of isoprene. In some embodiments, the volatile organic

fraction of the gas phase also has greater than about 2 mg of isoprene and greater than about 0.48 mg of ethanol.

[0572] In some embodiments, the invention also features systems that include any of the cells and/or compositions described herein. In some embodiments, the system includes a reactor that chamber comprises cells in oxygen-limited culture that produce greater than about 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, or more nmole/g_{wcm}/hr isoprene and greater than about 0.1, 0.25, 0.5, 1, 5, 10, 25, 50, 75, 100, 250, 500 or more mmol/L_{broth}/hr ethanol. In some embodiments, the system is not a closed system. In some embodiments, at least a portion of the isoprene is removed from the system. In some embodiments, the system includes a gas phase comprising isoprene and ethanol. In some embodiments,the system includes a gas phase comprising isoprene and a liquid phase comprising ethanol. In various embodiments, the gas phase comprises any of the compositions described herein. In various embodiments, the liquid phase comprises any of the compositions described herein.

[0573] In one aspect, the invention provides a tire comprising polyisoprene. In some embodiments, the polyisoprene is produced by (i) polymerizing isoprene in any of the compositions described herein or (ii) polymerizing isoprene recovered from any of the compositions described herein. In some embodiments, the polyisoprene comprises *cis*-1,4-polyisoprene.

[0574] In one aspect, the invention features a product produced by any of the compositions or methods described herein.

Exemplary Co-Production of Isoprene and 1,2-Propanediol or 1,3-Propanediol

[0575] In some embodiments, the C2-or C3-alcohol or diol is 1,2-propanediol. In some embodiments, the C2-or C3-alcohol or diol is 1,3-propanediol. In some embodiments, any of the isoprene-producing cells described herein that comprise one or more heterologous nucleic acids encoding an isoprene synthase polypeptide, a DXS polypeptide, an IDI polypeptide, and/or an MVA pathway polypeptide operably linked to a promoter further comprise a heterologous nucleic acid also operably linked to a promoter encoding one or more polypeptides in the glycerol pathway or the 1,3-propanediol pathway. Such cells can co-produce isoprene and 1,2-propanediol or 1,3-propanediol.

[0576] In some embodiments of any of the aspects of the invention, the cells are bacterial cells, such as gram-positive bacterial cells (*e.g.*, *Bacillus* cells such as *Bacillus subtilis* cells or *Streptomyces* cells such as *Streptomyces lividans*, *Streptomyces coelicolor*, or *Streptomyces griseus* cells). In some embodiments of any of the aspects of the invention, the cells are gram-negative bacterial cells (*e.g.*, *Escherichia* cells such as *Escherichia coli* cells, *Rhodopseudomonas sp.* such as *Rhodopseudomonas palustris* cells, *Pseudomonas sp.* such as *Pseudomonas fluorescens* cells or *Pseudomonas putida* cells, *Pantoea* cells such as *Pantoea citrea* cells, or *Zymomonas* cells such as *Zymomonas mobilis* cells). In some embodiments of any of the aspects of the invention, the gram-negative bacterial cells are *E. coli*. In some embodiments of any of the aspects of the invention, the gram-negative bacterial cells are *Zymomonas mobilis*. In some embodiments of any of the aspects of the invention, the cells are fungal, cells such as filamentous fungal cells (*e.g.*, *Trichoderma* cells such as *Trichoderma reesei* cells or *Aspergillus* cells such as *Aspergillus oryzae* and *Aspergillus niger*) or yeast cells (*e.g.*, *Yarrowia* cells such as *Yarrowia lipolytica* cells or *Saccharomyces* cells such as *Saccharomyces cerevisiae*). In some embodiments of any of the aspects of the invention, the yeast cells are *S. cerevisiae*.

[0577] In some embodiments of any of the aspects of the invention, the isoprene synthase polypeptide is a polypeptide from a plant such as *Pueraria* (*e.g., Pueraria montana* or *Pueraria lobata*)(also known as "Kudzu") or *Populus* (*e.g.*, *Populus tremuloides*, *Populus alba*, *Populus nigra*, *Populus trichocarpa*, or the hybrid, *Populus alba x Populus tremula*).

[0578] In some embodiments of any of the aspects of the invention, the cells further comprise a heterologous nucleic acid encoding an IDI polypeptide. In some embodiments of any of the aspects of the invention, the cells further comprise an insertion of a copy of an endogenous nucleic acid encoding an IDI polypeptide. In some embodiments of any of the aspects of the invention, the cells further comprise a heterologous nucleic acid encoding a DXS polypeptide. In some embodiments of any of the aspects of the invention, the cells further comprise an insertion of a copy of an endogenous nucleic acid encoding a DXS polypeptide. In some embodiments of any of the aspects of the invention, the cells further comprise one or more nucleic acids encoding an IDI polypeptide and a DXS polypeptide. In some embodiments of any of the aspects of the invention, one nucleic acid encodes the isoprene synthase polypeptide, IDI polypeptide, and DXS polypeptide. In some embodiments of any of the aspects of the invention, one vector encodes the isoprene synthase polypeptide,

IDI polypeptide, and DXS polypeptide. In some embodiments, the vector comprises a selective marker or a selectable marker, such as an antibiotic resistance nucleic acid.

[0579] In some embodiments of any of the aspects of the invention, the cells further comprise a heterologous nucleic acid encoding an MVA pathway polypeptide (such as an MVA pathway polypeptide from *Saccharomyces cerevisia* or *Enterococcus faecalis*). In some embodiments of any of the aspects of the invention, the cells further comprise an insertion of a copy of an endogenous nucleic acid encoding an MVA pathway polypeptide (such as an MVA pathway polypeptide from *Saccharomyces cerevisia* or *Enterococcus faecalis*). In some embodiments of any of the aspects of the invention, the cells comprise an isoprene synthase, DXS, and MVA pathway nucleic acid. In some embodiments of any of the aspects of the invention, the cells comprise an isoprene synthase nucleic acid, a DXS nucleic acid, an IDI nucleic acid, and a MVA pathway nucleic acid.

[0580] In some embodiments, the MVA pathway polypeptide is an upper MVA pathway polypeptide. In some embodiments, the MVA pathway polypeptide is a lower MVA pathway polypeptide. In some embodiments, the upper MVA pathway polypeptide is selected from the group consisting of: (i) an acetoacetyl-Coenzyme A synthase (thiolase) polypeptide; (ii) a 3-hydroxy-3-methylglutaryl-Coenzyme A synthase polypeptide; and (iii) a 3-hydroxy-3-methylglutaryl-Coenzyme A reductase polypeptide. In some embodiments, the upper MVA pathway polypeptide is from the genus *Enterococcus*. In some embodiments, the upper MVA pathway polypeptide is from *Enterococcus faecalis*. In some embodiments, the lower MVA pathway polypeptide is selected from the group consisting of: (i) mevalonate kinase (MVK); (ii) phosphomevalonate kinase (PMK); (iii) diphosphomevalonate decarboxylase (MVD); and (iv) isopentenyl diphosphate isomerase (IDI). In some embodiments, the lower MVA pathway polypeptide is an MVK polypeptide. In some embodiments, the MVK polypeptide is from the genus *Methanosarcina*. In some embodiments, the MVK polypeptide is from *Methanosarcina mazei*.

[0581] In some embodiments of any of the aspects of the invention, the cells further comprise a heterologous nucleic acid encoding one or more polypeptides in the glycerol pathway or the 1,3-propanediol pathway operably linked to a promoter. In some embodiments, the polypeptide involved in the glycerol pathway or the 1,3-propanediol pathway is dihydroxyacetone phosphate reductase (DAR1), glycerol-phosphate phosphatase (GPP2), glycerol dehydratase B1 (dhaB1), glycerol dehydratase B2 (dhaB2), glycerol

dehydratase B3 (dhaB3), dhaX, orfX, orfY, 1,3-propanediol oxidoreductase (dhaT), glycerol dehydrogenase (dhaD), or dihydroxyacetone kinase (dhaK) operably linked to a promoter. In some embodiments, the polypeptide involved in the glycerol pathway or the 1,3-propanediol pathway is dihydroxyacetone phosphate reductase (DAR1), glycerol-phosphate phosphatase (GPP2), glycerol dehydratase B1 (dhaB1), glycerol dehydratase B2 (dhaB2), glycerol dehydratase B3 (dhaB3), dhaX, orfX, and orfY operably linked to a promoter.

[0582] In some embodiments of any of the aspects of the invention, the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, glycerol pathway or the 1,3-propanediol pathway polypeptide or nucleic acid is operably linked to a T7 promoter, such as a T7 promoter contained in a medium or high copy plasmid. In some embodiments of any of the aspects of the invention, the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, glycerol pathway or the 1,3-propanediol pathway nucleic acid is operably linked to a Trc promoter, such as a Trc promoter contained in a medium or high copy plasmid. In some embodiments of any of the aspects of the invention, the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, glycerol pathway or the 1,3-propanediol pathway nucleic acid is operably linked to a Lac promoter, such as a Lac promoter contained in a low copy plasmid. In some embodiments of any of the aspects of the invention, the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, glycerol pathway or the 1,3-propanediol pathway nucleic acid is operably linked to an endogenous promoter, such as an endogenous alkaline serine protease promoter. In some embodiments, the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, glycerol pathway or the 1,3-propanediol pathway nucleic acid integrates into a chromosome of the cells without a selective marker or without a selectable marker.

[0583] In some embodiments, one or more MVA pathway, IDI, DXS, isoprene synthase, glycerol pathway or the 1,3-propanediol pathway nucleic acids are placed under the control of a promoter or factor that is more active in stationary phase than in the growth phase. For example, one or more MVA pathway, IDI, DXS, isoprene synthase, glycerol pathway or the 1,3-propanediol pathway nucleic acids may be placed under control of a stationary phase sigma factor, such as RpoS. In some embodiments, one or more MVA pathway, IDI, DXS, isoprene synthase, glycerol pathway or the 1,3-propanediol pathway nucleic acids are placed under control of a promoter inducible in stationary phase, such as a promoter inducible by a response regulator active in stationary phase.

[0584] In some embodiments of any of the aspects of the invention, at least a portion of the cells maintain the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, glycerol pathway or the 1,3-propanediol pathway nucleic acid for at least or about 5, 10, 20, 40, 50, 60, 65, or more cell divisions in a continuous culture (such as a continuous culture without dilution). In some embodiments of any of the aspects of the invention, the nucleic acid comprising the heterologous isoprene synthase, DXS polypeptide, IDI polypeptide, MVA pathway, glycerol pathway or the 1,3-propanediol pathway nucleic acid also comprises a selective marker or a selectable marker, such as an antibiotic resistance nucleic acid.

[0585] In some embodiments of any of the aspects of the invention, cells that co-produce isoprene and 1,2-propanediol are cultured in any of the culture media described herein, under oxygen-limited conditions to facilitate the co-production of isoprene and 1,2-propanediol by the cells. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells are grown in the presence of 0.5 moles of oxygen per mole of isoprene. In some embodiments, the cells are grown anaerobically, in the absence of oxygen.

[0586] In some embodiments, any of the cells described herein are grown in oxygen-limited culture and co-produce isoprene and 1,2-propanediol. In some embodiments, the cells in oxygen-limited culture have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of 1,2-propanediol greater than about 0.1 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have a peak volumetric productivity of isoprene greater than about 1000 mg/L_{broth}/hr and a peak volumetric productivity of 1,2-propanediol greater than about 1500 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have a peak volumetric productivity of isoprene greater than about 3000 mg/L_{broth}/hr and a peak volumetric productivity of 1,2-propanediol greater than about 4500 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have a peak volumetric productivity of isoprene greater than about 5000 mg/L_{broth}/hr and a peak volumetric productivity of 1,2-propanediol greater than about 7500 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have an average volumetric productivity of isoprene between about 0.1 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, and an average volumetric productivity of 1,2-propanediol between about 0.1 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have an average volumetric productivity of isoprene between about 1 mg/L_{broth}/hr and

about 5000 mg/L_{broth}/hr, between about 5 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 10 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 25 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 50 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 100 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 250 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 500 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 1000 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, and between about 2500 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, and an average volumetric productivity of 1,2-propanediol between about 0.1 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr, between about 1 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr, between about 10 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr, between about 100 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr, between about 500 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr, between about 1000 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr, between about 2500 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr, and between about 5000 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr.

[0587] In some embodiments, the cells in oxygen-limited culture comprise a heterologous nucleic acid encoding an isoprene synthase polypeptide, wherein the heterologous nucleic acid is operably linked to a promoter, and wherein the cells have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of 1,2-propanediol greater than about 0.1 mg/L_{broth}/hr. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide.

[0588] In some embodiments, provided herein are methods of co-producing isoprene and 1,2-propanediol, the methods comprising: (a) culturing cells under conditions suitable for the co-production of isoprene and 1,2-propanediol; and (b) co-producing isoprene and 1,2-propanediol, wherein the cells have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of 1,2-propanediol greater than about 0.1 mg/L_{broth}/hr.

[0589] In some embodiments, provided herein are compositions comprising 1,2-propanediol. In some embodiments, provided herein are compositions comprising isoprene. In some embodiments, the composition further comprises 1.0×10^{-4} molar percent or less of non-methane volatile impurities. In some embodiments, the non-methane volatile impurities comprise one or more of the following: 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, acetaldehyde, methanethiol, methyl acetate, 1-propanol, diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-

propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene, 2,3-cycloheptenolpyridine, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol) and citronellol (3,7-dimethyl-6-octen-1-ol) or a linear isoprene polymer (such as a linear isoprene dimer or a linear isoprene trimer derived from the polymerization of multiple isoprene units). In some embodiments, the non-methane volatile impurities comprise one or more of the following: the isoprene composition includes one or more of the following: an alcohol, an aldehyde, an ester or a ketone (such as any of the alcohols, aldehyes, esters or ketones described herein). In some embodiments, the isoprene composition includes (i) an alcohol and an aldehyde, (ii) an alcohol and a ketone, (iii) an aldehyde and a ketone, or (iv) an alcohol, an aldehyde, and a ketone. In some embodiments, the non-methane volatile impurities comprise one or more of the following: methanol, acetaldehyde, ethanol, methanethiol, 1-butanol, 3-methyl-1-propanol, acetone, acetic acid, 2-butanone, 2-methyl-1-butanol, or indole.

[0590] Also provided herein are methods of co-producing isoprene and 1,2-propanediol, the methods comprising: a) culturing cells under conditions suitable for the co-production of isoprene and 1,2-propanediol; and b) co-producing isoprene and 1,2-propanediol, wherein the peak concentration of the isoprene produced by the cells in oxygen-limited culture is greater than about 10 ng/L_{broth} and the 1,2-propanediol production rate of the cells is greater than about 0.002 mmol/L_{broth}/hour. In some embodiments of any of these methods, the 1,2-propanediol production rate is between about any of 0.002 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 0.01 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 0.05 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 0.1 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 0.5 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 1 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 5 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 10 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 25 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 50 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 75 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 100 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, and between about 150 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr.

[0591] In some embodiments of any of the aspects of the invention, cells that co-produce isoprene and 1,3-propanediol are cultured in any of the culture media described herein, under oxygen-limited conditions to facilitate the co-production of isoprene and 1,3-propanediol by the cells. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the cells are grown in the presence of 0.5 moles of oxygen per mole of isoprene. In some embodiments, the cells are grown anaerobically, in the absence of oxygen.

[0592] In some embodiments, any of the cells described herein are grown in oxygen-limited culture and co-produce isoprene and 1,3-propanediol. In some embodiments, the cells in oxygen-limited culture have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of 1,3-propanediol greater than about 0.1 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have a peak volumetric productivity of isoprene greater than about 1000 mg/L_{broth}/hr and a peak volumetric productivity of 1,3-propanediol greater than about 1500 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have a peak volumetric productivity of isoprene greater than about 3000 mg/L_{broth}/hr and a peak volumetric productivity of 1,3-propanediol greater than about 4500 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have a peak volumetric productivity of isoprene greater than about 5000 mg/L_{broth}/hr and a peak volumetric productivity of 1,3-propanediol greater than about 7500 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have an average volumetric productivity of isoprene between about 0.1 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, and an average volumetric productivity of 1,3-propanediol between about 0.1 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr. In some embodiments, the cells in oxygen-limited culture have an average volumetric productivity of isoprene between about 1 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 5 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 10 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 25 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 50 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 100 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 250 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 500 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, between about 1000 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, and between about 2500 mg/L_{broth}/hr and about 5000 mg/L_{broth}/hr, and an average volumetric productivity of 1,3-propanediol between about 0.1 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr, between about 1 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr, between about 10 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr, between about 100 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr, between about 500 mg/L_{broth}/hr and about 7500

mg/L_{broth}/hr, between about 1000 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr, between about 2500 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr, and between about 5000 mg/L_{broth}/hr and about 7500 mg/L_{broth}/hr.

[0593] In some embodiments, the cells in oxygen-limited culture comprise a heterologous nucleic acid encoding an isoprene synthase polypeptide, wherein the heterologous nucleic acid is operably linked to a promoter, and wherein the cells have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of 1,3-propanediol greater than about 0.1 mg/L_{broth}/hr. In some embodiments, the isoprene synthase polypeptide is a plant isoprene synthase polypeptide.

[0594] In some embodiments, provided herein are methods of co-producing isoprene and 1,3-propanediol, the methods comprising: (a) culturing cells under conditions suitable for the co-production of isoprene and 1,3-propanediol; and (b) co-producing isoprene and 1,3-propanediol, wherein the cells have an average volumetric productivity of isoprene greater than about 0.1 mg/L_{broth}/hr and an average volumetric productivity of 1,3-propanediol greater than about 0.1 mg/L_{broth}/hr.

[0595] In some embodiments, provided herein are compositions comprising 1,3-propanediol. In some embodiments, provided herein are compositions comprising isoprene. In some embodiments, the composition further comprises 1.0×10^{-4} molar percent or less of non-methane volatile impurities. In some embodiments, the non-methane volatile impurities comprise one or more of the following: 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, acetaldehyde, methanethiol, methyl acetate, 1-propanol, diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene, 2,3-cycloheptenolpyridine, 3-hexen-1-ol, 3-hexen-1-yl acetate, limonene, geraniol (trans-3,7-dimethyl-2,6-octadien-1-ol) and citronellol (3,7-dimethyl-6-octen-1-ol) or a linear isoprene polymer (such as a linear isoprene dimer or a linear isoprene trimer derived from the polymerization of multiple isoprene units). In some embodiments, the non-methane volatile impurities comprise one or more of the following: the isoprene composition includes one or more of the following: an alcohol, an aldehyde, an ester or a ketone (such as any of the alcohols, aldehyes, esters or ketones described herein). In

some embodiments, the isoprene composition includes (i) an alcohol and an aldehyde, (ii) an alcohol and a ketone, (iii) an aldehyde and a ketone, or (iv) an alcohol, an aldehyde, and a ketone. In some embodiments, the non-methane volatile impurities comprise one or more of the following: methanol, acetaldehyde, ethanol, methanethiol, 1-butanol, 3-methyl-1-propanol, acetone, acetic acid, 2-butanone, 2-methyl-1-butanol, or indole.

[0596] Also provided herein are methods of co-producing isoprene and 1,3-propanediol, the methods comprising: a) culturing cells under conditions suitable for the co-production of isoprene and 1,3-propanediol; and b) co-producing isoprene and 1,3-propanediol, wherein the peak concentration of the isoprene produced by the cells in oxygen-limited culture is greater than about 10 ng/L_{broth} and the 1,3-propanediol production rate of the cells is greater than about 0.002 mmol/L_{broth}/hour. In some embodiments of any of these methods, the 1,3-propanediol production rate is between about any of 0.002 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 0.01 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 0.05 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 0.1 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 0.5 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 1 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 5 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 10 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 25 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 50 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 75 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 100 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, and between about 150 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr.

[0597] Provided herein are also methods of co-producing isoprene and 1,3-propanediol comprising a) culturing cells under conditions suitable for the co-production of isoprene and 1,3-propanediol; and b) co-producing isoprene and 1,3-propanediol, wherein the liquid phase concentration of isoprene is less than about 200 mg/L, the cells produce greater than about 400 nmole/g_{wcm}/hour of isoprene, and the 1,3-propanediol production rate of the cells is greater than about 0.01 mmol/L_{broth}/hour. In some embodiments, the cells are grown in oxygen-limited culture. In some embodiments, the liquid phase concentration of isoprene in the culture is less than about any of 175 mg/L, 150 mg/L, 125 mg/L, 100 mg/L, 75 mg/L, 50 mg/L, 25 mg/L, 20 mg/L, 15 mg/L, 10 mg/L, 5 mg/L, or 2.5 mg/L. In some embodiments, the liquid phase concentration of isoprene in culture is between about any of 0.1 mg/L to 200 mg/L, 1 mg/L to 200 mg/L, 1 mg/L to 150 mg/L, 1 mg/L to 100 mg/L, 1 mg/L to 50 mg/L, 1

mg/L to 25 mg/L, 1 mg/L to 20 mg/L, or 10 mg/L to 20 mg/L. In some embodiments of any of these methods, the cells in oxygen-limited culture produce isoprene at a rate between about 400 nmole/g_{wcm}/hr and about 2.0×10^5 nmole/g_{wcm}/hr, between about 500 nmole/g_{wcm}/hr and about 1.5×10^5 nmole/g_{wcm}/hr, between about 750 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 1000 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 2500 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 5000 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, between about 7500 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, and between about 1×10^4 nmole/g_{wcm}/hr and about 1×10^5 nmole/g_{wcm}/hr, and produce 1,3-propanediol at a rate between about any of 0.002 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 0.01 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 0.05 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 0.1 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 0.5 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 1 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 5 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 10 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 25 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 50 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 75 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, between about 100 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr, and between about 150 mmol/L_{broth}/hr and about 200 mmol/L_{broth}/hr.

Exemplary Purification Methods

[0598] In some embodiments, any of the methods described herein further include recovering the co-produced compounds. In some embodiments, any of the methods described herein further include recovering the isoprene. In some embodiments, any of the methods described herein further include recovering the hydrogen by cryogenic membrane, adsorption matrix-based separation methods. In some embodiments, any of the methods described herein further include recovering the ethanol. In some embodiments, any of the methods described herein further include recovering the 1,3-propanediol.

[0599] The isoprene and co-products, for example, hydrogen, ethanol, 1,2-propanediol or 1,3-propanediol, produced using the compositions and methods of the invention can be recovered using standard techniques. such as gas stripping, membrane enhanced separation, fractionation, adsorption/desorption, pervaporation, thermal or vacuum desorption of isoprene from a solid phase, or extraction of isoprene immobilized or absorbed to a solid phase with a solvent (see, for example, U.S. Patent Nos. 4,703,007, 4,570,029, and 4,740,222

("Recovery and Purification of Hydrogen from Refinery and Petrochemical Off-gas Streams") which are each hereby incorporated by reference in their entireties, particularly with respect to isoprene recovery and purification methods ('007 and '029 patents) and with respect to hydrogen recovery and purification methods ('222 patent)). In particular embodiments, extractive distillation with an alcohol (such as ethanol, methanol, propanol, or a combination thereof) is used to recover the isoprene. In some embodiments, the recovery of isoprene involves the isolation of isoprene in a liquid form (such as a neat solution of isoprene or a solution of isoprene in a solvent). Gas stripping involves the removal of isoprene vapor from the fermentation off-gas stream in a continuous manner. Such removal can be achieved in several different ways including, but not limited to, adsorption to a solid phase, partition into a liquid phase, or direct condensation (such as condensation due to exposure to a condensation coil or do to an increase in pressure). In some embodiments, membrane enrichment of a dilute isoprene vapor stream above the dew point of the vapor resulting in the condensation of liquid isoprene. In some embodiments, the isoprene is compressed and condensed.

[0600] The recovery of isoprene may involve one step or multiple steps. In some embodiments, the removal of isoprene vapor from the fermentation off-gas and the conversion of isoprene to a liquid phase are performed simultaneously. For example, isoprene can be directly condensed from the off-gas stream to form a liquid. In some embodiments, the removal of isoprene vapor from the fermentation off-gas and the conversion of isoprene to a liquid phase are performed sequentially. For example, isoprene may be adsorbed to a solid phase and then extracted from the solid phase with a solvent.

[0601] The recovery of hydrogen may involve one step or multiple steps. In some embodiments, the removal of hydrogen gas from the fermentation off-gas and the conversion of hydrogen to a liquid phase are performed simultaneously. In some embodiments, the removal of hydrogen gas from the fermentation off-gas and the conversion of hydrogen to a liquid phase are performed sequentially. For example, hydrogen may be adsorbed to a solid phase and then desorbed from the solid phase by a pressure swing.

[0602] The recovery of ethanol may involve one step or multiple steps. In some embodiments, the ethanol is recovered from the fermentation broth by distillation. In some embodiments, the fermentation broth is first cleared of cells and debri by centrifugation, filtration or similar method.

[0603] The recovery of 1,2-propanediol or 1,3-propanediol may involve one step or multiple steps. In some embodiments, the 1,2-propanediol or 1,3-propanediol is recovered from the fermentation broth by distillation. In some embodiments, the 1,2-propanediol or 1,3-propanediol is recovered from the fermentation broth by chromatography or other standard methods. In some embodiments, the fermentation broth is first cleared of cells and debri by centrifugation, filtration or similar method.

[0604] In some embodiments, any of the methods described herein further include purifying the isoprene. For example, the isoprene produced using the compositions and methods of the invention can be purified using standard techniques. Purification refers to a process through which isoprene is separated from one or more components that are present when the isoprene is produced. In some embodiments, the isoprene is obtained as a substantially pure liquid. Examples of purification methods include (i) distillation from a solution in a liquid extractant and (ii) chromatography. As used herein, "purified isoprene" means isoprene that has been separated from one or more components that are present when the isoprene is produced. In some embodiments, the isoprene is at least about 20%, by weight, free from other components that are present when the isoprene is produced. In various embodiments, the isoprene is at least or about 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, 95%, or 99%, by weight, pure. Purity can be assayed by any appropriate method, *e.g.*, by column chromatography, HPLC analysis, or GC-MS analysis.

[0605] In some embodiments, any of the methods described herein further include purifying the hydrogen. For example, the hydrogen produced using the compositions and methods of the invention can be purified using standard techniques. Purification refers to a process through which hydrogen is separated from one or more components that are present when the hydrogen is produced. In some embodiments, the hydrogen is obtained as a substantially pure gas. In some embodiments, the hydrogen is obtained as a substantially pure liquid. Examples of purification methods include (i) cryogenic condensation and (ii) solid matrix adsorption. As used herein, "purified hydrogen" means hydrogen that has been separated from one or more components that are present when the hydrogen is produced. In some embodiments, the hydrogen is at least about 20%, by weight, free from other components that are present when the hydrogen is produced. In various embodiments, the hydrogen is at least or about 25%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, 95%, or

99%, by weight, pure. Purity can be assayed by any appropriate method, *e.g.*, by column chromatography or GC-MS analysis.

[0606] In some embodiments, at least a portion of the gas phase remaining after one or more recovery steps for the removal of isoprene is recycled by introducing the gas phase into a cell culture system (such as a fermentor) for the production of isoprene.

[0607] In some embodiments, any of the methods described herein further include polymerizing the isoprene. For example, standard methods can be used to polymerize the purified isoprene to form *cis*-polyisoprene or other down stream products using standard methods. Accordingly, the invention also features a tire comprising polyisoprene, such as *cis*-1,4- polyisoprene and/or *trans*-1,4- polyisoprene made from any of the isoprene compositions disclosed herein.

Cell Viability at High Isoprene Titer

[0608] Isoprene is a hydrophobic molecule secreted by many plants, animals, and microbes. Bacteria, such as Bacillus, produce isoprene at fairly low levels. While there is some evidence that plants secrete isoprene to help with thermoprotection, it has been hypothesized that isoprene may act antagonistically to cyanobacteria or fungi, or as an antimicrobial agent. *See, e.g.,* Ladygina et al., *Process Biochemistry* 41:1001-1014 (2006), which is incorporated by reference in its entirety, particularly with respect to isoprene acting antagonistically. Since the very low production levels happening in nature are sufficient to be anti-microbial, it was of great concern that the titers and productivity levels of isoprene necessary for commercialization of isoprene would kill the host microbe.

[0609] We have found methods for producing titers and productivity levels of isoprene for commercialization of isoprene while maintaining cell viability and/or metabolic activity as indicated by carbon dioxide evolution rate or total carbon dioxide evolution rate.

[0610] Provided herein are methods of producing isoprene comprising: a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein cells produce greater than about 400 nmole/g_{wcm}/hour of isoprene, and the carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. In some embodiments, the isoprene produced is any concentration or amount disclosed in the section entitled "Exemplary Production of Isoprene." In some embodiments, the amount of isoprene is

between about any of 400 nmole/g_{wcm}/hour to 1 mole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 1 mmole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 40 mmole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 4 mmole/g_{wcm}/hour, 1 mmole/g_{wcm}/hour to 1.5 mmole/g_{wcm}/hour, 1.5 mmole/g_{wcm}/hour to 3 mmole/g_{wcm}/hour, 3 mmole/g_{wcm}/hour to 5 mmole/g_{wcm}/hour, 5 mmole/g_{wcm}/hour to 25 mmole/g_{wcm}/hour, 25 mmole/g_{wcm}/hour to 100 mmole/g_{wcm}/hour, 100 mmole/g_{wcm}/hour to 500 mmole/g_{wcm}/hour, or 500 mmole/g_{wcm}/hour to 1000 mmole/g_{wcm}/hour. In some embodiments, the amount of isoprene is about any of 1 mmole/g_{wcm}/hour, 1.5 mmole/g_{wcm}/hour, 2 mmole/g_{wcm}/hour, 3 mmole/g_{wcm}/hour, 4 mmole/g_{wcm}/hour, or 5 mmole/g_{wcm}/hour. In some embodiments, the carbon dioxide evolution rate is between about any of 1 x 10^{-18} mmol/L/hour to about 1 mol/L/hour, 1 mmol/L/hour to 1 mol/L/hour, 25 mmol/L/hour to 750 mmol/L/hour, 25 mmol/L/hour to 75 mmol/L/hour, 250 mmol/L/hour to 750 mmol/L/hour, or 450 mmol/L/hour to 550 mmol/L/hour. In some embodiments, the carbon dioxide evolution rate is about any of 50 mmol/L/hour, 100 mmol/L/hour, 150 mmol/L/hour, 200 mmol/L/hour, 250 mmol/L/hour, 300 mmol/L/hour, 350 mmol/L/hour, 400 mmol/L/hour, 450 mmol/L/hour, or 500 mmol/L/hour.

[0611] Provided herein are also methods of producing isoprene comprising: a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein cells produce greater than about 400 nmole/g_{wcm}/hour of isoprene, and cell viability is reduced by less than about two-fold. In some embodiments, the isoprene produced is any concentration or amount disclosed in the section entitled "Exemplary Production of Isoprene." In some embodiments, the amount of isoprene is between about any of 400 nmole/g_{wcm}/hour to 1 mole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 1 mmole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 40 mmole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 4 mmole/g_{wcm}/hour, 1 mmole/g_{wcm}/hour to 1.5 mmole/g_{wcm}/hour, 1.5 mmole/g_{wcm}/hour to 3 mmole/g_{wcm}/hour, 3 mmole/g_{wcm}/hour to 5 mmole/g_{wcm}/hour, 5 mmole/g_{wcm}/hour to 25 mmole/g_{wcm}/hour, 25 mmole/g_{wcm}/hour to 100 mmole/g_{wcm}/hour, 100 mmole/g_{wcm}/hour to 500 mmole/g_{wcm}/hour, or 500 mmole/g_{wcm}/hour to 1000 mmole/g_{wcm}/hour. In some embodiments, the amount of isoprene is about any of 1 mmole/g_{wcm}/hour, 1.5 mmole/g_{wcm}/hour, 2 mmole/g_{wcm}/hour, 3 mmole/g_{wcm}/hour, 4 mmole/g_{wcm}/hour, or 5 mmole/g_{wcm}/hour. In some embodiments, cell viability is reduced by less than about any of 1.75-fold, 1.5-fold, 1.25-fold, 1-fold, 0.75-fold, 0.5-fold, or 0.25-fold. In some embodiments, cell viability is reduced by about 2-fold.

[0612] Further provided herein are methods of producing isoprene comprising: a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein the cumulative total productivity of the isoprene produced by the cells in culture is greater than about 0.2 mg/L_{broth}/hour and the carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. In some embodiments, the cumulative total productivity of isoprene is any concentration or amount disclosed in the section entitled "Exemplary Production of Isoprene." In some embodiments, the cumulative total productivity of the isoprene is between about any of 0.2 mg/L_{broth}/hour to 5 g/L_{broth}/hour, 0.2 mg/L_{broth}/hour to 1 g/L_{broth}/hour, 1 g/L_{broth}/hour to 2.5 g/L_{broth}/hour, 2.5 g/L_{broth}/hour to 5 g/L_{broth}/hour. In some embodiments, the carbon dioxide evolution rate is between about any of 1 x 10^{-18} mmol/L/hour to about 1 mol/L/hour, 1 mmol/L/hour to 1 mol/L/hour, 25 mmol/L/hour to 750 mmol/L/hour, 25 mmol/L/hour to 75 mmol/L/hour, 250 mmol/L/hour to 750 mmol/L/hour, or 450 mmol/L/hour to 550 mmol/L/hour. In some embodiments, the carbon dioxide evolution rate is about any of 50 mmol/L/hour, 100 mmol/L/hour, 150 mmol/L/hour, 200 mmol/L/hour, 250 mmol/L/hour, 300 mmol/L/hour, 350 mmol/L/hour, 400 mmol/L/hour, 450 mmol/L/hour, or 500 mmol/L/hour.

[0613] Provided herein are methods of producing isoprene comprising: a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein the cumulative total productivity of the isoprene produced by the cells in culture is greater than about 0.2 mg/L_{broth}/hour and cell viability is reduced by less than about two-fold. In some embodiments, the cumulative total productivity of isoprene is any concentration or amount disclosed in the section entitled "Exemplary Production of Isoprene." In some embodiments, the cumulative total productivity of the isoprene is between about any of 0.2 mg/L_{broth}/hour to 5 g/L_{broth}/hour, 0.2 mg/L_{broth}/hour to 1 g/L_{broth}/hour, 1 g/L_{broth}/hour to 2.5 g/L_{broth}/hour, 2.5 g/L_{broth}/hour to 5 g/L_{broth}/hour. In some embodiments, cell viability is reduced by less than about any of 1.75-fold, 1.5-fold, 1.25-fold, 1-fold, 0.75-fold, 0.5-fold, or 0.25-fold.

[0614] Methods of producing isoprene are also provided herein comprising: a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein the peak concentration of the isoprene produced by the cells in culture is greater than about 10 ng/L_{broth} and the carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. In some embodiments, the peak concentration of isoprene is any concentration or amount disclosed in the section entitled "Exemplary Production of Isoprene." In some

embodiments, the peak concentration of isoprene is between about any of 10 ng/L_{broth} to 500 ng/L_{broth}, 500 ng/L_{broth} to 1 μg/L_{broth}, 1 μg/L_{broth} to 5 μg/L_{broth}, 5 μg/L_{broth} to 50 μg/L_{broth}, 5 μg/L_{broth} to 100 μg/L_{broth}, 5 μg/L_{broth} to 250 μg/L_{broth}, 250 μg/L_{broth} to 500 μg/L_{broth}, 500 μg/L_{broth} to 1 mg/L_{broth}, 1 mg/L_{broth} to 50 mg/L_{broth}, 1 mg/L_{broth} to 100 mg/L_{broth}, 1 mg/L_{broth} to 200 mg/L_{broth}, 10 ng/L_{broth} to 200 mg/L_{broth}, 5 μg/L_{broth} to 100 mg/L_{broth}, or 5 μg/L_{broth} to 200 mg/L_{broth}. In some embodiments, the peak concentration is any of about 10 ng/L_{broth}, 100 ng/L_{broth}, 1 μg/L_{broth}, 5 μg/L_{broth}, 1 mg/L_{broth}, 30 mg/ L_{broth}, 100 mg/L_{broth}, or 200 mg/L_{broth}. In some embodiments, the carbon dioxide evolution rate is between about any of 1 x 10^{-18} mmol/L/hour to about 1 mol/L/hour, 1 mmol/L/hour to 1 mol/L/hour, 25 mmol/L/hour to 750 mmol/L/hour, 25 mmol/L/hour to 75 mmol/L/hour, 250 mmol/L/hour to 750 mmol/L/hour, or 450 mmol/L/hour to 550 mmol/L/hour. In some embodiments, the carbon dioxide evolution rate is about any of 50 mmol/L/hour, 100 mmol/L/hour, 150 mmol/L/hour, 200 mmol/L/hour, 250 mmol/L/hour, 300 mmol/L/hour, 350 mmol/L/hour, 400 mmol/L/hour, 450 mmol/L/hour, or 500 mmol/L/hour.

[0615] In addition, methods of producing isoprene are also provided herein comprising: a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein the peak concentration of the isoprene produced by the cells in culture is greater than about 10 ng/L_{broth} and cell viability is reduced by less than about two-fold. In some embodiments, the peak concentraion of isoprene is any concentration or amount disclosed in the section entitled "Exemplary Production of Isoprene." In some embodiments, the peak concentration of isoprene is between about any of 10 ng/L_{broth} to 500 ng/L_{broth}, 500 ng/L_{broth} to 1 μg/L_{broth}, 1 μg/L_{broth} to 5 μg/L_{broth}, 5 μg/L_{broth} to 50 μg/L_{broth}, 5 μg/L_{broth} to 100 μg/L_{broth}, 5 μg/L_{broth} to 250 μg/L_{broth}, 250 μg/L_{broth} to 500 μg/L_{broth}, 500 μg/L_{broth} to 1 mg/L_{broth}, 1 mg/L_{broth} to 50 mg/L_{broth}, 1 mg/L_{broth} to 100 mg/L_{broth}, 1 mg/L_{broth} to 200 mg/L_{broth}, 10 ng/L_{broth} to 200 mg/L_{broth}, 5 μg/L_{broth} to 100 mg/L_{broth}, or 5 μg/L_{broth} to 200 mg/L_{broth}. In some embodiments, the peak concentration is any of about 10 ng/L_{broth}, 100 ng/L_{broth}, 1 μg/L_{broth}, 5 μg/L_{broth}, 1 mg/L_{broth}, 30 mg/ L_{broth}, 100 mg/L_{broth}, or 200 mg/L_{broth}.In some embodiments, cell viability is reduced by less than about any of 1.75-fold, 1.5-fold, 1.25-fold, 1-fold, 0.75-fold, 0.5-fold, or 0.25-fold. In some embodiments, cell viability is reduced by about 2-fold.

[0616] Cells in culture are also provided herein comprising a nucleic acid encoding an isoprene synthase polypeptide, wherein the cells produce greater than about 400 nmole/g_{wcm}/hour of isoprene and carbon dioxide evolution rate of the cells is greater than

about 1 x 10^{-18} mmol/L/hour. In some embodiments, the isoprene produced is any concentration or amount disclosed in the section entitled "Exemplary Production of Isoprene." In some embodiments, the amount of isoprene is between about any of 400 nmole/g_{wcm}/hour to 1 mole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 1 mmole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 40 mmole/g_{wcm}/hour, 400 nmole/g_{wcm}/hour to 4 mmole/g_{wcm}/hour, 1 mmole/g_{wcm}/hour to 1.5 mmole/g_{wcm}/hour, 1.5 mmole/g_{wcm}/hour to 3 mmole/g_{wcm}/hour, 3 mmole/g_{wcm}/hour to 5 mmole/g_{wcm}/hour, 5 mmole/g_{wcm}/hour to 25 mmole/g_{wcm}/hour, 25 mmole/g_{wcm}/hour to 100 mmole/g_{wcm}/hour, 100 mmole/g_{wcm}/hour to 500 mmole/g_{wcm}/hour, or 500 mmole/g_{wcm}/hour to 1000 mmole/g_{wcm}/hour. In some embodiments, the amount of isoprene is about any of 1 mmole/g_{wcm}/hour, 1.5 mmole/g_{wcm}/hour, 2 mmole/g_{wcm}/hour, 3 mmole/g_{wcm}/hour, 4 mmole/g_{wcm}/hour, or 5 mmole/g_{wcm}/hour. In some embodiments, the carbon dioxide evolution rate is between about any of 1 x 10^{-18} mmol/L/hour to about 1 mol/L/hour, 1 mmol/L/hour to 1 mol/L/hour, 25 mmol/L/hour to 750 mmol/L/hour, 25 mmol/L/hour to 75 mmol/L/hour, 250 mmol/L/hour to 750 mmol/L/hour, or 450 mmol/L/hour to 550 mmol/L/hour. In some embodiments, the carbon dioxide evolution rate is about any of 50 mmol/L/hour, 100 mmol/L/hour, 150 mmol/L/hour, 200 mmol/L/hour, 250 mmol/L/hour, 300 mmol/L/hour, 350 mmol/L/hour, 400 mmol/L/hour, 450 mmol/L/hour, or 500 mmol/L/hour.

[0617] Provided herein are also cells in culture comprising a nucleic acid encoding an isoprene synthase polypeptide, wherein cumulative total productivity of the isoprene produced by the cells in culture is greater than about 0.2 mg/L_{broth}/hour and carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. In some embodiments, the cumulative total productivity of isoprene is any concentration or amount disclosed in the section entitled "Exemplary Production of Isoprene." In some embodiments, the cumulative total productivity of the isoprene is between about any of 0.2 mg/L_{broth}/hour to 5 g/L_{broth}/hour, 0.2 mg/L_{broth}/hour to 1 g/L_{broth}/hour, 1 g/L_{broth}/hour to 2.5 g/L_{broth}/hour, 2.5 g/L_{broth}/hour to 5 g/L_{broth}/hour. In some embodiments, the carbon dioxide evolution rate is between about any of 1 x 10^{-18} mmol/L/hour to about 1 mol/L/hour, 1 mmol/L/hour to 1 mol/L/hour, 25 mmol/L/hour to 750 mmol/L/hour, 25 mmol/L/hour to 75 mmol/L/hour, 250 mmol/L/hour to 750 mmol/L/hour, or 450 mmol/L/hour to 550 mmol/L/hour. In some embodiments, the carbon dioxide evolution rate is about any of 50 mmol/L/hour, 100 mmol/L/hour, 150 mmol/L/hour, 200 mmol/L/hour, 250 mmol/L/hour, 300 mmol/L/hour, 350 mmol/L/hour, 400 mmol/L/hour, 450 mmol/L/hour, or 500 mmol/L/hour.

[0618] In addition, provided herein are cells in culture comprising a nucleic acid encoding an isoprene synthase polypeptide, wherein peak concentration of the isoprene produced by the cells in culture is greater than about 10 ng/L_{broth} and carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. In some embodiments, the peak concentration of isoprene is any concentration or amount disclosed in the section entitled "Exemplary Production of Isoprene." In some embodiments, the peak concentration of isoprene is between about any of 10 ng/L_{broth} to 500 ng/L_{broth}, 500 ng/L_{broth} to 1 μg/L_{broth}, 1 μg/L_{broth} to 5 μg/L_{broth}, 5 μg/L_{broth} to 50 μg/L_{broth}, 5 μg/L_{broth} to 100 μg/L_{broth}, 5 μg/L_{broth} to 250 μg/L_{broth}, 250 μg/L_{broth} to 500 μg/L_{broth}, 500 μg/L_{broth} to 1 mg/L_{broth}, 1 mg/L_{broth} to 50 mg/L_{broth}, 1 mg/L_{broth} to 100 mg/L_{broth}, 1 mg/L_{broth} to 200 mg/L_{broth}, 10 ng/L_{broth} to 200 mg/L_{broth}, 5 μg/L_{broth} to 100 mg/L_{broth}, or 5 μg/L_{broth} to 200 mg/L_{broth}. In some embodiments, the peak concentration is any of about 10 ng/L_{broth}, 100 ng/L_{broth}, 1 μg/L_{broth}, 5 μg/L_{broth}, 1 mg/L_{broth}, 30 mg/ L_{broth}, 100 mg/L_{broth}, or 200 mg/L_{broth}. In some embodiments, the carbon dioxide evolution rate is between about any of 1 x 10^{-18} mmol/L/hour to about 1 mol/L/hour, 1 mmol/L/hour to 1 mol/L/hour, 25 mmol/L/hour to 750 mmol/L/hour, 25 mmol/L/hour to 75 mmol/L/hour, 250 mmol/L/hour to 750 mmol/L/hour, or 450 mmol/L/hour to 550 mmol/L/hour. In some embodiments, the carbon dioxide evolution rate is about any of 50 mmol/L/hour, 100 mmol/L/hour, 150 mmol/L/hour, 200 mmol/L/hour, 250 mmol/L/hour, 300 mmol/L/hour, 350 mmol/L/hour, 400 mmol/L/hour, 450 mmol/L/hour, or 500 mmol/L/hour.

[0619] In some embodiments of any of the methods and cells described herein, carbon dioxide evolution rate and/or cell viability of a cell expressing a MVA pathway and/or DXP pathway RNA and/or protein from one or more of a heterologous and/or duplicate copy of a MVA pathway and/or DXP pathway nucleic acid is compared to a control cell lacking one or more of a heterologous and/or duplicate copy of a MVA pathway and/or DXP pathway nucleic acid. In some embodiments, carbon dioxide evolution rate and/or cell viability of a cell expressing a MVA pathway and/or DXP pathway RNA and/or protein from one or more of a heterologous and/or duplicate copy of a MVA pathway and/or DXP pathway nucleic acid under the control of an inducible promoter, wherein the promotor is induced, is compared to a control cell containing one or more of a heterologous and/or duplicate copy of a MVA pathway and/or DXP pathway nucleic acid under the control of an inducible promoter, wherein the promotor is not induced (uninduced). In some embodiments, the inducible promoter is a beta-galactosidase promoter.

[0620] The invention provides methods of producing isoprene comprising: a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein cells produce greater than about 400 nmole/g_{wcm}/hour of isoprene, and the carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. Further provided herein are methods of producing isoprene comprising: a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein the cumulative total productivity of the isoprene produced by the cells in culture is greater than about 0.2 mg/L_{broth}/hour and the carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. Methods of producing isoprene are also provided herein comprising: a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein the peak concentration of the isoprene produced by the cells in culture is greater than about 10 ng/L_{broth} and the carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. In some embodiments of any of these methods, the carbon dioxide evolution rate is between about any of 1 x 10^{-18} mmol/L/hour to about 1 mol/L/hour, 1 mmol/L/hour to 1 mol/L/hour, 25 mmol/L/hour to 750 mmol/L/hour, 25 mmol/L/hour to 75 mmol/L/hour, 250 mmol/L/hour to 750 mmol/L/hour, or 450 mmol/L/hour to 550 mmol/L/hour. In some embodiments, the carbon dioxide evolution rate is about 50 mmol/L/hour or about 500 mmol/L/hour.

[0621] Further provided herein are cells in culture comprising a nucleic acid encoding an isoprene synthase polypeptide, wherein the cells produce greater than about 400 nmole/g_{wcm}/hour of isoprene and carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. Provided herein are also cells in culture comprising a nucleic acid encoding an isoprene synthase polypeptide, wherein cumulative total productivity of the isoprene produced by the cells in culture is greater than about 0.2 mg/L_{broth}/hour and carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. In addition, provided herein are cells in culture comprising a nucleic acid encoding an isoprene synthase polypeptide, wherein peak concentration of the isoprene produced by the cells in culture is greater than about 10 ng/L_{broth} and carbon dioxide evolution rate of the cells is greater than about 1 x 10^{-18} mmol/L/hour. In some embodiments of any of these cells in culture, the carbon dioxide evolution rate is between about any of 1 x 10^{-18} mmol/L/hour to about 1 mol/L/hour, 1 mmol/L/hour to 1 mol/L/hour, 25 mmol/L/hour to 750 mmol/L/hour, 25 mmol/L/hour to 75 mmol/L/hour, 250 mmol/L/hour to 750 mmol/L/hour, or 450

mmol/L/hour to 550 mmol/L/hour. In some embodiments, the carbon dioxide evolution rate is about 50 mmol/L/hour or about 500 mmol/L/hour.

[0622] Provided herein are also methods of producing isoprene comprising a) culturing cells under suitable conditions for production of isoprene; and b) producing isoprene, wherein the liquid phase concentration of isoprene is less than about 200 mg/L and the cells produce greater than about 400 nmole/g_{wcm}/hour of isoprene. In some embodiments, the liquid phase concentration of isoprene in the culture is less than about any of 175 mg/L, 150 mg/L, 125 mg/L, 100 mg/L, 75 mg/L, 50 mg/L, 25 mg/L, 20 mg/L, 15 mg/L, 10 mg/L, 5 mg/L, or 2.5 mg/L. In some embodiments, the liquid phase concentration of isoprene in culture is between about any of 0.1 mg/L to 200 mg/L, 1 mg/L to 200 mg/L, 1 mg/L to 150 mg/L, 1 mg/L to 100 mg/L, 1 mg/L to 50 mg/L, 1 mg/L to 25 mg/L, 1 mg/L to 20 mg/L, or 10 mg/L to 20 mg/L.

[0623] Also provided herein are methods of producing a compound, wherein the compound has one or more characteristics selected from the group consisting of (a) a Henry's law coefficient of less than about 250 M/atm and (b) a solubility in water of less than about 100 g/L. In some embodiments, the method comprises: a) culturing cells under suitable conditions for production of the compound, wherein gas is added (such as the addition of gas to a system such as a fermentation system) at a gas sparging rate between about 0.01 vvm to about 2 vvm; and b) producing the compound. In some embodiments, the Henry's law coefficient of the compound is less than about any of 200 M/atm, 150 M/atm, 100 M/atm, 75 M/atm, 50 M/atm, 25 M/atm, 10 M/atm, 5 M/atm, or 1 M/atm. In some embodiments, the solubility in water of the compound is less than about any of 75 g/L, 50 g/L, 25 g/L, 10 g/L, 5 g/L, or 1g/L. In some embodiments, the compound is selected from a group consisting of isoprene, an aldehyde (*e.g.*, acetaldehyde), a ketone (*e.g.,* acetone or 2-butanone), an alcohol (*e.g.*, methanol, ethanol, 1-butanol, or C5 alcohols such as 3-methyl-3-buten-1-ol or 3-methyl-2-buten-1-ol), an ester of an alcohol (*e.g.*, ethyl acetate or acetyl esters of C5 alcohols), a hemiterpene, a monoterpene, a sesquiterpene, and C1 to C5 hydrocarbons (*e.g.*, methane, ethane, ethylene, or propylene). In some embodiments, the C1 to C5 hydrocarbons are saturated, unsaturated, or branched. In particular embodiments, the compound is isoprene. In some embodiments of the methods of producing any of the compounds described above, the gas sparging rate is between about any of 0.1 vvm to 1 vvm, 0.2 vvm to 1 vvm, or 0.5 vvm to 1 vvm.

[0624] In one aspect, the invention features cells in culture that produce isoprene. In some embodiments, the invention provides cells in culture that produce greater than about 400 nmole of isoprene/gram of cells for the wet weight of the cells/hour (nmole/g_{wcm}/hr) of isoprene. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source, polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are cultured under limited glucose conditions.

[0625] In some embodiments, the invention provides cells in culture that convert more than about 0.002% of the carbon in a cell culture medium into isoprene. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source, polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are cultured under limited glucose conditions.

[0626] In some embodiments, the invention provides cells in culture that comprise a heterologous nucleic acid encoding an isoprene synthase polypeptide. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source, polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are cultured under limited glucose conditions.

[0627] In one aspect, the invention features methods of producing isoprene, such as methods of using any of the cells described herein to produce isoprene. In some embodiments, the method involves culturing cells under conditions sufficient to produce greater than about 400 nmole/g_{wcm}/hr of isoprene. In some embodiments, the method also includes recovering isoprene produced by the cells. In some embodiments, the method includes purifying isoprene produced by the cells. In some embodiments, the method includes polymerizing the isoprene. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil, animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source, polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are cultured under limited glucose conditions. In various embodiments, the amount of isoprene produced (such as the total amount of isoprene produced or the amount of isoprene produced per liter of broth per hour per OD_{600}) during stationary phase is greater than or about 2 or more times the amount of isoprene produced during the growth phase for the same length of time. In some embodiments, the gas phase comprises greater than or about 9.5 % (volume) oxygen, and the concentration of isoprene in the gas phase is less than the lower flammability limit or greater than the upper flammability limit. In particular embodiments, (i) the concentration of isoprene in the gas phase is less than the lower flammability limit or greater than the upper flammability limit, and (ii) the cells produce greater than about 400 nmole/g_{wcm}/hr of isoprene.

[0628] In some embodiments, the method includes culturing cells under conditions sufficient to convert more than about 0.002% of the carbon (mol/mol) in a cell culture medium into isoprene. In some embodiments, the method also includes recovering isoprene produced by the cells. In some embodiments, the method includes purifying isoprene produced by the cells. In some embodiments, the method includes polymerizing the isoprene. In some embodiments, the cells have a heterologous nucleic acid that (i) encodes an isoprene synthase polypeptide and (ii) is operably linked to a promoter. In some embodiments, the cells are cultured in a culture medium that includes one or more carbon sources, such as, but not limited to, a carbohydrate, glycerol, glycerine, dihydroxyacetone, one-carbon source, oil,

animal fat, animal oil, fatty acid, lipid, phospholipid, glycerolipid, monoglyceride, diglyceride, triglyceride, renewable carbon source, polypeptide (*e.g.,* a microbial or plant protein or peptide), yeast extract, or a component from a yeast extract. In some embodiments, the cells are cultured under limited glucose conditions.

[0629] In some embodiments, isoprene is only produced in stationary phase. In some embodiments, isoprene is produced in both the growth phase and stationary phase. In various embodiments, the amount of isoprene produced (such as the total amount of isoprene produced or the amount of isoprene produced per liter of broth per hour per OD_{600}) during stationary phase is greater than or about 2, 3, 4, 5, 10, 20, 30, 40, 50, or more times the amount of isoprene produced during the growth phase for the same length of time.

[0630] In one aspect, the invention features compositions and systems that comprise isoprene. In some embodiments, the composition comprises greater than or about 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 mg of isoprene. In some embodiments, the composition comprises greater than or about 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 g of isoprene(w/w) of the volatile organic fraction of the composition is isoprene.

[0631] In some embodiments, the composition comprises greater than or about 99.90, 99.92, 99.94, 99.96, 99.98, or 100% isoprene by weight compared to the total weight of all C5 hydrocarbons in the composition. In some embodiments, the composition comprises less than or about 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% C5 hydrocarbons other than isoprene (such 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 3-methyl-l-butyne, *trans*-piperylene, *cis*-piperylene, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne) by weight compared to the total weight of all C5 hydrocarbons in the composition. In some embodiments, the composition has less than or about 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% for 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 3-methyl-l-butyne, *trans*-piperylene, *cis*-piperylene, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne by weight compared to the total weight of all C5 hydrocarbons in the composition. In particular embodiments, the composition has greater than about 2 mg of isoprene and has greater than or about 99.90, 99.92, 99.94, 99.96, 99.98,

or 100% isoprene by weight compared to the total weight of all C5 hydrocarbons in the composition.

[0632] In some embodiments, the composition has less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ug/L of a compound that inhibits the polymerization of isoprene for any compound in the composition that inhibits the polymerization of isoprene. In particular embodiments, the composition also has greater than about 2 mg of isoprene.

[0633] In some embodiments, the composition has one or more compounds selected from the group consisting of ethanol, acetone, C5 prenyl alcohols, and isoprenoid compounds with 10 or more carbon atoms. In some embodiments, the composition has greater than or about 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 60, 80, 100, or 120 ug/L of ethanol, acetone, a C5 prenyl alcohol (such as 3-methyl-3-buten-1-ol or 3-methyl-2-buten-1-ol), or any two or more of the foregoing. In particular embodiments, the composition has greater than about 2 mg of isoprene and has one or more compounds selected from the group consisting of ethanol, acetone, C5 prenyl alcohols, and isoprenoid compounds with 10 or more carbon atoms.

[0634] In some embodiments, the composition includes isoprene and one or more second compounds selected from the group consisting of 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, acetaldehyde, methanethiol, methyl acetate, 1-propanol, diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene, and 2,3-cycloheptenolpyridine. In various embodiments, the amount of one of these second components relative to the amount of isoprene in units of percentage by weight (*i.e.*, weight of the component divided by the weight of isoprene times 100) is at greater than or about 0.01, 0.02, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, or 110% (w/w).

[0635] In some embodiments, the composition comprises (i) a gas phase that comprises isoprene and (ii) cells in culture that produce greater than about 400 nmole/g_{wcm}/hr of isoprene. In some embodiments, the composition comprises a closed system, and the gas phase comprises greater than or about 5. 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 ug/L of isoprene when normalized to 1 mL of 1 OD_{600} cultured for 1 hour. In some embodiments, the

composition comprises an open system, and the gas phase comprises greater than or about 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 ug/L of isoprene when sparged at a rate of 1 vvm. In some embodiments, the volatile organic fraction of the gas phase comprises greater than or about 99.90, 99.92, 99.94, 99.96, 99.98, or 100% isoprene by weight compared to the total weight of all C5 hydrocarbons in the volatile organic fraction. In some embodiments, the volatile organic fraction of the gas phase comprises less than or about 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% C5 hydrocarbons other than isoprene (such 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 3-methyl-l-butyne, *trans*-piperylene, *cis*-piperylene, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne) by weight compared to the total weight of all C5 hydrocarbons in the volatile organic fraction. In some embodiments, the volatile organic fraction of the gas phase has less than or about 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, or 0.00001% for 1,3-cyclopentadiene, *cis*-1,3-pentadiene, *trans*-1,3-pentadiene, 1-pentyne, 2-pentyne, 1-pentene, 2-methyl-1-butene, 3-methyl-l-butyne, *trans*-piperylene, *cis*-piperylene, pent-4-ene-1-yne, *trans*-pent-3-ene-1-yne, or *cis*-pent-3-ene-1-yne by weight compared to the total weight of all C5 hydrocarbons in the volatile organic fraction. In particular embodiments, the volatile organic fraction of the gas phase has greater than about 2 mg of isoprene and has greater than or about 99.90, 99.92, 99.94, 99.96, 99.98, or 100% isoprene by weight compared to the total weight of all C5 hydrocarbons in the volatile organic fraction.

[0636] In some embodiments, the volatile organic fraction of the gas phase has less than or about 50, 40, 30, 20, 10, 5, 1, 0.5, 0.1, 0.05, 0.01, or 0.005 ug/L of a compound that inhibits the polymerization of isoprene for any compound in the volatile organic fraction of the gas phase that inhibits the polymerization of isoprene. In particular embodiments, the volatile organic fraction of the gas phase also has greater than about 2 mg of isoprene.

[0637] In some embodiments, the volatile organic fraction of the gas phase has one or more compounds selected from the group consisting of ethanol, acetone, C5 prenyl alcohols, and isoprenoid compounds with 10 or more carbon atoms. In some embodiments, the volatile organic fraction of the gas phase has greater than or about 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 60, 80, 100, or 120 ug/L of ethanol, acetone, a C5 prenyl alcohol (such as 3-methyl-3-buten-1-ol or 3-methyl-2-buten-1-ol), or any two or more of the foregoing. In particular embodiments, the volatile organic fraction of the gas phase has greater than about 2

mg of isoprene and has one or more compounds selected from the group consisting of ethanol, acetone, C5 prenyl alcohols, and isoprenoid compounds with 10 or more carbon atoms.

[0638] In some embodiments, the volatile organic fraction of the gas phase has includes isoprene and one or more second compounds selected from the group consisting of 2-heptanone, 6-methyl-5-hepten-2-one, 2,4,5-trimethylpyridine, 2,3,5-trimethylpyrazine, citronellal, acetaldehyde, methanethiol, methyl acetate, 1-propanol, diacetyl, 2-butanone, 2-methyl-3-buten-2-ol, ethyl acetate, 2-methyl-1-propanol, 3-methyl-1-butanal, 3-methyl-2-butanone, 1-butanol, 2-pentanone, 3-methyl-1-butanol, ethyl isobutyrate, 3-methyl-2-butenal, butyl acetate, 3-methylbutyl acetate, 3-methyl-3-buten-1-yl acetate, 3-methyl-2-buten-1-yl acetate, (E)-3,7-dimethyl-1,3,6-octatriene, (Z)-3,7-dimethyl-1,3,6-octatriene, and 2,3-cycloheptenolpyridine. In various embodiments, the amount of one of these second components relative to amount of isoprene in units of percentage by weight (*i.e.*, weight of the component divided by the weight of isoprene times 100) is at greater than or about 0.01, 0.02, 0.05, 0.1, 0.5, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, or 110% (w/w) in the volatile organic fraction of the gas phase.

[0639] In some embodiments of any of the compositions of the invention, at least a portion of the isoprene is in a gas phase. In some embodiments, at least a portion of the isoprene is in a liquid phase (such as a condensate). In some embodiments, at least a portion of the isoprene is in a solid phase. In some embodiments, at least a portion of the isoprene is adsorbed to a solid support, such as a support that includes silica and/or activated carbon. In some embodiments, the composition includes ethanol. In some embodiments, the composition includes between about 75 to about 90% by weight of ethanol, such as between about 75 to about 80%, about 80 to about 85%, or about 85 to about 90% by weight of ethanol. In some embodiments, the composition includes between about 4 to about 15% by weight of isoprene, such as between about 4 to about 8%, about 8 to about 12%, or about 12 to about 15% by weight of isoprene.

[0640] In some embodiments, the invention also features systems that include any of the cells and/or compositions described herein. In some embodiments, the system includes a reactor that chamber comprises cells in culture that produce greater than about 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, or more nmole/g_{wcm}/hr isoprene. In some embodiments, the system is not a closed system. In some embodiments, at least a portion of the isoprene is removed from the system. In some

embodiments, the system includes a gas phase comprising isoprene. In various embodiments, the gas phase comprises any of the compositions described herein.

[0641] In one aspect, the invention provides a tire comprising polyisoprene. In some embodiments, the polyisoprene is produced by (i) polymerizing isoprene in any of the compositions described herein or (ii) polymerizing isoprene recovered from any of the compositions described herein. In some embodiments, the polyisoprene comprises *cis*-1,4-polyisoprene.

[0642] In some embodiments of any of the compositions, systems, and methods of the invention, a nonflammable concentration of isoprene in the gas phase is produced. In some embodiments, the gas phase comprises less than about 9.5 % (volume) oxygen. In some embodiments, the gas phase comprises greater than or about 9.5 % (volume) oxygen, and the concentration of isoprene in the gas phase is less than the lower flammability limit or greater than the upper flammability limit. In some embodiments, the portion of the gas phase other than isoprene comprises between about 0% to about 100% (volume) oxygen, such as between about 10% to about 100% (volume) oxygen. In some embodiments, the portion of the gas phase other than isoprene comprises between about 0% to about 99% (volume) nitrogen. In some embodiments, the portion of the gas phase other than isoprene comprises between about 1% to about 50% (volume) CO_2.

[0643] In some embodiments of any of the aspects of the invention, the cells in culture produce isoprene at greater than or about 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, or more nmole/g_{wcm}/hr isoprene. In some embodiments of any of the aspects of the invention, the cells in culture convert greater than or about 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.12, 0.14, 0.16, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.4, 1.6%, or more of the carbon in the cell culture medium into isoprene. In some embodiments of any of the aspects of the invention, the cells in culture produce isoprene at greater than or about 1, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, 10,000, 100,000, or more ng of isoprene/gram of cells for the wet weight of the cells /hr (ng/g_{wcm}/h). In some embodiments of any of the aspects of the invention, the cells in culture produce a cumulative titer (total amount) of isoprene at greater than or about 1, 10, 25, 50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1,000, 1,250, 1,500, 1,750, 2,000, 2,500, 3,000, 4,000, 5,000, 10,000, 50,000, 100,000, or more mg of isoprene/L of broth (mg/L_{broth}, wherein the volume

of broth includes the volume of the cells and the cell medium). Other exemplary rates of isoprene production and total amounts of isoprene production are disclosed herein.

[0644] In some embodiments of any of the aspects of the invention, the cells further comprise a heterologous nucleic acid encoding an IDI polypeptide. In some embodiments of any of the aspects of the invention, the cells further comprise an insertion of a copy of an endogenous nucleic acid encoding an IDI polypeptide. In some embodiments of any of the aspects of the invention, the cells further comprise a heterologous nucleic acid encoding a DXS polypeptide. In some embodiments of any of the aspects of the invention, the cells further comprise an insertion of a copy of an endogenous nucleic acid encoding a DXS polypeptide. In some embodiments of any of the aspects of the invention, the cells further comprise one or more nucleic acids encoding an IDI polypeptide and a DXS polypeptide. In some embodiments of any of the aspects of the invention, one nucleic acid encodes the isoprene synthase polypeptide, IDI polypeptide, and DXS polypeptide. In some embodiments of any of the aspects of the invention, one vector encodes the isoprene synthase polypeptide, IDI polypeptide, and DXS polypeptide. In some embodiments, the vector comprises a selective marker, such as an antibiotic resistance nucleic acid.

[0645] In some embodiments of any of the aspects of the invention, the heterologous isoprene synthase nucleic acid is operably linked to a T7 promoter, such as a T7 promoter contained in a medium or high copy plasmid. In some embodiments of any of the aspects of the invention, the heterologous isoprene synthase nucleic acid is operably linked to a Trc promoter, such as a Trc promoter contained in a medium or high copy plasmid. In some embodiments of any of the aspects of the invention, the heterologous isoprene synthase nucleic acid is operably linked to a Lac promoter, such as a Lac promoter contained in a low copy plasmid. In some embodiments of any of the aspects of the invention, the heterologous isoprene synthase nucleic acid is operably linked to an endogenous promoter, such as an endogenous alkaline serine protease promoter. In some embodiments, the heterologous isoprene synthase nucleic acid integrates into a chromosome of the cells without a selective marker.

[0646] In some embodiments, one or more MVA pathway, IDI, DXP, or isoprene synthase nucleic acids are placed under the control of a promoter or factor that is more active in stationary phase than in the growth phase. For example, one or more MVA pathway, IDI, DXP, or isoprene synthase nucleic acids may be placed under control of a stationary phase

sigma factor, such as RpoS. In some embodiments, one or more MVA pathway, IDI, DXP, or isoprene synthase nucleic acids are placed under control of a promoter inducible in stationary phase, such as a promoter inducible by a response regulator active in stationary phase.

[0647] In some embodiments of any of the aspects of the invention, at least a portion of the cells maintain the heterologous isoprene synthase nucleic acid for at least or about 5, 10, 20, 40, 50, 60, 65, or more cell divisions in a continuous culture (such as a continuous culture without dilution). In some embodiments of any of the aspects of the invention, the nucleic acid comprising the isoprene synthase, IDI, or DXS nucleic acid also comprises a selective marker, such as an antibiotic resistance nucleic acid.

[0648] In some embodiments of any of the aspects of the invention, the cells further comprise a heterologous nucleic acid encoding an MVA pathway polypeptide (such as an MVA pathway polypeptide from *Saccharomyces cerevisia* or *Enterococcus faecalis*). In some embodiments of any of the aspects of the invention, the cells further comprise an insertion of a copy of an endogenous nucleic acid encoding an MVA pathway polypeptide (such as an MVA pathway polypeptide from *Saccharomyces cerevisia* or *Enterococcus faecalis*). In some embodiments of any of the aspects of the invention, the cells comprise an isoprene synthase, DXS, and MVA pathway nucleic acid. In some embodiments of any of the aspects of the invention, the cells comprise an isoprene synthase nucleic acid, a DXS nucleic acid, an IDI nucleic acid, and a MVA pathway nucleic (in addition to the IDI nucleic acid).

[0649] In some embodiments of any of the aspects of the invention, the isoprene synthase polypeptide is a polypeptide from a plant such as *Pueraria* (*e.g., Pueraria montana or Pueraria lobata*) or *Populus* (*e.g.*, *Populus tremuloides*, *Populus alba*, *Populus nigra*, *Populus trichocarpa*, or the hybrid, *Populus alba x Populus tremula*).

[0650] In some embodiments of any of the aspects of the invention, the cells are bacterial cells, such as gram-positive bacterial cells (*e.g.*, *Bacillus* cells such as *Bacillus subtilis* cells or *Streptomyces* cells such as *Streptomyces lividans*, *Streptomyces coelicolor*, or *Streptomyces griseus* cells). In some embodiments of any of the aspects of the invention, the cells are gram-negative bacterial cells (*e.g.*, *Escherichia* cells such as *Escherichia coli* cells, *Rhodopseudomonas sp.* such as *Rhodopseudomonas palustris* cells, *Pseudomonas sp.* such as *Pseudomonas fluorescens* cells or *Pseudomonas putida* cells, or *Pantoea* cells such as *Pantoea citrea* cells). In some embodiments of any of the aspects of the invention, the cells

are fungal, cells such as filamentous fungal cells (*e.g.*, *Trichoderma* cells such as *Trichoderma reesei* cells or *Aspergillus* cells such as *Aspergillus oryzae* and *Aspergillus niger*) or yeast cells (*e.g.*, *Yarrowia* cells such as *Yarrowia lipolytica* cells or *Sacchraomyces* cells such as *Saccaromyces cerevisiae*).

[0651] In some embodiments of any of the aspects of the invention, the microbial polypeptide carbon source includes one or more polypeptides from yeast or bacteria. In some embodiments of any of the aspects of the invention, the plant polypeptide carbon source includes one or more polypeptides from soy, corn, canola, jatropha, palm, peanut, sunflower, coconut, mustard, rapeseed, cottonseed, palm kernel, olive, safflower, sesame, or linseed.

[0652] In one aspect, the invention features a product produced by any of the compositions or methods of the invention.

EXAMPLES

[0653] The examples, which are intended to be purely exemplary of the invention and should therefore not be considered to limit the invention in any way, also describe and detail aspects and embodiments of the invention discussed above. Unless indicated otherwise, temperature is in degrees Centigrade and pressure is at or near atmospheric. The foregoing examples and detailed description are offered by way of illustration and not by way of limitation.

[0654] All publications, patent applications, and patents cited in this specification are herein incorporated by reference as if each individual publication, patent application, or patent were specifically and individually indicated to be incorporated by reference. In particular, all publications cited herein are expressly incorporated herein by reference for the purpose of describing and disclosing compositions and methodologies which might be used in connection with the invention. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

Example 1: Production of isoprene in *E. coli* expressing recombinant kudzu isoprene synthase

I. Construction of vectors for expression of the kudzu isoprene synthase in *E. coli*.

[0655] The protein sequence for the kudzu (*Pueraria montana*) isoprene synthase gene (IspS) was obtained from GenBank (AAQ84170). A kudzu isoprene synthase gene, optimized for *E. coli* codon usage, was purchased from DNA2.0 (SEQ ID NO:1). The isoprene synthase gene was removed from the supplied plasmid by restriction endonuclease digestion with *Bsp*LU11I /*Pst*I, gel-purified, and ligated into pTrcHis2B (Invitrogen) that had been digested with *Nco*I/*Pst*I. The construct was designed such that the stop codon in the isoprene synthase gene 5' to the *Pst*I site. As a result, when the construct was expressed the His-Tag is not attached to the isoprene synthase protein. The resulting plasmid, pTrcKudzu, was verified by sequencing (Figures 2 and 3; SEQ ID NO:2).

[0656] The isoprene synthase gene was also cloned into pET16b (Novagen). In this case, the isoprene synthase gene was inserted into pET16b such that the recombinant isoprene synthase protein contained the N-terminal His tag. The isoprene synthase gene was amplified from pTrcKudzu by PCR using the primer set pET-His-Kudzu-2F: 5'-CGTGAGATCATATGTGTGCGACCTCTTCTCAATTTAC (SEQ ID NO:49) and pET-His-Kudzu-R: 5'-CGGTCGACGGATCCCTGCAGTTAGACATACATCAGCTG (SEQ ID NO:50). These primers added an *Nde*I site at the 5'-end and a *Bam*H1 site at the 3' end of the gene respectively. The plasmid pTrcKudzu, described above, was used as template DNA, Herculase polymerase (Stratagene) was used according to manufacture's directions, and primers were added at a concentration of 10 pMols. The PCR was carried out in a total volume of 25 µl. The PCR product was digested with *Nde*I/*Bam*H1 and cloned into pET16b digested with the same enzymes. The ligation mix was transformed into *E. coli* Top10 (Invitrogen) and the correct clone selected by sequencing. The resulting plasmid, in which the kudzu isoprene synthase gene was expressed from the T7 promoter, was designated pETNHisKudzu (Figures 4 and 5; SEQ ID NO:3).

[0657] The kudzu isoprene synthase gene was also cloned into the low copy number plasmid pCL1920. Primers were used to amplify the kudzu isoprene synthase gene from pTrcKudzu described above. The forward primer added a *Hin*dIII site and an *E. coli* consensus RBS to the 5' end. The *Pst*I cloning site was already present in pTrcKudzu just 3' of the stop codon so the reverse primer was constructed such that the final PCR product includes the *Pst*I site. The sequences of the primers were: HindIII-rbs-Kudzu F: 5'-

CATATGAAAGCTTGTATCGATTAAATAAGGAGGAATAAACC (SEQ ID NO:51) and BamH1-Kudzu R:

[0658] 5'- CGGTCGACGGATCCCTGCAGTTAGACATACATCAGCTG (SEQ ID NO:50). The PCR product was amplified using Herculase polymerase with primers at a concentration of 10 pmol and with 1 ng of template DNA (pTrcKudzu). The amplification protocol included 30 cycles of (95° C for 1 minute, 60° C for 1 minute, 72° C for 2 minutes). The product was digested with *Hin*dIII and *Pst*I and ligated into pCL1920 which had also been digested with *Hin*dIII and *Pst*I. The ligation mix was transformed into *E. coli* Top10. Several transformants were checked by sequencing. The resulting plasmid was designated pCL-lac-Kudzu (Figures 6 and 7; SEQ ID NO:4).

II. Determination of isoprene production.

[0659] For the shake flask cultures, one ml of a culture was transferred from shake flasks to 20 ml CTC headspace vials (Agilent vial cat# 5188 2753; cap cat# 5188 2759). The cap was screwed on tightly and the vials incubated at the equivalent temperature with shaking at 250 rpm. After 30 minutes the vials were removed from the incubator and analyzed as described below (*see* Table 1 for some experimental values from this assay).

[0660] In cases where isoprene production in fermentors was determined, samples were taken from the off-gas of the fermentor and analyzed directly as described below (*see* Table 2 for some experimental values from this assay).

[0661] The analysis was performed using an Agilent 6890 GC/MS system interfaced with a CTC Analytics (Switzerland) CombiPAL autosampler operating in headspace mode. An Agilent HP-5MS GC/MS column (30 m x 0.25 mm; 0.25 μm film thickness) was used for separation of analytes. The sampler was set up to inject 500 μL of headspace gas. The GC/MS method utilized helium as the carrier gas at a flow of 1 ml/min. The injection port was held at 250ºC with a split ratio of 50:1. The oven temperature was held at 37ºC for the 2 minute duration of the analysis. The Agilent 5793N mass selective detector was run in single ion monitoring (SIM) mode on m/z 67. The detector was switched off from 1.4 to 1.7 minutes to allow the elution of permanent gases. Under these conditions isoprene (2-methyl-1,3-butadiene) was observed to elute at 1.78 minutes. A calibration table was used to quantify the

absolute amount of isoprene and was found to be linear from 1 μg/L to 2000 μg/L. The limit of detection was estimated to be 50 to 100 ng/L using this method.

III. Production of isoprene in shake flasks containing *E. coli* cells expressing recombinant isoprene synthase.

[0662] The vectors described above were introduced to *E. coli* strain BL21 (Novagen) to produce strains BL21/ptrcKudzu, BL21/pCL-lac-Kudzu and BL21/pETHisKudzu. The strains were spread for isolation onto LA (Luria agar) + carbenicillin (50 μg/ml) and incubated overnight at 37ºC. Single colonies were inoculated into 250 ml baffled shake flasks containing 20 ml Luria Bertani broth (LB) and carbenicillin (100 μg/ml). Cultures were grown overnight at 20ºC with shaking at 200 rpm. The OD_{600} of the overnight cultures were measured and the cultures were diluted into a 250 ml baffled shake flask containing 30 ml MagicMedia (Invitrogen) + carbenicillin (100 μg/ml) to an OD_{600} ~ 0.05. The culture was incubated at 30ºC with shaking at 200 rpm. When the OD_{600} ~ 0.5 - 0.8, 400 μM IPTG was added and the cells were incubated for a further 6 hours at 30ºC with shaking at 200 rpm. At 0, 2, 4 and 6 hours after induction with IPTG, 1 ml aliquots of the cultures were collected, the OD_{600} was determined and the amount of isoprene produced was measured as described above. Results are shown in Figure 8.

IV. Production of Isoprene from BL21/ptrcKudzu in 14 liter fermentation.

[0663] Large scale production of isoprene from *E. coli* containing the recombinant kudzu isoprene synthase gene was determined from a fed-batch culture. The recipe for the fermentation media (TM2) per liter of fermentation medium was as follows: K_2HPO_4 13.6 g, KH_2PO_4 13.6 g, MgSO4 * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, $(NH_4)_2SO_4$ 3.2 g, yeast extract 5 g, 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in diH_2O. The pH was adjusted to 6.8 with potassium hydroxide (KOH) and q.s. to volume. The final product was filter sterilized with 0.22 μ filter (only, do not autoclave). The recipe for 1000X Modified Trace Metal Solution was as follows: Citric Acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, $NaMoO_4$ * $2H_2O$ 100 mg. Each component was dissolved one at a time in diH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 μ filter.

[0664] This experiment was carried out in 14 L bioreactor to monitor isoprene formation from glucose at the desired fermentation, pH 6.7 and temperature 34ºC. An inoculum of *E. coli* strain BL21/ptrcKudzu taken from a frozen vial was prepared in soytone-yeast extract-glucose medium. After the inoculum grew to OD_{550} = 0.6, two 600 ml flasks were centrifuged and the contents resuspended in 70 ml supernatant to transfer the cell pellet (70 ml of OD 3.1 material) to the bioreactor. At various times after inoculation, samples were removed and the amount of isoprene produced was determined as described above. Results are shown in Figure 9.

Example 2: Production of isoprene in *E. coli* expressing recombinant poplar isoprene synthase

[0665] The protein sequence for the poplar (*Populus alba* x *Populus tremula*) isoprene synthase (Schnitzler, J-P, *et al.* (2005) *Planta* 222:777-786) was obtained from GenBank (CAC35696). A gene, codon optimized for *E. coli*, was purchased from DNA2.0 (p9796-poplar, Figures 30 and 31; SEQ ID NO:14). The isoprene synthase gene was removed from the supplied plasmid by restriction endonuclease digestion with *Bsp*LU11I /*Pst*I, gel-purified, and ligated into pTrcHis2B that had been digested with *Nco*I/*Pst*I. The construct is cloned such that the stop codon in the insert is before the *Pst*I site, which results in a construct in which the His-Tag is not attached to the isoprene synthase protein. The resulting plasmid pTrcPoplar (Figures 32 and 33; SEQ ID NO:15), was verified by sequencing.

Example 2B: Demonstration of isoprene synthase activity from several *populus* isoprene synthases

[0666] The following isoprene synthases were examined; *Populus alba* (Accession number BAD98243; Figures 137A and B; SEQ ID NO:30), *Populus nigra* (Accession number CAL69918; Figures 137C and D; SEQ ID NO:31), *Populus tremuloides* (Accession number AAQ16588; Figure 137 E, F, and G; SEQ ID NOs:32-33), *Populus trichocarpa* (Accession number ACD70404; Figures 137H and I; SEQ ID NO:34), *Populus alba x Populus tremula* (Accession number CAJ29303; Figures 137J and K; SEQ ID NO:35), and MCM112-Kudzu.

[0667] pET24Kudzu (also referred to as MCM112) was constructed as follows: the kudzu isoprene synthase gene was subcloned into the pET24d vector (Novagen) from the pCR2.1 vector (Invitrogen). The kudzu IspS gene was amplified from pTrcKudzu template DNA

using primers MCM50 5'-GATCATGCAT TCGCCCTTAG GAGGTAAAAAAACATGTGTGCGACCTCTTC TCAATTTACT (SEQ ID NO:52); and MCM53 5'-CGGTCGACGGATCCCTGCAG TTAGACATAC ATCAGCTG (SEQ ID NO:50). PCR reactions were carried out using Taq DNA Polymerase (Invitrogen), and the resulting PCR product was cloned into pCR2.1-TOPO TA cloning vector (Invitrogen), and transformed into *E. coli* Top10 chemically competent cells (Invitrogen). Transformants were plated on L-agar containing carbenicillin (50 μg/ml) and incubated overnight at 37°C. Five ml Luria Broth cultures containing carbenicillin 50 μg/ml were inoculated with single transformants and grown overnight at 37°C. Five colonies were screened for the correct insert by sequencing of plasmid DNA isolated from 1 ml of liquid culture (Luria Broth) and purified using the QIAprep Spin Mini-prep Kit (Qiagen). The resulting plasmid, designated MCM93, contains the kudzu IspS coding sequence in a pCR2.1 backbone (Figure 137L). The sequence of MCM93 (SEQ ID NO:36) is shown in Figures 137M and N.

[0668] The kudzu coding sequence was removed by restriction endonuclease digestion with *Pci*I and *Bam*H1 (Roche) and gel purified using the QIAquick Gel Extraction kit (Qiagen). The pET24d vector DNA was digested with *Nco*I and *Bam*HI (Roche), treated with shrimp alkaline phosphatase (Roche), and purified using the QIAprep Spin Mini-prep Kit (Qiagen). The kudzu IspS fragment was ligated to the *Nco*I/*Bam*H1 digested pET24d using the Rapid DNA Ligation Kit (Roche) at a 5:1 fragment to vector ratio in a total volume of 20 μl. A portion of the ligation mixture (5 μl) was transformed into *E. coli* Top 10 chemically competent cells and plated on L agar containing kanamycin (50 μg/ml). The correct transformant was confirmed by sequencing and transformed into chemically competent BL21(λDE3)pLysS cells (Novagen). A single colony was selected after overnight growth at 37°C on L agar containing kanamycin (50 μg/ml). A map of the resulting plasmid designated as pET24D-Kudzu is shown in Figure 137O. The sequence of pET24D-Kudzu (SEQ ID NO:37) is shown in Figures 137P and Q.

[0669] *Escherichia coli* optimized isoprene synthase genes cloned into the pET24a expression vector (Novagen) were purchased from DNA2.0 (Menlo Park, CA) for *Populus tremuloides*, *Populus alba*, *Populus nigra* and *Populus trichocarpa*. Genes were synthesized with the chloroplast transit peptide sequence removed, resulting in expression of mature proteins.

[0670] The construct for the Kudzu isoprene synthase was used as control in this example. The plasmids were transformed into the *E. coli* expression host BL21(DE3)plysS and transformants were grown in 0.6 ml TM3 medium. The recipe for TM3 medium is as follows: K_2HPO_4 (13.6 g/l) KH_2PO_4 (13.6 g/l), $MgSO_4$ * $7H_2O$ (2 g/L) Citric Acid Monohydrate (2 g/L) Ferric Ammonium Citrate (0.3 g/L) $(NH_4)_2SO_4$ (3.2 g/L) yeast extract (0.2 g/L) 1 ml of 1000x Trace Elements solution, pH adjusted to 6.8 with ammonium hydroxide qs to volume with sterile DIH_2O and filter sterilized with a 0.22 micron filter. The recipe for 1000X Trace Elements solution is as follows: Citric Acids * H_2O (40 g/L), $MnSO_4$ * H_2O (30 g/L), NaCl (10 g/L), $FeSO_4$ * 7 H_2O (1 g/L), $CoCl_2$ * 6 H_2O (1 g/L), $ZnSO_4$ * 7 H_2O (1 g/L), $CuSO_4$ * 5 H_2O (100 mg/L), H_3BO_3 (100 mg/L), $NaMoO_4$ * 2 H_2O (100 mg/L). Each component was dissolved one at a time in DIH_2O, pH adjusted to 3.0 with HCl/NaOH, qs to volume and filter sterilized with a 0.22 micron filter.

[0671] The cultures were induced with 400 uM IPTG and growth was continued to OD_{600} of about 5. Aliquots of culture were transferred to a deep well glass plate and wells were sealed with aluminum plate sealer. The plate was incubated at 25°C for 30 minutes with shaking at 450 rpm. The reactions were heat inactivated by raising the temperature to 70°C for 5 minutes. Whole cell head space was measured by the GCMS method as described in Example 1, Part II.

[0672] K_m values were obtained from cultures grown in similar manner but cells were harvested and lysed by a freeze/thaw lysozyme protocol. A volume of 400 µL of culture was transferred into a new 96-well plate (Perkin Elmer, Catalog No. 6008290) and cells were harvested by centrifugation in a Beckman Coulter Allegra 6R centrifuge at 2500 x g. The pellet was resuspended in 200 mL of hypotonic buffer (5 mM $MgCL_2$, 5 mM Tris HCl, 5 mM DTT pH 8.0) and the plate was frozen at -80°C for a minimum time of 60 minutes. Cell lysate was prepared by thawing the plate and adding 32 mL of isoprene synthase DMAPP assay buffer (57 mM Tris HCl, 19 mM $MgCl_2$, 74 mg/mL DNase I (Sigma Catalog No. DN-25), $2.63x10^5$ U/mL of ReadyLyse lysozyme solution (Epicentre Catalog No. R1802M), and 5 mg/mL of molecular biology grade BSA. The plate was incubated with shaking at 25°C for 30 minutes and then placed on ice. DMAPP and lysate were added at desired concentration in a sealed deep well glass block for the whole cell head space assay described above. The reactions were allowed to proceed for 1 hour and then terminated by the heat step described above and head space activity was measured also as described.

[0673] In an alternate approach, the activity of the enzymes was measured from cells cultured in 25 mL volume and induced similarly as described above. Cells were harvested by centrifugation and the pellets were lysed by French pressing in buffer consisting of 50% glycerol mixed 1:1with 20 mM Tris/HCl pH 7.4, 20 mM $MgCl_2$, 200 mM KCl, 1 mM DTT. A lysate volume of 25 uL was assayed for isoprene synthase activity in 2 mL screw cap vials containing 75 uL of assay buffer (66.6 mM Tris/HCl pH 8, 6.66 mM DMAPP, 43 mM, $MgCl_2$). The reaction was incubated for 15 minutes at 30°C and was quenched by the addition of 100 uL of 250 mM EDTA through the septum of the vial. Isoprene was measured by GC/MS as described in Example 1, Part II.

[0674] All methods for the determination of activity showed that the poplar enzyme derived from the pure bred poplars were several-fold higher than the *Populus* [*alba x tremula*]. Figures 138 and 139 showed these results for the whole cell head space assay and the DMAPP assay, respectively, and surprisingly indicate that enzymes from *P. nigra, P. tremuloides, P. trichocarpa*, and *P. alba* all had significantly higher activity than hybrid [*P. alba x P.tremula*].

[0675] The DMAPP assay was performed as follows: a volume of 400 µL of culture was transferred into a new 96-well plate (Perkin Elmer, Catalog No. 6008290) and cells were harvested by centrifugation in a Beckman Coulter Allegra 6R centrifuge at 2500 x g. The pellet was resuspended in 200 mL of hypotonic buffer (5 mM $MgCL_2$, 5 mM Tris HCl, 5 mM DTT pH 8.0) and the plate was frozen at -80°C for a minimum time of 60 minutes. Cell lysate was prepared by thawing the plate and adding 32 mL of isoprene synthase DMAPP assay buffer (57 mM Tris HCl, 19 mM $MgCl_2$, 74 mg/mL DNase I (Sigma Catalog No. DN-25), $2.63x10^5$ U/mL of ReadyLyse lysozyme solution (Epicentre Catalog No. R1802M), and 5 mg/mL of molecular biology grade BSA. The plate was incubated with shaking at 25°C for 30 minutes and then placed on ice. For isoprene production an 80 mL aliquot of lysate was transferred to a 96-deep well glass plate (Zinsser Catalog No. 3600600) and 20 mL of a 10 mM DMAPP solution in 100 mM K_2HPO_4, pH 8.2 (Cayman Chemical Catalog No. 63180) was added. The plate was sealed with an aluminum plate seal (Beckman Coultor Catalog No. 538619) and incubated with shaking at 30°C for 60 minutes. The enzymatic reactions were terminated by heating the glass block (70°C for 5 minutes). The cell head space of each well was quantitatively analyzed as described in Example 1, Part II.

[0676] Notably, *P. alba, P. tremuloides, P. trichocarpa* had higher activity than the isoprene synthase from Kudzu. The enzyme from *P. alba* was expressed with the greatest activity of all enzymes tested. The higher activities observed with the cell lysate compared to the whole cell head space assay was likely due to limitations in DMAPP, the substrate for these enzymes, delivered by the endogenous deoxyxylulose 5-phosphate (DXP) pathway of the cell.

[0677] K_m kinetic parameter was measured to be about 2 to 3 mM for all enzymes for which the value was determined.

Example 3: Production of isoprene in *Panteoa citrea* expressing recombinant kudzu isoprene synthase

[0678] The pTrcKudzu and pCL-lac Kudzu plasmids described in Example 1 were electroporated into *P. citrea* (U.S. Pat. No. 7,241,587). Transformants were selected on LA containing carbenicillin (200 μg/ml) or spectinomycin (50 μg/ml) respectively. Production of isoprene from shake flasks and determination of the amount of isoprene produced was performed as described in Example 1 for *E. coli* strains expressing recombinant kudzu isoprene synthase. Results are shown in Figure 10.

Example 4: Production of isoprene in *Bacillus subtilis* expressing recombinant kudzu isoprene synthase

I. Construction of a *B. subtilis* replicating plasmid for the expression of kudzu isoprene synthase.

[0679] The kudzu isoprene synthase gene was expressed in *Bacillus subtilis aprEnprE Pxyl-comK* strain (BG3594comK) using a replicating plasmid (pBS19 with a chloramphenicol resistance cassette) under control of the *aprE* promoter. The isoprene synthase gene, the *aprE* promoter and the transcription terminator were amplified separately and fused using PCR. The construct was then cloned into pBS19 and transformed into *B. subtilis*.

a) Amplification of the *aprE* promoter

[0680] The *aprE* promoter was amplified from chromosomal DNA from *Bacillus subtilis* using the following primers:

CF 797 (+) Start *aprE* promoter MfeI
5'- GACATCAATTGCTCCATTTTCTTCTGCTATC (SEQ ID NO:53)

CF 07-43 (-) Fuse *aprE* promoter to Kudzu ispS
5'- ATTGAGAAGAGGTCGCACACACTCTTTACCCTCTCCTTTTA (SEQ ID NO:54)

b) Amplification of the isoprene synthase gene

[0681] The kudzu isoprene synthase gene was amplified from plasmid pTrcKudzu (SEQ ID NO:2). The gene had been codon optimized for *E. coli* and synthesized by DNA 2.0. The following primers were used:

CF 07-42 (+) Fuse the aprE promoter to kudzu isoprene synthase gene (GTG start codon)
5'- TAAAAGGAGAGGGTAAAGAGTGTGTGCGACCTCTTCTCAAT (SEQ ID NO:55)

CF 07-45 (-) Fuse the 3' end of kudzu isoprene synthase gene to the terminator
5'- CCAAGGCCGGTTTTTTTTAGACATACATCAGCTGGTTAATC (SEQ ID NO:56)

c) Amplification of the transcription terminator

[0682] The terminator from the alkaline serine protease of *Bacillus amyliquefaciens* was amplified from a previously sequenced plasmid pJHPms382 using the following primers:

CF 07-44 (+) Fuse the 3' end of kudzu isoprene synthase to the terminator
5'- GATTAACCAGCTGATGTATGTCTAAAAAAAACCGGCCTTGG (SEQ ID NO:57)

CF 07-46 (-) End of *B. amyliquefaciens* terminator (BamHI)
5'- GACATGACGGATCCGATTACGAATGCCGTCTC (SEQ ID NO:58)

[0683] The kudzu fragment was fused to the terminator fragment using PCR with the following primers:

CF 07-42 (+) Fuse the *aprE* promoter to kudzu isoprene synthase gene (GTG start codon)
5'- TAAAAGGAGAGGGTAAAGAGTGTGTGCGACCTCTTCTCAAT (SEQ ID NO:55)

CF 07-46 (-) End of *B. amyliquefaciens* terminator (BamHI)
5'- GACATGACGGATCCGATTACGAATGCCGTCTC (SEQ ID NO:58)

[0684] The kudzu-terminator fragment was fused to the promoter fragment using PCR with the following primers:

CF 797 (+) Start *aprE* promoter MfeI
5'- GACATCAATTGCTCCATTTTCTTCTGCTATC (SEQ ID NO:53)

CF 07-46 (-) End of *B. amyliquefaciens* terminator (BamHI)
5'- GACATGACGGATCCGATTACGAATGCCGTCTC (SEQ ID NO:58)

[0685] The fusion PCR fragment was purified using a Qiagen kit and digested with the restriction enzymes *Mfe*I and *Bam*HI. This digested DNA fragment was gel purified using a Qiagen kit and ligated to a vector known as pBS19, which had been digested with *Eco*RI and *Bam*HI and gel purified.

[0686] The ligation mix was transformed into *E. coli* Top 10 cells and colonies were selected on LA+50 carbenicillin plates. A total of six colonies were chosen and grown overnight in LB+50 carbenicillin and then plasmids were isolated using a Qiagen kit. The plasmids were digested with *Eco*RI and *Bam*HI to check for inserts and three of the correct plasmids were sent in for sequencing with the following primers:

CF 149 (+) EcoRI start of *aprE* promoter
5'- GACATGAATTCCTCCATTTTCTTCTGC (SEQ ID NO:59)

CF 847 (+) Sequence in pXX 049 (end of *aprE* promoter)
5'- AGGAGAGGGTAAAGAGTGAG (SEQ ID NO:60)

CF 07-45 (-) Fuse the 3' end of kudzu isoprene synthase to the terminator
5'- CCAAGGCCGGTTTTTTTTAGACATACATCAGCTGGTTAATC (SEQ ID NO:56)

CF 07-48 (+) Sequencing primer for kudzu isoprene synthase
5'- CTTTTCCATCACCCACCTGAAG (SEQ ID NO:61)

CF 07-49 (+) Sequencing in kudzu isoprene synthase
5'- GGCGAAATGGTCCAACAACAAAATTATC (SEQ ID NO:62)

[0687] The plasmid designated pBS Kudzu #2 (Figures 52 and 12; SEQ ID NO:5) was correct by sequencing and was transformed into BG 3594 comK, a *Bacillus subtilis* host strain. Selection was done on LA + 5 chloramphenicol plates. A transformant was chosen and struck to single colonies on LA + 5 chloramphenicol, then grown in LB+5 chloramphenicol until it reached an OD_{600} of 1.5. It was stored frozen in a vial at -80° C in the presence of glycerol. The resulting strain was designated CF 443.

II. Production of isoprene in shake flasks containing *B. subtilis* cells expressing recombinant isoprene synthase.

[0688] Overnight cultures were inoculated with a single colony of CF 443 from a LA + Chloramphenicol (Cm, 25 μg/ml). Cultures were grown in LB + Cm at 37° C with shaking at 200 rpm. These overnight cultures (1 ml) were used to inoculate 250 ml baffled shake flasks containing 25 ml Grants II media and chloramphenicol at a final concentration of 25 μg/ml. Grants II Media recipe was 10 g soytone, 3 ml 1M K_2HPO_4, 75 g glucose, 3.6 g urea, 100 ml 10X MOPS, q.s. to 1 L with H_2O, pH 7.2; 10X MOPS recipe was 83.72 g MOPS, 7.17 g tricine, 12 g KOH pellets, 10 ml 0.276M K_2SO_4 solution, 10 ml 0.528M $MgCl_2$ solution, 29.22 g NaCl, 100 ml 100X micronutrients, q.s. to 1 L with H_2O; and 100X micronutrients recipe was 1.47 g $CaCl_2*2H_2O$, 0.4 g $FeSO_4*7H_20$, 0.1 g $MnSO_4*H_20$, 0.1 g $ZnSO_4*H_2O$, 0.05 g $CuCl_2*2H_2O$, 0.1 g $CoCl_2*6H_2O$, 0.1 g $Na_2MoO_4*2H_2O$, q.s. to 1 L with H_2O. Shake flasks were incubated at 37° C and samples were taken at 18, 24, and 44 hours. At 18 hours the headspaces of CF443 and the control strain were sampled. This represented 18 hours of accumulation of isoprene. The amount of isoprene was determined by gas chromatography as described in Example 1. Production of isoprene was enhanced significantly by expressing recombinant isoprene synthase (Figure 11).

III. Production of isoprene by CF443 in 14 L fermentation.

[0689] Large scale production of isoprene from *B. subtilis* containing the recombinant kudzu isoprene synthase gene on a replication plasmid was determined from a fed-batch culture. Bacillus strain CF 443, expressing a kudzu isoprene synthase gene, or control stain which does not express a kudzu isoprene synthase gene were cultivated by conventional fed-

batch fermentation in a nutrient medium containing soy meal (Cargill), sodium and potassium phosphate, magnesium sulfate and a solution of citric acid, ferric chloride and manganese chloride. Prior to fermentation the media is macerated for 90 minutes using a mixture of enzymes including cellulases, hemicellulases and pectinases (see, WO95/04134). 14-L batch fermentations are fed with 60% wt/wt glucose (Cargill DE99 dextrose, ADM Versadex greens or Danisco invert sugar) and 99% wt/wt oil (Western Family soy oil, where the 99% wt/wt is the concentration of oil before it was added to the cell culture medium). Feed was started when glucose in the batch was non-detectable. The feed rate was ramped over several hours and was adjusted to add oil on an equal carbon basis. The pH was controlled at 6.8 – 7.4 using 28% w/v ammonium hydroxide. In case of foaming, antifoam agent was added to the media. The fermentation temperature was controlled at 37°C and the fermentation culture was agitated at 750 rpm. Various other parameters such as pH, DO%, airflow, and pressure were monitored throughout the entire process. The DO% is maintained above 20. Samples were taken over the time course of 36 hours and analyzed for cell growth (OD_{550}) and isoprene production. Results of these experiments are presented in Figures 53A and 53B.

IV. Integration of the kudzu isoprene synthase (ispS) in *B. subtilis*.

[0690] The kudzu isoprene synthase gene was cloned in an integrating plasmid (pJH101-cmpR) under the control of the *aprE* promoter. Under the conditions tested, no isoprene was detected.

Example 5: Production of isoprene in Trichoderma

I. Construction of vectors for expression of the kudzu isoprene synthase in *Trichoderma reesei*.

[0691] The *Yarrowia lipolytica* codon-optimized kudzu IS gene was synthesized by DNA 2.0 (SEQ ID NO:6) (Figure 13). This plasmid served as the template for the following PCR amplification reaction: 1 µl plasmid template (20 ng/ul), 1 µl Primer EL-945 (10 uM) 5'-GCTTATGGATCCTCTAGACTATTACACGTACATCAATTGG (SEQ ID NO:63), 1 µl Primer EL-965 (10uM) 5'-CACCATGTGTGCAACCTCCTCCCAGTTTAC (SEQ ID NO:64), 1 µl dNTP (10mM), 5 µl 10x PfuUltra II Fusion HS DNA Polymerase Buffer, 1 µl PfuUltra II Fusion HS DNA Polymerase, 40 µl water in a total reaction volume of 50 µl. The forward primer contained an additional 4 nucleotides at the 5'-end that did not correspond to

the *Y. lipolytica* codon-optimized kudzu isoprene synthase gene, but was required for cloning into the pENTR/D-TOPO vector. The reverse primer contained an additional 21 nucleotides at the 5'-end that did not correspond to the *Y. lipolytica* codon-optimized kudzu isoprene synthase gene, but were inserted for cloning into other vector backbones. Using the MJ Research PTC-200 Thermocycler, the PCR reaction was performed as follows: 95° C for 2 minutes (first cycle only), 95° C for 30 seconds, 55° C for 30 seconds, 72° C for 30 seconds (repeat for 27 cycles), 72° C for 1 minute after the last cycle. The PCR product was analyzed on a 1.2% E-gel to confirm successful amplification of the *Y. lipolytica* codon-optimized kudzu isoprene synthase gene.

[0692] The PCR product was then cloned using the TOPO pENTR/D-TOPO Cloning Kit following manufacturer's protocol: 1 μl PCR reaction, 1 μl Salt solution, 1 μl TOPO pENTR/D-TOPO vector and 3 μl water in a total reaction volume of 6 μl. The reaction was incubated at room temperature for 5 minutes. One microliter of TOPO reaction was transformed into TOP10 chemically competent *E. coli* cells. The transformants were selected on LA + 50 μg/ml kanamycin plates. Several colonies were picked and each was inoculated into a 5 ml tube containing LB + 50 μg/ml kanamycin and the cultures grown overnight at 37° C with shaking at 200 rpm. Plasmids were isolated from the overnight culture tubes using QIAprep Spin Miniprep Kit, following manufacturer's protocol. Several plasmids were sequenced to verify that the DNA sequence was correct.

[0693] A single pENTR/D-TOPO plasmid, encoding a *Y. lipolytica* codon-optimized kudzu isoprene synthase gene, was used for Gateway Cloning into a custom-made pTrex3g vector. Construction of pTrex3g is described in WO 2005/001036 A2. The reaction was performed following manufacturer's protocol for the Gateway LR Clonase II Enzyme Mix Kit (Invitrogen): 1 μl *Y. lipolytica* codon-optimized kudzu isoprene synthase gene pENTR/D-TOPO donor vector, 1 μl pTrex3g destination vector, 6 μl TE buffer, pH 8.0 in a total reaction volume of 8 μl. The reaction was incubated at room temperature for 1 hour and then 1 μl proteinase K solution was added and the incubation continued at 37° C for 10 minutes. Then 1 μl of reaction was transformed into TOP10 chemically competent *E. coli* cells. The transformants were selected on LA + 50 μg/ml carbenicillin plates. Several colonies were picked and each was inoculated into a 5 ml tube containing LB + 50 μlg/ml carbenicillin and the cultures were grown overnight at 37° C with shaking at 200 rpm. Plasmids were isolated from the overnight culture tubes using QIAprep Spin Miniprep Kit (Qiagen, Inc.), following

manufacturer's protocol. Several plasmids were sequenced to verify that the DNA sequence was correct.

[0694] Biolistic transformation of *Y. lipolytica* codon-optimized kudzu isoprene synthase pTrex3g plasmid (Figure 14) into a quad delete *Trichoderma reesei* strain was performed using the Biolistic PDS-1000/HE Particle Delivery System (see WO 2005/001036 A2). Isolation of stable transformants and shake flask evaluation was performed using protocol listed in Example 11 of patent publication WO 2005/001036 A2.

II. Production of isoprene in recombinant strains of *T. reesei*.

[0695] One ml of 15 and 36 hour old cultures of isoprene synthase transformants described above were transferred to head space vials. The vials were sealed and incubated for 5 hours at 30° C. Head space gas was measured and isoprene was identified by the method described in Example 1. Two of the transformants showed traces of isoprene. The amount of isoprene could be increased by a 14 hour incubation. The two positive samples showed isoprene at levels of about 0.5 μg/L for the 14 hour incubation. The untransformed control showed no detectable levels of isoprene. This experiment shows that *T. reesei* is capable of producing isoprene from endogenous precursor when supplied with an exogenous isoprene synthase.

Example 6: Production of isoprene in *Yarrowia*

I. Construction of vectors for expression of the kudzu isoprene synthase in *Yarrowia lipolytica*.

[0696] The starting point for the construction of vectors for the expression of the kudzu isoprene synthase gene in *Yarrowia lipolytica* was the vector pSPZ1(MAP29Spb). The complete sequence of this vector (SEQ ID NO:7) is shown in Figure 15.

[0697] The following fragments were amplified by PCR using chromosomal DNA of a *Y. lipolytica* strain GICC 120285 as the template: a promotorless form of the URA3 gene, a fragment of 18S ribosomal RNA gene, a transcription terminator of the *Y. lipolytica* XPR2 gene and two DNA fragments containing the promoters of XPR2 and ICL1 genes. The following PCR primers were used:

ICL1 3

5'- GGTGAATTCAGTCTACTGGGGATTCCCAAATCTATATATACTGCAGGTGAC (SEQ ID NO:65)

ICL1 5
5'- GCAGGTGGGAAACTATGCACTCC (SEQ ID NO:66)

XPR 3
5'- CCTGAATTCTGTTGGATTGGAGGATTGGATAGTGGG (SEQ ID NO:67)

XPR 5
5'- GGTGTCGACGTACGGTCGAGCTTATTGACC (SEQ ID NO:68)

XPRT3
5'- GGTGGGCCCGCATTTTGCCACCTACAAGCCAG (SEQ ID NO:69)

XPRT 5
5'- GGTGAATTCTAGAGGATCCCAACGCTGTTGCCTACAACGG (SEQ ID NO:70)

Y18S3
5'- GGTGCGGCCGCTGTCTGGACCTGGTGAGTTTCCCCG (SEQ ID NO:71)

Y18S 5
5'- GGTGGGCCCATTAAATCAGTTATCGTTTATTTGATAG (SEQ ID NO:72)

YURA3
5'- GGTGACCAGCAAGTCCATGGGTGGTTTGATCATGG (SEQ ID NO:73)

YURA 50
5'- GGTGCGGCCGCCTTTGGAGTACGACTCCAACTATG (SEQ ID NO:74)

YURA 51
5'- GCGGCCGCAGACTAAATTTATTTCAGTCTCC (SEQ ID NO:75)

[0698] For PCR amplification the PfuUltraII polymerase (Stratagene), supplier-provided buffer and dNTPs, 2.5 μM primers and the indicated template DNA were used as per the manufacturer's instructions. The amplification was done using the following cycle: 95° C for 1 min; 34x (95° C for 30 sec; 55° C for 30 sec; 72° C for 3 min) and 10 min at 72° C followed by a 4° C incubation.

[0699] Synthetic DNA molecules encoding the kudzu isoprene synthase gene, codon-optimized for expression in *Yarrowia,* was obtained from DNA 2.0 (Figure 16; SEQ ID NO:8). Full detail of the construction scheme of the plasmids pYLA(KZ1) and pYLI(KZ1) carrying the synthetic kudzu isoprene synthase gene under control of XPR2 and ICL1 promoters respectively is presented in Figure 18. Control plasmids in which a mating factor gene (MAP29) is inserted in place of an isoprene synthase gene were also constructed (Figure 18E and 18F).

[0700] A similar cloning procedure can be used to express a poplar (*Populus alba* x *Populus tremula*) isoprene synthase gene. The sequence of the poplar isoprene is described in Miller B. *et al.* (2001) *Planta* 213, 483-487 and shown in Figure 17 (SEQ ID NO:9). A construction scheme for the generation the plasmids pYLA(POP1) and pYLI(POP1) carrying synthetic poplar isoprene synthase gene under control of XPR2 and ICL1 promoters respectively is presented in Figure 18A and B.

II. Production of isoprene by recombinant strains of *Y. lipolytica*.

[0701] Vectors pYLA(KZ1), pYLI(KZ1), pYLA(MAP29) and pYLI(MAP29) were digested with *Sac*II and used to transform the strain *Y. lipolytica* CLIB 122 by a standard lithium acetate/polyethylene glycol procedure to uridine prototrophy. Briefly, the yeast cells grown in YEPD (1% yeast extract, 2% peptone, 2% glucose) overnight, were collected by centrifugation (4000 rpm, 10 min), washed once with sterile water and suspended in 0.1 M lithium acetate, pH 6.0. Two hundred μl aliquots of the cell suspension were mixed with linearized plasmid DNA solution (10-20 μg), incubated for 10 minutes at room temperature and mixed with 1 ml of 50% PEG 4000 in the same buffer. The suspensions were further incubated for 1 hour at room temperature followed by a 2 minutes heat shock at 42° C. Cells were then plated on SC his leu plates (0.67% yeast nitrogen base, 2% glucose, 100 mg/L each of leucine and histidine). Transformants appeared after 3-4 days of incubation at 30° C.

[0702] Three isolates from the pYLA(KZ1) transformation, three isolates from the pYLI(KZ1) transformation, two isolates from the pYLA(MAP29) transformation and two isolates from the pYLI(MAP29) transformation were grown for 24 hours in YEP7 medium (1% yeast extract, 2% peptone, pH 7.0) at 30° C with shaking. Cells from 10 ml of culture were collected by centrifugation, resuspended in 3 ml of fresh YEP7 and placed into 15 ml screw cap vials. The vials were incubated overnight at room temperature with gentle (60 rpm) shaking. Isoprene content in the headspace of these vials was analyzed by gas chromatography using mass-spectrometric detector as described in Example 1. All transformants obtained with pYLA(KZ1) and pYLI(KZ1) produced readily detectable amounts of isoprene (0.5 μg/L to 1 μg/L, Figure 20). No isoprene was detected in the headspace of the control strains carrying phytase gene instead of an isoprene synthase gene.

Example 7: Production of isoprene in *E. coli* expressing kudzu isoprene synthase and *idi*, or *dxs*, or *idi* and *dxs*

I. Construction of vectors encoding kudzu isoprene synthase and *idi*, or *dxs*, or *idi* and *dxs* for the production of isoprene in *E. coli*.

i) Construction of pTrcKudzuKan

[0703] The *bla* gene of pTrcKudzu (described in Example 1) was replaced with the gene conferring kanamycin resistance. To remove the *bla* gene, pTrcKudzu was digested with *Bsp*HI, treated with Shrimp Alkaline Phosphatase (SAP), heat killed at 65ºC, then end-filled with Klenow fragment and dNTPs. The 5 kbp large fragment was purified from an agarose gel and ligated to the kan^r gene which had been PCR amplified from pCR-Blunt-II-TOPO using primers MCM22 5'- GATCAAGCTTAACCGGAATTGCCAGCTG (SEQ ID NO:76) and MCM23 5'- GATCCGATCGTCAGAAGAACTCGTCAAGAAGGC (SEQ ID NO:77), digested with *Hin*dIII and *Pvu*I, and end-filled. A transformant carrying a plasmid conferring kanamycin resistance (pTrcKudzuKan) was selected on LA containing kanamycin 50 μg/ml.

ii) Construction of pTrcKudzu yIDI Kan

[0704] pTrcKudzuKan was digested with *Pst*I, treated with SAP, heat killed and gel purified. It was ligated to a PCR product encoding *idi* from *S. cerevisiae* with a synthetic RBS. The primers for PCR were NsiI-YIDI 1 F 5'-CATCAATGCATCGCCCTTAGGAGGTAAAAAAAAATGAC (SEQ ID NO:78) and PstI-

YIDI 1 R 5'- CCTTCTGCAGGACGCGTTGTTATAGC (SEQ ID NO:79); and the template was *S. cerevisiae* genomic DNA. The PCR product was digested with *Nsi*I and *Pst*I and gel purified prior to ligation. The ligation mixture was transformed into chemically competent TOP10 cells and selected on LA containing 50 μg/ml kanamycin. Several transformants were isolated and sequenced and the resulting plasmid was called pTrcKudzu-yIDI(kan) (Figures 34 and 35; SEQ ID NO:16).

iii) Construction of pTrcKudzu DXS Kan

[0705] Plasmid pTrcKudzuKan was digested with *Pst*I, treated with SAP, heat killed and gel purified. It was ligated to a PCR product encoding *dxs* from *E. coli* with a synthetic RBS. The primers for PCR were MCM13 5'-GATCATGCATTCGCCCTTAGGAGGTAAAAAAACATGAGTTTTGATATTGCCAAATACCCG (SEQ ID NO:80) and MCM14 5'-CATGCTGCAGTTATGCCAGCCAGGCCTTGAT (SEQ ID NO:81); and the template was *E. coli* genomic DNA. The PCR product was digested with *Nsi*I and *Pst*I and gel purified prior to ligation. The resulting transformation reaction was transformed into TOP10 cells and selected on LA with kanamycin 50 μg/ml. Several transformants were isolated and sequenced and the resulting plasmid was called pTrcKudzu-DXS(kan) (Figures 36 and 37; SEQ ID NO:17).

iv) Construction of pTrcKudzu-yIDI-dxs (kan)

[0706] pTrcKudzu-yIDI(kan) was digested with *Pst*I, treated with SAP, heat killed and gel purified. It was ligated to a PCR product encoding *E. coli dxs* with a synthetic RBS (primers MCM13 5'-GATCATGCATTCGCCCTTAGGAGGTAAAAAAACATGAGTTTTGATATTGCCAAATACCCG (SEQ ID NO:80) and MCM14 5'-CATGCTGCAGTTATGCCAGCCAGGCCTTGAT (SEQ ID NO:81); template TOP10 cells) which had been digested with *Nsi*I and *Pst*I and gel purified. The final plasmid was called pTrcKudzu-yIDI-dxs (kan) (Figures 21 and 22; SEQ ID NO:10).

v) Construction of pCL PtrcKudzu

[0707] A fragment of DNA containing the promoter, structural gene and terminator from Example 1 above was digested from pTrcKudzu using *Ssp*I and gel purified. It was ligated to

pCL1920 which had been digested with *Pvu*II, treated with SAP and heat killed. The resulting ligation mixture was transformed into TOP10 cells and selected in LA containing spectinomycin 50 µg/ml. Several clones were isolated and sequenced and two were selected. pCL PtrcKudzu and pCL PtrcKudzu (A3) have the insert in opposite orientations (Figures 38-41; SEQ ID NOs:18-19).

vi) Construction of pCL PtrcKudzu yIDI

[0708] The *Nsi*I-*Pst*I digested, gel purified, IDI PCR amplicon from (ii) above was ligated into pCL PtrcKudzu which had been digested with *Pst*I, treated with SAP, and heat killed. The ligation mixture was transformed into TOP10 cells and selected in LA containing spectinomycin 50 µg/ml. Several clones were isolated and sequenced and the resulting plasmid is called pCL PtrcKudzu yIDI (Figures 42 and 43; SEQ ID NO:20).

vii) Construction of pCL PtrcKudzu DXS

[0709] The *Nsi*I-*Pst*I digested, gel purified, DXS PCR amplicon from (iii) above was ligated into pCL PtrcKudzu (A3) which had been digested with *Pst*I, treated with SAP, and heat killed. The ligation mixture was transformed into TOP10 cells and selected in LA containing spectinomycin 50 µg/ml. Several clones were isolated and sequenced and the resulting plasmid is called pCL PtrcKudzu DXS (Figures 44 and 45; SEQ ID NO:21).

II. Measurement of isoprene in headspace from cultures expressing kudzu isoprene synthase, *idi*, and/or *dxs* at different copy numbers.

[0710] Cultures of *E. coli* BL21(λDE3) previously transformed with plasmids pTrcKudzu(kan) (A), pTrcKudzu-yIDI kan (B), pTrcKudzu-DXS kan (C), pTrcKudzu-yIDI-DXS kan (D) were grown in LB kanamycin 50 µg/mL. Cultures of pCL PtrcKudzu (E), pCL PtrcKudzu, pCL PtrcKudzu-yIDI (F) and pCL PtrcKudzu-DXS (G) were grown in LB spectinomycin 50 µg/mL. Cultures were induced with 400 µM IPTG at time 0 (OD_{600} approximately 0.5) and samples taken for isoprene headspace measurement (see Example 1). Results are shown in Figure 23A-23G.

[0711] Plasmid pTrcKudzu-yIDI-dxs (kan) was introduced into *E. coli* strain BL21 by transformation. The resulting strain BL21/pTrc Kudzu IDI DXS was grown overnight in LB containing kanamycin (50 µg/ml) at 20° C and used to inoculate shake flasks of TM3 (13.6 g

K_2PO_4, 13.6 g KH_2PO_4, 2.0 g $MgSO_4*7H_2O$), 2.0 g citric acid monohydrate, 0.3 g ferric ammonium citrate, 3.2 g $(NH_4)_2SO_4$, 0.2 g yeast extract, 1.0 ml 1000x Modified Trace Metal Solution, adjusted to pH 6.8 and q.s. to H_20, and filter sterilized) containing 1% glucose. Flasks were incubated at 30° C until an OD_{600} of 0.8 was reached, and then induced with 400 μM IPTG. Samples were taken at various times after induction and the amount of isoprene in the head space was measured as described in Example 1. Results are shown in Figure 23H.

III. Production of isoprene from biomass in *E. coli*/pTrcKudzu yIDI DXS.

[0712] The strain BL21 pTrcKudzuIDIDXS was tested for the ability to generate isoprene from three types of biomass; bagasse, corn stover and soft wood pulp with glucose as a control. Hydrolysates of the biomass were prepared by enzymatic hydrolysis (Brown, L and Torget, R., 1996, NREL standard assay method Lap-009 "Enzymatic Saccharification of Lignocellulosic Biomass") and used at a dilution based upon glucose equivalents. In this example, glucose equivalents were equal to 1% glucose. A single colony from a plate freshly transformed cells of BL21 (DE3) pTrcKudzu yIDI DXS (kan) was used to inoculate 5 ml of LB plus kanamycin (50 μg/ml). The culture was incubated overnight at 25° C with shaking. The following day the overnight culture was diluted to an OD_{600} of 0.05 in 25 ml of TM3 + 0.2% YE + 1% feedstock. The feedstock was corn stover, bagasse, or softwood pulp. Glucose was used as a positive control and no glucose was used as a negative control. Cultures were incubated at 30° C with shaking at 180 rpm. The culture was monitored for OD_{600} and when it reached an OD_{600} of ~0.8, cultures were analyzed at 1 and 3 hours for isoprene production as described in Example 1. Cultures are not induced. All cultures containing added feedstock produce isoprene equivalent to those of the glucose positive control. Experiments were done in duplicate and are shown in Figure 46.

IV. Production of isoprene from invert sugar in *E. coli*/pTrcKudzuIDIDXS.

[0713] A single colony from a plate freshly transformed cells of BL21 (λDE3)/pTrcKudzu yIDI DXS (kan) was used to inoculate 5 mL of LB + kanamycin (50 μg/ml). The culture was incubated overnight at 25° C with shaking. The following day the overnight culture was diluted to an OD_{600} of 0.05 in 25 ml of TM3 + 0.2% YE + 1% feedstock. Feedstock was glucose, inverted glucose or corn stover. The invert sugar feedstock (Danisco Invert Sugar) was prepared by enzymatically treating sucrose syrup. AFEX corn stover was prepared as described below (Part V). The cells were grown at 30° C and the first sample was measured

when the cultures reached an OD_{600} ~0.8-1.0 (0 hour). The cultures were analyzed for growth as measured by OD_{600} and for isoprene production as in Example 1 at 0, 1 and 3 hours. Results are shown in Figure 47.

V. Preparation of hydrolysate from AFEX pretreated corn stover.

[0714] AFEX pretreated corn stover was obtained from Michigan Biotechnology Institute. The pretreatment conditions were 60% moisture, 1:1 ammonia loading, and 90 °C for 30 minutes, then air dried. The moisture content in the AFEX pretreated corn stover was 21.27%. The contents of glucan and xylan in the AFEX pretreated corn stover were 31.7% and 19.1% (dry basis), respectively. The saccharification process was as follows; 20 g of AFEX pretreated corn stover was added into a 500 ml flask with 5 ml of 1 M sodium citrate buffer pH 4.8, 2.25 ml of Accellerase 1000, 0.1 ml of Grindamyl H121 (Danisco xylanase product from Aspergillus niger for bread-making industry), and 72.65 ml of DI water. The flask was put in an orbital shaker and incubated at 50° C for 96 hours. One sample was taken from the shaker and analyzed using HPLC. The hydrolysate contained 38.5 g/l of glucose, 21.8 g/l of xylose, and 10.3 g/l of oligomers of glucose and/or xylose.

VI. The effect of yeast extract on isoprene production in *E. coli* grown in fed-batch culture.

[0715] Fermentation was performed at the 14-L scale as previously described with *E. coli* cells containing the pTrcKudzu yIDI DXS plasmid described above. Yeast extract (Bio Springer, Montreal, Quebec, Canada) was fed at an exponential rate. The total amount of yeast extract delivered to the fermentor was varied between 70-830 g during the 40 hour fermentation. Optical density of the fermentation broth was measured at a wavelength of 550 nm. The final optical density within the fermentors was proportional to the amount of yeast extract added (Figure 48A). The isoprene level in the off-gas from the fermentor was determined as previously described. The isoprene titer increased over the course of the fermentation (Figure 48B). The amount of isoprene produced was linearly proportional to the amount of fed yeast extract (Figure 48C).

VII. Production of isoprene in 500 L fermentation of pTrcKudzu DXS yIDI.

[0716] A 500 liter fermentation of *E. coli* cells with a kudzu isoprene synthase, *S. cerevisiae* IDI, and *E. coli* DXS nucleic acids (*E. coli* BL21 (λDE3) pTrc Kudzu dxs yidi)

was used to produce isoprene. The levels of isoprene varied from 50 to 300 μg/L over a time period of 15 hours. On the basis of the average isoprene concentrations, the average flow through the device and the extent of isoprene breakthrough, the amount of isoprene collected was calculated to be approximately 17 g.

VIII. Production of isoprene in 500 L fermentation of E. coli grown in fed-batch culture.

Medium Recipe (per liter fermentation medium):

[0717] K_2HPO_4 7.5 g, $MgSO_4 * 7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium gas (NH_3) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotic were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0718] Citric Acids * H_2O 40 g, $MnSO_4 * H_2O$ 30 g, NaCl 10 g, $FeSO_4 * 7H_2O$ 1 g, $CoCl_2 * 6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4 * 5H_2O$ 100 mg, H_3BO_3 100 mg, $NaMoO_4 * 2H_2O$ 100 mg. Each component is dissolved one at a time in DIH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with 0.22 micron filter.

[0719] Fermentation was performed in a 500-L bioreactor with *E. coli* cells containing the pTrcKudzu yIDI DXS plasmid. This experiment was carried out to monitor isoprene formation from glucose and yeast extract at the desired fermentation pH 7.0 and temperature 30° C. An inoculum of *E. coli* strain taken from a frozen vial was prepared in soytone-yeast extract-glucose medium. After the inoculum grew to OD 0.15, measured at 550 nm, 20 ml was used to inoculate a bioreactor containing 2.5-L soytone-yeast extract-glucose medium. The 2.5-L bioreactor was grown at 30° C to OD 1.0 and 2.0-L was transferred to the 500-L bioreactor.

[0720] Yeast extract (Bio Springer, Montreal, Quebec, Canada) and glucose were fed at exponential rates. The total amount of glucose and yeast extract delivered to the bioreactor during the 50 hour fermentation was 181.2 kg and 17.6 kg, respectively. The optical density within the bioreactor over time is shown in Figure 49A. The isoprene level in the off-gas

from the bioreactor was determined as previously described. The isoprene titer increased over the course of the fermentation (Figure 49B). The total amount of isoprene produced during the 50 hour fermentation was 55.1 g and the time course of production is shown in Figure 49C.

Example 8: Production of isoprene in *E. coli* expressing kudzu isoprene synthase and recombinant mevalonic acid pathway genes

I. Cloning the lower MVA pathway.

[0721] The strategy for cloning the lower mevalonic pathway was as follows. Four genes of the mevalonic acid biosynthesis pathway; mevalonate kinase (MVK), phosphomevalonate kinase (PMK), diphosphomevalonte decarboxylase (MVD) and isopentenyl diphosphate isomerase genes were amplified by PCR from *S. cerevisiae* chromosomal DNA and cloned individually into the pCR BluntII TOPO plasmid (Invitrogen). In some cases, the *idi* gene was amplified from *E. coli* chromosomal DNA. The primers were designed such that an *E. coli* consensus RBS (AGGAGGT (SEQ ID NO:82) or AAGGAGG (SEQ ID NO:83)) was inserted at the 5' end, 8 bp upstream of the start codon and a *Pst*I site was added at the 3' end. The genes were then cloned one by one into the pTrcHis2B vector until the entire pathway was assembled.

[0722] Chromosomal DNA from *S. cerevisiae* S288C was obtained from ATCC (ATCC 204508D). The MVK gene was amplified from the chromosome of *S. cerevisiae* using primers MVKF (5'-AGGAGGTAAAAAAACATGTCATTACCGTTCTTAACTTCTGC, SEQ ID NO:84) and MVK-Pst1-R (5'-ATGGCTGCAGGCCTATCGCAAATTAGCTTATGAAGTCCATGGTAAATTCGTG, SEQ ID NO:85) using PfuTurbo as per manufacturer's instructions. The correct sized PCR product (1370 bp) was identified by electrophoresis through a 1.2% E-gel (Invitrogen) and cloned into pZeroBLUNT TOPO. The resulting plasmid was designated pMVK1. The plasmid pMVK1 was digested with *Sac*I and *Taq*1 restriction endonucleases and the fragment was gel purified and ligated into pTrcHis2B digested with *Sac*I and *Bst*BI. The resulting plasmid was named pTrcMVK1.

[0723] The second gene in the mevalonic acid biosynthesis pathway, PMK, was amplified by PCR using primers: PstI-PMK1 R (5'-GAATTCGCCCTTCTGCAGCTACC, SEQ ID

NO:86) and BsiHKA I-PMK1 F (5'-CGACTGGTGCACCCTTAAGGAGGAAAAAAACATGTCAG, SEQ ID NO:87). The PCR reaction was performed using Pfu Turbo polymerase (Stratagene) as per manufacturer's instructions. The correct sized product (1387 bp) was digested with *Pst*I and *Bsi*HKI and ligated into pTrcMVK1 digested with *Pst*I. The resulting plasmid was named pTrcKK. The MVD and the *idi* genes were cloned in the same manner. PCR was carried out using the primer pairs PstI-MVD 1 R (5'-GTGCTGGAATTCGCCCTTCTGCAGC, SEQ ID NO:88) and NsiI-MVD 1 F (5'-GTAGATGCATGCAGAATTCGCCCTTAAGGAGG, SEQ ID NO:89) to amplify the MVD gene and PstI-YIDI 1 R (5'-CCTTCTGCAGGACGCGTTGTTATAGC, SEQ ID NO:79) and NsiI-YIDI 1 F (5'-CATCAATGCATCGCCCTTAGGAGGTAAAAAAAAATGAC, SEQ ID NO:78) to amplify the yIDI gene. In some cases the IPP isomerase gene, *idi* from *E. coli* was used. To amplify *idi* from *E. coli* chromosomal DNA, the following primer set was used: PstI-CIDI 1 R (5'-GTGTGATGGATATCTGCAGAATTCG, SEQ ID NO:90) and NsiI-CIDI 1 F (5'-CATCAATGCATCGCCCTTAGGAGGTAAAAAAACATG, SEQ ID NO:91). Template DNA was chromosomal DNA isolated by standard methods from *E. coli* FM5 (WO 96/35796 and WO 2004/033646, which are each hereby incorporated by reference in their entireties, particularly with respect to isolation of nucleic acids). The final plasmids were named pKKDIy for the construct encoding the yeast *idi* gene or pKKDIc for the construct encoding the *E. coli idi* gene. The plasmids were transformed into *E. coli* hosts BL21 for subsequent analysis. In some cases the isoprene synthase from kudzu was cloned into pKKDIy yielding plasmid pKKDIyIS.

[0724] The lower MVA pathway was also cloned into pTrc containing a kanamycin antibiotic resistance marker. The plasmid pTrcKKDIy was digested with restriction endonucleases *Apa*I and *Pst*I, the 5930 bp fragment was separated on a 1.2% agarose E-gel and purified using the Qiagen Gel Purification kit according to the manufacturer's instructions. The plasmid pTrcKudzuKan, described in Example 7, was digested with restriction endonucleases *Apa*I and *Pst*I, and the 3338 bp fragment containing the vector was purified from a 1.2% E-gel using the Qiagen Gel Purification kit. The 3338 bp vector fragment and the 5930 bp lower MVA pathway fragment were ligated using the Roche Quick Ligation kit. The ligation mix was transformed into *E. coli* TOP10 cells and tranformants were grown at 37° C overnight with selection on LA containing kanamycin (50 μg/ml). The

transformants were verified by restriction enzyme digestion and one was frozen as a stock. The plasmid was designated pTrcKanKKDIy.

II. Cloning a kudzu isoprene synthase gene into pTrcKanKKDIy.

[0725] The kudzu isoprene synthase gene was amplified by PCR from pTrcKudzu, described in Example 1, using primers MCM50 5'-GATCATGCATTCGCCCTTAGGAGGTAAAAAAACATGTGTGCGACCTCTTCTCAATTTACT (SEQ ID NO:92) and MCM53 5'-CGGTCGACGGATCCCTGCAGTTAGACATACATCAGCTG (SEQ ID NO:50). The resulting PCR fragment was cloned into pCR2.1 and transformed into *E. coli* TOP10. This fragment contains the coding sequence for kudzu isoprene synthase and an upstream region containing a RBS from *E. coli*. Transformants were incubated overnight at 37° C with selection on LA containing carbenicillin (50 μg/ml). The correct insertion of the fragment was verified by sequencing and this strain was designated MCM93.

[0726] The plasmid from strain MCM93 was digested with restriction endonucleases *Nsi*I and *Pst*I to liberate a 1724 bp insert containing the RBS and kudzu isoprene synthase. The 1724 bp fragment was separated on a 1.2% agarose E-gel and purified using the Qiagen Gel Purification kit according to the manufacturer's instructions. Plasmid pTrcKanKKDIy was digested with the restriction endonuclease *Pst*I, treated with SAP for 30 minutes at 37° C and purified using the Qiagen PCR cleanup kit. The plasmid and kudzu isoprene synthase encoding DNA fragment were ligated using the Roche Quick Ligation kit. The ligation mix was transformed into *E. coli* TOP10 cells and transformants were grown overnight at 37° C with selection on LA containing Kanamycin at 50 μg/ml. The correct transformant was verified by restriction digestion and the plasmid was designated pTrcKKDyIkISKan (Figures 24 and 25; SEQ ID NO:11). This plasmid was transformed into BL21(λDE3) cells (Invitrogen).

III. Isoprene production from mevalonate in *E. coli* expressing the recombinant lower mevalonate pathway and isoprene synthase from kudzu.

[0727] Strain BL21/pTrcKKDyIkISKan was cultured in MOPS medium (Neidhardt *et al.*, (1974) *J. Bacteriology* 119:736-747) adjusted to pH 7.1 and supplemented with 0.5% glucose and 0.5% mevalonic acid. A control culture was also set up using identical conditions but

without the addition of 0.5% mevalonic acid. The culture was started from an overnight seed culture with a 1% inoculum and induced with 500 μM IPTG when the culture had reached an OD_{600} of 0.3 to 0.5. The cultures were grown at 30° C with shaking at 250 rpm. The production of isoprene was analyzed 3 hours after induction by using the head space assay described in Example 1. Maximum production of isoprene was 6.67×10^{-4} $mol/L_{broth}/OD_{600}/hr$ where L_{broth} is the volume of broth and includes both the volume of the cell medium and the volume of the cells. The control culture not supplemented with mevalonic acid did not produce measurable isoprene.

IV. Cloning the upper MVA pathway.

[0728] The upper mevalonate biosynthetic pathway, comprising two genes encoding three enzymatic activities, was cloned from *Enterococcus faecalis*. The *mvaE* gene encodes a protein with the enzymatic activities of both acetyl-CoA acetyltransferase and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the first and third proteins in the pathway, and the *mvaS* gene encodes second enzyme in the pathway, HMG-CoA synthase. The *mvaE* gene was amplified from *E. faecalis* genomic DNA (ATCC 700802D-5) with an *E. coli* ribosome binding site and a spacer in front using the following primers:

CF 07-60 (+) Start of *mvaE* w/ RBS + ATG start codon *Sac*I
5' - GAGACATGAGCTCAGGAGGTAAAAAAACATGAAAACAGTAGTTATTATTG
(SEQ ID NO:93)

CF 07-62 (-) Fuse *mvaE* to *mvaS* with RBS in between
5' - TTTATCAATCCCAATTGTCATGTTTTTTTACCTCCTTTATTGTTTTCTTAAATC
(SEQ ID NO:94)

[0729] The *mvaS* gene was amplified from *E. faecalis* genomic DNA (ATCC 700802D-5) with a RBS and spacer from *E. coli* in front using the following primers:

CF 07-61 (+) Fuse *mvaE* to *mvaS* with RBS in between
5' -
GATTTAAGAAAACAATAAAGGAGGTAAAAAAACATGACAATTGGGATTGATAAA
(SEQ ID NO:95)

CF 07-102 (-) End of *mvaS* gene *Bgl*II
5' -GACATGACATAGATCTTTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:96)

[0730] The PCR fragments were fused together with PCR using the following primers:

CF 07-60 (+) Start of *mvaE* w/ RBS + ATG start codon *Sac*I
5' -GAGACATGAGCTCAGGAGGTAAAAAAACATGAAAACAGTAGTTATTATTG (SEQ ID NO:93)

CF 07-102 (-) End of *mvaS* gene *Bgl*II
5'-GACATGACATAGATCTTTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:96)

[0731] The fusion PCR fragment was purified using a Qiagen kit and digested with the restriction enzymes *Sac*I and *Bgl*II. This digested DNA fragment was gel purified using a Qiagen kit and ligated into the commercially available vector pTrcHis2A, which had been digested with *Sac*I and *Bgl*II and gel purified.

[0732] The ligation mix was transformed into *E. coli* Top 10 cells and colonies were selected on LA+50 μg/ml carbenicillin plates. A total of six colonies were chosen and grown overnight in LB+50 μg/ml carbenicillin and plasmids were isolated using a Qiagen kit. The plasmids were digested with *Sac*I and *Bgl*II to check for inserts and one correct plasmid was sequenced with the following primers:

CF 07-58 (+) Start of *mvaE* gene
5' – ATGAAAACAGTAGTTATTATTGATGC (SEQ ID NO:97)

CF 07-59 (-) End of *mvaE* gene
5' – ATGTTATTGTTTTCTTAAATCATTTAAAATAGC (SEQ ID NO:98)

CF 07-82 (+) Start of *mvaS* gene
5' – ATGACAATTGGGATTGATAAAATTAG (SEQ ID NO:99)

CF 07-83 (-) End of *mvaS* gene
5' – TTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:100)

CF 07-86 (+) Sequence in *mvaE*
5' – GAAATAGCCCCATTAGAAGTATC (SEQ ID NO:101)

CF 07-87 (+) Sequence in *mvaE*
5' – TTGCCAATCATATGATTGAAAATC (SEQ ID NO:102)

CF 07-88 (+) Sequence in *mvaE*
5' – GCTATGCTTCATTAGATCCTTATCG (SEQ ID NO:103)

CF 07-89 (+) Sequence *mvaS*
5' – GAAACCTACATCCAATCTTTTGCCC (SEQ ID NO:104)

[0733] The plasmid called pTrcHis2AUpperPathway#1 was correct by sequencing and was transformed into the commercially available *E. coli* strain BL21. Selection was done on LA+ 50 μg/ml carbenicillin. Two transformants were chosen and grown in LB+ 50 μg/ml carbenicillin until they reached an OD_{600} of 1.5. Both strains were frozen in a vial at -80° C in the presence of glycerol. Strains were designated CF 449 for pTrcHis2AUpperPathway#1 in BL21, isolate #1 and CF 450 for pTrcHis2AUpperPathway#1 in BL21, isolate #2. Both clones were found to behave identically when analyzed.

V. Cloning of UpperMVA Pathway into pCL1920.

[0734] The plasmid pTrcHis2AUpperPathway was digested with the restriction endonuclease *Ssp*I to release a fragment containing pTrc-*mvaE-mvaS*-(His tag)-terminator. In this fragment, the his-tag was not translated. This blunt ended 4.5 kbp fragment was purified from a 1.2% E-gel using the Qiagen Gel Purification kit. A dephosphorylated, blunt ended 4.2 kbp fragment from pCL1920 was prepared by digesting the vector with the restriction endonuclease *Pvu*II, treating with SAP and gel purifying from a 1.2% E-gel using the Qiagen Gel Purification kit. The two fragments were ligated using the Roche Quick Ligation Kit and transformed into TOP10 chemically competent cells. Transformants were selected on LA containing spectinomycin (50 μg/ml). A correct colony was identified by screening for the presence of the insert by PCR. The plasmid was designated pCL PtrcUpperPathway (Figures 26 and 27A-27D; SEQ ID NO:12).

VI. Strains expressing the combined Upper and Lower Mevalonic Acid Pathways.

[0735] To obtain a strain with a complete mevalonic acid pathway plus kudzu isoprene synthase, plasmids pTrcKKDyIkISkan and pCLpTrcUpperPathway were both transformed into BL21(λDE3) competent cells (Invitrogen) and transformants were selected on LA containing kanamycin (50 μg/ml) and Spectinomycin (50 μg/ml). The transformants were checked by plasmid prep to ensure that both plasmids were retained in the host. The strain was designated MCM127.

VII. Production of mevalonic acid from glucose in *E. coli*/pUpperpathway.

[0736] Single colonies of the BL21/pTrcHis2A-*mvaE/mvaS* or FM5/p pTrcHis2A-*mvaE/mvaS* are inoculated into LB + carbenicillin (100 μg/ml) and are grown overnight at 37° C with shaking at 200 rpm. These cultures were diluted into 50 ml medium in 250 ml baffled flasks to an OD_{600} of 0.1. The medium was TM3 + 1 or 2% glucose + carbenicillin (100 ug/ml) or TM3 + 1% glucose + hydrolyzed soy oil + carbenicillin (100 ug/ml) or TM3 + biomass (prepared bagasse, corn stover or switchgrass). Cultures were grown at 30° C with shaking at 200 rpm for approximately 2-3 hours until an OD_{600} of 0.4 was reached. At this point the expression from the *mvaE mvaS* construct was induced by the addition of IPTG (400 μM). Cultures were incubated for a further 20 or 40 hours with samples taken at 2 hour intervals to 6 hour post induction and then at 24, 36 and 48 hours as needed. Sampling was done by removing 1 ml of culture, measuring the OD_{600}, pelleting the cells in a microfuge, removing the supernatant and analyzing it for mevalonic acid.

[0737] A 14 liter fermentation of *E. coli* cells with nucleic acids encoding *Enterococcus faecalis* AA-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase polypeptides produced 22 grams of mevalonic acid with TM3 medium and 2% glucose as the cell medium. A shake flask of these cells produced 2-4 grams of mevalonic acid per liter with LB medium and 1% glucose as the cell culture medium. The production of mevalonic acid in these strains indicated that the MVA pathway was functional in *E. coli*.

VIII. Production of isoprene from *E. coli* BL21 containing the upper and lower MVA pathway plus kudzu isoprene synthase.

[0738] The following strains were created by transforming in various combinations of plasmids containing the upper and lower MVA pathway and the kudzu isoprene synthase

gene as described above and the plasmids containing the idi, dxs, and dxr and isoprene synthase genes described in Example 7. The host cells used were chemically competent BL21(λDE3) and the transformations were done by standard methods. Transformants were selected on L agar containing kanamycin (50 μg/ml) or kanamycin plus spectinomycin (both at a concentration of 50 μg/ml). Plates were grown at 37° C. The resulting strains were designated as follows:

Grown on Kanamycin plus Spectinomycin (50 μg/ml each)
MCM127 - pCL Upper MVA + pTrcKKDyIkIS (kan) in BL21(λDE3)
MCM131 - pCL1920 + pTrcKKDyIkIS (kan) in BL21(λDE3)
MCM125 - pCL Upper MVA + pTrcHis2B (kan) in BL21(λDE3)

Grown on Kanamycin (50 μg/ml)
MCM64 - pTrcKudzu yIDI DXS (kan) in BL21(λDE3)
MCM50 - pTrcKudzu (kan) in BL21(λDE3)
MCM123 - pTrcKudzu yIDI DXS DXR (kan) in BL21(λDE3)

[0739] The above strains were streaked from freezer stocks to LA + appropriate antibiotic and grown overnight at 37° C. A single colony from each plate was used to inoculate shake flasks (25 ml LB + the appropriate antibiotic). The flasks were incubated at 22° C overnight with shaking at 200 rpm. The next morning the flasks were transferred to a 37° C incubator and grown for a further 4.5 hours with shaking at 200 rpm. The 25 ml cultures were centrifuged to pellet the cells and the cells were resuspended in 5 ml LB + the appropriate antibiotic. The cultures were then diluted into 25 ml LB+1% glucose + the appropriate antibiotic to an OD_{600} of 0.1. Two flasks for each strain were set up, one set for induction with IPTG (800 μM) the second set was not induced. The cultures were incubated at 37° C with shaking at 250 rpm. One set of the cultures were induced after 1.50 hours (immediately following sampling time point 1). At each sampling time point, the OD_{600} was measured and the amount of isoprene determined as described in Example 1. Results are presented in Table 3. The amount of isoprene made is presented as the amount at the peak production for the particular strain.

Table 3. Production of isoprene in *E. coli* strains

Strain	Isoprene (μg/liter/OD/hr)
MCM50	23.8
MCM64	289
MCM125	ND
MCM131	Trace
MCM127	874

ND: not detected

Trace: peak present but not integrable.

IX. Analysis of mevalonic acid.

[0740] Mevalonolactone (1.0 g, 7.7 mmol) (CAS# 503-48-0) was supplied from Sigma-Aldrich (WI, USA) as a syrup that was dissolved in water (7.7 mL) and was treated with potassium hydroxide (7.7 mmol) in order to generate the potassium salt of mevalonic acid. The conversion to mevalonic acid was confirmed by ^{1}H NMR analysis. Samples for HPLC analysis were prepared by centrifugation at 14,000 rpm for 5 minutes to remove cells, followed by the addition of a 300 μl aliquot of supernatant to 900 μl of H_2O. Perchloric acid (36 μl of a 70% solution) was then added followed by mixing and cooling on ice for 5 minutes. The samples were then centrifuged again (14,000 rpm for 5 min) and the supernatant transferred to HPLC. Mevalonic acid standards (20, 10, 5, 1 and 0.5 g/L) were prepared in the same fashion. Analysis of mevalonic acid (20 uL injection volume) was performed by HPLC using a BioRad Aminex 87-H+ column (300 mm by 7.0 mm) eluted with 5 mM sulfuric acid at 0.6 mL/min with refractive index (RI) detection. Under these conditions mevalonic acid eluted as the lactone form at 18.5 minutes.

X. Production of isoprene from *E. coli* BL21 containing the upper MVA pathway plus kudzu isoprene synthase.

[0741] A 15-L scale fermentation of *E. coli* expressing mevalonic acid pathway polypeptides and Kudzu isoprene synthase was used to produce isoprene from cells in fed-batch culture. This experiment demonstrates that growing cells under glucose limiting conditions resulted in the production of 2.2 g/L of isoprene.

Medium Recipe (per liter fermentation medium):

[0742] The medium was generated using the following components per liter fermentation medium: K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X modified trace metal solution 1 ml. All of the

components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0743] The 1000X modified trace metal solution was generated using the following components: citric acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4$ * $2H_2O$ 100 mg. Each component was dissolved one at a time in diH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume, and filter sterilized with a 0.22 micron filter.

[0744] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the pCL PtrcUpperPathway (Figure 26) and pTrcKKDyIkIS plasmids. This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into soytone-yeast extract-glucose medium. After the inoculum grew to OD 1.0 when measured at 550 nm, 500 mL was used to inoculate a 5-L bioreactor.

[0745] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 54 hour fermentation was 3.7 kg. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). The IPTG concentration was brought to 25 uM when the optical density at 550 nm (OD_{550}) reached a value of 10. The IPTG concentration was raised to 50 uM when OD_{550} reached 190. IPTG concentration was raised to 100 uM at 38 hours of fermentation. The OD_{550} profile within the bioreactor over time is shown in Figure 54. The isoprene level in the off gas from the bioreactor was determined as described herein. The isoprene titer increased over the course of the fermentation to a final value of 2.2 g/L (Figure 55). The total amount of isoprene produced during the 54 hour fermentation was 15.9 g, and the time course of production is shown in Figure 56.

XI. Isoprene fermentation from *E. coli* expressing genes from the mevalonic acid pathway and grown in fed-batch culture at the 15-L scale.

[0746] A 15-L scale fermentation of *E. coli* expressing mevalonic acid pathway polypeptides and Kudzu isoprene synthase was used to produce isoprene from cells in fed-batch culture. This experiment demonstrates that growing cells under glucose limiting conditions resulted in the production of 3.0 g/L of isoprene.

Medium Recipe (per liter fermentation medium):

[0747] The medium was generated using the following components per liter fermentation medium: K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0748] The 1000X modified trace metal solution was generated using the following components: citric acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4$ * $2H_2O$ 100 mg. Each component was dissolved one at a time in diH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume, and filter sterilized with a 0.22 micron filter.

[0749] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids. This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 500 mL was used to inoculate a 5-L bioreactor.

[0750] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time, the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 59 hour fermentation was 2.2 kg. Induction

was achieved by adding IPTG. The IPTG concentration was brought to 25 uM when the optical density at 550 nm (OD_{550}) reached a value of 10. The IPTG concentration was raised to 50 uM when OD_{550} reached 190. The OD_{550} profile within the bioreactor over time is shown in Figure 93. The isoprene level in the off gas from the bioreactor was determined as described herein. The isoprene titer increased over the course of the fermentation to a final value of 3.0 g/L (Figure 94). The total amount of isoprene produced during the 59 hour fermentation was 22.8 g, and the time course of production is shown in Figure 95. The molar yield of utilized carbon that went into producing isoprene during fermentation was 2.2%. The weight percent yield of isoprene from glucose was 1.0%.

XII. Isoprene fermentation from *E. coli* expressing genes from the mevalonic acid pathway and grown in fed-batch culture at the 15-L scale.

[0751] A 15-L scale fermentation of *E. coli* expressing mevalonic acid pathway polypeptides, *Pueraria lobata* isoprene synthase, and Kudzu isoprene synthase was used to produce isoprene from cells in fed-batch culture. This experiment demonstrates that growing cells under glucose limiting conditions resulted in the production of 3.3 g/L of isoprene.

i) Construction of pCLPtrcUpperPathwayHGS2

[0752] The gene encoding isoprene synthase from *Pueraria lobata* was PCR-amplified using primers NsiI-RBS-HGS F (CTTGATGCATCCTGCATTCGCCCTTAGGAGG, SEQ ID NO:105) and pTrcR (CCAGGCAAATTCTGTTTTATCAG, SEQ ID NO:106), and pTrcKKDyIkIS as a template. The PCR product thus obtained was restriction-digested with *Nsi*I and *Pst*I and gel-purified. The plasmid pCL PtrcUpperPathway was restriction-digested with *Pst*I and dephosphorylated using rAPid alkaline phosphatase (Roche) according to manufacturer's instructions.

[0753] These DNA fragments were ligated together and the ligation reaction was transformed into *E. coli* Top10 chemcially competent cells (Invitrogen), plated on L agar containing spectinomycin (50 ug/ml) and incubated overnight at 37°C. Plasmid DNA was prepared from 6 clones using the Qiaquick Spin Mini-prep kit. The plasmid DNA was digested with restriction enzymes *Eco*RV and *Mlu*I to identify a clone in which the insert had the right orientation (*i.e.*, the gene oriented in the same way as the pTrc promoter).

[0754] The resulting correct plasmid was designated pCLPtrcUpperPathwayHGS2. This plasmid was assayed using the headspace assay described herein and found to produce isoprene in *E. coli* Top10, thus validating the functionality of the gene. The plasmid was transformed into BL21(LDE3) containing pTrcKKDyIkIS to yield the strain BL21/pCLPtrcUpperPathwayHGS2-pTrcKKDyIkIS. This strain has an extra copy of the isoprene synthase compared to the BL21/pCL PtrcUpperMVA and pTrc KKDyIkIS strain (Example 8, part XI). This strain also had increased expression and activity of HMGS compared to the BL21/pCL PtrcUpperMVA and pTrc KKDyIkIS strain used in Example 8, part XI.

ii) Isoprene fermentation from *E. coli* expressing pCLPtrcUpperPathwayHGS2-pTrcKKDyIkIS and grown in fed-batch culture at the 15-L scale

Medium Recipe (per liter fermentation medium):

[0755] The medium was generated using the following components per liter fermentation medium: K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X modified trace metal solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0756] The 1000X modified trace metal solution was generated using the following components: citric acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4$ * $2H_2O$ 100 mg. Each component is dissolved one at a time in DiH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with 0.22 micron filter.

[0757] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the pCLPtrcUpperPathwayHGS2 and pTrc KKDyIkIS plasmids. This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single

colony was inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0 measured at 550 nm, 500 mL was used to inoculate a 5-L bioreactor.

[0758] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 58 hour fermentation was 2.1 kg. Induction was achieved by adding IPTG. The IPTG concentration was brought to 25 uM when the optical density at 550 nm (OD_{550}) reached a value of 9. The IPTG concentration was raised to 50 uM when OD_{550} reached 170. The OD_{550} profile within the bioreactor over time is shown in Figure 104. The isoprene level in the off gas from the bioreactor was determined as described herein. The isoprene titer increased over the course of the fermentation to a final value of 3.3 g/L (Figure 105). The total amount of isoprene produced during the 58 hour fermentation was 24.5 g and the time course of production is shown in Figure 106. The molar yield of utilized carbon that went into producing isoprene during fermentation was 2.5%. The weight percent yield of isoprene from glucose was 1.2%. Analysis showed that the activity of the isoprene synthase was increased by approximately 3-4 times that compared to BL21 expressing CL PtrcUpperMVA and pTrc KKDyIkIS plasmids (data not shown).

XIII. Chromosomal Integration of the Lower Mevalonate Pathway in *E. coli.*

[0759] A synthetic operon containing mevalonate kinase, mevalonate phosphate kinase, mevalonate pyrophosphate decarboxylase, and the IPP isomerase was integerated into the chromosome of *E. coli*. If desired, expression may be altered by integrating different promoters 5' of the operon.

[0760] Table 4 lists primers used for this experiment.

Table 4. Primers

MCM78	attTn7 up rev for integration construct	gcatgctcgagcggccgcTTTTAATCAAACATCCTGCCAACTC (SEQ ID NO:107)
MCM79	attTn7 down rev for integration construct	gatcgaagggcgatcgTGTCACAGTCTGGCGAAACCG (SEQ ID NO:108)
MCM88	attTn7 up forw for integration construct	ctgaattctgcagatatcTGTTTTTCCACTCTTCGTTCACTTT (SEQ ID NO:109)
MCM89	attTn7 down forw for integration construct	tctagagggcccAAGAAAAATGCCCCGCTTACG (SEQ ID NO:110)
MCM104	GI1.2 promoter – MVK	Gatcgcggccgcgcccttgacgatgccacatcctgagcaaataattcaaccactaattgtgagc ggataacacaaggaggaaacagctatgtcattaccgttcttaacttc (SEQ ID NO:111)
MCM105	aspA terminator – yIDI	Gatcgggccccaagaaaaaaggcacgtcatctgacgtgccttttttatttgtagacgcgttgttata gcattcta (SEQ ID NO:112)
MCM120	Forward of attTn7: attTn7	aaagtagccgaagatgacggtttgtcacatggagttggcaggatgtttgattaaaagcAATTA

	homology, GB marker homology	ACCCTCACTAAAGGGCGG (SEQ ID NO:113)
MCM127	Rev complement of 1.2 GI: GB marker homology(extra long), promoter, RBS, ATG	AGAGTGTTCACCAAAAATAATAACCTTTCCCGGTGCAgaagtt aagaacggtaatgacatagctgtttcctccttgtgttatccgctcacaattagtggttgaattatttgct caggatgtggcatcgtcaagggcTAATACGACTCACTATAGGGCTCG (SEQ ID NO:114)

i) Target vector construction

[0761] The attTn7 site was selected for integration. Regions of homology upstream (attTn7 up) (primers MCM78 and MCM79) and downstream (attTn7 down) (primers MCM88 and MCM89) were amplified by PCR from MG1655 cells. A 50 uL reaction with 1uL 10uM primers, 3uL ddH2O, 45uL Invitrogen Platinum PCR Supermix High Fidelity, and a scraped colony of MG1655 was denatured for 2:00 at 94°C, cycled 25 times (2:00 at 94°C, 0:30 at 50°C, and 1:00 at 68°C), extended for 7:00 at 72°C, and cooled to 4°C. This resulting DNA was cloned into pCR2.1 (Invitrogen) according to the manufacturer's instructions, resulting in plasmids MCM278 (attTn7 up) and MCM252 (attTn7 down). The 832bp *Apa*I-*Pvu*I fragment digested and gel purified from MCM252 was cloned into *Apa*I-*Pvu*I digested and gel purified plasmid pR6K, creating plasmid MCM276. The 825bp *Pst*I-*Not*I fragment digested and gel purified from MCM278 was cloned into *Pst*I-*Not*I digested and gel purified MCM276, creating plasmid MCM281.

ii) Cloning of lower pathway and promoter

[0762] MVK-PMK-MVD-IDI genes were amplified from pTrcKKDyIkIS with primers MCM104 and MCM105 using Roche Expand Long PCR System according to the manufacturer's instructions. This product was digested with *Not*I and *Apa*I and cloned into MCM281 which had been digested with *Not*I and *Apa*I and gel purified. Primers MCM120 and MCM127 were used to amplify CMR cassette from the GeneBridges FRT-gb2-Cm-FRT template DNA using Stratagene Pfu Ultra II. A PCR program of denaturing at 95°C for 4:00, 5 cycles of 95°C for 0:20, 55°C for 0:20, 72°C for 2:00, 25 cycles of 95°C for 0:20, 58°C for 0:20, 72°C for 2:00, 72°C for 10:00, and then cooling to 4°C was used with four 50 µL PCR reactions containing 1uL ~10 ng/µL template, 1 µL each primer, 1.25 µL 10mM dNTPs, 5 µL 10X buffer, 1 µL enzyme, and 39.75 µL ddH20. Reactions were pooled, purified on a Qiagen PCR cleanup column, and used to electroporate water-washed Pir1 cells containing plasmid MCM296. Electroporation was carried out in 2 mM cuvettes at 2.5V and 200 ohms. Electroporation reactions were recovered in LB for 3hr at 30°C. Transformant MCM330 was selected on LA with CMP5, Kan50 (Figures 107 and 108A-108C; SEQ ID NO:25).

iii) Integration into *E. coli* chromosome

[0763] Miniprepped DNA (Qiaquick Spin kit) from MCM330 was digested with *Sna*BI and used to electroporate BL21(DE3) (Novagen) or MG1655 containing GeneBridges plasmid pRedET Carb. Cells were grown at 30°C to ~OD1 then induced with 0.4% L-arabinose at 37°C for 1.5 hours. These cells were washed three times in 4°C ddH2O before electroporation with 2 μL of DNA. Integrants were selected on L agar with containing chloramphenicol (5 μg/ml) and subsequently confirmed to not grow on L agar + Kanamycin (50 ug/ml). BL21 integrant MCM331 and MG1655 integrant MCM333 were frozen.

iv) Construction of pET24D-Kudzu encoding Kudzu isoprene synthase

[0764] The kudzu isoprene synthase gene was subcloned into the pET24d vector (Novagen) from the pCR2.1 vector (Invitrogen). In particular, the kudzu isoprene synthase gene was amplified from the pTrcKudzu template DNA using primers MCM50 5'-GATCATGCAT TCGCCCTTAG GAGGTAAAAA AACATGTGTG CGACCTCTTC TCAATTTACT (SEQ ID NO:52) and MCM53 5'-CGGTCGACGG ATCCCTGCAG TTAGACATAC ATCAGCTG (SEQ ID NO:50). PCR reactions were carried out using Taq DNA Polymerase (Invitrogen), and the resulting PCR product was cloned into pCR2.1-TOPO TA cloning vector (Invitrogen), and transformed into *E. coli* Top10 chemically competent cells (Invitrogen). Transformants were plated on L agar containing carbenicillin (50 μg/ml) and incubated overnight at 37°C. Five ml Luria Broth cultures containing carbenicillin 50 μg/ml were inoculated with single transformants and grown overnight at 37°C. Five colonies were screened for the correct insert by sequencing of plasmid DNA isolated from 1 ml of liquid culture (Luria Broth) and purified using the QIAprep Spin Mini-prep Kit (Qiagen). The resulting plasmid, designated MCM93, contains the kudzu isoprene synthase coding sequence in a pCR2.1 backbone.

[0765] The kudzu coding sequence was removed by restriction endonuclease digestion with *Pci*I and *Bam*H1 (Roche) and gel purified using the QIAquick Gel Extraction kit (Qiagen). The pET24d vector DNA was digested with *Nco*I and *Bam*HI (Roche), treated with shrimp alkaline phosphatase (Roche), and purified using the QIAprep Spin Mini-prep Kit (Qiagen). The kudzu isoprene synthase fragment was ligated to the *Nco*I/*Bam*H1 digested pET24d using the Rapid DNA Ligation Kit (Roche) at a 5:1 fragment to vector ratio in a total volume of 20 μl. A portion of the ligation mixture (5 μl) was transformed into *E. coli* Top 10

chemically competent cells and plated on L agar containing kanamycin (50 μg/ml). The correct transformant was confirmed by sequencing and transformed into chemically competent BL21(λDE3)pLysS cells (Novagen). A single colony was selected after overnight growth at 37°C on L agar containing kanamycin (50 μg/ml). A map of the resulting plasmid designated as pET24D-Kudzu is shown in Figure 109. The sequence of pET24D-Kudzu (SEQ ID NO:26) is shown in Figures 110A and 110B. Isoprene synthase activity was confirmed using a headspace assay.

v) Production strains

[0766] Strains MCM331 and MCM333 were cotransformed with plasmids pCLPtrcupperpathway and either pTrcKudzu or pETKudzu, resulting in the strains shown in Table 5.

Table 5. Production Strains

Background	Integrated Lower	Upper MVA plasmid	Isoprene synthase plasmid	Production Stain
BL21(DE3)	MCM331	pCLPtrcUpper Pathway	pTrcKudzu	MCM343
BL21(DE3)	MCM331	pCLPtrcUpper Pathway	pET24D-Kudzu	MCM335
MG1655	MCM333	pCLPtrcUpper Pathway	pTrcKudzu	MCM345

vi) Isoprene fermentation from *E. coli* expressing genes from the mevalonic acid pathway and grown in fed-batch culture at the 15-L scale.

Medium Recipe (per liter fermentation medium):

[0767] The medium was generated using the following components per liter fermentation medium: K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X modified trace metal solution 1 ml. All of the components were added together and dissolved in diH2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0768] The 1000X modified trace metal solution was generated using the following components: citric acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4$ * $2H_2O$ 100 mg. Each component is dissolved one at a time in DiH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 micron filter.

[0769] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the gi1.2 integrated lower MVA pathway described above and the pCL PtrcUpperMVA and pTrcKudzu plasmids. This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 500 mL was used to inoculate a 5-L bioreactor.

[0770] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time, the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 57 hour fermentation was 3.9 kg. Induction was achieved by adding IPTG. The IPTG concentration was brought to 100 uM when the carbon dioxide evolution rate reached 100 mmol/L/hr. The OD_{550} profile within the bioreactor over time is shown in Figure 111A. The isoprene level in the off gas from the bioreactor was determined as described herein. The isoprene titer increased over the course of the fermentation to a final value of 1.6 g/L (Figure 111B). The specific productivity of isoprene over the course of the fermentation is shown in Figure 111C and peaked at 1.2 mg/OD/hr. The total amount of isoprene produced during the 57 hour fermentation was 16.2 g. The molar yield of utilized carbon that went into producing isoprene during fermentation was 0.9%. The weight percent yield of isoprene from glucose was 0.4%.

XIV. Production of isoprene from *E. coli* BL21 containing the kudzu isoprene synthase using glycerol as a carbon source.

[0771] A 15-L scale fermentation of *E. coli* expressing Kudzu isoprene synthase was used to produce isoprene from cells fed glycerol in fed-batch culture. This experiment

demonstrates that growing cells in the presence of glycerol (without glucose) resulted in the production of 2.2 mg/L of isoprene.

Medium Recipe (per liter fermentation medium):

[0772] The medium was generated using the following components per liter fermentation medium: K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, and 1000X modified trace metal solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glycerol 5.1 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0773] The medium was generated using the following components per liter fermentation medium: citric acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4$ * $2H_2O$ 100 mg. Each component was dissolved one at a time in diH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 micron filter.

[0774] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the pTrcKudzu plasmid. This experiment was carried out to monitor isoprene formation from glycerol at the desired fermentation pH 7.0 and temperature 35°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LA broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into soytone-yeast extract-glucose medium and grown at 35°C. After the inoculum grew to OD 1.0, measured at 550 nm, 600 mL was used to inoculate a 7.5-L bioreactor.

[0775] Glycerol was fed at an exponential rate until cells reached an optical density at 550 nm (OD_{550}) of 153. The total amount of glycerol delivered to the bioreactor during the 36 hour fermentation was 1.7 kg. Other than the glucose in the inoculum, no glucose was added to the bioreactor. Induction was achieved by adding IPTG. The IPTG concentration was brought to 20 uM when the OD_{550} reached a value of 50. The OD_{550} profile within the bioreactor over time is shown in Figure 57. The isoprene level in the off gas from the bioreactor was determined as described herein. The isoprene titer increased over the course of the fermentation to a final value of 2.2 mg/L (Figure 58). The total amount of isoprene

produced during the 54 hour fermentation was 20.9 mg, and the time course of production is shown in Figure 59.

XV. Isoprene fermentation from *E. coli* expressing genes from the mevalonic acid pathway and grown in fed-batch culture at the 15-L scale using invert sugar as a carbon source.

[0776] A 15-L scale fermentation of *E. coli* expressing mevalonic acid pathway polypeptides and Kudzu isoprene synthase was used to produce isoprene from cells fed invert sugar in fed-batch culture. This experiment demonstrates that growing cells in the presence of invert sugar resulted in the production of 2.4 g/L of isoprene.

Medium Recipe (per liter fermentation medium):

[0777] The medium was generated using the following components per liter fermentation medium: K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Invert sugar 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0778] The 1000X modified trace metal solution was generated using the following components: citric acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4$ * $2H_2O$ 100 mg. Each component is dissolved one at a time in DiH2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with 0.22 micron filter.

[0779] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids. This experiment was carried out to monitor isoprene formation from invert sugar at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 500 mL was used to inoculate a 5-L bioreactor.

[0780] Invert sugar was fed at an exponential rate until cells reached the stationary phase. After this time the invert sugar feed was decreased to meet metabolic demands. The total amount of invert sugar delivered to the bioreactor during the 44 hour fermentation was 2.4 kg. Induction was achieved by adding IPTG. The IPTG concentration was brought to 25 uM when the optical density at 550 nm (OD_{550}) reached a value of 9. The IPTG concentration was raised to 50 uM when OD_{550} reached 200. The OD_{550} profile within the bioreactor over time is shown in Figure 96. The isoprene level in the off gas from the bioreactor was determined as described herein. The isoprene titer increased over the course of the fermentation to a final value of 2.4 g/L (Figure 97). The total amount of isoprene produced during the 44 hour fermentation was 18.4 g and the time course of production is shown in Figure 98. The molar yield of utilized carbon that went into producing isoprene during fermentation was 1.7%. The weight percent yield of isoprene from glucose was 0.8%.

Example 9. Construction of the upper and lower MVA pathway for integration into *Bacillus subtilis*

I. Construction of the Upper MVA pathway in *Bacillus subtilis*.

[0781] The upper pathway from *Enterococcus faecalis* is integrated into *B. subtilis* under control of the *aprE* promoter. The upper pathway consists of two genes; *mvaE*, which encodes for AACT and HMGR, and *mvaS*, which encodes for HMGS. The two genes are fused together with a stop codon in between, an RBS site in front of *mvaS*, and are under the control of the *aprE* promoter. A terminator is situated after the *mvaE* gene. The chloramphenicol resistance marker is cloned after the *mvaE* gene and the construct is integrated at the *aprE* locus by double cross over using flanking regions of homology.

[0782] Four DNA fragments are amplified by PCR such that they contain overhangs that will allowed them to be fused together by a PCR reaction. PCR amplifications are carried out using Herculase polymerase according to manufacturer's instructions.

1. PaprE

CF 07-134 (+) Start of *aprE* promoter PstI

5'- GACATCTGCAGCTCCATTTTCTTCTGC (SEQ ID NO:115)

CF 07-94 (-) Fuse PaprE to *mvaE*

5'- CAATAATAACTACTGTTTTCACTCTTTACCCTCTCCTTTTAA (SEQ ID NO:116)
Template: *Bacillus subtilis* chromosomal DNA

2. *mvaE*
CF 07-93 (+) fuse *mvaE* to the *aprE* promoter (GTG start codon)
5'- TTAAAAGGAGAGGGTAAAGAGTGAAAACAGTAGTTATTATTG (SEQ ID NO:117)

CF 07-62 (-) Fuse *mvaE* to *mvaS* with RBS in between
5'- TTTATCAATCCCAATTGTCATGTTTTTTTACCTCCTTTATTGTTTTCTTAAATC (SEQ ID NO:94)

Template: *Enterococcus faecalis* chromosomal DNA (from ATCC)

3. *mvaS*
CF 07-61 (+) Fuse *mvaE* to *mvaS* with RBS in between
5'-
GATTTAAGAAAACAATAAAGGAGGTAAAAAAACATGACAATTGGGATTGATAAA (SEQ ID NO:95)

CF 07-124 (-) Fuse the end of *mvaS* to the terminator
5'- CGGGGCCAAGGCCGGTTTTTTTTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:118)
Template: *Enterococcus faecalis* chromosomal DNA

4. *B. amyliquefaciens* alkaline serine protease terminator
CF 07-123 (+) Fuse the end of mvaS to the terminator
5'- ACCGTTCGTTCTTATCGAAACTAAAAAAAACCGGCCTTGGCCCCG (SEQ ID NO:119)

CF 07-46 (-) End of *B. amyliquefaciens* terminator BamHI
5'- GACATGACGGATCCGATTACGAATGCCGTCTC (SEQ ID NO:58)

Template: *Bacillus amyliquefaciens* chromosomal DNA

PCR Fusion Reactions

5. Fuse *mvaE* to *mvaS*

CF 07-93 (+) fuse *mvaE* to the *aprE* promoter (GTG start codon)

5'- TTAAAAGGAGAGGGTAAAGAGTGAAAACAGTAGTTATTATTG (SEQ ID NO:117)

CF 07-124 (-) Fuse the end of *mvaS* to the terminator

5'- CGGGGCCAAGGCCGGTTTTTTTTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:118)

Template: #2 and 3 from above

6. Fuse *mvaE-mvaS* to *aprE* promoter

CF 07-134 (+) Start of *aprE* promoter PstI

5'- GACATCTGCAGCTCCATTTTCTTCTGC (SEQ ID NO:115)

CF 07-124 (-) Fuse the end of *mvaS* to the terminator

5'- CGGGGCCAAGGCCGGTTTTTTTTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:118)

Template #1 and #4 from above

7. Fuse PaprE-*mvaE-mvaS* to terminator

CF 07-134 (+) Start of *aprE* promoter PstI

5'- GACATCTGCAGCTCCATTTTCTTCTGC (SEQ ID NO:115)

CF 07-46 (-) End of *B. amyliquefaciens* terminator BamHI

5'- GACATGACGGATCCGATTACGAATGCCGTCTC (SEQ ID NO:58)

Template: #4 and #6

[0783] The product is digested with restriction endonucleases *Pst*I/*Bam*HI and ligated to pJM102 (Perego, M. 1993. Integrational vectors for genetic manipulation in *Bacillus subtilis*, p. 615–624. In A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington,D.C.) which is digested with *Pst*I/*Bam*HI. The ligation is transformed into *E.coli* TOP 10 chemically competent cells and transformants are selected on LA containing carbenicillin (50 μg/ml). The correct plasmid is identified by sequencing and is designated pJMUpperpathway2 (Figures 50 and 51; SEQ ID NO:22). Purified plasmid DNA is transformed into *Bacillus subtilis aprEnprE* Pxyl-*comK* and transformants are selected on L agar containing chloramphenicol (5 μg/ml). A correct colony is selected and is plated sequentially on L agar containing chloramphenicol 10, 15 and 25 μg/ml to amplify the number of copies of the cassette containing the upper pathway.

[0784] The resulting strain is tested for mevalonic acid production by growing in LB containing 1% glucose and 1%. Cultures are analyzed by GC for the production of mevalonic acid.

[0785] This strain is used subsequently as a host for the integration of the lower mevalonic acid pathway.

[0786] The following primers are used to sequence the various constructs above.

Sequencing primers:
CF 07-134 (+) Start of *aprE* promoter PstI
5'- GACATCTGCAGCTCCATTTTCTTCTGC (SEQ ID NO:115)

CF 07-58 (+) Start of *mvaE* gene
5'- ATGAAAACAGTAGTTATTATTGATGC (SEQ ID NO:97)

CF 07-59 (-) End of *mvaE* gene
5'- ATGTTATTGTTTTCTTAAATCATTTAAAATAGC (SEQ ID NO:98)

CF 07-82 (+) Start of *mvaS* gene
5'- ATGACAATTGGGATTGATAAAATTAG (SEQ ID NO:99)

CF 07-83 (-) End of *mvaS* gene
5'- TTAGTTTCGATAAGAACGAACGGT (SEQ ID NO:100)

CF 07-86 (+) Sequence in *mvaE*
5'- GAAATAGCCCCATTAGAAGTATC (SEQ ID NO:101)

CF 07-87 (+) Sequence in *mvaE*
5'- TTGCCAATCATATGATTGAAAATC (SEQ ID NO:102)

CF 07-88 (+) Sequence in *mvaE*
5'- GCTATGCTTCATTAGATCCTTATCG (SEQ ID NO:103)

CF 07-89 (+) Sequence *mvaS*
5'- GAAACCTACATCCAATCTTTTGCCC (SEQ ID NO:104)

[0787] Transformants are selected on LA containing chloramphenicol at a concentration of 5 μg/ml. One colony is confirmed to have the correct integration by sequencing and is plated on LA containing increasing concentrations of chloramphenicol over several days, to a final level of 25 μg/ml. This results in amplification of the cassette containing the genes of interest. The resulting strain is designated CF 455: pJMupperpathway#1 X *Bacillus subtilis aprEnprE* Pxyl *comK* (amplified to grow on LA containing chloramphenicol 25 μg/ml).

II. Construction of the Lower MVA pathway in *Bacillus subtilis*.

[0788] The lower MVA pathway, consisting of the genes mvk1, pmk, mpd and idi are combined in a cassette consisting of flanking DNA regions from the nprE region of the *B. subtilis* chromosome (site of integration), the *aprE* promoter, and the spectinomycin resistance marker (see Figures 28 and 29; SEQ ID NO:13). This cassette is synthesized by DNA2.0 and is integrated into the chromosome of *B. subtilis* containing the upper MVA pathway integrated at the aprE locus. The kudzu isoprene synthase gene is expressed from the

replicating plasmid described in Example 4 and is transformed into the strain with both upper and lower pathways integrated.

Example 10: Exemplary isoprene compositions and methods of making them

I. Compositional analysis of fermentation off-gas containing isoprene.

[0789] A 14 L scale fermentation was performed with a recombinant *E. coli* BL21 (DE3) strain containing two plasmids (pCL upperMev; pTrcKKDyIkIS encoding the full mevalonate pathway for isoprenoid precursor biosynthesis, an isoprenyl pyrophosphate isomerase from yeast, and an isoprene synthase from Kudzu. Fermentation off-gas from the 14 L tank was collected into 20 mL headspace vials at around the time of peak isoprene productivity (27.9 hours elapsed fermentation time, "EFT") and analyzed by headspace GC/MS for volatile components.

[0790] Headspace analysis was performed with an Agilent 6890 GC/MS system fitted with an Agilent HP-5MS GC/MS column (30 m x 250 μm; 0.25 μm film thickness). A combiPAL autoinjector was used for sampling 500 uL aliquots from 20 mL headspace vials. The GC/MS method utilized helium as the carrier gas at a flow of 1 mL/min. The injection port was held at 250 ^{o}C with a split ratio of 50:1. The oven temperature was held at 37 ^{o}C for an initial 2 minute period, followed an increase to 237 ^{o}C at a rate of 25 ^{o}C/min for a total method time of 10 minutes. The Agilent 5793N mass selective detector scanned from m/z 29 to m/z 300. The limit of detection of this system is approximately 0.1 ug/L_{gas} or approximately 0.1 ppm. If desired, more sensitive equipment with a lower limit of detection may be used.

[0791] The off-gas consisted of 99.925 % (v/v) permanent gases (N_2, CO_2 and O_2), approximately 0.075% isoprene (2-methyl-1,3-butadiene) (~750 ppmv, 2100 μg/L) and minor amounts (<50 ppmv) of ethanol, acetone, and two C5 prenyl alcohols. The amount of water vapor was not determined but was estimated to be equal to the equilibrium vapor pressure at 0 ^{o}C. The composition of the volatile organic fraction was determined by integration of the area under the peaks in the GC/MS chromatogram (Figs. 86A and 86B) and is listed in Table 6. Calibration curves for ethanol and acetone standards enabled the conversion of GC area to gas phase concentration in units of ug/L using standard methods.

Table 6. Composition of volatile organic components in fermentation off-gas. The off-gas was analyzed at the 27.9 hour time point of a fermentation using an E. coli BL21 (DE3) strain

expressing a heterologous mevalonate pathway, an isoprenyl pyrophosphate isomerase from yeast, and an isoprene synthase from Kudzu.

Compound	RT (min)	GC area	Area %	Conc. (ug/L)
Ethanol	1.669	239005	0.84	62 +/- 6
Acetone	1.703	288352	1.02	42 +/- 4
Isoprene (2-methyl-1,3-butadiene)	1.829	27764544	97.81	2000 +/- 200
3-methyl-3-buten-1-ol	3.493	35060	0.12	<10
3-methyl-2-buten-1-ol	4.116	58153	0.20	<10

II. Measurement of trace volatile organic compounds (VOCs) co-produced with isoprene during fermentation of a recombinant E. coli strain.

[0792] A 14 L scale fermentation was performed with a recombinant *E. coli* BL21 (DE3) strain containing two plasmids (pCL upperMev; pTrcKKDyIkIS) encoding the full mevalonate pathway for isoprenoid precursor biosynthesis, an isoprenyl pyrophosphate isomerase from yeast, and an isoprene synthase from Kudzu.

[0793] Fermentation off-gas was passed through cooled headspace vials in order to concentrate and identify trace volatile organic components. The off-gas from this fermentation was sampled at a rate of 1 L/min for 10 minutes through a 20 mL headspace vial packed with quartz wool (2g) and cooled to –78 ^{o}C with dry ice. The vial was recapped with a fresh vial cap and analyzed by headspace GC/MS for trapped VOCs using the conditions described in Example 10, part I. The ratios of compounds observed in Figs. 87A-87D are a combination of overall level in the fermentation off-gas, the relative vapor pressure at -78 ^{o}C, and the detector response of the mass spectrometer. For example, the low level of isoprene relative to oxygenated volatiles (*e.g.*, acetone and ethanol) is a function of the high volatility of this material such that it does not accumulate in the headspace vial at -78 ^{o}C.

[0794] The presence of many of these compounds is unique to isoprene compositions derived from biological sources. The results are depicted in Figs. 87A-87D and summarized in Tables 7A and 7B.

Table 7A: Trace volatiles present in off-gas produced by E. coli BL21 (DE3) (pCL upperMev; pTrcKKDyIkIS) following cryo-trapping at -78°C.

Compound	RT (min)	GC Area1	Area%2	Ratio%3

Compound	RT (min)	GC Area1	Area%2	Ratio%3
Acetaldehyde	1.542	4019861	4.841	40.14
Ethanol	1.634	10553620	12.708	105.39
Acetone	1.727	7236323	8.714	72.26
2-methyl-1,3-butadiene	1.777	10013714	12.058	100.00
1-propanol	1.987	163574	0.197	1.63
Diacetyl	2.156	221078	0.266	2.21
2-methyl-3-buten-2-ol	2.316	902735	1.087	9.01
2-methyl-1-propanol	2.451	446387	0.538	4.46
3-methyl-1-butanal	2.7	165162	0.199	1.65
1-butanol	2.791	231738	0.279	2.31
3-methyl-3-buten-1-ol	3.514	14851860	17.884	148.32
3-methyl-1-butanol	3.557	8458483	10.185	84.47
3-methyl-2-buten-1-ol	4.042	18201341	21.917	181.76
3-methyl-2-butenal	4.153	1837273	2.212	18.35
3-methylbutyl acetate	5.197	196136	0.236	1.96
3-methyl-3-buten-1-yl acetate	5.284	652132	0.785	6.51
2-heptanone	5.348	67224	0.081	0.67
2,5-dimethylpyrazine	5.591	58029	0.070	0.58
3-methyl-2-buten-1-yl acetate	5.676	1686507	2.031	16.84
6-methyl-5-hepten-2-one	6.307	101797	0.123	1.02
2,4,5-trimethylpyridine	6.39	68477	0.082	0.68
2,3,5-trimethylpyrazine	6.485	30420	0.037	0.30
(E)-3,7-dimethyl-1,3,6-octatriene	6.766	848928	1.022	8.48
(Z)-3,7-dimethyl-1,3,6-octatriene	6.864	448810	0.540	4.48
3-methyl-2-but-1-enyl butyrate	7.294	105356	0.127	1.05
Citronellal	7.756	208092	0.251	2.08
2,3-cycloheptenolpyridine	8.98	1119947	1.349	11.18

1 GC area is the uncorrected area under the peak corresponding to the listed compound.
2 Area % is the peak area expressed as a % relative to the total peak area of all compounds.
3 Ratio % is the peak area expressed as a % relative to the peak area of 2-methyl-1,3-butadiene.

Table 7B. Trace volatiles present in off-gas produced by E. coli BL21 (DE3) (pCL upperMev; pTrcKKDyIkIS) following cryo-trapping at -196°C.

Compound	RT (min)	GC Area1	Area%2	Ratio%3
Acetaldehyde	1.54	1655710	0.276	0.33
Methanethiol	1.584	173620	0.029	0.03
Ethanol	1.631	10259680	1.707	2.03

Compound	RT (min)	GC Area1	Area%2	Ratio%3
Acetone	1.722	73089100	12.164	14.43
2-methyl-1,3-butadiene	1.771	506349429	84.269	100.00
methyl acetate	1.852	320112	0.053	0.06
1-propanol	1.983	156752	0.026	0.03
Diacetyl	2.148	67635	0.011	0.01
2-butanone	2.216	254364	0.042	0.05
2-methyl-3-buten-2-ol	2.312	684708	0.114	0.14
ethyl acetate	2.345	2226391	0.371	0.44
2-methyl-1-propanol	2.451	187719	0.031	0.04
3-methyl-1-butanal	2.696	115723	0.019	0.02
3-methyl-2-butanone	2.751	116861	0.019	0.02
1-butanol	2.792	54555	0.009	0.01
2-pentanone	3.034	66520	0.011	0.01
3-methyl-3-buten-1-ol	3.516	1123520	0.187	0.22
3-methyl-1-butanol	3.561	572836	0.095	0.11
ethyl isobutyrate	3.861	142056	0.024	0.03
3-methyl-2-buten-1-ol	4.048	302558	0.050	0.06
3-methyl-2-butenal	4.152	585690	0.097	0.12
butyl acetate	4.502	29665	0.005	0.01
3-methylbutyl acetate	5.194	271797	0.045	0.05
3-methyl-3-buten-1-yl acetate	5.281	705366	0.117	0.14
3-methyl-2-buten-1-yl acetate	5.675	815186	0.136	0.16
(E)-3,7-dimethyl-1,3,6-octatriene	6.766	207061	0.034	0.04
(Z)-3,7-dimethyl-1,3,6-octatriene	6.863	94294	0.016	0.02
2,3-cycloheptenolpyridine	8.983	135104	0.022	0.03

1 GC area is the uncorrected area under the peak corresponding to the listed compound.
2 Area % is the peak area expressed as a % relative to the total peak area of all compounds.
3 Ratio % is the peak area expressed as a % relative to the peak area of 2-methyl-1,3-butadiene.

III. Absence of C5 hydrocarbon isomers in isoprene derived from fermentation.

[0795] Cryo-trapping of isoprene present in fermentation off-gas was performed using a 2 mL headspace vial cooled in liquid nitrogen. The off-gas (1 L/min) was first passed through a 20 mL vial containing sodium hydroxide pellets in order to minimize the accumulation of ice and solid CO_2 in the 2 mL vial (-196 ^{o}C). Approximately 10L of off-gas was passed through the vial, after which it was allowed to warm to -78 ^{o}C with venting, followed by resealing with a fresh vial cap and analysis by GC/MS.

[0796] GC/MS headspace analysis was performed with an Agilent 6890 GC/MS system using a 100uL gas tight syringe in headspace mode. A Zebron ZB-624 GC/MS column (30 m x 250 μm; 1.40 μm film thickness) was used for separation of analytes. The GC autoinjector was fitted with a gas-tight 100 uL syringe, and the needle height was adjusted to allow the injection of a 50 uL headspace sample from a 2 mL GC vial. The GC/MS method utilized helium as the carrier gas at a flow of 1 mL/min. The injection port was held at 200°C with a split ratio of 20:1. The oven temperature was held at 37 °C for the 5 minute duration of the analysis. The Agilent 5793N mass selective detector was run in single ion monitoring (SIM) mode on m/z 55, 66, 67 and 70. Under these conditions, isoprene was observed to elute at 2.966 minutes (Figure 88B). A standard of petroleum derived isoprene (Sigma-Aldrich) was also analyzed using this method and was found to contain additional C5 hydrocarbon isomers, which eluted shortly before or after the main peak and were quantified based on corrected GC area (Figure 88A).

Table 8A: GC/MS analysis of petroleum-derived isoprene

Compound	RT (min)	GC area	Area % of total C5 hydrocarbons
2-methyl-1-butene	2.689	18.2 x 103	0.017%
(Z)-2-pentene	2.835	10.6x 104	0.101%
Isoprene	2.966	10.4x 107	99.869%
1,3-cyclopentadiene (CPD)	3.297	12.8 x 103	0.012%

Table 8B: GC/MS analysis of fermentation-derived isoprene (% total C5 hydrocarbons)

Compound	RT (min)	Corrected GC Area	% of total C5 hydrocarbons
Isoprene	2.966	8.1 x 107	100%

[0797] In a separate experiment, a standard mixture of C5 hydocarbons was analyzed to determine if the detector response was the same for each of the compounds. The compounds were 2-methyl-1-butene, 2-methyl-1,3-butadiene, (E)-2-pentene, (Z)-2-pentene and (E)-1,3-pentadiene. In this case, the analysis was performed on an Agilent DB-Petro column (100 m x 0.25 mm, 0.50 um film thickness) held at 50 °C for 15 minutes. The GC/MS method utilized helium as the carrier gas at a flow of 1 mL/min. The injection port was held at 200 °C with a split ratio of 50:1. The Agilent 5793N mass selective detector was run in full scan

mode from m/z 19 to m/z 250. Under these conditions, a 100 ug/L concentration of each standard produced the same detector response within experimental error.

IV. Compositions comprising isoprene adsorbed to a solid phase.

[0798] Biologically-produced isoprene was adsorped to activated carbon resulting in a solid phase containing 50 to 99.9% carbon, 0.1% to 50% isoprene, 0.01% to 5% water, and minor amounts (<0.1%) of other volatile organic components.

[0799] Fermentation off-gas was run through a copper condensation coil held at 0 ^{o}C, followed by a granulated silica desiccant filter in order to remove water vapor. The dehumidified off-gas was then run through carbon containing filters (Koby Jr, Koby Filters, MA) to the point at which breakthrough of isoprene was detected in the filter exhaust by GC/MS. The amount of isoprene adsorped to the cartridge can be determined indirectly by calculating the concentration in the off-gas, the overall flow rate and the percent breakthrough over the collection period. Alternately the adsorped isoprene can be recovered from the filters by thermal, vacuum, or solvent-mediated desorption.

V. Collection and analysis of condensed isoprene.

[0800] Fermentation off-gas is dehumidified, and the CO_2 removed by filtration through a suitable adsorbant (*e.g.*, ascarite). The resulting off-gas stream is then run through a liquid nitrogen-cooled condenser in order to condense the VOCs in the stream. The collection vessel contains t-butyl catechol to inhibit the resulting isoprene condensate. The condensate is analyzed by GC/MS and NMR in order to determine purity using standard methods, such as those described herein.

VI. Production of prenyl alcohols by fermentation.

[0801] Analysis of off-gas from an *E. coli* BL21 (DE3) strain expressing a Kudzu isoprene synthase revealed the presence of both isoprene and 3-methyl-3-buten-1-ol (isoprenol). The levels of the two compounds in the fermentation off-gas over the fermentation are shown in Figure 89 as determined by headspace GC/MS. Levels of isoprenol (3-methyl-3-buten-1-ol, 3-MBA) attained was nearly 10 ug/L_{offgas} in this experiment. Additional experiments produced levels of approximately 20 ug/L_{offgas} in the fermentation off-gas.

Example 11: The decoupling of growth and production of isoprene in *E. coli* expressing genes from the mevalonic acid pathway and fermented in a fed-batch culture

[0802] Example 11 illustrates the decoupling of cell growth from mevalonic acid and isoprene production.

I. Fermentation Conditions

Medium Recipe (per liter fermentation medium):

[0803] The medium was generated using the following components per liter fermentation medium: K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X modified trace metal solution 1 ml. All of the components were added together and dissolved in diH2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0804] The 1000X modified trace metal solution was generated using the following components: citric acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, CoCl2 * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4$ * $2H_2O$ 100 mg. Each component was dissolved one at a time in DiH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume, and filter sterilized with a 0.22 micron filter.

[0805] Fermentation was performed with *E. coli* cells containing the pTrcHis2AUpperPathway (also called pTrcUpperMVA, Figures 91 and 92A-92C; SEQ ID NO:23) (50 μg/ml carbenicillin) or the pCL PtrcUpperMVA (also called pCL PtrcUpperPathway (Figure 26)) (50 μg/ml spectinomycin) plasmids. For experiments in which isoprene was produced, the *E. coli* cells also contained the pTrc KKDyIkIS (50 μg/ml kanamycin) plasmid. These experiments were carried out to monitor mevalonic acid or isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of an *E. coli* strain taken from a frozen vial was streaked onto an LA broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into tryptone-yeast extract medium. After the inoculum grew to optical density 1.0 when measured at 550 nm, it was used to inoculate the bioreactor.

[0806] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. Induction was achieved by adding IPTG. The mevalonic acid concentration in fermentation broth was determined by applying perchloric acid (Sigma-Aldrich # 244252) treated samples (0.3 M incubated at 4°C for 5 minutes) to an organic acids HPLC column (BioRad # 125-0140). The concentration was determined by comparing the broth mevalonic acid peak size to a calibration curve generated from mevalonolacetone (Sigma-Aldrich # M4667) treated with perchloric acid to form D,L-mevalonate. The isoprene level in the off gas from the bioreactor was determined as described herein. The isoprene titer is defined as the amount of isoprene produced per liter of fermentation broth.

II. Mevalonic acid production from *E. coli* BL21 (DE3) cells expressing the pTrcUpperMVA plasmid at a 150-L scale

[0807] BL21 (DE3) cells that were grown on a plate as explained above in Example 11, part I were inoculated into a flask containing 45 mL of tryptone-yeast extract medium and incubated at 30°C with shaking at 170 rpm for 5 hours. This solution was transferred to a 5-L bioreactor of tryptone-yeast extract medium, and the cells were grown at 30 °C and 27.5 rpm until the culture reached an OD_{550} of 1.0. The 5 L of inoculum was seeded into a 150-L bioreactor containing 45-kg of medium. The IPTG concentration was brought to 1.1 mM when the OD_{550} reached a value of 10. The OD_{550} profile within the bioreactor over time is shown in Figure 60A. The mevalonic acid titer increased over the course of the fermentation to a final value of 61.3 g/L (Figure 60B). The specific productivity profile throughout the fermentation is shown in Figure 60C and a comparison to Figure 60A illustrates the decoupling of growth and mevalonic acid production. The total amount of mevalonic acid produced during the 52.5 hour fermentation was 4.0 kg from 14.1 kg of utilized glucose. The molar yield of utilized carbon that went into producing mevalonic acid during fermentation was 34.2%.

III. Mevalonic acid production from *E. coli* BL21 (DE3) cells expressing the pTrcUpperMVA plasmid at a 15-L scale

[0808] BL21 (DE3) cells that were grown on a plate as explained above in Example 11, part I were inoculated into a flask containing 500 mL of tryptone-yeast extract medium and grown at 30 °C at 160 rpm to OD_{550} 1.0. This material was seeded into a 15-L bioreactor

containing 4.5-kg of medium. The IPTG concentration was brought to 1.0 mM when the OD_{550} reached a value of 10. The OD_{550} profile within the bioreactor over time is shown in Figure 61A. The mevalonic acid titer increased over the course of the fermentation to a final value of 53.9 g/L (Figure 61B). The specific productivity profile throughout the fermentation is shown in Figure 61C and a comparison to Figure 61A illustrates the decoupling of growth and mevalonic acid production. The total amount of mevalonic acid produced during the 46.6 hour fermentation was 491 g from 2.1 kg of utilized glucose. The molar yield of utilized carbon that went into producing mevalonic acid during fermentation was 28.8%.

IV. Mevalonic acid production from *E. coli* FM5 cells expressing the pTrcUpperMVA plasmid at a 15-L scale

[0809] FM5 cells that were grown on a plate as explained above in Example 11, part I were inoculated into a flask containing 500 mL of tryptone-yeast extract medium and grown at 30 °C at 160 rpm to OD_{550} 1.0. This material was seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration was brought to 1.0 mM when the OD_{550} reached a value of 30. The OD_{550} profile within the bioreactor over time is shown in Figure 62A. The mevalonic acid titer increased over the course of the fermentation to a final value of 23.7 g/L (Figure 62B). The specific productivity profile throughout the fermentation is shown in Figure 62C and a comparison to Figure 62A illustrates the decoupling of growth and mevalonic acid production. The total amount of mevalonic acid produced during the 51.2 hour fermentation was 140 g from 1.1 kg of utilized glucose. The molar yield of utilized carbon that went into producing mevalonic acid during fermentation was 15.2%.

V. Isoprene production from *E. coli* BL21 (DE3) cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids at a 15-L scale

[0810] BL21 (DE3) cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids that were grown on a plate as explained above in Example 11, part I were inoculated into a flask containing 500 mL of tryptone-yeast extract medium and grown at 30 °C at 160 rpm to OD_{550} 1.0. This material was seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration was brought to 25 μM when the OD_{550} reached a value of 10. The IPTG concentration was raised to 50 uM when OD_{550} reached 190. The IPTG concentration was raised to 100 uM at 38 hours of fermentation. The OD_{550} profile within the bioreactor over time is shown in Figure 63A. The isoprene titer increased over the

course of the fermentation to a final value of 2.2 g/L broth (Figure 63B). The specific productivity profile throughout the fermentation is shown in Figure 63C and a comparison to Figure 63A illustrates the decoupling of growth and isoprene production. The total amount of isoprene produced during the 54.4 hour fermentation was 15.9 g from 2.3 kg of utilized glucose. The molar yield of utilized carbon that went into producing isoprene during fermentation was 1.53%.

VI. Isoprene production from *E. coli* BL21 (DE3) tuner cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids at a 15-L scale

[0811] BL21 (DE3) tuner cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids that were grown on a plate as explained above in Example 11, part I were inoculated into a flask containing 500 mL of tryptone-yeast extract medium and grown at 30 °C at 160 rpm to OD_{550} 1.0. This material was seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration was brought to 26 μM when the OD_{550} reached a value of 10. The IPTG concentration was raised to 50 uM when OD_{550} reached 175. The OD_{550} profile within the bioreactor over time is shown in Figure 64A. The isoprene titer increased over the course of the fermentation to a final value of 1.3 g/L broth (Figure 64B). The specific productivity profile throughout the fermentation is shown in Figure 64C and a comparison to Figure 64A illustrates the decoupling of growth and isoprene production. The total amount of isoprene produced during the 48.6 hour fermentation was 9.9 g from 1.6 kg of utilized glucose. The molar yield of utilized carbon that went into producing isoprene during fermentation was 1.34%.

VII. Isoprene production from *E. coli* MG1655 cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids at a 15-L scale

[0812] MG1655 cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids that were grown on a plate as explained above in Example 11, part I were inoculated into a flask containing 500 mL of tryptone-yeast extract medium and grown at 30 °C at 160 rpm to OD_{550} 1.0. This material was seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration was brought to 24 μM when the OD_{550} reached a value of 45. The OD_{550} profile within the bioreactor over time is shown in Figure 65A. The isoprene titer increased over the course of the fermentation to a final value of 393 mg/L broth (Figure 65B). The specific productivity profile throughout the fermentation is shown in Figure 65C and a

comparison to Figure 65A illustrates the decoupling of growth and isoprene production. The total amount of isoprene produced during the 67.4 hour fermentation was 2.2 g from 520 g of utilized glucose. The molar yield of utilized carbon that went into producing isoprene during fermentation was 0.92%.

VIII. Isoprene production from *E. coli* MG1655ack-pta cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids at a 15-L scale

[0813] MG1655ack-pta cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids that were grown on a plate as explained above in Example 11, part I were inoculated into a flask containing 500 mL of tryptone-yeast extract medium and grown at 30 °C at 160 rpm to OD_{550} 1.0. This material was seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration was brought to 30 μM when the OD_{550} reached a value of 10. The OD_{550} profile within the bioreactor over time is shown in Figure 66A. The isoprene titer increased over the course of the fermentation to a final value of 368 mg/L broth (Figure 66B). The specific productivity profile throughout the fermentation is shown in Figure 66C and a comparison to Figure 66A illustrates the decoupling of growth and isoprene production. The total amount of isoprene produced during the 56.7 hour fermentation was 1.8 g from 531 g of utilized glucose. The molar yield of utilized carbon that went into producing isoprene during fermentation was 0.73%.

IX. Isoprene production from *E. coli* FM5 cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids at a 15-L scale

[0814] FM5 cells expressing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids that were grown on a plate as explained above in Example 11, part I were inoculated into a flask containing 500 mL of tryptone-yeast extract medium and grown at 30 °C at 160 rpm to OD_{550} 1.0. This material was seeded into a 15-L bioreactor containing 4.5-kg of medium. The IPTG concentration was brought to 27 μM when the OD_{550} reached a value of 15. The OD_{550} profile within the bioreactor over time is shown in Figure 67A. The isoprene titer increased over the course of the fermentation to a final value of 235 mg/L broth (Figure 67B). The specific productivity profile throughout the fermentation is shown in Figure 67C and a comparison to Figure 67A illustrates the decoupling of growth and isoprene production. The total amount of isoprene produced during the 52.3 hour fermentation was 1.4 g from 948 g of

utilized glucose. The molar yield of utilized carbon that went into producing isoprene during fermentation was 0.32%.

Example 12: Production of isoprene during the exponential growth phase of *E. coli* expressing genes from the mevalonic acid pathway and fermented in a fed-batch culture

[0815] Example 12 illustrates the production of isoprene during the exponential growth phase of cells.

Medium Recipe (per liter fermentation medium):

[0816] The medium was generated using the following components per liter fermentation medium: K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X modified trace metal solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0817] The 1000X modified trace metal solution was generated using the following components: citric acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, CoCl2 * $6H_2O$ 1 g, ZnSO * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4$ * $2H_2O$ 100 mg. Each component is dissolved one at a time in DiH2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with 0.22 micron filter.

[0818] Fermentation was performed in a 15-L bioreactor with ATCC11303 *E. coli* cells containing the pCL PtrcUpperMVA and pTrc KKDyIkIS plasmids. This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 500 mL was used to inoculate a 5-L bioreactor.

[0819] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 50 hour fermentation was 2.0 kg. Induction

was achieved by adding IPTG. The IPTG concentration was brought to 25 uM when the optical density at 550 nm (OD_{550}) reached a value of 10. The IPTG concentration was raised to 50 uM when OD_{550} reached 190. The OD_{550} profile within the bioreactor over time is shown in Figure 99. The isoprene level in the off gas from the bioreactor was determined as described herein. The isoprene titer increased over the course of the fermentation to a final value of 1.4 g/L (Figure 100). The total amount of isoprene produced during the 50 hour fermentation was 10.0 g. The profile of the isoprene specific productivity over time within the bioreactor is shown in Figure 101. The molar yield of utilized carbon that contributed to producing isoprene during fermentation was 1.1%. The weight percent yield of isoprene from glucose was 0.5%.

Example 13: Flammability modeling and testing of isoprene

I. Summary of flammability modeling and testing of isoprene

[0820] Flammability modeling and experiments were performed for various hydrocarbon/oxygen/nitrogen/water/carbon dioxide mixtures. This modeling and experimental tested was aimed at defining isoprene and oxygen/nitrogen flammability curves under specified steam and carbon monoxide concentrations at a fixed pressure and temperature. A matrix of the model conditions is shown in Table 9, and a matrix of the experiments performed is shown in Table 5.

Table 9. Summary of Modeled Isoprene Flammability

Series	Temperature (°C)	Pressure (psig)	Steam Concentration (wt%)	Carbon Dioxide Concentration (wt. %)	Isoprene Concentration (vol. %)	Oxygen Concentration (vol. %)
A	40	0	0	0	Varying	Varying
B	40	0	4	0	Varying	Varying
C	40	0	0	5	Varying	Varying
D	40	0	0	10	Varying	Varying
E	40	0	0	15	Varying	Varying
F	40	0	0	20	Varying	Varying
G	40	0	0	30	Varying	Varying

Table 10. Summary of Isoprene Flammability Tests

Series Number	Temperature (°C)	Pressure (psig)	Steam Concentration (vol. %)	Isoprene Concentration (vol. %)	Oxygen Concentration (vol. %)
1	40	0	0	Varying	Varying
2	40	0	4	Varying	Varying

II. Description of calculated adiabatic flame temperature (CAFT) model

[0821] Calculated adiabatic flame temperatures (CAFT) along with a selected limit flame temperature for combustion propagation were used to determine the flammability envelope for isoprene. The computer program used in this study to calculate the flame temperatures is the NASA Glenn Research Center CEA (Chemical Equilibrium with Applications) software.

[0822] There are five steps involved in determining the flammability envelope using an adiabatic flame temperature model for a homogeneous combustion mechanism (where both the fuel and oxidant are in the gaseous state): selection of the desired reactants, selection of the test condition, selection of the limit flame temperature, modification of the reactants, and construction of a flammability envelope from calculations.

[0823] In this first step, selection of desired reactants, a decision must be made as to the reactant species that will be present in the system and the quantities of each. In many cases the computer programs used for the calculations have a list of reactant and product species. If any of the data for the species to be studied are not found in the program, they may be obtained from other sources such as the JANAF tables or from the internet. In this current model data for water, nitrogen, oxygen and carbon dioxide were present in the program database. The program database did not have isoprene as a species; therefore the thermodynamic properties were incorporated manually.

[0824] The next step is to decide whether the initial pressure and temperature conditions that the combustion process is taking place in. In this model the pressure was 1 atmosphere (absolute) and the temperature was 40°C, the boiling point of isoprene.

[0825] The limit flame temperature for combustion can be either selected based on theoretical principles or determined experimentally. Each method has its own limitations.

[0826] Based on prior studies, the limit flame temperatures of hydrocarbons fall in the range of 1000 K to 1500 K. For this model, the value of 1500 K was selected. This is the temperature at which the reaction of carbon monoxide to carbon dioxide (a highly exothermic reaction and constitutes a significant proportion of the flame energy) becomes self sustaining.

[0827] Once the limit flame temperature has been decided upon, model calculations are performed on the given reactant mixture (species concentrations) and the adiabatic flame temperature is determined. Flame propagation is considered to have occurred only if the temperature is greater than the limit flame temperature. The reactant mixture composition is then modified to create data sets for propagation and non-propagation mixtures.

[0828] This type of model shows good agreement with the experimentally determined flammability limits. Regions outside the derived envelope are nonflammable and regions within it are flammable. The shape of the envelope forms a nose. The nose of the envelope is related to the limiting oxygen concentration (LOC) for gaseous fuels.

III. Results from calculated adiabatic flame temperature (CAFT) model

[0829] Plotted in Figs. 68 through 74 are the CAFT model results for Series A to G, respectively. The figures plot the calculated adiabatic flame temperature (using the NASA CEA program) as a function of fuel concentration (by weight) for several oxygen/nitrogen ratios (by weight). The parts of the curve that are above 1500 K, the selected limit flame temperature, contain fuel levels sufficient for flame propagation. The results may be difficult to interpret in the form presented in Figs. 68 through 74. Additionally, the current form is not conducive to comparison with experimental data which is generally presented in terms of volume percent.

[0830] Using Series A as an example the data in Figure 68 can be plotted in the form of a traditional flammability envelope. Using Figure 68 and reading across the 1500 K temperature line on the ordinate one can determine the fuel concentration for this limit flame temperature by dropping a tangent to the abscissa for each curve (oxygen to nitrogen ratio) that it intersects. These values can then be tabulated as weight percent of fuel for a given weight percent of oxidizer (Figure 75A). Then knowing the composition of the fuel (100

wt.% isoprene) and the composition of the oxidizer (relative content of water, oxygen and nitrogen) molar quantities can be established.

[0831] From these molar quantities percentage volume concentrations can be calculated. The concentrations in terms of volume percent can then be plotted to generate a flammability envelope (Figure 75B). The area bounded by the envelope is the explosible range and the area excluded is the non-explosible range. The "nose" of the envelope is the limiting oxygen concentration. Figs. 76A and 76B contain the calculated volume concentrations for the flammability envelope for Series B generated from data presented in Figure 69. A similar approach can be used on data presented in Figs. 70-74.

IV. Flammability testing experimental equipment and procedure

[0832] Flammability testing was conducted in a 4 liter high pressure vessel. The vessel was cylindrical in shape with an inner diameter of 6" and an internal height of 8.625". The temperature of the vessel (and the gases inside) was maintained using external heaters that were controlled by a PID controller. To prevent heat losses, ceramic wool and reflective insulation were wrapped around the pressure vessel. Type K thermocouples were used the measure the temperature of the gas space as well as the temperature of the vessel itself. Figure 77 illustrates the test vessel.

[0833] Before a test was run, the vessel was evacuated and purged with nitrogen to ensure that any gases from previous tests were removed. A vacuum was then pulled on the vessel. The pressure after this had been done was typically around 0.06 bar(a). Due to the nitrogen purging, the gas responsible for this initial pressure was assumed to be nitrogen. Using partial pressures, water, isoprene, nitrogen, and oxygen were then added in the appropriate amounts to achieve the test conditions in question. A magnetically driven mixing fan within the vessel ensured mixing of the gaseous contents. The gases were allowed to mix for about 2 minutes with the fan being turned off approximately 1 minute prior to ignition.

[0834] The igniter was comprised of a 1.5 ohm nicrome coil and an AC voltage source on a timer circuit. Using an oscilloscope, it was determined that 34.4 VAC were delivered to the igniter for 3.2 seconds. A maximum current of 3.8 amps occurred approximately halfway into the ignition cycle. Thus, the maximum power was 131 W and the total energy provided over the ignition cycle was approximately 210 J.

[0835] Deflagration data was acquired using a variable reluctance Validyne DP215 pressure transducer connected to a data acquisition system. A gas mixture was considered to have deflagrated if the pressure rise was greater than or equal to 5%.

V. Results of flammability testing

[0836] The first experimental series (Series 1) was run at 40°C and 0 psig with no steam. Running tests at varying concentrations of isoprene and oxygen produced the flammability curve shown in Figure 78A. The data points shown in this curve are only those that border the curve. A detailed list of all the data points taken for this series is shown in Figs. 80A and 80B.

[0837] Figure 78B summarizes the explosibility data points shown in Figure 78A. Figure 78C is a comparison of the experimental data with the CAFT model predicted flammability envelope. The model agrees very well with the experimental data. Discrepancies may be due to the non-adiabatic nature of the test chamber and limitations of the model. The model looks at an infinite time horizon for the oxidation reaction and does not take into consideration any reaction kinetic limitation.

[0838] Additionally, the model is limited by the number of equilibrium chemical species that are in its database and thus may not properly predict pyrolytic species. Also, the flammability envelope developed by the model uses one value for a limit flame temperature (1500K). The limit flame temperature can be a range of values from 1,000K to 1,500K depending on the reacting chemical species. The complex nature of pyrolytic chemical species formed at fuel concentrations above the stoichiometric fuel/oxidizer level is one reason why the model may not accurately predict the upper flammable limit for this system.

[0839] The second experimental series (Series 2) was run at 40°C and 0 psig with a fixed steam concentration of 4%. Running tests at varying concentrations of isoprene and oxygen produced the flammability curve shown in Figure 79A. The data points shown in this curve are only those that border the curve. A detailed list of all the data points taken for this series is shown in Figure 81. Due to the similarity between the data in Series 1 only the key points of lower flammable limit, limiting oxygen concentration, and upper flammable limits were tested. The addition of 4% steam to the test mixture did not significantly change the key

limits of the flammability envelope. It should be noted that higher concentrations of steam/water and or other inertants may influence the flammability envelope.

[0840] Figure 79B summarizes the explosibility data points shown in Figure 79A. Figure 79C is a comparison of the experimental data with the CAFT model predicted flammability envelope. The model agrees very well with the experimental data. Discrepancies may be due to the same factors described in Series 1

V. Calculation of Flammability Limits of Isoprene in Air at 3 Atmospheres of Pressure

[0841] The methods described in Example 13, parts I to IV were also used to calculate the flammability limits of isoprene at an absolute system pressure of 3 atmospheres and 40°C. These results were compared to those of Example 13, parts I to IV at an absolute system pressure of 1 atmosphere and 40°C. This higher pressure was tested because the flammability envelope expands or grows larger as the initial system pressure is increased. The upper flammability limit is affected the most, followed by the limiting oxygen composition. The lower flammability limit is the least affected (*see*, for example, "Bulletin 627 – Flammability Characteristics of Combustible Gases and Vapors" written by Michael G. Zabetakis and published by the former US Bureau of Mines (1965), which is hereby incorporated by reference in its entirety, particular with respect to the calculation of flammability limits).

[0842] In Figure 82, the calculated adiabatic flame temperature is plotted as a function of isoprene (fuel) concentration, expressed in weight percent of the total fuel/nitrogen/oxygen, where the system pressure was initially 3 atmospheres. The calculated flame temperatures are very similar to those determined initially in the 1 atmosphere system (Figure 83). As a result, when flammability envelopes are generated using the calculated adiabatic flammability data, the curves are very similar (see Figs. 84 and 85). Therefore, based on these theoretical calculations, a system pressure increase from 1 atmosphere to 3 atmosphere does not result in a significant increase/broadening of the flammability envelope. If desired, these model results may be validated using experimental testing (such as the experimental testing described herein at a pressure of 1 atmosphere).

VII. Summary of flammability studies

[0843] A calculated adiabatic temperature model was developed for the flammability envelope of the isoprene/oxygen/nitrogen/water/ carbon dioxide system at 40°C and 0 psig. The CAFT model that was developed agreed well with the experimental data generated by the tests conducted in this work. The experimental results from Series 1 and 2 validated the model results from Series A and B.

Example 14: Expression Constructs and Strains

I. Construction of plasmids encoding mevalonate kinase.

[0844] A construct encoding the *Methanosarcina mazei* lower MVA pathway (Accession numbers NC_003901.1, NC_003901.1, NC_003901.1, and NC_003901.1, which are each hereby incorporated by reference in their entireties) was synthesized with codon optimization for expression in *E. coli*. This construct is named *M. mazei* archeal Lower Pathway operon (Figures 112A-112C; SEQ ID NO:27) and encodes *M. mazei* MVK, a putative decarboxylase, IPK, and IDI enzymes. The gene encoding MVK (Accession number NC_003901.1) was PCR amplified using primers MCM165 and MCM177 (Table 11) using the Strategene Herculase II Fusion kit according to the manufacturer's protocol using 30 cycles with an annealing temperature of 55°C and extension time of 60 seconds. This amplicon was purified using a Qiagen PCR column and then digested at 37°C in a 10 μL reaction with PmeI (in the presence of NEB buffer 4 and BSA). After one hour, NsiI and Roche buffer H were added for an additional hour at 37°C. The digested DNA was purified over a Qiagen PCR column and ligated to a similarly digested and purified plasmid MCM29 (MCM29 is *E. coli* TOP10 (Invitrogen) transformed with pTrcKudzu encoding Kudzu isoprene synthase) in an 11 uL reaction 5uL Roche Quick Ligase buffer 1, 1 uL buffer 2, 1 uL plasmid, 3 uL amplicon, and 1 uL ligase (1 hour at room temperature). MCM 29 is pTrcKudzuKan. The ligation reaction was introduced into Invitrogen TOP10 cells and transformants selected on LA/kan50 plates incubated at 37 °C overnight. The MVK insert in the resulting plasmid MCM382 was sequenced (Figures 113A-113C; SEQ ID NO: 28).

Table 11. Oligonucleotides.

MCM161	*M.mazei* MVK for	CACCATGGTATCCTGTTCTGCG (SEQ ID NO:120)
MCM162	*M.mazei* MVK rev	TTAATCTACTTTCAGACCTTGC (SEQ ID NO:121)
MCM165	*M.mazei* MVK for w/	gcgaacgATGCATaaaggaggtaaaaaaacATGGTATCCTGTTCTG

	RBS	CGCCGGGTAAGATTTACCTG (SEQ ID NO:122)
MCM177	*M.mazei* MVK rev Pst	gggcccgtttaaactttaactagactTTAATCTACTTTCAGACCTTGC (SEQ ID NO:123)

II. Creation of strains overexpressing mevalonate kinase and isoprene synthase.

[0845] Plasmid MCM382 was transformed into MCM331 cells (which contains chromosomal construct gi1.2KKDyI encoding *S. cerevisiae* mevalonate kinase, mevalonate phosphate kinase, mevalonate pyrophosphate decarboxylase, and IPP isomerase) that had been grown to midlog in LB medium and washed three times in iced, sterile water. One μL of DNA was added to 50 μL of cell suspension, and this mixture was electroporated in a 2 mm cuvette at 2.5 volts, 25 uFd followed immediately by recovery in 500 μL LB medium for one hour at 37°C. Transformant was selected on LA/kan50 and named MCM391. Plasmid MCM82 was introduced into this strain by the same electroporation protocol followed by selection on LA/kan50/spec50. The resulting strain MCM401 contains a cmp-marked chromosomal construct gi1.2KKDyI, kan-marked plasmid MCM382, and spec-marked plasmid MCM82 (which is pCL PtrcUpperPathway encoding *E. faecalis* mvaE and mvaS). *See* Table 12.

Table 12. Strains overexpressing mevalonate kinase and isoprene synthase.

MCM382	E. coli BL21 (lambdaDE3) pTrcKudzuMVK(M. mazei)GI1.2KKDyI
MCM391	MCM331 pTrcKudzuMVK(M. mazei)
MCM401	MCM331pTrcKudzuMVK(M.mazei)pCLPtrcUpperpathway
MCM396	MCM333pTrcKudzuMVK(M. mazei)
MCM406	MCM333pTrcKudzuMVK(M.mazei)pCLPtrcUpperpathway

III. Construction of plasmid MCM376 - MVK from *M. mazei* archeal Lower in pET200D.

[0846] The MVK ORF from the *M. mazei* archeal Lower Pathway operon (Figures 112A-112C; SEQ ID NO:27) was PCR amplified using primers MCM161 and MCM162 (Table 11) using the Invitrogen Platinum HiFi PCR mix. 45 uL of PCR mix was combined with 1 uL template, 1 uL of each primer at 10 uM, and 2 uL water. The reaction was cycled as follows: 94°C for 2:00; 30 cycles of 94°C for 0:30, 55°C for 0:30, and 68°C for 1:15; and then 72°C for 7:00, and 4°C until cool. 3 uL of this PCR reaction was ligated to Invitrogen pET200D

plasmid according to the manufacturer's protocol. 3 uL of this ligation was introduced into Invitrogen TOP10 cells, and transformants were selected on LA/kan50. A plasmid from a transformant was isolated and the insert sequenced, resulting in MCM376 (Figures 114A-114C; SEQ ID NO:29).

V. Creation of expression strain MCM378.

[0847] Plasmid MCM376 was transformed into Invitrogen BL21(DE3) pLysS cells according to the manufacturer's protocol. Transformant MCM378 was selected on LA/kan50.

Example 15: Production of isoprene by *E. coli* expressing the upper mevalonic acid (MVA) pathway, the integrated lower MVA pathway (gi1.2KKDyI), mevalonate kinase from *M. mazei,* and isoprene synthase from Kudzu and grown in fed-batch culture at the 20 mL batch scale

Medium Recipe (per liter fermentation medium):

[0848] Each liter of fermentation medium contained K_2HPO_4 13.6 g, KH_2PO_4 13.6 g, $MgSO_4 * 7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, $(NH_4)_2SO_4$ 3.2 g, yeast extract 1 g, and 1000X Trace Metal Solution 1 ml. All of the components were added together and dissolved in diH_2O. The pH was adjusted to 6.8 with ammonium hydroxide (30%) and brought to volume. Media was filter sterilized with a 0.22 micron filter. Glucose (2.5 g) and antibiotics were added after sterilization and pH adjustment.

1000X Trace Metal Solution:

[0849] 1000X Trace Metal Solution contained citric Acids * H_2O 40 g, $MnSO_4 * H_2O$ 30 g, NaCl 10 g, $FeSO_4 * 7H_2O$ 1 g, $CoCl_2 * 6H_2O$ 1 g, $ZnSO_4 * 7H_2O$ 1 g, $CuSO_4 * 5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4 * 2H_2O$ 100 mg. Each component was dissolved one at a time in diH_2O, pH to 3.0 with HCl/NaOH, then brought to volume and filter sterilized with a 0.22 micron filter.

Strains:

[0850] MCM343 cells are BL21 (DE3) *E. coli* cells containing the upper mevalonic acid (MVA) pathway (pCL Upper), the integrated lower MVA pathway (gi1.2KKDyI), and isoprene synthase from Kudzu (pTrcKudzu).

[0851] MCM401 cells are BL21 (DE3) *E. coli* cells containing the upper mevalonic acid (MVA) pathway (pCL PtrcUpperPathway), the integrated lower MVA pathway (gi1.2KKDyI), and high expression of mevalonate kinase from *M. mazei* and isoprene synthase from Kudzu (pTrcKudzuMVK(M.mazei)).

[0852] Isoprene production was analyzed by growing the strains in 100 mL bioreactors with a 20 mL working volume at a temperature of 30°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 30°C. A single colony was inoculated into media and grown overnight. The bacteria were diluted into 20 mL of media to reach an optical density of 0.05 measured at 550 nm. The 100 mL bioreactors were sealed, and air was pumped through at a rate of 8 mL/min. Adequate agitation of the media was obtained by stirring at 600 rpm using magnetic stir bars. The off-gas from the bioreactors was analyzed using an on-line Hiden HPR-20 mass spectrometer. Masses corresponding to isoprene, CO_2, and other gasses naturally occurring in air were monitored. Accumulated isoprene and CO_2 production were calculated by summing the concentration (in percent) of the respective gasses over time. Atmospheric CO_2 was subtracted from the total in order to estimate the CO_2 released due to metabolic activity.

[0853] Isoprene production from a strain expressing the full mevalonic acid pathway and Kudzu isoprene synthase (MCM343) was compared to a strain that in addition over-expressed MVK from *M. mazei* and Kudzu isoprene synthase (MCM401) in 100 mL bioreactors. The bacteria were grown under identical conditions in defined media with glucose as carbon source. Induction of isoprene production was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG) to a final concentration of either 100 uM or 200 uM. Off-gas measurements revealed that the strain over-expressing both MVK and isoprene synthase (MCM401) produced significantly more isoprene compared to the strain expressing only the mevalonic acid pathway and Kudzu isoprene synthase (MCM343) as shown in Figures 115A-115D. At 100 uM induction, the MCM401 strain produced 2-fold more isoprene compared to the MCM343 strain. At 200 uM IPTG induction, the MCM401 strain produced 3.4-fold more isoprene when compared to the MCM343 strain. Analysis of CO_2 in the off-gas from the bioreactors, which is a measure of metabolic activity, indicates that metabolic activity was independent from IPTG induction and isoprene production.

Example 16: Production of isoprene by *E. coli* expressing the upper mevalonic acid (MVA) pathway, the integrated lower MVA pathway (gi1.2KKDyI), mevalonate kinase from *M. mazei,* and isoprene synthase from Kudzu and grown in fed-batch culture at the 15-L scale

Medium Recipe (per liter fermentation medium):

[0854] Each liter of fermentation medium contained K_2HPO_4 7.5 g, $MgSO_4 * 7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in DIH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0855] 1000X Modified Trace Metal Solution contained citric Acids * H_2O 40 g, $MnSO_4 * H_2O$ 30 g, NaCl 10 g, $FeSO_4 * 7H_2O$ 1 g, $CoCl_2 * 6H_2O$ 1 g, $ZnSO_4 * 7H_2O$ 1 g, $CuSO_4 * 5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4 * 2H_2O$ 100 mg. Each component was dissolved one at a time in DIH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 micron filter.

[0856] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the upper mevalonic acid (MVA) pathway (pCL PtrcUpperPathway encoding *E. faecalis* mvaE and mvaS), the integrated lower MVA pathway (gi1.2KKDyI encoding *S. cerevisiae* mevalonate kinase, mevalonate phosphate kinase, mevalonate pyrophosphate decarboxylase, and IPP isomerase), and high expression of mevalonate kinase from *M. mazei* and isoprene synthase from Kudzu (pTrcKudzuMVK(M.mazei)). This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 500 mL was used to inoculate 5-L of medium in a 15-L bioreactor.

[0857] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount

of glucose delivered to the bioreactor during the 68 hour fermentation was 3.8 kg. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). The IPTG concentration was brought to 51 uM when the optical density at 550 nm (OD_{550}) reached a value of 9. The IPTG concentration was raised to 88 uM when OD_{550} reached 149. Additional IPTG additions raised the concentration to 119 uM at OD_{550} = 195 and 152 uM at OD_{550} = 210. The OD_{550} profile within the bioreactor over time is shown in Figure 116. The isoprene level in the off gas from the bioreactor was determined using a Hiden mass spectrometer. The isoprene titer increased over the course of the fermentation to a final value of 23.8 g/L (Figure 117). The total amount of isoprene produced during the 68 hour fermentation was 227.2 g and the time course of production is shown in Figure 118. The metabolic activity profile, as measured by TCER, is shown in Figure 119. The total viable count (total colony forming units) decreased by two orders of magnitude between 10 and 39 hours of fermentation (Figure 120). The molar yield of utilized carbon that went into producing isoprene during fermentation was 13.0%. The weight percent yield of isoprene from glucose was 6.3%.

[0858]

Example 17: Production of isoprene by *E. coli* expressing the upper mevalonic acid (MVA) pathway, the integrated lower MVA pathway (gi1.2KKDyI), mevalonate kinase from *M. mazei,* and isoprene synthase from Kudzu and grown in fed-batch culture at the 15-L scale (2x 100μM IPTG induction)

Medium Recipe (per liter fermentation medium):

[0859] Each liter of fermentation medium contained K_2HPO_4 7.5 g, $MgSO_4 * 7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in DIH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0860] 1000X Modified Trace Metal Solution contained citric Acids * H_2O 40 g, $MnSO_4 * H_2O$ 30 g, NaCl 10 g, $FeSO_4 * 7H_2O$ 1 g, $CoCl_2 * 6H_2O$ 1 g, $ZnSO_4 * 7H_2O$ 1 g, $CuSO_4 *$

$5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4 * 2H_2O$ 100 mg. Each component was dissolved one at a time in DIH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 micron filter.

[0861] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the upper mevalonic acid (MVA) pathway (pCL PtrcUpperPathway encoding *E. faecalis* mvaE and mvaS), the integrated lower MVA pathway (gi1.2KKDyI encoding *S. cerevisiae* mevalonate kinase, mevalonate phosphate kinase, mevalonate pyrophosphate decarboxylase, and IPP isomerase), and high expression of mevalonate kinase from *M. mazei* and isoprene synthase from Kudzu (pTrcKudzuMVK(*M.mazei*)). This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 500 mL was used to inoculate 5-L medium in a 15-L bioreactor.

[0862] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 55 hour fermentation was 1.9 kg. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). The IPTG concentration was brought to 111 uM when the optical density at 550 nm (OD_{550}) reached a value of 9. The IPTG concentration was raised to 193 uM when OD_{550} reached 155. The OD_{550} profile within the bioreactor over time is shown in Figure 121. The isoprene level in the off gas from the bioreactor was determined using a Hiden mass spectrometer. The isoprene titer increased over the course of the fermentation to a final value of 19.5 g/L (Figure 122). The total amount of isoprene produced during the 55 hour fermentation was 133.8 g and the time course of production is shown in Figure 123. Instantaneous volumetric productivity levels reached values as high as 1.5 g isoprene/L broth/hr (Figure 124). Instantaneous yield levels reached as high as 17.7% w/w (Figure 125). The metabolic activity profile, as measured by TCER, is shown in Figure 126. The total viable count (total colony forming units) decreased by two orders of magnitude between 8 and 36 hours of fermentation (Figure 127). The molar yield of utilized carbon that went into producing isoprene during fermentation was 15.8%. The weight percent yield of isoprene from glucose over the entire fermentation was 7.4%.

[0863] In addition, as a control, fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the upper mevalonic acid (MVA) pathway (pCL PtrcUpperPathway encoding *E. faecalis* mvaE and mvaS), the integrated lower MVA pathway (gi1.2KKDyI encoding *S. cerevisiae* mevalonate kinase, mevalonate phosphate kinase, mevalonate pyrophosphate decarboxylase, and IPP isomerase), and high expression of mevalonate kinase from *M. mazei* and isoprene synthase from Kudzu (pTrcKudzuMVK(*M.mazei*)). This experiment was carried out to monitor uninduced cell metabolic activity as measured by CER from glucose at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain (MCM401 described above) taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 500 mL was used to inoculate 5-L medium in a 15-L bioreactor. Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands.

[0864] Figure 148 compares the CER profiles for the uninduced cells described above and the cells induced by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG) in Examples 16 and 17.

Example 18: Production of isoprene by *E. coli* expressing the upper mevalonic acid (MVA) pathway, the integrated lower MVA pathway (gi1.2KKDyI), mevalonate kinase from *M. mazei,* and isoprene synthase from Kudzu and grown in fed-batch culture at the 15-L scale (1x 50μM IPTG + 150μM IPTG fed induction)

Medium Recipe (per liter fermentation medium):

[0865] Each liter of fermentation medium contained K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0866] 1000X Modified Trace Metal Solution contained citric Acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, $ZnSO_4$ * $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4$ * $2H_2O$ 100 mg. Each component was dissolved one at a time in DIH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 micron filter.

[0867] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the upper mevalonic acid (MVA) pathway (pCL PtrcUpperPathway encoding *E. faecalis* mvaE and mvaS), the integrated lower MVA pathway (gi1.2KKDyI encoding *S. cerevisiae* mevalonate kinase, mevalonate phosphate kinase, mevalonate pyrophosphate decarboxylase, and IPP isomerase), and high expression of mevalonate kinase from *M. mazei* and isoprene synthase from Kudzu (pTrcKudzuMVK(M.mazei)). This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 37°C. A single colony was inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 500 mL was used to inoculate 5-L medium in a 15-L bioreactor.

[0868] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 55 hour fermentation was 2.2 kg. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). The IPTG concentration was brought to 51 uM when the optical density at 550 nm (OD_{550}) reached a value of 10. In addition to the IPTG spike, at OD_{550} = 10 a constant feed began and delivered 164 mg of IPTG over 18 hours. The OD_{550} profile within the bioreactor over time is shown in Figure 128. The isoprene level in the off gas from the bioreactor was determined using a Hiden mass spectrometer. The isoprene titer increased over the course of the fermentation to a final value of 22.0 g/L (Figure 129). The total amount of isoprene produced during the 55 hour fermentation was 170.5 g and the time course of production is shown in Figure 130. The metabolic activity profile, as measured by TCER, is shown in Figure 131. When the airflow to the bioreactor was decreased from 8 slpm to 4 slpm for a period of about 1.7 hours, the concentration of isoprene in the offgas increased from 0.51 to 0.92 w/w % (Figure 132). These elevated levels of isoprene did not appear to have any negative impact on cell metabolic activity as measured by the total carbon dioxide evolution rate (TCER), since

TCER declined only 7% between 37.2 and 39.3 hours (Figure 132). The total viable count (total colony forming units) decreased by two orders of magnitude between 7 and 36 hours of fermentation (Figure 133). The molar yield of utilized carbon that went into producing isoprene during fermentation was 16.6%. The weight percent yield of isoprene from glucose over the entire fermentation was 7.7%.

Example 19: The effect of externally applied isoprene on a wild-type *E. coli* grown in fed-batch culture at the 1-L scale

Medium Recipe (per liter fermentation medium):

[0869] Each liter of fermentation medium contained K_2HPO_4 7.5 g, $MgSO_4 * 7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, and 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

1000X Modified Trace Metal Solution:

[0870] 1000X Modified Trace Metal Solution contained citric Acids * H_2O 40 g, $MnSO_4 * H_2O$ 30 g, NaCl 10 g, $FeSO_4 * 7H_2O$ 1 g, $CoCl_2 * 6H_2O$ 1 g, $ZnSO_4 * 7H_2O$ 1 g, $CuSO_4 * 5H_2O$ 100 mg, H_3BO_3 100 mg, and $NaMoO_4 * 2H_2O$ 100 mg. Each component was dissolved one at a time in DIH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 micron filter.

[0871] Fermentation was performed in a 1-L bioreactor with BL21 (DE3) *E. coli* cells. This experiment was carried out to monitor the effects of isoprene on cell viability and metabolic activity in a glucose fed-batch bioreactor at the desired fermentation pH 7.0 and temperature 30°C. An inoculum of *E. coli* strain from a frozen vial was inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 50 mL was used to inoculate 0.5-L medium in a 1-L bioreactor.

[0872] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was fed to meet metabolic demands. Isoprene was fed into the bioreactor using nitrogen gas as a carrier. The rate of isoprene feeding was 1 g/L/hr

during mid-growth phase (OD_{550} = 31-44) and lasted for a total of 75 minutes (13.2 to 14.4 hours). The OD_{550} profile within the bioreactor over time is shown in Figure 134. The metabolic activity profile, as measured by TCER, is shown in Figure 135. The total viable count (total colony forming units) increased by 14-fold during the period when isoprene was introduced into the bioreactor (Figure 136).

Example 20: Production of isoprene and expression of isoprene synthase by *Saccharomyces cerevisiae*

[0873] The Kudzu isoprene synthase enzyme was optimized for expression according to a hybrid *Saccharomyces cerevisiae/Pichia pastoris* codon usage table, synthesized, and cloned into pDONR221:19430 (by DNA 2.0, Figure 140 for map and Figure 141 for sequence (SEQ ID NO:38)). A Gateway® Cloning (Invitrogen) reaction was performed according to the manufacturer's protocol: Since pDONR221:19430 was an "entry" vector, the LR Clonase II enzyme (the LR Reaction) was used to introduce the codon-optimized isoprene synthase into the "destination" vector pYES-DEST52 (Invitrogen).

[0874] The LR Reaction was then transformed into Top10 chemically competent cells (Invitrogen) according to the manufacturer's protocol, and bacteria harboring pYES-DEST52 plasmids with the isoprene synthase ORF were selected for on LA plates containing 50 μg/ml carbenicillin. Individual positive transformants were tested by colony PCR (see below for primer concentrations and thermocycling parameters) using illustra PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare) with the T7 forward primer and the Yeast isoprene synthase -Rev2 primer (*See* Table 13).

Table 13. Primer sequences for amplifying isoprene synthase.

Primer Name	Sequence (5´ to 3´)	Purpose
Yeast HGS -For2	CACCAAAGACTTCATAGACT (SEQ ID NO:124)	Forward primer for yeast optimized isoprene synthase
Yeast HGS -Rev2	AGAGATATCTTCCTGCTGCT (SEQ ID NO:125)	Reverse primer for yeast optimized isoprene synthase
T7 Forward	TAATACGACTCACTATAGGG (SEQ ID NO:126)	PCR and sequencing primer

[0875] Plasmids that yielded a PCR fragment of the correct size (1354 bp) were purified by miniprep (Qiagen) and sent for sequencing (Quintara Biosciences, Berkeley, CA) with the T7 Forward and Yeast isoprene synthase-For2 primers (*See* Table 13). Results from sequencing

runs were compared to the known sequence of pDONR221:19430 (using Vector NTI software, Invitrogen), and a single plasmid, pDW14, was selected for further study (Figure 142A for map and Figures 142B and C for the complete sequence (SEQ ID NO:39)). The sequence of pDW14 diverged from that of pDONR221:19430 by a single nucleotide (marked in bold in Figure 142B). The single nucleotide change (G to A) did not result in a change in the ORF, since it was in the third position of a lysine-encoding codon. It is unknown whether this base change was introduced in the LR cloning reaction, or was an error in the original sequence that was synthesized by DNA 2.0. All sequenced plasmids contained this base change.

[0876] Purified pDW14 was transformed into *Saccharomyces cerevisiae* strain INVSc-1 using the protocol described in the S. c. EasyComp Transformation kit (Invitrogen). INVSc-1 strains harboring pDW14 or pYES-DEST52 (which contains an intact URA3 gene) were selected for and maintained on SC Minimal Medium with 2% glucose without uracil, as described in the pYES-DEST52 Gateway Vector manual (Invitrogen). Two independent isolates of INVSc-1 containing pDW14 and a single control strain with pYES-DEST52 were chosen for further analysis.

[0877] To induce isoprene synthase expression, cultures were grown overnight in liquid SC Minimal Medium. The cultures were then diluted to an OD_{600} of approximately 0.2 and grown for 2-3 hours. Cultures were spun by centrifugation, washed once, resuspended in an equal volume (10 ml) of SC minimal medium with 1% raffinose, 2% galactose without uracil, and grown overnight to induce the expression of isoprene synthase. The OD_{600} of the strains was determined (Figure 144A), and strains were harvested by centrifugation and resuspended in 2 ml of lysis buffer (a 1:1 mix of 50% glycerol and PEB pH 7.4: Tris Base 2.423 g/L, $MgCl_2$ (Anhydrous) 1.904 g/L, KCl 14.910 g/L, DTT 0.154 g/L,Glycerol 50 mL/L).

[0878] The lysis mixtures were passed through a french press three times, and lysates were analyzed by SDS-PAGE. For Coomassie gel analysis (Figure 143A), samples were diluted 1:1 with 2X SDS loading buffer with reducing agent, loaded (20 μl total volume) onto a 4-12% bis-tris gel, run in MES buffer, and stained using SimplyBlue SafeStain according to the manufacturer's protocol (the Invitrogen Novex system).

[0879] The WesternBreeze kit (Invitrogen) was used for transfer and chromogenic detection of isoprene synthase on a nitrocellulose membrane. The primary antibody was

1799A 10 week diluted 1:1000 in Invitrogen antibody diluent. Primary antibody binding was followed by development with a secondary antibody labeled with Alexa Fluor 488 (Invitrogen Catalog No. A-11008) to permit quantitative signal determination. The western blot procedure was carried out as described by Invitrogen. The fluorescence signal was recorded with a Molecular Dynamics Storm instrument using the blue filter setting and quantitatively analyzed with the Molecular Dynamics ImageQuant image analysis software package. Specific activity of the library members was calculated from the ratio of the amount of isoprene produced divided by either the A600 of the induction cultures or the isoprene synthase protein concentration determined by western blot. Figure 143B shows that isoprene synthase was present in the induced INVSc-1 strains harboring pDW14 (lanes 2 and 3) in comparison to the control harboring pYES-DEST52 (lane 1).

[0880] The DMAPP assay for isoprene synthase headspace was performed on 25 μL of the lysate from each strain to which 5 μL 1 M $MgCl_2$, 5 μL 100 mM DMAPP, and 65 μL 50 mM Tris pH 8 were added. The reaction was performed at 30°C for 15 minutes in a gas tight 1.8 mL GC tube. Reactions were terminated by addition of 100 uL 250 mM EDTA pH 8. Figure 144B showed the specific activity values (in μg HG/L/OD) of the induced strains harboring pDW14 in comparison to the control. Induced strains harboring pDW14 displayed approximately 20X higher activity than the control lacking isoprene synthase.

PCR Cycling Parameters

[0881] Illustra PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare) were used with oligonucleotide primer pairs at a concentration of 0.4 μM each in 25 μl total volume/reaction. For analysis of plasmids resulting from the LR Clonase reaction (Invitrogen), a small amount of bacteria from individual colonies on a selective plate was added to each tube containing the PCR mix described above. The reaction cycle was as follows: 1) 95°C for 4 minutes; 2) 95°C for 20 seconds; 3) 52°C for 20 seconds; 4) 72°C for 30 seconds; 5 cycles of steps 2 through 4; 5) 95°C for 20 seconds; 6) 55°C for 20 seconds; 7) 72°C for 30 seconds; 25 cycles of steps 5 through 7, 72°C for 10 minutes, and 4°C until cool.

Example 21: Production of isoprene in *Pseudomonas* and other Gram negative bacteria Construction of pBBR5HGSOpt2_2, conjugation in *Pseudomonas* and measurement of isoprene synthase activity

[0882] A gene encoding isoprene synthase from *Pueraria lobata* (Kudzu plant) was codon-optimized for different microbial species of interest (Table 14; fluo-opt2v2 was the sequence chosen) and was synthesized by DNA2.0, Menlo Park, CA. The map and sequence of fluo-opt2v2 can be found in Figures 145A and 145B (SEQ ID NO:40). HindIII and BamHI restriction sites were added to the synthesized sequence for easier cloning, and a RBS was added in front of the ATG to enhance transcription.

[0883] Number of rare codons, as a function of the microbial species, in different versions of codon-optimized isoprene synthase from *Pueraria lobata*. Several rounds of optimization led to a gene with no rare codons in the all the species of interest.

Table 14. Number of rare codons.

Organism	fluo-opt1 (quote)	fluo-opt2	fluo-opt3	E. coli opt	fluo-opt2v2
Pseudomonas fluorescens Pf-5	19	X	X	57	0
Phodopseudomonas palustris CGA009	37	13	3	74	0
Pseudomonas putida F1	0	0	0	29	0
Corynebacterium glutamicum (ATCC)	4 (Ser)	0	0	0	0
Pseudomonas fluorescens PfO-1	1 (Val)	0	0	57	0

[0884] The gene was provided by DNA2.0 in a cloning vector. The vector was digested with HindIII/BamHI, the band corresponding to the insert of interest was gel-purified, and relegated with HindIII/BamHI-digested pBBR1MCS5 (Kovach et al, *Gene* 166:175-176, 1995, which is incorporated by reference in its entirety, particularly with respect to pBBR1MCS5), Figure 146A for map and Figures 146B and C for sequence (SEQ ID NO:41). This resulted in plasmid pBBR5HGSOpt2_2 (Figure 147A for map and Figures 147B and C for sequence (SEQ ID NO:42)) in which isoprene synthase was expressed from the lac promoter presented in pBBR1MCS5.

[0885] The vector was transformed in *E. coli* S17-1 and mated with *Pseudomonas putida* F1 ATCC700007 and *Pseudomonas fluorescens* ATCC 13525. After conjugation on LB, selection for plasmid-harboring Pseudomonas strains was on M9 + 16 mM sodium citrate +Gentamicin 50 ug/ml. Presence of the plasmid in the strains thus generated was checked by plasmid preparation using the Qiagen kit (Valencia, CA).

[0886] Isoprene synthase activities of the recombinant strains *P. putida*, pBBR5HGSOpt2_2 and *P. fluorescens*, pBBR5HGSOpt2_2 were assayed by growing the strains in TM3 medium (as described in Example 1 Part II) + 10 g/L glucose, harvesting the biomass in mid-log phase, breaking the cells by French Press and proceeding with the DMAPP assay**.** Results of the assay were presented in Table 15. The presence of activity measured by the DMAPP assay confirmed that isoprene synthase was expressed in *Pseudomonas*.

[0887] Isoprene synthase activity was examined in *Pseudomonas putida* and *Pseudomonas fluorescens* expressing isoprene synthase from the lac promoter, using plasmid pBBR5HGSOpt2_2

Table 15. Isoprene synthase activity in *Pseudomonas putida* and *Pseudomonas fluorescens.*

Strain	OD	Isoprene synthase activity mg isoprene/(L.h.OD)
P. fluorescens, pBBR5HGSOpt2_2	1.46	0.96
P. putida, pBBR5HGSOpt2_2	3.44	0.65
Control (*P. putida* w/o plasmid)	8.32	To be determined

Example 22: Growth of *E. coli* and *Pseudomonas* strains on sugar cane compared to glucose, and expression of isoprene synthase using both substrates

I. Preparation of liquid sugar cane.

[0888] Crystallized raw cane sugar was dissolved in water in the following way: 750 g H_2O was added to 250 g sugar. The solution was stirred and gently heated until dissolution. Some material was not soluble. The weight of the solution was adjusted to 1 kg after dissolution to replenish the evaporated water. The volume of the solution was measured to be 940 mL. Hence the concentration of the solution was 265 g/L. The product label claimed 14 g of carbohydrate for 15 g of raw sugar cane. Hence the carbohydrate concentration of the solution was 248 g/L. Dry solids were measured to be 24.03 %, close enough of the expected 250 g/kg. pH of the solution was 5.49. Glucose concentration was measured using an enzymatic/spectrophotometric assay, with glucose oxidase. The glucose concentration was 17.4 g/L.

[0889] As a majority of microorganisms do not use sucrose, but can use glucose and fructose, the solution was split in two. One half was autoclaved once for 30 minutes (sugar cane as is). Some inversion resulted, as the glucose content increased to 29.75 g/L (*See* Figure 149). The other half of the solution was adjusted to pH 4.0 using phosphoric acid, then the solution was inverted by autoclaving (inverted sugar cane). Three cycles of 30 min were sufficient to obtain complete inversion, as shown on Figure 149. Both solutions were used for the growth curves described below.

II. Growth curves of different strains of *E. coli* and *Pseudomonas* on sugar cane compared to glucose.

[0890] One colony of each of the strains presented in Table 16 was inoculated in 25 ml TM3 + 10 g/L glucose, and was grown overnight at 30°C and 200 rpm. TM3 is described in Example 7, Section II. The morning after, 1 ml of each culture was used to inoculate flasks containing 25 mL TM3 and 10 g/L glucose, 10 g/L sugar cane as is, or 10 g/L inverted sugar cane (sugar cane solutions described above). The flasks were incubated at 30°C and 200 rpm and samples were taken regularly to measure OD600. Figures 150 and 151 show that growth rate and biomass yield were comparable for glucose and inverted sugar cane, both for *Pseudomonas* and *E. coli* strains. *P. fluorescens* showed some signs of being able to use sugar cane which has not been inverted too.

Table 16. Strains used in this study.

	Strain
Escherichia coli	BL21
	MG1655
	ATCC11303
	B REL 606
Pseudomonas	*putida* F1 (ATCC700007)
	Fluorescens (ATCC13525)

III. Comparison of isoprene production from *E. coli* expressing isoprene synthase when grown on glucose or sugar cane.

[0891] *E. coli* MCM401 (BL21(DE3)) containing the full MVA pathway, mevalonate kinase from *M. mazei* and isoprene synthase from *Pueraria lobata*, as described in Example 14, Section II was grown in TM3 + either 10 g/L glucose or 10 g/L inverted sugar cane (based on carbohydrate concentration of the syrup). Flasks were inoculated from an overnight

culture on TM3 + 10 g/L glucose at an OD_{600} = 0.2. Antibiotics were added where needed. After two hours, the *E. coli* cultures were induced with 400 μM IPTG. After 6 hours of growth, isoprene production and isoprene synthase activities, using the DMAPP assay as described in Example 2B, were measured. Results are presented in Table 17 and illustrate clearly that inverted sugar cane is equivalent to glucose in terms of isoprene and isoprene synthase production on a per cell basis.

Table 17.

Strain	Carbon Source	OD	Isoprene synthase activity mg isoprene/(L.h.OD)	Isoprene production mg isoprene/(L.h.OD)
MCM401	Glucose	2.20	21.06	8.98
MCM401	Sugar cane inverted	2.32	20.20	9.23

Example 23: Construction of *E. coli* strains expressing the *S. cerevisiae* gi1.2KKDyI operon, *P. alba* isoprene synthase, *M. mazei* mevalonate kinase, pCL Upper MVA (*E. faecalis* mvaE and mvaS) and ybhE (pgl)

(i) Construction of strain EWL201 (BL21, Cm-GI1.2-KKDyI)

[0892] *E. coli* BL21 (Novagen brand, EMD Biosciences, Inc.) was a recipient strain, transduced with MCM331 P1 lysate (lysate prepared according to the method described in Ausubel, *et al., Current Protocols in Molecular Biology*. John Wiley and Sons, Inc.). MCM331 cells contain chromosomal construct gi1.2KKDyI encoding *S. cerevisiae* mevalonate kinase, mevalonate phosphate kinase, mevalonate pyrophosphate decarboxylase, and IPP isomerase (*i.e.*, the gi1.2-KKDyI operon from *S. cerevisiae*). Transductants were selected for by spreading cells onto L Agar and 20 μg/μl chloramphenicol. The plates were incubated overnight at 30°C. Analysis of transductants showed no colonies on control plates (water + cells control plate for reversion and water and P1 lysate control plate for lysate contamination.

[0893] Four transductants were picked and used to inoculate 5 mL L Broth and 20 μg/μl chloramphenicol. The cultures were grown overnight at 30°C with shaking at 200 rpm. To make genomic DNA preps of each transductant for PCR analysis, 1.5mL of overnight cell culture were centrifuged. The cell pellet was resuspended with 400μl Resuspension Buffer

(20mM Tris, 1mM EDTA, 50mM NaCl, pH 7.5) and 4µl RNase, DNase-free (Roche) was added. The tubes were incubated at 37°C for 30 minutes followed by the addition of 4µl 10% SDS and 4µl of 10mg/ml Proteinase K stock solution (Sigma-Aldrich). The tubes were incubated at 37°C for 1 hour. The cell lysate was transferred into 2 ml Phase Lock Light Gel tubes (Eppendorf) and 200µl each of saturated phenol pH7.9 (Ambion Inc.) and chloroform were added. The tubes were mixed well and microcentrifuged for 5 minutes. A second extraction was done with 400µl chloroform and the aqueous layer was transferred to a new eppendorf tube. The genomic DNA was precipitated by the addition of 1ml of 100% ethanol and centrifugation for 5 minutes. The genomic DNA pellet was washed with 1ml 70% ethanol. The ethanol was removed and the genomic DNA pellet was allowed to air dry briefly. The genomic DNA pellet was resuspended with 200µl TE.

[0894] Using Pfu Ultra II DNA polymerase (Stratagene) and 200ng/µl of genomic DNA as template, 2 different sets of PCR reaction tubes were prepared according to manufacturer's protocol. For set 1, primers MCM130 and GB Cm-Rev (Table 18) were used to ensure transductants were successfully integrated into the attTn7 locus. PCR parameters for set 1 were 95°C for 2 minutes (first cycle only), 95°C for 25 seconds, 55°C for 25 seconds, 72°C for 25 seconds (repeat steps 2-4 for 28 cycles), 72°C for 1 minute. For set 2, primers MVD For and MVD Rev (Table 18) were used to ensure that the gi1.2-KKDyI operon integrated properly. PCR parameters for set 2 were 95°C for 2 minutes (first cycle only), 95°C for 25 seconds, 55°C for 25 seconds, 72°C for 10 seconds (repeat steps 2-4 for 28 cycles), 72°C for 1 minute. Analysis of PCR amplicons on a 1.2% E-gel (Invitrogen Corp.) showed that all 4 transductant clones were correct. One was picked and designated as strain EWL201.

(ii) Construction of Strain EWL204 (BL21, loopout-GI1.2-KKDyI)

[0895] The chloramphenicol marker was looped out of strain EWL201 using plasmid pCP20 as described by Datsenko and Wanner (2000) (Datsenko *et al.*, *Proc Natl. Acad. Sci* USA 97:6640-6645, 2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. (Datsenko *et al.*, *PNAS,* 97: 6640-6645, 2000). EWL201 cells were grown in L Broth to midlog phase and then washed three times in ice-cold, sterile water. An aliquot of 50µl of cell suspension was mixed with 1µl of pCP20 and the cell suspension mixture was electroporated in a 2mm cuvette (Invitrogen Corp.) at 2.5 Volts and 25uFd using

a Gene Pulser Electroporator (Bio-Rad Inc.). 1ml of LB was immediately added to the cells, then transferred to a 14ml polypropylene tube (Sarstedt) with a metal cap. Cells were allowed to recover by growing for 1 hour at 30°C. Transformants were selected on L Agar and 20μg/μl chloramphenicol and 50μg/μl carbenicillin and incubated at 30°C overnight. The next day, a single clone was grown in 10ml L Broth and 50μg/μl carbenicillin at 30°C until early log phase. The temperature of the growing culture was then shifted to 42°C for 2 hours. Serial dilutions were made, the cells were then spread onto LA plates (no antibiotic selection), and incubated overnight at 30°C. The next day, 20 colonies were picked and patched onto L Agar (no antibiotics) and LA and 20μg/μl chloramphenicol plates. Plates were then incubated overnight at 30°C. Cells able to grow on LA plates, but not LA and 20μg/μl chloramphenicol plates, were deemed to have the chloramphenicol marker looped out (picked one and designated as strain EWL204).

(iii) Construction of plasmid pEWL230 (pTrc P. alba)

[0896] Generation of a synthetic gene encoding *Populus alba* isoprene synthase (*P. alba* HGS) was outsourced to DNA2.0 Inc. (Menlo Park, CA) based on their codon optimization method for *E. coli* expression. The synthetic gene was custom cloned into plasmid pET24a (Novagen brand, EMD Biosciences, Inc.) and delivered lyophilized (Figures 152, 153A-B; SEQ ID NO:43).

[0897] A PCR reaction was performed to amplify the *P. alba* isoprene synthase (*P. alba* HGS) gene using pET24a P. alba HGS as the template, primers MCM182 and MCM192, and Herculase II Fusion DNA polymerase (Stratagene) according to manufacturer's protocol. PCR conditions were as follows: 95°C for 2 minutes (first cycle only), 95°C for 25 seconds, 55°C for 20 seconds, 72°C for 1 minute, repeat for 25 cycles, with final extension at 72°C for 3 minutes. The *P. alba* isoprene synthase PCR product was purified using QIAquick PCR Purification Kit (Qiagen Inc.).

[0898] *P. alba* isoprene synthase PCR product was then digested in a 20μl reaction containing 1μl *Bsp*HI endonuclease (New England Biolabs) with 2μl 10X NEB Buffer 4. The reaction was incubated for 2 hours at 37°C. The digested PCR fragment was then purified using the QIAquick PCR Purification Kit. A secondary restriction digest was performed in a 20μl reaction containing 1μl *Pst*I endonuclease (Roche) with 2μl 10X Buffer

H. The reaction was incubated for 2 hours at 37°C. The digested PCR fragment was then purified using the QIAquick PCR Purification Kit. Plasmid pTrcHis2B (Invitrogen Corp.) was digested in a 20µl reaction containing 1µl *Nco*I endonuclease (Roche), 1µl *Pst*I endonuclease, and 2µl 10X Buffer H. The reaction was incubated for 2 hours at 37°C. The digested pTrcHis2B vector was gel purified using a 1.2% E-gel (Invitrogen Corp.) and extracted using the QIAquick Gel Extraction Kit (Qiagen) (Figure 154). Using the compatible cohesive ends of *Bsp*HI and *Nco*I sites, a 20µl ligation reaction was prepared containing 5µl *P. alba* isoprene synthase insert, 2µl pTrc vector, 1µl T4 DNA ligase (New England Biolabs), 2µl 10X ligase buffer, and 10µl ddH_2O. The ligation mixture was incubated at room temperature for 40 minutes. The ligation mixture was desalted by floating a 0.025µm nitrocellulose membrane filter (Millipore) in a petri dish of ddH_2O and applying the ligation mixture gently on top of the nitrocellulose membrane filter for 30 minutes at room temperature. MCM446 cells (*see* Section II) were grown in LB to midlog phase and then washed three times in ice-cold, sterile water. An aliquot of 50µl of cell suspension was mixed with 5µl of desalted pTrc P.alba HGS ligation mix. The cell suspension mixture was electroporated in a 2mm cuvette at 2.5 Volts and 25uFd using a Gene Pulser Electroporator. 1ml of LB is immediately added to the cells, then transferred to a 14ml polypropylene tube (Sarstedt) with a metal cap. Cells were allowed to recover by growing for 2 hour at 30°C. Transformants were selected on L Agar and 50µg/µl carbenicillin and 10mM mevalonic acid and incubated at 30°C. The next day, 6 transformants were picked and grown in 5ml L Broth and 50µg/µl carbenicillin tubes overnight at 30°C. Plasmid preps were performed on the overnight cultures using QIAquick Spin Miniprep Kit (Qiagen). Due to the use of BL21 cells for propagating plasmids, a modification of washing the spin columns with PB Buffer 5X and PE Buffer 3X was incorporated to the standard manufacturer's protocol for achieving high quality plasmid DNA. Plasmids were digested with *Pst*I in a 20µl reaction to ensure the correct sized linear fragment. All 6 plasmids were the correct size and shipped to Quintara Biosciences (Berkeley, CA) for sequencing with primers MCM65, MCM66, EL1000 (Table 18). DNA sequencing results showed all 6 plasmids were correct. One plasmid was picked designated as plasmid EWL230 (Figures 155, 156A-B; SEQ ID NO:44).

(iv) Construction of plasmid pEWL244 (pTrc P. alba-mMVK)

[0899] A PCR reaction was performed to amplify the *Methanosarcina mazei* (*M. mazei*) MVK gene using MCM376 as the template (see section (v) below), primers MCM165 and MCM177 (see Table 18), and Pfu Ultra II Fusion DNA polymerase (Stratagene) according to manufacturer's protocol. PCR conditions were as follows: 95°C for 2 minutes (first cycle only), 95°C for 25 seconds, 55°C for 25 seconds, 72°C for 18 seconds, repeat for 28 cycles, with final extension at 72°C for 1 minute. The *M. mazei* MVK PCR product was purified using QIAquick PCR Purification Kit (Qiagen Inc.).

[0900] The *M. mazei* MVK PCR product was then digested in a 40μl reaction containing 8μl PCR product, 2μl *Pme*I endonuclease (New England Biolabs), 4μl 10X NEB Buffer 4, 4μl 10X NEB BSA, and 22μl of ddH_2O. The reaction was incubated for 3 hours at 37°C. The digested PCR fragment was then purified using the QIAquick PCR Purification Kit. A secondary restriction digest was performed in a 47μl reaction containing 2μl *Nsi*I endonuclease (Roche), 4.7μl 10X Buffer H, and 40μl of PmeI digested *M. mazei* MVK fragment. The reaction was incubated for 3 hours at 37°C. The digested PCR fragment was then gel purified using a 1.2% E-gel and extracted using the QIAquick Gel Extraction Kit. Plasmid EWL230 was digested in a 40μl reaction containing 10μl plasmid, 2μl PmeI endonuclease, 4μl 10X NEB Buffer 4, 4μl 10X NEB BSA, and 20μl of ddH_2O. The reaction was incubated for 3 hours at 37°C. The digested PCR fragment was then purified using the QIAquick PCR Purification Kit. A secondary restriction digest was performed in a 47μl reaction containing 2μl *Pst*I endonuclease, 4.7μl 10X Buffer H, and 40μl of *Pme*I digested EWL230 linear fragment. The reaction was incubated for 3 hours at 37°C. The digested PCR fragment was then gel purified using a 1.2% E-gel and extracted using the QIAquick Gel Extraction Kit (Figure 157). Using the compatible cohesive ends of *Nsi*I and *Pst*I sites, a 20μl ligation reaction was prepared containing 8μl *M. mazei* MVK insert, 3μl EWL230 plasmid, 1μl T4 DNA ligase, 2μl 10X ligase buffer, and 6μl ddH_2O. The ligation mixture was incubated overnight at 16°C. The next day, the ligation mixture was desalted by floating a 0.025μm nitrocellulose membrane filter in a petri dish of ddH_2O and applying the ligation mixture gently on top of the nitrocellulose membrane filter for 30 minutes at room temperature. MCM446 cells were grown in LB to midlog phase and then washed three times in ice-cold, sterile water. An aliquot of 50μl of cell suspension was mixed with 5μl of desalted pTrc P.alba-mMVK ligation mix. The cell suspension mixture was electroporated in

a 2mm cuvette at 2.5 Volts and 25uFd using a Gene Pulser Electroporator. 1ml of LB is immediately added to the cells, then the cells are transferred to a 14ml polypropylene tube with a metal cap. Cells were allowed to recover by growing for 2 hour at 30°C. Transformants were selected on LA and 50μg/μl carbenicillin and 5mM mevalonic acid plates and incubated at 30°C. The next day, 6 transformants were picked and grown in 5ml LB and 50μg/μl carbenicillin tubes overnight at 30°C. Plasmid preps were performed on the overnight cultures using QIAquick Spin Miniprep Kit. Due to the use of BL21 cells for propagating plasmids, a modification of washing the spin columns with PB Buffer 5X and PE Buffer 3X was incorporated to the standard manufacturer's protocol for achieving high quality plasmid DNA. Plasmids were digested with PstI in a 20μl reaction to ensure the correct sized linear fragment. Three of the 6 plasmids were the correct size and shipped to Quintara Biosciences for sequencing with primers MCM65, MCM66, EL1000, EL1003, and EL1006 (Table 18). DNA sequencing results showed all 3 plasmids were correct. One was picked and designated as plasmid EWL244 (Figures 158 and 159A-B; SEQ ID NO:45).

(v) Construction of plasmid MCM376 - MVK from *M. mazei* archaeal Lower in pET200D.

[0901] The MVK ORF from the *M. mazei* archaeal Lower Pathway operon (Figures 160A-C; SEQ ID NO:46) was PCR amplified using primers MCM161 and MCM162 (Table 18) using the Invitrogen Platinum HiFi PCR mix. 45 uL of PCR mix was combined with 1 uL template, 1 uL of each primer at 10 uM, and 2 uL water. The reaction was cycled as follows: 94 °C for 2:00 minutes; 30 cycles of 94 °C for 0:30 minutes, 55 °C for 0:30 minutes and 68 °C for 1:15 minutes; and then 72 °C for 7:00 minutes, and 4 °C until cool. 3 uL of this PCR reaction was ligated to Invitrogen pET200D plasmid according to the manufacturer's protocol. 3 uL of this ligation was introduced into Invitrogen TOP10 cells, and transformants were selected on LA/kan50. A plasmid from a transformant was isolated and the insert sequenced, resulting in MCM376 (Figures 161A-C; SEQ ID NO:47).

(vi) Construction of strain EWL251 (BL21(DE3), Cm-GI1.2-KKDyI, pTrc P.alba-mMVK)

[0902] MCM331 cells (which contain chromosomal construct gi1.2KKDyI encoding *S. cerevisiae* mevalonate kinase, mevalonate phosphate kinase, mevalonate pyrophosphate decarboxylase, and IPP isomerase) were grown in LB to midlog phase and then washed three

times in ice-cold, sterile water. Mixed 50μl of cell suspension with 1μl of plasmid EWL244. The cell suspension mixture was electroporated in a 2mm cuvette at 2.5 Volts and 25uFd using a Gene Pulser Electroporator. 1ml of LB is immediately added to the cells, and then the cells were transferred to a 14ml polypropylene tube with a metal cap. Cells were allowed to recover by growing for 2 hours at 30°C. Transformants were selected on LA and 50μg/μl carbenicillin and 5mM mevalonic acid plates and incubated at 37°C. One colony was selected and designated as strain EWL251.

(vii) Construction of strain EWL256 (BL21(DE3), Cm-GI1.2-KKDyI, pTrc P.alba-mMVK, pCL Upper MVA)

[0903] EWL251 cells were grown in LB to midlog phase and then washed three times in ice-cold, sterile water. Mixed 50μl of cell suspension with 1μl of plasmid MCM82 (comprising pCL PtrcUpperPathway (also known as "pCL Upper MVA"), encoding *E. faecalis* mvaE and mvaS). Plasmid pCL Ptrc Upper Pathway was constructed as described in Example 8 above. The cell suspension mixture was electroporated in a 2mm cuvette at 2.5 Volts and 25 μFd using a Gene Pulser Electroporator. 1ml of LB was immediately added to the cells. Cells were then transferred to a 14ml polypropylene tube with a metal cap. Cells were allowed to recover by growing for 2 hours at 30°C. Transformants were selected on LA and 50μg/μl carbenicillin and 50μg/μl spectinomycin plates and incubated at 37°C. One colony was picked and designated as strain EWL256.

Table 18: Primer Sequences

Primer name	Primer sequence
MCM130	ACCAATTGCACCCGGCAGA (SEQ ID NO:127)
GB Cm Rev	GCTAAAGCGCATGCTCCAGAC (SEQ ID NO:128)
MVD For	GACTGGCCTCAGATGAAAGC (SEQ ID NO:129)
MVD Rev	CAAACATGTGGCATGGAAAG (SEQ ID NO:130)
MCM182	GGGCCCGTTTAAACTTTAACTAGACTCTGCAGTTAGCGTTCAAACGGCAGAA (SEQ ID NO:131)
MCM192	CGCATGCATGTCATGAGATGTAGCGTGTCCACCGAAAA (SEQ ID NO:132)
MCM65	ACAATTTCACACAGGAAACAGC (SEQ ID NO:133)
MCM66	CCAGGCAAATTCTGTTTTATCAG (SEQ ID NO:106)

EL1000	GCACTGTCTTTCCGTCTGCTGC (SEQ ID NO:134)
MCM165	GCGAACGATGCATAAAGGAGGTAAAAAAACATGGTATCCTGTTCTGCGCCGGC TAAGATTTACCTG (SEQ ID NO:135)
MCM177	GGGCCCGTTTAAACTTTAACTAGACTTTAATCTACTTTCAGACCTTGC (SEQ ID NO:136)
EL1003	GATAGTAACGGCTGCGCTGCTACC (SEQ ID NO:137)
EL1006	GACAGCTTATCATCGACTGCACG (SEQ ID NO:138)
MCM161	CACCATGGTATCCTGTTCTGCG (SEQ ID NO:120)
MCM162	TTAATCTACTTTCAGACCTTGC (SEQ ID NO:121)

(viii) Construction of strain RM111608-2 (Cm-GI1.2-KKDyI, pTrc P.alba-mMVK, pCL Upper MVA, pBBRCMPGI1.5-pgl)

[0904] The BL21 strain of *E. coli* producing isoprene (EWL256) was constructed with constitutive expression of the *ybhE* gene (encoding *E. coli* 6-phosphogluconolactonase) on a replicating plasmid pBBR1MCS5(Gentamycin) (obtained from Dr. K. Peterson, Louisiana State University).

[0905] FRT-based recombination cassettes, and plasmids for Red/ET-mediated integration and antibiotic marker loopout were obtained from Gene Bridges GmbH (Germany). Procedures using these materials were carried out according to Gene Bridges protocols. Primers Pgl-F (SEQ ID NO:139) and PglGI1.5-R (SEQ ID NO:140) were used to amplify the resistance cassette from the FRT-gb2-Cm-FRT template using Stratagene Herculase II Fusion kit according to the manufacturer's protocol. The PCR reaction (50 uL final volume) contained: 5 uL buffer, 1 uL template DNA (FRT-gb2-Cm-F from Gene Bridges), 10 pmols of each primer, and 1.5 uL 25mM dNTP mix, made to 50 uL with dH_2O. The reaction was cycled as follows: 1 x 2 minutes, 95°C then 30 cycles of (30 seconds at 95°C; 30 seconds at 63°C; 3 minutes at 72°C).

[0906] The resulting PCR product was purified using the QiaQick PCR purification kit (Qiagen) and electroporated into electrocompetent MG1655 cells harboring the pRed-ET recombinase-containing plasmid as follows. Cells were prepared by growing in 5 mLs of L broth to and OD600~0.6 at 30°C. The cells were induced for recombinase expression by the addition of 4% arabinose and allowed to grow for 30 minutes at 30°C followed by 30 minutes

of growth at 37°C. An aliquot of 1.5 mLs of the cells was washed 3-4 times in ice cold dH_2O. The final cell pellet was resuspended in 40 uL of ice cold dH_2O and 2-5 uL of the PCR product was added. The electroporation was carried out in 1-mm gap cuvettes, at 1.3 kV in a Gene Pulser Electroporator (Bio-Rad Inc.). Cells were recovered for 1-2 hours at 30°C and plated on L agar containing chloramphenicol (5 ug/mL). Five transformants were analyzed by PCR and sequencing using primers flanking the integration site (2 primer sets: pgl and 49 rev and 3' EcoRV-pglstop; Bottom Pgb2 and Top GB's CMP (946)). A correct transformant was selected and this strain was designated MG1655 GI1.5-pgl::CMP.

[0907] The chromosomal DNA of MG1655 GI1.5-pgl::CMP was used as template to generate a PCR fragment containing the FRT-CMP-FRT-GI1.5 - ybhE construct. This construct was cloned into pBBR1MCS5(Gentamycin) as follows. The fragment, here on referred to as CMP-GI1.5-pgl, was amplified using the 5' primer Pglconfirm-F (SEQ ID NO:141) and 3' primer 3' EcoRV-pglstop (SEQ ID NO:142). The resulting fragment was cloned using the Invitrogen TOPO-Blunt cloning kit into the plasmid vector pCR-Blunt II-TOPO as suggested from the manufacturer. The *Nsi*I fragment harboring the CMP-GI1.5-pgl fragment was cloned into the *Pst*I site of pBBR1MCS5 (Gentamycin). A 20µl ligation reaction was prepared containing 5µl CMP-GI1.5-pgl insert, 2µl pBBR1MCS5 (Gentamycin) vector, 1µl T4 DNA ligase (New England Biolabs), 2µl 10X ligase buffer, and 10µl ddH_2O. The ligation mixture was incubated at room temperature for 40 minutes then 2-4 uL were electroporated into electrocompetent Top10 cells (Invitrogen) using the parameters disclosed above. Transformants were selected on L agar containing 10 ug/ml chloramphenicol and 5 ug/ml Gentamycin. The sequence of the selected clone was determined using a number of the primers described above as well as with the in-house T3 and Reverse primers provided by Sequetech, CA. This plasmid was designated pBBRCMPGI1.5-pgl (Figures 162, 163A-B and SEQ ID NO:48).

[0908] Plasmid pBBRCMPGI1.5-pgl was electroporated into EWL256, as described herein and transformants were plated on L agar containing Chloramphenicol (10 ug/mL), Gentamycin (5 ug/mL), spectinomycin (50 ug/mL), and carbenicillin (50 ug/mL). One transformant was selected and designated strain RM111608-2.

Primers:

Pgl-F

5'-
ACCGCCAAAAGCGACTAATTTTAGCTGTTACAGTCAGTTGAATTAACCCTCACTA
AAGGGCGGCCGC-3' (SEQ ID NO:139)

PglGI1.5-R
5'-
GCTGGCGATATAAACTGTTTGCTTCATGAATGCTCCTTTGGGTTACCTCCGGGAA
ACGCGGTTGATTTGTTTAGTGGTTGAATTATTTGCTCAGGATGTGGCATAGTCAA
GGGCGTGACGGCTCGCTAATACGACTCACTATAGGGCTCGAG-3' (SEQ ID NO:140)

3' EcoRV-pglstop:

5'-CTT GAT ATC TTA GTG TGC GTT AAC CAC CAC (SEQ ID NO:142)

pgl +49 rev: CGTGAATTTGCTGGCTCTCAG (SEQ ID NO:143)

Bottom Pgb2: GGTTTAGTTCCTCACCTTGTC (SEQ ID NO:144)

Top GB's CMP (946): ACTGAAACGTTTTCATCGCTC (SEQ ID NO:145)

Pglconfirm-F

5'-ACCGCCAAAAGCGACTAATTTTAGCT-3' (SEQ ID NO:141)

Example 24: Improvement of isoprene production by constitutive expression of *ybhE* (pgl) in *E. coli*.

[0909] This example shows production of isoprene in a strain constitutively expressing *E. coli ybhE* (pgl) compared to a control strain expressing *ybhE* at wild-type levels (*i.e.*, EWL256). The gene *ybhE* (pgl) encodes *E. coli* 6-phosphogluconolactonase that suppresses posttranslational gluconylation of heterologously expressed proteins and improves product solubility and yield while also improving biomass yield and flux through the pentose phosphate pathway (Aon *et al.*, *Applied and Environmental Microbiology*, 74(4): 950-958, 2008).

Small scale analysis

[0910] **Media Recipe (per liter fermentation media)**: K_2HPO_4 13.6 g, KH_2PO_4 13.6 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, $(NH_4)_2SO_4$ 3.2 g, yeast extract 1 g, 1000X Trace Metals Solution 1 ml. All of the components were

added together and dissolved in diH_2O. The pH was adjusted to 6.8 with ammonium hydroxide (30%) and brought to volume. Media was filter-sterilized with a 0.22 micron filter. Glucose 5.0 g and antibiotics were added after sterilization and pH adjustment.

[0911] 1000X Trace Metal Solution (per liter fermentation media): Citric Acid * H_2O 40g, $MnSO_4$ * H_2O 30g, NaCl 10g, $FeSO_4$ * $7H_2O$ 1g, $CoCl_2$ * $6H_2O$ 1g, $ZnSO_4$ * $7H_2O$ 1g, $CuSO_4$ * $5H_2O$ 100mg, H_3BO_3 100mg, $NaMoO_4$ * $2H_2O$ 100mg. Each component is dissolved one at a time in diH_2O. The pH is adjusted to 3.0 with HCl/NaOH, and then the solution is brought to volume and filter-sterilized with a 0.22 micron filter.

(a) Experimental procedure

[0912] Isoprene production was analyzed by growing the strains in a Cellerator™ from MicroReactor Technologies, Inc. The working volume in each of the 24 wells was 4.5 mL. The temperature was maintained at 30°C, the pH setpoint was 7.0, the oxygen flow setpoint was 20 sccm and the agitation rate was 800 rpm. An inoculum of *E. coli* strain taken from a frozen vial was streaked onto an LB broth agar plate (with antibiotics) and incubated at 30°C. A single colony was inoculated into media with antibiotics and grown overnight. The bacteria were diluted into 4.5 mL of media with antibiotics to reach an optical density of 0.05 measured at 550 nm.

[0913] Off-gas analysis of isoprene was performed using a gas chromatograph-mass spectrometer (GC-MS) (Agilent) headspace assay. Sample preparation was as follows: 100 μL of whole broth was placed in a sealed GC vial and incubated at 30°C for a fixed time of 30 minutes. Following a heat kill step, consisting of incubation at 70°C for 5 minutes, the sample was loaded on the GC.

[0914] Optical density (OD) at a wavelength of 550 nm was obtained using a microplate reader (Spectramax) during the course of the run. Specific productivity was obtained by dividing the isoprene concentration (μg/L) by the OD reading and the time (hour).

[0915] The two strains EWL256 and RM11608-2 were assessed at 200 and 400 uM IPTG induction levels. Samples were analyzed for isoprene production and cell growth (OD550) at 1, 2.5, 4.75, and 8 hours post-induction. Samples were done in duplicate.

(b) Results

[0916] The experiment demonstrated that at 2 different concentrations of IPTG the strain expressing the *ybhE* (pgl) had a dramatic 2-3 fold increase in specific productivity of isoprene compared to the control strain.

[0917] Isoprene fermentation from *E. coli* expressing Cm-GI1.2-KKDyI, *M. mazei* mevalonate kinase, *P. alba* isoprene synthase, and *ybhE* (pgl) (RM111608-2) and grown in fed-batch culture at the 15-L scale

[0918] **Medium Recipe (per liter fermentation medium)**: K_2HPO_4 7.5 g, $MgSO_4$ * $7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, yeast extract 0.5 g, 1000X Modified Trace Metal Solution 1 ml. All of the components were added together and dissolved in diH_2O. This solution was autoclaved. The pH was adjusted to 7.0 with ammonium hydroxide (30%) and q.s. to volume. Glucose 10 g, thiamine * HCl 0.1 g, and antibiotics were added after sterilization and pH adjustment.

[0919] **1000X Modified trace Metal Solution**: Citric Acids * H_2O 40 g, $MnSO_4$ * H_2O 30 g, NaCl 10 g, $FeSO_4$ * $7H_2O$ 1 g, $CoCl_2$ * $6H_2O$ 1 g, $ZnSO_4$* $7H_2O$ 1 g, $CuSO_4$ * $5H_2O$ 100 mg, H_3BO_3 100 mg, $NaMoO_4$ * $2H_2O$ 100 mg. Each component is dissolved one at a time in DiH_2O, pH to 3.0 with HCl/NaOH, then q.s. to volume and filter sterilized with a 0.22 micron filter

[0920] Fermentation was performed in a 15-L bioreactor with BL21 (DE3) *E. coli* cells containing the upper mevalonic acid (MVA) pathway (pCL Upper), the integrated lower MVA pathway (gi1.2KKDyI), high expression of mevalonate kinase from *M. mazei* and isoprene synthase from *P. alba* (pTrcAlba-mMVK), and high expression of *E. coli pgl* (pBBR-pgl). This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation pH 7.0 and temperature 34°C. A frozen vial of the *E. coli* strain was thawed and inoculated into tryptone-yeast extract medium. After the inoculum grew to OD 1.0, measured at 550 nm, 500 mL was used to inoculate a 15-L bioreactor bringing the initial volume to 5-L.

[0921] Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 40 hour (59 hour) fermentation was 3.1 kg (4.2 kg at 59 hour). Induction was achieved by adding IPTG. The IPTG concentration was

brought to 110 uM when the optical density at 550 nm (OD_{550}) reached a value of 4. The IPTG concentration was raised to 192 uM when OD_{550} reached 150. The OD_{550} profile within the bioreactor over time is shown in Figure 164A. The isoprene level in the off gas from the bioreactor was determined using a Hiden mass spectrometer. The isoprene titer increased over the course of the fermentation to a maximum value of 33.2 g/L at 40 hours (48.6 g/L at 59 hours) (Figure 164B). The isoprene titer increased over the course of the fermentation to a maximum value of 40.0 g/L at 40 hours (60.5 g/L at 59 hours) (Figure 164C). The total amount of isoprene produced during the 40-hour (59-hour) fermentation was 281.3 g (451.0 g at 59 hours) and the time course of production is shown in Figure 164D. The time course of volumetric productivity is shown in Figure 164E and shows that an average rate of 1.0 g/L/hr was maintained between 0 and 40 hours (1.4 g/L/hour between 19 and 59 hour). The metabolic activity profile, as measured by CER, is shown in Figure 164F. The molar yield of utilized carbon that went into producing isoprene during fermentation was 19.6% at 40 hours (23.6% at 59 hours). The weight percent yield of isoprene from glucose was 8.9% at 40 hours (10.7% at 59 hours).

Example 25: Co-production of isoprene and hydrogen in *E. coli* strains expressing *M. mazei* mevalonate kinase, *P. alba* isoprene synthase, pCL Upper MVA (*E. faecalis* mvaE and mvaS) and *ybhE* (pgl)

Collection and analysis of fermentation off-gas for hydrogen and isoprene levels

[0922] Fermentations were performed using strains RM111608-2 (*E. coli* BL21 (DE3), pCL Upper MVA, cmR-gi1.2-yKKDyI, pTrcAlba-mMVK, pBBR cmR-gi1.5-pgl) and EWL 256 (*E. coli* BL21 (DE3), pCL Upper MVA, cmR-gi1.2-yKKDyI, pTrcAlba-mMVK). Construction of bacterial strains is described in Example 23 above.

[0923] Large scale production of isoprene from *E. coli* was determined from a fed-batch culture of *E. coli* strains EWL256 and RM111608-2 expressing *M. mazei* mevalonate kinase, *P. alba* isoprene synthase, pCL Upper MVA (*E. faecalis mvaE* and *mvaS*) and either constitutively expressing *ybhE* (pgl) (RM111608-2) or normally expressing *ybhE* (pgl) (EWL256). This experiment demonstrates that growing cells in the presence of glucose resulted in the co-production of isoprene and hydrogen.

[0924] The recipe for the fermentation medium (TM2) per liter of TM2 fermentation medium was as follows: K_2HPO_4 13.6 g, KH_2PO_4 13.6 g, $MgSO_4 * 7H_2O$ 2 g, citric acid monohydrate 2 g, ferric ammonium citrate 0.3 g, $(NH_4)_2SO_4$ 3.2 g, yeast extract 5 g, 1000X Modified Trace Metal Solution 1 ml. 1000X Modified Trace Metal Solution: Citric Acids * H_2O 40 g, $MnSO_4 * H_2O$ 30 g, NaCl 10 g, $FeSO_4 * 7H_2O$ 1 g, $CoCl_2 * 6H_2O$ 1 g, $ZnSO_4 * 7H_2O$ 1 g, $CuSO_4 * 5H_2O$ 100 mg, H_3BO_3 100 mg, $NaMoO_4 * 2H_2O$ 100 mg. For the 1000X Modified Trace Metal Solution, each component is dissolved one at a time in DiH_2O, pH to 3.0 with HCl/NaOH, then brought to final volume in distilled water and filter sterilized with a 0.22 micron (μm) filter (this solution is not autoclaved). For the TM2 fermentation medium, all of the components were added together, dissolved in diH_2O, the pH was adjusted to 6.8 with potassium hydroxide (KOH), q.s. to volume, and the medium was filter sterilized with a 0.22 micron (μm) filter. Glucose was sourced from Cargill as 99DE (dextrose equivalent), 71% DS (dry solids) syrup.

[0925] Fermentations were performed in 15-L bioreactors with *E. coli* strains EWL256 or RM111608-2, containing the upper mevalonic acid (MVA) pathway (pCL Upper MVA), the integrated lower MVA pathway (cmR-gi1.2-yKKDyI), mevalonate kinase from *M. mazei* and isoprene synthase from *P. alba* (pTrcAlba-mMVK), and constitutively expressing *ybhE* (pgl) (RM111608-2) or normally expressing *ybhE* (pgl) (EWL256). This experiment was carried out to monitor isoprene formation from glucose at the desired fermentation conditions (pH 7.0 and temperature 34°C).

[0926] An inoculum of the appropriate *E. coli* strain taken from a frozen vial was prepared in peptone-yeast extract medium. After the inoculum grew to $OD_{550} = 0.6$, 600 mL was used to inoculate a 15-L bioreactor containing TM2 medium. Glucose was fed at an exponential rate until cells reached the stationary phase. After this time the glucose feed was decreased to meet metabolic demands. The total amount of glucose delivered to the bioreactor during the 67 hour fermentation was 3.9 kg. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG). The IPTG concentration was brought to 102 uM when the optical density at 550 nm (OD_{550}) reached a value of 9. The IPTG concentration was raised to 192 uM when OD_{550} reached 140. At various times after inoculation, samples were removed and the amount of isoprene produced was determined as described below. Levels of hydrogen, nitrogen, oxygen, carbon dioxide, and isoprene in the off gas from the bioreactor were determined using a Hiden HPR-20 mass spectrometer as discussed below.

[0927] Samples of fermentation off-gas from 15-L bioreactors were collected into 20 mL glass headspace vials by sparging the vials at 1 L_{offgas}/min for 10 seconds and sealed with metal screw caps fitted with teflon-coated septa (Agilent, CA). The vials were analyzed within 30 minutes of collection.

[0928] Analysis of the two samples was performed by infusion into a Hiden HPR-20 mass spectrometer (Hiden Analytics, U.K.) at a rate of 4 scc/min (4 mL/min) by placing the inlet tube of the mass spectrometer into the uncapped headspace vials for 1-2 minutes. The HPR-20 instrument was configured to scan masses corresponding to hydrogen (*m/z* 2), nitrogen (*m/z* 28), oxygen (*m/z* 32), carbon dioxide (*m/z* 44) and isoprene (*m/z* 67). The Faraday detector was used for masses 28, 32, 44 and 67. The SEM detector was used for hydrogen (*m/z* 2). Detector response was measured in arbitrary units of pressure (Torr). Absolute hydrogen levels were estimated by comparison to an authentic hydrogen gas standard. Results were recorded using MASsoft V 6.21.0.51 software (Hiden Analytics, United Kingdom).

Results

[0929] Off-gas samples were taken from two fermentation runs and analyzed as described above:

[0930] A) Strain RM111608-2 (*E. coli* BL21 (DE3), pCL upper, cmR-gi1.2-yKKDyI, pTrcAlba-mMVK, pBBR cmR-gi1.5-pgl). Sample was taken at 64.8 hours into the run during which time the fermentation was being run anaerobically with a nitrogen sparge at 1 vvm.

[0931] B) Strain EWL256 (*E. coli* BL21 (DE3), pCL upper, cmR-gi1.2-yKKDyI, pTrcAlba-mMVK). Sample was taken at 34.5 hours into the run during which time the fermentation was being run aerobically with an air sparge at 1 vvm.

[0932] The results are depicted in Figures 165A-B. In both cases low levels of hydrogen were detected, in addition to isoprene, oxygen and carbon dioxide. The baseline reading for hydrogen was 0.95×10^{-8} Torr. Both Sample A and B gave reading of around 1.3×10^{-8} Torr. Based on a comparison to a hydrogen standard, the amount of hydrogen present in the off-gas for samples A and B was estimated to be less than 10 ppmv (parts per million volume) but

above the baseline. As shown in Figures 165A-B, both samples A and B also contained significant amounts of isoprene and carbon dioxide.

Example 26: Co-production of isoprene and hydrogen in *E. coli* strains expressing *M. mazei* mevalonate kinase, *P. alba* isoprene synthase, pCL Upper MVA (*E. faecalis* mvaE and mvaS) and *ybhE* (pgl)

Collection and analysis of fermentation off-gas for hydrogen and isoprene levels

[0933] The objective of this experiment is to co-produce hydrogen and isoprene in an engineered strain of *E. coli*. For this purpose, a portion of the *hyc* operon encoding *E. coli* hydrogenase-3 will be expressed in strain EWL256 [BL21 (DE3), pCL upper, cmR-gi1.2-yKKDyI, pTrcAlba-mMVK], prepared as described herein, although any of the bacterial strains described herein, such as RM111608-2, can be similarly modified. An expression construct comprising *hyc* operon genes *hycB* (gi|16130631), *hycC* (gi|16130630), *hycD* (gi|16130629), *hycE* (gi|16130628), *hycF* (gi|16130627), and *hycG* (gi|16130626) is prepared by standard cloning methods known in the art based upon publicly available gene sequences, and introduced into strain EWL256 to produce new strain EWL256+Hyd-3.

[0934] The impact of additional mutations on co-production of hydrogen and isoprene is assessed alone or in combination in EWL256+Hyd-3, by introducing genes involved in the maturation or regulation of hydrogenase-3 (*e.g.*, hycH (gi|16130625) and hycI (gi|16130624)), by inactivating or deleting genes involved in hydrogen uptake or transport (*e.g.*, *E. coli* hydrogenase-1 (*hya* operon) and hydrogenase-2 (*hyb* operon)) or related proteins (*e.g.*, formate dehydrogenase (*fdhF* (gi|16130624)), repressor of formate lyase (*hycA* (gi|16130632)), formate dehydrogenase N, alpha subunit (*fdnG* (gi|16129433)), formate dehydrogenase O, large subunit (*fdoG* (gi|16131734)), nitrate reductase (*narG* (gi|16129187)), fumarate reductase regulator (*fnr* (gi|16129295)), and acetyl-coenzyme A synthetase (*acs* (gi|16131895))), by activating genes involved in upregulation of hydrogenases (*e.g.*, activator of formate hydrogen lyase (*fhlA* (gi|16130638)), by inactivating or deleting genes involved in the production of fermentation side products (*e.g.*, lactate dehydrogenase (*ldhA* (gi|16129341)), fumarate reductase membrane protein (*frdC* (gi|16131977)), alcohol dehydrogenase (*adhE* (gi|16129202)), pyruvate oxidase (*poxB* (gi|16128839)), pyruvate dehydrogenase E1 component *ackA/pta* (*aceE* (gi|16128107)), formate dehydrogenase regulatory protein (*hycA* (gi|16130632)), and formate transporters A

and B (*FocA* (gi|16128871) and *FocB* (gi|16130417)), or by expression of heterologous genes involved in hydrogen metabolism (*e.g.*, glyceraldehyde-3-phosphate dehydrogenase from *Clostridium acetobutylicum* (*gapC* (gi|15893997)).

[0935] Fermentations are performed using engineered variants of strain EWL 256+Hyd-3 (BL21 (DE3), pCL upper, cmR-gi1.2-yKKDyI, pTrcAlba-mMVK and *hycB-F*), modified to comprise one or more additional mutations as described herein, either alone or in combination, essentially as described in Example 25 above. Co-production of hydrogen and isoprene is assessed by analysis of off-gas samples essentially as described above. Strains are selected for further analysis based upon the rate of isoprene and hydrogen co-production.

Example 27: Co-Production of Ethanol and Isoprene by *Saccharomyces cerevisiae*

[0936] To determine the feasibility of co-production of isoprene and ethanol in a *Saccharomyces cerevisiae* strain, IspS (Isoprene Synthase)-expressing *S. cerevisiae* was grown anaerobically under inducing conditions for 48 hours, and the production of isoprene and ethanol was measured.

[0937] **Strains used in this Example**. (1) DW112: *S. cerevisiae* (InvSC1) + pDW14 encoding codon-optimized IspS (Kudzu) on 2-micron plasmid (ura); and (2) DW114: *S. cerevisiae* (InvSC1) + pYES-DEST52 - empty vector control (ura). **Abbreviations used in this Example**. (1) 112G (112gal): strain DW112 induced (0.5% glucose, 2% galactose); (2) 112R (112raf): strain DW112 uninduced (0.5% glucose, 1% raffinose); (3) 114G (114gal): strain DW114 induced (0.5% glucose, 2% galactose); and (4) 114R (114raf): strain DW114 uninduced (0.5% glucose, 1% raffinose).

[0938] **Growth and Induction conditions**. INVSc-1 strains harboring pDW14 (strain DW112) or pYES-DEST52 (strain DW114) (*see* Example 20 for details on vector construction) were selected for on SC Minimal Medium with 2% glucose without uracil, as described in the pYES-DEST52 Gateway Vector manual (Invitrogen). SC Minimal Medium without uracil contains: 0.67% yeast nitrogen base (without amino acids; with ammonium sulfate); 2 % carbon source (*e.g.*, glucose for propagation or galactose for induction); 0.01% (adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan); 0.005% (aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, valine); and optionally 2% agar (for plates). Figure 141 shows the sequence of the codon-optimized IspS

from Kudzu; Figure 142A shows a map of the replicating vector for galactose-inducible expression in yeast; and Figures 142B-C show the complete nucleotide sequence of the expression vector for galactose-inducible expression of Kudzu IspS in *S. cerevisiae* (SEQ ID NO:39).

[0939] For propagation, strains were grown aerobically at 30°C in either solid or liquid SC minimal medium without uracil with 2% glucose. After overnight incubation, strains were diluted to an OD_{600} of 1.0 in 20 ml of SC minimal medium without uracil with 0.5% glucose, and grown for an additional 2 hours. At an OD_{600} of approximately 1.4 (see Figure 166), 10 ml of each strain were transferred to sealed 20 ml gas chromatography (GC) vials and either raffinose or galactose was added to a final concentration of 1% or 2%, respectively. This resulted in both uninduced (R, for raffinose) and induced (G, for galactose) growth of both DW112 and DW114. The GC vials were sealed and incubated for 48 hours at 30°C.

[0940] **Detection of isoprene by GC-MS**. After 48 hours of incubation in sealed GC vials, strains were assayed for isoprene production via GC-MS (*see* below for method). Control samples (112R, 114G, and 114R) displayed no isoprene production, whereas the strain containing Kudzu IspS, grown in the presence of 2% galactose (112G), produced a detectable level of isoprene (*see* Table 19 and Figure 167). For the controls, there was no detectable peak at the retention time for isoprene (0.49 min), so it was not possible to generate an integrated value for isoprene (*see* Figure 167). Using a calibration factor of 888/µg, the peak area for 112G corresponds to a concentration of isoprene in the headspace gas of 4.97 µg/L.

Table 19 – GC-MS data

Sample	Retention time (min)	Peak height	Corrected area	Calibration factor	Concentration (area/cal. factor)
112G	0.49	1025	4413	888/µg	4.97 µg/L

[0941] **GC-MS Method for Isoprene Detection**. The analysis was performed using an Agilent 6890 GC/MS system interfaced with a CTC Analytics (Switzerland) CombiPAL autosampler operating in headspace mode. An Agilent HP-5MS GC/MS column (15 m x 0.25 mm; 0.25 µm film thickness) was used for separation of analytes. The sampler was set up to inject 100 µL of headspace gas. The GC/MS method used helium as the carrier gas at a flow rate of 2 mL/minute. The injection port was held at 250°C with a split ratio of 50:1.

The oven temperature was held at 37ºC for the 2 minute duration of the analysis. The Agilent 5793N mass selective detector was run in single ion monitoring (SIM) mode on *m/z* 67. The detector was switched off from 0.01 to 0.45 minutes to allow the elution of permanent gases. Under these conditions isoprene (2-methyl-1,3-butadiene) was observed to elute at 0.49 minutes. A calibration table was used to quantify the absolute amount of isoprene and was found to be linear from 1 μg/L to 20000 μg/L. The limit of detection was estimated to be 50 to 100 ng/L using this method.

[0942] **Detection of Ethanol by HPLC**. After detection of isoprene by GC-MS, vials were opened and the OD_{600} of each culture was measured. OD_{600} values were between 5.0 and 5.7, indicating that for all strains, growth occurred during the 48 hour incubation (*see* Figure 166). Production of ethanol in all samples was then measured by HPLC (see below). All four cultures produced ethanol. Figure 168 shows peaks and integrated values (g/L) for ethanol, and Table 20 shows all data from the HPLC protocol.

[0943] **Organic Acids HPLC Method**. This method was developed to separate and quantify typical organic acids from fermentation processes. Running Buffer was 0.01 N H_2SO_4 buffer (equivalent to 5mM). Running buffer was prepared as follows: using a 4 L graduated cylinder, add 17.75 ml of the 10% H_2SO_4 stock solution to 4.0 L deionized water. This solution will be used to refill the HPLC buffer bottle. **Detector**. A Knauer K2301 RI detector was used to quantify the peaks as they came off the column.

[0944] **Preparation of broth samples for HPLC**. Broth samples were prepared for HPLC as follows: (1) Broth was poured into a labeled Eppendorf tube (~1.8 mL); (2) Tubes were centrifuged at 14,000 rpm for 5 minutes to pellet cells; (3) 300 μL of supernatant was transferred to a fresh Eppendorf tube; (4) 900 μL of H_2O was added to the supernatant. One can dilutethe sample less if one wants to see low concentration analytes, however, this will dirty the column more. The samples should be limited to those diluted less than 4X; (5) 36 μL of 70% perchloric acid (Sigma, Catalog No. 244252) was added and the tube inverted several times to mix; (6) Tubes were incubated on ice for 5 min to precipitate proteins, and then centrifuged at 14,000 rpm for 5 minutes. At this point, the supernatant was ready to be analyzed.

[0945] The supernatant was then poured into a plastic, conical bottom HPLC vial. The cap was screwed onto the vial, the vial was tapped on a hard surface to remove bubbles from the

bottom (otherwise, the HPLC injection needle would remove only air), and the samples were run on the HPLC loaded with an Aminex HPX-87H Ion Exclusion Column 300 mm x 7.8mm (Catalog # 125-0140; Bio Rad, Hercules, CA), run at 50°C, equipped with a Microguard Cation H refill 30 mm x 4.6 mm guard column (Catalog # 125-0129; Bio Rad, Herculues, CA), using 0.01 N H_2SO_4 as running buffer, at a flow rate of 0.6 ml/minute, at an approximate running pressure of ~950 psi, with an injection volume of 20 microliters. Run time was about 26 to 36 minutes; for example, the void came off at about 6 minutes; glucose came off at 8.5 minutes; acetic acid came off at 14 minutes, and ethanol came off at 21 minutes. When the column and guard column are not in use, they are stored in 0.01N H_2SO_4.

Table 20 – HPLC data

Sample Name	Name	Amount (g/L)	Retention Time	Area	% Area	Height
112G			6.539274	3038675	21.90461	499230.5
112G			7.799441	242845.5	1.750578	9319.017
112G	glucose		9.194			
112G			9.910056	9733369	70.16403	548067.4
112G			12.07399	6070	0.043756	397.731
112G	lactic		13.044			
112G	glycerol	0.258157	13.84041	107065	0.771789	5342.111
112G	acetate	0.045332	15.59951	11295	0.081421	704.9043
112G	mva	0.022844	18.39964	10628	0.076613	588.5367
112G			19.92238	71118	0.512662	2513.014
112G	**ethanol**	**3.056254**	**22.55683**	**651241.5**	**4.694544**	**21362**
112R			6.679975	6760286	62.07404	673489.1
112R			7.11816	157055.5	1.442109	14593.57
112R			7.808459	2616853	24.02837	179605.7
112R	glucose		9.194			
112R			9.664757	304552.7	2.796452	10171.72
112R			10.26993	104312.8	0.957817	5681.594
112R	lactic		13.044			
112R	glycerol	0.241637	14.13761	100214	0.920181	4987.541
112R	acetate	0.055357	15.94772	13793	0.12665	683.0312
112R	mva	0.022971	18.77139	10687	0.09813	580.4755
112R			20.33575	33230.5	0.305128	1384.247
112R	**ethanol**	**3.706021**	**23.0497**	**789697**	**7.251126**	**25052.53**
114G			6.605895	4805009	30.84022	599783.9
114G			7.882295	241598	1.55066	9176.059
114G	glucose	0.37193	9.569593	188423.8	1.20937	10212.97
114G			10.02025	9492665	60.92723	535906.8
114G	lactic		13.044			
114G	glycerol	0.257023	14.00157	106595	0.684164	5186.41
114G	acetate	0.051408	15.79813	12809	0.082213	704.3561
114G	mva	0.023238	18.60869	10811.5	0.069392	579.3297
114G			20.16202	52365	0.336097	1935.451
114G	**ethanol**	**3.144548**	**22.82513**	**670055.5**	**4.30065**	**21595.97**

Sample Name	Name	Amount (g/L)	Retention Time	Area	% Area	Height
114R			6.622579	5297638	56.41625	627059
114R			7.055373	157610.6	1.678446	15390.13
114R			7.738859	2558683	27.24824	175422.1
114R	glucose	0.607738	9.565885	307886.2	3.278779	10379.93
114R			10.16476	71425.78	0.760636	4133.971
114R	lactic		13.044			
114R	glycerol	0.245692	14.01421	101895.5	1.085118	4966.784
114R	acetate	0.049779	15.81667	12403	0.132084	599.6038
114R	mva	0.105887	18.61106	49263	0.524618	1138.927
114R			20.13147	46185	0.491839	1616.316
114R	**ethanol**	**3.694678**	**22.83235**	**787280**	**8.383998**	**25084.6**
114R-2			6.614597	5118040	55.89791	606469.3
114R-2			7.053017	176053.7	1.922813	16687.26
114R-2			7.734239	2544066	27.78563	172083.4
114R-2	glucose	0.60747	9.553156	307750.8	3.361175	10424.68
114R-2			10.15617	61748.18	0.674398	3441.637
114R-2	lactic		13.044			
114R-2	glycerol	0.242708	14.00365	100658	1.099361	4942.726
114R-2	acetate	0.056583	15.77043	14098.5	0.15398	628.3414
114R-2	mva	0.029084	18.60404	13531	0.147782	658.1601
114R-2			20.15232	38940	0.425293	1504.547
114R-2	**ethanol**	**3.665969**	**22.81841**	**781162.5**	**8.531655**	**25133.2**

Example 28: Co-generation of Isoprene via the DXP Pathway and Ethanol in *E. coli*.

[0946] As shown in Figure 169, cogeneration of isoprene and ethanol is a way of increasing the theoretical yield of isoprene from glucose by the DXP pathway, as the ATP generated in the production of ethanol can be utilized in the pathway to make isoprene. Maximum theoretical mass yield (without counting carbon used for building biomass) is then 32.3%. Assuming a CPI (cell productivity index) of 5, mass yield would be 29% in comparison to 27% for the DXP pathway only. Thus, when the process runs anaerobically, the capital investment decreases for oxygen transfer. The process could run in existing ethanol plants, in terms of tank stirring.

[0947] Although *E. coli* can produce ethanol when it is grown anaerobically, using the enzyme adhE to go from acetyl-CoA to ethanol via acetaldehyde, ethanol production is greatly improved by expressing pyruvate decarboxylase (pdc) from *Zymomonas*. As shown on Figure 170, pdc uses pyruvate as a substrate, has a low Km, and production of ethanol through pdc and adhE (*E. coli*) or adhB (*Zymomonas*) requires less reducing equivalents than through pfl and adhE. Although pdc expression alone already significantly increases the

amount of ethanol produced by *E. coli*, adding adhB from *Zymomonas* has been shown to increase the concentration of ethanol produced more than 20 times (Ingram et al. 1987).

[0948] Cloning of *Zymomonas mobilis* pyruvate decarboxylase (pdc) behind a Trc promoter in pBBR1-MCS5. Pyruvate decarboxylase (pdc) was amplified from *Zymomonas mobilis* ZM4 genomic DNA (ATCC31821) using primers SpeI-PTrc-rbs-pdcF (5'-gttactACTAGTGTTGACAATTAATCATCCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTaggaggaaaaaaaaATGAGTTATACTGTCGGTACCTATTTAG-3'; SEQ ID NO:146) and PstI-pdcR (5'-gttagatCTGCAGgtttatttaaaaactagaggagcttg-3'; SEQ ID NO:147). The resulting PCR product was purified, digested with SpeI/PstI and religated with SpeI/PstI-digested pBBR1-MCS5 (Kovach et al., *Biotechniques* (1994) 5:800-802). The plasmid was extracted from a white colony selected on LB + Gentamicin (5 ppm) + Xgal and was found to be correct by sequencing. This plasmid clone was named pBBR5-Ptrcpdc (Figure 171A shows a map of pBBR5-Ptrcpdc; Figures 171B-C show the sequence of pBBR5-Ptrcpdc; SEQ ID NO:148).

Construction of an *E. coli* strain co-expressing pdc and the DXP pathway.

[0949] Construction of strain MCM597 (BL21(DE3), pET24(MEA)alba+DXS+yIDI). *Construction of pDU-39*. Primer sequences: (1) **Alba TRC(MEA)-NdeI-F:** 5'-gaaactgaaaccCATATGgaagctcgtcgttctgc-3' (SEQ ID NO:149); (2) **Alba FLTRC (-) TER-R:** 5'-cccgcgcttaCTCGAGgcgttcaaacggcagaatcggttcagtg-3' (SEQ ID NO:150). A truncated version of the *Populus alba* isoprene synthase was created by amplifying the gene using the primer set Alba TRC(MEA)-NdeI-F/Alba FLTRC(-) TER-R and the template pET24a P. alba HGS (*see, e.g.*, Example 23(iii), SEQ ID NO:43, and Figures 152, 153A, and 153B). The PCR reaction was set up as follows: 1 μl (pET24a-*P.alba*); 5 μl 10X PfuUltraII Fusion buffer; 1 μl 10 mM dNTPs; 1 μl primer (50 μM) Set #1 forward; 1 μl primer (50 μM) Set #1 reverse; 41 μl ddiH2O; + 1μl of *PfuUltra* II Fusion DNA Polymerase from Stratagene. Cycling parameters were 95°C/1 minute, followed by 29 cycles of 95°C/30 seconds, 55°C/20 seconds, 72°C/25 seconds, followed by 72°C/3 minutes. The reactions were then held at 4°C until cool (Eppendorf Mastercycler).

[0950] The PCR products were digested with *Nde*I-*Xho*I restiction endonucleases (Roche) and gel purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. An aliquot of 3 μl of the purified product was ligated using T4

ligase (New England BioLabs) to pET-24a vector (Invitrogen) that was previously digested with *Nde*I-*Xho*I, gel purified and treated with Shrimp Alkaline Phosphatase (SAP, Roche). The ligation was carried out overnight at16°C.

[0951] An aliquot of 5 uL of the overnight ligation mixture was transformed into TOP10 cells (Invitrogen) and transformants were selected on L agar containing kanamycin (50 μg/ml) at 37°C overnight. Plasmids were isolated from a few of the transformants using the QiaQuick Spin Kit (Qiagen) according to the manufacturer's instructions. The insert was verified by *Nde*I-*Xho*I restriction endonuclease digestion and the clones were sequenced with the commercially available T7 promoter and T7 terminator primers (Quintara Bio Sequencing Service, Berkeley, CA). The correct plasmid was designated pDu-39 (Figure 172; SEQ ID NO:151)

[0952] *Construction of MCM597.* Primer Sequences: (1) MCM270 5'-GATCGGATCCATTCGCCCTTAGGAGGTAAA-3' (SEQ ID NO:152); and (2) MCM271 5'-GATCGCGGCCGCCAGCTGCAGGACGCGTTGTTATAGCATT-3' (SEQ ID NO:153).

[0953] The DXS-yIDI genes were amplified by PCR using primers MCM270/MCM271 and the template pMCM72 (Figure 173 is a map of pMCM72). Two identical PCR reactions were set up according to the manufacturer's protocol for Herculase II Fusion (Stratagene): 35 μL water, 10 μL buffer, 1.25 μL each primer, 0.5 μL dNTPs, 1 μL polymerase. Reactions were cycled: 95°C/2:00, followed by thirty cycles of 95°C/15 seconds, 55°C/15 seconds, 72°C/1 minute, 45 seconds, followed by 72°C/3 minutes. The reactions were then kept at 4°C until cold.

[0954] The resulting PCR fragment was digested with *Bam*HI and *Not*I (Roche), and then ligated with Roche Rapid Ligation Kit into pDu39 that had been digested with the same restriction endonucleases. The ligation reaction was set up in 10 μL containing 5 μL Buffer 1, 1 μL vector, 3 μL insert and 1 μL ligase, then incubated for 1 hour at room temperature. An aliquot of 5 μL was transformed into *E. coli* Top10 chemically competent cells (Invitrogen). Transformants were selected on L agar containing kanamycin (50 μg/ml) at 37°C overnight. Plasmids were purified from a few transformants and screened for the presence of insert by PCR using Herculase II Fusion (Stratagene): 17.5 μL water, 5 μL buffer, 0.625 μL each primer, 0.25 μL dNTPs, 0.5 μL polymerase. Reactions were cycled as follows: 95°C/2

minutes, followed by 30 cycles of 95°C/15 seconds, 52°C/15 seconds, 72°C/45 seconds, then held at 72°C/3 minutes. The reactions were then held at 4°C until cold. Clones with a PCR product near 1.5 kbp in length were sequenced (Quintara Biosciences, Berkeley CA). A correct plasmid was designated pMCM596 (Figure 174A is a map of pMCM596; Figures 174B-D are the sequence of pMCM596 (SEQ ID NO: 154)). The plasmid was then transformed into electrocompetent BL21(DE3)pLysS cells (Invitrogen) and transformants were selected on L agar containing kanamycin (50 μg/ml) and chloramphenicol (35 μg/mL). One colony was selected and designated MCM597.

[0955] MCM597 (BL21(DE3), pET24(MEA)alba+DXS+yIDI) was transformed with pBBR5-Ptrcpdc and pBBR1-MCS5 as a control. Colonies were selected on LB + Kanamycin 50 ppm, Chloramphenicol (35 ppm), Gentamycin (5 ppm). One colony of each was selected and named strain CMP182 and strain CMP183 respectively.

[0956] **Co-production of isoprene and ethanol in *E. coli*.** One colony each of strains CMP182 and CMP183 was incubated overnight in TM3 medium with 1% glucose and 1% yeast extract, and appropriate antibiotics, at 30°C and 170 rpm. The morning after, cultures were diluted in the 20 mL of the same medium, containing 0.5% glucose, and 0.11% or 1% yeast extract, to an OD=1 and incubated at 30°C and 170 rpm. The 1% yeast extracts flasks were done in duplicate. Duplicates were highly similar so results from only one set of flasks were presented. After 2 h, 200 uM IPTG was added and agitation was reduced to 40 rpm. Samples were taken 2 hours and 5 hours after induction, and analyzed for OD, organic acids by HPLC (Ion exclusion column Aminex HPX-87H, 300 mm × 7.8 mm) and specific productivity of isoprene. Isoprene concentration was measured in the offgas by GC/MS as described herein. The specific productivity of each strain is reported as μg/L per OD per hour. Note the ratio of 1900 μl headspace:100 μl broth in assay vials for 30 minutes incubation results in the following conversion of isoprene μg/L of culture to specific productivity: (isoprene/L determined by GC-MS) × (38)/(OD 600nm of the culture). With 1% yeast extract, glucose was depleted at 5 hours after induction.

[0957] Figure 175 shows that growth was not affected by the expression of pdc. Cultures containing 1 % yeast extract grew to a higher OD.

[0958] Figure 176 shows ethanol concentration and isoprene specific productivity (in arbitrary units) in the flasks containing 0.1% (A) (5 hours after induction) and 1 % (B) (2

hours after induction) yeast extract. It can be seen that the strains are directing carbon both towards isoprene and ethanol. As expected from a functional pdc, more ethanol is produced in the strain harboring pdc. Specific productivity of isoprene is lower in the strain harboring pdc, since more carbon flux is going to ethanol, but it is still significant, showing that dxs (using pyruvate and glyceraldehyde as substrates) can take carbon flux from pdc.

[0959] Figure 177 shows fermentation products after 5 hours of induction in the 1 % yeast extract flasks. The strain expressing pdc shows a higher concentration in ethanol, confirming the fact that pdc was expressed and active. As expected from comparing K_ms for ldhA and pdc, pyruvate flux to lactate is interrupted once pdc is expressed. Also, in the strain expressing pdc, more carbon is going towards acetaldehyde than towards acetyl-CoA, leading to a decrease of acetate.

Example 29: Coproduction of Isoprene and Ethanol in *Zymomonas mobilis*.

[0960] **Construction of a plasmid for production of isoprene in *Zymomonas mobilis*.** *Zymomonas mobilis* ZM4 (ATCC31821) was obtained from ATCC (Manassas, VA). It was routinely grown in 10 ml tubes containing RM medium (20 g/L glucose, yeast extract 10 g/L, KH_2PO_4 2 g/L, adjusted to pH 6.0), at 30°C without shaking. *Zymomonas mobilis* is well-known for its capacity to produce ethanol. J. Swings et al. (1977) *Bacteriol. Rev.* 41:1-46.

[0961] A PCR product containing the *Z. mobilis* pdc promoter in front of a gene coding for a truncated isoprene synthase from *Populus alba* was generated by PCR SOEing (Polymerase Chain Reaction - Splicing by Overlapping Extension) using primer XbaI-PpdcF (5'-ctaaacTCTAGAGC TCA TGA TCG CGG CAT GTT CTG-3'; SEQ ID NO:155) and primer FusPpdc-HGSR (5'-gcagaacgacgagcttcggtcattgcttactccatatattcaaaacactatg-3'; SEQ ID NO:156) for amplifying the pdc promoter, and using primer PstI-MTEARRalbahpR (5'-ctacgaCTGCAGCCGGATATAGTTCCTCCTTTCAGC-3'; SEQ ID NO:157) and FusPpdc-HGSF (5'-catagtgttttgaatatatggagtaagcaAtgaccgaagctcgtcgttctgc-3'; SEQ ID NO:158) for amplifying the isoprene synthase gene, followed by a PCR reaction on a mixture of the two products obtained in step 1, with primers XbaI-PpdcF (SEQ ID NO:155) and PstI-MTEARRalbahpR (SEQ ID NO:157). Template for the pdc promoter was genomic DNA of *Z. mobilis* ZM4 and the truncated isoprene synthase was amplified from plasmid pDU47 (a map of pDU47 is shown in Figure 178; the sequence of plasmid pDU47 (SEQ ID NO:159) is shown in Figure 179). The codon bias for that gene has been optimized for *E. coli*.

[0962] The PCR product obtained was digested with XbaI/PstI and religated with XbaI/PstI-digested pBBR1-MCS (Kovach et al., *Biotechniques* (1994) 5:800-802). pBBR1-MCS is a broad-host range plasmid shown to be stably replicating in *Zymomonas* (Jeon et al., *FEMS Microbiol. Letters* (2005) 244:85-92). The plasmid obtained was named pBBR-Ppdc-HGS1 (a map of plasmid pBBR-Ppdc-HGS1 is shown in Figure 180; the sequence of plasmid pBBR-Ppdc-HGS1 (SEQ ID NO:160) is shown in Figure 181).

[0963] **Transformation of *Z. mobilis* ZM4 with pBBR1-MCS or pBBR1-Ppdc-HGS1**. Plasmids pBBR1-MCS and pBBR1-Ppdc-HGS1 were transformed into *Z. mobilis* ZM4 by biparental mating (via *E. coli* S17-1) according to Conway et al. (Conway et al., *Appl. Environ. Microbiol.* (1987) 53:235-241), or electroporation according to Jeon et al. (Jeon et al., *FEMS Microbiol. Letters* (2005) 244:85-92). Transformants were selected on RM + chloramphenicol 100 μg/ml following electroporation and appeared after 48 h at 30°C in anaerobic conditions. Cultures for conjugation were incubated on RM overnight then restreaked on YPG (yeast extract, 10 g/L, peptone, 10 g/L, glucose, 70 g/L, pH 6.0) + 40 μg/ml nalidixic acid + chloramphenicol 100 μg/ml.

[0964] Both methods yielded an abundance of colonies on RM + chloramphenicol 100 μg/ml or YPG + 40 μg/ml nalidixic acid + chloramphenicol 100 μg/ml. Plasmid was extracted from the *Zymomonas* cells using a Qiagen mini-prep kit (Qiagen, Valencia, CA) and shown to be present by gel electrophoresis.

[0965] **Production of Isoprene and Coproduction of Isoprene and Ethanol by *Z. mobilis* ZM4, pBBR1-Ppdc-HGS1**. One colony each of *Zymomonas mobilis* ZM4, pBBR1-MCS and *Zymomonas mobilis* ZM4, pBBR1-Ppdc-HGS1 were inoculated in 10 ml RM + chloramphenicol 100 μg/ml in a 20 ml headspace vial sealed and incubated overnight standing at 30°C. After 16 hours, a vial was removed and analyzed for isoprene concentration in the headspace, OD and organic acids or alcohols. Isoprene production in that same vial was measured using a headspace assay as follows. The analysis was performed using an Agilent 6890 GC/MS system interfaced with a CTC Analytics (Switzerland) CombiPAL autosampler operating in headspace mode. An Agilent HP-5MS GC/MS column (30 m x 0.25 mm; 0.25 μm film thickness) was used for separation of analytes. The sampler was set up to inject 500 μL of headspace gas. The GC/MS method used helium as the carrier gas at a flow rate of 1 ml/minute. The injection port was held at 250°C with a split ratio of 50:1. The

oven temperature was held at 37ºC for the 2 minute duration of the analysis. The Agilent 5793N mass selective detector was run in single ion monitoring (SIM) mode on *m/z* 67. The detector was switched off from 1.4 to 1.7 minutes to allow the elution of permanent gases. Under these conditions isoprene (2-methyl-1,3-butadiene) was observed to elute at 1.78 minutes. A calibration table was used to quantify the absolute amount of isoprene, which was found to be linear from 1 μg/L to 200 μg/L. The limit of detection was estimated to be 50 to 100 ng/L using this method.

[0966] Organic acids and alcohols were analyzed by HPLC (Ion exclusion column Aminex HPX-87H, 300 mm × 7.8 mm, 0.005 M H_2SO_4, 0.6 mL/min as the mobile phase). Cells of *Zymomonas mobilis* ZM4 transformed with pBBR1-MCS were growing faster than cells of *Zymomonas mobilis* ZM4 transformed with pBBR1-Ppdc-HGS1 (data not shown). At the end of the culture, cells were harvested, lysed by two passages through a French press, and extracts were analyzed by a Western blot probed with a monoclonal antibody against purified *Populus alba* isoprene synthase. The protein was detected in cells of *Zymomonas mobilis* ZM4 transformed with pBBR1-Ppdc-HGS1 but not in cells of *Zymomonas mobilis* ZM4 transformed with pBBR1-MCS.

[0967] Figure 182 shows the amount of isoprene detected divided by OD. OD_{600} was 1.9 and 2.1 for *Zymomonas mobilis* ZM4, pBBR1-MCS and *Zymomonas mobilis* ZM4, pBBR1-Ppdc-HGS1 respectively (not significantly different). *Zymomonas mobilis* ZM4, pBBR1-Ppdc-HGS1 produced 16X more isoprene/OD than *Zymomonas mobilis* ZM4, pBBR1-MCS. Table 21 presents relative OD and relative ethanol production from *Zymomonas mobilis* ZM4, pBBR1-Ppdc-HGS1 compared to *Zymomonas mobilis* ZM4, pBBR1-MCS. Lactate was not detected in those cultures, and acetate levels were less than 0.1 g/L. Ethanol (more than 9 g/L) was produced at equal concentrations for both *Zymomonas mobilis* ZM4, pBBR1-MCS and *Zymomonas mobilis* ZM4, pBBR1-Ppdc-HGS1.

Table 21: Relative OD and relative ethanol production of *Zymomonas mobilis* ZM4, pBBR1-MCS compared to *Zymomonas mobilis* ZM4, pBBR1-Ppdc-HGS1

Relative OD of *Zymomonas mobilis* ZM4, pBBR1-MCS compared to *Zymomonas mobilis* ZM4, pBBR1-Ppdc-HGS1	Relative EtOH production of *Zymomonas mobilis* ZM4, pBBR1-MCS compared to *Zymomonas mobilis* ZM4, pBBR1-Ppdc-HGS1
0.89	1.03

[0968] It has been found that an increased amount of isoprene per OD can be obtained in the same setting if the inoculum consists of a growing culture (data not shown).

Example 30: Coproduction of Isoprene and 1,3-propanediol.

[0969] Other two- (C2) and three-carbon (C3) alcohols and diols such as, for example, 1,2-propane diol or 1,3-propanediol (1,3-PDO), are co-produced with isoprene in a variety of organisms, including yeasts, such as *S. cerevisiae*, and bacteria, such as *Escherichia sp.* (*e.g.*, *E. coli*) and *Zymomonas sp.* (*e.g.*, *Z. mobilis*). Yield of isoprene and 1,3-PDO is estimated from the following equations:

(1): 1.5Glc + 3ATP → 3AcCoA + $3CO_2$ + 6NAD(P)H

(2): 3AcCoA + 2NAD(P)H → MVA

(3): MVA + 3ATP → HG + CO_2 + H2O

(1)+(2)+(3)=(4): 1.5Glc → HG + $3CO_2$ + 4NAD(P)H

(5): 1/18Glc + $1/3O_2$ → 2 ATP + 1/3 H_2O

(6): 0.5Glc + ATP + 2NAD(P)H → PDO

(4)+(5)+2*(6) = (7): 23/9Glc + $1/3O_2$ → HG + 2PDO

23/9Glc($C_6H_{12}O_6$) + $1/3O_2$ → C_5H_8 + $2C_3H_8O_2$ + $4.33CO_2$ + $3.33H_2O$

At a CPI (cell productivity index) of 6, isoprene yield on sugar = 13%; PDO yield on sugar = 30%.

[0970] ***Construction of CMP250, an E. coli strain expressing the pathways for isoprene production via the MVA pathway and 1,3-propanediol production.*** *Construction of pDW15.* Plasmid pBBr1-MCS5 (Kovach et al, *Gene* 166:175-176, 1995) was digested with XhoI/XbaI and religated with a Ptrc Upper MVA piece amplified from pTrcUpperPathway (Figures 26 and 27A-27D; SEQ ID NO:12). The resulting plasmid was named pDW15 (SEQ ID NO:161; *see* Figure 183A for a plasmid map, and Figures 183B-D for the plasmid sequence). Plasmid pDW15 expressed the upper mevalonic acid pathway polypeptides *mvaE* and *mvaS* from *Enterobacter faecalis*.

[0971] *Construction of E. coli strain CMP250.* Strain EWL204 was electroporated (protocol described in Example 23) with pEWL244 and pDW15 (*see* Example 23 for information regarding the construction of strain EWL204 and plasmid pEWL244, as well as for the electroporation protocol). Transformants were selected on LB + Carbenicillin (50 μg/mL) + Gentamycin (5 μg/mL). The resulting strain was then electroporated with plasmid pSYCO109 (*see* U.S. Patent No. 7,371,558, which is incorporated herein by reference, particularly with respect to construction of the plasmids designated pSYCO101 *et seq.*, including pSYCO109)(SEQ ID NO:162; *see* Figure 184A for a plasmid map and Figures 184B-F for the sequence). The plasmid pSYCO109 encodes (1) DAR1 (dihydroxyacetone phosphate reductase) and GPP2 (glycerol-phosphate phosphatase), both genes from the glycerol pathway; (2) dhaB1-3, dhaX, orfX, and orfY, all genes from the 1,3-propanediol pathway. The plasmid pSYCO109 also includes an *E. coli* threonine operator attenuator (Thr atten), an *E. coli* TonB terminator (TonB term), a trc promoter (trc), and an aspartate ammonia lyase gene terminator (AspA term). Transformants were selected on LB + Carbenicillin (50 μg/mL) + Gentamycin (5 μg/mL) + Spectinomycin 50 μg/mL. The resulting strain was designated CMP250.

[0972] Alternatively, a cassette containing, in this order, the *E. coli* MG1655 native pgl promoter, *E. coli* MG1655 native pgl gene (ybhE, not present in BL21), and an FRT-Chloramphenicol-FRT cassette (GeneBridges, Heidelberg, Germany) was recombined in the chromosome of EWL204 by Red/ET recombination (GeneBridges, Heidelberg, Germany). The chosen site of integration was between *ybhJ* and *ybhC*. The marker was looped out using 706-Flp (GeneBridges, Heidelberg, Germany) and the strain thus generated was named CMP251. Strain CMP251 was electroporated with plasmids pEWL244, pDW15 and pSYCO109 in the order described in the paragraph above, to produce strain CMP252.

[0973] Additional strains are constructed with deletions in a variety of genes involved with metabolic pathways relating to the production of C2- or C3-alcohols or diols, such as 1,3-propanediol, including: (1) a strain in which the *glpK* and *gldA* genes are deleted; (2) a strain in which the *tpiA* gene is deleted; or (3) a strain in which *ptsHIcrr* is deleted, and in which the *galP* and *glk* genes are constitutively expressed (*see, e.g.*, US Patent Publication No. 2009/0142843-A1, entitled "Glucose Transport Mutants For Production Of Biomaterial," which is hereby incorporated by reference, in particular with respect to construction of various bacterial strains). Other strains are constructed with one or more useful mutations,

including, for example, deletions of *edd, ndh, arcA*, *mgsA, qor, ackA-pta, poxB, ldhA*, or mutations that result in the downregulation of *gapA* or upregulation of *ppc*. These and other deletions are constructed by commonly-used methods, such as making lysates from the Keio mutant having the deletion of interest (Baba et al., *Mol. Syst. Biol*. 2:2006.0008 (published online February 2006) and transducing the mutation of interest into the desired bacterial strain, such as, for example, CMP250 or CMP252.

[0974] ***Coproduction of isoprene and 1,3-propanediol***. Strains CMP250, CMP252, or other strains derived from strains CMP250 and CMP252 but incorporating one or more of the additional mutations described above, are grown anaerobically in HM1 medium and expression of the various plasmids incorporating MVA pathway polypeptides and/or other heterologous polypeptides as described elsewhere herein is induced. Isoprene concentrations in the offgas are measured by GC/MS as described elsewhere herein, and 1,3-propanediol concentrations in the fermentation broth are measured by HPLC as described elsewhere herein, or by other suitable method known to one skilled in the art. The various bacterial strains are shown to have a high productivity of both isoprene and 1,3-propanediol, produced in a mass ratio of approximately 13:30.

Example 31: Construction of *E. coli* strain CMP249 for co-production of isoprene and 1,3-propanediol.

[0975] Yield calculations for co-production of isoprene and 1,3-PDO, based on the following equations:

(1): 1.5 Glc + 3 ATP → 3 AcCoA + 3 CO_2 + 6 NAD(P)H

(2): 3 AcCoA + 2 NAD(P)H → MVA

(3): MVA + 3 ATP → HG + CO_2 + H_2O

(1)+(2)+(3)=(4): 1.5 Glc → HG + 4 CO_2 + 4 NAD(P)H

(5): 1/18 Glc + 1/3 O_2 → 2 ATP + 1/3 H_2O

(6): 0.5 Glc + ATP + 2 NAD(P)H → PDO

(4)+(5)+2*(6) = (7): 23/9 Glc + 1/3 O_2 → HG + 2 PDO + 4.33 CO_2 + 3.33 H_2O

23/9 C $_6H_{12}O_6$ + 1/3 O_2 → C_5H_8 + 2 $C_3H_8O_2$ + 4.33 CO_2 + 3.33 H_2O

[0976] At a cell productivity index (CPI) of 6, the isoprene yield on sugar = 13%, with a maximum of 14.8%; the 1,3-propanediol yield on sugar = 30%, with a maximum of 33%. The peak oxygen uptake rate (OUR) ~ 17 was 3 g/L/hr.

[0977] **Construction of Co-production Strain CMP249 (BL21 PL.2 mKKDyI*gldAglpK::Kan* tpgl, pDW15, pEWL244, pSYCO109).** *Plasmids pEWL244, pSYCO109 and pDW15.* The plasmid pSYCO109 (SEQ ID NO:162; Figure 184A for a plasmid map; Figures 184B-F for the sequence) contains all of the necessary pathway genes to convert dihydroxyacetone-phosphate into 1,3-propanediol via glycerol, and is described in U.S. Patent No. 7,371,558. pSYCO109F1.1 (SEQ ID NO:163; *see* Figure 185A for a plasmid map and Figures 185B-F for the plasmid sequence) contains a fusion of two subunits of the glycerol dehydratase enzyme with an amino acid change. The plasmid EWL244 (pTrcAlba-mMVK) contains the genes encoding *P. alba* isoprene synthase and *Methanosarcina mazei* mevalonate kinase (mMVK) transcribed from the *trc* promoter, constructed as described in Example 23 herein (SEQ ID NO:45; *see* Figure 158 for a map of plasmid EWL244 and Figures 159A-B for the plasmid sequence).

[0978] *Construction of pDW15 (Ptrc-upper MVA pathway on pBBR1MCS-5).* To insert the upper MVA pathway onto the pBBR1MCS-5 vector, the entire expression cassette containing pTrc, *mvaE, mvaS*, and the rrn terminator was amplified by PCR from plasmid MCM82 (described in Example 23 herein) using the primers Upper5'XhoI (SEQ ID NO:164) and Upper3'XbaI (SEQ ID NO:165). The PCR reaction contained 1 µl MCM82 (approx. 30 ng), 10 µl 5X Herculase® Buffer (Stratagene, La Jolla, CA), 0.5 µl dNTPs (100 mM), 1 µl Upper5'XhoI (SEQ ID NO:164) (20 uM), 1 µl Upper3'XbaI (SEQ ID NO:165) (20 uM), 35.5 µl diH_2O, and 1 µl Herculase DNA Polymerase (Stratagene). The reactions were cycled as follows: 95°C/4 minutes; 5 cycles of 95°C / 20 minutes, 52°C 20 seconds, 72°C 4 minutes; 25 cycles of 95°C / 20 minutes, 55°C 20 seconds, 72°C / 4 minutes; 72°C / 10 minutes, then cooled to 4°C.

[0979] The size of the approximately 4.2 kb PCR product was confirmed by gel electrophoresis (E-Gel, Invitrogen, Carlsbad, CA) and then purified using QiaQuick purification columns (Qiagen, La Jolla, CA) according to the manufacturer's recommended protocol. Purified PCR product and the pBBR1MCS-5 vector were then treated with XbaI

and XhoI restriction endonucleases overnight at 37°C. Digestions were performed as follows: 6 µl diH$_2$O, 2 µl 10X Buffer H (Roche), 10 µl DNA (pBBR1MCS-5 or PCR insert), 1 µl XhoI (Roche), and 1 µl XbaI (Roche) were incubated overnight at 37°C. The restriction enzymes were then heat inactivated for 20 minutes at 65°C, before ligation.

[0980] Ligation reactions were carried out at 4°C overnight as follows: 2 µl diH20, 1 µl 10X ligase buffer (New England Biolabs), 1 µl T4 DNA ligase (NEB), 2 µl vector (pBBR1MCS-5), and 4 µl insert (upper MVA expression cassette). The reaction mixture was then microdialyzed (Millipore, Billerica, MA). Approximately 5 µl of the ligation reactions was transformed into chemically competent *E. coli* TOP10 cells (Invitrogen, Carlsbad, CA) according to the manufacturer's recommended protocol, recovered at 37°C in LB for 1 hour, and then plated onto LB plates containing X-gal and Gentamicin at 10 µg/ml. Colonies displaying no β-galactosidase activity were selected for further analysis by PCR using primers M13 Reverse and MCM163 to confirm the presence of the insert. The plasmid from one of these colonies was purified (Qiagen) and completely sequenced (Quintara Biosciences, see Table 1 for primer sequences) to verify that it contained the complete upper MVA pathway expression cassette in the correct orientation. Figure 183A shows a map of plasmid pDW15 (SEQ ID NO:161), expressing the upper MVA pathway polypeptides *mvaE* and *mvaS* from *Enterobacter faecalis*. Figures 183B-D show the sequence of pDW15.

Table 22. PCR and Sequencing Primers

Upper5'XhoI	atgctcgagctgttgacaattaatcatccggctc (SEQ ID NO:164)
Upper3'XbaI	cgatctagaaaggcccagtctttcgactgagcc (SEQ ID NO:165)
MCM163	GGATTTTGGCCATTTCCAGCTT (SEQ ID NO:166)
CF07-58	atgaaaacagtagttattattgatgc (SEQ ID NO:167)
CF07-59	cttaaatcatttaaaatagc (SEQ ID NO:168)
CF07-82	atgacaattgggattgataaaattag (SEQ ID NO:169)
CF07-86	gaaatagccccattagaagtatc (SEQ ID NO:170)
CF07-87	ttgccaatcatatgattgaaaatc (SEQ ID NO:171)
CF07-88	gctatgcttcattagatccttatcg (SEQ ID NO:172)
CF07-89	gaaacctacatccaatcttttgccc (SEQ ID NO:173)

[0981] *Construction of Strains MCM518-521 and 528-531: Lambda promoters driving integrated mKKDyI.* Primers MCM120 and MCM224 were used to amplify the resistance cassette from the GeneBridges FRT-gb2-Cm-FRT template using Stratagene Herculase II Fusion kit according to the manufacturer's protocol. Four 50 µL PCR reactions were cycled as follows: 95°C /2 minutes; 30 cycles of 95°C / 20 seconds, 55°C / 20 seconds, 72°C / 1

minute; 72°C / 3 minutes; and cooled to 4°C. The four reactions were pooled and purified on a Qiagen PCR column according to the manufacturer's protocol and eluted with 60 μL Elution Buffer at 55°C.

[0982] Plasmid pRedET-carb (GeneBridges) was electroporated into MCM446 (*E. coli* BL21 cmR-gi1.6mKKDyI A1-3 (A), constructed as described in International Publication No. WO 2009/076676 A2, which is incorporated herein by reference) as described elsewhere herein. Transformants were recovered by shaking for one hour in SOC medium (Invitrogen) at 30°C and then selected on L agar containing carbenicillin (50 ug/mL) plates at 30°C overnight. A carbenicillin resistant colony was frozen as MCM508.

[0983] Strain MCM508 was grown from a fresh streak in 5 mL L Broth containing carbenicillin (50 μg/mL) at 30°C to an OD600 of ~0.5. 40 mM L-arabinose was added and culture was incubated at 37°C for 1.5 hours. Cells were harvested and electroporated with 3 μL of purified amplicons as previously, and then recovered in 500 μL SOC at 37°C for 1.5-3 hours. Transformants were selected on L agar plates containing 10 μg/ml kanamycin at 37°C.

[0984] Recombination of the amplicon at the target locus was confirmed by PCR with primers GB-DW (SEQ ID NO:177) and MCM208 (SEQ ID NO:175). The resulting amplicons were sequenced to identify four clones with the sequences below. Carbenicillin-sensitive clones were frozen as strains MCM518-521.

[0985] MCM518-521 were restreaked on L agar containing 10 μg/ml kanamycin and grown overnight at 37°C.

[0986] Strains MCM518-521 were cultured in L Broth containing kanamycin (10 μg/mL) at 37°C and then electrotransformed with plasmid pCP20. Cells were recovered in 500 μL SOC, shaking at 30°C for 1 hour. Transformants were selected on L agar containing carbenicillin (50 μg/mL) plates at 30°C overnight. The following morning a colony from each transformation was grown at 30°C in liquid LB/carbenicillin (50 μg/mL) until visibly turbid. The culture was then shifted to 37°C for at least 3 hours. Cells were streaked from this culture onto L agar plates and grown overnight at 37°C.

[0987] The following day colonies were patched to L agar, L agar containing carbenicillin (50 μg/mL), and L agar containing kanamycin (10 μg/ml). Clones that grew on neither carbenicillin (50 μg/mL) nor kanamycin (10 μg/ml) were cultured in liquid LB from the patch on L agar and frozen as MCM528-531.

Strain genotypes

Strain	Description	Parent
MCM508	BL21 gi1.6-mKKDyI + predet.-carb	MCM446
MCM518	BL21 neo-PL.6-mKKDyI, clone10	MCM508
MCM519	BL21 neo-PL.0-mKKDyI, clone11	MCM508
MCM520	BL21 neo-PL.0-mKKDyI (bad RBS in front of mMVK), clone13	MCM508
MCM521	BL21 neo-PL.2-mKKDyI, clone15	MCM508
MCM528	BL21 PL.6-mKKDyI, loopedout	MCM518
MCM529	BL21 PL.0-mKKDyI, loopedout	MCM519
MCM530	BL21 PL.0-mKKDyI (bad RBS in front of mMVK), loopedout	MCM520
MCM531	BL21 PL.2-mKKDyI, loopedout	MCM521

Primers

MCM120	aaagtagccgaagatgacggtttgtcacatggagttggcaggatgtttgattaaaagcAATTAACCCTCACTAAAGGGCGG (SEQ ID NO:174)
MCM208	GCTCTGAATAGTGATAGAGTCA (SEQ ID NO:175)
MCM224	taaatcttacccggcgcagaacaggataccatgttttttacctcctttgcaccttcatggtggtcagtgcgtcctgctgatgtgctcagtatcac cgccagtggtatttaNgtcaacaccgccagagataatttatcaccgcagatggttatctgtatgtttttatatgaatttaatacgactcactatag ggctcg (SEQ ID NO176)
GB-DW	Aaagaccgaccaagcgacgtctga (SEQ ID NO:177)

[0988] These assemblies include the new promoters inserted on the chromosome in strains MCM518-521, as well as the very beginning of the mMVK ORF. Upstream of these assemblies is sequence from the GeneBridges FRT-gb2-Cm-FRT cassette. Downstream is the remainder of the mMVK ORF and then the rest of the lower MVA pathway integron from strain MCM508.

[0989] MCM518:

aaagaccgaccaagcgacgtctgagagctccctggcgaattcggtaccaataaaagagctttattttcatgatctgtgtgttggtttttgtg tgcggcgcggaagttcctattctctagaaagtataggaacttcctcgagccctatagtgagtcgtattaaattcatataaaaaacatacag ataaccatctgcggtgataaattatctctggcggtgttgacataaataccactggcggtgatactgagcacatcagcaggacgcactga ccaccatgaaggtgcaaaggaggtaaaaaaacatggtatcctgttctgcgccgggtaagatttacctgttcggtgaacacgccgtagtt tatggcgaaactgcaattgcgtgtgcggtggaactgcgtacccgtgttcgcgcggaactcaatgactctatcactattcagagc (SEQ ID NO:178).

[0990] MCM519:

aaagaccgaccaagcgacgtctgagagctccctggcgaattcggtaccaataaaagagctttattttcatgatctgtgtgttggtttttgtg

tgcggcgcggaagttcctattctctagaaagtataggaacttcctcgagccctatagtgagtcgtattaaattcatataaaaaacatacag ataaccatctgcggtgataaattatctctggcggtgttgacctaaataccactggcggtgatactgagcacatcagcaggacgcactga ccaccatgaaggtgcaaaggaggtaaaaaaacatggtatcctgttctgcgccgggtaagatttacctgttcggtgaacacgccgtagtt tatggcgaaactgcaattgcgtgtgcggtggaactgcgtacccgtgttcgcgcggaactcaatgactctatcactattcagagc (SEQ ID NO:179).

[0991] MCM520:

aaagaccgaccaagcgacgtctgagagctccctggcgaattcggtaccaataaaagagctttattttcatgatctgtgtgttggtttttgtg tgcggcgcggaagttcctattctctagaaagtataggaacttcctcgagccctatagtgagtcgtattaaattcatataaaaaacatacag ataaccatctgcggtgataaattatctctggcggtgttgacctaaataccactggcggtgatactgagcacatcagcaggacgcactga ccaccatgaaggtgcaaaggtaaaaaaacatggtatcctgttctgcgccgggtaagatttacctgttcggtgaacacgccgtagtttatg gcgaaactgcaattgcgtgtgcggtggaactgcgtacccgtgttcgcgcggaactcaatgactctatcactattcagagc (SEQ ID NO:180).

[0992] MCM521:

aaagaccgaccaagcgacgtctgagagctccctggcgaattcggtaccaataaaagagctttattttcatgatctgtgtgttggtttttgtg tgcggcgcggaagttcctattctctagaaagtataggaacttcctcgagccctatagtgagtcgtattaaattcatataaaaaacatacag ataaccatctgcggtgataaattatctctggcggtgttgacgtaaataccactggcggtgatactgagcacatcagcaggacgcactga ccaccatgaaggtgcaaaggaggtaaaaaaacatggtatcctgttctgcgccgggtaagatttacctgttcggtgaacacgccgtagtt tatggcgaaactgcaattgcgtgtgcggtggaactgcgtacccgtgttcgcgcggaactcaatgactctatcactattcagagc (SEQ ID NO:181).

[0993] *Deletion of glpK and gldA in MCM531.* A P1 lysate was made of strain JW3897 (glpK::Kan) or JW5556 (gldA::Kan) from the Keio collection (Baba et al. 2006). The *gldA:Kan* P1 lysate was used to transduce MCM531 and transductants were selected on L agar containing kanamycin (10 μg/mL). 3 colonies were screened by PCR using primers CMP5 (SEQ ID NO:184) and CMP6 (SEQ ID NO:185) to confirm the deletion of *gldA*. One correct colony was selected and designated CMP212 (MCM531*gldA::Kan*). The Kan antibiotic resistance marker was looped out by transforming CMP212 with pCP20, selecting transformants at 30°C on LB + carbenicillin 50 μg/L, streaking two transformants on LB at 42°C overnight, and, from that, selecting a colony sensitive to kanamycin and carbenicillin. The resultant strain was designated CMP219 (MCM531 *gldA* ML). The *glpK:Kan* P1 lysate was used to transduce strain CMP219 and transductants were selected on L agar containing kanamycin (10 μg/mL). Three colonies were screened by PCR using primers CMP1 (SEQ

ID NO:182) and CMP3 (SEQ ID NO:183) to confirm the deletion of *glpK*. One correct colony was selected and designated CMP229 (CMP219 *glpK::Kan)*.

Primers
CMP1 GCTATTCTGATGGGGCTGATCC (SEQ ID NO:182)
CMP3 GCCTTTATCGCCTACTGCCAGC (SEQ ID NO:183)
CMP5 CGTAGCGCATCAGGCAATTTTGCG (SEQ ID NO:184)
CMP6 GTGACTTCCGAAGGTCTGGCAGC (SEQ ID NO:185)

[0994] *Construction of CMP239. E. coli* strain CMP239 is derived from *E. coli* BL21 harboring the pathway for the production of isoprene and the pathway for production of 1,3-propanediol. Strain CMP229 (constructed as described above) was electroporated with pEWL244 (SEQ ID NO:45 and Figures 158-159) and pDW15 (SEQ ID NO:161 and Figure 183). Transformants were selected on L agar containing Carbenicillin (50 μg/mL) and Gentamycin (5 μg/mL). The resulting strain was electroporated with plasmid pSYCO109F1.1 (SEQ ID NO:163 and Figure 185; *see also* US Patent Publication No. US 2008/0293119 A1, which is incorporated herein by reference, particularly with respect to disclosure relating to plasmid pSYCO109F1.1) and transformants were selected on L agar containing carbenicillin (50 μg/mL), gentamycin (5 μg/mL) and spectinomycin (50 μg/mL). The strain thus generated was named CMP239.

[0995] *Restoration of pgl in CMP229.* This example describes the construction of *Escherichia coli* strains derived from BL21 transduced with P1 phage containing *E. coli* MG1655 genomic DNA and selected for recombination of a 17,257 bp piece present in MG1655 but absent in BL21 and BL21(DE3).

[0996] A P1 lysate was made of *E. coli* strain MG1655. The lysate was used to infect strain CMP229. Transductants were selected for by plating the cells on M9 medium supplemented with 0.5% (w/v) galactose (the galactose utilization operon is adjacent to the pgl gene). Each liter of M9 medium contains 200 ml of M9 salts, 2 ml of sterile 1 M $MgSO_4$, and 100 μl of sterile 1 M $CaCl_2$. The volume is adjusted to 1000 ml with distilled H_2O, and the solution is filter sterilized. Each liter of M9 salts contains 64 g Na_2HPO_4-$7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, and 5.0 g NH_4Cl. The solution is stirred until the salts dissolve. Volume is then adjusted to 1000 ml with distilled H_2O and the solution is sterilized by autoclaving. Integration of the 17,257 bp fragment in colonies which grew on the M9 + galactose was confirmed by PCR with the galMF primer (5'-GAC GCT TTC GCC AAG TCA GG; SEQ ID

NO:186) and the galMR primer (5'-GTCAGGCTGGAATACTCTTCG; SEQ ID NO:187), which anneal to the galM gene as shown on Figure 186, using the protocol. One colony was stirred in 30 μL H_2O and heated to 95°C for 5 minutes. The resulting solution was spun down and 2 μL of the supernatant were used as the template in the following PCR reaction: 2μl colony in H_2O, 5 μl Herculase® Buffer, 1 μl 100 mM dNTPs, 1 μl 10 μM Forward primer, 1 μl 10 μM Reverse primer, 39.5 μL H_2O + 0.5 μL of Herculase® Enhanced DNA Polymerase from Stratagene (La Jolla, CA). The reactions were cycled as follows: 95°C / 2 minutes; 30 cycles of 95°C / 30 seconds, 52°C (3°C lower than lower T^m of primers) / 30 seconds, 72°C / 60 seconds (~60 seconds/kbp); 72°C / 7 minutes; then cooled to 4°C (PCRExpress Thermocycler from ThermoHybaid). A PCR using those primers does not give a product if the template is chromosomal DNA of BL21, which lacks the 17,257 bp fragment obtained from *E. coli* strain MG1655 (*see* Figure 186).

[0996] The size of the resulting PCR fragments was determined on a 0.8% E-gel (Invitrogen), using DNA Molecular Weight X (Roche) as a ladder. A correct colony was selected and designated CMP241 (BL21 PL.2 mKKDyI (MCM531) t gldAML t glpK::Kan t pgl+4).

[0997] *Construction of E. coli strain CMP249.* This experiment describes the construction of a strain derived from BL21 harboring the pathway for the production of isoprene and the pathway for production of 1,3-propanediol. This strain also contain a 17,257 bp piece present in *E. coli* K12 strain MG1655 but absent in BL21 and BL21(DE3). Strain CMP241 (constructed as described above) was electroporated with pEWL244 (SEQ ID NO:45 and Figures 158-159) and pDW15 (SEQ ID NO:161 and Figure 183). Transformants were selected on L agar containing Carbenicillin (50 μg/mL) and Gentamycin (5 μg/mL). The resulting strain was electroporated with plasmid pSYCO109F1.1 and transformants were selected on L agar containing carbenicillin (50 μg/mL), gentamycin (5 μg/mL) and spectinomycin (50 μg/mL). The strain thus generated was named CMP249.

[0998] *Co-Production of isoprene and 1,3-propanediol (1,3-PDO) by E. coli strain CMP249.* CMP249 was tested in shake flasks for the production of both isoprene and 1,3-PDO.

[0999] *Culture conditions*. Shake flask experiments were done in 250 ml Erlenmeyer flasks containing 25 mL TM3 medium (per liter: 13.6 g K_2PO_4, 13.6 g KH_2PO_4, 2.0 g $MgSO_4*7H_2O$, 2.0 g citric acid monohydrate, 0.3 g ferric ammonium citrate, 3.2 g $(NH_4)_2SO_4$, 0.2 g yeast extract, 1.0 ml 1000X Modified Trace Metal Solution, adjusted to pH 6.8, brought to final volume with H_20, and filter sterilized) containing 2% glucose, 200 μM IPTG and extra yeast extract to reach a total of 1 g/L. Shake flasks were run with and without vitamin B12. Each liter of 1000X Modified Trace Metal Solution contains: citric acid*H_2O (4.0 g/L), $MnSO_4*H_2O$ (3.0 g/L), NaCl (1.0 g/L), $FeSO_4*7H_2O$ (0.10 g/L), $COCl_2*6H_2O$ (0.10 g/L), $ZnSO_4*7H_2O$ (0.10 g/L), $CuSO_4*5H_2O$ (0.010 g/L), H_3BO_3 (0.010 g/L), and $Na_2MoO_4*2H_2O$ (0.010 g/L). Cultures were grown at 30°C with shaking at 250 rpm in an Infors Multitron shaker. The production of glycerol from pSYCO109 was driven by an IPTG-inducible Trc promoter (inducible in the presence of the product of the lacI gene), while the production of 1,3-PDO was induced by the addition of vitamin B_{12} (at concentrations ranging from 5-125 mg/mL). The production of isoprene was induced by the addition of IPTG (for example, at 200 μM). The growth of the culture was followed by monitoring optical density (OD) at a wavelength of 600 nM.

[1000] *Detection of glycerol and 1,3-PDO*. The methods used are described in US Patent Publication No. US 2008/0176302 A1, which is incorporated herein by reference, particular with respect to methods of detecting production of 1,3-PDO by HPLC. Briefly, the conversion of glucose to 1,3-propanediol was monitored by HPLC. Analyses were performed using standard chromatography. One suitable method utilized a Waters Alliance HPLC system using R1 detection. Samples were injected onto an Aminex HPX87H column (7.8 mm × 300 mm, Biorad, Hercules, CA) equipped with a Cation H Refill Cartridge precolumn (4.6 mm × 30 mm, Biorad, Hercules, CA), temperature controlled at 50°C, using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.4 mL/minute. The system was calibrated weekly against standards of known concentration. Typically, the retention times of glucose, glycerol, 1,3-propanediol, and acetic acid were 12.7 min, 19.0 min, 25.2 min, and 21.5 min, respectively.

[1001] *Headspace analysis for the detection of isoprene*. The headspace analysis was performed as described in International Patent Publication No. WO 2009/076676 A2, which is incorporated herein by reference, particularly with respect to methods of headspace analysis to detect isoprene. Briefly, one ml of a shake flask culture was transferred from to a

20 ml CTC headspace vial (Agilent vial cat# 5188 2753; cap cat# 5188 2759). The cap was screwed on tightly and the vial was incubated at the equivalent temperature with shaking at 250 rpm. After 30 minutes, the vials were removed from the incubator and analyzed as follows. The analysis was performed using an Agilent 6890 GC/MS system interfaced with a CTC Analytics (Switzerland) CombiPAL autosampler operating in headspace mode. An Agilent HP-5MS GC/MS column (30 m × 0.25 mm; 0.25 μm film thickness) was used for separation of analytes. The sampler was set up to inject 500 μL of headspace gas. The GC/MS method utilized helium as the carrier gas at a flow of 1 ml/minutes The injection port was held at 250°C with a split ratio of 50: 1. The oven temperature was held at 37°C for the 2 minute duration of the analysis. The Agilent 5793N mass selective detector was run in single ion monitoring (SIM) mode on *m/z* 67. The detector was switched off from 1.4 to 1.7 minutes to allow the elution of permanent gases. Under these conditions isoprene (2-methyl-1,3-butadiene) was observed to elute at 1.78 minutes. A calibration table was used to quantify the absolute amount of isoprene and was found to be linear from 1 μg/L to 200 μg/L. The limit of detection was estimated to be 50 to 100 ng/L using this method.

[1002] *Shake flask experiments*. Shake flask experiments were run with strain CMP249. Different concentrations of vitamin B12 were tested (data not shown), with 125 mg/L and above showing the same amount of 1,3-propanediol (lower concentrations resulted in production of less 1,3-propanediol). The simultaneous production of isoprene and glycerol or 1,3-propanediol was measured. Figures 187A-D present data from the same set of shake flasks. Figure 187A shows production of glycerol and 1,3-propanediol by *E. coli* strain CMP249 in the presence of 200 μM IPTG. Figure 187B shows production of isoprene by *E. coli* strain CMP249 in the presence of 200 μM IPTG, plus or minus 125 mg/L vitamin B12. Figure 187C shows an OD profile and glucose consumption by *E. coli* strain CMP249 in the presence of 200 μM IPTG, plus or minus 125 mg/L vitamin B12. Figure 187D shows molar yield of 1,3-propanediol and glycerol from strain CMP249 in the presence of 200 μM IPTG, plus or minus 125 mg/L vitamin B12. Glycerol/1,3-propanediol molar yield is calculated as follows: Molar yield = (glycerol produced (g/L)/92 + 1,3-propanediol produced (g/L)/76)/(glucose consumed (g/L)/180).

Example 32: Co-production of isoprene and 1,3-propanediol in *E. coli* BL21 grown in fed-batch culture at the 15-L scale.

[1003] *Medium Recipe (per liter fermentation medium)*: 7.5 g K_2HPO_4, 2 g $MgSO_4*7H_2O$, 2 g citric acid monohydrate, 0.3 g ferric ammonium citrate, 0.5 g yeast extract, 1 ml 1000X Modified Trace Metal Solution. All of the components were added together and dissolved in diH $_2O$. The solution was heat sterilized at 121°C for 20 minutes, then the pH was adjusted to 7.0 with 28% ammonium hydroxide and brought to final volume. Ten g of glucose, 8 mL of Mercury Vitamin Solution, and appropriate antibiotics were added after sterilization and pH adjustment.

[1004] *1000X Modified Trace Metal Solution (per liter)*: 40 g Citric Acid*H_2O, 30 g $MnSO_4*H_2O$, 10 g NaCl, 1 g $FeSO_4*7H_2O$, 1 g $CoCl_2*6H_2O$, 1 g $ZnSO_4*7H_2O$, 100 mg $CuSO_4*5H_2O$, 100 mg H_3BO_3, 100 mg $NaMoO_4*2H_2O$. Each component was dissolved one at a time in diH $_2O$, the pH was adjusted to 3.0 with HCl/NaOH, the solution was brought to final volume, and then filter sterilized with a 0.22 micron filter.

[1005] *Mercury Vitamin Solution (per liter)*: 1.0 g Thiamine hydrochloride, 1.0 g D-(+)-biotin, 1.0 g nicotinic acid, 4.8 g D-pantothenic acid, 4.0 g pyridoxine hydrochloride. Each component was dissolved one at a time in diH2O, the pH was adjusted to 3.0 with HCl/NaOH, the solution was brought to final volume and filter sterilized with a 0.22 micron filter.

[1006] *Feed solution (per kilogram)*: 0.57 kg Glucose, 0.38 kg diH_2O, 7.5 g K_2HPO_4, and 10 g 100% Foamblast. All components were mixed together and autoclaved. 5.6 mL Macro Salt Solution, 0.8 mL 1000X Modified Trace Metal Solution, and 6.7 mL Mercury Vitamin Solution were added after the solution had cooled to 25°C.

[1007] *Macro Salt Solution (per liter)*: 296 g $MgSO_4*7H_2O$, 296 g citric acid monohydrate, and 49.6 g ferric ammonium citrate were dissolved in water, brought to final volume and filter sterilized with a 0.22 micron filter.

[1008] This experiment monitors isoprene and 1,3-propanediol formation from glucose at the desired fermentation pH (7.0) and temperature (34°C). Fermentation was performed in a 15-L bioreactor with *E. coli* BL21 cells of strain CMP239 (prepared as described above). Strain CMP239 expresses the upper mevalonic acid (MVA) pathway (pDW15; *see* Example

30 above), the integrated lower MVA pathway (PL.2 mKKDyI), mevalonate kinase from *M. mazei* and truncated isoprene synthase from *P. alba* (pTrcAlba(MEA) mMVK (pDW34)), and the genes required for 1,3-propanediol production (pSYCO109F1.1), without restoration of the *pgl* gene. A frozen vial of *E. coli* strain CMP239 was thawed and inoculated into tryptone-yeast extract medium. After the inoculum grew to optical density 1.0, measured at 550 nm (OD_{550}), 500 mL was used to inoculate a 15-L bioreactor and bring the initial tank volume to 5-L.

[1009] The feed solution was fed at an exponential rate until a top feed rate of 5.8 g/minute was reached. After this time, the glucose feed was adjusted to meet metabolic demands at rates less than or equal to 5.8 g/min. The total amount of glucose delivered to the bioreactor was 4.2 kg over 37 hours of fermentation. Induction was achieved by adding isopropyl-beta-D-1-thiogalactopyranoside (IPTG) in a stepwise fashion shown in Table 23.

[1010] Two shots of 208 mg of vitamin B12 were administered at 12.8 and 35.8 hours. The OD_{550} profile within the bioreactor over time is shown in Figure 188. The isoprene level in the off gas from the bioreactor was determined using a Hiden mass spectrometer. The isoprene titer increased over the course of the fermentation to a maximum value of 2.2 g/L at 37 hrs (Figure 189). The total amount of isoprene produced during the 37 hour fermentation was 17.7 g and the time course of production is shown in Figure 190. The time course of isoprene specific productivity is shown in Figure 191. The 1,3-propanediol titer increased over the course of the fermentation to a maximum value of 53.3 g/L at 37 hrs (Figure 192). The total amount of 1,3-propanediol produced during the 37 hour fermentation was 507.9 g and the time course of production is shown in Figure 193. The time course of 1,3-propanediol specific productivity is shown in Figure 194. The glycerol titer increased over the course of the fermentation to a maximum value of 27.3 g/L at 37 hours (Figure 195). The total amount of glycerol produced during the 37 hour fermentation was 259.8 g and the time course of production is shown in Figure 196. The time course of glycerol specific productivity is shown in Figure 197. Final product yields are shown in Table 24.

Table 23. IPTG additions during the fermentation.

Time (hr)	Optical Density (550 nm)	IPTG concentration (uM)
5.3	25.0	51.1
17.8	110.0	95.9
18.4	122.0	138.4
24.3	182.0	207.0

31.1	195.0	261.0

Table 24. Product yields after 37 hours of fermentation.

	Isoprene	1,3-Propanediol	Glycerol
Mole carbon %, product C/total C * 100	1.0	14.9	7.2
Weight %, g product/g glucose * 100	0.4	12.7	6.5

Appendix 1

Exemplary 1-deoxy-D-xylulose-5-phosphate synthase nucleic acids and polypeptides

ATH: AT3G21500(DXPS1)
AT4G15560(CLA1) AT5G11380(DXPS3)
OSA: 4338768 4340090 4342614
CME: CMF089C
PFA: MAL13P1.186
TAN: TA20470
TPV: TP01_0516
ECO: b0420(dxs)
ECJ: JW0410(dxs)
ECE: Z0523(dxs)
ECS: ECs0474
ECC: c0531(dxs)
ECI: UTI89_C0443(dxs)
ECP: ECP_0479
ECV: APECO1_1590(dxs)
ECW: EcE24377A_0451(dxs)
ECX: EcHS_A0491
STY: STY0461(dxs)
STT: t2441(dxs)
SPT: SPA2301(dxs)
SEC: SC0463(dxs)
STM: STM0422(dxs)
YPE: YPO3177(dxs)
YPK: y1008(dxs)
YPM: YP_0754(dxs)
YPA: YPA_2671
YPN: YPN_0911
YPP: YPDSF_2812
YPS: YPTB0939(dxs)
YPI: YpsIP31758_3112(dxs)
SFL: SF0357(dxs)
SFX: S0365(dxs)
SFV: SFV_0385(dxs)
SSN: SSON_0397(dxs)
SBO: SBO_0314(dxs)
SDY: SDY_0310(dxs)
ECA: ECA1131(dxs)
PLU: plu3887(dxs)
BUC: BU464(dxs)
BAS: BUsg448(dxs)
WBR: WGLp144(dxs)
SGL: SG0656
KPN: KPN_00372(dxs)
BFL: Bfl238(dxs)
BPN: BPEN_244(dxs)
HIN: HI1439(dxs)
HIT: NTHI1691(dxs)
HIP: CGSHiEE_04795
HIQ: CGSHiGG_01080
HDU: HD0441(dxs)
HSO: HS_0905(dxs)
PMU: PM0532(dxs)

MSU: MS1059(dxs)
APL: APL_0207(dxs)
XFA: XF2249
XFT: PD1293(dxs)
XCC: XCC2434(dxs)
XCB: XC_1678
XCV: XCV2764(dxs)
XAC: XAC2565(dxs)
XOO: XOO2017(dxs)
XOM: XOO_1900(XOO1900)
VCH: VC0889
VVU: VV1_0315
VVY: VV0868
VPA: VP0686
VFI: VF0711
PPR: PBPRA0805
PAE: PA4044(dxs)
PAU: PA14_11550(dxs)
PAP: PSPA7_1057(dxs)
PPU: PP_0527(dxs)
PST: PSPTO_0698(dxs)
PSB: Psyr_0604
PSP: PSPPH_0599(dxs)
PFL: PFL_5510(dxs)
PFO: Pfl_5007
PEN: PSEEN0600(dxs)
PMY: Pmen_3844
PAR: Psyc_0221(dxs)
PCR: Pcryo_0245
ACI: ACIAD3247(dxs)
SON: SO_1525(dxs)
SDN: Sden_2571
SFR: Sfri_2790
SAZ: Sama_2436
SBL: Sbal_1357
SLO: Shew_2771
SHE: Shewmr4_2731
SHM: Shewmr7_2804
SHN: Shewana3_2901
SHW: Sputw3181_2831
ILO: IL2138(dxs)
CPS: CPS_1088(dxs)
PHA: PSHAa2366(dxs)
PAT: Patl_1319
SDE: Sde_3381
PIN: Ping_2240
MAQ: Maqu_2438
MCA: MCA0817(dxs)
FTU: FTT1018c(dxs)
FTF: FTF1018c(dxs)
FTW: FTW_0925(dxs)
FTL: FTL_1072
FTH: FTH_1047(dxs)
FTA: FTA_1131(dxs)
FTN: FTN_0896(dxs)
NOC: Noc_1743
AEH: Mlg_1381
HCH: HCH_05866(dxs)

CSA: Csal_0099
ABO: ABO_2166(dxs)
AHA: AHA_3321(dxs)
BCI: BCI_0275(dxs)
RMA: Rmag_0386
VOK: COSY_0360(dxs)
NME: NMB1867
NMA: NMA0589(dxs)
NMC: NMC0352(dxs)
NGO: NGO0036
CVI: CV_2692(dxs)
RSO: RSc2221(dxs)
REU: Reut_A0882
REH: H16_A2732(dxs)
RME: Rmet_2615
BMA: BMAA0330(dxs)
BMV: BMASAVP1_1512(dxs)
BML: BMA10299_1706(dxs)
BMN: BMA10247_A0364(dxs)
BXE: Bxe_B2827
BUR: Bcep18194_B2211
BCN: Bcen_4486
BCH: Bcen2424_3879
BAM: Bamb_3250
BPS: BPSS1762(dxs)
BPM: BURPS1710b_A0842(dxs)
BPL: BURPS1106A_A2392(dxs)
BPD: BURPS668_A2534(dxs)
BTE: BTH_II0614(dxs)
BPE: BP2798(dxs)
BPA: BPP2464(dxs)
BBR: BB1912(dxs)
RFR: Rfer_2875
POL: Bpro_1747
PNA: Pnap_1501
AJS: Ajs_1038
MPT: Mpe_A2631
HAR: HEAR0279(dxs)
MMS: mma_0331
NEU: NE1161(dxs)
NET: Neut_1501
NMU: Nmul_A0236
EBA: ebA4439(dxs)
AZO: azo1198(dxs)
DAR: Daro_3061
TBD: Tbd_0879
MFA: Mfla_2133
HPY: HP0354(dxs)
HPJ: jhp0328(dxs)
HPA: HPAG1_0349
HHE: HH0608(dxs)
HAC: Hac_0968(dxs)
WSU: WS1996
TDN: Tmden_0475
CJE: Cj0321(dxs)
CJR: CJE0366(dxs)
CJJ: CJJ81176_0343(dxs)
CJU: C8J_0298(dxs)

CJD: JJD26997_1642(dxs)
CFF: CFF8240_0264(dxs)
CCV: CCV52592_1671(dxs)
CCV52592_1722
CHA: CHAB381_1297(dxs)
CCO: CCC13826_1594(dxs)
ABU: Abu_2139(dxs)
NIS: NIS_0391(dxs)
SUN: SUN_2055(dxs)
GSU: GSU0686(dxs-1) GSU1764(dxs-2)
GME: Gmet_1934 Gmet_2822
PCA: Pcar_1667
PPD: Ppro_1191 Ppro_2403
DVU: DVU1350(dxs)
DVL: Dvul_1718
DDE: Dde_2200
LIP: LI0408(dsx)
DPS: DP2700
ADE: Adeh_1097
MXA: MXAN_4643(dxs)
SAT: SYN_02456
SFU: Sfum_1418
PUB: SAR11_0611(dxs)
MLO: mlr7474
MES: Meso_0735
SME: SMc00972(dxs)
ATU: Atu0745(dxs)
ATC: AGR_C_1351
RET: RHE_CH00913(dxs)
RLE: RL0973(dxs)
BME: BMEI1498
BMF: BAB1_0462(dxs)
BMS: BR0436(dxs)
BMB: BruAb1_0458(dxs)
BOV: BOV_0443(dxs)
BJA: bll2651(dxs)
BRA: BRADO2161(dxs)
BBT: BBta_2479(dxs)
RPA: RPA0952(dxs)
RPB: RPB_4460
RPC: RPC_1149
RPD: RPD_4305
RPE: RPE_1067
NWI: Nwi_0633
NHA: Nham_0778
BHE: BH04350(dxs)
BQU: BQ03540(dxs)
BBK: BARBAKC583_0400(dxs)
CCR: CC_2068
SIL: SPO0247(dxs)
SIT: TM1040_2920
RSP: RSP_0254(dxsA) RSP_1134(dxs)
JAN: Jann_0088 Jann_0170
RDE: RD1_0101(dxs) RD1_0548(dxs)
MMR: Mmar10_0849
HNE: HNE_1838(dxs)
ZMO: ZMO1234(dxs) ZMO1598(dxs)
NAR: Saro_0161

SAL: Sala_2354
ELI: ELI_12520
GOX: GOX0252
GBE: GbCGDNIH1_0221
GbCGDNIH1_2404
RRU: Rru_A0054 Rru_A2619
MAG: amb2904
MGM: Mmc1_1048
SUS: Acid_1783
BSU: BG11715(dxs)
BHA: BH2779
BAN: BA4400(dxs)
BAR: GBAA4400(dxs)
BAA: BA_4853
BAT: BAS4081
BCE: BC4176(dxs)
BCA: BCE_4249(dxs)
BCZ: BCZK3930(dxs)
BTK: BT9727_3919(dxs)
BTL: BALH_3785(dxs)
BLI: BL01523(dxs)
BLD: BLi02598(dxs)
BCL: ABC2462(dxs)
BAY: RBAM_022600
BPU: BPUM_2159
GKA: GK2392
GTN: GTNG_2322
LMO: lmo1365(tktB)
LMF: LMOf2365_1382(dxs)
LIN: lin1402(tktB)
LWE: lwe1380(tktB)
LLA: L108911(dxsA) L123365(dxsB)
LLC: LACR_1572 LACR_1843
LLM: llmg_0749(dxsB)
SAK: SAK_0263
LPL: lp_2610(dxs)
LJO: LJ0406
LAC: LBA0356
LSL: LSL_0209(dxs)
LGA: LGAS_0350
STH: STH1842
CAC: CAC2077 CA_P0106(dxs)
CPE: CPE1819
CPF: CPF_2073(dxs)
CPR: CPR_1787(dxs)
CTC: CTC01575
CNO: NT01CX_1983
CTH: Cthe_0828
CDF: CD1207(dxs)
CBO: CBO1881(dxs)
CBA: CLB_1818(dxs)
CBH: CLC_1825(dxs)
CBF: CLI_1945(dxs)
CKL: CKL_1231(dxs)
CHY: CHY_1985(dxs)
DSY: DSY2348
DRM: Dred_1078
PTH: PTH_1196(dxs)

SWO: Swol_0582
CSC: Csac_1853
TTE: TTE1298(dxs)
MTA: Moth_1511
MPE: MYPE730
MGA: MGA_1268(dxs)
MTU: Rv2682c(dxs1) Rv3379c(dxs2)
MTC: MT2756(dxs)
MBO: Mb2701c(dxs1) Mb3413c(dxs2)
MLE: ML1038(dxs)
MPA: MAP2803c(dxs)
MAV: MAV_3577(dxs)
MSM: MSMEG_2776(dxs)
MMC: Mmcs_2208
CGL: NCgl1827(cgl1902)
CGB: cg2083(dxs)
CEF: CE1796
CDI: DIP1397(dxs)
CJK: jk1078(dxs)
NFA: nfa37410(dxs)
RHA: RHA1_ro06843
SCO: SCO6013(SC1C3.01)
SCO6768(SC6A5.17)
SMA: SAV1646(dxs1) SAV2244(dxs2)
TWH: TWT484
TWS: TW280(Dxs)
LXX: Lxx10450(dxs)
CMI: CMM_1660(dxsA)
AAU: AAur_1790(dxs)
PAC: PPA1062
TFU: Tfu_1917
FRA: Francci3_1326
FAL: FRAAL2088(dxs)
ACE: Acel_1393
SEN: SACE_1815(dxs) SACE_4351
BLO: BL1132(dxs)
BAD: BAD_0513(dxs)
FNU: FN1208 FN1464
RBA: RB2143(dxs)
CTR: CT331(dxs)
CTA: CTA_0359(dxs)
CMU: TC0608
CPN: CPn1060(tktB_2)
CPA: CP0790
CPJ: CPj1060(tktB_2)
CPT: CpB1102
CCA: CCA00304(dxs)
CAB: CAB301(dxs)
CFE: CF0699(dxs)
PCU: pc0619(dxs)
TPA: TP0824
TDE: TDE1910(dxs)
LIL: LA3285(dxs)
LIC: LIC10863(dxs)
LBJ: LBJ_0917(dxs)
LBL: LBL_0932(dxs)
SYN: sll1945(dxs)
SYW: SYNW1292(Dxs)

SYC: syc1087_c(dxs)
SYF: Synpcc7942_0430
SYD: Syncc9605_1430
SYE: Syncc9902_1069
SYG: sync_1410(dxs)
SYR: SynRCC307_1390(dxs)
SYX: SynWH7803_1223(dxs)
CYA: CYA_1701(dxs)
CYB: CYB_1983(dxs)
TEL: tll0623
GVI: gll0194
ANA: alr0599
AVA: Ava_4532
PMA: Pro0928(dxs)
PMM: PMM0907(Dxs)
PMT: PMT0685(dxs)
PMN: PMN2A_0300
PMI: PMT9312_0893
PMB: A9601_09541(dxs)
PMC: P9515_09901(dxs)
PMF: P9303_15371(dxs)
PMG: P9301_09521(dxs)
PMH: P9215_09851
PMJ: P9211_08521
PME: NATL1_09721(dxs)
TER: Tery_3042
BTH: BT_1403 BT_4099
BFR: BF0873 BF4306
BFS: BF0796(dxs) BF4114
PGI: PG2217(dxs)
CHU: CHU_3643(dxs)
GFO: GFO_3470(dxs)
FPS: FP0279(dxs)
CTE: CT0337(dxs)
CPH: Cpha266_0671
PVI: Cvib_0498
PLT: Plut_0450
DET: DET0745(dxs)
DEH: cbdb_A720(dxs)
DRA: DR_1475
DGE: Dgeo_0994
TTH: TTC1614
TTJ: TTHA0006
AAE: aq_881
TMA: TM1770
PMO: Pmob_1001

Exemplary acetyl-CoA-acetyltransferase nucleic acids and polypeptides

HSA: 38(ACAT1) 39(ACAT2)
PTR: 451528(ACAT1)
MCC: 707653(ACAT1) 708750(ACAT2)
MMU: 110446(Acat1) 110460(Acat2)
RNO: 25014(Acat1)
CFA: 484063(ACAT2) 489421(ACAT1)
GGA: 418968(ACAT1)
421587(RCJMB04_34i5)
XLA: 379569(MGC69098)
414622(MGC81403) 414639(MGC81256)
444457(MGC83664)
XTR: 394562(acat2)
DRE: 30643(acat2)
SPU: 759502(LOC759502)
DME: Dmel_CG10932 Dmel_CG9149
CEL: T02G5.4 T02G5.7 T02G5.8(kat-1)
ATH: AT5G48230(ACAT2/EMB1276)
OSA: 4326136 4346520
CME: CMA042C CME087C
SCE: YPL028W(ERG10)
AGO: AGOS_ADR165C
PIC: PICST_31707(ERG10)
CAL: CaO19.1591(erg10)
CGR: CAGL0L12364g
SPO: SPBC215.09c
MGR: MGG_01755 MGG_13499
ANI: AN1409.2

AFM: AFUA_6G14200 AFUA_8G04000
AOR: AO090103000012 AO090103000406
CNE: CNC05280
UMA: UM03571.1
DDI: DDB_0231621
PFA: PF14_0484
TET: TTHERM_00091590
TTHERM_00277470 TTHERM_00926980
TCR: 511003.60
ECO: b2224(atoB)
ECJ: JW2218(atoB) JW5453(yqeF)
ECE: Z4164(yqeF)
ECS: ECs3701
ECC: c2767(atoB) c3441(yqeF)
ECI: UTI89_C2506(atoB)
UTI89_C3247(yqeF)
ECP: ECP_2268 ECP_2857
ECV: APECO1_3662(yqeF)
APECO1_4335(atoB)
APECO1_43352(atoB)
ECX: EcHS_A2365
STY: STY3164(yqeF)
STT: t2929(yqeF)
SPT: SPA2886(yqeF)
SEC: SC2958(yqeF)
STM: STM3019(yqeF)
SFL: SF2854(yqeF)

SFX: S3052(yqeF)
SFV: SFV_2922(yqeF)
SSN: SSON_2283(atoB) SSON_3004(yqeF)
SBO: SBO_2736(yqeF)
ECA: ECA1282(atoB)
ENT: Ent638_3299
SPE: Spro_0592
HIT: NTHI0932(atoB)
XCC: XCC1297(atoB)
XCB: XC_2943
XCV: XCV1401(thlA)
XAC: XAC1348(atoB)
XOO: XOO1881(atoB)
XOM: XOO_1778(XOO1778)
VCH: VCA0690
VCO: VC0395_0630
VVU: VV2_0494 VV2_0741
VVY: VVA1043 VVA1210
VPA: VPA0620 VPA1123 VPA1204
PPR: PBPRB1112 PBPRB1840
PAE: PA2001(atoB) PA2553 PA3454 PA3589 PA3925
PAU: PA14_38630(atoB)
PPU: PP_2051(atoB) PP_2215(fadAx) PP_3754 PP_4636
PPF: Pput_2009 Pput_2403 Pput_3523 Pput_4498
PST: PSPTO_0957(phbA-1) PSPTO_3164(phbA-2)
PSB: Psyr_0824 Psyr_3031
PSP: PSPPH_0850(phbA1) PSPPH_2209(phbA2)
PFL: PFL_1478(atoB-2) PFL_2321 PFL_3066 PFL_4330(atoB-2) PFL_5283
PFO: Pfl_1269 Pfl_1739 Pfl_2074 Pfl_2868
PEN: PSEEN3197 PSEEN3547(fadAx) PSEEN4635(phbA)
PMY: Pmen_1138 Pmen_2036 Pmen_3597 Pmen_3662 Pmen_3820
PAR: Psyc_0252 Psyc_1169
PCR: Pcryo_0278 Pcryo_1236 Pcryo_1260
PRW: PsycPRwf_2011
ACI: ACIAD0694 ACIAD1612 ACIAD2516(atoB)
SON: SO_1677(atoB)
SDN: Sden_1943
SFR: Sfri_1338 Sfri_2063
SAZ: Sama_1375
SBL: Sbal_1495
SBM: Shew185_1489
SBN: Sbal195_1525
SLO: Shew_1667 Shew_2858
SPC: Sputcn32_1397
SSE: Ssed_1473 Ssed_3533
SPL: Spea_2783
SHE: Shewmr4_2597
SHM: Shewmr7_2664
SHN: Shewana3_2771

SHW: Sputw3181_2704

ILO: IL0872

CPS: CPS_1605 CPS_2626

PHA: PSHAa0908 PSHAa1454(atoB) PSHAa1586(atoB)

PAT: Patl_2923

SDE: Sde_3149

PIN: Ping_0659 Ping_2401

MAQ: Maqu_2117 Maqu_2489 Maqu_2696 Maqu_3162

CBU: CBU_0974

LPN: lpg1825(atoB)

LPF: lpl1789

LPP: lpp1788

NOC: Noc_1891

AEH: Mlg_0688 Mlg_2706

HHA: Hhal_1685

HCH: HCH_05299

CSA: Csal_0301 Csal_3068

ABO: ABO_0648(fadAx)

MMW: Mmwyl1_0073 Mmwyl1_3021 Mmwyl1_3053 Mmwyl1_3097 Mmwyl1_4182

AHA: AHA_2143(atoB)

CVI: CV_2088(atoB) CV_2790(phaA)

RSO: RSc0276(atoB) RSc1632(phbA) RSc1637(bktB) RSc1761(RS02948)

REU: Reut_A0138 Reut_A1348 Reut_A1353 Reut_B4561 Reut_B4738 Reut_B5587 Reut_C5943 Reut_C6062

REH: H16_A0170 H16_A0867 H16_A0868 H16_A0872 H16_A1297 H16_A1438(phaA) H16_A1445(bktB) H16_A1528 H16_A1713 H16_A1720 H16_A1887 H16_A2148 H16_B0380 H16_B0381 H16_B0406 H16_B0662 H16_B0668 H16_B0759 H16_B1369 H16_B1771

RME: Rmet_0106 Rmet_1357 Rmet_1362 Rmet_5156

BMA: BMA1316 BMA1321(phbA) BMA1436

BMV: BMASAVP1_A1805(bktB) BMASAVP1_A1810(phbA)

BML: BMA10299_A0086(phbA) BMA10299_A0091

BMN: BMA10247_1076(bktB) BMA10247_1081(phbA)

BXE: Bxe_A2273 Bxe_A2335 Bxe_A2342 Bxe_A4255 Bxe_B0377 Bxe_B0739 Bxe_C0332 Bxe_C0574 Bxe_C0915

BVI: Bcep1808_0519 Bcep1808_1717 Bcep1808_2877 Bcep1808_3594 Bcep1808_4015 Bcep1808_5507 Bcep1808_5644

BUR: Bcep18194_A3629 Bcep18194_A5080 Bcep18194_A5091 Bcep18194_A6102 Bcep18194_B0263 Bcep18194_B1439 Bcep18194_C6652 Bcep18194_C6802 Bcep18194_C6874 Bcep18194_C7118 Bcep18194_C7151 Bcep18194_C7332

BCN: Bcen_1553 Bcen_1599 Bcen_2158 Bcen_2563 Bcen_2998 Bcen_6289

BCH: Bcen2424_0542 Bcen2424_1790 Bcen2424_2772 Bcen2424_5368 Bcen2424_6232 Bcen2424_6276

BAM: Bamb_0447 Bamb_1728 Bamb_2824 Bamb_4717 Bamb_5771 Bamb_5969

BPS: BPSL1426 BPSL1535(phbA) BPSL1540

BPM: BURPS1710b_2325(bktB) BURPS1710b_2330(phbA) BURPS1710b_2453(atoB-2)

BPL: BURPS1106A_2197(bktB) BURPS1106A_2202(phbA)

BPD: BURPS668_2160(bktB) BURPS668_2165(phbA)

BTE: BTH_I2144 BTH_I2256 BTH_I2261

PNU: Pnuc_0927

BPE: BP0447 BP0668 BP2059

BPA: BPP0608 BPP1744 BPP3805 BPP4216 BPP4361

BBR: BB0614 BB3364 BB4250 BB4804 BB4947

RFR: Rfer_0272 Rfer_1000 Rfer_1871 Rfer_2273 Rfer_2561 Rfer_2594 Rfer_3839

POL: Bpro_1577 Bpro_2140 Bpro_3113 Bpro_4187

PNA: Pnap_0060 Pnap_0458 Pnap_0867 Pnap_1159 Pnap_2136 Pnap_2804

AAV: Aave_0031 Aave_2478 Aave_3944 Aave_4368

AJS: Ajs_0014 Ajs_0124 Ajs_1931 Ajs_2073 Ajs_2317 Ajs_3548 Ajs_3738 Ajs_3776

VEI: Veis_1331 Veis_3818 Veis_4193

DAC: Daci_0025 Daci_0192 Daci_3601 Daci_5988

MPT: Mpe_A1536 Mpe_A1776 Mpe_A1869 Mpe_A3367

HAR: HEAR0577(phbA)

MMS: mma_0555

NEU: NE2262(bktB)

NET: Neut_0610

EBA: ebA5202 p2A409(tioL)

AZO: azo0464(fadA1) azo0469(fadA2) azo2172(thlA)

DAR: Daro_0098 Daro_3022

HPA: HPAG1_0675

HAC: Hac_0958(atoB)

GME: Gmet_1719 Gmet_2074 Gmet_2213 Gmet_2268 Gmet_3302

GUR: Gura_3043

BBA: Bd0404(atoB) Bd2095
DOL: Dole_0671 Dole_1778 Dole_2160 Dole_2187
ADE: Adeh_0062 Adeh_2365
AFW: Anae109_0064 Anae109_1504
MXA: MXAN_3791
SAT: SYN_02642
SFU: Sfum_2280 Sfum_3582
RPR: RP737
RCO: RC1134 RC1135
RFE: RF_0163(paaJ)
RBE: RBE_0139(paaJ)
RAK: A1C_05820
RBO: A1I_07215
RCM: A1E_04760
PUB: SAR11_0428(thlA)
MLO: mlr3847
MES: Meso_3374
PLA: Plav_1573 Plav_2783
SME: SMa1450 SMc03879(phbA)
SMD: Smed_0499 Smed_3117 Smed_5094 Smed_5096
ATU: Atu2769(atoB) Atu3475
ATC: AGR_C_5022(phbA) AGR_L_2713
RET: RHE_CH04018(phbAch) RHE_PC00068(ypc00040) RHE_PF00014(phbAf)
RLE: RL4621(phaA) pRL100301 pRL120369
BME: BMEI0274 BMEII0817
BMF: BAB1_1783(phbA-1) BAB2_0790(phbA-2)
BMS: BR1772(phbA-1) BRA0448(phbA-2)
BMB: BruAb1_1756(phbA-1) BruAb2_0774(phbA-2)
BOV: BOV_1707(phbA-1)
OAN: Oant_1130 Oant_3107 Oant_3718 Oant_4020
BJA: bll0226(atoB) bll3949 bll7400 bll7819 blr3724(phbA)
BRA: BRADO0562(phbA) BRADO0983(pimB) BRADO3110 BRADO3134(atoB)
BBT: BBta_3558 BBta_3575(atoB) BBta_5147(pimB) BBta_7072(pimB) BBta_7614(phbA)
RPA: RPA0513(pcaF) RPA0531 RPA3715(pimB)
RPB: RPB_0509 RPB_0525 RPB_1748
RPC: RPC_0504 RPC_0636 RPC_0641 RPC_0832 RPC_1050 RPC_2005 RPC_2194 RPC_2228
RPD: RPD_0306 RPD_0320 RPD_3105 RPD_3306
RPE: RPE_0168 RPE_0248 RPE_3827
NWI: Nwi_3060
XAU: Xaut_3108 Xaut_4665
CCR: CC_0510 CC_0894 CC_3462

SIL: SPO0142(bktB) SPO0326(phbA) SPO0773 SPO3408
SIT: TM1040_0067 TM1040_2790 TM1040_3026 TM1040_3735
RSP: RSP_0745 RSP_1354 RSP_3184
RSH: Rsph17029_0022 Rsph17029_2401 Rsph17029_3179 Rsph17029_3921
RSQ: Rsph17025_0012 Rsph17025_2466 Rsph17025_2833
JAN: Jann_0262 Jann_0493 Jann_4050
RDE: RD1_0025 RD1_0201(bktB) RD1_3394(phbA)
PDE: Pden_2026 Pden_2663 Pden_2870 Pden_2907 Pden_4811 Pden_5022
DSH: Dshi_0074 Dshi_3066 Dshi_3331
MMR: Mmar10_0697
HNE: HNE_2706 HNE_3065 HNE_3133
NAR: Saro_0809 Saro_1069 Saro_1222 Saro_2306 Saro_2349
SAL: Sala_0781 Sala_1244 Sala_2896 Sala_3158
SWI: Swit_0632 Swit_0752 Swit_2893 Swit_3602 Swit_4887 Swit_5019 Swit_5309
ELI: ELI_01475 ELI_06705 ELI_12035
GBE: GbCGDNIH1_0447
ACR: Acry_1847 Acry_2256
RRU: Rru_A0274 Rru_A1380 Rru_A1469 Rru_A1946 Rru_A3387
MAG: amb0842
MGM: Mmc1_1165
ABA: Acid345_3239
BSU: BG11319(mmgA) BG13063(yhfS)
BHA: BH1997 BH2029 BH3801(mmgA)
BAN: BA3687 BA4240 BA5589
BAR: GBAA3687 GBAA4240 GBAA5589
BAA: BA_0445 BA_4172 BA_4700
BAT: BAS3418 BAS3932 BAS5193
BCE: BC3627 BC4023 BC5344
BCA: BCE_3646 BCE_4076 BCE_5475
BCZ: BCZK3329(mmgA) BCZK3780(thl) BCZK5044(atoB)
BCY: Bcer98_2722 Bcer98_3865
BTK: BT9727_3379(mmgA) BT9727_3765(thl) BT9727_5028(atoB)
BTL: BALH_3262(mmgA) BALH_3642(fadA) BALH_4843(atoB)
BLI: BL03925(mmgA)
BLD: BLi03968(mmgA)
BCL: ABC0345 ABC2989 ABC3617 ABC3891(mmgA)
BAY: RBAM_022450
BPU: BPUM_2374(yhfS) BPUM_2941 BPUM_3373
OIH: OB0676 OB0689 OB2632 OB3013
GKA: GK1658 GK3397
SAU: SA0342 SA0534(vraB)
SAV: SAV0354 SAV0576(vraB)

SAM: MW0330 MW0531(vraB)
SAR: SAR0351(thl) SAR0581
SAS: SAS0330 SAS0534
SAC: SACOL0426 SACOL0622(atoB)
SAB: SAB0304(th1) SAB0526
SAA: SAUSA300_0355
SAUSA300_0560(vraB)
SAO: SAOUHSC_00336 SAOUHSC_00558
SAJ: SaurJH9_0402
SAH: SaurJH1_0412
SEP: SE0346 SE2384
SER: SERP0032 SERP0220
SHA: SH0510(mvaC) SH2417
SSP: SSP0325 SSP2145
LMO: lmo1414
LMF: LMOf2365_1433
LIN: lin1453
LWE: lwe1431
LLA: L11745(thiL) L25946(fadA)
LLC: LACR_1665 LACR_1956
LLM: llmg_0930(thiL)
SPY: SPy_0140 SPy_1637(atoB)
SPZ: M5005_Spy_0119 M5005_Spy_0432
M5005_Spy_1344(atoB)
SPM: spyM18_0136 spyM18_1645(atoB)
SPG: SpyM3_0108 SpyM3_1378(atoB)
SPS: SPs0110 SPs0484
SPH: MGAS10270_Spy0121
MGAS10270_Spy0433
MGAS10270_Spy1461(atoB)
SPI: MGAS10750_Spy0124
MGAS10750_Spy0452
MGAS10750_Spy1453(atoB)
SPJ: MGAS2096_Spy0123
MGAS2096_Spy0451
MGAS2096_Spy1365(atoB)
SPK: MGAS9429_Spy0121
MGAS9429_Spy0431
MGAS9429_Spy1339(atoB)
SPF: SpyM50447(atoB2)
SPA: M6_Spy0166 M6_Spy0466
M6_Spy1390
SPB: M28_Spy0117 M28_Spy0420
M28_Spy1385(atoB)
SAK: SAK_0568
LJO: LJ1609
LAC: LBA0626(thiL)
LSA: LSA1486
LDB: Ldb0879
LBU: LBUL_0804
LBR: LVIS_2218
LCA: LSEI_1787
LGA: LGAS_1374
LRE: Lreu_0052
EFA: EF1364
OOE: OEOE_0529

STH: STH2913 STH725 STH804
CAC: CAC2873 CA_P0078(thiL)
CPE: CPE2195(atoB)
CPF: CPF_2460
CPR: CPR_2170
CTC: CTC00312
CNO: NT01CX_0538 NT01CX_0603
CDF: CD1059(thlA1) CD2676(thlA2)
CBO: CBO3200(thl)
CBE: Cbei_0411 Cbei_3630
CKL: CKL_3696(thlA1) CKL_3697(thlA2) CKL_3698(thlA3)
AMT: Amet_4630
AOE: Clos_0084 Clos_0258
CHY: CHY_1288 CHY_1355(atoB) CHY_1604 CHY_1738
DSY: DSY0632 DSY0639 DSY1567 DSY1710 DSY2402 DSY3302
DRM: Dred_0400 Dred_1491 Dred_1784 Dred_1892
SWO: Swol_0308 Swol_0675 Swol_0789 Swol_1486 Swol_1934 Swol_2051
TTE: TTE0549(paaJ)
MTA: Moth_1260
MTU: Rv1135A Rv1323(fadA4) Rv3546(fadA5)
MTC: MT1365(phbA)
MBO: Mb1167 Mb1358(fadA4) Mb3576(fadA5) Mb3586c(fadA6)
MBB: BCG_1197 BCG_1385(fadA4) BCG_3610(fadA5) BCG_3620c(fadA6)
MLE: ML1158(fadA4)
MPA: MAP2407c(fadA3) MAP2436c(fadA4)
MAV: MAV_1544 MAV_1573 MAV_1863 MAV_5081
MSM: MSMEG_2224 MSMEG_4920
MUL: MUL_0357
MVA: Mvan_1976 Mvan_1988 Mvan_4305 Mvan_4677 Mvan_4891
MGI: Mflv_1347 Mflv_1484 Mflv_2040 Mflv_2340 Mflv_4356 Mflv_4368
MMC: Mmcs_1758 Mmcs_1769 Mmcs_3796 Mmcs_3864
MKM: Mkms_0251 Mkms_1540 Mkms_1805 Mkms_1816 Mkms_2836 Mkms_3159 Mkms_3286 Mkms_3869 Mkms_3938 Mkms_4227 Mkms_4411 Mkms_4580 Mkms_4724 Mkms_4764 Mkms_4776
MJL: Mjls_0231 Mjls_1739 Mjls_1750 Mjls_2819 Mjls_3119 Mjls_3235 Mjls_3800 Mjls_3850 Mjls_4110 Mjls_4383 Mjls_4705 Mjls_4876 Mjls_5018 Mjls_5063 Mjls_5075
CGL: NCgl2309(cgl2392)
CGB: cg2625(pcaF)
CEF: CE0731 CE2295
CJK: jk1543(fadA3)

NFA: nfa10750(fadA4)
RHA: RHA1_ro01455 RHA1_ro01623 RHA1_ro01876 RHA1_ro02517(catF) RHA1_ro03022 RHA1_ro03024 RHA1_ro03391 RHA1_ro03892 RHA1_ro04599 RHA1_ro05257 RHA1_ro08871
SCO: SCO5399(SC8F4.03)
SMA: SAV1384(fadA5) SAV2856(fadA1)
ART: Arth_1160 Arth_2986 Arth_3268 Arth_4073
NCA: Noca_1371 Noca_1797 Noca_1828 Noca_2764 Noca_4142
TFU: Tfu_1520 Tfu_2394
FRA: Francci3_3687
FRE: Franean1_1044 Franean1_2711 Franean1_2726 Franean1_3929 Franean1_4037 Franean1_4577
FAL: FRAAL2514 FRAAL2618 FRAAL5910(atoB)
ACE: Acel_0626 Acel_0672
SEN: SACE_1192(mmgA) SACE_2736(fadA6) SACE_4011(catF) SACE_6236(fadA4)
STP: Strop_3610
SAQ: Sare_1316 Sare_3991
RXY: Rxyl_1582 Rxyl_1842 Rxyl_2389 Rxyl_2530
FNU: FN0495
BGA: BG0110(fadA)
BAF: BAPKO_0110(fadA)
LIL: LA0457(thiL1) LA0828(thiL2) LA4139(fadA)
LIC: LIC10396(phbA)
LBJ: LBJ_2862(paaJ-4)
LBL: LBL_0209(paaJ-4)
SYN: slr1993(phaA)
SRU: SRU_1211(atoB) SRU_1547
CHU: CHU_1910(atoB)
GFO: GFO_1507(atoB)
FJO: Fjoh_4612
FPS: FP0770 FP1586 FP1725
RRS: RoseRS_3911 RoseRS_4348
RCA: Rcas_0702 Rcas_3206
HAU: Haur_0522
DRA: DR_1072 DR_1428 DR_1960 DR_2480 DR_A0053
DGE: Dgeo_0755 Dgeo_1305 Dgeo_1441 Dgeo_1883
TTH: TTC0191 TTC0330
TTJ: TTHA0559
TME: Tmel_1134
FNO: Fnod_0314
PMO: Pmob_0515
HMA: rrnAC0896(acaB3) rrnAC2815(aca2) rrnAC3497(yqeF) rrnB0240(aca1) rrnB0242(acaB2) rrnB0309(acaB1)
TAC: Ta0582

TVO: TVN0649

PTO: PTO1505

APE: APE_2108

SSO: SSO2377(acaB-4)

STO: ST0514

SAI: Saci_0963 Saci_1361(acaB1)

MSE: Msed_0656

PAI: PAE1220

PIS: Pisl_0029 Pisl_1301

PCL: Pcal_0781

PAS: Pars_0309 Pars_1071

CMA: Cmaq_1941

Exemplary HMG-CoA synthase nucleic acids and polypeptides

HSA: 3157(HMGCS1) 3158(HMGCS2)
PTR: 457169(HMGCS2) 461892(HMGCS1)
MCC: 702553(HMGCS1) 713541(HMGCS2)
MMU: 15360(Hmgcs2) 208715(Hmgcs1)
RNO: 24450(Hmgcs2) 29637(Hmgcs1)
CFA: 479344(HMGCS1) 607923(HMGCS2)
BTA: 407767(HMGCS1)
SSC: 397673(CH242-38B5.1)
GGA: 396379(HMGCS1)
XLA: 380091(hmgcs1) 447204(MGC80816)
DRE: 394060(hmgcs1)
SPU: 578259(LOC578259)
DME: Dmel_CG4311(Hmgs)
CEL: F25B4.6
ATH: AT4G11820(BAP1)
OSA: 4331418 4347614
CME: CMM189C
SCE: YML126C(ERG13)
AGO: AGOS_ADL356C
PIC: PICST_83020
CAL: CaO19_7312(CaO19.7312)
CGR: CAGL0H04081g
SPO: SPAC4F8.14c(hcs)
MGR: MGG_01026
ANI: AN4923.2
AFM: AFUA_3G10660 AFUA_8G07210
AOR: AO090003000611 AO090010000487
CNE: CNC05080 CNG02670
UMA: UM05362.1
ECU: ECU10_0510
DDI: DDBDRAFT_0217522
DDB_0219924(hgsA)
TET: TTHERM_00691190
TBR: Tb927.8.6110
YPE: YPO1457
YPK: y2712(pksG)
YPM: YP_1349(pksG)
YPA: YPA_0750
YPN: YPN_2521
YPP: YPDSF_1517
YPS: YPTB1475
CBD: COXBU7E912_1931
TCX: Tcr_1719
DNO: DNO_0799
BMA: BMAA1212
BPS: BPSS1002
BPM: BURPS1710b_A2613
BPL: BURPS1106A_A1384
BPD: BURPS668_A1470
BTE: BTH_II1670
MXA: MXAN_3948(tac)
MXAN_4267(mvaS)
BSU: BG10926(pksG)
OIH: OB2248

SAU: SA2334(mvaS)
SAV: SAV2546(mvaS)
SAM: MW2467(mvaS)
SAR: SAR2626(mvaS)
SAS: SAS2432
SAC: SACOL2561
SAB: SAB2420(mvaS)
SAA: SAUSA300_2484
SAO: SAOUHSC_02860
SAJ: SaurJH9_2569
SAH: SaurJH1_2622
SEP: SE2110
SER: SERP2122
SHA: SH0508(mvaS)
SSP: SSP0324
LMO: lmo1415
LMF: LMOf2365_1434(mvaS)
LIN: lin1454
LWE: lwe1432(mvaS)
LLA: L13187(hmcM)
LLC: LACR_1666
LLM: llmg_0929(hmcM)
SPY: SPy_0881(mvaS.2)
SPZ: M5005_Spy_0687(mvaS.1)
SPM: spyM18_0942(mvaS2)
SPG: SpyM3_0600(mvaS.2)
SPS: SPs1253
SPH: MGAS10270_Spy0745(mvaS1)
SPI: MGAS10750_Spy0779(mvaS1)
SPJ: MGAS2096_Spy0759(mvaS1)
SPK: MGAS9429_Spy0743(mvaS1)
SPF: SpyM51121(mvaS)
SPA: M6_Spy0704
SPB: M28_Spy0667(mvaS.1)
SPN: SP_1727
SPR: spr1571(mvaS)
SPD: SPD_1537(mvaS)
SAG: SAG1316
SAN: gbs1386
SAK: SAK_1347
SMU: SMU.943c
STC: str0577(mvaS)
STL: stu0577(mvaS)
STE: STER_0621
SSA: SSA_0338(mvaS)
SSU: SSU05_1641
SSV: SSU98_1652
SGO: SGO_0244
LPL: lp_2067(mvaS)
LJO: LJ1607
LAC: LBA0628(hmcS)
LSA: LSA1484(mvaS)
LSL: LSL_0526
LDB: Ldb0881(mvaS)
LBU: LBUL_0806
LBR: LVIS_1363
LCA: LSEI_1785
LGA: LGAS_1372

LRE: Lreu_0676

PPE: PEPE_0868

EFA: EF1363

OOE: OEOE_0968

LME: LEUM_1184

NFA: nfa22120

SEN: SACE_4570(pksG)

BBU: BB0683

BGA: BG0706

BAF: BAPKO_0727

FJO: Fjoh_0678

HAL: VNG1615G(mvaB)

HMA: rrnAC1740(mvaS)

HWA: HQ2868A(mvaB)

NPH: NP2608A(mvaB_1)

NP4836A(mvaB_2)

Exemplary hydroxymethylglutaryl-CoA reductase nucleic acids and polypeptides

HSA: 3156(HMGCR)
PTR: 471516(HMGCR)
MCC: 705479(HMGCR)
MMU: 15357(Hmgcr)
RNO: 25675(Hmgcr)
CFA: 479182(HMGCR)
BTA: 407159(HMGCR)
GGA: 395145(RCJMB04_14m24)
SPU: 373355(LOC373355)
DME: Dmel_CG10367(Hmgcr)
CEL: F08F8.2
OSA: 4347443
SCE: YLR450W(HMG2) YML075C(HMG1)
AGO: AGOS_AER152W
CGR: CAGL0L11506g
SPO: SPCC162.09c(hmg1)
ANI: AN3817.2
AFM: AFUA_1G11230 AFUA_2G03700
AOR: AO090103000311 AO090120000217
CNE: CNF04830
UMA: UM03014.1
ECU: ECU10_1720
DDI: DDB_0191125(hmgA)
DDB_0215357(hmgB)
TBR: Tb927.6.4540
TCR: 506831.40 509167.20
LMA: LmjF30.3190
VCH: VCA0723
VCO: VC0395_0662
VVU: VV2_0117
VVY: VVA0625
VPA: VPA0968
VFI: VFA0841
PAT: Patl_0427
CBU: CBU_0030 CBU_0610
CBD: COXBU7E912_0151
COXBU7E912_0622(hmgA)
TCX: Tcr_1717
DNO: DNO_0797
CVI: CV_1806
SUS: Acid_5728 Acid_6132
SAU: SA2333(mvaA)
SAV: SAV2545(mvaA)
SAM: MW2466(mvaA)
SAB: SAB2419c(mvaA)
SEP: SE2109
LWE: lwe0819(mvaA)
LLA: L10433(mvaA)
LLC: LACR_1664
LLM: llmg_0931(mvaA)
SPY: SPy_0880(mvaS.1)
SPM: spyM18_0941(mvaS1)
SPG: SpyM3_0599(mvaS.1)
SPS: SPs1254

SPH: MGAS10270_Spy0744
SPI: MGAS10750_Spy0778
SPJ: MGAS2096_Spy0758
SPK: MGAS9429_Spy0742
SPA: M6_Spy0703
SPN: SP_1726
SAG: SAG1317
SAN: gbs1387
STC: str0576(mvaA)
STL: stu0576(mvaA)
STE: STER_0620
SSA: SSA_0337(mvaA)
LPL: lp_0447(mvaA)
LJO: LJ1608
LSL: LSL_0224
LBR: LVIS_0450
LGA: LGAS_1373
EFA: EF1364
NFA: nfa22110
BGA: BG0708(mvaA)
SRU: SRU_2422
FPS: FP2341
MMP: MMP0087(hmgA)
MMQ: MmarC5_1589
MAC: MA3073(hmgA)
MBA: Mbar_A1972
MMA: MM_0335
MBU: Mbur_1098
MHU: Mhun_3004
MEM: Memar_2365
MBN: Mboo_0137
MTH: MTH562
MST: Msp_0584(hmgA)
MSI: Msm_0227
MKA: MK0355(HMG1)
AFU: AF1736(mvaA)
HAL: VNG1875G(mvaA)
HMA: rrnAC3412(mvaA)
HWA: HQ3215A(hmgR)
NPH: NP0368A(mvaA_2)
NP2422A(mvaA_1)
TAC: Ta0406m
TVO: TVN1168
PTO: PTO1143
PAB: PAB2106(mvaA)
PFU: PF1848
TKO: TK0914
RCI: RCIX1027(hmgA) RCIX376(hmgA)
APE: APE_1869
IHO: Igni_0476
HBU: Hbut_1531
SSO: SSO0531
STO: ST1352
SAI: Saci_1359
PAI: PAE2182
PIS: Pisl_0814
PCL: Pcal_1085
PAS: Pars_0796

Exemplary mevalonate kinase nucleic acids and polypeptides

HSA: 4598(MVK)
MCC: 707645(MVK)
MMU: 17855(Mvk)
RNO: 81727(Mvk)
CFA: 486309(MVK)
BTA: 505792(MVK)
GGA: 768555(MVK)
DRE: 492477(zgc:103473)
SPU: 585785(LOC585785)
DME: Dmel_CG33671
OSA: 4348331
SCE: YMR208W(ERG12)
AGO: AGOS_AER335W
PIC: PICST_40742(ERG12)
CGR: CAGL0F03861g
SPO: SPAC13G6.11c
MGR: MGG_06946
ANI: AN3869.2
AFM: AFUA_4G07780
AOR: AO090023000793
CNE: CNK01740
ECU: ECU09_1780
DDI: DDBDRAFT_0168621
TET: TTHERM_00637680
TBR: Tb927.4.4070
TCR: 436521.9 509237.10
LMA: LmjF31.0560

CBU: CBU_0608 CBU_0609
CBD: COXBU7E912_0620(mvk)
LPN: lpg2039
LPF: lpl2017
LPP: lpp2022
BBA: Bd1027(lmbP) Bd1630(mvk)
MXA: MXAN_5019(mvk)
OIH: OB0225
SAU: SA0547(mvaK1)
SAV: SAV0590(mvaK1)
SAM: MW0545(mvaK1)
SAR: SAR0596(mvaK1)
SAS: SAS0549
SAC: SACOL0636(mvk)
SAB: SAB0540(mvaK1)
SAA: SAUSA300_0572(mvk)
SAO: SAOUHSC_00577
SEP: SE0361
SER: SERP0238(mvk)
SHA: SH2402(mvaK1)
SSP: SSP2122
LMO: lmo0010
LMF: LMOf2365_0011
LIN: lin0010
LWE: lwe0011(mvk)
LLA: L7866(yeaG)
LLC: LACR_0454

LLM: llmg_0425(mvk)
SPY: SPy_0876(mvaK1)
SPZ: M5005_Spy_0682(mvaK1)
SPM: spyM18_0937(mvaK1)
SPG: SpyM3_0595(mvaK1)
SPS: SPs1258
SPH: MGAS10270_Spy0740(mvaK1)
SPI: MGAS10750_Spy0774(mvaK1)
SPJ: MGAS2096_Spy0753(mvaK1)
SPK: MGAS9429_Spy0737(mvaK1)
SPF: SpyM51126(mvaK1)
SPA: M6_Spy0699
SPB: M28_Spy0662(mvaK1)
SPN: SP_0381
SPR: spr0338(mvk)
SPD: SPD_0346(mvk)
SAG: SAG1326
SAN: gbs1396
SAK: SAK_1357(mvk)
SMU: SMU.181
STC: str0559(mvaK1)
STL: stu0559(mvaK1)
STE: STER_0598
SSA: SSA_0333(mvaK1)
SSU: SSU05_0289
SSV: SSU98_0285
SGO: SGO_0239(mvk)
LPL: lp_1735(mvaK1)
LJO: LJ1205
LAC: LBA1167(mvaK)
LSA: LSA0908(mvaK1)
LSL: LSL_0685(eRG)
LDB: Ldb0999(mvk)
LBU: LBUL_0906
LBR: LVIS_0858
LCA: LSEI_1491
LGA: LGAS_1033
LRE: Lreu_0915
PPE: PEPE_0927
EFA: EF0904(mvk)
OOE: OEOE_1100
LME: LEUM_1385
NFA: nfa22070
BGA: BG0711
BAF: BAPKO_0732
FPS: FP0313
MMP: MMP1335
MAE: Maeo_0775
MAC: MA0602(mvk)
MBA: Mbar_A1421
MMA: MM_1762
MBU: Mbur_2395
MHU: Mhun_2890
MEM: Memar_1812
MBN: Mboo_2213
MST: Msp_0858(mvk)
MSI: Msm_1439
MKA: MK0993(ERG12)

HAL: VNG1145G(mvk)

HMA: rrnAC0077(mvk)

HWA: HQ2925A(mvk)

NPH: NP2850A(mvk)

PTO: PTO1352

PHO: PH1625

PAB: PAB0372(mvk)

PFU: PF1637(mvk)

TKO: TK1474

RCI: LRC399(mvk)

APE: APE_2439

HBU: Hbut_0877

SSO: SSO0383

STO: ST2185

SAI: Saci_2365(mvk)

MSE: Msed_1602

PAI: PAE3108

PIS: Pisl_0467

PCL: Pcal_1835

Exemplary phosphomevalonate kinase nucleic acids and polypeptides

HSA: 10654(PMVK)
PTR: 457350(PMVK)
MCC: 717014(PMVK)
MMU: 68603(Pmvk)
CFA: 612251(PMVK)
BTA: 513533(PMVK)
DME: Dmel_CG10268
ATH: AT1G31910
OSA: 4332275
SCE: YMR220W(ERG8)
AGO: AGOS_AER354W
PIC: PICST_52257(ERG8)
CGR: CAGL0F03993g
SPO: SPAC343.01c
MGR: MGG_05812
ANI: AN2311.2
AFM: AFUA_5G10680
AOR: AO090010000471
CNE: CNM00100
UMA: UM00760.1
DDI: DDBDRAFT_0184512
TBR: Tb09.160.3690
TCR: 507913.20 508277.140
LMA: LmjF15.1460
MXA: MXAN_5017
OIH: OB0227
SAU: SA0549(mvaK2)
SAV: SAV0592(mvaK2)
SAM: MW0547(mvaK2)
SAR: SAR0598(mvaK2)
SAS: SAS0551
SAC: SACOL0638
SAB: SAB0542(mvaK2)
SAA: SAUSA300_0574
SAO: SAOUHSC_00579
SAJ: SaurJH9_0615
SEP: SE0363
SER: SERP0240
SHA: SH2400(mvaK2)
SSP: SSP2120
LMO: lmo0012
LMF: LMOf2365_0013
LIN: lin0012
LWE: lwe0013
LLA: L10014(yebA)
LLC: LACR_0456
LLM: llmg_0427
SPY: SPy_0878(mvaK2)
SPZ: M5005_Spy_0684(mvaK2)
SPM: spyM18_0939
SPG: SpyM3_0597(mvaK2)
SPS: SPs1256
SPH: MGAS10270_Spy0742(mvaK2)
SPI: MGAS10750_Spy0776(mvaK2)

SPJ: MGAS2096_Spy0755(mvaK2)
SPK: MGAS9429_Spy0739(mvaK2)
SPF: SpyM51124(mvaK2)
SPA: M6_Spy0701
SPB: M28_Spy0664(mvaK2)
SPN: SP_0383
SPR: spr0340(mvaK2)
SPD: SPD_0348(mvaK2)
SAG: SAG1324
SAN: gbs1394
SAK: SAK_1355
SMU: SMU.938
STC: str0561(mvaK2)
STL: stu0561(mvaK2)
STE: STER_0600
SSA: SSA_0335(mvaK2)
SSU: SSU05_0291
SSV: SSU98_0287
SGO: SGO_0241
LPL: lp_1733(mvaK2)
LJO: LJ1207
LAC: LBA1169
LSA: LSA0906(mvaK2)
LSL: LSL_0683
LDB: Ldb0997(mvaK)
LBU: LBUL_0904
LBR: LVIS_0860
LCA: LSEI_1092
LGA: LGAS_1035
LRE: Lreu_0913
PPE: PEPE_0925
EFA: EF0902
NFA: nfa22090
BGA: BG0710
BAF: BAPKO_0731
NPH: NP2852A
SSO: SSO2988
STO: ST0978
SAI: Saci_1244

Exemplary diphosphomevalonate decarboxylase nucleic acids and polypeptides

HSA: 4597(MVD)
PTR: 468069(MVD)
MCC: 696865(MVD)
MMU: 192156(Mvd)
RNO: 81726(Mvd)
CFA: 489663(MVD)
GGA: 425359(MVD)
DME: Dmel_CG8239
SCE: YNR043W(MVD1)
AGO: AGOS_AGL232C
PIC: PICST_90752
CGR: CAGL0C03630g
SPO: SPAC24C9.03
MGR: MGG_09750
ANI: AN4414.2
AFM: AFUA_4G07130
AOR: AO090023000862
CNE: CNL04950
UMA: UM05179.1
DDI: DDBDRAFT_0218058
TET: TTHERM_00849200
TBR: Tb10.05.0010 Tb10.61.2745
TCR: 507993.330 511281.40
LMA: LmjF18.0020
CBU: CBU_0607(mvaD)
CBD: COXBU7E912_0619(mvaD)
LPN: lpg2040
LPF: lpl2018
LPP: lpp2023
TCX: Tcr_1734
DNO: DNO_0504(mvaD)
BBA: Bd1629
MXA: MXAN_5018(mvaD)
OIH: OB0226
SAU: SA0548(mvaD)
SAV: SAV0591(mvaD)
SAM: MW0546(mvaD)
SAR: SAR0597(mvaD)
SAS: SAS0550
SAC: SACOL0637(mvaD)
SAB: SAB0541(mvaD)
SAA: SAUSA300_0573(mvaD)
SAO: SAOUHSC_00578
SAJ: SaurJH9_0614
SAH: SaurJH1_0629
SEP: SE0362
SER: SERP0239(mvaD)
SHA: SH2401(mvaD)
SSP: SSP2121
LMO: lmo0011
LMF: LMOf2365_0012(mvaD)
LIN: lin0011
LWE: lwe0012(mvaD)
LLA: L9089(yeaH)

LLC: LACR_0455
LLM: llmg_0426(mvaD)
SPY: SPy_0877(mvaD)
SPZ: M5005_Spy_0683(mvaD)
SPM: spyM18_0938(mvd)
SPG: SpyM3_0596(mvaD)
SPS: SPs1257
SPH: MGAS10270_Spy0741(mvaD)
SPI: MGAS10750_Spy0775(mvaD)
SPJ: MGAS2096_Spy0754(mvaD)
SPK: MGAS9429_Spy0738(mvaD)
SPF: SpyM51125(mvaD)
SPA: M6_Spy0700
SPB: M28_Spy0663(mvaD)
SPN: SP_0382
SPR: spr0339(mvd1)
SPD: SPD_0347(mvaD)
SAG: SAG1325(mvaD)
SAN: gbs1395
SAK: SAK_1356(mvaD)
SMU: SMU.937
STC: str0560(mvaD)
STL: stu0560(mvaD)
STE: STER_0599
SSA: SSA_0334(mvaD)
SSU: SSU05_0290
SSV: SSU98_0286
SGO: SGO_0240(mvaD)
LPL: lp_1734(mvaD)
LJO: LJ1206
LAC: LBA1168(mvaD)
LSA: LSA0907(mvaD)
LSL: LSL_0684
LDB: Ldb0998(mvaD)
LBU: LBUL_0905
LBR: LVIS_0859
LCA: LSEI_1492
LGA: LGAS_1034
LRE: Lreu_0914
PPE: PEPE_0926
EFA: EF0903(mvaD)
LME: LEUM_1386
NFA: nfa22080
BBU: BB0686
BGA: BG0709
BAF: BAPKO_0730
GFO: GFO_3632
FPS: FP0310(mvaD)
HAU: Haur_1612
HAL: VNG0593G(dmd)
HMA: rrnAC1489(dmd)
HWA: HQ1525A(mvaD)
NPH: NP1580A(mvaD)
PTO: PTO0478 PTO1356
SSO: SSO2989
STO: ST0977
SAI: Saci_1245(mvd)
MSE: Msed_1576

Exemplary isopentenyl phosphate kinases (IPK) nucleic acids and polypeptides

Methanobacterium thermoautotrophicum gil2621082

Methanococcus jannaschii DSM 2661 gil1590842 ;

Methanocaldococcus jannaschii gil1590842

Methanothermobacter thermautotrophicus gil2621082

Picrophilus torridus DSM9790 (IG-57) gil48477569

Pyrococcus abyssi gil14520758

Pyrococcus horikoshii OT3 gil3258052

Archaeoglobus fulgidus DSM4304 gil2648231

Exemplary isopentenyl-diphosphate Delta-isomerase (IDI) nucleic acids and polypeptides

HSA: 3422(IDI1) 91734(IDI2)
PTR: 450262(IDI2) 450263(IDI1)
MCC: 710052(LOC710052)
721730(LOC721730)
MMU: 319554(Idi1)
RNO: 89784(Idi1)
GGA: 420459(IDI1)
XLA: 494671(LOC494671)
XTR: 496783(idi2)
SPU: 586184(LOC586184)
CEL: K06H7.9(idi-1)
ATH: AT3G02780(IPP2)
OSA: 4338791 4343523
CME: CMB062C
SCE: YPL117C(IDI1)
AGO: AGOS_ADL268C
PIC: PICST_68990(IDI1)
CGR: CAGL0J06952g
SPO: SPBC106.15(idi1)
ANI: AN0579.2
AFM: AFUA_6G11160
AOR: AO090023000500
CNE: CNA02550
UMA: UM04838.1
ECU: ECU02_0230
DDI: DDB_0191342(ipi)

TET: TTHERM_00237280
TTHERM_00438860
TBR: Tb09.211.0700
TCR: 408799.19 510431.10
LMA: LmjF35.5330
EHI: 46.t00025
ECO: b2889(idi)
ECJ: JW2857(idi)
ECE: Z4227
ECS: ECs3761
ECC: c3467
ECI: UTI89_C3274
ECP: ECP_2882
ECV: APECO1_3638
ECW: EcE24377A_3215(idi)
ECX: EcHS_A3048
STY: STY3195
STT: t2957
SPT: SPA2907(idi)
SEC: SC2979(idi)
STM: STM3039(idi)
SFL: SF2875(idi)
SFX: S3074
SFV: SFV_2937
SSN: SSON_3042 SSON_3489(yhfK)
SBO: SBO_3103
SDY: SDY_3193

ECA: ECA2789
PLU: plu3987
ENT: Ent638_3307
SPE: Spro_2201
VPA: VPA0278
VFI: VF0403
PPR: PBPRA0469(mvaD)
PEN: PSEEN4850
CBU: CBU_0607(mvaD)
CBD: COXBU7E912_0619(mvaD)
LPN: lpg2051
LPF: lpl2029
LPP: lpp2034
TCX: Tcr_1718
HHA: Hhal_1623
DNO: DNO_0798
EBA: ebA5678 p2A143
DVU: DVU1679(idi)
DDE: Dde_1991
LIP: LI1134
BBA: Bd1626
AFW: Anae109_4082
MXA: MXAN_5021(fni)
RPR: RP452
RTY: RT0439(idi)
RCO: RC0744
RFE: RF_0785(fni)
RBE: RBE_0731(fni)
RAK: A1C_04190
RBO: A1I_04755
RCM: A1E_02555
RRI: A1G_04195
MLO: mlr6371
RET: RHE_PD00245(ypd00046)
XAU: Xaut_4134
SIL: SPO0131
SIT: TM1040_3442
RSP: RSP_0276
RSH: Rsph17029_1919
RSQ: Rsph17025_1019
JAN: Jann_0168
RDE: RD1_0147(idi)
DSH: Dshi_3527
BSU: BG11440(ypgA)
BAN: BA1520
BAR: GBAA1520
BAA: BA_2041
BAT: BAS1409
BCE: BC1499
BCA: BCE_1626
BCZ: BCZK1380(fni)
BCY: Bcer98_1222
BTK: BT9727_1381(fni)
BTL: BALH_1354
BLI: BL02217(fni)
BLD: BLi02426
BAY: RBAM_021020(fni)
BPU: BPUM_2020(fni)

OIH: OB0537
SAU: SA2136(fni)
SAV: SAV2346(fni)
SAM: MW2267(fni)
SAR: SAR2431(fni)
SAS: SAS2237
SAC: SACOL2341(fni)
SAB: SAB2225c(fni)
SAA: SAUSA300_2292(fni)
SAO: SAOUHSC_02623
SEP: SE1925
SER: SERP1937(fni-2)
SHA: SH0712(fni)
SSP: SSP0556
LMO: lmo1383
LMF: LMOf2365_1402(fni)
LIN: lin1420
LWE: lwe1399(fni)
LLA: L11083(yebB)
LLC: LACR_0457
LLM: llmg_0428(fni)
SPY: SPy_0879
SPZ: M5005_Spy_0685
SPM: spyM18_0940
SPG: SpyM3_0598
SPS: SPs1255
SPH: MGAS10270_Spy0743
SPI: MGAS10750_Spy0777
SPJ: MGAS2096_Spy0756
SPK: MGAS9429_Spy0740
SPF: SpyM51123(fni)
SPA: M6_Spy0702
SPB: M28_Spy0665
SPN: SP_0384
SPR: spr0341(fni)
SPD: SPD_0349(fni)
SAG: SAG1323
SAN: gbs1393
SAK: SAK_1354(fni)
SMU: SMU.939
STC: str0562(idi)
STL: stu0562(idi)
STE: STER_0601
SSA: SSA_0336
SGO: SGO_0242
LPL: lp_1732(idi1)
LJO: LJ1208
LAC: LBA1171
LSA: LSA0905(idi)
LSL: LSL_0682
LDB: Ldb0996(fni)
LBU: LBUL_0903
LBR: LVIS_0861
LCA: LSEI_1493
LGA: LGAS_1036
LRE: Lreu_0912
EFA: EF0901
OOE: OEOE_1103

STH: STH1674
CBE: Cbei_3081
DRM: Dred_0474
SWO: Swol_1341
MTA: Moth_1328
MTU: Rv1745c(idi)
MTC: MT1787(idi)
MBO: Mb1774c(idi)
MBB: BCG_1784c(idi)
MPA: MAP3079c
MAV: MAV_3894(fni)
MSM: MSMEG_1057(fni)
MSMEG_2337(fni)
MUL: MUL_0380(idi2)
MVA: Mvan_1582 Mvan_2176
MGI: Mflv_1842 Mflv_4187
MMC: Mmcs_1954
MKM: Mkms_2000
MJL: Mjls_1934
CGL: NCgl2223(cgl2305)
CGB: cg2531(idi)
CEF: CE2207
CDI: DIP1730(idi)
NFA: nfa19790 nfa22100
RHA: RHA1_ro00239
SCO: SCO6750(SC5F2A.33c)
SMA: SAV1663(idi)
LXX: Lxx23810(idi)
CMI: CMM_2889(idiA)
AAU: AAur_0321(idi)
PAC: PPA2115
FRA: Francci3_4188
FRE: Franean1_5570
FAL: FRAAL6504(idi)
KRA: Krad_3991
SEN: SACE_2627(idiB_2) SACE_5210(idi)
STP: Strop_4438
SAQ: Sare_4564 Sare_4928
RXY: Rxyl_0400
BBU: BB0684
BGA: BG0707
SYN: sll1556
SYC: syc2161_c
SYF: Synpcc7942_1933
CYA: CYA_2395(fni)
CYB: CYB_2691(fni)
TEL: tll1403
ANA: all4591
AVA: Ava_2461 Ava_B0346
TER: Tery_1589
SRU: SRU_1900(idi)
CHU: CHU_0674(idi)
GFO: GFO_2363(idi)
FJO: Fjoh_0269
FPS: FP1792(idi)
CTE: CT0257
CCH: Cag_1445
CPH: Cpha266_0385

PVI: Cvib_1545
PLT: Plut_1764
RRS: RoseRS_2437
RCA: Rcas_2215
HAU: Haur_4687
DRA: DR_1087
DGE: Dgeo_1381
TTH: TT_P0067
TTJ: TTHB110
MJA: MJ0862
MMP: MMP0043
MMQ: MmarC5_1637
MMX: MmarC6_0906
MMZ: MmarC7_1040
MAE: Maeo_1184
MVN: Mevan_1058
MAC: MA0604(idi)
MBA: Mbar_A1419
MMA: MM_1764
MBU: Mbur_2397
MTP: Mthe_0474
MHU: Mhun_2888
MLA: Mlab_1665
MEM: Memar_1814
MBN: Mboo_2211
MTH: MTH48
MST: Msp_0856(fni)
MSI: Msm_1441
MKA: MK0776(lldD)
AFU: AF2287
HAL: VNG1818G(idi) VNG6081G(crt_1) VNG6445G(crt_2) VNG7060 VNG7149
HMA: rrnAC3484(idi)
HWA: HQ2772A(idiA) HQ2847A(idiB)
NPH: NP0360A(idiB_1) NP4826A(idiA) NP5124A(idiB_2)
TAC: Ta0102
TVO: TVN0179
PTO: PTO0496
PHO: PH1202
PAB: PAB1662
PFU: PF0856
TKO: TK1470
RCI: LRC397(fni)
APE: APE_1765.1
SMR: Smar_0822
IHO: Igni_0804
HBU: Hbut_0539
SSO: SSO0063
STO: ST2059
SAI: Saci_0091
MSE: Msed_2136
PAI: PAE0801
PIS: Pisl_1093
PCL: Pcal_0017
PAS: Pars_0051
TPE: Tpen_0272

Exemplary isoprene synthase nucleic acids and polypeptides

Genbank Accession Nos.

AY341431

AY316691

AY279379

AJ457070

AY182241

CLAIMS:

1. Recombinant host cells comprising (a) a heterologous nucleic acid encoding an isoprene synthase polypeptide, (b) a nucleic acid encoding an isopentenyl-diphosphate delta-isomerase (IDI) polypeptide, (c) nucleic acid(s) encoding a 1-Deoxyxylulose-5-phosphate synthase (DXS) polypeptide and/or one or more mevalonate (MVA) pathway polypeptides and (d) nucleic acid(s) encoding one or more polypeptides involved in ethanol fermentation, wherein the cells co-produce isoprene and ethanol under oxygen-limited culture conditions, and wherein the oxygen transfer rate (OTR) is less than the oxygen uptake rate (OUR)

2. The cells of claim 1, wherein the heterologous nucleic acid encoding an isoprene synthase polypeptide is operably linked to a promoter.

3. The cells of claim 2, wherein the isoprene synthase polypeptide is a plant isoprene synthase polypeptide.

4. The cells of claim 3, wherein the plant isoprene synthase polypeptide is from *Populus alba*.

5. The cells of claim 1, wherein the polypeptide involved in ethanol fermentation is an alcohol dehydrogenase B (adhB) polypeptide, an alcohol dehydrogenase E (adhE) polypeptide, or a pyruvate decarboxylase (pdc) polypeptide.

6. The cells of claim 1, wherein the cells comprise nucleic acids encoding one or more MVA pathway polypeptides selected from group: (i) an acetoacetyl-Coenzyme A synthase (thiolase) polypeptide; (ii) a 3-hydroxy-3-methylglutaryl-Coenzyme A synthase polypeptide (iii) a 3-hydroxy-3-methylglutaryl-Coenzyme A reductase polypeptide, (iv) mevalonate kinase (MVK); (v) phosphomevalonate kinase (PMK); or (vi) diphosphomevalonate decarboxylase (MVD).

7. The cells of claim 1, wherein the cells have an average volumetric productivity of isoprene greater than 0.1 mg/L_{broth}/hr and an average volumetric productivity of ethanol greater than 0.1 mg/L_{broth}/hr.

8. The cells of claim 1, wherein the cells produce isoprene at a rate between 400 nmole/g_{wcm}/hr to 2.0×10^5 nmole/g_{wcm}/hr and produce ethanol at a rate between 0.01 mmol/L_{broth}/hr and 200 mmol/L_{broth}/hr.

9. The cells of claim 1, wherein the cells are *E. coli* cells.

10. The cells of claim 9, wherein the cells (i) have an average volumetric productivity of isoprene greater than 0.1 mg/L_{broth}/hr and an average volumetric productivity of ethanol greater than 0.1 mg/L_{broth}/hr; or (ii) produce isoprene at a rate between 400 nmole/g_{wcm}/hr to 2.0×10^5 nmole/g_{wcm}/hr and produce ethanol at a rate between 0.01 mmol/L_{broth}/hr and 200 mmol/L_{broth}/hr.

11. A method of co-producing isoprene and ethanol, the method comprising:

(a) culturing cells capable of co-producing isoprene and under oxygen-limited culture conditions wherein the oxygen transfer rate (OTR) is less than the oxygen uptake rate (OUR), and wherein the cells comprise (i) a heterologous nucleic acid encoding an isoprene synthase polypeptide; (ii) a nucleic acid encoding an isopentenyl-diphosphate delta-isomerase (IDI) polypeptide; (iii) nucleic acid(s) encoding a 1-Deoxyxylulose-5-phosphate synthase (DXS) polypeptide and/or one or more mevalonate (MVA) pathway polypeptides; and (iv) nucleic acid(s) encoding one or more polypeptides involved in ethanol fermentation; and

(b) co-producing isoprene and ethanol.

12. The method of claim 11, wherein the heterologous nucleic acid encoding an isoprene synthase polypeptide is operably linked to a promoter.

13. The method of claim 12, wherein the isoprene synthase polypeptide is a plant isoprene synthase polypeptide.

14. The method of claim 13, wherein the plant isoprene synthase polypeptide is from *Populus alba*.

15. The method of claim 11, wherein the polypeptide involved in ethanol fermentation is an alcohol dehydrogenase B (adhB) polypeptide, an alcohol dehydrogenase E (adhE) polypeptide, or a pyruvate decarboxylase (pdc) polypeptide.

16. The method of claim 11, wherein the cells comprise one or more MVA pathway polypeptides selected from group: (a) an acetoacetyl-Coenzyme A synthase (thiolase) polypeptide; (b) a 3-hydroxy-3-methylglutaryl-Coenzyme A synthase polypeptide (c) a 3-hydroxy-3-methylglutaryl-Coenzyme A reductase polypeptide, (d) mevalonate kinase

(MVK); (e) phosphomevalonate kinase (PMK); or (f) diphosphomevalonate decarboxylase (MVD).

17. The method of claim 11, wherein the cells have an average volumetric productivity of isoprene greater than 0.1 mg/L_{broth}/hr and an average volumetric productivity of ethanol greater than 0.1 mg/L_{broth}/hr.

18. The method of claim 11, wherein the cells produce isoprene at a rate between 400 nmole/g_{wcm}/hr to 2.0×10^5 nmole/g_{wcm}/hr and produce ethanol at a rate between 0.01 mmol/L_{broth}/hr and 200 mmol/L_{broth}/hr.

19. The method of claim 11, wherein the cells are *E. coli* cells.

20. The cells of claim 19, wherein the cells (a) have an average volumetric productivity of isoprene greater than 0.1 mg/L_{broth}/hr and an average volumetric productivity of ethanol greater than 0.1 mg/L_{broth}/hr; or (b) produce isoprene at a rate between 400 nmole/g_{wcm}/hr to 2.0×10^5 nmole/g_{wcm}/hr and produce ethanol at a rate between 0.01 mmol/L_{broth}/hr and 200 mmol/L_{broth}/hr.